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The second cysteine-rich domain of Mac1p is a potent transactivator that modulates DNA binding efficiency and functionality of the protein

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Abstract Mac1p is a *Saccharomyces cerevisiae* DNA binding transcription factor that activates genes involved in copper uptake. A copper-induced N–C-terminal intramolecular interaction and copper-independent homodimerization affect its function. Here, we present a functional analysis of Mac1p deletion derivatives that attributes new roles to the second cysteine-rich (REPII) domain of the protein. This domain exhibits the copper-responsive potent transactivation function when assayed independently and, in the context of the entire protein, modulates the efficiency of Mac1p binding to DNA. The efficiency of binding to both copper-response promoter elements can determine the in vivo functionality of Mac1p independent of homodimerization.

Key words: Mac1p; Transcriptional activation/repression; DNA binding; Yeast two-hybrid; Metalloregulation; Copper homeostasis

1. Introduction

Copper is an essential nutrient for all living organisms serving as catalytic co-factor of various enzymes required for respiration, oxidative damage response and iron uptake. Excess copper accumulated in the cell catalyzes the generation of reactive hydroxyl radicals causing damage to DNA, proteins and lipids. In *Saccharomyces cerevisiae*, copper homeostasis is maintained through both regulated cellular uptake and intracellular sequestration of copper ions [1,2]. Copper uptake is mediated by the plasma membrane high affinity permeases Ctr1p and Ctr3p and the Cu(II)/Fe(III) reductases Fre1p/Fre2p [3–6]. *CTR1*, *CTR3* and *FRE1* genes are transcriptionally upregulated under copper starvation conditions (below 1 nM) and downregulated in copper-replete cells [7–9]. The metal-sensitive transcription factor Mac1p (metal binding activator) binds to two CuRE (copper-response element, 5′-TTTG(T/G)C(A/G)-3′) DNA sequences on the promoters of the responding genes and mediates activation [8–10].

Mac1p was found within the nucleus of both copper-deficient and copper-loaded cells [11,12]. It contains different functional domains within its 417 amino acid residues [12]. Its DNA binding portion (residues 1–159) contains a zinc and copper binding motif (Cys-X_2-Cys-X_8-Cys-X-His) homologous to the metallothionein transactivators Ace1p (*S. cerevisiae*) and Amt1 (*Candida glabrata*) [13,14]. In vitro DNA binding of Mac1p was found copper-independent [9] but in vivo footprint was feasible only in copper-deficient cells, extinguished in 10 nM of copper ions [8]. Its C-terminal region (residues 252–341), that includes two cysteine-rich repeats (Cys-X-Cys-X_4-Cys-X-Cys-X_2-Cys-X_2-His), termed REPI (residues 264–279) and REPII (residues 322–337), respectively, exhibits the transactivation function and possibly binds eight copper ions [11]. The transcriptional activity of Mac1p is regulated by copper [6,15]. Two-hybrid interaction between the DNA binding and transactivation domains proposes that copper binding induces an allosteric interaction that inhibits both functions [11]. An unknown detoxification mechanism ensures elimination of the Cu(I) transport system, by rapid degradation of Mac1p at high copper ion concentrations (over 10 μM) [16]. Mac1p activation domain contains two functionally distinct regions. Mutations in the REPI motif resulted in constitutively active peptides, incapable of intramolecular interaction and more resistant to copper-dependent degradation [11,16]. Mutations in the REPII motif failed to activate *CTR3* transcription and did not rescue the respiratory deficiency of *mac1* [16]. However, they did not affect the copper-regulated transcriptional activity of Gal4pDBD-Mac1p fusion proteins on *GAL1*-UAS-driven *lacZ* [15] and resulted in peptides degraded with similar to wild-type kinetics [16]. Therefore the function of REPII was not clear. Finally, a copper-independent two-hybrid Mac1p–Mac1p interaction was attributed to a predicted helix in the C-terminal residues 388–406 [12] and in vitro DNA shift assays proposed that Mac1p homodimerization facilitates binding to two CuRE elements of the responsive promoters [17]. In this paper, our functional analysis of Mac1p deletion derivatives investigates the role of the REPII-containing region and identifies indispensable regions for a copper-regulated in vivo functional Mac1p molecule.

