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Regulated Expression of the *Saccharomyces cerevisiae* Fre1p/Fre2p Fe/Cu Reductase Related Genes

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The *Saccharomyces cerevisiae* genome contains nine open reading frames (ORFs)—*YLR214w* (*FRE1*), *YKL220c* (*FRE2*), *YOR381w*, *YNR060w*, *YOR384w*, *YLL051c*, *YOL152w*, *YGL160w* and *YLR047c*—which, based on amino acid sequence similarity, fall in the category of iron/copper reductase-related genes. *FRE1* and *FRE2* are the first identified and studied genes of this family. They both encode for plasma membrane ferric/cupric reductases and their expression is regulated by iron and copper availability, mediated by the transcription factors Aft1p and Mac1p, respectively. We have studied the expression of the seven ORFs of unknown function by monitoring mRNA accumulation under different growth conditions, namely, their response to iron and copper availability in the medium, as well as the involvement of transcription factors Aft1p and Mac1p in their expression. A compilation of these results, together with sequence comparison data, permits a first classification of these genes under three major groups: genes mainly regulated by iron availability, genes mainly regulated by copper availability and genes not regulated by either metal. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — iron/copper homeostasis; multigene families; ferric/cupric reductases; metalloregulation

INTRODUCTION

The complete *Saccharomyces cerevisiae* genome sequence provides a precious source of information for our understanding of both the genome structure and complexity and the physiology of this unicellular free-living eukaryotic organism. A particularly interesting observation was that in the relatively small *S. cerevisiae* genome several distinct regions have been duplicated bringing, up the question of genetic redundancy (Dujon, 1996; Wolfe and Shields, 1997). In addition to the large subtelomeric sequence segments which are almost identical between several chromosomes (Louis, 1995) and the tandem repeats of certain genes, such as rDNA and *CUP1* (Keil and McWilliams, 1993), many other genes have one or more homologues. One-third of the ORFs (~2000) can be assigned to about 800 multigene families of two to >10 members (B. Dujon, personal communication). It is thus of great interest to understand the particular function of each multigene family member, in order to comprehend the role (or the necessity) of genetic redundancy in evolution.

The genes encoding for the externally directed plasma membrane Fre1p and Fre2p ferric/cupric reductases fall in this category (Alexandraki and Tzermia, 1994; Georgatsou and Alexandraki, 1994) since seven more ORFs scattered throughout the *S. cerevisiae* genome exhibit varying extents of sequence similarity to them (MIPS, SGD, YPD). The Fre1p and Fre2p have been shown to play an important role in iron and copper uptake, since reduction of these metals occurs prior to their transport through the plasma membrane (Dancis *et al*., 1992; Hassett and Kosman, 1995; Knight *et al*., 1996; Georgatsou *et al*., 1997). Iron and copper are transition metals very frequently involved in electron transfer reactions that play key roles in many processes of fundamental biological significance. They are most commonly used

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by electron transfer proteins, where their valence state is important, and they can be found under different oxidation states, depending on the environment. Iron, for example, is bivalent when incorporated in a heme molecule and trivalent when it is stored in ferritin. In *S. cerevisiae*, several ferric reductase activities have been detected in different subcellular extracts (Lesuisse *et al*., 1990). It is thus possible that at least some of the unknown function Fe/Cu reductase-related ORFs encode for proteins that ensure the proper partition of metal ions under different valence states in cellular compartments, according to particular physiological demands.

The expression of *FRE1* and *FRE2* genes is transcriptionally regulated by iron and copper availability (Dancis *et al*., 1992; Georgatsou and Alexandraki, 1994; Hassett and Kosman, 1995; Georgatsou *et al*., 1997). This regulation is distinct for each gene and is mainly exerted by the metal-affected transcription factors Aft1p and Mac1p (Yamagushi-Iwai *et al*., 1995; Jungmann *et al*., 1993). The iron-affected Aft1p upregulates both genes (Yamagushi-Iwai *et al*., 1996) but the copper modulated Mac1p upregulates *FRE1* (Graden and Winge, 1997; Labbe *et al*., 1997; Yamagushi-Iwai *et al*., 1997) and downregulates *FRE2* (Georgatsou *et al*., 1997). *YOL152w* (*FRE7*) mRNA accumulation has also been reported to increase under Fe/Cu chelation conditions (Casas *et al*., 1997). In order to initiate a functional analysis of the *FRE1*/*FRE2* homologue ORFs, we have examined their expression in respect to iron and copper availability during cell growth, as well as the involvement of Aft1p and Mac1p factors on their expression.

