Structure-Function Analysis of the Protein-binding Domains of Mac1p, a Copper-dependent Transcriptional Activator of Copper Uptake in Saccharomyces cerevisiae*

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The Mac1 protein in Saccharomyces cerevisiae is essential for the expression of yeast high affinity copper uptake. A positive transcription factor, Mac1p binds via its N-terminal domain to GCTC elements in the promoters of CTR1 and FRE1, encoding a copper permease and metal reductase, respectively. Mac1p-dependent transcriptional activation is negatively regulated by copper. We have mapped the domains in Mac1p responsible for its nuclear localization and for the protein-protein interactions that underlie its transcriptional activity. Immunofluorescence studies indicate that Mac1p contains two nuclear localization signals, one each in the N- and C-terminal halves of the protein. Yeast one-hybrid analysis demonstrates that the copper-dependent transcriptional activity in Mac1p resides primarily in a cysteine-rich element encompassing residues 264–279. Two-hybrid analysis indicates that copper-independent Mac1p-Mac1p interaction linked to DNA binding is due primarily to a predicted helix in the C-terminal region of the protein encompassing residues 388–406. Point mutations within this putative helix abrogate the Mac1-Mac1 interaction in vitro and formation of a ternary (Mac1p)2-DNA complex in vitro. When produced in normal abundance, Mac1p396D and Mac1p400D helix mutants do not support transcriptional activation in vivo consistent with an essential Mac1p dimerization in transcriptional activation. Lastly, the one- and two-hybrid data indicate that an intramolecular interaction between the DNA-binding and transactivation domains negatively modulates Mac1p activity.

In conditions of low cell copper the Mac1 protein in the yeast Saccharomyces cerevisiae activates the expression of genes encoding the high affinity copper uptake system (1–11). Principal among these genes are CTR1 (3–6, 8–11) and FRE1 (1, 4, 7, 10), which encode the copper permease and metal reductase that are required for copper uptake. Expression of the CTR3 locus, encoding a second copper permease, is Mac1p-dependent as well. However, CTR3 is not expressed in most laboratory strains of yeast due to a 6-kilobase Ty2 insertion between the TATA element and the transcription start site (3). Expression of these genes due to Mac1p is strongly reduced by addition of copper to a copper-depleted yeast culture medium; the [copper] that results in 50% reduction of this expression is ~20 nm (11).

Both in vivo Mac1p DNA binding (5) and the transcriptional activity of Mac1p in fusions to heterologous DNA-binding domains, e.g. the Gal4 DNA-binding domain (TAD) (6, 11) and LexA protein (7), also are strongly reduced at medium copper concentrations >100 nM. These data indicate that Mac1p-DNA binding and transcriptional activity share a common dependence on copper. What the structural features in Mac1p are that underlie this copper dependence remain largely uncharacterized. However, some data suggest that one component of this regulation by copper may be linked to an intramolecular interaction between the N- and C-terminal domains of the protein (9, 11).

The Mac1p sequence suggests that the N- and C-terminal halves of the protein may play distinct functional roles. For example, at neutral pH, the N-terminal domain is predicted to have a net +10 charge (pI = 9.9) while the C-terminal half would have a net charge of −12 (pI = 4.7). The basic nature of the N-terminal domain suggests that it would be involved in DNA binding. Indeed, it contains a possible zinc finger element (ZF) (Scheme 1) which shares ~50% sequence identity with the N-terminal domains in two other copper-dependent trans factors, Ace1p in S. cerevisiae (53%, Refs. 12 and 13) and Amt1p in Candida glabrata (48%, Ref. 14). This domain is required for DNA binding in all three proteins. In the case of Amt1p, NMR analysis demonstrated that this domain forms a zinc ribbon structure with two short helical segments projecting at one end; basic residues projecting from this end most likely make essential DNA contacts (15). The N-terminal half of Mac1p (designated NT, residues 1–200) also includes the sequence KKKRXX10KKXX (residues 155–177). This sequence conforms to a nuclear localization signal sequence (NLS) (16).

As noted, the C-terminal half of Mac1p is acidic (designated here as C, Scheme 1). This domain contains two cysteine-rich repeats, Rep I and Rep II (17). Each of these repeats is terminated by a histidine residue. Mutation of the first of these to glutamine, i.e. H279Q, yielded Mac1UP1 (1), a mutant protein whose DNA binding (5) and transcriptional (1, 4–6) activities were essentially independent of copper. A second mutation in the Rep I motif, C271Y, or Mac1UP2, results in an identical gain-of-function copper uptake phenotype (4). These results indicate that Rep I is associated with the copper regulation of the protein (9, 11). In contrast, a corresponding mutation in Rep II, H337Q, has no effect on Mac1p transcriptional activity in a fusion to the Gal4 DBD (6). The role of Rep II in Mac1p structure–function is unknown.

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The abbreviations used are: DBD, DNA-binding domain; TAD, transactivation domain; NLS, nuclear localization sequence; NT, N-terminal portion of Mac1p, residues 1–200; PCR, polymerase chain reaction; ORF, open reading frame; HA, hemagglutinin (epitope); EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; ZF, zinc finger.

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The Mac1p-binding site in the promoters of genes regulated by this protein is a repeated cis element containing a core sequence, GTCCT (4, 5, 8, 11). Deletion (4, 5, 8, 10, 18) or mutation (5, 10) of one of the elements impairs Mac1p binding and inactivates the upstream activating sequence demonstrating that the tandem repeat is required for full promoter activation. The requirement for two copies of the GTCCT element suggested that Mac1p binds to the DNA as a dimer. We affirmed this hypothesis by a combination of EMSA and promoter analysis (11). The protein-protein interaction dependence of the binding of a truncated form of Mac1p to DNA also indicated the formation of both binary and ternary protein-DNA complexes (8).

Thus, Mac1p function requires multiple intermolecular protein-protein interactions. A further role for an intramolecular interaction in Mac1p is suggested by the cooperativity seen in binding of a truncated form of Mac1p to DNA also indicated the formation of both binary and ternary protein-DNA complexes (8).

