Identification of a copper-induced intramolecular interaction in the transcription factor Mac1 from *Saccharomyces cerevisiae*

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Mac1 mediates copper (Cu)-dependent expression of genes involved in high-affinity uptake of copper ions in *Saccharomyces cerevisiae*. Mac1 is a transcriptional activator in Cu-deficient cells, but is inhibited in Cu-replete cells. Mac1 resides within the nucleus in both Cu-deficient and Cu-loaded cells. Cu inhibition of Mac1 appears to result from binding of eight copper ions within a C-terminal segment consisting of two Cys-rich motifs. In addition, two zinc ions are bound within the N-terminal DNA-binding domain. Only 4–5 mol. eq. Cu are bound to a mutant Mac1 (His279Gln substitution) that is impervious to Cu inhibition. The CuMac1 complex is luminescent, indicative of copper bound in the Cu(I) state. Cu binding induces a molecular switch resulting in an intramolecular interaction in Mac1 between the N-terminal DNA-binding domain and the C-terminal activation domain. This allosteric interaction is Cu dependent and is not observed when Mac1 contained the mutant His279Gln substitution. Fusion of the minimal DNA-binding domain of Mac1 (residues 1–159) to the minimal Cu-binding activation domain (residues 252–341) yields a functional Cu-regulated transcriptional activator. These results suggest that Cu repression of Mac1 arises from a Cu-induced intramolecular interaction that inhibits both DNA binding and transactivation activities.

Keywords: copper/intramolecular interaction/Mac1/metalloregulation

Introduction

Although copper (Cu) is required for normal physiology (Vulpe and Packman, 1995; Linder and Hazegh-Azam, 1996), abnormally high levels of copper can be toxic and result in severe damage of tissues and organs. Transition metal ions such as copper can catalyze the formation of a highly reactive hydroxyl radical via the Fenton reaction (Fridovich, 1978). Copper-induced toxicity may arise from cell damage caused by this and other reactive oxygen intermediates (Imlay and Linn, 1988; Farr and Kogoma, 1991).

The budding yeast *Saccharomyces cerevisiae* has been used as a model system to study the mechanisms of copper detoxification, transport and distribution. Copper ion homeostasis in yeast is attained, in part, through regulated expression of genes involved in copper ion uptake and sequestration (Dancis *et al.*, 1994b; Hassett and Kosman,

1995). The mechanism of high-affinity copper uptake in *S.cerevisiae* includes cell surface permeases and metal ion reductases (Dancis *et al.*, 1990, 1994c; Knight *et al.*, 1996; Georgatsou *et al.*, 1997). Cu(II) is reduced to Cu(I) at the cell surface by the membrane-associated NADPH-dependent ferric and cupric reductases, Fre1 and Fre2 (Hassett and Kosman, 1995; Finegold *et al.*, 1996; Shatwell *et al.*, 1996; Georgatsou *et al.*, 1997). Cu(I) is then transported across the plasma membrane by the high-affinity copper transporters Ctr1 and Ctr3 (Dancis *et al.*, 1994c; Knight *et al.*, 1996). Under copper-limiting condition *CTR1*, *CTR3*, *FRE1* and a *FRE1*-homolog *FRE7* gene are highly expressed (Dancis *et al.*, 1994a; Hassett and Kosman, 1995; Labbe *et al.*, 1997; Yamaguchi-Iwai *et al.*, 1997; Martins *et al.*, 1998). However, the presence of elevated external concentrations of Cu(II) leads to the downregulation of these genes. Expression of these genes is mediated by the transcription factor Mac1 (metal-binding activator) (Jungmann *et al.*, 1993). Mac1 is an active transcriptional activator under Cu-limiting conditions, but is inhibited under Cu-replete conditions (Georgatsou *et al.*, 1997; Graden and Winge, 1997; Labbe *et al.*, 1997; Yamaguchi-Iwai *et al.*, 1997).

Transcription factors serve as integration centers for different signal transduction pathways that modulate gene expression (Ptashne, 1988; Mitchell and Tjian, 1989). The regulation of these regulators themselves is important for differential gene expression. Transcription factors can be regulated at two levels, concentration and activity. Many transcription factors are unstable and rapidly degraded (Kornitzer *et al.*, 1994). Degradation can occur constitutively or in response to stimuli. The activity of a transcription factor can also be regulated in its localization, DNA-binding or transactivation activity (Furst *et al.*, 1988; Mitchell and Tjian, 1989; Whiteside and Goodbourn, 1993; Zhang and Guarente, 1995; Vandromme *et al.*, 1996). Masking of the nuclear localization signal by a sequestering protein or by phosphorylation may hinder the factor from reaching the nucleus (Vandromme *et al.*, 1996). Induced nuclear export occurs for several transcription factors (Wen *et al.*, 1995). Alternatively, the DNA-binding and transactivation functions can be affected in many ways including post-translational modification (Mitchell and Tjian, 1989), ligand binding (Zhang and Guarente, 1995; Wang *et al.*, 1997; Yano and Fukasawa, 1997), intramolecular interactions (Johansen and Prywes, 1993; Li and Green, 1996; Wang *et al.*, 1997) and interaction with other proteins (Hahn, 1993; Momand *et al.*, 1992).

DNA-binding and transactivation activities map to distinct segments of the Mac1 polypeptide. The minimal DNA-binding domain maps to the N-terminal 159 residues (Jensen *et al.*, 1998). The activation domain resides in the C-terminal segment of the polypeptide (Graden and Winge,

1997; Zhu *et al.*, 1998). The mechanism of Cu inhibition of Mac1 function involves both Cu-dependent loss of *in vivo* DNA-binding activity (Labbe *et al.*, 1997) and Cu-dependent inhibition of transactivation function (Georgatsou *et al.*, 1997; Graden and Winge, 1997). Although Mac1 does not bind DNA *in vivo* in Cu-replete cells, it is curious that DNA binding *in vitro* does not show a Cu dependency using either *in vitro*-translated Mac1, yeast extracts containing epitope-tagged Mac1, or the purified minimal DNA-binding domain (Yamaguchi-Iwai *et al.*, 1997; Jensen *et al.*, 1998). Loss of *in vivo* DNA-binding activity of Mac1 may arise from Cu-dependent efflux of Mac1 from the nucleus. A Mac1- β -galactosidase fusion was shown previously to be localized in the nucleus (Jungmann *et al.*, 1993). Since the localization was not tested in Cu-deficient and Cu-supplemented cells, we re-investigated the localization of Mac1.