MATERIALS AND METHODS

Yeast strains, media and growth conditions

The yeast strains used in this study are all derivatives of the S288C strain. The wild-type and *mac1*Δ strains have been previously described (Georgatsou *et al*., 1997). The *aft1*Δ strain was produced by replacement of the chromosomal wild-type copy of *AFT1* gene in our wild-type strain by the deleted version of *AFT1* gene in pCM24 (Casas *et al*., 1997).

Growth media and conditions were as previously described (Georgatsou *et al*., 1997). YNBFC medium was purchased from Bio 101 and SD medium from Difco.

RNA blot hybridization and DNA fragments used as probes

Total RNA was prepared as described previously (Georgatsou *et al*., 1997). 50 μg of total RNA was loaded on each lane of 1·2% agarose gels and transferred onto nylon membranes. The following genomic DNA fragments were used as radiolabelled probes for the RNA blot hybridizations: *FRE1* (from −680 to +2280 bp); *FRE2* (from −2041 to +2340 bp); *FRE3* (from +405 to +1782 bp); *FRE4* (from −934 to +2411 bp); *FRE5* (from +426 to +2368 bp); *FRE6* (from +274 to +1416 bp); *FRE7* (from +106 to +1758 bp); *YGL160w* (from −1059 to +1888 bp); and *YLR047c* (from −1018 to +2266 bp). DNA fragments were generated from genomic DNA by PCR and used following subcloning into pBluescript, except for *FRE4*, *YLR047c* and *YGL160w*, which were used directly as PCR products. Standard hybridization conditions were used (Ausubel *et al*., 1987–1998), during which we did not observe any cross-hybridization between the *FRE2* and *FRE3* sequences, which exhibit some homologies at the nucleotide level also. Synthetic oligonucleotides were purchased from MWG-BIOTECH GmbH.

Sequence analysis

DNA and protein sequence information was obtained from the yeast databases: MIPS (Munich Information Centre for Protein Sequences, http://speedy.mips.biochem.mpg.de/mips/yeast); SGD (Saccharomyces Genome Database, http://genome-www.stanford.edu/Saccharomyces); and YPD (Yeast Protein Database) (http://quest7.proteome.com/YPDhome.html). Additional sequence information was obtained from the sites: PSORT II programme (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences, http://psort.nibb.ac.jp:8800); and Yeast Gene Duplications (http://acer.gen.tcd.ie/khwolfe/yeast/topmenu.html). Sequence analysis was also performed by the GCG package software (Bestfit, Clustal, Pileup programmes for sequence alignments).

RESULTS AND DISCUSSION

Seven ORFs in the S. cerevisiae *genome share amino acid sequence similarities to the Fre1p/Fre2p Fe/Cu reductases*

The predicted amino acid sequences of seven ORFs in the *S. cerevisiae* genome show significant

Table 1. Summarized features of the Fre1p/Fre2p Fe/Cu-related ORFs.

ORF name	Other name	ORF size (no. of amino acids)	Trans-membrane regions*	N-end signal peptide (no. of amino acids)	FAD binding motif	NADPH binding motif	bis-H-eme binding motif
YLR214w	*FRE1*	686	7	20	HPFTVLS	GPYG, CGP	+
YKL220c	*FRE2*	711	6+1	20	HPFTVLDS	GPYG, CGP	+
YOR381w	*FRE3*	711	5	20	HPFTVLDS	GPYG, CGP	+
YNR060w	*FRE4*	719	5+1	18	HPFTVMDS	GPYG, CGP	+
YOR384w	*FRE5*	694	6+1	19	HPFTVMDS	GPYG, CGS	+
YLL051c	*FRE6*	712	5+1	17	HPFTIID	GPYG, CGP	+
YOL152w	*FRE7*	629	7	—	HPFSIFPS	GPYG	+
YGL160w		570	7+1	—	HPYTIAS	CGP	+
YLR047c		686	6+1	—	HPFTIAS	—	+

*First number indicates integral membranes, second number indicates peripheral membranes (PSORT II).

Table 2. Percentage of amino acid residue identity or similarity (*italics*) between the products of the Fe/Cu reductase-related genes, as calculated by the Bestfit algorithm of the GCG package software.

	FRE1	*FRE2*	*FRE3*	*FRE4*	*FRE5*	*FRE6*	*FRE7*	*YGL160w*	*YLR047c*
FRE1		*50·4*	*51·2*	*49·3*	*50·8*	*48·0*	*51·9*	*47·3*	*44·0*
FRE2	25·8		*86·9*	*74·8*	*60·1*	*61·0*	*52·9*	*47·8*	*42·7*
FRE3	26·1	75·9		*75·4*	*59·7*	*60·6*	*50·6*	*48·0*	*45·6*
FRE4	27·2	57·3	56·5		*59·6*	*61·7*	*48·4*	*47·9*	*48·4*
FRE5	26·1	38·5	37·0	37·8		*59·2*	*51·2*	*51·8*	*48·5*
FRE6	22·2	38·0	37·5	36·2	34·1		*50·6*	*51·9*	*47·5*
FRE7	23·9	23·2	22·7	22·0	24·2	22·6		*47·0*	*45·7*
YGL160w	20·4	21·3	21·7	24·5	24·1	23·1	20·0		*56·5*
YLR047c	15·5	20·0	17·9	19·2	21·8	19·8	18·1	30·6	