**Mac1p Protein Domains**

**Yeast Strains and Culture Conditions—**A MAC1 deletion (11) in wild type strain DEY1457 (MATa ade6 can1 his3 leu2 trp1) (19) was used as host for plasmids producing wild type and mutant forms of Mac1p. Strain SFY526 (MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3, 112 can1 gal4-542 gpd1 808-598 TRP1::GAL1-locZ, CLONTECH, Palo Alto, CA) (20) was used as host in the one- and two-hybrid analyses. The following media were used for culture growth: YPD was used for routine growth of wild type strains (2% yeast extract, 2% peptone, 2% glucose); SC medium was used for routine selective growth of trans-acting, Chelex-treated medium was used that was prepared as described previously (1). This medium contained an estimated 0.5 nm residual copper based on the amount of copper in the individual media components (as determined by flameless atomic absorption spectrophotometry) and the affinity of the Chelex resin for copper. This medium was subsequently supplemented with a trace metal mixture lacking copper, to which copper was added as required for a given experiment. Glycerol was used as carbon source replacing glucose to test for the respiration competence of DEY1457 (mac1Δ) when performing functional tests of mutant forms of Mac1p. This is a standard test for mac1Δ-complementing clones that takes advantage of the iron (and, consequently, respiratory) deficiency in this background (1). The Mac1p fragments C1C2 (residues 203–417) and C3 (residues 287–417) were PCR-generated and subcloned into the NcoI/SalI sites in pGCTH and pGAD424. The fragents with a STOP codon following Asn286 (C3-deleted) were constructed by removing the endogenous 197-base pair EcoRI fragment from the MAC1 ORF and religating the filled-in ends. The N-terminal fragments of Mac1p, encompassing residues 1–51, 1–200, and 42–200, respectively, were generated by PCR, also, and subcloned into the NcoI/SalI sites of the bait and catch vectors.

The Mac1p D-helix mutants were functionally tested by complementation in DEY1457mac1Δ in the following manner. The MAC1 ORF (Saccharomyces cerevisiae MAC1 [1967]) from MAC16 was subcloned into pRS414 and pRS424 to generate MAC14 and MAC24. These constructs were modified by site-directed mutagenesis using the Quick-Change kit from Stratagene (La Jolla, CA). The resulting recombinants, Mac1p388D, Mac1p400D, and Mac1pΔD, in both centromeric (CEN) and 2-µm vectors, were subsequently transferred into the Mac1p minus strain and the transformants tested for respiration competence as described above. Similarly, a set of complementary primers were used to engineer the HA epitope coding sequence by oligonucleotide-mediated mutagenesis. Both single HA and HA2 recombinants were isolated.

**Western Blotting—**In all cases the amount of Mac1 protein species, whether full-length, a truncation epitope-tagged, or a fusion to the Gal4DBD, was assessed by Western blot analysis (data not shown in text). Cells were treated with trichloroacetic acid, homogenized with glass beads, and the pellet collected by centrifugation. This pellet was then subjected to two cycles of freezing, thawing, and sonication in the presence of 1% Triton X-100 prior to adding SDS sample buffer. Detection was achieved by use of one (in some cases, two) of the following antibodies: a mouse monoclonal to the Gal4 DBD, a rabbit polyclonal to the HA epitope (both from Santa Cruz Biotechnology, Santa Cruz, CA), or a goat polyclonal raised against a synthetic peptide corresponding to the COOH terminus of Mac1p (Pharmingen, San Diego, CA). Immunocomplexes were visualized using chemiluminescent detection (Ultra, Pierce Chemical Co., Rockford, IL) using the appropriate horseradish peroxidase-conjugated secondary antibody and Kodak BIOMAX MR film. The one- and two-hybrid clones, yeast transformants carrying either one or both parental (control) or recombinant bait (fusions in pGBT9) and catch (fusions in pGAD424) vectors were grown in the appropriate Chelex-treated/copper-supplemented defined medium for at least 5 doublings until the cultures reached early-log phase (OD660 nm = 1.0). Samples (2 × 107 cells) were assayed in triplicate for β-galactosidase activity which was expressed in Miller units in the standard fashion (23). In general, the results given were from at least three independent experiments and were analyzed with the use of InStat (GraphPad, San Diego, CA).

**EMSAs—**Two double stranded (ds) oligonucleotides were used in the EMSA shown in Fig. 5. One was the 44-mer that conformed to the Mac1p-dependent, CTR1 binding element, TTTGCTC, was randomized (designated CTR1-R) from MAC16 and 0.6 mM dithiothreitol (11). This 15-mer was then annealed together to form a double-stranded probe (typically pGEM3ZF+) or with vector containing either wild type or mutated Mac1p-encoding sequences. Binding reactions were performed by preincubation of all components except labeled probe for 10 min at room temperature; 2 fmol of radiolabeled probe was then added and the mixture was incubated for another 10 min at room temperature. The mixture was chilled on ice and then electrophoretically resolved on a

dogenous NcoI (+121) site in MAC16 was destroyed by PCR mutagenesis and a new NcoI site was engineered at the +1 nucleotide (translation start). This modified NcoI(+1VPstI (+210) fragment from MAC16 was exchanged with the NcoI (+121)VPstI (+210) fragment in pGB-ΔZF to generate pGB-MAC, comprising the whole Mac1p ORF. The downstream constant NcoI–1 fragment from pGB-MAC was then subcloned into the catch vector pGAD424 (giving pGAD-MAC) and pGEM3ZF+ (giving pGEM-MAC) as above. The double mutant ZF*Mac1p (C235H25N) was engineered by two rounds of PCR mutagenesis using pGEM-MAC as the template. The NcoI(+1HinDI (+1927) fragment from pGEM-ZF*MAC was filled-in and subcloned into yeast SnaI sites in pGBT9 and pGAD424 to make plasmids pGB-ZF*MAC and pGAD-ZF*MAC, respectively.