2. Materials and methods

2.1. Yeast strains and growth conditions

Construction of *mac1*Δ strain was previously described [6]. PJ69-4α (*MAT*α) used for one- and two-hybrid experiments derived from PJ69-4A (*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ UASGAL2-ADE2 *LYS2*::UASGAL1-HIS3 met2::UASGAL7-*lacZ*)

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Abbreviations: DBD, DNA binding domain; AD, DNA activation domain; REPI/REPII, repeat I/II; CuRE, copper-response element; BCS, bathocuproine disulfonate

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[18,19] (gifts from D. Lockshon and J.-C. Jauniaux). FY105 (*ura3-52 his3*Δ200 *trp1-54*) was used for gene isolation.

Standard bacterial and yeast growth media and methods were used as previously described [20–22]. SC, synthetic complete medium, contains 0.67% yeast nitrogen base (Difco), all amino acids, uracil, adenine and 2% glucose. SC was supplemented with 100 μM bathocuproine disulfonic acid-Na_2 salt (BCS) as Cu(I) chelator [4]. For all assays described, cells were cultured in SC medium to OD_{600} of 1.0, aliquots were resuspended to OD_{600} of 0.1 into the desired medium and cultured for 6 h at 30°C.

2.2. Plasmid construction

MAC1 protein coding sequence and deletion derivatives were generated by PCR using Vent Polymerase (New England Biolabs) and synthetic oligonucleotides (Microchemistry Lab at FORTH, Greece, and MWG-Biotech AG, Germany). pGBT9 (Clontech), pACTII (Clontech) were used for one- and two-hybrid assays, pYX142 (Novagen) and pRS315 [23] for *mac1*Δ complementation and pBluescript II for in vitro transcription/translation. The levels of *TPI*-driven *MAC1* RNAs in pYX142 were 8–9-fold higher than those expressed from *MAC1* promoter (data not shown).

MAC1 genomic sequence [6] (including 939 bp upstream of the AUG, the coding region and 674 bp downstream of the stop codon) was subcloned into the *Apa*I–*Hin*dIII sites of pRS315. A *MAC1* PCR-generated fragment using 5′-GGATCTCGAGACAATAAGCTGC-3′ and 5′-GGGGTACCTGAAGTGGTGGCATCGCTTA-3′, containing the coding region and 100 bp upstream was subcloned into the *Kpn*I–*Xho*I sites of pBluescript II KS. Primers 5′-ttcccccgggATAATATTTAATGGGAACA-3′ and 5′-ggggtaccTGAAGTGGTGGCATCGCTTA-3′ generated *MAC1* coding fragment, subsequently subcloned bluntly into (a) *Sal*I site of pGBT9 filled in with dNTPs by the Klenow fragment of DNA polymerase I, (b) *Sma*I site of pACTII and (c) *Nco*I–*Hin*dIII sites of pYX142 filled in with dNTPs.

Mac1p(1–337) was generated using primers 5′-cgggatccgtATGATAATATTTAATGGGA-3′ and 5′-cggaattcAGAGTGAGAAAAACATCC-3′ and subcloned into (a) *Bam*HI and *Pst*I filled in sites of pGBT9 and (b) *Bam*HI–*Sma*I sites of pYX142; the *Bam*HI–(*Nhe*I blunt) fragment from this was subcloned into the *Bam*HI–*Sma*I sites of pBluescript II KS.