homology to the Fre1p/Fre2p amino acid sequences by the commonly used Blastp search analysis (Altschul *et al.*, 1995). These are, *YOR381w*, *YNR060w*, *YOR384w*, *YLL051c*, *YOL152w*, *YGL160w* and *YLR047c* (Table 1). Considering the degree of similarity, at least for the first five ORFs (Table 2), and although their function has not yet been determined, it has been agreed by yeast researchers to name them in the order of homology as *FRE3–7* (Dennis R. Winge, Saccharomyces Genome Database; Table 1).

Based on our knowledge on Fre1p and Fre2p, and in order to evaluate the relationships of the nine ORFs and to deduce information for their structure and function, we first analysed the available sequence data in the yeast databases (MIPS, SGD and YPD). Comparisons of the entire length of amino acid sequences of the nine ORFs using multiple alignment programmes (see Table 2, where we show the results from the Bestfit programme, which compares sequences at their entire length and measures both identities and similarities) showed that:

1. Most of the new members of this family exhibit higher similarity to the Fre2p amino acid sequence, mainly Fre3p and Fre4p and significantly Fre5p and Fre6p.
2. Fre1p and Fre7p are similarly distant from all other members.
3. YGL160w and YLR047c are the most distantly related ORFs to all other members but they are somewhat more similar to each other.

All putative peptide sequences contain potential transmembrane domains and all members, except Fre7p, YGL160w and YLR047c, contain a 17–20 residue N-terminal potentially cleavable signal peptide (Table 1; PSORT II programme). All

putative peptide sequences contain, at similar positions, sequence motifs indicative of electron transfer proteins (Segal and Abo, 1993; Finegold *et al.*, 1996), i.e. they contain the HPFTXXS box or a closely related sequence, proposed to be binding site for the FAD co-factor and (except for YLR047c) they also contain sequence motifs for the electron donor NADPH (glycine-rich motif and/or cysteine–glycine motif) (Table 1). Fre1p has been shown to be a heme-containing protein and four histidine residues are predicted to coordinate a bis-heme structure between transmembrane domains of the protein (Finegold *et al.*, 1996). These histidines are also conserved, similarly spaced, in all members of the family. It is worth noting, however, that Fre2p, which displays this histidine motif, does not seem to contain heme (Lesuisse *et al.*, 1996). All putative peptide sequences contain several (6–11) potential *N*-glycosylation sites (YPD). Finally, a possible vacuolar targeting motif (KLPN) exists in YLR047c sequence (PRORT II).

Expression of the FRE1/FRE2 related genes under iron and copper depletion conditions

We have previously shown that *FRE1* and *FRE2* genes are distinctly regulated under iron and copper depletion conditions (Georgatsou and Alexandraki, 1994; Georgatsou *et al.*, 1997). Expression of both genes is induced in the presence of the Fe/Cu chelator BPS but with different kinetics (earlier induction of *FRE1* compared to that of *FRE2*), whereas only *FRE1* expression is induced in the presence of the Cu-specific chelator BCS. Moreover, it has been reported that *FRE7* mRNA levels are also increased when the Fe/Cu chelator ferrozine is added in the medium (Casas *et al.*, 1997). Thus, we examined the mRNA accumulation of the seven *FRE1*/*FRE2*-related genes, at particular time points of cell growth, under iron and copper depletion conditions, to find out whether the new genes are transcribed at all, or whether their transcription is regulated, and attempt to correlate their expression patterns with their sequence similarities.

Our results show that all *FRE1*/*FRE2*-related ORFs are transcribed (Figure 1). Moreover, we can classify the nine genes into three groups according to their expression under the examined conditions:

1. The *FRE2–FRE6* genes, whose mRNA levels were induced only in the presence of BPS. These genes are also distinguished in two subcategories of gene expression patterns: *FRE2* and *FRE3*, which were highly induced under these conditions, and *FRE4*, *FRE5* and *FRE6*, which were moderately induced. It should be noted that although Fre4p sequence is more similar to Fre2p and Fre3p (~57% identity) than to Fre5p and Fre6p (~37% identity), *FRE4* gene expression resembles more that of *FRE5* and *FRE6*.
2. The *FRE1* and *FRE7* genes which, although not particularly similar, were transcriptionally induced by both chelators.
3. *YGL160w* and *YLR047c*, whose expression was not affected by the presence of Fe/Cu chelators in the medium, at least according to our detection conditions.

