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MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast

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The related transcription factors ACE1 of *Saccharomyces cerevisiae* and AMT1 of *Candida glabrata* are involved in copper metabolism by activating the transcription of copper metallothionein genes. ACE1 and AMT1 are 'copper-fist' transcription factors which possess a conserved cysteine-rich copper binding domain required for DNA binding. Here we report the identification of a nuclear protein from *S.cerevisiae*, MAC1, whose N-terminal region is highly similar to the copper and DNA binding domains of ACE1 and AMT1. Loss-of-function mutants of *MAC1* have a defect in the plasma membrane Cu(II) and Fe(III) reductase activity, are slow growing, respiratory deficient, and hypersensitive to heat and exposure to cadmium, zinc, lead and H_2O_2. Conversely, a dominant gain-of-function mutant of *MAC1* shows an elevated reductase activity and is hypersensitive to copper. We have identified two target genes of MAC1 whose altered expression in mutants of *MAC1* can account for some of the observed mutant phenotypes. First, *MAC1* is involved in basal level transcription of *FRE1*, encoding a plasma membrane component associated with both Cu(II) and Fe(III) reduction. Second, *MAC1* is involved in the H_2O_2-induced transcription of *CTT1*, encoding the cytosolic catalase. This suggests that *MAC1* may encode a novel metal-fist transcription factor required for both basal and regulated transcription of genes involved in Cu/Fe utilization and the stress response.

Key words: ACE1/copper/iron/stress response/transcription factor

Introduction

Copper and iron are essential cofactors for a large variety of proteins. In enzymes these cofactors participate in catalytic functions or fulfil structural purposes. Deprivation of these metals can result in discrete deficiency states (for a review see Wilson *et al.*, 1991). Copper deficiency in animals can lead for example to anemia, faulty development of bones and the nervous system and an impaired immune response. Iron deficiency is the most common cause of anemia in man. Conversely, abnormally high levels of iron, and of copper in particular, can be toxic, resulting in a severe damage of tissues and organs. The reason for this toxicity is thought to be due to metal-dependent oxy radical generation and metal ion antagonism, i.e. through competition of different metal ions for the same biochemical sites. The importance of tightly regulated intracellular copper and iron levels is intelligible by the severe symptoms observed in patients with genetic disorders in copper or iron metabolism (Wilson *et al.*, 1991). Hemochromatosis is an iron storage disease in which an increase of intestinal iron absorption results in damage to liver, heart, pancreas and pituitary. Wilson's disease is an inherited abnormality in the hepatic excretion of copper which results in toxic accumulations of the metal in the liver and also in the brain. Whereas the genetic basis for these diseases has remained elusive, the gene mutated in patients with Menkes disease, a copper metabolism disorder, has been recently identified. It encodes a putative copper export protein and its loss of (or reduced) function in Menkes patients leads to a decreased copper efflux from cells causing rapid nervous system and arterial degeneration (Davis, 1993).

In an oxygen-containing environment, iron and copper are predominantly in the form of Fe(III) and Cu(II) and are reduced to Fe(II) and Cu(I) for biological function. In animal cells, ferric iron is taken up as a complex with transferrin, is reduced in the endosomal compartment and finally delivered to the cytoplasm (Klausner *et al.*, 1993). Iron utilization in yeast is mediated by a different path. In this organism, ferric iron reduction is mediated at the cell surface by the *FRE1* gene product and ferrous iron is taken into the cell (Dancis *et al.*, 1990, 1992). Transcription from *FRE1* is repressed by iron, allowing a feedback control of iron utilization (Dancis *et al.*, 1992). Comparatively little is known about the elements required for copper metabolism. Among the few known components of copper metabolism pathways in eukaryotes is the yeast metallothionein-like *CUP1* gene product which is involved in copper sequestration (reviewed by Hamer, 1986) and ACE1, a copper binding transcription factor (also known as CUP2) which regulates *CUP1* gene expression (Thiele, 1988; Huibregtse *et al.*, 1989; Szyczypka and Thiele, 1989; Welch *et al.*, 1989).

Here we describe the cloning and characterization of a yeast gene, *MAC1*, encoding an ACE1-related nuclear regulatory protein involved in copper and iron utilization and resistance to stress, including H_2O_2. We show that MAC1 regulates the transcription of *FRE1*, whose function is linked to copper and iron reduction and uptake, and of *CTT1* (Spevak *et al.*, 1983), encoding the cytosolic catalase (which decomposes H_2O_2 to H_2O and O_2). The relatedness of MAC1 to the copper-binding transcription factor ACE1 indicates that MAC1 may function by an analogous

mechanism. This further suggests that in yeast, two essential functions of copper metabolism, copper utilization and sequestration, may be regulated by two related copper-binding transcription factors which act on distinct target genes.

Results

MAC1 is related to ACE1 and AMT1 transcription factors

In the course of our studies on *S.cerevisiae* ubiquitin-conjugating enzymes, we identified an open reading frame upstream from *UBC7* (Jungmann *et al.*, 1993). This gene, designated *MAC1* (**m**etal binding **ac**tivator), is separated from *UBC7* by 261 bp and is divergently transcribed. Northern analysis showed that *MAC1* expresses a 1.4 kb transcript (not shown). DNA sequence analysis predicts that *MAC1* encodes a protein of 46.5 kDa (417 amino acids) with significant sequence similarity to the transcription factors ACE1 of *S.cerevisiae* (Szyczypka and Thiele, 1989; Welch *et al.*, 1989) and AMT1 of *Candida glabrata* (Zhou and Thiele, 1991) (Figure 1). ACE1 and AMT1 are cysteine-rich, copper-dependent transcription factors (called 'copper-fist' transcription factors; Fürst and Hamer, 1989) which activate the transcription of the metallothionein genes of both yeasts (Thiele, 1988; Welch *et al.*, 1989; Zhou *et al.*, 1992). The region of similarity of MAC1 to ACE1 and AMT1 is confined to the N-terminal ends of the proteins (Figure 1B

