A Copper-responsive Transcription Factor, CRF1, Mediates Copper and Cadmium Resistance in *Yarrowia lipolytica* *

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The dimorphic yeast *Yarrowia lipolytica* is more resistant to high copper concentrations than *Saccharomyces cerevisiae*. This differential tolerance to copper ions has been observed in several strains arising from non-related isolates. To investigate the molecular basis of this resistance, we obtained several copper-sensitive mutants. By complementation of one of them, we isolated the *YICRF1* gene encoding for a copper-binding transcription factor of 411 amino acids homologous to *ScAce1p*, *GgAmtlp*, and *ScMac1p*. Naturally occurring copper-sensitive strains lack the *CRF1* allele. The *YICRF1* transcript is not induced by the addition of copper to the medium. Gene disruption demonstrated that *YICRF1* is responsible for a 4- to 5-fold increase in *Y. lipolytica* copper tolerance. We further show that strain Δcrf1 is more sensitive to cadmium but not to other metals. The role of *YICrf1p* as a copper-sensitive transcription factor is supported by the finding that the protein is immunolocalized in the nucleus during growth in copper-supplemented but not in copper-free medium. However, in contrast to the *S. cerevisiae* strain mutated in the metallothionein transcription activator *ACE1*, *Y. lipolytica* strain Δcrf1 is still able to increase metallothionein (MTP) mRNA levels in response to copper addition. *CRF1* deletion does not affect superoxide dismutase (SOD) activity either. Our data suggest the existence of one or more different target genes for *Crf1p*, other than *MTP* or *SOD1*, and support its role as a novel copper-responsive transcription factor involved in metal detoxification.

Copper is an essential element for life due to its ability to catalyze redox reactions, and as such is the ideal cofactor of many enzymes such as cytochrome c oxidase, Cu/Zn-superoxide dismutase (SOD)§, and several ATPases (1, 2). This property, easy electron transport, can, however, be deleterious when copper reacts with oxygen and produces toxic-free radicals (Fenton reaction), which induce severe cellular damage through the oxidation of proteins, the cleavage of DNA and RNA, and lipid peroxidation (3). The importance of copper homeostasis is further underscored by the existence of several human genetic disorders linked to improper copper distribution. Amyotrophic lateral sclerosis is caused by a mutation in human *SOD1*, and copper-binding proteins are known to be involved in both Menkes and Wilson’s syndromes (4–6). Study of the genetics of copper homeostasis in a relatively simple model, i.e. yeast, has led to significant advances in our understanding of human disease, because some of the human genes involved were first identified in a transformed yeast host (7).

To maintain the delicate balance between essential and toxic levels of copper, organisms must ensure copper uptake through the plasma membrane as well as its distribution to the appropriate sub-cellular compartments, but must also have the appropriate mechanisms to detoxify copper ions when they are present in excess. Copper uptake in *Saccharomyces cerevisiae* is mediated by two high affinity transporters: Ctr1p and Ctr3p. Copper detoxification involves the synthesis of small cysteine-rich proteins, the metallothioneins (MT), which are able to chelate several metal ions (Cu(I), Zn(II), and Cd(II)), sequestering them in a non-toxic form. In addition, increased SOD1 activity neutralizes the free radicals produced by copper oxidation. In *S. cerevisiae*, metallothioneins are encoded by two small genes, *CUP1* and *CRS5*, whose expression increases rapidly after exposure to elevated copper concentrations (8, 9). Both nutritional and detoxification requirements involve a system able to “sense” the copper status within the yeast cells and activate or repress, via different transcription factors, the expression of the corresponding genes (10). The nutritional copper-responsive transcription factor Mac1p binds to the *CTR1* and *CTR3* promoters in the absence of copper and activates their transcription (11–13). Mac1p, therefore, ensures expression of the two copper transporters when the metal is scarce. If copper concentration within the cell increases, Mac1p binds to copper, resulting in a conformational change that releases the protein from the *CTR1* promoter and prevents further copper uptake (14). The toxic copper-responsive transcription factor Ace1p (15, 16) functions in the opposite way: in the presence of copper it is activated and binds to the *CUP1* and *CRS5* promoters, increasing their rate of transcription by as much as 50-fold. Ace1p is also the transcriptional activator of *SOD1* (17, 18). Deletion of *ACE1* results in a decrease of both basal and copper-induced MT mRNAs to undetectable levels, and cells lose their ability to resist high copper concentrations (16).