Since BPS is a chelator of both metals and induction in its presence cannot be attributed to either iron or copper depletion alone, we have additionally examined RNA accumulation in cells grown in YNBFC medium (SD medium without iron and copper), adding either iron or copper at standard concentrations (SD medium; Difco). This medium was not used at the experiments shown in Figure 1, because induction of ferric reductase activity occurred later and at lower levels than those obtained using metal chelators (data not shown). Our results, presented in Figure 2, show that *FRE2–FRE6* gene expression was induced only when iron was absent from the medium and the relative magnitude of induction agreed with that observed in cells grown in BPS-containing medium. On the other hand, *FRE7* gene expression was clearly induced only in the absence of copper, in contrast to *FRE1*, which was induced in the absence of either metal.

We have previously shown (Georgatsou *et al.*, 1997), and have confirmed in this study, that the observed induction of *FRE1* gene expression by the presence of BPS is due to chelation of both metals, with copper depletion affecting early induction and iron depletion being evident at later time points. In fact, if one monitors for *CTR1* mRNA levels (*CTR1* encodes for a copper transporter that is transcriptionally induced in the absence of copper; Dancis et al., 1994) during growth of a wild-type strain in the presence of BPS, one can observe induction during the first 4–6 h of growth in minimal medium and a subsequent drop, indicating that copper is available for the cell after this

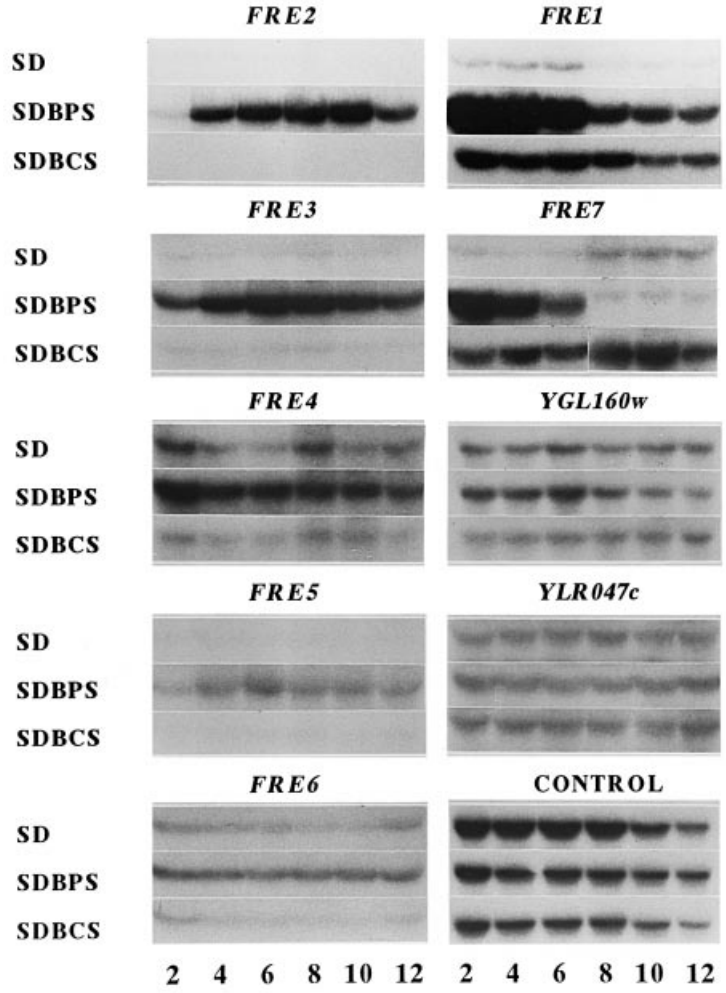

Figure 1. RNA accumulation of the Fe/Cu reductase-related genes in cells grown in the presence of Fe/Cu chelators. Cells were grown exponentially in SD medium and, following dilution to OD_{600} of 0·45, they were shifted to either SD or SDBPS or SDBCS medium and grown for another 12 h. Total RNA was prepared at the time points indicated (hours). RNAs were separated by electrophoresis on four identical agarose gels, transferred and hybridized with the indicated probes (described in Materials and Methods). Filters were boiled in water for 5 min and rehybridized. Hybridization intensity differences between the different probes should not be considered as a direct quantitative measure due to differences in specific activities. *YLR048w* (40S ribosomal protein SOB) RNA accumulation (included in the probe used for *YLR047c*) is shown as control. Note: the decrease of *YLR048w* RNA observed after 10 h of growth was not seen with *ACTIN* or *HIS3* gene probes (data not shown).

