THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 12, Issue of March 23, pp. 8793–8797, 2001

Printed in U.S.A.

Phosphorylation and Cu^+ Coordination-dependent DNA Binding of the Transcription Factor Mac1p in the Regulation of Copper Transport*

Received for publication, September 7, 2000, and in revised form, December 21, 2000
Published, JBC Papers in Press, December 28, 2000, DOI 10.1074/jbc.M008179200

Jose Heredia‡, Matthew Crooks, and Zhiwu Zhu§

From the Department of Environmental Toxicology, University of California, Santa Cruz, California 95064

Copper ions are essential at a proper level yet toxic when present in excess. To maintain a proper intracellular level, cells must be able to sense the changes in copper ion concentrations. The yeast transcription factor Mac1p plays a critical role in the transcriptional regulation of *CTR1* and *CTR3*, both encoding high affinity copper ion transporters. Here we report that the Mac1p binding of the copper ion-responsive elements (CuREs) in the promoters of *CTR1* and *CTR3* is affected by copper ions. On one hand, the Mac1p DNA binding is Cu^+ coordination-dependent, and on the other hand, exogenous Cu^+ and isoelectronic Ag^+ ions disrupt the DNA binding of Mac1p. These results suggest that the Mac1p is able to sense two different levels of copper ions. These two levels are probably the physiological and toxic copper levels in yeast cells. Furthermore, we found that Mac1p undergoes posttranslational phosphorylation modification in yeast and that the phosphorylation is required for the Mac1p to become DNA-binding active. Nonphosphorylated Mac1p is unable to bind the *CTR1* promoter DNA. The data support the model of intradomain interactions and indicate further that the phosphorylation probably prevents the inhibition of DNA-binding domain activity by the activation domain of Mac1p. Taken together, these findings demonstrate that Mac1p functions critically in maintaining a proper intracellular concentration of copper ions.

Due to its essential yet toxic nature in biological systems (1–3), the intracellular concentration of copper ions is tightly controlled in the yeast *Saccharomyces cerevisiae*, in part, by regulating the expression of the genes *CTR1* and *CTR3* that encode copper ion transporters at both transcriptional and posttranslational levels (4–10). The *CTR1* and *CTR3* transcription is enhanced by copper starvation (addition of chelator bathocuproinedisulfonate (BCS)[1] in the growth medium) and inactivated by even a slight increase in the copper ion concentrations (of picomolar range) (9). The transporter Ctr1p is induced to undergo degradation by toxic copper levels (of 10 μM or higher), which is thought to further inhibit copper uptake under toxic conditions (6). Interestingly, a recent study (33) reports that the Ctr3p, unlike the Ctr1p, does not undergo degradation in response to toxic copper levels. This latest finding may explain the earlier report that *CTR3* is disrupted by a transposon element in most yeast laboratory strains (5). These concentration-dependent responses noted above indicate that cells are able to sense and differentiate the physiological from toxic levels of copper ions. How the degradation of Ctr1p is controlled is currently unknown. The transcription of *CTR1* and *CTR3* is regulated by the copper-sensing factor Mac1p through the cis-acting CuREs in the promoters (9). However, how the copper ion signal, particularly that of physiological and toxic levels, is propagated to the promoters of *CTR1* and *CTR3* is currently poorly understood.

The primary sequence indicates that the Mac1p may sense copper ions through direct copper ion coordination (7), similar to the two other known copper-sensing factors Ace1p and Amt1p (11–14). Biochemical studies show that the separated DNA-binding domain and the activation domain of Mac1p expressed in the *Escherichia coli* both bind Cu^+ ions (15). The bacteria-expressed DNA-binding domain was also found to coordinate Zn^{2+}; a zinc-module was then postulated to also exist in the Mac1p DNA-binding domain (15). The DNA-binding domain of Amt1p expressed in the bacteria was the first copper-sensing factor found to be Zn^{2+}-coordinated (16). However, this Zn^{2+} coordination was not required for the Amt1p to bind DNA (16). DNA binding activities at the CuREs in the promoters of *CTR1* and *CTR3* have been detected in the yeast extracts prepared from the cells expressing various forms of the truncated Mac1 protein (17). The purified truncate of Mac1p(1–159), which lacks the activation domain, was also found able to bind the CuREs (15). *In vitro* translated Mac1p has also been shown to be able to bind the *CTR1* promoter (8). These studies have demonstrated that Mac1p is able to bind the CuREs. However, whether or not copper affects Mac1p DNA binding has yet to be resolved. *In vivo* footprinting showed that upon $CuSO_4$ treatment, the CuREs in the *CTR3* promoter became more accessible to the methylation reagent (9), indicative of possible dissociation of Mac1p. The DNA binding activities of the yeast extracts, prepared from the cells expressing the truncated Mac1p(1–194) and grown in the medium containing either BCS or $CuSO_4$, exhibited no difference (15). There is no report on the copper effect on Mac1p DNA binding by the addition of $CuSO_4$ to the binding reactions using yeast cell extracts or purified proteins.