Mac1p(1–322) was generated by *Nde*I–*Xho*I digestion of *MAC1* subcloned into pYX142, filling in and religation. From this construct, the *Eco*RV–*Sac*I fragment was subcloned into the *Nco*I filled in and *Sac*I sites of pACTII, the *Eco*RV–*Sac*I (filled in) fragment was subcloned into the *Sal*I filled in site of pGBT9 and the *Eco*RI–(*Nhe*I blunt) fragment into the *Eco*RI–*Xba*I sites of pBluescript II KS.

Mac1pΔ(323–337) was generated from ligation of two DNA fragments (*Bam*HI–*Nsi*I from Mac1p(1–337) and a PCR fragment from 5′-aaactgcagcACAAATATAATTCCATTTGAA-3′ and 5′-ggggtaccTGAAGTGGTGGCATCGCTTA-3′ primers), and subcloning into (a) *Bam*HI and *Pst*I filled in sites of pGBT9, (b) *Bam*HI and *Xho*I filled in sites of pACTII and (c) *Bam*HI–*Sma*I sites of pYX142; from this construct, a *Bam*HI–(*Kpn*I blunt) fragment was subcloned to *Bam*HI–*Sma*I sites of pBluescript II KS.

Mac1p(281–417) was generated using 5′-tcccccgggAGCGAAGAGGAACTGAATTC-3′ and 5′-ggggtaccTGAAGTGGTGGCATCGCTTA-3′ primers and subcloning into the *Sal*I filled in site of pGBT9.

Mac1p(338–417) was synthesized using 5′-aaactgcagcACAAATATAATTCCATTTGAA-3′ and 5′-ggggtaccTGAAGTGGTGGCATCGCTTA-3′ and subcloning into the *Xma*I filled in site of pGBT9. Mac1p(1–159) was generated from Mac1pΔ(338–417) as *Bam*HI–*Nae*I fragment and subcloned into *Bam*HI and *Pst*I filled in sites of pGBT9 or into *Bam*HI–*Eco*RV sites of pBluescript II KS.

2.3. RNA blot hybridization and DNA mobility shift assays

Samples of 50 μg total RNA [6] were electrophoresed on 1.5% agarose gels, transferred onto nylon membranes and used for hybridizations. Electrophoretic mobility shift assays were conducted using a radiolabelled double-stranded 40-mer oligonucleotide as probe, containing *CTR1* promoter sequences extending from −339 to −300 and including all sequence context needed for strong Mac1p binding [17]. Mac1p peptides were produced in vitro by the coupled TNT wheat germ extract system (Promega), using *MAC1* deletion derivatives as DNA templates, in the presence of [^{35}S]methionine. Visual detection and quantitation of the protein products were possible by electrophoresis on sodium dodecyl sulfate (SDS) 10% polyacrylamide gels followed by autoradiography. DNA–protein binding reactions were carried out in 15 μl of binding buffered solution (12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris–HCl (pH 8.0), 0.6 mM EDTA and 0.6 mM dithiothreitol [17]) containing 0.5 μg of herring sperm DNA, 5 fmol of ^{32}P-end-labelled probe and 1 μl of wheat germ extract. Mixtures were incubated for 20 min at room temperature and then electrophoresed on 1.5% agarose gels run at 120 V, at 4°C [24] and analyzed by autoradiography.

2.4. One- and two-hybrid assays and Western blot analysis

Mac1p hybrid derivatives were expressed from pGBT9 (Gal4pDBD-Mac1p) and pACTII (Gal4pAD) vectors. PJ69-4α transformed with the appropriate vectors was grown on SC selective medium. PJ69-4α strain has three distinct reporter genes, *ADE2*, *HIS3* and *lacZ*, each under the control of a different Gal4p-dependent UAS, *GAL2*, *GAL1* and *GAL7*, respectively [19]. One- and two-hybrid assays were performed by measuring β-galactosidase activity in liquid assays [20]. Protein levels of Gal4pDBD-Mac1p fusion derivatives were detected using a polyclonal antibody to Gal4p DNA binding domain (DBD). PJ69-4A transformed with Gal4pDBD-Mac1p derivatives was grown to $OD_{550} = 1$. Whole cell extracts were prepared from 10 OD_{550} units of cells by glass bead disruption [25]. Proteins were electrophoresed on a 10% SDS polyacrylamide gel, transferred onto nitrocellulose membranes and analyzed with anti-Gal4pDBD (Santa Cruz, cat # *sc-577*) (1:100 dilution) and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:40 000 dilution).