A

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-141                                               CTCGTTTTCGGGTAATGGCGA
-120 AAAGGAGTTATAAGAAGGATCTCGAGACAATAAGCTGCTGCATCTTCGTGAGGTAGATGC
 -60 GATGAGGCGCCTTGTTTTAAATCTGAAACAGTCTGGTAAGTTCTTCAAGCTCTATCAGAG

   1 ATGATAATATTTAATGGGAACAAATATGCGTGTGCATCGTGCATCAGAGGGCATCGCTCT
     MetIleIlePheAsnGlyAsnLysTyrAlaCysAlaSerCysIleArgGlyHisArgSer

  61 TCAACATGCAGGCATTCTCACCGAATGCTAATTAAGGTGAGAACTAGAGGAAGACCTTCA
     SerThrCysArgHisSerHisArgMetLeuIleLysValArgThrArgGlyArgProSer

 121 CCCATGGCTATCAGAGACGCTATTTTAGTAGACTCTACATCGCAAAGTACAGAATACGAA
     ProMetAlaIleArgAspAlaIleLeuValAspSerThrSerGlnSerThrGluTyrGlu

 181 AATGGTGCACAAATTGAAGGTGACTGCTGCAGCGCAATGAATCAACAGCCAATACTGTTT
     AsnGlyAlaGlnIleGluGlyAspCysCysSerAlaMetAsnGlnGlnProIleLeuPhe

 241 GTACGTGCCTCTGCTGTTAGAAAGGCAAGAATGATAAACGGAAAATTGCATATATTAATG
     ValArgAlaSerAlaValArgLysAlaArgMetIleAsnGlyLysLeuHisIleLeuMet

 301 GAGGAAGGTTTCACTGCTCATGAGCCTAAAGATATTAGCACATTTACCGATGATGGTAAC
     GluGluGlyPheThrAlaHisGluProLysAspIleSerThrPheThrAspAspGlyAsn

 361 AAATATATCACCGAAACGGAGTTTCTTAGGAAACACTCTCCCAAAGCTCCGGCAACAGGA
     LysTyrIleThrGluThrGluPheLeuArgLysHisSerProLysAlaProAlaThrGly

 421 ACAATATCTCCGGACTCTACCAAGTCATCTTCTTCAAGTGAGAAGAAAGAGCGAAGCCGG
     ThrIleSerProAspSerThrLysSerSerSerSerSerGluLysLysGluArgSerArg

 481 CTTCAACAGGAGCCTATACGACATTTTTCAAACTGCTGTAAGAAAGATAAATCACAGAAC
     LeuGlnGlnGluProIleArgHisPheSerAsnCysCysLysLysAspLysSerGlnAsn

 541 CCAGCTTCTAATGGCAAGACGAACAAGGCACCGTCTGATGACATATTTACCGCATACGGC
     ProAlaSerAsnGlyLysThrAsnLysAlaProSerAspAspIlePheThrAlaTyrGly

 601 TCCTTGGAATCTACGTCCGCTTTTAACGATATTTTACAAGAAAACTACAATAGTTCTGTT
     SerLeuGluSerThrSerAlaPheAsnAspIleLeuGlnGluAsnTyrAsnSerSerVal

 661 CCTGGTGCGCATGACAGTTCAGAAACACTCACCCCACAAAGTACAACAACGATTGCTGCT
     ProGlyAlaHisAspSerSerGluThrLeuThrProGlnSerThrThrThrIleAlaAla

 721 CCTCATTCAAGCGACGTTGCTTCGAAAGTTGAAGTCCTGACTCATAAGGGCATTTTTTTA
     ProHisSerSerAspValAlaSerLysValGluValLeuThrHisLysGlyIlePheLeu

 781 AGCACGCAGTGCTCTTGTGAAGATGAAAGCTGCCCATGTGTTAATTGTCTAATCCATAGA
     SerThrGlnCysSerCysGluAspGluSerCysProCysValAsnCysLeuIleHisArg
                                                              Gln

 841 AGCGAAGAGGAACTGAATTCTTATATTCAACAAAGTGGTGTTCCTTTAACCAATATTGGT
     SerGluGluGluLeuAsnSerTyrIleGlnGlnSerGlyValProLeuThrAsnIleGly

 901 GAAGCTCAAATTACTGATAAGATGATGGATTATTTGGATGATTGTAAATGCACTGACAAG
     GluAlaGlnIleThrAspLysMetMetAspTyrLeuAspAspCysLysCysThrAspLys

 961 GAATGCATATGTCCTCCGGATAACTGCACTTGTGATGGATGTTTTTCTCACTCTACAAAT
     GluCysIleCysProProAspAsnCysThrCysAspGlyCysPheSerHisSerThrAsn

1021 ATAATTCCATTTGAAAAATTCTTTTTCTATGGAATTCTGAATGCAAGATTAACAAGGAAA
     IleIleProPheGluLysPhePhePheTyrGlyIleLeuAsnAlaArgLeuThrArgLys

1081 ACTCAAATAAAATTCAAAGGTAAATTGGTGCCGTCAAAATATTGGTGGGATTTTTTGAAG
     ThrGlnIleLysPheLysGlyLysLeuValProSerLysTyrTrpTrpAspPheLeuLys

1141 TTGCAAGTGCCATTGATGACAGATGCTCAGTTAGAATTACTTGACATTCATGCGTGGTTT
     LeuGlnValProLeuMetThrAspAlaGlnLeuGluLeuLeuAspIleHisAlaTrpPhe