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- **The abbreviations used are: SOD, superoxide dismutase; CRF1, Copper Responsive Factor 1; MTP, Y. lipolytica metallothionein; MT, metallothioneins; NBT, nitro blue tetrazolium; ACE1, activator of CUP metallothioneins expression; CRS5, copper-resistant suppressor; Crtr1 and Ctr3, copper transport proteins; AMT1, activator of metallothioneins; MAC1, metal binding activator; FITC, fluorescein isothiocyanate; MIC, minimum inhibitory concentration; Cup, copper-sensitive; ORF, open reading frame; TEMED, N,N,N’,N’-tetramethylethylenediamine; DAPI, 4’,6-diamidino-2-phenylindole; SC, medium supplemented with the required amino acids.**

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Mac1p and Ace1p transcription factors exhibit a number of common features (10). Both share the same N-terminal domain, which is responsible for DNA binding. It has been shown that Ace1p N-terminal domain binds Cu(I) cooperatively to form a poly-Cu(I) triolate cluster through specific cysteine residues (19, 20). Copper binding leads to a conformational change in this domain, which protects the protein from proteolytic degradation and results in specific binding to the metal response elements in CUP1 and CRS5 promoters (9, 21–23). However, whereas the copper modulation of Ace1p activity depends on the formation of a copper cluster in the DNA-binding domain, copper metalloregulation in Mac1p occurs through copper-specific repression of the C-terminal activation domain (14).

Yarrowia lipolytica is a dimorphic heterothallic fungus located phylogenetically in an isolated branch that is clearly separated from the bulk of most of the ascomycetous yeasts. Y. lipolytica secretes large amounts of various metabolites and enzymes, is amenable to genetic analysis, DNA-mediated integration, and autonomous replication systems have been developed, its genomic organization is reasonably well known (24). Our group has developed systems for inducing the yeast-hypha transition in more than 90% of the cells of a culture in a reproducible and easy way, and thus it can be used as an alternative model to Candida albicans to analyze the yeast-hypha transition (25). Here we introduced Y. lipolytica as an alternative model for the study of copper homeostasis. The copper sensitivity of different strains of this yeast was characterized, and most were found to be highly resistant. To characterize the molecular basis of this unusual resistance to copper salts, we identified the CRFI gene, coding for a transcription factor that confers resistance to copper and cadmium. Our study of copper-regulated targets in a Acr1 mutant suggests a novel metal detoxification circuit in this yeast.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Media, and Growth Conditions—**Y. lipolytica strains used in this study are listed in Table I. The S. cerevisiae strain Xc270, a S. cerevisiae melA2::mel2UTA CUP1 was obtained from the Yeast Genetic Stock Centre. Strains were grown either on YPD medium (1% yeast extract, 2% peptone, 2% glucose) or minimal YNB medium (0.67% yeast nitrogen base, 2%, glucose) supplemented with the required amino acids (SC), as described previously. Metal salts were added at the required concentrations from a 0.2 M stock.

Induction with copper was carried out as follows. Yeast strains were inoculated into the corresponding media at 28 °C for 5 days. The log phase (A600 = 0.8–1.2). The exponential-phase culture was divided into two aliquots, and CuSO4 was added to one of them at the required concentration.

Copper and cadmium resistance was defined as the minimum concentration of metal (MIC) that inhibits colony growth after 4 days of incubation at 28 °C on YNB plates. Other compounds were added to the plates at different concentrations: NaCl (0.5, 1, and 1.5 M), SDS (0.005, 0.01, and 0.05%), calcofluor white (0.5, 3, and 5 μg/ml), and caffeine (5, 10, and 15 mM).

**Yeast Cell Mutagenesis—**Copper-sensitive mutants were screened after exposure to a suspension of cells (1.4 × 109 cells/ml in 0.5% NaCl) in a Petri dish to 2.82 J/m2 of UV light for 70 s. Different dilutions of this suspension were plated onto YPD medium. After incubation for 3 days at 28 °C, the colonies arising from the YPD plates were subcultured in copper-supplemented YPD.

**Yeast Cell Random Spore Analysis—**The sporulation of MATA/MATB crosses and diploid strains was carried out as described elsewhere (24). To obtain spore-rich samples, two sporulations of the sporulated cultures were resuspended in 4 ml of 50 mM sodium citrate buffer, sonicated for 5–4 h in 2 ml of buffer containing 5% glycerol, 180 m units of β-glucuronidase (12 mg), and 12 mg of β-glucuronidase (12 mg) at 37 °C. This suspension was washed three times in 0.85% NaCl, resuspended in 20% glycerol/0.85% NaCl, and sonicated 4 × 30 s to kill vegetative cells. Dilutions were plated onto YPD and grown at 28 °C for 7 days for further auxotrophy analysis.

**Plasmid Constructions—**Plasmid pMP3 is an isolate from the Y. lipolytica genomic library containing a 16-kb Sau3AII fragment inserted into the BamHI site of the integrative plasmid pIN2A6 (25). pMP7 contains a fragment (including the CRFI gene) that complements the MP7 strain mutation and was obtained by digesting pMP3 with BamHI and by cloning the 4-kb fragment at the same sites of pIN2A6. The insertion of the Cia-BamHI fragment into the corresponding sites of pBluescript-SK generated the plasmid pMP13. Plasmid pM25, containing the CRFI gene deletion cassette, was originated by inserting a 2.4-kb BglII-SphI fragment with the YLEU2 marker from pMP23, a variant of pIN2A40 (26), into the BciI and SphI sites of pMP13.