The previous study using Mac1 fusion proteins with Gal4p (either DNA-binding or activation domain) and VP16 provided indirect evidence that there are interactions between the DNA-binding and activation domain of the Mac1p (17). It is postulated that copper-induced intramolecular interactions inhibit

* This work was supported by National Science Foundation Grant MCB-9807786 and National Institutes of Health Grant GM58082-01 (to Z. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported in part by Minority Biomedical Research Support (MBRS) program.

§ To whom correspondence should be addressed. Tel.: 831-459-3987; Fax: 831-459-3524; E-mail: zhu@biology.ucsc.edu.

[1] The abbreviations used are: BCS, bathocuproinedisulfonate; CuRE or CuREs, copper ion-responsive element(s); HA, hemagglutinin; MES, 4-morpholineethanesulfonic acid.

both DNA binding and transactivation activities of the Mac1p (17, 18). A more recent study using one- and two-hybrid methods also suggests that the intramolecular interaction negatively modulates Mac1p activity (19).

In this report, we describe the finding that the Mac1p undergoes posttranslational phosphorylation and that the phosphorylation is required for the Mac1p to bind to the CuRE in the *CTR1* promoter. The dephosphorylated Mac1p is unable to bind the DNA. We also found that the Mac1p DNA binding, on one hand, is dependent on Cu^+ coordination and, on the other hand, is disrupted by the addition of copper ions. These studies indicate that Mac1p is able to sense two different copper levels and that the posttranslational phosphorylation of Mac1p is probably a key event in the regulation of copper transport.

EXPERIMENTAL PROCEDURES

Plasmid Construction, Cell Growth, and Protein Analysis—A high copy plasmid pGPD-Mac1HA(opt) was constructed by cloning a *Bam*HI-*Xho*I fragment of an artificial *MAC1* into the p423GPD vector at the same sites (20). The artificial *MAC1* has optimal codons and is tagged with a single copy of HA epitope at the carboxyl terminus. The DNA was synthesized *in vitro* (GeneMed).

A single copy plasmid pRSMac1(3HA) was constructed essentially the same as pRSMac1(HA) as described previously (10). To introduce 3 × HA epitopes in the *MAC1* coding region, a *Nar*I site was generated by silent mutations at the DNA encoding residues Gly-222 and Ala-223. Complementary oligonucleotides were synthesized for a DNA fragment encoding three copies of the nine amino acids of the HA epitope with flanking *Nar*I sites. The oligonucleotides were annealed and cloned into the *Nar*I site of the *MAC1*.

Plasmids were transformed into the SLY2(*mac1*Δ) cells as described previously (10). The cells were grown in the copper ion-depleted medium treated with Chelex (Bio-Rad) (6). Yeast whole cell extracts were prepared using the previously described method (6) or by using the glass bead disruption method (21). The protein concentrations of the extracts were determined using the Bradford protein assay (21). The lysis buffer contained 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM dithiothreitol. The protein extracts were treated with λ-protein phosphatase (New England Biolabs). After the treatment, proteins were precipitated using trichloroacetic acid, fractionated on 8.5% SDS-polyacrylamide gels, and then transferred to a nitrocellulose membrane as described previously (21). Western blotting analyses were then carried out using a polyclonal anti-HA antibody, Y11 (Santa Cruz Biotechnology Inc.) (10).