point (data not shown). It is possible that, since BPS retains copper only when it is in its bivalent (oxidized) form, the action of the reductases during growth transforms copper to its monovalent form, which is not retained by BPS and is thus available for uptake. Accordingly, we noticed that the difference between *FRE1* and *FRE7* induction can be discerned even under BPS chelation conditions. *FRE7* mRNA induction was evident during the initial 6 h of growth and then it was diminished (induction due to copper absence), whereas *FRE1* mRNA induction persisted at later time points

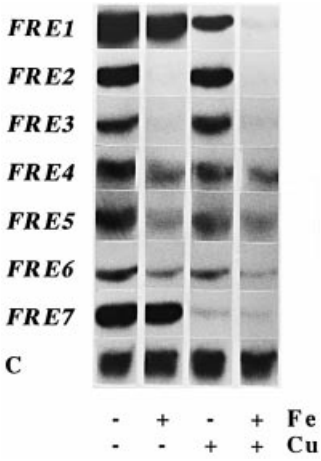

Figure 2. RNA accumulation of the Fe/Cu reductase-related genes in cells grown in the absence of iron and/or copper. Cells were grown in YNBFC medium or YNBFC supplied with copper (0·25 μM) or iron (1·25 μM), as indicated, for 10 h, diluted at the initial cell concentration and grown for another 8 h in the same medium. Total RNA was prepared and analysed as described on the legend to Figure 1.

(induction due to both copper and iron absence) (Figure 1).

Dependence of FRE1/FRE2 related gene expression on the Aft1p and Mac1p transcriptional activators

It was previously shown that the regulated expression of *FRE1* and *FRE2* genes by iron and copper is affected by Aft1p and Mac1p transcription factors, respectively. Aft1p is a transcriptional activator with iron-modulated DNA binding properties required for *FRE1* and *FRE2* gene induction by iron depletion (Yamagushi-Iwai *et al.*, 1995, 1996). On the other hand, Mac1p is a copper, modulated transcriptional activator (Georgatsou *et al.*, 1997; Graden and Winge, 1997) which affects differently the expression of the two genes. In its absence, *FRE1* is only very weakly induced by copper depletion (Jungmann *et al.*, 1993). However, Mac1p is a direct or indirect repressor of the *FRE2* gene. Whereas *FRE2* gene is not induced by copper starvation, it can be moderately induced in a *mac1*Δ strain (Georgatsou *et al.*, 1997). (As previously shown by Georgatsou *et al.*, 1997, this differential action of Mac1p accounts for the temporally distinct expression patterns of *FRE1* and *FRE2* genes in cells depleted from both metals.) We have examined the requirement of the two transcription factors on the inducible expression of the *FRE1/FRE2*-related genes using strains disrupted for either *AFT1* or *MAC1* gene. The results presented on Figure 3 show that: (a) the iron-regulated induction of *FRE3, FRE5* and *FRE6* genes is dependent on the presence of Aft1p (similarly to *FRE1* and *FRE2* gene induction). *FRE4* gene expression does not seem to be significantly affected by the absence of Aft1p, at least not at a level that we could detect. Also, *FRE5* and *FRE6* gene expression may not be entirely dependent on Aft1p. The Aft1p dependence of *FRE1* expression was evident at the late time points of growth in BPS, when iron absence persists (according to our earlier discussion). *FRE1* dependence on Aft1p has been questioned in a study where ferrozine was used as iron chelator because *FRE1* mRNA was still present in the absence of Aft1p (Casas *et al.*, 1997). However, since ferrozine also retains copper (Stookey, 1970; Kundra *et al.*, 1974), this induction was probably due to copper depletion (and Mac1p function); (b) the copper-regulated *FRE7* gene was dependent on Mac1p for its induction (similarly to *FRE1*); (c) The *FRE2*-related genes (*FRE3–6*) were influenced negatively by Mac1p (like *FRE2*), since their expression was induced by copper depletion only when Mac1p was absent.

Promoter elements of the Fe/Cu reductase-related genes

Studies on Aft1p and Mac1p have shown that both proteins are metal-regulated DNA binding transcriptional activators. *In vivo* footprinting assays have demonstrated that Aft1p occupies its binding site when cells are deprived of iron and the consensus binding site on the promoters of its known target genes is the PyPuCACCCPu sequence (Yamagushi-Iwai *et al.*, 1996). On the other hand, Mac1p has been shown to bind on the promoter of *FRE1* gene (Yamagushi-Iwai *et al.*, 1997) at a site which is common in the other known Mac1p-regulated genes (*CTR1, CTR3*), termed CuRE consensus (TTTGCTC; Labbe *et al.*, 1997). *In vivo* footprinting studies have shown that occupation at this element is evident only in the absence of copper (Labbe *et al.*, 1997). This element is present on the promoters of the target genes as either a direct or an inverted repeat. Mac1p transcriptional activation capacity itself is increased in the absence of copper (Georgatsou *et al.*, 1997; Graden and Winge, 1997).