**To perform the CRFI-LacZ translational fusion, the LacZ-coding region was excised from pMC187 (27) by digestion with BamHI and was inserted into the pMP13 BciI site (pMP37). The correct reading frame of the CRFI Asp97 codon with respect to the LacZ Pro4′ from pMC187 was maintained. A 7-kb BamHI-CiaI fragment from pMP37 containing the CRFI-LacZ fusion was introduced between the CiaI and BamHI sites of pIN2A6 to generate pMP40.**

**DNA Sequencing—**Sequencing of the pMP7 4-kb insert containing the CRFI gene was accomplished using an ABI PRISM 377 automated DNA sequencer (PerkinElmer Life Sciences).

**Yeast Transformation—**Y. lipolytica transformation was performed using the lithium acetate protocol developed by Xuan et al. (26). Sometimes, a faster low efficiency protocol was employed (28).

**Total yeast DNA—**isolated from 10 ml of stationary-phase cultures grown overnight in YPD medium (29). Restriction endonuclease cleavage, agarose gel electrophoresis, blotting to nylon, and hybridization were performed according to standard protocols Sambrook et al. (30).

**Northern Blot Analysis and Quantification—**Yeast cultures were grown in the presence or absence of copper, as described above, and 50 ml of culture were centrifuged at the indicated times. Total RNAs were prepared from each sample as described previously (31). RNA samples (5 μg) were denatured and separated on 1.2% agarose gels containing 2.2 M formaldehyde (32). The RNA was transferred to Hybond-N (Amersham Biosciences) filters and hybridization with 32P-labeled probes (Rediprime kit, Amersham Biosciences) was accomplished in 50% formamide/5 × SSC, 0.1% SDS. 125 μg/ml denatured salmon sperm DNA at 42 °C overnight. The filters were washed four times in 0.1% SSC, 0.1% SDS at room temperature followed by one wash at 55 °C for 30 min. When a second hybridization was required, filters were striped by immersing them twice in boiling 0.1% SDS for 15 min. For the dot blots, serial dilutions (60, 15, 3.75, and 0.93 ng) of the DNA used as a template for each labeling reaction were spotted onto the filters and hybridized together with the Northern blot.

Radioactive signal was captured in a phosphor screen (Molecular Dynamics, model 445) and quantified as pixel density units by ImageQuant software (version 5.1). Relative contributions of each of two highly homologous metallothionoines (MTP1–MTP11 and MTP14–MTP14) were obtained as follows: cross-hybridization percentage (percent c) of each of two MTPs was quantified on the DNA dot-blot; MTP densities in Fig. 5B were normalized within each hybridization by the average (equals the normalized density, nD), and relative contributions of each MTP gene were calculated from the formulae,

\[ nD_{MTP1–MTP11} \times 100 = c_{MTP1} \times nD_{MTP1} + c_{MTP11} \times nD_{MTP11} \]  
\[ nD_{MTP14–MTP14} \times 100 = c_{MTP14} \times nD_{MTP14} + c_{MTP14} \times nD_{MTP14} \]  

for hybridization with the MTP11 probe and identically for the MTP14–MTP14 couple.

**SOD Activity Gels—**For the analysis of SOD activity by NBT (33), cells were grown in 50 ml of YNB plus Leu medium in the presence or absence of CuSO4 up to an A of 1.0, harvested, and washed once in cold H2O. The resulting cell pellet was resuspended in 0.5 ml of lysis buffer containing 10 mg NaHPO4 (pH 7.8), 1.0 mg EDTA, 0.1% Triton, 20 μg/ml leupeptin, 10 μg/ml pepstatin, and 1.0 mg phenylmethylsulfonl fluoride. Cells were broken with glass beads and homogenized, and glycerol was added at a final concentration of 5%. 50 μg of the resulting cell extract was applied directly without boiling to a non-denaturing 12% polyacrylamide gel. Following electrophoresis, the gel was stained with 0.01% K3Fe(CN)6 (pH 7.8), 0.01% TMB, 150 μg/ml NBT, 65 μg/ml riboflavin, and 3.2 μg/ml TEMED. After 45-min incubation in the dark, the blue NBT stain for O2 was developed by exposure to light. Staining was absent at sites of O2 scavenging.

**β-Galactosidase Activity Assay—**Relative β-galactosidase levels were measured in yeast cell extracts (34).

**Immunolocalization of the CRFI-LacZ Fusion Protein—**To deter-
The most sensitive strains fail to grow in 0.1 mM CuSO₄, whereas resistant strains are able to grow in up to 1.75 mM CuSO₄. The most sensitive strain, B204—supplemented cultures.