3. Results

3.1. A Mac1p derivative lacking both the REPII cysteine-rich and dimerization domains is copper-responsive and functional in vivo

To investigate the role of the REPII region in Mac1p function in vivo, we examined the ability of Mac1p deletion derivatives to complement the respiratory deficiency of a *mac1*Δ strain [7]. Our analysis included Mac1p(1–337) lacking the dimerization domain, Mac1pΔ(323–337) lacking the REPII motif and Mac1p(1–322) lacking both the REPII and dimerization domains. Expression of all derivatives and full-length Mac1p(1–417) was driven from the constitutive *TPI1* promoter of the centromeric plasmid pYX142. Mac1p expressed from *MAC1* promoter on the centromeric pRS315 was also examined. As shown in Fig. 1, Mac1pΔ(323–337) complemented the growth defect as well as full-length Mac1p, indicating that deletion of the REPII domain had no dramatic effects on the examined Mac1p function. Mac1p(1–337) did not complement the growth defect, in agreement to previous results, suggesting that dimerization of Mac1p was essential for that function [12]. However, complementation was also obtained by Mac1p(1–322) that contains neither the REPII

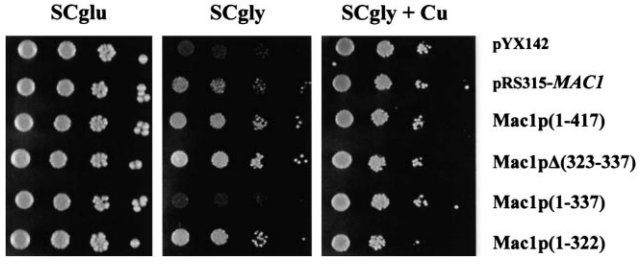

Fig. 1. Phenotypic complementation of *mac1*Δ strain by Mac1p deletion derivatives. *mac1*Δ cells were transformed with pYX142 expressing the indicated Mac1p derivatives, pYX142 vector and pRS315-*MAC1*. 10 μl of serially diluted cells from exponentially grown cultures in SC glucose medium were spotted (from left to right) on solid media SC glucose, SC glycerol (containing 3% glycerol instead of 2% glucose) or SC glycerol plus 100 μM of $CuSO_4$ and grown for 2, 5 and 5 days, respectively, at 30°C.

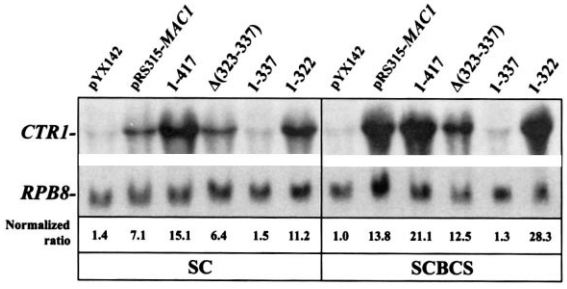

Fig. 2. CTR1 mRNA accumulation regulated by Mac1p deletion derivatives. *mac1*Δ cells transformed with pYX142 expressing the indicated Mac1p derivatives, pYX142 vector and pRS315-*MAC1* were grown in copper-replete (SC) or copper-deplete (SCBCS) medium. Extracted RNA was subjected to Northern analysis using *CTR1* and *RPB8* (internal quantitative control) radiolabelled DNA probes. Normalized ratios of *CTR1*/*RPB8* mRNA levels were measured using the Scion Image program on various autoradiography exposures.

nor the dimerization domains, indicating that this derivative was functional. Growth defects were rescued under high copper ion conditions known to rescue the inability of *mac1*Δ to utilize glycerol as a sole carbon source.