A semi-dominant mutation in *MAC1* (designated *MAC1^{up1}*) that is not inactivated by copper treatment contains a His→Gln substitution next to one of two Cys-rich motifs in the C-terminal domain of Mac1 (Jungmann *et al.*, 1993). The Cys-rich motifs consisting of Cys-X-Cys-X₄-Cys-X-Cys-X₂-Cys-X₂-His (where X is any amino acid) resemble Cu(I)-binding cysteinyl sequences found in metallothioneins and the Cu-activated transcription factors Ace1 from *S.cerevisiae* and Amt1 from *Candida glabrata*. The Mac1^{up1} H279Q substitution and Cys-rich motifs in Mac1 lie within the activation domain of Mac1 (Graden and Winge, 1997; Zhu *et al.*, 1998). The presence of the Cys-rich motifs similar to those present in other Cu-binding proteins suggests that Mac1 may also bind Cu ions. We show that Mac1 is a Cu-binding protein and that Cu binding to Mac1 induces a repressive intramolecular interaction.

Results

Mac1 is localized within the nucleus in Cu-deficient and Cu-replete yeast

The possibility that Cu modulation of Mac1 activity involves regulation of nuclear localization was re-investigated. The strategy was to fuse Mac1 with the green fluorescent protein (GFP) and monitor the distribution of the GFP fluorescence. A vector containing GFP fused to the 3' end of the *MAC1* ORF controlled by the *ADHI* promoter was transformed into YJJ1 cells lacking a functional Mac1. The Mac1-GFP fusion is a functional protein in that it activates expression of a *CTR1-lacZ* reporter gene in cells deficient in Cu due to growth in the presence of the Cu(I)-specific chelator bathocuproine sulfonate (BCS) (Figure 1A). Addition of excess copper to the medium results in the loss of activation, as is seen in wild-type *MAC1* cells (Figure 1A).

To evaluate localization of the Mac1-GFP, the fusion gene was placed under the control of the *GALI* promoter and transformed into the *ctr1Δ* strain YPH499. Fluorescence in cells expressing the Mac1-GFP fusion was concentrated into single spots that stained positively with the DNA-stain reagent 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI). The co-localization of Mac1-GFP and DNA suggests that Mac1-GFP is localized within the nuclei of the cells under both Cu-limiting and Cu-replete conditions (Figure 1B). As a control, cells

harboring only GFP showed uniform fluorescence throughout the cell, independent of the Cu levels in the growth medium. It is unlikely that the nuclear localization of Mac1 in Cu-replete cells is an artifact of overexpression of Mac1-GFP. First, the activity of the fusion protein shows the same fold Cu inhibition as does the chromosomally encoded Mac1. Secondly, there was no evidence of low-abundance diffuse cytoplasmic fluorescence in these cells.

Mac1 is a Cu-binding protein

Cu regulation of Mac1 activity within the nucleus may arise from direct interaction of Cu ions with Mac1. To test whether Mac1 is a Cu-binding protein, a *MAC1-GST* fusion gene was constructed for bacterial expression of Mac1-GST (C-terminal GST). The fusion gene was active in yeast and the activity was Cu modulated (data not shown). The expressed fusion protein was recovered as an insoluble inclusion body. The particulate material isolated from *Escherichia coli* cultured in Cu-containing medium was highly emissive upon ultraviolet irradiation. Orange luminescence is indicative of Cu(I) coordination. The luminescent particulate material was dependent on induction of the Mac1-GST fusion. Co-transformation of bacteria with the vector containing the *MAC1-GST* fusion and a second vector containing the *E.coli* thioredoxin vector resulted in limited expression of soluble Mac1-GST. The fusion protein, purified by affinity chromatography on glutathione-Sepharose, gave a single band on SDS-PAGE which was consistent with the calculated molecular mass of 73 kDa. Metal analysis on the purified fusion protein revealed 7.9 ± 0.2 mol eq. bound Cu and 1.9 ± 0.1 mol. eq. Zn (Table I). The two Zn ions are presumably bound within the N-terminal DNA-binding domain, since the N-terminal 159 residues of Mac1 were shown previously to bind 2 mol. eq. Zn(II) (Jensen *et al.*, 1998). The Cu content of the Mac1^{up1} mutant containing a H279G substitution was only 4.4 ± 0.2 mol. eq. Significantly less soluble protein was isolated from cells cultured in low Cu-containing medium, suggesting that Cu ions stabilized the expressed protein.

The candidate Cu-binding domain of Mac1 consists of the two Cys-rich motifs comprising residues 252–341. This peptide expressed in *E.coli* was also isolated within insoluble inclusion bodies. Co-expression with thioredoxin resulted in the production of soluble protein. Glutathione-Sepharose was used to affinity purify Mac1(252–341) and H279Q Mac1(252–341) peptides as N-terminal GST fusions. The purified fusion proteins gave a single band by SDS-PAGE consistent with the calculated molecular mass of 36 kDa. Metal analysis revealed that the GST-Mac1(252–341) fusion protein contained 7.7 ± 0.4 bound Cu ions and, as expected, no bound Zn ions (Table I). The metal content of the GST-H279Q Mac1(252–341) fusion was determined to be 4.5 ± 0.5 Cu ions and, unexpectedly, two Zn ions per protein (Table 1).

The Cu complex of Mac1(252–341) was luminescent with an emission maximum near 570 nm (Figure 2). Similar emission is observed in a series of proteins with polycopper thiolate clusters (Winge *et al.*, 1994). The emission is indicative that Mac1(252–341) and, presumably, Mac1 bind copper ions as Cu(I). With equal concentrations of copper, the emission of the H279Q protein was

Fig. 1. Cellular localization of Mac1. (A) A C-terminal fusion of Mac1 with the GFP (Mac1-GFP) activates expression of a *CTR1-lacZ* reporter gene. (B) Fluorescence of cells harboring the Mac1-GFP fusion cultured in Cu-deficient (30 μM bathocuproine sulfonate, BCS) and Cu-replete (100 μM CuSO₄) medium. Cells harboring the fusion gene and GFP under the control of the *GALI* promoter were grown at 30°C in synthetic complete media containing galactose in place of dextrose. GFP was excited at 488 nm and detected at 520 nm; DAPI was excited at 345 nm and detected at 465 nm.

lower than that seen for wild-type Mac1(252–341). The observed lower emission in the mutant molecule suggests greater solvent quenching of the Cu-thiolate fluorophore that may arise from a less stably folded conformer.