Acids were stained by DAPI (Sigma Chemical Co.). Cells were imaged using microscopy (35). Incubation with mouse anti-EScherichia coli LacZp was carried out with a goat anti-mouse-IgG antibody conjugated to FITC (Roche Molecular Biochemicals). Nucleic acid detection were performed following the recommendations of Amersham International. Western blot and fractions were resuspended in equivalent volumes. Western blot and detection were performed following the recommendations of Amersham International.

 mine the sub-cellular location of the CRF1 gene product, a hybrid construction consisting of the CRF1 promoter and coding regions (399 amino acids of 411) fused in-frame with the Escherichia coli LacZ gene was introduced in a Y. lipolytica integrative plasmid (pMP40). Strain MP73 was transformed with pMP40 and two independent transformants (strains MP7340-1 and MP7340-2) were grown in minimal or MP73 was transformed with pMP4 and two independent transformants (strains MP7340-1 and MP7340-2) were grown in minimal or copper-supplemented medium and prepared for immunofluorescence microscopy (35). Incubation with mouse anti-β-galactosidase (Roche Molecular Biochemicals) diluted 1/100 was performed for 16 h, and detection of Crf1-LacZp was carried out with a goat anti-mouse-IgG antibody conjugated to FITC (Roche Molecular Biochemicals). Nucleic acid stains were stained by DAPI (Sigma Chemical Co.). Cells were imaged under a Leica DMXRA microscope, and fluorescence was detected using the recommended filters. The exposure times for FITC fluorescence in copper-free cultures were increased 2- to 10-fold with respect to copper-supplemented cultures.

Cell Fractionation and Western Blot—For obtaining protein extracts, cells were subjected to mechanical breakage (24). After separation of glass beads, the crude extract was centrifuged (5 min at 4 °C and 600 × g) for removing cell walls. Membranes were separated from cytosolic content by ultracentrifugation at 40,000 × g during 30 min. All fractions were resuspended in equivalent volumes. Western blot and detection were performed following the recommendations of Amersham Biosciences (ECL Western blotting Systems).

RESULTS

Characterization of Copper Resistance in Y. lipolytica—S. cerevisiae strains exhibit a differential resistance to copper: the most sensitive strains fail to grow in 0.1 mM CuSO₄, whereas resistant strains are able to grow in up to 1.75 mM CuSO₄ (36). Using the copper-resistant S. cerevisiae strain X2180-1A as reference, we screened several unrelated strains of Y. lipolytica for their ability to grow on YNB plates containing CuSO₄ (Table I). The minimum inhibitory concentration to growth (MIC) was determined for each strain. Most of the strains showed a higher resistance to copper than S. cerevisiae and grew up to 3 mM CuSO₄. The most sensitive strain, B204-12D, had an MIC of 0.80 mM CuSO₄, 8-fold higher than the maximum tolerated by naturally occurring sensitive S. cerevisiae strains. The MIC of the wild-type strain W29 was >4 mM, which represents a copper resistance of at least two times higher than the S. cerevisiae X2180-1A strain. These values are also higher than for Candida glabrata, another yeast in which copper resistance processes have been studied (37, 38).

Isolation and Characterization of Copper-sensitive Mutants—On the assumption that the above differences reflected a peculiar genetic system for mediating metal resistance, Y. lipolytica strain 11606-1B (MIC > 4 mM) was mutagenized by UV irradiation. Of 23,082 colonies screened, 5 copper-sensitive mutants (MIC < 0.3 mM CuSO₄) were isolated.

The Cup⁺ strains were crossed with Y. lipolytica strain 21501-13 (Table I). Only the mutant MPC7V86 was able to produce viable spores. Because tetrad analysis is quite difficult in Y. lipolytica (24), segregation of the Cup⁺ phenotype was analyzed in random spores. Spore MP73 was originated from the cross MPC7V86 × 21501-13 and exhibited the following features: MIC = 0.3 mM, high transformability, and leu2 auxotrophy; accordingly this was selected for further study. MP73 was crossed with the parental strain 11606-1B. The copper sensitivity character segregated 2:2 (by random analysis), indicating a recessive mutation designated as crf1-1, which segregates during meiosis as a single nuclear genetic locus.