To further investigate the copper responsiveness of Mac1p(1–322), we examined its ability to promote *CTR1* mRNA accumulation in *mac1*Δ cells grown in the presence or absence of copper. In the presence of 0.25 μM $CuSO_4$ (SC medium), full-length Mac1p expressed from its own promoter activated *CTR1* whereas the *TPI*-driven full-length Mac1p promoted even higher *CTR1* activation (Fig. 2). In the absence of copper (100 μM BCS) both Mac1p-expressing strains displayed even higher *CTR1* activation. In agreement to the phenotypic complementation results, Mac1pΔ(323–337) activated *CTR1* in response to copper depletion, however, at lower (about 2.5-fold) levels than those induced by the full-length Mac1p. This could indicate a contribution of the REPII domain to the transactivation potential of Mac1p, not revealed by the phenotypic test. Mac1p(1–337) did not activate transcription at any condition. Mac1p(1–322) responded to copper depletion similarly to full-length Mac1p. Therefore, the N-terminal truncated derivative containing the DNA binding and REPI domains was sufficient for copper-modulated in vivo Mac1p function, although the other derivatives indicated that the dimerization domain was essential and the contribution of REPII could be significant for *CTR1* activation.

3.2. The REPII cysteine-rich region exhibits independent copper-regulated potent transactivation function

To investigate the contribution of the REPII domain in the transcriptional induction of *CTR1*, we examined the transactivation potential and copper responsiveness of the described and other derivatives in a one-hybrid system. All peptides were fused to the Gal4pDBD and examined for regulation of the *GAL7*-UAS-driven *lacZ* gene, in response to both copper repletion (100 μM $CuSO_4$) and copper starvation (100 μM BCS) conditions. As shown in Fig. 3A, a C-terminal derivative containing only the REPII and dimerization domains, Mac1p(281–417), exhibited high transactivation. This function was regulated by copper, indicative of an inhibitory effect from copper binding. Another C-terminal derivative containing only the dimerization domain, Mac1p(338–417), gave insignificant levels of activation, attributing the previous result to the remaining REPII-containing sequences. However, the