1201 CAAAAATTAGTCTCTAATTATGCACCACATCTAAGCGATGCCACCACTTCATAAAATCAC
     GlnLysLeuValSerAsnTyrAlaProHisLeuSerAspAlaThrThrSer

1261 GTAAATACAGATAGAAAACACATTGAGGCAACAGGAATGAAAATGCACCCTACTACTCTC
1321 TGAACCACCATATGATAATATTCCAATCTATTGTGCTTAACATTAAAATTGTTCAAACTT
1381 GGTATAAGCAACAAGTGGGAGAATTAGGTGTACCTCAAATTTTTGAAAAAGCACAGAGTG
1441 TTACTAAAAATTTTAATATGTATGTATATCCTTTTCTAGCCGTCAAAAGTGTAAGTAATT
1501 TCGAAAAAATAGATTCTATTTCAGTAAGTCAGAGATTCGAGTCGCCTCCCGTCTTCCGCT
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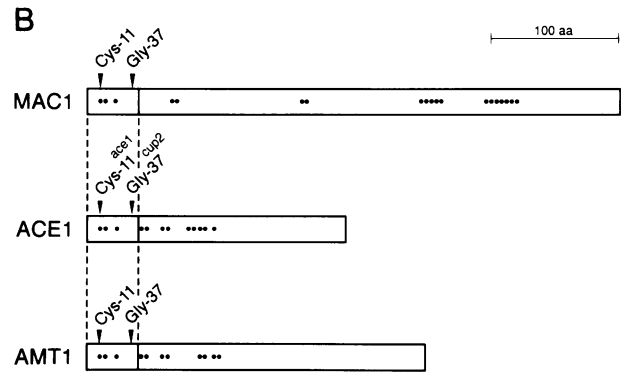

C

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MAC1  M I I F N G N K Y A C A S C I R G H R S S T C R H S H R M L I K V R T R G R P S
ACE1  M V V I N G V K Y A C E T C I R G H R A A Q C T H T D G P L Q M I R R K G R P S
AMT1  M V V I N G V K Y A C D S C I K S H K A A Q C E H N D R P L K I L K P R G R P P
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Fig. 1. MAC1 is related to ACE1 and AMT1. (**A**) Nucleotide sequence of the *MAC1* gene and the predicted amino acid sequence of the encoded protein. Cysteine residues are shown in bold. The duplicated cysteine-rich repeat is underlined. The His at the end of the first repeat is replaced by Gln in the MAC1up1 protein with gain-of-function properties, as indicated. Nucleotide numbers starting at the first nucleotide of the coding region are given on the left. The DNA sequence shown upstream from *MAC1* continues with the reversed strand of the published upstream sequence of *UBC7* (Jungmann *et al.*, 1993). The EMBL data library accession number of the sequence reported is X74551. (**B**) Comparison of the MAC1, ACE1 and AMT1 proteins (boxes). Cysteine residues are symbolized as dots. The conserved region is indicated by the stippled lines. The conserved Cys11 and Gly37 residues that are mutated in the *ace1-1* and *cup2* alleles of the *ACE1* gene are indicated. A bar representing the length of 100 amino acids is given as a reference. (**C**) Enlargement of the conserved region of the three proteins. Amino acids (single letter code) in ACE1 and AMT1 identical to those of MAC1 are boxed.

and C). In this region MAC1 exhibits 53% amino acid identity to ACE1 and 48% identity to AMT1. As this domain of ACE1 is known to be required for both DNA and copper binding (Fürst *et al.*, 1988; Hu *et al.*, 1990), MAC1 probably belongs to the same family of Cu-dependent transcription factors. Although in MAC1 only three of the 11 cysteines required for ACE1 function are conserved, MAC1 has additional 16 cysteine residues which are organized in two pairs of vicinal cysteines and in a cluster with a duplicated $CysXCysX_4CysXCysX_2CysX_2His$ motif (Figure 1A; underlined). These unique features suggest that MAC1 may be more functionally complex than either ACE1 or AMT1.

Support for a nuclear function of MAC1 comes from immunofluorescence studies. We constructed a gene encoding a MAC1−β-galactosidase fusion protein and expressed the gene in yeast (this fusion is functional as it can rescue *mac1* null mutant phenotypes; not shown). As shown in Figure 2, indirect immunofluorescence images obtained with anti-β-galactosidase antibodies demonstrate that the fusion protein localizes to the nucleus.

Loss-of-function mutant

To assess the *in vivo* function of MAC1, we constructed *mac1* null alleles. Gene disruption by inserting the yeast

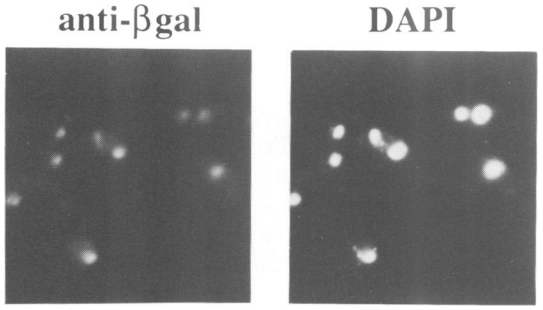

Fig. 2. Nuclear localization of a MAC1−β-galactosidase fusion protein. Wild-type yeast cells expressing the fusion protein were stained either with antibodies against β-galactosidase (indirect immunofluorescence; left) or with a DNA stain (DAPI; right).