Mac1 is stabilized in Cu-replete cells

We attempted to show that Mac1 is a Cu-binding protein in Cu-replete yeast using affinity purification of Mac1-GST. Although we were unable to purify the fusion protein, we obtained indirect evidence using Western analysis showing that Cu ions enhanced the *in vivo* stability of Mac1. Yeast expression vectors were constructed to express either *MAC1* or *MAC1^{up1}* with 3' myc epitope tags. The presence of the C-terminal myc tag did not impair Mac1 function. *MAC1*-myc epitope fusions under the control of the *ADHI* promoter were co-transformed with the *CTR1-lacZ* reporter into the *mac1-1* strain YJJ1. Both Mac1-myc and H279Q Mac1-myc stimulated *lacZ* expression under Cu-deficient conditions (Figure 3A). With the addition of excess copper to the growth medium, β-galactosidase activity was substantially reduced in cells expressing Mac1-myc. In contrast, β-galactosidase activity of cells expressing H279Q Mac1-myc was unchanged (Figure 3A).

Table I. Metal-binding stoichiometry of Mac1 and Mac1 domains

	Mol. eq. metal ions bound	
	Cu	Zn
Mac1	7.9 ± 0.2	1.9 ± 0.1
Mac1 ^{up1}	4.4 ± 0.2	2.0 ± 0.1
Mac1 (252–341)	7.7 ± 0.4	ND
Mac1 ^{up1} (252–341)	4.5 ± 0.5	1.9 ± 0.3

ND, none detected.

Metal to protein stoichiometry of the purified Mac1-GST fusions were determined from at least two independent isolates. Protein concentrations were determined using quantitative amino acid analysis and metal ion concentrations by atomic absorption spectroscopy. Errors shown are the standard deviations of triplicate measurements. The minimal DNA-binding domain of Mac1 (residues 1–159) binds two Zn(II) ions per molecule (Jensen *et al.*, 1998).

To assess protein stability *in vivo*, *MAC1* and *MAC1^{up1}* with 3' myc epitope tags were subcloned into an expression vector controlled by the *GALI* promoter. The fusion genes were preinduced by a short, 3 h incubation in galactose-containing medium. Glucose was then added to repress further transcription. Whole-cell extracts were examined

Fig. 2. Luminescence of CuMac1(252–341). The Cu(I) luminescence of purified Mac1(252–341) and H279Q Mac1(252–341) was measured using equimolar Cu(I) concentrations (100 nmol Cu/ml). Excitation was at 300 nm, and emission was scanned from 510–620 nm with maximal emission occurring near 570 nm.

at various times after the addition of glucose ($\pm$ CuSO₄) by immunoblotting, using a monoclonal anti-c-myc antibody. Under Cu-deficient conditions Mac1 was significantly degraded within 90 min, but in the presence of elevated levels of CuSO₄, remained stable for at least 3 h (Figure 3B). The increase in stability of Mac1 was also seen in wild-type cells cultured in standard medium or in *ctr1Δ* cells cultured in as little as 10 μ M CuSO₄ (data not shown). The H279Q Mac1^{up1} mutant protein was significantly more stable than the wild-type molecule under Cu-deficient conditions, although the turnover of the H279Q Mac1 protein was unaffected by exposure to elevated levels of CuSO₄ (Figure 3C).

Cu induces an intramolecular interaction in Mac1

In vivo DNA-binding and transactivation activities of Mac1 are inhibited in Cu-replete cells (Georgatsou *et al.*, 1997; Graden and Winge, 1997; Labbe *et al.*, 1997). The two activities are independent and separable. The minimal DNA-binding domain (DBD) of Mac1 consists of residues 1–159 (Jensen *et al.*, 1998). Although the exact boundaries of the transactivation domain are not known, residues 240–417 contain transactivation activity (Graden and Winge, 1997). Neither of these minimal modules are regulated by Cu ions. Cu-modulation of transactivation activity requires sequences upstream of the actual activation domain (AD) (Graden and Winge, 1997). These results led to the hypothesis that Cu binding to Mac1 may result in direct inhibition of both activities through a Cu-induced intramolecular interaction.

To test for an intramolecular interaction between the N-terminal DBD and the C-terminal activation domain, a two-hybrid-based assay was used. The Mac1 DBD (residues 1–159) was fused to the minimal Gal4 DBD (lacking any transactivation activity) in the first expression vector [Mac1(1–159)–Gal4]. The second vector consisted of the Mac1 AD fused to the VP16 activation domain of the herpes simplex virus (Sadowski *et al.*, 1988) (Figure 4). Two constructs were engineered for the AD vector, one consisting of Mac1(240–417), and the second H279Q Mac1(240–417). The Mac1(1–159)–Gal4 DBD vector was co-transformed into yeast strain Y190 with AD vectors

Fig. 3. Copper-induced stabilization of Mac1. (A) Fusions of a myc epitope to either Mac1 or Mac1^{up1} activate expression of a *CTR1-lacZ* reporter gene. Expression of Mac1–myc (B) or Mac1^{up1}–myc (C) was induced with galactose for 3 h prior to addition of glucose. Cultures were then split; one aliquot was untreated and 100 μ M CuSO₄ was added to the other. Samples from each culture were removed at 45 min intervals. Cell extracts were separated by SDS–PAGE, transferred to nitrocellulose and then immunoblotted with a monoclonal anti-c-myc antibody and an anti-actin antibody (to control for gel loading).

encoding Mac1(240–417)–VP16, H279Q Mac1(240–417)–VP16 or the empty plasmid. Expression of the *GAL1-lacZ* reporter gene was assessed.

If a Cu-induced Mac1 intramolecular interaction occurs, Cu may promote the heterodimerization of the Mac1 DBD and AD expressed as separate fusion proteins and yield expression of *lacZ*. Therefore, β -galactosidase activity would be observed in Cu-treated cells containing both Mac1(1–159)–Gal4 and Mac1(240–417)–VP16. No β -galactosidase activity would be expected in cells containing Mac1(1–159)–Gal4 and H279Q Mac1(240–417)–VP16 as the H279Q substitution in Mac1^{up1} abrogates Cu-repression (Jungmann *et al.*, 1993; Graden and Winge, 1997; Labbe *et al.*, 1997).

β -galactosidase was assayed in co-transformants cultured with either the Cu-specific chelator BCS added to generate Cu-deficient conditions or with CuSO₄ added to the growth media. Only low levels of β -galactosidase were observed in Cu-deficient cells (Figure 4). However, with the addition of CuSO₄, substantial β -galactosidase activity was observed in cells containing Mac1(1–159)/Gal4 and Mac1(240–417)/VP16. As predicted, the Cu-induced interaction was precluded with either H279Q Mac1(240–417)/VP16 or the VP16 control plasmid.