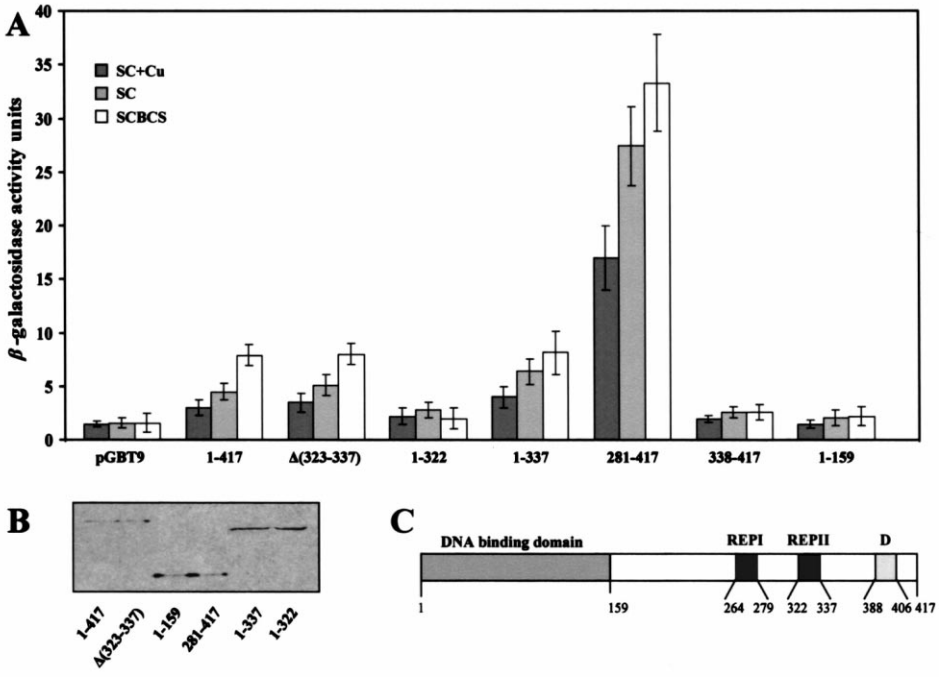

Fig. 3. Copper-modulated Mac1p transactivation function. A: Diagrammatic representation of β-galactosidase activity units obtained from a met2::UASGAL7-*lacZ* reporter gene, in PJ69-4α cells transformed with pGBT9 vector or the indicated Mac1p derivatives fused to the Gal4pDBD of pGBT9. Cells were cultured under three different conditions (SC supplemented with 100 μM of $CuSO_4$, SC and SC supplemented with 100 μM of BCS) as described in Section 2. Standard deviation of unit values from five independent experiments is indicated. B: Protein levels of Gal4pDBD-Mac1p derivatives analyzed by Western blotting using anti-Gal4pDBD as described in Section 2. C: Diagrammatic representation of Mac1p domains.

full-length Mac1p(1–417), the REPII deletant Mac1pΔ(323–337) and Mac1p(1–337) lacking the dimerization domain all exhibited comparable levels of copper-responsive transactivation. This result suggested that the activation function of the REPII domain was not significant in the context of these derivatives. Therefore, the REPII-containing region (most likely residues 322–337) has a potent activation function that is apparently reduced in the context of the entire protein. The copper-regulated in vivo functional Mac1p(1–322) exhibited very low levels of activation and this was not due to lower amounts of protein, since all fusion peptides were expressed at comparable amounts (Fig. 3B). It was rather indicative of low activation potential of the REPI domain in the context of this construct.

3.3. The REPII domain modulates DNA binding efficiency of Mac1p derivatives

Since the above presented transactivation potential of Mac1p derivatives did not correlate directly to their in vivo functionality, we further examined the DNA binding efficiency of each peptide. Previous reports have shown that binding to both CuRE elements present in all Mac1p-regulated promoters is needed for transcriptional activation [8,9]. The inability of a D-helix deletion derivative to form $(Mac1p)_2$–DNA ternary complex in vitro and complement *mac1* respiratory deficiency in vivo led to the hypothesis that homodimerization of Mac1p facilitates its binding to the two promoter elements [17].

We tested equal amounts of in vitro-synthesized Mac1p and derivatives (Fig. 4A) for binding on a DNA fragment containing both CuRE elements of the *CTR1* promoter. According to previous in vitro electrophoretic mobility shift assays [13,17], we considered the resolution of a shifted DNA band as binding to one element and the resolution of an additional band with lower mobility as binding to both elements. Mac1p showed binding to one and both consensus elements, indicated by two shifted bands (Fig. 4B) and increased amounts of input protein resulted in almost exclusive binding to both elements (Fig. 4C). The minimal DBD alone, Mac1p(1–159), showed binding to one and both elements and Mac1p(1–337), lacking dimerization domain, bound to one element only as previously observed. Under the same experimental conditions, the REPII deletant Mac1pΔ(323–337) showed significantly higher binding to both elements, compared to the full-length Mac1p, and Mac1p(1–322) bound to one element with much higher efficiency than any other tested peptide; increased amounts of input protein increased binding to both elements. This finding implied that, although Mac1p(1–322) is not as potent a transactivator as the full-length Mac1p, it binds to both CuRE elements on target promoters much more efficiently, namely in higher quantities, and therefore provides comparable functionality. It also implied that Mac1p(1–322) and Mac1pΔ(323–337) bind better because they lack the REPII domain.