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6.0% polyacrylamide gel at 4 °C. The gel was dried and exposed to a PhosphorImager screen and to Kodak IMAGER™ MR film. The screen was then read using a Bio-Rad model GS-505 PhosphorImager and the digitized intensity data were then quantitated using Molecular Analyst 1.5. The EMSA figure (Fig. 5) was imaged directly from these digitized intensities.

To determine if the amount of input Mac1p protein was independent of the MAC1 construct used to program the wheat germ extract, each construct was expressed in the presence of [35S]methionine, the reaction mixture resolved on SDS-polyacrylamide gel electrophoresis and the dried gel developed by autoradiography. In all cases, a single Mac1p product was detected of appropriate molecular mass. Based on quantitation of the film by image analysis (Bio-Rad Gel Doc system) equivalent amounts of protein were produced from all templates.

**Immunofluorescence Localization of Mac1p and Its Domains—** Yeast transformants producing Mac1p-HA constructs were prepared for indirect immunofluorescence in a standard fashion (24). An equal volume of a fresh solution of paraformaldehyde (4%) was added to 20 ml of a log-phase culture (OD660 nm = 1.5-2.5). Cells were collected by filtration (0.8-μm filters) and resuspended in 4% paraformaldehyde. After 90 min, the fixed cells were washed three times by centrifugation in 40 mM potassium phosphate (pH 6.5). Cell walls were digested in a 1-h treatment with 1 mg of Zymolyase 20T (ICN, Costa Mesa, CA) in 1 ml of solution of 40 mM potassium phosphate, 1.2 mM sorbitol, 0.5 mM MgCl2, 2 mM diethiothreitol, and 10 μl of β-mercaptoethanol. The fixed spheroplasts were washed twice with a phosphate-sorbitol buffer and acetone (-20 °C, 30 min) and acetone (-20 °C, 30 s) followed by three PBS/Mg washes. The spheroplasts were treated with Triton X-100 (0.1% in PBS/Mg, 5 min), washed again three times with PBS/Mg, and then incubated with DNase-free RNase A (50 μg/ml in PBS/Mg, 30 min). Following three PBS/Mg washes, the cells were blocked with bovine serum albumin (1% in PBS/Mg, overnight) and with normal goat serum (1:200 in PBS/Mg, 30 min). The polyclonal rabbit anti-HA antibody (preadsorbed by incubation with fixed, digested, and blocked Mac1p minus yeast cells) was diluted 1:500 with 0.1% bovine serum albumin in PBS/Mg and incubated with the samples for 1 h. Following washing (5 times, 0.1% bovine serum albumin in PBS/Mg), the samples were incubated with Cy3-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA; 1:300 in 0.1% PBS/Mg, 1 h) and then washed as above. The coverslips were stained with 2.4 nM YOYO-1 in PBS for 30 min (Molecular Probes, Eugene, OR; 1:300 in 0.1% PBS/Mg, 1 h) and then washed again three times with PBS/Mg, and then incubated with Cy3-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA; 1:300 in 0.1% PBS/Mg, 1 h) and then washed as above. The coverslips were stained with 2.4 nM YOYO-1 in PBS for 30 min (Molecular Probes, Eugene, OR), rinsed with water, and mounted with Elvanol for subsequent phase-contrast and confocal fluorescence microscopy.

**RESULTS**

**Mac1p Transactivation Domain: Mac1p Interaction(s) with General Transcription Factors—** Previous studies have shown that in a one-hybrid fusion to the Gal4 DBD, the C1C2 region of Mac1p had a strong transcriptional activity. However, this activity was not strongly copper-dependent (6). We constructed additional fusions to the Gal4 DBD to map systematically the region(s) in Mac1p that must interact with one or more of the general transcription factors needed for the assembly of a preinitiation complex (26).

In Fig. 1, we demonstrate the following. First, although Mac1p itself exhibited a minimal transcriptional activity in the fusion with Gal4 DBD (Fig. 1, row 2), this activity increased ~20-fold following deletion of the N-terminal zinc finger homology motif that is essential for Mac1p DNA binding (ΔZFMac1p, row 3). Second, the activity seen in the ΔZFMac1p fusion was due to the C1C2 domain since a fusion to this domain alone yielded the most active hybrid (row 4) confirming a prior finding (6). Third, the C1C2 domain was essential to transcriptional activity since constructs containing only C1 retained (some) activity (rows 5 and 7) while any construct that lacked C1 did not (row 6 and data not shown). However, an obvious synergism of some type occurred between C1 and C2 inasmuch as the C1C2 hybrid (row 4) had ~20-40-fold greater activity than fusions that contained C1 only (rows 5 and 7). Western blot analysis using antibodies to the Gal4 DBD or to Mac1p (residues 42-417) showed that the different fusions were produced at comparable levels (“Experimental Procedures”). These results showed that the data in Fig. 1 represented differences in the specific transcriptional activities of the fusion proteins and not their relative amounts.

The transcriptional activities of all fusion proteins were up-regulated in cells grown in the absence of copper (Fig. 1, compare −Cu to +Cu). The fold increase in activity upon copper depletion varied from 4 to 13, with the former value found for the C1C2. With this one exception, however, the copper dependent observed in these one-hybrid assays was stronger than that reported previously (6), and similar to that observed in Mac1p-dependent expression from the CTR1 promoter (5, 6, 11). Furthermore, the data indicated that the copper modulation occurred within the same domain that supported transcriptional activity, namely, the C1, Rep I-containing domain. For example, the transcriptional activity of C1 alone was copper-dependent to the same degree as the activity of the ΔZFT Mac1p fusion (Fig. 1, compare rows 3 to rows 5 and 7).

The data indicate that the N-terminal half of the molecule masked the transcriptional activity of the C1 domain. This conclusion obtained from the increasing transcriptional activity observed in the fusions following sequential N-terminal deletions (Mac1p, ΔZFT Mac1p and ΔZFTN-C1, and C1C2, or rows 2, 3 and 4, respectively). This result deserves particular emphasis. As noted above, ΔZFT Mac1p was ~20-fold more active than Mac1p itself. In contrast, the fusion of ZF*Mac1p, in which two of the five Cys-His residues in the (zinc ribbon) DNA-binding domain of Mac1p were mutated, was equivalent to wild type in having limited activity (Fig. 1, last entry). Although inactive in DNA binding activity (11), the N-terminal domain of this C23S/H25N mutant was nonetheless capable of masking the ability of the C-terminal half of the molecule to promote the assembly of a preinitiation complex. As developed below, our results suggest that this inhibition is due to an intramolecular interaction between the DNA-binding and C2 domains.