We undertook a thorough examination of the upstream sequences of the nine genes for the

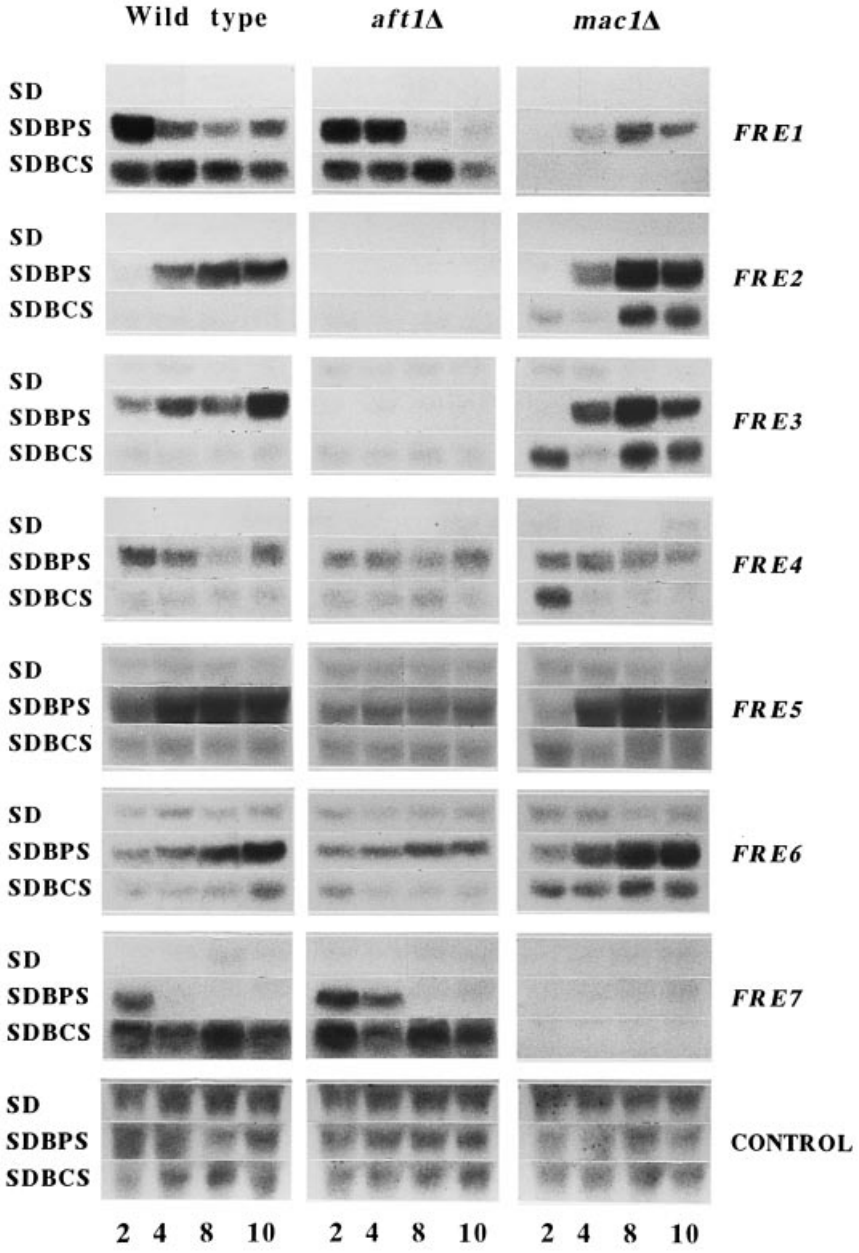

Figure 3. RNA accumulation of the *FRE1–7* genes in *aft1*Δ and *mac1*Δ strains. RNA blot hybridization analysis (as described on the legend to Figure 1) of total RNA samples prepared from wild-type and mutant cells grown exponentially in SD medium and, following dilution, shifted to either SD or SDBPS or SDBCS and further grown for the indicated time points (hours). Note: the decrease of *FRE* RNAs observed in the *mac1*Δ strain at 4 h of growth in SDBCS is a non-reproducible artifact, probably due to slight degradation of larger RNAs in this particular sample.

Table 3. Upstream regions of the Fe/Cu reductase-related genes that match either the Aft1p or the Mac1p (CuRE consensus) binding core element.

