Independent Metalloregulation of Ace1 and Mac1 in *Saccharomyces cerevisiae*

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Ace1 and Mac1 undergo reciprocal copper metalloregulation in yeast cells. Mac1 is functional as a transcriptional activator in copper-deficient cells, whereas Ace1 is a transcriptional activator in copper-replete cells. Cells undergoing a transition from copper-deficient to copper-sufficient conditions through a switch in the growth medium show a rapid inactivation of Mac1 and a corresponding rise in Ace1 activation. Cells analyzed after the transition show a massive accumulation of cellular copper. Under these copper shock conditions we show, using two epitope-tagged variants of Mac1, that copper-mediated inhibition of Mac function is independent of induced protein turnover. The transcription activity of Mac1 is rapidly inhibited in the copper-replete cells, whereas chromatin immunoprecipitation studies showed only partial copper-induced loss of DNA binding. Thus, the initial event in copper inhibition of Mac1 function is likely copper inhibition of the transcriptional activity. Copper inhibition of Mac1 in transition experiments is largely unaffected in cells overexpressing copper-binding proteins within the nucleus. Likewise, high expression of a copper-binding, non-DNA-binding Mac1 mutant is without effect on the copper activation of Ace1. Thus, metalloregulation of Ace1 and Mac1 occurs independently.

The essentiality of copper for normal *Saccharomyces cerevisiae* physiology is consistent with the known mechanisms available to *S. cerevisiae* to ensure that adequate but not excessive cellular copper levels are maintained. Growth conditions of copper deficiency result in the induction of a family of genes whose products are involved in cellular uptake of copper (11, 18, 29, 41). Two copper uptake genes encode the plasma membrane Ctr1 and Ctr3 Cu(I) ion permeases (10, 29). A third gene encodes the Fre1 metalloreductase, which generates a diffusible reductant in the culture medium to mobilize Cu(II) ions (30). Homeostatic mechanisms also prevent excessive accumulation of cellular copper that can be deleterious to growth. Three genes are induced in cells exposed to elevated extracellular copper levels (>1 μM copper). Two detoxification genes (*CUP1* and *CRS5*) encode metallothioneins that buffer Cu(I) ions in the cytoplasm. *CUP1* is the dominant locus that confers the ability of yeast cells to propagate in medium containing copper salts. The third gene (*SOD1*) encodes superoxide dismutase, which is also effective in buffering cellular copper levels (8).

Genes induced in copper-deficient cells are regulated by the Mac1 transcriptional activator (26, 29, 41). *MAC1* was originally identified as a partially dominant mutation designated *MAC1up1* (26). Cells harboring the *MAC1up1* allele are incapable of down-regulation of copper uptake genes in copper-replete medium, resulting in a copper hypersensitivity phenotype (18, 26, 41). The copper sensitivity of *MAC1up1* cells demonstrates the critical importance of down-regulating the copper uptake system in copper-replete cells.

Copper metalloregulation of the high-affinity copper uptake system occurs at both the transcriptional level through Mac1 and posttranscriptionally through copper-induced degradation of Ctrl1 (32, 42). The copper-induced degradation of Ctrl1 appears to require the product of an unidentified Mac1 target gene as well as a C-terminal Cys-rich sequence motif in Ctrl1 (42).

Copper inhibition of Mac1 function involves copper attenuation of DNA binding to the CuRE promoter element in copper-deficient but not copper-replete cells (29). Transactivation activity maps to two carboxyl-terminal cysteine-rich motifs that bind up to eight Cu(I) ions (25). This activity is repressed in copper-supplemented cells (17). Mac1 is believed to be a direct Cu(I) sensor. Cu(I) binding to the Cys-rich motifs induces an intramolecular interaction with the N-terminal DNA-binding domain (24, 25). Copper inhibition of Mac1 function does not alter the nuclear localization of the protein (25, 37). However, discrepant results have been reported on whether the Mac1 protein level is altered in a transition from copper-deficient to copper-replete conditions. Overexpressed Mac1 is copper-inhibited in its transcriptional activity, yet the protein accumulated, presumably due to Cu(I) binding stabilization (25). Low-copy Mac1 with a C-terminal epitope tag was shown to undergo copper-mediated degradation (43). Copper metalloregulation of Mac1 is also believed to involve signal transduction pathways. DNA binding by Mac1 was reported to be dependent on phosphorylation (19).