β -galactosidase activity in Cu-treated cells with the latter two combinations was no greater than the activity observed for Cu-deficient cells (BCS-treated) (Figure 4).

Fusion of the minimal DBD and AD segments creates a functional Mac1

To determine whether a functional copper-regulated Mac1 could be generated by using the minimal DBD and Cu-

Fig. 4. Identification of a Cu-induced intramolecular interaction in Mac1. An intramolecular interaction between the N-terminal DNA-binding domain (DBD) and the C-terminal activation domain of Mac1 was identified using a two-hybrid approach. The Mac1 DBD Mac1(1–159) was fused to the Gal4 DBD, while Mac1(240–417) and H279Q Mac1(240–417) were fused to the VP16 activation domain. The dots shown in the fusion construct are cysteinyl residues in Mac1. Cells were grown under Cu-deficient conditions (30 μ M BCS), or with excess copper added to the growth media. The β -galactosidase activity of a *GAL1-lacZ* reporter was measured in each case. Each sample was assayed in triplicate.

binding domains, the minimal Mac1 DBD (residues 1–159) was fused in-frame to the minimal Cu-binding activation domain (residues 252–341) yielding Mac1-(DBD+AD). This minimal Mac1(DBD+AD) contains 248 residues, unlike the 417 residues in the intact Mac1. The ability of *MAC1* (DBD+AD) and wild-type *MAC1* both under control of the *ADH1* promoter to activate a *CTR1-lacZ* reporter were compared (Figure 5). The activity of Mac1(DBD+AD) was similar to that of wild-type Mac1. Mac1(DBD+AD) expressed high levels of β -galactosidase under Cu-deficient conditions. The level of β -galactosidase expression was substantially reduced in Cu-replete cells.

Discussion

Mac1 is responsible for Cu-dependent regulation of *CTR1*, *FRE1*, *CTR3* and *FRE7* (Labbe *et al.*, 1997; Martins *et al.*, 1997; Yamaguchi-Iwai *et al.*, 1997). Mac1 is a transcriptional activator of these genes in Cu-deficient cells, but is inhibited in Cu-replete cells. The mechanism of Cu repression of Mac1 has been investigated. Two observations reported previously include Cu-dependent loss of *in vivo* DNA-binding activity (Labbe *et al.*, 1997) and Cu-dependent repression of transactivation function (Georgatsou *et al.*, 1997; Graden and Winge, 1997). The mechanism of Cu-inhibition of *in vivo* DNA binding by Mac1 was unclear, since DNA binding *in vitro* does not show a Cu dependency using either *in vitro* translated Mac1, yeast extracts containing epitope-tagged minimal DBD of Mac1 or the purified minimal DBD (Yamaguchi-Iwai *et al.*, 1997; Jensen *et al.*, 1998).

The loss of *in vivo* DNA-binding activity of Mac1 may arise from Cu-dependent efflux of Mac1 from the nucleus. This was found to be not true, as Mac1 is localized within the nucleus in both Cu-deficient and Cu-replete cells. The nuclear localization of Mac1 during Cu deprivation and when excess copper is present was determined using the GFP fused to the C-terminus of Mac1. Fluorescence due to the Mac1-GFP fusion was co-localized with DAPI staining of DNA and showed clearly that Mac1-GFP is concentrated in the nuclei of the cells, independent of the cellular Cu status. This is agreement with an earlier report

Fig. 5. Minimal Mac1. A minimal Mac1 construct was generated by fusing the Mac1 minimal DNA-binding domain (DBD) (residues 1–159) with the Cu-binding activation domain (residues 252–341) and placed under control of the *ADH1* promoter and terminator. The dots in the scheme of the constructs represent cysteinyl residues in Mac1. β -galactosidase activities of Mac1 and Mac1(DBD+AD) were assayed in the *mac1-1* strain YJJ1 using a *CTR1-lacZ* reporter. Each sample was assayed in triplicate.

showing nuclear localization of a Mac1- β -galactosidase fusion (Jungmann *et al.*, 1993).

Cu-repression of Mac1 function may arise from a direct effect of Cu ions on Mac1, or alternatively from an indirect effect of a Cu-regulatory molecule. We observe that Mac1 is itself a Cu-binding protein. Cu binding occurs in the C-terminal Cys-rich motifs of Mac1. Two repeats of a Cys-X-Cys-X₄-Cys-X-Cys-X₂-Cys-X₂-His sequence motif are present; the second repeat contains an additional upstream Cys-X-Cys motif. A peptide containing both Cys-rich motifs (residues 252–341) binds eight Cu(I) ions. This is the same Cu-binding stoichiometry of full-length Mac1. Intact Mac1 binds two Zn(II) ions, as does the minimal DBD (residues 1–159) (Jensen *et al.*, 1998). The His279Gln substitution in the first Cys-rich motif (Mac1^{up1} allele) that abrogates Cu-repression of Mac1 function *in vivo* results in a diminution in bound Cu(I) ions to 4.4 mol. eq. The possibility exists that the two Cys-rich repeats enfold separate Cu-thiolate clusters. The significant decrease in bound Cu in the Mac1^{up1} molecule may suggest that the remaining four to five Cu ions are associated with the second repeat. Whether His279 is a metal ligand or a structurally important residue remains unclear.

The bound Cu ions are in the Cu(I) valence state by virtue of the observed luminescence of the CuMac1 complexes with Mac1(252–341) and H279Q Mac1(252–

341) peptides. Similar emission is seen in proteins with Cu-thiolate clusters such as Cu-metallothioneins and CuAce1. With equal concentrations of copper the emission of the H279Q protein was lower than that seen for wild-type Mac1(252–341). The intensity of Cu(I) luminescence has been correlated with the solvent accessibility of the Cu(I) ions. The low emission of H279Q CuMac1 complex suggests the mutant molecule is structurally different from the wild-type protein.

Although we were unable to purify Mac1 from Cu-treated yeast to verify Cu binding, we demonstrated that the turnover of Mac1 was retarded in Cu-replete cells. Using a Mac1–myc fusion we observed that Mac1 was significantly degraded within 90 min in Cu-deficient cells after a pulse of Mac1 synthesis. However, in the presence of elevated levels of CuSO₄, Mac1 remained stable for at least 3 h. The Cu-induced stabilization of Mac1 is suggestive of direct Cu binding to Mac1 in yeast. The observed stabilization presumably arises from the Cu-induced allosteric interaction in Mac1. In contrast, H279Q Mac1–myc was not stabilized by the addition of CuSO₄ to the growth medium. While the H279Q Mac1 protein was more stable than the wild-type under Cu-deficient conditions, the turnover of H279Q Mac1 was unaffected by exposure to elevated levels of copper. The lack of Cu stabilization of H279Q Mac1 is consistent with the inability of Cu to induce the allosteric switch in the mutant protein. In addition, these results argue against degradation of Mac1 being responsible for the loss of Mac1 activity in Cu-replete cells. This is in agreement with a recently published study (Zhu *et al.*, 1998). However, increasing the Cu load of cells results in degradation of Mac1 only when Mac1 is expressed at low levels (Zhu *et al.*, 1998).