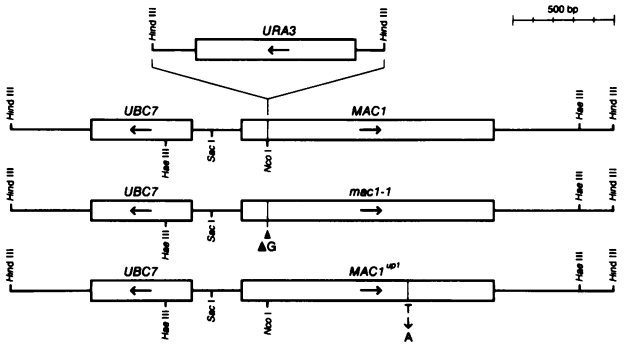

Fig. 3. Construction of mutant *MAC1* alleles. The *MAC1* gene (large box) is closely linked to *UBC7* (small box). The transcription of the genes is indicated by arrows. Gene disruption (top) was carried out by inserting a fragment containing the *URA3* gene. This insertion (and various others; not shown) also affects transcription from *UBC7*, probably by affecting *cis*-acting elements required for *UBC7* transcription (see text). Deletion of a guanine nucleotide (ΔG) at the *Nco*I site of *MAC1* results in the *mac1-1* frameshift mutant (middle). The $MAC1^{up1}$ allele (bottom) has a single T-A transversion. The constructed *mac1-1* and $MAC1^{up1}$ strains are isogenic to the wild-type strain except for the single point mutations in *MAC1*.

URA3 gene into the *MAC1* open reading frame (Figure 3, top) resulted in viable but slowly growing cells. Subsequent studies, however, indicated that insertions (independent of its orientation) also affected the level of the transcript of the closely linked gene *UBC7*. To test whether the reduced transcription of *UBC7* in this strain is directly linked to MAC1 loss of function or, as we were concerned, is the result of alteration of *cis*-acting DNA sequences which affect *UBC7* transcription, we constructed an additional *mac1* null allele. We introduced a frameshift mutation close to the 5′ end of the *MAC1* open reading frame without any further DNA alterations (see Figure 3 centre; Materials and methods). This mutant, designated *mac1-1*, was viable and grew at the same rate as the gene disruption mutant (not shown; *ubc7* deletion mutants grow at wild-type rates). However, in contrast to the *mac1* gene disruption mutant the transcription of *UBC7* was not altered in the *mac1-1* frameshift mutant, indicating that indeed *cis*-acting sequences required for normal *UBC7* transcription may have been altered by the *URA3* insertion. To avoid any interference with *UBC7*-linked functions (Jungmann *et al.*, 1993), we used the *mac1-1* mutant in all further studies.

In addition to slow growth (on YPD plates), we observed that *mac1-1* mutants are respiratory-deficient as indicated by their inability to grow on nonfermentable carbon sources (Figure 4) and the severe reduction of O_2 uptake in mutant cells (Figure 5). Moreover, *mac1-1* mutants are hypersensitive to a surprising variety of stress conditions including heat, H_2O_2, and exposure to cadmium (Figure 4), zinc and lead (not shown). Yet, in contrast to mutants in the related gene *ACE1* (Thiele, 1988; Welch *et al.*, 1989), *mac1-1* mutants are not copper hypersensitive. On the contrary, the slow growth of *mac1-1* mutants can be rescued by the addition of copper (Figure 4). Moreover, we observed that not only the growth defect but also the stress phenotypes of *mac1-1* could be rescued by the addition of copper, and surprisingly, also by iron into the medium. [As Cu(II) or Fe(III) catalyse the decomposition of H_2O_2, similar rescue studies for the peroxide sensitivity of *mac1-1* would be ambiguous.] This suggests that a primary defect of *mac1-1* mutants is a deficiency in a copper and/or iron utilization pathway and that some other pathways can compensate for the deficiency when elevated copper or iron levels are present in the medium. As the respiratory deficiency could only be rescued by added copper but not by iron (Figures 4 and 5), certain processes required for respiration might be particularly sensitive to low copper levels.

Gain-of-function mutant

Genetic linkage studies indicated that *MAC1* is allelic to a previously identified dominant mutation (see Materials and methods) which leads to a copper hypersensitive phenotype (not shown). We cloned this allele, designated $MAC1^{up1}$, and found that it differed from the wild-type *MAC1* coding sequence by a single T-A transversion which results in a histidine-glutamine substitution at residue 279 in the primary sequence (Figures 1A and 3). Interestingly, this histidine is the terminal residue of the first $CysXCysX_4CysXCysX_2$ $CysX_2His$ repeat of the MAC1 protein, suggesting that this motif does have functional importance.

Using the cloned $MAC1^{up1}$ allele we constructed a mutant strain congenic to our wild-type and *mac1-1* mutant strains. Similar to the original isolate this strain is hypersensitive