Copper-dependent activation of gene expression is mediated by the Ace1 transcriptional activator (Activator of *CUP1 Expression* in *S. cerevisiae*) (40). Ace1 is an inactive, nuclear protein in cells cultured in copper-deficient medium. Cu activation is achieved through the formation of a tetracopper-thiolate cluster within the copper regulatory domain of Ace1 (9, 15). Cu(I) binding converts Ace1 from an inactive, non-DNA-binding molecule into a functional transcriptional activator.

The copper activation of Ace1 and copper inhibition of Mac1 appear to occur within the yeast nucleus, yet it is unclear how copper ions are transported to the nucleus. Intracellular...
copper ion transport occurs by specific protein-mediated Cu(I) shuttling to the copper-requiring Sod1 in the cytoplasm and Ccc2 P-type ATPase in trans-Golgi vesicles (22). Since Ace1 and Mac1 are both direct Cu(I) sensors that reside within the nucleus, the question is whether independent, specific routes of Cu(I) shuttling exist for Ace1 and Mac1. Competition studies were conducted to determine whether changes in the protein level of one of these sensors influences the activity of the other. We show presently that Cu(I) sensing by Ace1 and Mac1 occurs independently. We show that the observed copper inhibition of Mac1 function is independent of Mac1 protein turnover and likely arises primarily from copper inhibition of transactivation function.

MATERIALS AND METHODS

**Strains and culture conditions.** The strains used in this study include BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), DY513 (W303) MAC1-TAP (MATa ade2 can1 his3 leu2 trp1Δ1 MAC1-TAP::TRP1) ace1Δ (BY4742: MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0), and mac1Δ in the BY4741 background. Yeast strains were cultured in standard YPD (1% yeast extract, 2% peptone, and 2% dextrose) medium, synthetic complete (SC), or low-copper complete medium (LCM) containing copper and iron-limiting yeast nitrogen base (BIO 101) and the copper-specific chelator bathocuproine sulfonate (BCS). In certain experiments SC medium containing the low-copper nitrogen base was used as indicated in the figure legends. SC medium contains 0.7 μM copper, whereas SC medium supplemented with copper under the conditions specified in the text. To terminate cytoplasmic translation, 0.1 mM cycloheximide was added for 30 min prior to the switch in culture medium.

**Plasmid constructions and transformation.** Green fluorescent protein (GFP) fusions of Ace1 and Mac1 were constructed using the galactose-inducible expression vector GFP-pYeF2 (7). DNA sequences coding for MAC1 (codons 1 to 417) and ACE1 (codons 1 to 123) were PCR amplified, creating 5′ BamHI and 3′ XhoI sites. Chromosomal MAC1 and ACE1 TAP (5′) tag fusions were created by homologous recombination as described previously (34). An internal Myc-tagged Mac1 was engineered by inserting a triple Myc epitope tag repeat in frame to MAC1 between codons 183 and 184 using overlap extension PCR (20). Amino acid substitutions were introduced into expression vectors using the QuikChange method (Stratagene). All plasmids were confirmed by restriction digestion and sequencing. DNA transformations were performed using a lithium acetate procedure.

**mRNA quantitation by S1 nuclease analysis.** Total RNA isolated from cultures harvested at mid-log phase and total RNA was extracted using the hot acidic phenol method (1). Hybridizations with 32P-labeled, single-stranded DNA molecules harvested at mid-log phase and total RNA was extracted using the hot acetate procedure.

**RESULTS**

**Metalloregulation during steady-state and transition conditions.** Ace1 and Mac1 are known to exhibit reciprocal copper regulation of their target genes. In copper-replete cells, Ace1 target genes such as CUP1 are maximally induced (Fig. 1A). In contrast, Mac1 target genes such as CTR1 are mainly induced during copper deficiency (Fig. 1A). Under these steady-state growth conditions, maximal CUP1 expression occurs in cells treated with >0.05 mM Cu(II). Maximal CTR1 expression in laboratory cultures requires a reduction in the availability of copper in the growth medium below the nanomolar level. This is usually achieved by incubation of cells with a Cu(I) chelator such as bathocuproine sulfonate. The low-copper medium (LCM) used in the present study to activate Mac1 consists of Cu-depleted yeast nitrogen base with the addition of 0.1 mM BCS.