Gene	Aft1p binding site	Mac1p binding site
FRE1	− 223 TACACCCA − 216	− 286 TTTGCTC − 280 − 269 TTTGCTC − 263
FRE2	− 265 TGGGTGTA − 258 − 430 TGCACCCA − 423	—
FRE3	− 255 TGGGTGTA − 248 − 398 CGGGTGTA − 391 − 427 TGCACCCA − 420	—
FRE4	− 437 TGCACCCT − 430	—
FRE5	− 331 TGCACCCT − 323	—
FRE6	− 169 TACACCCC − 162	—
FRE7	—	—
YGL160w	—	—
YLR047c	—	—

Nucleotide numbering considers the AUG translation start site as +1.

CACCC Aft1p binding core element, as well as the TTTGCTG CuRE Mac1p binding element, which identified the sequences shown on Table 3. We have examined sequences up to − 600 (+1 being the AUG translation start site), considering that due to the compactness of the yeast genome (as revealed after the completion of the yeast genome sequencing), a typical yeast gene is preceded by an average upstream region of 309 bp (Dujon, 1996).

The Aft1 binding core element was found on the promoter regions of all the iron-responsive genes and is absent from the promoters of the non-responsive genes. Furthermore, the highly iron-inducible genes harbour Aft1 binding elements perfectly matching the consensus sequence, whereas the elements of the moderately induced *FRE4-6* genes do not match at the last Pu residue of the consensus. A Pu–Py mutation at this nucleotide was shown to be competent for DNA binding, albeit not as efficient (Yamagushi-Iwai *et al.*, 1996).

The Mac1p binding consensus was found only on the *FRE1* gene promoter. However, the *FRE7* copper-regulated expression seems to be Mac1p-dependent, similarly to that of *FRE1*. A deletion mutant of the *FRE7* gene containing only 285 nucleotides upstream of the AUG exhibits Mac1p-dependent regulation in the presence of BCS (data not shown). The only element resembling the Mac1p binding site on this promoter region is a TTTGCGCAAA palindromic element (situated at − 175). This sequence does not exactly match the Mac1p binding consensus, since the sixth nucleotide is not a T. However, this nucleotide has not been tested for its importance on Mac1p binding, and since the CuRE element is deduced based only on the elements of three target genes, it might be that the mentioned sequence also constitutes a Mac1p binding site.[1]

On the other hand, the *FRE2*-similar genes (*FRE2–6*), which have been shown to be negatively affected by Mac1p, do not contain any CuRE elements. Moreover, although Mac1p does affect their expression similarly, we were not able to detect any motifs uniquely shared in their promoter regions (within 1 kb upstream of their AUG). The action of Mac1p on the *FRE2* related gene promoters either by direct binding or via mediators requires further investigation.

CONCLUSIONS

Combining the sequence comparison data with those of RNA accumulation and promoter examination (summarized in Table 4), *FRE1*/*FRE2*-related genes could be classified in the following three groups:

- Group I Mainly iron-dependent genes, *FRE2, FRE3, FRE4, FRE5* and *FRE6*. These genes are transcriptionally induced in the absence of iron, Aft1p being necessary for this induction. They can also be weakly induced in the absence of

[1]See Note added in proof.

Table 4. Summarized results of the Fe/Cu reductase-related gene expression.

Gene	Iron-regulated		Copper-regulated	
	+Aft1p	−Aft1p	+Mac1p	−Mac1p
FRE1	+	−	+++	−
FRE2	+++	−	−	+
FRE3	+++	−	−	+
FRE4	+	−(±)	−	+
FRE5	+	−(±)	−	+
FRE6	+	−(±)	−	+
FRE7	−	−	+++	−
YGL160w	−	n.d.	−	n.d.
YLR047c	−	n.d.	−	n.d.

−, No induction; +, low induction; +++, high induction; n.d., not determined.

copper, Mac1p being a repressor of this induction. (*FRE4* gene expression may also be affected by Aft1p since, although it did not appear significantly affected in its absence, it contains a potential Aft1p binding site.) This group can be subdivided into the *FRE2* and *FRE3* gene class, which is highly inducible by iron depletion, and the *FRE4, FRE5* and *FRE6* gene class, which is only moderately induced.

- Group II mainly copper-dependent genes, *FRE1* and *FRE7*. These genes are induced in the absence of copper, Mac1p being necessary for this induction. This group can be further divided into two subclasses, since *FRE1* is also induced by iron depletion, although at lower levels, Aft1p being necessary for this induction, whereas *FRE7* is not.
- Group III genes that are not inducible by either iron or copper availability, *YGL160w* and *YLR047c*.

The presented functional analysis results provide a clear example of how peptide sequence similarities may be informative for similarity of function. The two most distantly related ORFs, *YGL160w* and *YLR047c*, although they possibly encode electron transfer proteins, may not be related at all with iron or copper metabolism. The necessity for such functional tests is obvious, especially when dealing with redundant genes whose deletion from the genome does not result in easily resolved phenotypes (Georgatsou and Alexandraki, unpublished results).