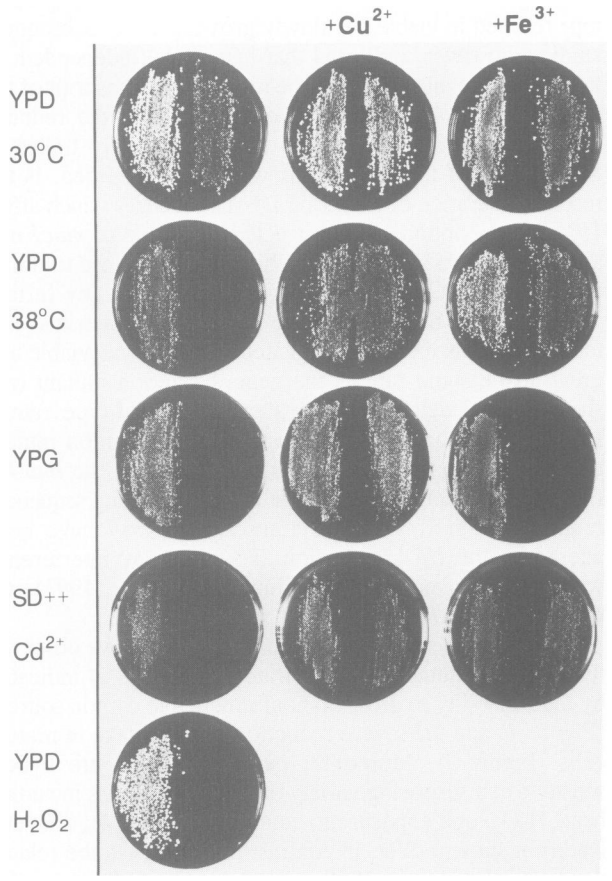

Fig. 4. Growth and viability of *mac1-1* mutants. Compared with wild-type (always on the left side of the plates) *mac1-1* mutants (on the right side of the plates) are slow growing on YPD plates at 30°C and inviable at 38°C (from top to bottom). The *mac1-1* mutant cannot grow on nonfermentable carbon sources (YPG) or on SD^{++} plates containing 25 μM Cd^{2+} or YPD plates supplemented with 0.008% H_2O_2. Addition of either copper (Cu^{2+}, 200 μM; middle row) or iron (Fe^{3+}, 1 mM; right row) can rescue the growth defect, the heat sensitivity and the Cd sensitivity. The defect in respiration is only rescued by the addition of copper. Rescue studies for the peroxide sensitivity of *mac1-1* would be ambiguous (see text).

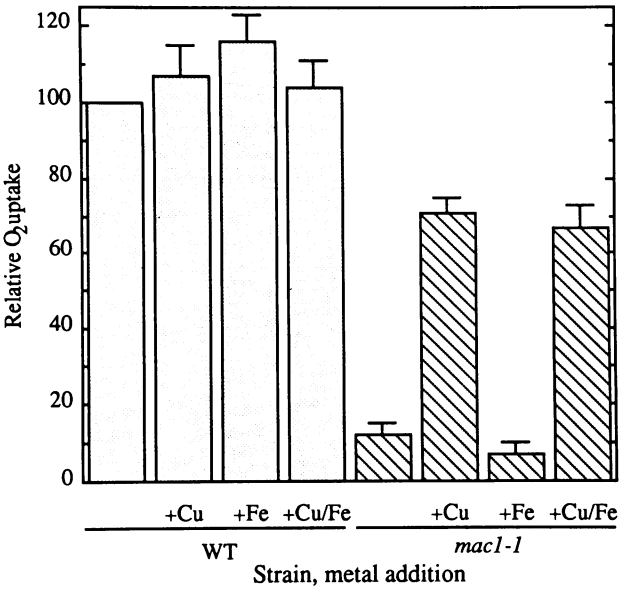

Fig. 5. Respiration deficiency of *mac1-1* and rescue by copper. Relative O_2 uptake of wild-type (WT; shaded bars) and *mac1-1* mutants (striped bars) without or with added copper (Cu^{2+}, 50 μM), iron (Fe^{3+}, 50 μM), or copper and iron (Cu^{2+}/Fe^{3+}, 50 μM each). The data (standard deviation is indicated by error bars, mean $n = 3$) are presented as a percent of wild-type without added copper and iron. The respiration defect of *mac1-1* can be rescued by added copper but not by iron.

to copper. The reason for the difference in the copper phenotypes of $MAC1^{up1}$ and *mac1-1* mutants became clear by the finding that the $MAC1^{up1}$ allele is a gain-of-function mutant of *MAC1* (see below). Although $MAC1^{up1}$ is a dominant allele, overexpression of wild-type *MAC1* can suppress the phenotypes associated with the $MAC1^{up1}$ allele (not shown).

In accordance with the copper sensitive phenotype, $MAC1^{up1}$ mutants exhibit a strongly elevated Cu uptake (R.Hassett and D.Kosman, unpublished). In particular, gain-of-function mutants show a markedly elevated plasma membrane reductase activity for both Cu and Fe (Figure 6A). In the *mac1-1* loss-of-function mutant, however, Cu(II) and Fe reduction(III) (and uptake; R.Hassett and D.Kosman, unpublished) was strongly reduced. We observed a similar defect in both Cu and Fe reduction in a strain mutant for *FRE1* (Figure 6A), a locus previously shown to encode a component of the plasma membrane reductase necessary for Fe(III) reduction and Fe(II) uptake (Dancis *et al.*, 1992). This indicates that the FRE1 protein may also participate in Cu(II) reduction and Cu(I) uptake.

Targets of MAC1

The observed defect in Cu/Fe reductase activity in *mac1-1* and the increase in the reductase activity in the strain with the dominant $MAC1^{up1}$ allele suggests that MAC1 may regulate the transcription of *FRE1*, and that $MAC1^{up1}$ encodes a transcription factor with gain-of-function properties. In fact, as shown in Figure 6B, the level of *FRE1* transcription is low in the *mac1-1* strain and a few-fold higher compared with wild-type in the congenic $MAC1^{up1}$ strain. In wild-type cells, transcription of *FRE1* is under a feedback control, as indicated by the strong repression of *FRE1* transcription by iron (Dancis *et al.*, 1992 and Figure 6B). We found that *FRE1* transcription is also negatively regulated by elevated copper levels (unpublished data), indicating that this negative feedback loop is operative with both metals. Interestingly, this repression (by both metals) was only moderate in the $MAC1^{up1}$ gain-of-function mutant (Figure 6B). We thus conclude that the *FRE1* gene is a target of MAC1 and that MAC1 is required for basal level and possibly regulated transcription of *FRE1*.