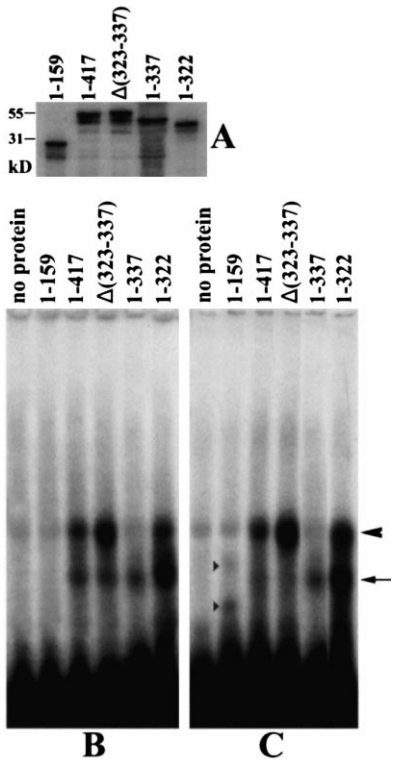

Fig. 4. In vitro binding of Mac1p deletion derivatives to *CTR1* promoter. A: Autoradiogram of Mac1p derivatives synthesized in wheat germ extract, in the presence of [^{35}S]methionine and analyzed by SDS–PAGE. 0.5 μl of the indicated peptides were loaded in each lane. B: Autoradiogram of mobility band shift assays performed by mixing in vitro-synthesized Mac1p derivatives with 5 fmol of a ^{32}P-end-labelled *CTR1* promoter 40-mer DNA. 0.5 μl of input proteins plus 0.5 μl of wheat germ extract were added to each binding reaction. C: as in B but 1 μl of input protein was added to each reaction. 'No protein' lanes contain 1 μl wheat germ extract alone and *CTR1* probe. Migration sites for the Mac1p–DNA binary (arrow) and $(Mac1p)_2$–DNA ternary (arrowhead) complexes are indicated. Small arrowheads indicate the corresponding sites for the smallest Mac1p(1–159) derivative.

These results also indicated that dimerization was not a prerequisite for DNA binding and functionality of Mac1p(1–322). Indeed, examination of protein interactions in a two-hybrid system showed that, while all derivatives con-

Table 1
Two-hybrid interactions between Mac1p deletion derivatives

	pACTII	1–417	1–322	Δ(323–337)	240–417	293–417
pGBT9	1.50	2.96	3.00	2.00	1.60	2.30
1–417	4.50	71.00	–	74.00	50.00	132.00
1–322	3.20	–	3.80	–	4.15	–
1–337	6.70	14.00	–	13.00	10.00	15.00
338–417	2.60	12.00	–	7.60	98.00	145.00
281–417	45.00	72.00	–	68.00	52.00	129.00

β-Galactosidase activity units obtained from a met2::UASGAL7-*lacZ* reporter gene, in PJ69-4α cells co-transformed with the indicated Mac1p derivatives fused to the Gal4pDBD of pGBT9 and to the Gal4pAD of pACTII. Derivatives 240–417 and 293–417 are clones identified in a pACTII genomic DNA library. Cells were cultured in SC medium. Unit values from a representative out of five independent experiments are shown.

taining dimerization domain exhibited strong two-hybrid interactions, both derivatives lacking dimerization domain, Mac1p(1–322) and Mac1p(1–337), exhibited very low levels of β-galactosidase activity, indicating that they do not use alternative domains to homodimerize (Table 1).