Microbes in nature often show reduced growth rates as nutrient levels are much lower than those in laboratory conditions. Thus, microbes are often nutrient deprived and face a “feast or famine” existence (12). Metal ion availability is often limited, therefore metal ion deficiency may be commonly encountered. In an attempt to mimic a growth transition from deficiency to sufficiency, yeast were first cultured in LCM and then cultured in LCM supplemented with copper under the conditions specified in the text. To terminate cytoplasmic translation, 0.1 mM cycloheximide was added for 30 min prior to the switch in culture medium.

**Copper levels were determined on**
Metalloregulation occurs within the nucleus. Mac1 and Ace1 are reported to exist within the nucleus independent of the copper status of cells (38) (37). Thus, copper metalloregulation occurs on molecules within the nucleus or during nascent chain biosynthesis within the cytoplasm. To determine whether copper metalloregulation occurs in preexisting proteins, cells precultured in LCM were treated with cycloheximide for 30 min prior to copper shock. Cycloheximide treatment did not impair the copper inactivation of Mac1 or copper activation of Ace1 (Fig. 2). These results are consistent with copper modulation of Mac1 and Ace1 function occurring in existing molecules within the nucleus.

Mechanism of metalloregulation. Multiple mechanisms have been suggested to account for the observed copper inhibition of Mac1. These mechanisms include attenuated DNA binding, inhibition of transactivation activity, copper-induced proteolytic degradation, and posttranslational modification. To readdress the mechanism of copper inhibition of Mac1 function, two epitope-tagged Mac1 variants were generated for mechanistic studies. First, an internal Myc epitope-tagged Mac1 was generated by the insertion of a triple Myc tag between the N-terminal DNA binding domain and C-terminal activation domain. Second, the chromosomal allele of MAC1 was TAP tagged by homologous recombination yielding a C-terminal TAP tag. Transformation of mac1/H9004 cells with the MAC1-myc chimera on a YCp plasmid yielded normal BCS induction of CTR1 under steady-state conditions (Fig. 3A) and wild-type copper inhibition of CTR1 expression during a transition from copper deficiency to copper-replete medium (Fig. 1B, right panel). These experiments confirm the functional state of the internal tagged molecule (Fig. 3A).

MAC1-TAP cells showed BCS-induced CTR1 expression, but the induction was partially attenuated relative to wild-type cells (Fig. 3A). To evaluate whether Mac1 undergoes copper-induced proteolysis, cells with Mac1-Myc or Mac1-TAP were cultured under steady-state conditions in copper-deficient or -replete conditions (Fig. 3B). Mac1 protein levels were elevated in copper-supplemented cells compared to copper-deficient cells or SC-cultured cells. Inhibition of Mac1 function is not mediated through proteolysis as was reported earlier with a C-terminal hemagglutinin-tagged Mac1 chimera (43). The stabilization of Mac1 in copper-replete cells was also observed in Mac1-TAP cells supplemented with CuSO4.
copper inhibition of Mac1 function is independent of copper-induced proteolysis.

The DNA-binding function of Mac1 was shown previously to be copper dependent using an in vivo methylation analysis (29). To confirm this type of metalloregulation, cells with the epitope-tagged Mac1 were subjected to chromatin immunoprecipitation analysis. Wild-type and Myc-tagged MAC1 cells were cultured under steady-state conditions with either BCS or CuSO4 as a supplement. Chromatin immunoprecipitation analysis on Mac1-Myc cells revealed specific Mac1 DNA binding in LCM cultures (Fig. 4A). DNA binding activity to CTR1, CTR3, and FRE1 was attenuated in both CM and copper-supplemented cultures.

Although Mac1 shows specific binding to the CuRE in CTR3, the presence of a transposon downstream of the CuRE precludes expression of CTR3 in these cells as is known in many yeast strains (28). Chromatin immunoprecipitation analysis of Mac1 target genes was repeated in transition cultures undergoing the medium switch. Mac1 engagement at CTR1, CTR3, and FRE1 was attenuated by 15 min of the transition to SC medium (Fig. 4B). Thus, as was shown previously (29), DNA binding by Mac1 is inhibited in copper-replete cells. DNA binding by Mac1-TAP was attenuated to a similar extent in copper-replete cells, confirming that the attenuation was independent of the epitope used (data not shown). However, comparing the transcriptional activity of the internal Myc-tagged Mac1 in Fig. 1B to the DNA binding activity in Fig. 4B reveals that expression of CTR1 is inhibited in transition cultures to a greater extent than attenuation in DNA binding. Since the Mac1 protein level is elevated in copper-shocked cells, the obvious suggestion is that inhibition of the Mac1 transactivating activity likely accounts for much of the observed attenuation in CTR1 expression.