**Mac1p-Mac1p Interaction Domain—** EMSAs and in vivo expression studies described previously (11) strongly suggest that Mac1p binds productively to the CTR1 promoter as a homodimer. Here we have delineated the regions in Mac1p that...
Indeed, this interaction was the strongest of all assay (92% of those detected, including that for the positive control in this observation that the interaction involving C2 was lost when this domain, residues 389–417. This result followed from the interaction was further localized to the C-terminal region of the D-helix (Scheme 1). In fact, D-helix deletion region folds into an amphipathic helix (see below) which we secondary structure prediction suggested that this sequence may fold into an amphipathic helix; a model that includes Ile396 and Phe400 (modeling studies not shown).

The data in Fig. 2 summarize the results of this systematic two-hybrid mapping of these Mac1p-Mac1p interactions. In striking contrast to the one-hybrid data (Fig. 1), all of the interactions observed in the two-hybrid assays were copper-independent (see legend, Fig. 2). Since many of the same fusions to the Gal4 DBD were used in the one-hybrid experiments and there exhibited copper-dependent activity, the lack of a copper dependence of any Mac1p-Mac1p interaction was a meaningful result.

The data from these experiments also indicated that the intermolecular Mac1p-Mac1p interaction was mediated by the C2 domain. This conclusion followed from the observation that C2 alone exhibited a symmetrical interaction (Fig. 2, row 4, column 5). Indeed, this interaction was the strongest of all those detected, including that for the positive control in this assay (92% versus 39 Miller units; see legend, Fig. 2). This interaction was further localized to the C-terminal region of this domain, residues 389–417. This result followed from the observation that the interaction involving C2 was lost when residues 389–417 were deleted (row 3, column 5 versus row 8, column 5). Secondary structure prediction suggested that this region folds into an amphipathic helix (see below) which we have denoted the D-helix (Scheme 1). In fact, D-helix deletion in either the bait or the catch fusion uniformly led to a loss of the two-hybrid interaction, as in ΔZFMac1p (shown) or in any of the other interacting constructs listed in Fig. 2 (not shown).

The results summarized in Fig. 2 also supported the conclusion that the N-terminal half of Mac1p masked the ability of the C terminus to bind intermolecularly with a fusion protein partner. A clear indication of this masking was the progressive increase in the interaction between the C2 domain used as bait and fusions of Mac1p (2.4 Miller units), ΔZFMac1p (14.4 units), and C2C89 (89 units; Fig. 2, row 4, columns 2–4). A reasonable explanation for this pattern was that in the Mac1p catch proteins that included all or portions of the N-terminal domain, the C2 domain in those fusions was masked for interaction with the interacting partner (C2) in the bait fusion. In summary, the two-hybrid analysis indicated that the C2 domain in a catch protein fusion participated in two interactions: an intermolecular one with the C2 bait, and an intramolecular interaction with the N-terminal half of the same catch protein.

Thus, the data suggested a striking similarity between the one- and two-hybrid analyses that N-terminal truncations of Mac1p, whether as bait or catch, consistently gave a stronger signal in a given assay. For example, whereas Mac1p-Mac1p exhibited a weak interaction, the ΔZFMac1p-Mac1p and ΔZFMac1p-ΔZFMac1p interactions were 10- and 40-fold greater, respectively (0.4, 4.2, and 16.3 Miller units, respectively, Fig. 2, rows 2 and 3, columns 2 and 3). In contrast, in this two-hybrid screen the ZF*Mac1p fusion (0.5 Miller units; Fig. 2, row 7, last entry) was equivalent to wild type in exhibiting a weak interaction (0.4 Miller units; Fig. 2, row 2, first entry), also similar to the pattern in the one-hybrid analyses. The inference drawn from this parallel behavior was that it had a common structural basis: whatever in the N-terminal domain masked the ability of Mac1p to associate with general transcription factors also interfered with intermolecular self-association.

### The Mac1p-Mac1p Interaction Occurs Within the D-Helix—

Computer modeling of residues 387–407 within C2 indicated that this sequence may fold into an amphipathic helix; a model is shown in Scheme 2. Further modeling studies demonstrated that two such helices could stably dock along the non-polar face that includes Ile396 and Phe400 (modeling studies not shown). To test the hypothesis that the apparent intermolecular Mac1p-Mac1p interaction was due to an interaction at this helix face, I396D and F400D mutants were constructed and tested in three assays. First, production of these mutant Mac1 proteins in a mac1Δ-containing background demonstrated that when expressed from a single/low copy CEN vector, neither mutant could effectively complement the loss of the endogenous, wild type Mac1p (Fig. 3, row 3; only data for the I396D mutant is shown). On the other hand, the I396D mutant could complement the respiration-deficient, mac1Δ phenotype when overexpressed from a high copy, 2-μm vector (Fig. 3, row 4). In contrast, the Mac1pΔTR mutation, which lacked the entire D-helix, failed to complement even when overproduced (Fig. 3, row 4). Although Western blotting showed that the amount of either of the two mutant proteins was ~50% of wild type, protein abundance in all three cases positively correlated with plasmid copy number (data not shown). Furthermore, these mutants proteins, like wild type Mac1p, were targeted to the nucleus. This was demonstrated by immunofluorescence analysis of cells expressing triple HA-tagged Mac1 proteins (Fig. 4). Wild type Mac1p (panel B) and the I396D mutant protein (panel E) appeared exclusively in the nucleus, although the