We showed previously that Cu-inhibition of transactivation activity of Mac1 in a Gal4–Mac1 fusion requires sequences within the Mac1 DBD (Graden and Winge, 1997). A Gal4(DBD)–Mac1 fusion containing residues 42–417 of Mac1 can activate transcription of a *GAL1–lacZ* reporter gene under Cu-deficient conditions and was inactivated by the addition of copper (Graden and Winge, 1997). However, a Gal4–Mac1 fusion containing residues 176–417 of Mac1 was not inactivated in Cu-replete cells. These results demonstrated that neither DNA binding nor transactivation activity of Mac1 was Cu modulated with isolated domains. We postulate that Cu repression arises from a Cu-induced intramolecular interaction involving DBD and AD sequences.

A Cu-induced interaction of the C-terminal activation domain and the N-terminal DBD was observed in a two-hybrid assay system. Co-expression of two fusion proteins consisting of the Mac1 DBD (residues 1–159) fused to the Gal4 DBD and the C-terminus activation domain of Mac1 (residues 240–417) fused to the VP16 AD resulted in an *in vivo* protein–protein interaction as determined by expression of the *GAL1–lacZ* reporter. This protein–protein interaction was Cu induced and, as expected, was precluded under Cu-starvation conditions. No intramolecular interaction was seen when the Mac1 AD contained the H279Q substitution that abrogates Cu-repression of Mac1 in the intact protein.

The first Cys-rich motif (residues 264–279) appears to be an important determinant of the intramolecular interaction. Mutations in each of the Cys codons in this

Fig. 6. Model of the Cu-induced intramolecular interaction. DNA-binding and transactivation domains are shown by hatching and shaded boxes, respectively. The dots in the figures represent cysteinyl residues.

first repeat abolish Cu regulation of Mac1 function (Graden and Winge, 1997). Surprisingly, Cys codon mutations in the second repeat do not alter Cu modulation of Mac1. The two Cys-rich repeats may have different functions. Cu binding to the first Cys-rich motif may induce the molecular switch, leading to the repressive interaction. According to this model, the attenuation in Cu(I) binding in H279Q Mac1 may preclude the Cu-induced conformational change.

To narrow further the regions of Mac1 necessary for Cu repression, the minimal N-terminal DBD (residues 1–159) was fused to the C-terminal Cu-binding activation domain (residues 252–341). This minimal construct was sufficient to activate expression of a *CTR1–lacZ* reporter and was inactivated by the addition of copper. This demonstrates that these two domains are necessary and sufficient for activation of Mac1 as well as Cu-modulated inactivation. A stretch of 93 residues separating the two functional domains are dispensable and apparently form a connecting link between the two functional regions of Mac1. A model of the Cu-induced intramolecular interaction is shown in Figure 6.

Inhibitory intramolecular interactions are important in the regulation of the transcription factors, Leu3, ATF-2 and HSF1 (Zhou and Kohlhaw, 1990; Jakobsen and Pelham, 1991; Bonner *et al.*, 1992; Li and Green, 1996; Wang *et al.*, 1997). The *S.cerevisiae* Leu3 is ligand-modulated by α -isopropylmalate (Zhou and Kohlhaw, 1990; Wang *et al.*, 1997). Without this signal molecule the activation domain is masked by an intramolecular interaction. Similarly, the transcriptionally inactive state of ATF-2 results from a suppression of the activation activity by the DBD (Li and Green, 1996). The intramolecular interaction in HSF1 can also repress a heterologous activation domain fused to the protein (Bonner *et al.*, 1992). Inducers of Leu3, ATF-2 and HSF1 disrupt these interactions to activate transcription (Jakobsen and Pelham, 1991; Li and Green, 1996; Wang *et al.*, 1997). In contrast, Cu(I) binding by Mac1 induces the repressive intramolecular interaction.

A Cu-dependent interaction between the N-terminal DBD and the C-terminal activation domain can explain the observed loss of transactivation activity and *in vivo* DNA-binding activity in response to copper. Activation domains tend to be flexible modules (Cho *et al.*, 1996; Kussie *et al.*, 1996; Shen *et al.*, 1996). Under Cu-deficient conditions the lack of a repressive interaction between the N- and C-terminal segments in Mac1 may leave the activation domain solvent exposed and poised for interactions with general transcription factors within the mediator complex (Zawel and Reinberg, 1995). In contrast, the Cu-induced repressive interaction may stabilize the

structure in the AD region, resulting in decreased susceptibility to proteolysis of Mac1 and masking of AD activity.

The Cu-dependent intramolecular interaction likely results in the masking of the DBD preventing the formation of the Mac1–DNA complex and abolishing high-affinity DNA binding in response to copper. The clear prediction is that DNA binding by full-length Mac1 will be attenuated *in vitro*. We were unsuccessful in verifying this prediction with purified intact Mac1 as the complex was unstable during gel electrophoretic conditions. Further mapping studies are in progress to map the residues important in stabilizing the repressive intramolecular interaction.

Materials and methods

Strains and culture conditions

The strains used in this study include YPH499Δ *ctr1* (*MATa*, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *lys2-801*, *trp1-Δ63*, *ura3-52*, *Δctr1::LEU2*) (Dancis *et al.*, 1994c), YW02 (*MATa* *his3-Δ200 leu2-3,112*, *lys2-801 trp1-1*, *ura3-52*), YJ11 (*MATa*, *his3-Δ200*, *leu2-3, 2-112*, *lys2-801*, *trp1-1*, *ura3-52*, *mac1-1*) (Jungmann *et al.*, 1993) and Y190 (*MATa*, *ade2-101*, *cyh2*, *his3*, *leu2-3,112*, *trp1-901*, *ura3-52*, *gal4*, *gal80*, *LYS2:pGAL-HIS3*, *URA3:pGAL-lacZ*) (Bai and Elledge, 1997). DNA transformations were performed using the lithium acetate procedure. Cells were propagated either in YPD or synthetic complete medium plus dextrose agar plates. Transformed cells were grown in liquid complete medium lacking the appropriate nutrients.