Complementation Analysis—Strain MP73 was transformed with a Y. lipolytica gene bank constructed in plasmid pDNA22 (26, 31). The library was linearized with NotI for integration, and leucine prototrophs were selected on SC agar lacking uracil. Copper-resistant transformants were identified by replica plating of colonies onto SC agar containing 2.0 mM CuSO₄. Of the 10⁴ transformants analyzed, three copper-resistant isolates were recovered. Plasmid DNAs from these isolates were rescued by transforming E. coli with NotI-digested and religated DNA from all three yeast transformants. Each of them contained the same plasmid, pMP3, including a 16-kb insert. pMP3 was able to confer copper resistance to strain MP73 when reintroduced by transformation. A shorter 4-kb fragment able to complement crf1-1 mutation was sub-cloned and sequenced (GenBank™/EMBL accession number Z23265). Sequence analysis revealed an open reading frame of 1233 bp coding for a putative copper-sensitive transcription factor, which was designated YICRF1.
The YlCRF1 Gene Codes for a Transcription Factor Homologous to Ace1p and Mac1p——The YlCRF1 ORF yielded a 411-amino acid polypeptide, rich in serine and proline residues, with an asymmetric charge distribution. The N-terminal domain (residues 1–114) is basic, and the C-terminal region is acidic (residues 220–411). On comparison with the protein sequences available in the databases (Fig. 1), the N-terminal domain of the YlCrf1p shows a significant degree of identity with the copper-dependent transcription factors ScAce1p, CgAmt1p, and ScMac1p (39.5%, 35.9% and 34.2% identity, respectively). Another S. cerevisiae protein of unknown function (SPTREMBL: Q12753) was found to be 35.1% identical. The Crf1p glutamine-rich region is underlined.

The ClustalW method. Asterisks represent identical residues; double and single dots, conservative and semi-conservative substitutions. Aligned cysteines are shown in boldface. The conserved structure of the Zn(II) module is boxed. The Crf1p glutamine-rich region is underlined.

**Fig. 1.** Comparison of the YlCRF1, Ace1, AMT1, and MAC1 gene products. The sequences were aligned using the ClustalW method. Asterisks represent identical residues; double and single dots, conservative and semi-conservative substitutions. Aligned cysteines are shown in boldface. The conserved structure of the Zn(II) module is boxed. The Crf1p glutamine-rich region is underlined.

[2] Y. Wang, A. Ahmed, H. Bussey, N. Fortin, J. D. Friesen, J. Hall, R. A. Storms, D. H. Vo, and E. Winnett, submitted (August 1995) to the EMBL/GenBank/DDBJ databases.

[3] S. García, M. Prado, and A. Domínguez, manuscript in preparation.
YICRF1 Transcription Is Not Affected by the Addition of Copper—To test whether copper can modulate CRF1 transcription, we performed a Northern blot analysis of the CRF1 transcript. Total RNA from strain JM12 growing in liquid medium with or without CuSO₄ was isolated at different times after copper addition and hybridized with a CRF1 probe (Fig. 3A). Methylene blue staining was performed on the membrane as a control immunofluorescence using anti-Crf1p-associated fluorescence observed in YNB medium in the absence of copper could be due to an instability and rapid degradation of the protein in the cytoplasm. To evaluate this hypothesis, a Western blot of protein extracts fractionated by centrifugation of the supernatant, which contains mostly cytosolic proteins, and of the pellet, enriched in cell membranes, from both copper-treated and copper-free cultures was carried out. Anti-β-galactosidase detection reveals a band of 165 kDa, corresponding to the expected size of Crf1-LacZ fusion (Fig. 5). This band is present in both copper-free and copper-supplemented cultures, disproving the hypothesis of degradation of Crf1 protein in the absence of copper.

Deletion of YICRF1 Results in Copper and Cadmium Sensitivity and in Decreased Efficiency to Survive UV Radiation—To directly address the role of YICRF1 in metal tolerance, we deleted this gene from the Y. lipolytica genome by homologous recombination. The deletion cassette replaced 1.6 kb from the CRF1 ORF by the LEU2 marker and retained 1.5 and 1.4 kb from the CRF1 flanking regions (Fig. 6A). Competent JM12 cells were transformed with the linearized ClaI-BamHI cassette and leucine prototrophs were selected on SC-Leu plates and analyzed by Southern blotting. Of the 10 Leu⁺ colonies analyzed, one of them, transformant SGSDJ3, had integrated the LEU2 gene into the CRF1 locus (Fig. 6B). Deletion of most of the CRF1 ORF was confirmed in a second Southern blot using a CRF1 probe (Fig. 6C). Expression of CRF1 mRNA was totally abolished in this strain (Fig. 3A, lane 2).

The phenotypes of SGSDJ3 and pINA240-transformed JM12 (empty plasmid control) strains were compared in copper-supplemented media. The disruptant showed a growth curve similar to that of the wild-type in YPD or YNB media, but the
addition of CuSO₄ resulted in a striking decrease of the growth rate in both solid (Fig. 7A) and liquid media (not shown). The MIC of SGSDJ3 was determined as 0.8 mM CuSO₄, 4–5 times lower than that of the parental strain. Similarly, the effect of other metals such as cadmium and zinc was tested. Cadmium, but not zinc, was found to decrease the growth of the mutant severalfold when added as CdSO₄ to YNB plates (Fig. 7B). To rule out the possibility of a general defect in stress response or some weakness in the cell wall, the growth of both strains was compared in the presence of compounds that increase osmotic stress or that compromise cell wall stability. No differences were found NaCl-, SDS-, Calcofluor White-, or caffeine-supplemented SC plates. Therefore, YiCrf1p appeared to be responsible for the observed 4-fold increase in tolerance to copper and cadmium intoxication through a metal-specific mechanism.