Evidence that *FRE1* is not the only target of MAC1 came from studies indicating that a *fre1* null mutant (*fre1::URA3*) does not show the defect in respiration and the broad stress phenotype of the *mac1-1* mutant (not shown). In fact, the H_2O_2 hypersensitivity of *mac1-1* could be linked to an altered transcription of the gene for a catalase which decomposes H_2O_2. Transcription of the cytosolic catalase gene of yeast, *CTT1*, is known to be normally low (in YPD) but inducible by H_2O_2 (Marchler *et al.*, 1993). As shown in Figure 7, induction of *CTT1* transcription by H_2O_2 is almost absent in *mac1-1*. Since the $MAC1^{up1}$ mutation does not cause an increased transcription of *CTT1* in the absence of H_2O_2, MAC1 appears to be required, but may not be sufficient for the H_2O_2-induced transcription of *CTT1*.

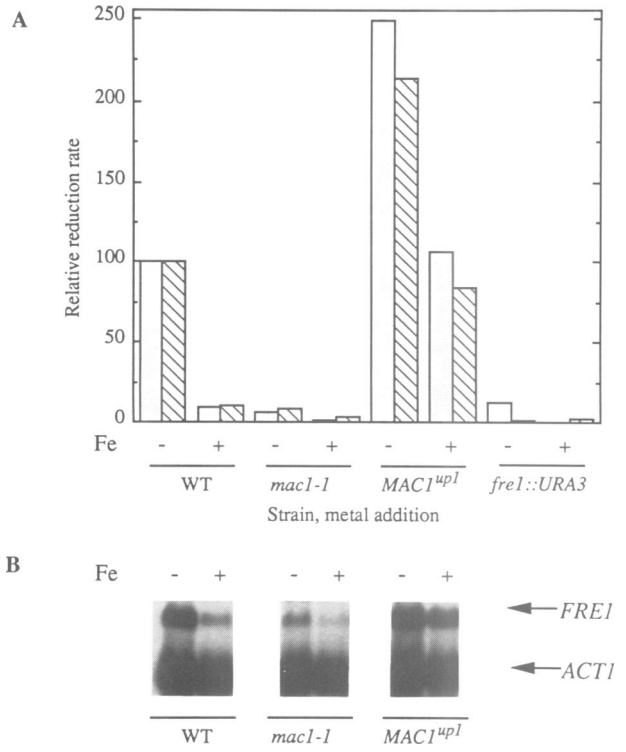

Fig. 6. MAC1 acts on FRE1-dependent reductase activity and *FRE1* transcription. (**A**) Bar diagram showing the reduction of iron (shaded bars) and copper (striped bars) in (from left to right) wild-type, *mac1-1* and *MAC1*^{up1} mutants, and *fre1::URA3* mutant cells. The data (mean $n = 3$) are presented as a percent of wild-type activity (without added iron). Addition of iron (40 μM $FeCl_3$, final) leads to a strong repression of both Cu and Fe reduction in wild-type, and to a moderate repression in *MAC1*^{up1}. (**B**) Northern analysis of *FRE1* transcript levels (*ACT1*, actin as control) of total RNA of wild-type, *mac1-1* and *MAC1*^{up1} mutant cells. Cultures were grown without (−) or with (+) added iron as described above (Figure 6A) and equal amounts of total RNA were loaded.

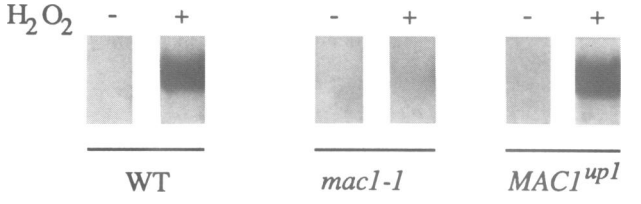

Fig. 7. MAC1 is required for *CTT1* mRNA induction by H_2O_2. Northern blot of RNA isolated from cultures (YPD medium, $A_{660nm} = 0.8$) grown in the absence of H_2O_2 (−) or after a 1 h incubation (+) with H_2O_2 (500 μM) hybridized with a labelled *CTT1* fragment. Equal amounts of total RNA were loaded.

Discussion

In this work we have identified a yeast protein related to the copper-fist transcription factors ACE1 and AMT1, which activate the metallothionein genes of *S.cerevisiae* and *C.glabrata*, respectively. Apart from the structural relatedness, further evidence strongly supports the concept that MAC1 is a transcription factor. First, immunofluorescence data indicate that MAC1 is a nuclear protein. Second, we have identified two target genes that are apparently transactivated by MAC1. Using loss-of-function and gain-of-function mutants of *MAC1*, we could show that MAC1 is required for normal basal level transcription of *FRE1*, encoding a plasma membrane component associated with Cu/Fe reduction. The other identified target of MAC1 is *CTT1*, encoding the cytosolic catalase. Interestingly, MAC1 is involved in the regulated, H_2O_2-induced expression of that gene. This could indicate that the *trans*-activating function of MAC1 may be modulated by other factors which cooperate with MAC1 and general transcription factors.

The primary sequence of the MAC1 protein encourages speculations about the protein's mode of action. As noted, the N-terminal 40 residues are highly similar to the N-terminal residues of ACE1 and AMT1 (Figure 1B and C). Within this domain are two amino acid residues essential for ACE1 function that are mutated in two loss-of-function alleles of the *ACE1* locus, *ace1-1* and *cup2* (Buchman *et al.*, 1989, 1990). These two residues, Cys11 and Gly37, are required in ACE1 for normal copper and DNA binding and are found at identical positions in the MAC1 sequence (Figure 1B and C). Binding of several Cu(I) ions to the ACE1 protein occurs in a highly cooperative fashion and results in a conformational change of the protein ('copper fist') required for the interaction of the protein to the correct target sites on the DNA (Fürst *et al.*, 1988; Fürst and Hamer, 1989; Hu *et al.*, 1990). In the ACE1 protein, 11 N-terminal cysteine residues are thought to be required for Cu(I) binding and DNA target site recognition (Hu *et al.*, 1990) but only the N-terminal three of them are conserved in the MAC1 protein. However, the MAC1 protein has additional cysteine-rich domains that are likely to participate in metal coordination. Particularly striking is a duplicated $CysXCysX_4CysXCysX_2CysX_2His$ motif in the C-terminal region of MAC1. As the terminal histidine residue of the first repeat is mutated in the dominant *MAC1*^{up1} allele, these repeats probably provide important functions, e.g. they may act as sensors for intracellular Cu or Fe ion concentrations or their redox states.