Plasmid construction

Mac1–GFP fusions. Sequences coding for the GFP were amplified by PCR using vector pEGFP–C1 (Clontech) as a template creating 5' *Bam*HI and *Cl*aI sites and 3' *Not*I and *Sal*I sites. The PCR product was digested with *Bam*HI–*Not*I and subcloned into vector pYeF2 which uses the *GAL*I promoter generating GFP–pYeF2. *MAC1* and *MAC1^{up1}* were amplified by PCR creating 5' *Bam*HI and *Afl*III sites, 3' *Cl*aI and *Hind*III sites, and removing the stop codon. The PCR product was digested *Bam*HI–*Hind*III, subcloned into pHolly (a pBluescript derivative kindly provided by R.D.Palmiter with a small insert creating sites for *Nde*I, *Nco*I and *Sna*I) and sequenced. *MAC1* was excised from pHolly by digestion with *Bam*HI and *Cl*aI and subcloned into GFP–pYeF2 generating Mac1GFP–pYeF2.

Mac1–myc tag fusions. Six copies of the c-myc epitope were excised from vector CS2+MT (Rupp *et al.*, 1994) as a *Bam*HI–*Sna*I fragment and subcloned into pHolly to pick up a stop codon and a 3' *Sal*I site. The myc epitope was then removed from pHolly as a *Bam*HI–*Sal*I fragment and ligated into GFP–pYeF2 digested *Bam*HI–*Sal*I, thus removing sequences for GFP. *MAC1* and *MAC1^{up1}* were excised from pHolly by digestion with *Bam*HI and *Cl*aI and subcloned into Myc–pYeF2 generating Mac1–myc–pYeF2 and H279Q Mac1–myc–pYeF2.

pVT102-U vectors. The *MAC1* ORF was PCR-amplified with 5' *Bam*HI and 3' *Sal*I sites retaining the stop codon. The PCR product digested with *Bam*HI–*Sal*I was subcloned into pHolly and sequenced. *MAC1*, *MAC1*–myc and *MAC1*–GFP were excised from pHolly using *Bam*HI and *Sal*I and subcloned into pVT102-U resulting in vectors Mac1–pVT102-U, Mac1–myc–pVT102-U and Mac1–GFP–pVT102-U. GST was excised from pAED4–GST as a *Cl*aI–*Not*I fragment and subcloned into Mac1–myc–pVT102-U replacing the myc-tag with sequences for GST generating Mac1–GST–pVT102-U. Sequences coding for codons 252–341 of *MAC1* were amplified by PCR, creating a 5' *Bam*HI and *Not*I sites and 3' *Sal*I and *Hind*III sites and stop codon after codon 341. The PCR product was subcloned into pVT102-U using *Bam*HI and *Hind*III sites generating vector Mac1(252–341)pVT102-U. *MAC1* (codons 1–159) was excised from Mac1(1–159)–myc (Jensen *et al.*, 1998) as a *Bam*HI–*Not*I fragment and subcloned into Mac1(252–341)pVT102-U generating Mac1(DBD+AD)pVT102-U. The expression of constructs in vector pVT102-U is constitutive under the control of the *ADH1* promoter.

Escherichia coli expression vectors. *MAC1* and *MAC1^{up1}* were excised from pHolly by digestion with *Nco*I and *Cl*aI and subcloned into a T7-based vector Mac1(1–159)pAED4–GST (Jensen *et al.*, 1998) to generate C-terminal GST fusion expression vectors. The codons for the first 24 amino acids were optimized for expression in *E.coli* (Anderson and

Kurland, 1990). DNA sequences coding for residues 252–341 of Mac1 as well as H279Q Mac1 were amplified by PCR, generating a 5' *Bam*HI site with a ATG start codon and a 3' *Not*I site with an upstream stop codon. The PCR products were subcloned into pGEX-4T-1 (Pharmacia) to generate N-terminal GST fusions.

Two-hybrid vectors. Sequences coding for codons 240–417 of the *MAC1* ORF were PCR-amplified. The PCR products contained 5' *Bam*HI and 3' *Cl*aI sites and the stop codon was removed. The PCR products were subcloned into vector pVT–VP16 (Jensen *et al.*, 1998), generating vectors Mac1(240–417)–VP16 and H279QMac1(240–417)–VP16 with a fusion of Mac1(240–417) with the minimal activation domain (AD) from the herpes simplex virus-1 VP16 protein (Sadowski *et al.*, 1988). Sequences coding for amino acids 1–159 were excised from vector Mac1(1–159)–VP16 as a *Bam*HI–*Xho*I fragment and subcloned into pBG4D-1 cut *Bam*HI–*Sal*I, destroying both the *Sal*I and *Xho*I sites creating vector Mac1(1–159)pBG4D-1.

Immunofluorescence

Mac1–GFP–pYeF2 and GFP–pYeF2 in YPH499Δ *ctr1* were grown in low-copper synthetic complete media lacking uracil with 2% raffinose. Cells were grown at 30°C to OD₆₀₀ of 1.0, diluted 100-fold and then split into three, 10 ml aliquots. One aliquot was the untreated control, galactose (2% final) and 30 μM BCS (Cu-specific chelator bathocuproine sulfonate) were added to the second, and galactose and 100 μM CuSO₄ were added to the third. After growth to OD₆₀₀ of 1.0, 0.5 ml of 37% formaldehyde was added and the cells were incubated for an additional 10 min. The cells were harvested, resuspended in 5 ml of buffer A (40 mM KPO₄ pH 6.5, 0.5 M MgCl₂), plus 0.5 ml of 37% formaldehyde and grown for an additional 1 h. The cells were then washed twice in buffer A and once in buffer A plus 1.2 M sorbitol. Spheroplasts were prepared and spotted onto polylysine-coated slides and incubated at 25°C for 10 min. Mount solution (1 mg/ml *p*-phenylenediamine, 20 mM sodium carbonate pH 9, 90% glycerol, 50 ng/ml DAPI) was overlaid on the slides. Cells were viewed using a fluorescence microscope. The GFP was excited at 488 nm and detected at 520 nm; DAPI was excited at 345 nm and detected at 465 nm.