### Mac1 Protein Domains

| Row | Gold4 DNA Binding Domain | Mac1 Protein Fusion | β-Galactosidase activity, Miller units |
|-----|--------------------------|--------------------|-------------------------------------|
| 1   | Vector Alone             |                    | (0.0)                              |
| 2   | Mac1 (1–417)             |                    | 0.4  3.2  13.4  47.4  0.1  0.1  0.1 |
| 3   | ΔZF (42–417)             |                    | 4.2  16.3  31.6  43.8  0.4  0.5  0.5 |
| 4   | C2 (289–417)             |                    | 2.4  14.4  89.1  92.1  0.1  0.1  0.1 |
| 5   | ZF (1–54)                |                    | 0.1  0.1  0.2  0.2  0.1  0.1  0.1 |
| 6   | NT (1–300)               |                    | •    •    •    •    0.0    •    • |
| 7   | ZF*Mac1                  |                    | •    •    •    •    •    •    •    • 0.5 |
| 8   | ΔZF*ΔD                 |                    | •    •    •    •    •    •    •    • |

- The Mac1p-Mac1p interaction is localized to the D-helix (Scheme 1).
- Secondary structure prediction suggested that this sequence may fold into an amphipathic helix; a model that includes Ile396 and Phe400 (modeling studies not shown).
- To test the hypothesis that the apparent intermolecular Mac1p-Mac1p interaction was due to an interaction at this helix face, I396D and F400D mutants were constructed and tested in three assays. First, production of these mutant Mac1 proteins in a mac1Δ-containing background demonstrated that when expressed from a single/low copy CEN vector, neither mutant could effectively complement the loss of the endogenous, wild type Mac1p (Fig. 3, row 3; only data for the I396D mutant is shown). On the other hand, the I396D mutant could complement the respiration-deficient, mac1Δ phenotype when overexpressed from a high copy, 2-μm vector (Fig. 3, row 4). In contrast, the Mac1pΔTR mutation, which lacked the entire D-helix, failed to complement even when overproduced (Fig. 3, row 4). Although Western blotting showed that the amount of either of the two mutant proteins was ~50% of wild type, protein abundance in all three cases positively correlated with plasmid copy number (data not shown). Furthermore, these mutants proteins, like wild type Mac1p, were targeted to the nucleus. This was demonstrated by immunofluorescence analysis of cells expressing triple HA-tagged Mac1 proteins (Fig. 4). Wild type Mac1p (panel B) and the I396D mutant protein (panel E) appeared exclusively in the nucleus, although the

- The data summarized in Fig. 2 also supported the conclusion that the N-terminal half of Mac1p masked the ability of the C terminus to bind intermolecularly with a fusion protein partner. A clear indication of this masking was the progressive increase in the interaction between the C2 domain used as bait and fusions of Mac1p (2.4 Miller units), ΔZFMac1p (14.4 units), and C2C89 (89 units; Fig. 2, row 4, columns 2–4). A reasonable explanation for this pattern was that in the Mac1p catch proteins that included all or portions of the N-terminal domain, the C2 domain in those fusions was masked for interaction with the interacting partner (C2) in the bait fusion. In summary, the two-hybrid analysis indicated that the C2 domain in a catch protein fusion participated in two interactions: an intermolecular one with the C2 bait, and an intramolecular interaction with the N-terminal half of the same catch protein.
The requirement for the putative D-helix in Mac1p function was tested by hybrid analysis (Fig. 5). Introduction of the I396D mutation decreased in the protein-protein interaction detected by two-produced in this background from either low copy (CEN) or high copy (2 strain, DEY1457 (Mac1p).moter as probe (Fig. 6). On this template, wild type Mac1p using the Mac1p DNA-binding element from the CTR1 site (Fig. 6, lanes 4–6). EMSA also indicated that the D-helix is required for Mac1p self-association and the formation of the proposed (Mac1p)_2 DNA ternary complex (11). This was demonstrated using the Mac1p DNA-binding element from the CTR1 promoter as probe (Fig. 6). On this template, wild type Mac1p forms both Mac1p-DNA and (Mac1p)_2-DNA complexes (Fig. 6, lane 3). In contrast, Mac1pΔD, Mac1pI396D, and Mac1pF400D form only the corresponding Mac1p-DNA binary complexes (Fig. 6, lanes 4–6). This same binary complex forms to a typical nuclear localization signal (Scheme 1). Immunofluorescence was used also to map regions in Mac1p that conferred nuclear targeting, presumably due to interaction with importin-α (27). In these experiments, regions of Mac1p individually fused to the HA epitope were produced in a mac1Δ background and the cells were subsequently fixed and stained. As in Western blotting (above), the immunofluorescence results indicated that the various Mac1 proteins were present at similar levels. The results suggest that Mac1p contains at least two regions that confer nuclear targeting. This was indicated by the finding that both NT-C1 (Mac1p residues 1–287) and C2 (residues 289–417) were strongly localized to the nucleus (Fig. 7, panels B, D, and F, respectively). In contrast, the extreme N-terminal region, containing the DNA-binding, ZF element (residues 1–70) was seen only in the cytoplasm (Fig. 7, panel H). These results suggest that one NLS resides in the region 70–287 and one resides in C2, or residues 289–417. The putative NLS noted in Scheme 1 is found within the former region, although definitive proof that these residues represent an NLS will require additional studies.

**DISCUSSION**

The data presented in this and in other published work indicate that the division of Mac1p into discrete structural domains based on sequence analysis (e.g. Scheme 1) does have a functional complement. First, the data suggest that the N-terminal half of the molecule is associated uniquely with DNA binding (1, 8, 11). Second, the transcriptional activity and its modulation by copper are features of Mac1p that reside in the C-terminal half of the protein (6, 9, 11). Furthermore, while the data demonstrate that the two halves of the protein can function independently of one another (as indicated by one-hybrid assay, for example) they also strongly suggest that there is a significant degree of cooperativity between the two domains in the expression of their individual activities. We propose that this cooperativity results from an intramolecular interaction that occurs between the N- and C-terminal domains as suggested by the analysis of the one- and two-hybrid data presented herein. Other studies have led to a similar conclusion (9, 11).