The data demonstrate several phenotypes of *mac1* loss-of-function mutants not obviously linked to either *FRE1* or *CTT1* expression. These include sensitivity to heat and heavy metals, and a respiration incompetence. Interestingly, these phenotypes can be suppressed by added copper or iron (in case of respiration, only by copper), suggesting that they could be secondary to a primary defect in a Cu/Fe utilization pathway. Thus, the respiratory deficiency may be the result of an insufficient supply of mitochondrial copper levels, leading to inactive enzymes of the respiratory chain such as cytochrome *c* oxidase, a copper binding protein. It is also possible that the heat and heavy metal hypersensitive phenotype of the *mac1-1* mutant could be the consequence of the generation of aberrant metal binding proteins caused by low intracellular copper or iron levels. These defects are not linked to *FRE1* expression, however, as the *fre1* null mutant (with the exception of a measurable cadmium sensitivity; J.Jungman and S.Jentsch, unpublished data) does not exhibit these phenotypes. We cannot rule out, however, that targets of MAC1 also include stress resistance genes. One obvious candidate is the closely linked gene *UBC7*, encoding a ubiquitin-conjugating enzyme required for cadmium tolerance (Jungmann *et al.*, 1993). However, transcription studies using *mac1-1* and *MAC1*^{up1} mutants have failed to provide evidence that *UBC7* is regulated by MAC1.

The identification here of two target genes of the MAC1 transactivator will help to localize the *cis* elements in the promoter regions of *FRE1* and *CTT1* through which MAC1

exerts its function. Recently, an upstream activating sequence has been described which mediates transcriptional activation of *CTT1* by osmotic and heat shock, and importantly, H_2O_2 (Marchler *et al.*, 1993). However, we obtained no evidence so far that this element directly serves as a binding site for MAC1. More extensive DNA binding studies and the elucidation of the structure–function relationships in the MAC1 protein are the goals of continuing study.

Materials and methods

Gene cloning and analysis

Full-length *MAC1* located upstream of *UBC7* (Jungmann *et al.*, 1993) was isolated from a genomic λEMBL library (kindly provided by Rick Young, MIT). Both strands were sequenced completely with a Sequenase kit (United States Biochemicals). Standard protocols were followed for DNA techniques, Northern and Southern blots and DNA sequencing (Ausubel *et al.*, 1993). For Northern blots, equal loads of total RNA were separated on agarose gels and probed with random-primed fragments internal to the corresponding coding sequences. To study the intracellular localization, *MAC1* including 390 bp of the promoter region was fused in frame to the *LacZ* gene of plasmid YEp354 (Myers *et al.*, 1986).

Yeast media and techniques

Yeast stocks were maintained on YPD medium (Ausubel *et al.*, 1993). YP is the same medium which lacks glucose. YPG is YP medium with 3% glycerol. SD^{++} is a synthetic medium (Guthrie and Fink, 1991) supplemented for auxotrophic growth. For the experiments described in Figure 6, cultures were grown in a synthetic, complete medium prepared without sulfate (Difco Laboratories, Detroit, MI; Lin and Kosman, 1990) from which Fe and Cu were extracted using dithizone (Holmquist, 1988) prior to the addition of the remaining trace metals. This medium contained ~15 nM copper and 170 nM iron as determined by flameless atomic absorption spectrophotometry and is referred to as Cu/Fe-free media in the text. Bathophenanthroline sulfonate (BPS, 50 μM) was included also to limit further the bioavailability of residual Fe and Cu. BPS strongly inhibits uptake of both metals. In case of Fe(III) and Cu(II) addition, BPS was omitted. The carbon source in this medium was 2% glucose.

Standard protocols were followed for growth of yeast strains, yeast transformation by the lithium acetate method, preparation of total DNA and RNA, and for the construction of yeast plasmids (Guthrie and Fink, 1991; Ausubel *et al.*, 1993). Indirect immunofluorescence with anti-β-galactosidase antibodies (Promega) was done as described (Harlow and Lane, 1988; Guthrie and Fink, 1991). 4′,6′-diamidino-2-phenyl-indole (DAPI; Sigma) was used stain to DNA.

Yeast strains and construction of mutants

The constructed haploid *S.cerevisiae* strains used in this study are congenic to diploid DF5 (Finley *et al.*, 1987). The original $MAC1^{up1}$ isolate (*MAT***a** *ade8 his3 trp1-1 gal1* $MAC1^{up1}$; obtained from Dean Hamer, NIH), the *fre1* null mutant strain (strain FΔFe; F113 derivative; *MAT***a** *ura3-52 ino1-1 fre1::URA3*) and its congenic parental strain (strain F113; both strains obtained from Andrew Dancis and Rick Klausner, NIH) have a different genetic background.