Western blot analysis

Strain YPH499Δ *ctr1* transformed with either Mac1–myc–pYeF2 or H279Q Mac1–myc–pYeF2 under the control of the *GAL*I promoter were grown in low-copper synthetic complete media lacking uracil containing 2% raffinose in place of dextrose to an OD₆₀₀ of 1.0. Mac1–myc and H279Q Mac1–myc expression was induced by the addition of galactose (2%) to the growth medium. Cells were grown for 3 h, at which time glucose was added to 2% to stop induction, and the culture was split. To one aliquot was added 100 μM CuSO₄ (at the same time as glucose), while the other aliquot was untreated. At 45 min intervals, 10 ml of each culture was removed and cells were harvested, washed and flash-frozen. The cells were lysed with glass beads and centrifuged at 4°C for 5 min at 10 000 g. Total protein was quantified by the method of Bradford (1976). SDS–PAGE was carried out with 20 μg protein using a 7.5% gel. Proteins were transferred to nitrocellulose and the blots incubated with monoclonal anti-c-myc and anti-actin antibodies in blocking buffer (10% non-fat dry milk, phosphate-buffered saline, 0.1% Tween-20). Detection by enhanced chemiluminescence (ECL) was performed after incubation with a horseradish peroxidase-conjugated anti-mouse antibody.

Purification of Mac1 and Mac1(252–341)

Mac1 expressed in *E.coli* was insoluble and present in inclusion bodies, as was Mac1(252–341). Co-expression of *E.coli* thioredoxin increases the solubility of some proteins expressed in *E.coli* (Lutsenko and Kaplan, 1995). Expression of either Mac1–GST or GST–Mac1(252–341) fusions with thioredoxin increased the level of soluble protein. GST fusions were co-expressed with thioredoxin in BL21 (DE3) cells pregrown at 30°C to an OD₆₀₀ of 0.5 prior to the addition of IPTG. At 30 min after IPTG induction, 1.4 mM CuSO₄ was added and the cells were grown for an additional 4 h. The harvested cells were washed with 0.25 M sucrose and resuspended in buffer (20 mM NaH₂PO₄ pH 7.5, 250 mM NaCl, 10% glycerol, 5 mM DTT) for sonication. The lysate was clarified at 100 000 g for 30 min at 4°C. Triton X-100 was added to 1% and the extract was purified on a glutathione–Sepharose column. The Mac1–GST fusion proteins were eluted with 20 mM glutathione in sonication buffer. The eluted proteins were monitored by SDS–PAGE and a single band at the expected molecular weight was observed for each fusion.

Protein and metal analyses

Protein samples were quantified by amino acid analysis following hydrolysis in 5.7 M HCl *in vacuo*. Metal analyses were performed on a Perkin-Elmer Analyst 100 atomic absorption spectrometer. Cu(I) luminescence measurements were performed on a Perkin-Elmer 650-10S fluorometer with a band-pass filter. Excitation was at 300 nm.

β -galactosidase assays

Plasmids Mac1-pVT102-U, Mac1-myc-pVT102-U, H279Q Mac1-myc-pVT102-U, Mac1-GFP-pVT102-U and Mac1(DBD+AD)-pVT102-U were co-transformed with a *CTR1-lacZ* reporter into the *mac1-1* yeast strain YJJ1. Plasmids Mac1(240–417)/VP16, H279Q Mac1(240–417)-VP16 or pVT-VP16 were co-transformed with Mac1(1–159)pBG4D-1 into yeast strain Y190 which contains an integrated *GAL-lacZ* reporter gene. Yeast transformants were grown in low-copper synthetic complete media with 2% dextrose and lacking either leucine and uracil for YJJ1 transformants or leucine and tryptophan for Y190 transformants. The cells were grown to an OD₆₀₀ of 1.0. The cultures were then diluted 100-fold with the addition of either 30 μ M BCS or 100 μ M CuSO₄ added to the growth medium and grown to a final OD₆₀₀ of 1.0. Harvested cells were washed with buffer (85 mM Na₂HPO₄, 45 mM NaH₂PO₄, 10 mM KCl, 85 mM β -mercaptoethanol, 1 mM MgSO₄) and frozen at –70°C. Cells were resuspended in the same buffer and lysed by vortexing with glass beads. β -galactosidase activities were assayed using *o*-nitrophenyl β -D galactopyranoside as a substrate. The absorbance at 420 nm was recorded and protein concentrations were determined by the method of Bradford (1976). Each sample was assayed in triplicate.