EMSA data have shown that the Ace1p/Amt1p homology domain at the N terminus of Mac1p is required for the specific Mac1p binding to its cognate cis element. This follows from the finding that neither ZFMac1p nor the ZF mutant, ZF*Mac1p, forms a DNA complex in vitro (11). Furthermore, neither protein is active in vivo (11). This subdomain encompasses residues 1–41 based on the truncations and single amino acid mutants used here and elsewhere (6, 9, 11). We believe that this unit is the primary DNA-binding element in the N-terminal domain. As noted in the Introduction, the N-terminal half of Mac1p is quite basic.

Another feature of the N-terminal domain is a consensus bipartite nuclear localization sequence (NLS), KK_{17}KKK (16, 27). The N terminus of this motif, KKRRXR, is similar to the NLS in the yeast PHO2 gene product (KKRRKKK) while the C-terminal KKKK element is seen in yeast RNA Pol I (16). The homologous sequences in Mac1p (residues 155–177) may support its nuclear localization since Mac1p (1–287) is efficiently targeted to the nucleus while Mac1p (1–70) is not. However, the

**FIG. 3.** D-helix dependence of Mac1p function in vivo. The requirement for the putative D-helix in Mac1p function was tested by complementation of the respiration deficiency of a mac1Δ-containing strain, DEY1457Δmac1. Wild type and mutant forms of Mac1p were produced in this background from either low copy (CEN) or high copy (2 μm) vectors as indicated. The transformants were plated on media containing glycerol as the sole carbon source. Growth requires the Mac1p-dependent copper uptake via Ctr1p that is required for respiratory capacity in S. cerevisiae in the absence of added copper. Addition of excess copper (100 μm, right panel) suppresses this Ctr1p dependence, and, therefore, the dependence on Mac1p.

staining of the latter cells was less efficient overall (see legend, Fig. 4).

Thus, these results were compatible with the model that a Mac1p-DNA interaction is stabilized by a Mac1p-Mac1p interaction; that this protein-protein interaction involves the putative D-helix; and that impairment of this interaction by a single amino acid substitution can be suppressed by overproduction of the mutant protein. In contrast, protein overproduction could not overcome the complete loss of this putative interaction due to deletion of this structural motif.

Consistent with this model, introduction of the I396D mutation into one C2 fusion protein (Fig. 2) resulted in a 10-fold decrease in the protein-protein interaction detected by two-hybrid analysis (Fig. 5). Introduction of the I396D mutation into both the bait and catch proteins led to an >100-fold decrease in the two-hybrid signal. Fusion protein abundance was equivalent in all cases indicating that the results reflected differences in intrinsic binding activity (data not shown).

EMSA also indicated that the D-helix is required for Mac1p self-association and the formation of the proposed (Mac1p)_2 DNA ternary complex (11). This was demonstrated using the Mac1p DNA-binding element from the CTR1 promoter as probe (Fig. 6). On this template, wild type Mac1p forms both Mac1p-DNA and (Mac1p)_2-DNA complexes (Fig. 6, lane 3). In contrast, Mac1pΔD, Mac1pI396D, and Mac1pF400D form only the corresponding Mac1p-DNA binary complexes (Fig. 6, lanes 4–6). This same binary complex forms when wild type Mac1p is added to a probe that has a single Mac1p-binding site (Fig. 6, lane 7 and Ref. 11). This result indicates that the tandem repeat on the DNA template and the Mac1p-Mac1p interaction are both required for stable formation of the (Mac1p)_2-DNA species. These several data are consistent with the model that the loss of Mac1p in vivo activity due to either single mutation in the putative D-helix, or to its deletion, stems from a strongly reduced ability of Mac1p to form a stable ternary complex at Mac1p-dependent upstream activating sequences (11). Furthermore, the data support the inference that formation of this complex requires residues 388–406.

**Mac1p Contains Two Nuclear Localization Signals**—Mac1p may contain a sequence in its N-terminal domain that conforms to a typical nuclear localization signal (Scheme 1). Immunofluorescence was used also to map regions in Mac1p that conferred nuclear targeting, presumably due to interaction with importin-α (27). In these experiments, regions of Mac1p individually fused to the HA epitope were produced in a mac1Δ background and the cells were subsequently fixed and stained. As in Western blotting (above), the immunofluorescence results indicated that the various Mac1 proteins were present at similar levels. The results suggest that Mac1p contains at least two regions that confer nuclear targeting. This was indicated by the finding that both NT-C1 (Mac1p residues 1–287) and C2 (residues 289–417) were strongly localized to the nucleus (Fig. 7, panels B, D, and F, respectively). In contrast, the extreme N-terminal region, containing the DNA-binding, ZF element (residues 1–70) was seen only in the cytoplasm (Fig. 7, panel H). These results suggest that one NLS resides in the region 70–287 and one resides in C2, or residues 289–417. The putative NLS noted in Scheme 1 is found within the former region, although definitive proof that these residues represent an NLS will require additional studies.

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![Diagram](image-url)
data indicate that an efficient NLS resides in C2 also since this domain alone (residues 289–417) is equivalently localized. Mac1p is not unique in having two apparent nuclear targeting elements. Indeed, another yeast protein, Upf3p, has three independent NLSs (28).

The C-terminal domain is acidic (pI 5 4.7). However, a cluster of basic residues appears with residues 356–368 which contain 6 of 16 K/R residues in the C-terminal half of Mac1p. This region is within the C2 domain which was shown to be nuclear localized. This potential NLS (R\textsubscript{X}2RK\textsubscript{X}3K\textsubscript{X}K) is bracketed by the cysteine-rich repeat Rep II (residues 322–337) and the apparent Mac1p dimerization region at the N terminus of Mac1p that we have designated the D-helix (residues 388–406).