MAC1 gene disruption was carried out by inserting the yeast *URA3* gene (*Hin*dIII fragment) into the unique *Nco*I site of *MAC1* (Figure 3, top). Linear DNA of the resulting plasmid was used for transformation of YWO1, a *MATα* segregant of diploid DF5 (Finley *et al.*, 1987) for marker prototrophy. The *mac1-1* frameshift allele was constructed by deletion of a guanine nucleotide at the *Nco*I site (position 126; Figures 1 and 3). This leads to codons at the *Nco*I site for a sequence ...*PSPM*LSETLF (single letter code; wild-type-derived amino acids are italicized), followed by two UAG stop codons. Linear DNA of this mutated clone was used for transformation of the *mac1::URA3* disruption strain and homologous recombinants were selected for ura^- using 5-fluoroorotic acid (5-FOA). The correct recombinant (strain YJJ1) was identified by Southern hybridization. The $MAC1^{up1}$ allele (Figure 3, bottom) was cloned from *Hae*III-restricted genomic DNA from the original dominant strain. DNA fragments which hybridized with *MAC1* probes were subcloned into *Escherichia coli* vectors for colony hybridization. Positive clones were isolated and sequenced. Linear DNA bearing the $MAC1^{up1}$ allele was used for transformation of the *mac1::URA3* disruption strain. The correct recombinant (strain YJJ2) was selected for the ura^- (5-FOA resistant), copper hypersensitive (200 μM in SD), and heat resistant phenotype.

O_2 uptake

Respiratory competence was evaluated on cultures first grown (to $A_{660\,nm}$ = 0.6) in YPD. Cells were collected and resuspended in YP to the initial culture volume. In testing for the Cu or Fe rescue of respiration competence, $CuCl_2$ and $FeCl_3$ (50 μM each) were added to both the YPD and YP media. The cells were then incubated for 4 h, pelleted again and washed with O_2 uptake buffer (0.1 M potassium phosphate buffer, pH 7.8, 0.1% glucose) and resuspended in O_2 uptake buffer. Oxygen uptake was followed (Yellow Springs model 53 oxygen monitor with Clark-type electrode) at 30°C in air-saturated cultures which contained ~3×10^7 cells/ml.

Reductase activity

Iron and copper reductase activities in whole cells were done essentially as described (Lesuisse *et al.*, 1989). Cells were grown in Cu/Fe-free medium (see above) and concentrated to 5×10^8 cells in reductase assay buffer (50 mM sodium citrate, 5% glucose, pH 6). Metal reductase assays were carried out at 30°C using BPS to monitor Fe(III) reduction and bathocuproine sulfonate (BCS) to monitor Cu(II) reduction. The metal reduction rates were determined as nmol(Cu or Fe)/min per mg total protein.

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References

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1993) *Current Protocols in Molecular Biology*. John Wiley and Sons, New York.

Buchman,C., Skroch,P., Welch,J., Fogel,S. and Karin,M. (1989) *Mol. Cell. Biol.*, **9**, 4091–4095.

Buchman,C., Skroch,P., Dixon,W., Tullius,T.D. and Karin,M. (1990) *Mol. Cell. Biol.*, **10**, 4778–4787.

Dancis,A., Klausner,R.D., Hinnebusch,A.G. and Barriocanal,J.G. (1990) *Mol. Cell. Biol.*, **10**, 2294–2301.

Dancis,A., Roman,D.G., Anderson,G.J., Hinnebusch,A.G. and Klausner,R.D. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3869–3873.

Davis,K. (1993) *Nature*, **361**, 98.

Finley,D., Özkaynak,E. and Varshavsky,A. (1987) *Cell*, **48**, 1035–1046.

Fürst,P. and Hamer,D. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 5267–5271.

Fürst,P., Hu,S., Hackett,R. and Hamer,D. (1988) *Cell*, **55**, 705–717.

Guthrie,C. and Fink,G.R. (eds) (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press, San Diego.

Hamer,D.H. (1986) *Annu. Rev. Biochem.*, **55**, 913–951.

Harlow,E. and Lane,D. (1988) *Antibodies. A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Holmquist,B. (1988) *Methods Enzymol.*, **158**, 6–12.

Hu,S., Fürst,P. and Hamer,D. (1990) *New Biol.*, **2**, 544–555.

Huibregtse,J.M., Engelke,D.R. and Thiele,D.J. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 65–69.

Jungmann,J., Reins,H.-A., Schobert,C. and Jentsch,S. (1993) *Nature*, **361**, 369–371.

Klausner,R.D., Rouault,T.A. and Harford,J.B. (1993) *Cell*, **72**, 19–28.

Lesuisse,E. and Labbe,P. (1989) *J. Gen. Microbiol.*, **135**, 257–263.

Lin,C.-M. and Kosman,D.J. (1990) *J. Biol. Chem.*, **265**, 9194–9200.

Marchler,G., Schüller,R., Adam,G. and Ruis,H. (1993) *EMBO J.*, **12**, 1997–2003.

Myers,A.M., Tzagoloff,A., Kinney,D.M. and Lusty,C.J. (1986) *Gene*, **45**, 299–310.

Spevak,W., Fessl,F., Rytka,J., Traczyk,A., Skoneczny,M. and Ruis,H. (1983) *Mol. Cell. Biol.*, **3**, 1545–1551.

Szczypka,M.S. and Thiele,D.J. (1989) *Mol. Cell. Biol.*, **9**, 421–429.

Thiele,D.J. (1988) *Mol. Cell. Biol.*, **8**, 2745–2752.

Welch,J., Fogel,S., Buchman,C. and Karin,M. (1989) *EMBO J.*, **8**, 255–260.

Wilson,J.D., Braunwald,E., Isselbacher,K.J., Petersdorf,R.G., Martin,J.B., Fauci,A.S. and Root,J.R (eds) (1991) *Harrison's Principles of Internal Medicine*. McGraw Hill, New York.

Zhou,P. and Thiele,D.J. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 6112–6116.

Zhou,P., Szczypka,M.S., Sosinowski,T. and Thiele,D.J. (1992) *Mol. Cell. Biol.*, **12**, 3766–3775.

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