Consistent with the reciprocal copper metalloregulation of Mac1 and Ace1, S1 nuclease analysis of cells containing a chromosomal 3’ TAP tag to ACE1 showed the expected enhanced CUP1 expression (Fig. 5A) and chromatin immunoprecipitation analysis showed enhanced CUP1 DNA binding in copper shocked cells (Fig. 5B). The induction of CUP1 expression arises without change in the Ace1 protein level (data not shown). These results confirm previous studies showing that copper activation of Ace1 occurs by enhanced DNA binding (15).

**Metalloregulation is specific.** The mechanism of copper translocation to the nucleus for metalloregulation of Ace1 and Mac1 is unknown. To address whether Ace1 or Mac1 perturb the function of each other, nonfunctional variants of each gene
were engineered and placed under the control of the \textit{GAL1} promoter on a high-copy vector, permitting overexpression of each gene. To evaluate whether the Ace1 copper regulatory domain can alter Cu(I) sensing by Mac1, the DNA-binding domain (DBD) of Ace1 (codons 1 to 123) fused to green fluorescent protein was subcloned into a \textit{GAL1} expression vector. The Ace1 DNA binding domain consists of a conserved N-terminal Zn module, AT-hook motif, and the Cu(I) binding domain (4, 16).

Control studies were initially undertaken to evaluate the effect of this construct on the function of wild-type Ace1. Transformants were markedly compromised in \textit{CUP1} expression (Fig. 6A). The overexpressed Ace1 DBD fused to GFP showed prominent nuclear localization, equivalent to full-length Ace1/GFP, based on epifluorescence of the GFP moiety (Fig. 7).

\[ \text{CUP1}^{\text{expression}} \text{ is inhibited when a mutant Ace1/GFP chimera was used with a Gly}^{\text{37}}\text{Gln substitution that is known to attenuate DNA binding in Ace1 (4)} \]

\[ \text{Panel C: Galactose was added to cells cultured overnight in raffinose SC medium (prepared with BIO 101 low-copper nitrogen base) to induce expression of the \textit{ACE1} DBD. Cells were harvested at 0, 45 or 90 min after the addition of galactose. RNA was extracted for S1 nuclease analysis to quantify \textit{CTR1} expression levels. Wild-type control cells were cultured in raffinose SC medium in the presence of 0.1 mM BCS to maximally induce \textit{CTR1}.} \]

To address the effect of overexpression of the \textit{ACE1} DBD variant on Mac1 function, transformants were cultured in galactose LCM medium to induce the Ace1 DBD. After 1.5 h in galactose, cells were switched to SC medium or SC medium containing 10 \(\mu\text{M CuSO}_4\) for 15 min before harvest. RNA extracted from these cells was used for quantitation of \textit{CTR1} transcript levels. As can be seen in Fig. 6B, the induction of Ace1 DBD was without effect on the copper attenuation of \textit{CTR1} mRNA levels. In a second type of competition experiment, cells cultured in SC with raffinose were induced by the addition of galactose for 45 and 90 min. Induction of the \textit{ACE1} DBD failed in this steady-state experiment to induce \textit{CTR1} expression (Fig. 6C). These results suggest that Mac1 regulation is independent of the Cu(I)-binding Ace1 protein level.

To address whether overexpression of Mac1 was able to inhibit copper activation of Ace1, we created a nonfunctional \textit{MAC1} allele that was transcriptionally inactive but accumulated normally within the nucleus. The DNA binding domain of Mac1 contains a similar N-terminal Zn module and AT-hook motif as Ace1 (24). Lysine substitution of two Arg residues (R16 and R19) within the Zn module of Mac1 abrogates DNA binding, thereby inactivating Mac1 (Fig. 8A). The dominant role of the AT-hook motif of Mac1 in DNA binding has
not been reported previously. The mutant protein fused to GFP was expressed well and gave intense nuclearly localized fluorescence, as does WT Mac1 (Fig. 7). Unlike Ace1, the Mac1-GFP chimera was localized in punctae within the nucleus. The Mac1-like factor from <i>Schizosaccharomyces pombe</i> Cuf1 showed the same punctate localization as a GFP fusion when expressed in <i>S. cerevisiae</i> (data not shown).