The cysteine-rich motifs, Rep I and Rep II, and the regions immediately adjacent to them have a net charge at neutral pH of \( -2 \) and \( -4 \), respectively. The domain C1, which includes Rep I, productively recruits a preinitiation complex to the chimeric GAL4\textsubscript{DB}-lac\textsubscript{Z} reporter template (Fig. 1). Therefore, Mac1p might be considered an acidic transactivator (26). The charge on C2 may also be functionally important, inasmuch as the two-hybrid data suggest an intramolecular interaction between this motif and the basic N-terminal domain. However, we did not investigate separately the potential inter- and intramolecular interactions of the cysteine-rich element itself (Rep II), contiguous residues (the region including residues 300–350, for example), and those specific to the strongly basic region immediately following Rep II noted above (residues 356–368).

Our data show that a region included within the extreme C-terminal domain of Mac1p, residues 388–417, is equivalent to a Mac1p-Mac1p intermolecular interaction that is critical to full transcriptional activity. This region contains the predicted D-helix. Within this region, at least two non-polar residues, Ile\textsubscript{396} and Phe\textsubscript{400}, are required for this apparent Mac1p dimerization. The suppression of dimerization following from mutation of either of these residues suggests that this protein-protein interaction is due in part to the hydrophobic effect of burying the non-polar face of this helix in the two interacting partners. Our interpretation of the effect of D-helix mutation...
on Mac1p in vivo activity and in vitro DNA binding is at variance with Martins et al. (10) who concluded that Mac1p dimerization was not required for transactivation. However, these workers based their conclusion in part on EMSA experiments employing a truncated form of Mac1p that lacked the D-helix region (8). As a result their experiments did not explore the possibility of the synergism inherent to linked protein-protein and protein-DNA interactions.

A second major conclusion that follows from the data presented here and elsewhere (6, 9, 11) is that Mac1p activity is modulated by an intramolecular interaction between the N- and C-terminal domains. The primary consequence of this interaction is a suppression of the inherent transcriptional activity of Mac1p as measured in the one-hybrid assay, which reports the interaction of Mac1p with general transcription factors (Fig. 1). In this interpretation, this intramolecular interaction between the DNA-binding region and C2 prevents the recruitment by Mac1p of general transcription factors to the promoter. Our data show that upon removal of the ZF domain, the resulting fusions are now up to 20-fold more active in intermolecular binding to general transcription factors (see Scheme 3). Removal of the entire N-terminal half of the molecule results in additional transactivity (in the fusion to C1C2) suggesting that an additional portion of the N-terminal domain may be associated with the stabilization of this "closed" or transcriptionally silent state of the protein.

This model of Mac1p has precedent. For example, the ability of yeast TFIIB to form a stable, transcriptionally competent DNA-TBP-TFIIB ternary complex may involve a conformational change in TFIIB that exposes a domain essential to the required intermolecular protein-protein interaction(s) (29). Indeed, a 42-amino acid segment of human TFIIB has been shown to interact physically (in trans) with the core domain of this transcription factor (30). Furthermore, this segment in the human protein is homologous to the region in yeast TFIIB potentially involved in modulating the activity of this latter protein (29). We propose that the N-terminal segment of Mac1p could modulate the activity of this transcription factor in a similar fashion.

We suggest also that the Gal4-DBD fusions to N-terminal truncations of Mac1p which exhibit greater transactivity are equivalent to endogenous Mac1p bound to its sequence-specific upstream activating sequence, GCTC, as in the CTRI promoter. That is, DNA binding (Reaction 1, Scheme 3) stabilizes an "open" form, or one in which the C-terminal domain is fully required intermolecular protein-protein interaction(s) (29). Indeed, a 42-amino acid segment of human TFIIB has been shown to interact physically (in trans) with the core domain of this transcription factor (30). Furthermore, this segment in the human protein is homologous to the region in yeast TFIIB potentially involved in modulating the activity of this latter protein (29). We propose that the N-terminal segment of Mac1p could modulate the activity of this transcription factor in a similar fashion.

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competent for intermolecular protein-protein interactions with another Mac1p and with general transcription factors. As an
example of this model, the C-terminal domain of p53 appears to mask the N-terminal-dependent DNA binding of
this transfactor; this intramolecular interaction is modulated by reversible acetylation of the p53 C terminus (31). Although
there is no evidence at present that Mac1p undergoes post-
translational modification, its iron-dependent sister trans-
factor, Aft1p, is phosphorylated in vivo (32). Mac1p carries consen-
sus PKA and PKC phosphorylation sites; whether the serine residues within any of these sites are phosphorylated,
and if so, whether such modification modulates Mac1p func-
tion, are currently being investigated.

We were unable to detect an N/C-terminal interaction by in vivo two-hybrid analysis (using the TAD from Gal4p as in Fig.
2) and in vitro by immunologic methods. However, Jensen and Winge (9) were successful in measuring a weak interaction
between regions of the N- and C-terminal domains of Mac1p
using the stronger transactivation domain from VP16 to en-
hance the signal in a two-hybrid assay. The VP16 TAD has an
estimated 5-fold greater inherent transcriptional activity than
the Gal4 TAD (33). Based on these published data, we estimate
that the strength of the intramolecular, masking interaction
between the N- and C-terminal domains was about 100-fold
less than the intermolecular interaction between the C2
domains involved in the Mac1p dimerization we have charac-
terized. With reference to Scheme 3, for Mac1p to bind to DNA efficiently the stability of the closed conformation (specifically,
the binding energy of the intramolecular interaction) would be
of necessity orders of magnitude less than that of the stability
of the energy state defined by the open conformation. This latter state would equal the sum of the binding energies of the intramolecular interactions in the ternary (Mac1p)2-DNA com-
plex. Indeed, the 500-fold increase in the transactivity in
Mac1p-Gal4 DBD fusions resulting from successive N-terminal truncation (Fig. 1) can be equated to an alteration in the equilibrium constant between closed and open conformations
(e.g. Reaction 2, Scheme 3). This change in equilibrium would
be equivalent to a loss of intramolecular binding energy of
about 4 Kcal/mol, equivalent to an equilibrium dissociation
constant of 2 mM. A weak interaction like this one is not
commonly detectable in a two-hybrid assay with the protein
partners interacting in trans (34). Furthermore, this 500-fold
increase is comparable to the estimated 100-fold difference
between the binding of the ZF and C2 regions of Mac1p (9) in
comparison to the interaction between two C2 domains (this
work), consistent with our model.