Our study has confirmed that five out of the seven *FRE1*/*FRE2*-related genes are regulated by iron and/or copper, therefore it is almost certain that they encode for enzymes functioning in the homeostasis of these two metals. However, since we have examined only the mRNA accumulation of these genes, the question is raised as to whether their RNAs produce functional proteins. We know that at least *FRE6* and *FRE7* do, since their overexpression in yeast produces distinguishable phenotypes (data not shown). Assuming that most other genes of this family would not be pseudogenes, the immediate question is that of the cellular localization of their products. Examination of their peptide sequences indicates that they are all membrane proteins but does not reveal any clear-cut motif indicative of a specific cellular compartment. Except for Fre1p and Fre2p, no other ferric reductase activity can be detected on the plasma membrane of *S. cerevisiae* (Georgatsou and Alexandraki, 1994). A third, copper-specific, reductase activity that has been detected does not seem to be regulated by either iron or copper availability (Hassett and Kosman, 1995; Georgatsou *et al.*, 1997). One of the two non-Fe/Cu-regulated ORFs might account for this activity. Therefore, the five metal-regulated ORFs, if functional, most probably produce enzymes that are not found on the plasma membrane. Alternatively, some of these enzymes could use as substrates metals other than the ferric and cupric ions or even other compounds (as shown for Fre1p by Hassett and Kosman, 1995). On the other hand, ferric reductase activities have been shown to exist in several subcellular fractions of *S. cerevisiae* (Lesuisse *et al.*, 1990). In fact, cellular compartments like the vacuole (Amillet *et al.*, 1996; Szczypka *et al.*, 1997) or mitochondria (Li and

Kaplan, 1997) are involved in Fe and/or Cu intracellular trafficking and they probably harbour Fe/Cu reductases at their external membranes. Therefore, some of the *FRE1*/*FRE2*-related ORFs should account for these activities.

It is worth noticing that four out of the five metal-regulated new genes fall into the same group as Fre2p, both in sequence similarity and expression pattern in respect to metal regulation (*FRE3–6*). Previously identified differences between *FRE1* and *FRE2* gene expression were unable to clearly point towards different functions of the two proteins. However, in view of the classification in the present study, it emerges that distinct specializations are attributed to *FRE1* and *FRE2*, since *FRE1* belongs to the mainly copper-related gene group (II) and *FRE2* to the mainly iron-related gene group (I).

Concerning the valence state of the two metals in the cell, a difference between the two exists. It is known that iron needs to be reoxidized by the Fet3p protein (Askwith *et al*., 1994) in order to be transported by Ftr1p permease (Stearman *et al*., 1996; Kaplan and O'Halloran, 1996). It thus enters the cell in its trivalent form and is reduced in order to be used in its divalent form. No such mechanism has been described for copper, which is transported in its reduced state (Eide, 1998). Moreover iron is stored in ferritin in its trivalent form and needs to be reduced in order to be mobilized (Crichton and Charloteaux-Wauters, 1987). Although *S. cerevisiae* does not seem to use this storage mechanism, a ferritin-like molecule nevertheless exists in its cytosol, complexed with iron (Raguzzi *et al*., 1988). Considering that the gene family dedicated to iron response contains as many as five members, their common ancestor could encode for an intracellular iron reductase which, following subsequent gene duplications, provided the cell with new molecules located at different compartments, in that way increasing the efficiency of iron utilization. Fre2p, for example, has been localized on the plasma membrane, where it adds its ferric/cupric reductase activity to that of Fre1p. On the other hand, most copper sensing/binding proteins contain copper in its monovalent form (metalothioneins, transcription factors, blue copper proteins, Mac1p, Atx1p) (Pufahl *et al*., 1997; Winge, 1998). Therefore, *FRE7*, for example, would be capable of covering all the intracellular copper reductase requirements.

Compartmental localization of the protein products of the *FRE1*/*FRE2* related genes, as well as study of strains harbouring combinations of their disrupted alleles, will help us understand their specific roles in metal metabolism in yeast. Unravelling the discrete functions of this relatively large gene family in yeast may provide new insights in the important biological role of metals in multicellular eukaryotic systems such as plants and humans (Eide and Guerinot, 1997; Askwith and Kaplan, 1998).

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NOTE ADDED IN PROOF

When this work was submitted for publication, results identical to ours had recently been published (Martins, L. J., Jensen, L. T., Simons, J. R., Keller, G. L. and Winge, D. R. (1998). Metalloregulation of *FRE1* and *FRE2* homologues in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 23 716–23 721. In this publication, a new consensus nucleotide sequence for Mac1p binding, TTTGC(T/G)C(A/G), was also defined, based on the regulation of *FRE7* promoter.

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