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References

- Anderson, S.G.E. and Kurland, C.G. (1990) Codon preferences in free-living microorganisms. *Microbiol. Rev.*, **54**, 198–210.
- Bai, C. and Elledge, S.J. (1997) Gene identification using the yeast two-hybrid system. *Methods Enzymol.*, **283**, 141–156.
- Bonner, J.J., Heywood, S. and Fackenthal, D.L. (1992) Temperature-dependent regulation of a heterologous transcriptional activation domain fused to yeast shock transcription factor. *Mol. Cell. Biol.*, **12**, 1021–1030.
- Bradford, N.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
- Cho, H.S., Liu, C.W., Damberger, F.F., Pelton, J.G., Nelson, H.C.M. and Wemmer, D.E. (1996) Yeast heat shock transcription factor N-terminal activation domains are unstructured as probed by heteronuclear NMR spectroscopy. *Protein Sci.*, **5**, 262–269.
- Dancis, A., Roman, D.G., Anderson, G.J., Hinnebusch, A.G. and Klausner, R.D. (1990) Ferric reductase of *Saccharomyces cerevisiae*: molecular characterization, role in iron uptake, and transcriptional control by iron. *Proc. Natl Acad. Sci. USA*, **89**, 3869–3873.
- Dancis, A., Haile, D., Yuan, D.S. and Klausner, R.D. (1994a) The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). *J. Biol. Chem.*, **269**, 25660–25667.
- Dancis, A., Haile, D., Yuan, D.S. and Klausner, R.D. (1994b) The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. *J. Biol. Chem.*, **269**, 25660–25667.
- Dancis, A., Yuan, D.S., Haile, D., Askwith, C., Elde, D., Moehle, C., Kaplan, J. and Klausner, R.D. (1994c) Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. *Cell*, **76**, 393–402.
- Farr, S.B. and Kogoma, T. (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol. Rev.*, **55**, 561–585.
- Finegold, A.A., Shatwell, K.P., Segal, A.W., Klausner, R.D. and Dancis, A. (1996) Intramembrane bis-heme motif for transmembrane electron transport conserved in a yeast iron reductase and the human NADPH oxidase. *J. Biol. Chem.*, **271**, 31021–31024.
- Fridovich, I. (1978) The biology of oxygen radicals. *Science*, **201**, 875–880.
- Furst, P., Hu, S., Hackett, R. and Hamer, D. (1988) Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein. *Cell*, **55**, 705–717.
- Georgatsou, E., Mavrogianis, L.A., Fragiadakis, G.S. and Alexandraki, D. (1997) The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *J. Biol. Chem.*, **272**, 13786–13792.
- Graden, J.A. and Winge, D.R. (1997) Copper-mediated repression of the activation domain in the yeast Mac1p transcription factor. *Proc. Natl Acad. Sci. USA*, **94**, 5550–5555.
- Hahn, S. (1993) Structure (?) and function of acidic transcription activators. *Cell*, **72**, 481–483.
- Hassett, R. and Kosman, D.J. (1995) Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **270**, 128–134.
- Imlay, J.A. and Linn, S. (1988) DNA damage and oxygen radical toxicity. *Science*, **240**, 1302–1309.
- Jakobsen, B.K. and Pelham, H.R.B. (1991) A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor. *EMBO J.*, **10**, 369–375.
- Jensen, L.T., Posewitz, M.C., Srinivasan, C. and Winge, D.R. (1998) Mapping of the DNA binding domain of the copper-responsive transcription factor Mac1 from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, in press.
- Johansen, F.-E. and Prywes, R. (1993) Identification of transcriptional activation and inhibitory domains in serum response factor (SRF) by using GAL4-SRF constructs. *Mol. Cell. Biol.*, **13**, 4640–4647.
- Jungmann, J., Reins, H.A., Lee, J., Romeo, A., Hassett, R., Kosman, D. and Jentsch, S. (1993) MAC1, a nuclear regulatory protein related to Cu dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. *EMBO J.*, **12**, 5051–5056.
- Knight, S.A.B., Labbe, S., Kwon, L.F., Kosman, D.J. and Thiele, D.J. (1996) A widespread transposable element masks expression of a yeast copper transport gene. *Genes Dev.*, **10**, 1917–1929.
- Kornitzer, D., Raboy, B., Kulka, R.G. and Fink, G.R. (1994) Regulated degradation of the transcription factor Gcn4. *EMBO J.*, **13**, 6021–6030.
- Kussie, P.H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A.J. and Pavletich, N.P. (1996) Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science*, **274**, 948–953.
- Labbe, S., Zhu, Z. and Thiele, D.J. (1997) Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. *J. Biol. Chem.*, **272**, 15951–15958.
- Li, X.-Y. and Green, M.R. (1996) Intramolecular inhibition of activating transcription factor-2 function by its DNA-binding domain. *Genes Dev.*, **10**, 517–527.
- Linder, M.C. and Hazegh-Azam, M. (1996) Copper biochemistry and molecular biology. *Am. J. Clin. Nutr.*, **63**, 797S–818S.
- Lutsenko, S. and Kaplan, J.H. (1995) Organization of P-type ATPases: significance of structural diversity. *Biochemistry*, **34**, 15607–15613.
- Martins, L., Jensen, L.T., Simons, J.R., Keller, G. and Winge, D.R. (1998) Metalloregulation of *FRE1* and *FRE2* homologs in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, in press.
- Mitchell, P.J. and Tjian, R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science*, **245**, 371–378.
- Momand, J., Zambetti, G.P., Olson, D.C., George, D. and Levine, A.J. (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, **69**, 1237–1245.
- Ptashne, M. (1988) How eukaryotic transcriptional activators work. *Nature*, **335**, 683–689.
- Rupp, R.A.W., Snider, L. and Weintraub, H. (1994) *Xenopus* embryos regulate the nuclear localization of XMyoD. *Genes Dev.*, **8**, 1311–1323.
- Sadowski, I., Triezenberg, S. and Ptashne, M. (1988) GAL4-VP16 is an unusually potent transcriptional activator. *Nature*, **335**, 563–564.
- Shatwell, K.P., Dancis, A., Cross, A.R., Klausner, R.D. and Segal, A.W. (1996) The *FRE1* ferric reductase of *Saccharomyces cerevisiae* is a cytochrome *b* similar to that of NADPH oxidase. *J. Biol. Chem.*, **271**, 14240–14244.
- Shen, F., Triezenberg, S.J., Hensley, P., Porter, D. and Knutson, J.R. (1996) Critical amino acids in the transcriptional activation domain of the herpesvirus protein VP16 are solvent-exposed in highly mobile protein segments. *J. Biol. Chem.*, **271**, 4819–4826.

- Vandromme,M., Gauthier-Rouviere,C., Lamb,N. and Fernandez,A. (1996) Regulation of transcription factor localization: fine-tuning of gene expression. *Trends Biol. Sci.*, **21**, 59–64.
- Vulpe,C.D. and Packman,S. (1995) Cellular copper transport. *Annu. Rev. Nutr.*, **15**, 293–322.
- Wang,D., Hu,Y., Zheng,F., Zhou,K. and Kohlhaw,G. (1997) Evidence that intramolecular interactions are involved in masking the activation domain of transcriptional activator Leu3p. *J. Biol. Chem.*, **272**, 19383–19392.
- Wen,W., Meinkoth,J.L., Tsien,R.Y. and Taylor,S.S. (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell*, **82**, 463–473.
- Whiteside,S.T. and Goodbourn,S. (1993) Signal transduction and nuclear targeting: regulation of transcription factor activity by subcellular localisation. *J. Cell Sci.*, **104**, 949–955.
- Winge,D.R., Dameron,C.T. and George,G.N. (1994) The metallothionein structural motif in gene expression. *Adv. Inorg. Biochem.*, **10**, 1–48.
- Yamaguchi-Iwai,Y., Serpe,M., Haile,D., Yang,W., Kosman,D.J., Klausner,R.D. and Dancis,A. (1997) Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of *FRE1* and *CTR1*. *J. Biol. Chem.*, **272**, 17711–17718.
- Yano,K.-I. and Fukasawa,T. (1997) Galactose-dependent reversible interaction of Gal3p with Gal80p in the induction pathway of Gal4p-activated genes of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **94**, 1721–1726.
- Zawel,L. and Reinberg,D. (1995) Common themes in assembly and function of eukaryotic transcription factors. *Annu. Rev. Biochem.*, **64**, 533–561.
- Zhang,L. and Guarente,L. (1995) Heme binds to a short sequence that serves a regulatory function in diverse proteins. *EMBO J.*, **14**, 313–320.
- Zhou,K. and Kohlhaw,G.B. (1990) Transcriptional activator *LEU3* of yeast. *J. Biol. Chem.*, **265**, 17409–17412.
- Zhu,Z., Labbe,S., Pena,M.M.O. and Thiele,D.J. (1998) Copper differentially regulates the activity and degradation of yeast Mac1 transcription factor. *J. Biol. Chem.*, **273**, 1277–1280.

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