The mutant form of Mac1 retains a wild-type Cu(I) binding domain in the C-terminal segment of the molecule. Expression of the nonfunctional allele in wild-type cells attenuated the function of endogenous Mac1 not through DNA binding competition, but presumably through the known interaction of Mac1 molecules through the C-terminal helix (37). This mode of inhibition was confirmed by the observation that a triple mutation in the C-terminal helix abrogated the effect (data not shown).

To evaluate the effect of the nonfunctional nuclear <i>MAC1</i> allele on the copper activation of Ace1, transformants overexpressing inactive Mac1 were cultured in LCM prior to a growth medium switch. The transition to SC medium resulted in a modest but reproducible diminution in <i>CUP1</i> induction in a transition to SC medium but no apparent effect in a transition to SC medium containing 10 μM CuSO<sub>4</sub> suggesting that Mac1 overexpression has only a modest effect on copper activation of Ace1 within the nucleus (Fig. 8B). In the transfer studies to SC medium, the pool of Mac1 may be sufficient to partially compete for Cu(I); whereas in the transfer experiment to 10 μM CuSO<sub>4</sub> the Mac1 pool may be insufficient to compete for the available copper resulting in elevated Ace1-mediated <i>CUP1</i> expression. Overexpression of Mac1 using a similar galactose-inducible construct had a similar effect to the inactive Mac1 in transition studies. Thus, both Ace1 and Mac1 sense Cu(I) largely independently of the other protein, although a modest effect of Mac1 overexpression on Ace1 function may exist.

Although overexpression of the Ace1 DBD did not activate Mac1, overexpression of the yeast metallothionein Crs5p did result in elevated <i>CTR1</i> expression consistent with Mac1 activation. Overexpression of <i>CRS5</i> from <i>GAL1</i>-regulated expression vectors or overexpression of a nucleus-targeted <i>CRS5</i> containing an appended nuclear localization sequence (NLS) resulted in elevated <i>CTR1</i> expression in steady-state cultures in a time-dependent manner (Fig. 9). The addition of the NLS motif resulted in unambiguous nuclear localization of the GFP-tagged Crs5. In contrast, copper attenuation of Mac1 function was not altered in transition experiments by overexpression of NLS-Crs5 or Crs5 (data not shown). The absence

FIG. 7. Immunofluorescence of Mac1-GFP and Ace1-GFP chimeras. Cells transformed with GFP fusions of <i>MAC1</i> or <i>ACE1</i> were grown in raffinose-containing medium with either 100 μM BCS or 100 μM CuSO<sub>4</sub>. When the cells reached an optical density of 1, galactose was added to a final concentration of 2% and the cells were incubated for an additional 2 h. Glucose was then added to a final concentration of 2% and the cells grown for an additional hour. The cultures were fixed by the direct addition of formaldehyde (4% final concentration) for 1 h with gentle shaking. The cells were harvested, resuspended in buffered formaldehyde (4% formaldehyde, 50 mM potassium phosphate pH 7.2, 0.5 mM MgCl<sub>2</sub>) and incubated overnight at 30°C with shaking. The fixed cells were harvested and washed with PBS three times. Upon the addition of 4',6'-diamidino-2-phenylindole (DAPI) (50 ng/ml) the cells were incubated at 4°C for 48 h. Aliquots of cells were placed on polylysine-coated slides, mounted with FluorSave (Calbiochem), and sealed with a coverslip for microscopy. The overlay of the DAPI image and the GFP fluorescence is shown for both Ace1 and Mac1.
used for S1 nuclease analysis to quantify CTR1 cultures and cells were harvested 7 h later. RNA was extracted and cells were transitioned to SC medium or SC medium containing 10 μM CuSO4 with raffinose as the carbon source. Galactose was added and 1.5 h later the observed increase in CUP1 expression correlates with copper ac-

FIG. 8. Effects of overexpression of MAC1 or a mutant MAC1 containing mutations at codons 16 and 19 resulting in Arg to Lys substitutions. Panel A. mac1Δ cells transformed with either MAC1, mutant MAC1(m) containing the R>K substitutions, or a vector control (vec) were cultured overnight in either LCM or SC plus 10 μM CuSO4 with raffinose as the carbon source. Galactose was added to the cultures and cells were harvested at either 0, 45, or 90 min later to assess the effect of overexpression of the MAC1 alleles on expression of CTR1 by S1 nuclease analysis.

of a competitive effect on copper inhibition of Mac1 in transition experiments was not due to nonfunctional molecules. When the same vectors were transformed into ace1Δ cells, both Crs5 variants conferred marked copper resistance on the ace1Δ cells.