Most of the damaging effects of irradiation are due to the production of free radicals in the cell, and such effects are increased in the presence of heavy metals. It has also been described that after exposure to UV light certain cells produce several metallothionein-inducing factors (42, 43). Accordingly, we compared the ability of Δcrf1 and wild-type strains to survive UV irradiation. Cells were plated onto YEPD or YNB and exposed to two different doses of radiation (4.5 and 9.5 mJ), and the percentages of survival were calculated against unexposed cells. In all cases, the survival of the strain Δcrf1 was at least 50% lower than that of the wild-type (Fig. 7C).

Yicrf1-deleted Strain Is Able to Increase Metallothionein MTPI–II Expression in Response to the Addition of Copper—In view of the homology of YiCrf1 with ScACE1, we decided to analyze the expression of the main targets of this transcriptional activator: the metallothionein-encoding genes. We anticipated that the copper-inducible expression of these genes would be abolished in the crf1 mutant, because to date metallothioneins are the only proteins described in S. cerevisiae that have a direct role in copper detoxification at high metal concentrations (44).

There are four different highly homologous metallothionein genes in Y. lipolytica: MTPI–II and MTPIII–IV. MTPI and MTPII are located contiguously in the chromosome, separated by 0.9 kb of a bidirectional promoter. Both proteins present an identity of 42%. Two nearly identical MTP genes are found on a different chromosome, following a similar arrangement; homologies between MTPI–MTPII and MTIII–MTPIV are, respectively, 96 and 90%, differing only in three nucleotides length stretches. For this reason specific probes for each MTP gene cross-hybridize, making difficult the analysis of single gene expression by Northern blot.

RNA from JM12 and SGSDJ3 strains was purified after the
addition of 0.4 mM CuSO₄ to exponentially growing cultures. This concentration was chosen to minimize the toxic effect of copper on the growth of the /H9004 crf1 strain. Following hybridization to each MTP gene probe, the membranes were re-probed with a ACT1-labeled fragment (Fig. 8A). MTP signal was quantified and normalized with respect to the ACT1 signal, corresponding to 5/H9262 g of loaded RNA (Fig. 8B). Contribution of each MTP probe to the cross-hybridization was determined by dot-blot quantification (Fig. 8C). Contributions of the two less homologous MTPs to the cross-hybridization were always 6%, hence for simplifying the analysis they were not considered. On the basis of the calculated cross-hybridization percentages, signal represented in Fig. 8B was split in the single signals corresponding to the two most homologous MTP genes (Fig. 8D).

Surprising, the ACT1-relative normalization showed that the increase of MTP transcription in response to copper occurs in both wild-type and the crf1-deleted strain (Fig. 8E). Even more overall MTP expression in the first hour after copper addition is higher in the crf1 mutant than in the wild-type, and this is true also for each single MTP gene (Fig. 8D). This result indicates that Crf1p is not essential for metal-induced activation of MTP, moreover deletion of CRF1 can affect positively this activation in the early times after copper addition. However, crf1 mutants are more sensitive, not more resistant, to copper. If crf1 mutation is affecting copper resistance by a different process to metallothionein activation, the increase of MTP expression in the first hour could be the consequence of the higher copper sensibility in the crf1 mutant, inducing an MTP response by a different mechanism.

Individual contributions of each MTP gene can also be evaluated (Fig. 8D). In the wild-type MTPI and MTPIII copper-induced expression oscillate alternatively: two peaks of MTP expression can be observed at 2 and 6 h, whereas the MTPI peak appears at 4 h. However, expression of MTPII and MTPIV seems to follow an increasing kinetics, with maximal expression between 4 and 6 h. In the crf1 mutant kinetics of the MTP genes expression are more similar. An increase of MTP induction occurs in the first hour followed by a progressive decrease at longer incubation times. If Crf1p is affecting directly copper-mediated induction of MTPs, its effect occurs clearly at longer times, because the points of maximal induction, 4 h for MTPI, 6 h for MTPIII, and 4–6 h for MTPII, do not occur in the copper-treated crf1 mutant. The overall effect along the time of crf1 mutation on MTP expression would be indicated by the total area between the copper-free and the copper-added curves. Quantification of this area (not shown) indicates that