Nonetheless, our view of Mac1p function is a model only.
Many questions remain about this copper responsive transac-
tor. Although Mac1p may share structure-function elements
with many other metal sensors and transcription factors, it
appears to combine them in a novel fashion. Thus, Mac1p
apparently contains potential copper clusters similar to those
found in Ace1p (12, 13) and Amt1p (14) and most likely binds to
its upstream activating sequence via an homologous zinc rib-
bon motif. It forms a transcriptionally active dimer as the
mercury-dependent MerR protein does (35). The open and
closed conformations illustrated in Scheme 3 are comparable to
what has been proposed for TFIIIB (29, 30) and p53 (31). An-
other potential element is regulation by protein modification as
has been suggested for Aft1p (32) and, as noted above, for p53
(31). How these multiple protein-protein and protein-DNA in-
teractions are modulated by copper now remains the principal
unanswered question about the mechanism of action of this
interesting regulatory protein.

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REFERENCES
1. Jungmann, J., Reins, H.-A., Lee, J., Romeo, A., Hassett, R., Kosman, D., and
Jentusch, S. (1993) EMBO J. 12, 5051–5056
2. Hassett, R., and Kosman, D. J. (1995) J. Biol. Chem. 270, 128–134
3. Knight, S. A. B., Labbe, S., Kwan, L. F., Kosman, D. J., and Thiele, D. J. (1996)
Genes Dev. 10, 1917–1929
4. Yamaguchi-Iwai, Y., Serpe, M., Haile, D., Yang, W., Kosman, D. J., Klauser,
R. D., and Danzi, D. (1997) J. Biol. Chem. 272, 17711–17718
5. Labbe, S., Zhu, Z., and Thiele, D. J. (1997) J. Biol. Chem. 272, 15951–15958
6. Graden, J. A., and Winge, D. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94,
5550–5555
7. Georgatsou, E., Mavrogiannis, L. A., Fragiadakis, G. S., and Alexandraki, D.
(1997) J. Biol. Chem. 272, 13786–13792
8. Jensen, L. T., Posewitz, M. C., Srinivasan, C., and Winge, D. R. (1998) J. Biol.
Chem. 273, 23805–23811
9. Jensen, L. T., and Winge, D. R. (1998) EMBO J. 17, 5400–5408
10. Martins, L. J., Jensen, L. T., Simon, J. R., Keller, G. L., and Winge, D. R. (1998)
J. Biol. Chem. 273, 23716–23721
11. Joshi, A., Serpe, M., and Kosman, D. J. (1999) J. Biol. Chem. 274, 218–226
12. Draper, C. T., George, G. N. Arnold, P., Santanagopaalan, V., and Winge, D.
R. (1993) Biochemistry 32, 7924–7931
13. Dobi, A., Dameron, C. T., Hu, S., Hamer, D., and Winge, D. R. (1995) J. Biol.
Chem. 270, 10171–10178
14. Farrell, R. A., Thoraldsen, J. L., and Winge, D. R. (1996) Biochemistry 35,
1571–1580
15. Turner, R. B., Smith, D. L., Zawrotny, M. E., Summers, M. F., Posewitz, M. C.,
and Winge, D. R. (1998) Nature Struct. Biol. 5, 551–555
16. Dingwall, C., and Laskey, R. A. (1991) Trends Biochem. Sci. 16, 478–481
17. Zhu, Z., Labbe, S., Peña, M. M. O., and Thiele, D. J. (1998) J. Biol. Chem. 273,
1277–1280
18. Dancis, A., Roman, D. G., Anderson, G. J., Hinnebusch, A. G., and Klauser,
R. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3869–3873
19. Dix, D. R., Bridgham, J. T., Broderius, M. A., Byersdorfer, C. A., and Eide, D.
(1994) J. Biol. Chem. 269, 26092–26099
20. Bartel, P. L., Chen, C.-T., Sternglanz, R., and Fields, S. (1993) in Cellular Inter-
actions in Development: A Practical Approach (Hartley, D., ed.) pp. 153–179, Oxford
University Press, Oxford
21. Sikorski, R. S., and Heter, P. (1989) Genetics 122, 19–27
22. Bartel, P. L., Chen, C.-T., Sternglanz, R., and Fields, S. (1993) in Biotechniques
14, 92–924
23. Miller, J. H. (1972) in Experiments in Molecular Genetics, pp. 352–355, Cold
Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Aris, J. P., and Blobel, G. (1988) J. Cell Biol. 107, 17–31
25. Harlow, E., and Lane, D. (1988) in Antibodies: A Laboratory Manual (Harow,
E., and Lane, D., eds) pp. 359–420, Cold Spring Harbor Laboratory, Cold
Spring Harbor, NY
26. Orphanides, G., Lagrange, T., and Reinberg, D. (1996) Genes Dev. 10,
2657–2683
27. Nigg, E. A. (1997) Nature 386, 779–787
28. Shirley, R. L., Leilveldt, M. J., Schenkmam, L. R., Dahlseid, J. N., and

Mac1 Protein Domains

Culbertson, M. R. (1998) J. Cell Sci. 111, 3129–3143
29. Bangur, C. S., Pardee, T. S., and Ponticelli, A. S. (1997) Mol. Cell. Biol. 17, 6784–6793
30. Roberts, S. G., and Green, M. R. (1994) Nature 371, 717–720
31. Gu, W., and Roeder, R. G. (1997) Cell 90, 595–606
32. Casas, C., Aldea, M., Espinet, C., Gallego, C., Gil, R., and Herrero, E. (1997) Yeast 13, 621–637
33. Melcher, K., and Johnston, S. A. (1995) Mol. Cell. Biol. 15, 2839–2848
34. Estojak, J., Brent, R., and Golemis, E. A. (1995) Mol. Cell. Biol. 15, 5820–5829
35. Summers, A. O. (1992) J. Bacteriol. 174, 3097–3101