DISCUSSION

Ace1 and Mac1 are known to undergo reciprocal copper metalloregulation (33). Mac1 is functional as a transcriptional activator in copper-deficient cells, whereas Ace1 is inactive. Cells cultured in standard laboratory culture conditions contain both Ace1 and Mac1 in transcriptionally silent states. Ace1 becomes activated as the cellular copper content is elevated, as seen in copper-supplemented cells. We show presently that cells undergoing a transition from copper-deficient to copper-sufficient conditions through a switch in the growth medium show a dramatic rise in Ace1 activation reported by enhanced CUP1 expression. CUP1 expression correlates with copper activation of Ace1 (15). Exposure to copper-deficient conditions results in an up-regulation of the copper uptake system, consisting of the copper permeases Ctr1 and Ctr3 and the Fre1 metalloreductase, necessary to mobilize Cu(I) (33). Transition of cells in this state to conditions of copper sufficiency results in massive accumulation of cellular copper. Ace1 activation and induction of metallothioneins are likely a protective response to minimize any deleterious effect of the copper shock conditions.

This condition of copper shock resembles a typical situation for microbes in the environment that often experience a shift from nutrient deficiency to repletion. Such transitions result in stress from the nutrient shock (2, 6). The stress imposed by copper shock can be deleterious, as copper is capable of catalyzing toxic reactions within the cell. The evolutionary pressure for the selection of the Ace1/Cup1 system of copper detoxification may not have been from exposure of cells to high exogenous copper salts in the environment or use in copper culinary vats, but rather from the need to protect cells from copper shock resulting from a change in nutrient availability.

The homeostatic mechanism protecting cells against zinc shock differs from that for copper shock. Cells encountering zinc deficiency up-regulate the plasma membrane zinc uptake transporters and also induce the vacuolar influx zinc transporter Zrc1 (31). Induction of ZRC1 is a proactive response poising the cell to respond to any subsequent transition to zinc-sufficient conditions in which rapid sequestration of zinc within the yeast vacuole is an important detoxification response. Although copper is sequestered within the yeast vacuole (35), yeast cells do not appear to induce vacuolar copper sequestration during copper shock conditions.

Cu(I) binding activates the DNA binding function in Ace1 (14, 15). We confirm that cells undergoing the copper deficiency to sufficiency transition show enhanced Ace1 DNA binding and the corresponding elevation in CUP1 expression. The mechanism of copper inhibition of Mac1 function is more complex. Previous studies suggest copper inhibition of Mac1 to involve attenuated DNA binding and transactivation activity in addition to copper-induced proteolysis. We show presently, using two epitope-tagged variants of Mac1, that copper inhibition is independent of protein turnover. Addition of a TAP tag chromosomally to MAC1 resulted in a chimeric protein level that was increased in copper-replete cells rather than reduced by proteolysis. Likewise, the Myc-tagged chimera consisting of an internal triple Myc tag separating the DNA binding and transactivation domains of Mac1 was also increased, consistent with our earlier study using an overexpressed C-terminal Myc-tagged protein (23).

The observed increase in protein levels in copper-replete cells may arise either from copper-induced stabilization of the Mac1 protein or alternatively from reduced stability of the transcriptionally active state (36). We conclude that copper-induced proteolysis is not the mechanism responsible for copper inhibition of Mac1 function. The observed copper-induced

FIG. 9. Effects of overexpressing CRS5 or CRS5 containing a NLS (CRS5-NLS). Wild-type cells transformed with either CRS5, CRS5-NLS or a vector control were cultured in SC medium (prepared with BIO 101 low-copper nitrogen base) containing raffinose. Galactose was added to these cultures and cells were harvested at either 0, 45, or 90 min later to assess the effect of overexpression of the CRS5 alleles on expression of CTR1 by S1 nuclease analysis.
degradation of Mac1 reported previously (43) may have arisen from copper-dependent removal of the C-terminal hemagglutin epitope tag, or alternatively Mac1 degradation is only observed in certain genetic backgrounds. It should be pointed out that Mac1 stabilization was observed presently in two distinct genetic backgrounds (W303 and BY4741).