**Fig. 7. Phenotypic analysis of SGSDJ3 (Δcrf1) and empty plasmid-transformed JM12 strains.** A, growth on solid YNB medium plus increasing copper (A) and cadmium (B) concentrations, indicated on the left. C, survival percentages to UV irradiation of both strains. Exponential growth cultures were diluted and plated on YPD or YNB medium. Plates were opened to the air and exposed to different doses of UV light (45 and 90 µJ ×100) in a Stratalinker. After 3–4 days, colonies emerging form each plate were counted (about 2 × 10⁸ colonies/sample) and averaged. Survival was expressed as a percentage relative to the colonies counted in unexposed plates (0 µJ ×100). Black columns and white columns represent the survival of SGDJ3 and wild-type strains, respectively. D, assay for SOD activity present in fresh cell extracts. JM12 and of SGSDJ3 strains were grown for 4 h in liquid medium plus 0.4 or 8.0 mM CuSO₄, after which cell extracts were prepared and loaded in a non-denaturing acrylamide gel. Gels were stained with NBT for 45 min, and antioxidant activities were developed by exposure to light. SOD activity corresponds to the lower 30-kDa unstained band, which is the estimated size for several Cu,Zn-SOD proteins.
only MTPI and MTPII are significantly more expressed in the wild-type than in the mutant; MTPIII expression does not differ between both strains, and MTPIV expression is higher in the mutant than the wild-type.

The existence of a copper-induced activation of MTP in strain \(/H9004 crf1\) was confirmed by the use of a reporter gene fused to the MTPII promoter. An autonomous plasmid, pSGS017, carrying an in-frame fusion of the promoter region and the first codon of MTPII with the \(E. coli\) LacZ ORF, was introduced into the wild-type and \(/H9004 crf1\) strains, and \(/H9252\)-galactosidase activity was measured under the same growth conditions as in the previous experiment (Fig. 9). Two hours after copper addition, the \(/H9252\)-galactosidase activity of strain \(/H9004 crf1\) was 9-fold higher in the copper-supplemented than in the copper-free cultures. Because the drastic reduction in copper tolerance of the \(/H9004 crf1\) strain was not accompanied by the total abolishment in MTP expression, our data pointed to the existence of one or more other target genes for \(YlCRF1\) different from the \(MTP\) genes.

The \(Ylcrf1\)-deleted Strain Is Not Affected in SOD Activity—

The second target gene for Ace1p described in \(S. cerevisiae\) is the SOD1 gene. Copper induces an increase in both mRNA

**Fig. 8.** Transcription of \(Y. lipolytica\) MTP genes in the \(\Delta crf1\) strain. A, exponential cultures were collected, washed, and resuspended in YNB (\(- CuSO4\) lanes) or in YNB plus 0.4 mM CuSO4 (\(+ CuSO4\) lanes). Total RNA from wild-type (CRF1) and SGSDJ3 (\(\Delta crf1\)) strains was isolated from non-induced and copper-induced cultures at 0, 1, 2, 4, and 6 h. 5 \(\mu\)g of RNA samples was loaded in four agarose gels, transferred to nylon filters, and analyzed by Northern blot using probes specific for MTPI, MTPII, MTPIII, and MTPIV. A second hybridization was performed with a control ACT1 probe. Ethidium bromide stain of the RNA is also shown (EtBr). B, quantification of MTP radioactive signals with respect to the loading control ACT1 for each hybridized membrane. Both MTP- and ACT1-specific signals were captured in a phosphor screen and quantified as pixel density units (ImageQuant software). Amount of loaded RNA in each line was estimated from the ACT1 quantification. On the y-axis MTP expression is represented as density units/5 \(\mu\)g of total RNA. On the x-axis time in hours is represented. C, dot blot showing the cross-hybridization between the four MTP probes. Serial dilutions of each gene template were spotted onto the filters. Dot blots were hybridized and exposed in the same conditions as their corresponding Northern blots. I-IV indicates the MTP gene spotted in each case. Quantification was performed by ImageQuant and expressed as a percentage ("Experimental Procedures"). D, relative contribution of each single MTP gene to the radioactive signal represented in B after considering the cross-hybridization percentage. Values represented in B were normalized within each hybridization, single contributions were calculated as described under "Experimental Procedures," and results are expressed as normalized units (1 unit = average MTP density corresponding to 5 \(\mu\)g of total RNA). Black squares, CRF1 with copper; open squares, CRF1 without copper; black circles, \(\Delta crf1\) with copper; open circles, \(\Delta crf1\) without copper.

**Fig. 9.** \(\beta\)-Galactosidase reporter expression promoted by the MTPII promoter in the absence of CRF1. Plasmid pSGS017 containing a translational MTPII promoter-LacZ fusion was introduced into CRF1 and \(\Delta crf1\) strains, and \(\beta\)-galactosidase activity was determined at the indicated times after the addition of 0.4 mM CuSO4. Activity is given as units/\(\mu\)g/min. Lanes A and B, \(\Delta crf1\) and CRF1 strains in copper-free medium; lanes C and D, transformed strains in copper-supplemented medium.
transcription and activity of Cu,Zn-SOD in a parallel fashion (45). Deletion of certain genes required for copper metabolism results in the “insensitivity” of SOD to copper activation that disappears in the presence of extremely high copper concentrations (46). We therefore decided to analyze the superoxide dismutase activity of the ∆crf1 strain under very high copper concentrations. Cells were grown for 4 h in liquid SC ± 0.4 or 8.0 mM CuSO4 and crude protein extracts were analyzed by gel assays for SOD activity. No significant differences in SOD activity were observed between strains, indicating that the role of YICrf1p is not related to the activation of SOD expression (Fig. 7D).