4. Discussion

Mac1p is a multidomain protein and its function involves both inter- and intramolecular interactions. The presented data on functionality in vivo, transactivation potential and DNA binding efficiency of Mac1p truncated derivatives provide new insights on the role of the second cysteine-rich domain. We found that this domain, when assayed independently, was a potent transactivator responsive to copper. In the context of the entire protein, REPII domain mostly affected the efficiency of the protein to bind to both CuRE promoter elements, pertinent for Mac1p function; in the absence of REPII domain, DNA binding efficiency was increased. Mac1p(1–322), that lacks REPII and dimerization domains, was copper-responsive and fully functional in vivo due to its higher efficiency of binding to both CuRE elements and possibly better exposure of the REPI-containing activation domains, compared to the full-length Mac1p homodimer. Homodimerization was not detected between Mac1p(1–322) peptides and therefore did not play any role in their binding on promoters and their functionality. The in vivo functional REPII deletant Mac1pΔ(323–337) also exhibited more efficient in vitro binding to both CuRE elements compared to the full-length homodimer. The negative role of REPII in DNA binding could be exherted through potential cysteine–cysteine interactions between REPII and DBDs. As previously proposed, such interactions can occur primarily via REPI cysteines and copper ions [11]. We suggest that, in the absence of REPII domain, the probability of N–C-terminal intramolecular interactions is reduced and DNA is better stabilized in its DNA-bound conformation. In fact, our one-hybrid results are in agreement with this hypothesis. The Gal4pDBD fusion N-terminal derivatives contain Mac1pDBD in a non-DNA-bound conformation, possibly more available for intramolecular interactions. All N derivatives in which REPI was followed by additional 'stabilizing' sequences (REPII or REPII-D or D) exhibited *lacZ* transactivation; Mac1p(1–322), containing only the first cysteine-rich domain REPI, showed practically no activation, possibly because REPI was mostly involved in interactions with Mac1pDBD. On the other hand, the Gal4pDBD–REPII-D fusion, containing no Mac1pDBD, exhibited its full activation potential.

Previous studies investigating the effects of point mutations in REPII in one-hybrid context suggested a minor role for this domain in the activation function of Mac1p [15], similarly to our results with Gal4pDBD-Mac1pΔ(323–337). Other studies have shown that a Mac1p derivative bearing Cys–Ser substitutions in all Cys sequence positions of REPII was not functional in vivo when expressed from a low copy number plasmid [16]. Our Mac1pΔ(323–337) was functional but overexpressed and this could explain the apparent difference concerning the necessity of REPII on Mac1p function. Alternatively, Cys–Ser substitutions could have different effects on Mac1p DNA binding than REPII deletion.

Mac1pΔ(323–337), despite its better DNA binding potential, activated *CTR1* expression less than Mac1p. This could possibly be due to interference of the more proximal dimerization domain with the REPI transactivation function.

The non-functional Mac1p(1–337) that lacks the dimerization but contains the REPII domain showed in vitro binding to one CuRE element only, indicating an essential positive role of dimerization when REPII is included in the protein context. In other studies, the need for dimerization could also be bypassed when the very strong activation domain of VP16 was fused to the DBD of Mac1p [13].

Overall, our results showed that REPII exerts a negative effect on Mac1p DNA binding. Neutralization of its effects results in increased DNA binding efficiency that overrides even the necessity for homodimerization. The REPII domain effects indicate that Mac1p function could be altered in vivo by structural modulations affecting its DNA binding properties. Modulations that resemble the deletion of REPII or REPII-D regions could be mediated by intermolecular interactions. The fact that (a) truncated peptides lacking the REPII or REPII-D domains are functional and regulated by copper in vivo, (b) the N–C intramolecular modulation by copper involves primarily REPI and (c) the REPII-D-containing region, when isolated, exhibits high transactivation function, indicate that this latter region may be involved in independent molecular interactions with other proteins or metals. We have preliminary two-hybrid results indicating such interactions between Mac1p and several proteins of known or unknown function. Analysis of their biological significance may reveal new aspects of Mac1p function[1].

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[1] Note added in proof: Keller et al. [26] have also identified functional independence of the two cysteine-rich activation domains of Mac1p.

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