Copper-induced diminution of DNA binding was observed with both epitope-tagged variants of Mac1, consistent with an earlier report (29). Copper-induced dissociation of Mac1 from target genes is partially responsible for the observed copper inhibition of CTR1 expression. Since chromatin immunoprecipitation analyses reveal only partial Mac1 dissociation, copper inhibition of Mac1 function must also involve copper inhibition of the transactivation activity. We showed previously that transactivation activity in Mac1 is copper regulated (17) and the transactivation domain is embedded within two Cu(I) binding subdomains (3, 25).

Inhibition of the Mac1 transactivation function may arise from direct copper binding or a copper-mediated posttranslational modification. Mutational data on Ace1 and Mac1 are consistent with each protein being a direct copper sensor (21, 27). Since each protein resides solely within the nucleus, copper metalloregulation may occur by Cu(I) binding to nascent chains emerging from the cytosolic ribosomes or copper translocation to the nucleus. We show presently that copper metalloregulation of Ace1 and Mac1 occurs in cycloheximide-treated cells suggesting that metalloregulation of Ace1 and Mac1 must occur within the nucleus.

Phosphorylation of Mac1 was reported to be essential for DNA binding suggesting that metalloregulation may arise from by a copper-regulated phosphatase (19). Heredia et al. showed that the addition of calf intestinal phosphatase enhanced the electrophoretic mobility of an epitope-tagged Mac1 (19). We failed to see any change in the electrophoretic mobility of Mac1 extracted from copper-deficient or copper-supplemented cells. In the absence of identification of a copper-regulated phosphatase in yeast, we focused in the present study on direct copper metalloregulation of Mac1.

One intriguing question arising from copper metalloregulation of Ace1 and Mac1 within the nucleus concerns the pathway of copper shuttling to the nucleus and presentation to the two proteins. Although we have not identified the mechanism of copper delivery to the nucleus, we show for the first time that copper metalloregulation of Ace1 and Mac1 is specific and not altered by high expression of the other copper-binding proteins within the nucleus. High expression of the copper-binding N-terminal domain of Ace1 attenuates the function of endogenous Ace1 but is without effect on the copper inhibition of Mac1 function. Likewise, high expression of a copper-binding, non-DNA-binding Mac1 mutant is without effect on the copper activation of Ace1. Furthermore, the absence of cross-competition and absence of any effect of nuclear Crs5 on copper metalloregulation of Mac1 in transition studies suggest that diffusion of a reactive copper pool such as Cu(I)-GSH is not the source of copper for metalloregulation. A reactive pool of copper exists in cells, as shown by the efficient copper metabolism of human Sod1 in yeast cells lacking the Ces1 metallochaperone (5).

Overexpression of CUP1 or CRS5 resulted in enhanced CTR1 expression levels in steady state cultures. Presumably, high levels of Cup1 or Crs5 metallothioneins perturb intracellular copper pools resulting in Mac1 activation. However, overexpression of Crs5 either in the cytoplasm or in the nucleus did not yield the same extent of Mac1 activation as did treatment of cells with the copper chelator BCS. The reason for the greater efficacy of the extracellular BCS chelator compared to intracellular Crs5 in Mac1 activation may relate to the ability of metallothioneins to bind various metal ions. The overexpressed Crs5 may bind metal ions other than copper, thus lowering its ability to deplete copper pools.

No cross competition between Ace1 and Mac1 was observed in cell transition experiments. The lack of cross competition may arise from one of three scenarios. First, if the initial event of Mac1 inhibition is the inhibition of the Mac1 transactivation activity by Cu(I) binding to the C-terminal Cys motifs, the absence of cross competition implies a highly specific route of copper ion presentation to Mac1. This may occur through a specific metallochaperone within the nucleus. Second, copper inhibition of Mac1 function may occur through a signal transduction pathway. Cu(I) binding to Mac1 may be only a late event, perhaps reinforcing the repressed state. High expression levels of Ace1 may not be expected to modulate Mac1 function if signal transduction is the primary inhibitory pathway. Third, the lack of cross competition may arise if Ace1 and Mac1 reside within different subnuclear localizations. The nuclear punctate staining of GFP-Mac1 is clearly distinct from the diffuse nuclear staining of GFP-Ace1.

A rigorous proof of direct copper metalloregulation of Mac1 in cells would require documentation of Cu(I) binding to Mac1 extracted from cells. Due to the low abundance of Mac1, this demonstration has been possible only in cells overexpressing Mac1 (25). Resolution of this question will require future studies to identify additional proteins that modulate metalloregulation of Mac1.

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