**DISCUSSION**

The essential yet toxic nature of copper represents a paradox for the living organism. Cells must have a number of homeostatic mechanisms that sense copper levels and “make the decision” to activate the assimilation or detoxification of the metal. One of the best-understood mechanisms of copper detoxification is metallothionein activation in *S. cerevisiae* (8, 9, 47, 48). Here we provide strong evidence that despite certain similarities observed between the known components of the copper detoxification machinery in fungi, the molecular mechanisms involved in this process differ from one to another species.

*Y. lipolytica* is a remarkably copper-resistant yeast that tolerates metal concentrations several times higher than *S. cerevisiae* does. Here we demonstrate that the YICRF1 gene plays an essential role in this resistant phenotype and that variations in the copper resistance of different *Y. lipolytica* strains are correlated with naturally occurring modifications of the CRF1 allele. Based on sequence homology, we propose that YICrf1p would act as a copper-responsive transcription factor. The N-terminal domain of YICrf1p exhibits all the features of the DNA activation domain in copper-sensitive transcription activators: i.e. a conserved DNA-binding Zn-module and abundant cysteine residues typically arranged in Cys-X1,2-Cys patterns. This arrangement is also the signature pattern of metallothioneins and allows covalent binding of the proteins to copper. Immunofluorescence microscopy revealed that the location of YICrf1p is nuclear in the presence of copper but not in its absence, supporting the idea that the function of YICrf1p is mediated by a copper-responsive domain in the protein. This result leads to two alternative hypotheses: first, protein is cytosolic and metal binding contributes to its import to the nucleus. In this instance a diffuse cytosolic contribution may be hard to detect by microscopy. Second, the protein is cytosolic but the copper-unbound form is unstable and degraded. Western analysis reveals that Crf1p is present in the cells even in the absence of copper, supporting the first hypothesis.

The specific role of CRF1 in metal detoxification was demonstrated by the fact that gene disruption led to a remarkable increase in sensitivity to copper and cadmium. This phenotype is not the result of a defect in the general response to stress or toxic conditions, because strain ∆crf1 exhibits the same resistance as the wild-type to other toxic treatments. Moreover, the metal specificity of Crf1p is restricted to copper and cadmium but not other heavy metals.

In an effort to further elucidate the mechanism of action of Crf1p, the expression of metal detoxification-related target genes was analyzed in a ∆crf1 strain. *MTPI* genes were able to increase their transcription in response to copper treatment. Moreover, transcription is higher during the first hour after copper addition; after this time, copper-induced expression of the reporter protein increases both in the deleted and full-length promoter.3 It is therefore possible that after this time a secondary activator protein could induce *MTPI* expression. One interesting hypothesis is that Crf1p acts both like an early repressor on *MTPI* expression (in agreement with observed early over-induction of *MTPI* expression in the mutant) and like a late activator (in agreement with observed late under-induction in the mutant). However, evaluation of this hypothesis in terms of copper-resistance phenotype is difficult. Considering the overall effect along the time of crf1 mutation on *MTPI* expression, only *MTPI* and *MTPII* are significantly more expressed in the wild-type than in the mutant; *MTPIII* expression does not differ between both strains; however, *MTPIV* expression is higher in the mutant than the wild-type. Relative contributions to each of these four metallothioneins to copper resistance are not known. 2) Crf1p could be the transcriptional activator of a target gene other than *MTPI*, with an important role in copper detoxification. Although this gene has not been described in *S. cerevisiae*, the existence of a novel ATP-dependent copper extrusion pump has recently been reported in another dimorphic yeast, the pathogen *Candida albicans*. This gene is responsible for the usual resistance of this yeast to copper (39). Expression of this extrusion pump would be regulated by some kind of copper-sensing factor, because active extrusion of copper is an expensive process in terms of metabolic waste. To date no such regulatory factor has been reported.

Both mechanisms could coexist in *Y. lipolytica*, ensuring a more efficient defense against the copper poisoning. During the first moments after copper intoxication, a mechanism that rapidly increases the production of small, relatively simple copper-chelating proteins by de-repression of their promoters could represent an advantage for the cell. The persistence of toxic metal concentrations would require later expression of these or more complex proteins for the active extrusion of copper. Further study of these mechanisms in *Y. lipolytica* is currently an ongoing project in our laboratory.

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