In vivo ^{32}P-Labeling and Immunoprecipitation—SLY2 cells transformed with pRSMac1(3HA) or control plasmid pRS313 were used to carry out *in vivo* ^{32}P-labeling (22). Cells were pre-grown overnight in the copper-depleted selective medium synthetic complete medium lacking histidine without phosphate and reinoculated into 5 ml of synthetic complete medium lacking histidine medium of 0.02 of the normal phosphate level with a starting OD_{650} of 0.4. ^{32}P-Inorganic phosphate (ICN) was added to a final concentration of 200 μCi/ml. Cells were grown until the OD_{650} reached ~1.5 and then harvested. Cell pellets were washed with H_2O, and whole cell extracts were prepared using the method described previously (6). The proteins were dissolved in buffer A (50 mM Na_3PO_4, pH 7.0, 25 mM MES, pH 7.0, 1% SDS, 3 M urea, 0.5% β-mercaptoethanol, 50 mM NaF). Protein concentrations in the extracts were measured as described above. For immunoprecipitation, 250 μg of total proteins in 100 μl of buffer A was mixed with 700 μl of buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA), and 5 μl of anti-HA antibody 12CA5 was added. The reactions were incubated on a rotating wheel for 2 h at 4 °C. Protein A-Sepharose CL-4B beads were added with 10 μl of 100 mM bovine serum albumin, and the reactions were incubated overnight at 4 °C on a rotating wheel. Immunocomplexes were precipitated by microcentrifuge at 13,000 rpm and washed five times with the buffer B. The complexes were eluted in 40 μl of SDS-polyacrylamide gel electrophoresis sample buffer by heating at 80 °C for 10 min. Twenty microliters of elutes were analyzed on a 6% SDS-polyacrylamide gel. The gel was fixed and dried and exposed to Kodak Biomax film at −80 °C with an intensifying screen. The exposure time that was required for autoradiographic detection of Mac1p phosphorylation was 2–4 days.

DNA Binding Experiments—Yeast extracts were prepared from cells of SLY2 harboring pGPD-Mac1HA(opt) or vector p423GPD grown in copper-depleted medium, using the glass bead disruption method (21), in lysis buffer of 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol. ^{32}P-Labeled *CTR1* promoter DNA fragment was generated by polymerase chain reactions using [^{32}P]dATP. The primers used for the wild type DNA were 5′-CATGTATTGATGCAA-3′ and 5′-CTTGAAAAGTGCTC-3′, and primers used for the mutant probe were 5′-CATGTATTGATGCAAATCATGGGATAGGGATGAAAGACG-ACGGTAAAA-3′ and 5′-CTTGAAAAGTGCTCTTTTCAGGATCGTGC-CATTGGGATGAATTTTACCGTC-3′. The radioactive DNAs were purified by the agarose gel electrophoresis. Because of the observed interference of the Mn^{2+} on Mac1p DNA binding, calf intestinal phosphatase (New England Biolabs) was used to dephosphorylate the Mac1p in the protein extracts. The phosphatase inhibitors, glycerol phosphate (40 mM), NaF (50 mM), and okadeic acid (2 μM), were used as previously described (24). The binding reactions were carried out as described previously (23).

RESULTS

Mac1p Undergoes Posttranslational Phosphorylation in S. cerevisiae—Probably because of the low physiological level of Mac1p in the yeast cells, our repeated efforts to detect the CuRE binding activity of Mac1p in the yeast extracts did not produce conclusive results. An artificial *MAC1* of optimized codons, tagged with a single copy of HA epitope at the carboxyl terminus, was then synthesized *in vitro*, and the Mac1-HA protein was expressed in the SLY2 cells carrying the synthetic gene on a high copy plasmid pGPG-Mac1HA(opt) as described under "Experimental Procedures." As shown in Fig. 1*A*, three proteins (two labeled with p-Mac1-HA and one with Mac1-HA) were detected by immunoblotting using polyclonal anti-HA antibody Y11 and found only in cells harboring the plasmid pGPD-Mac1HA(opt). Compared with the *E. coli*-expressed Mac1-HA, the two proteins had higher molecular masses. Upon protein phosphatase treatment, the two proteins collapsed to the protein Mac-HAp, which migrated at 57 kDa. The *E. coli*-expressed Mac1-HA also migrated at 57 kDa. Protein phosphatase treatment did not change the *E. coli*-expressed Mac1-HA (data not shown). This result indicates that the Mac1-HAp when overexpressed undergoes phosphorylation modification in the yeast cells but not in the bacteria.

We observed that the overexpression causes hyperphosphorylation of the Mac1-HA protein (data not shown). In SLY2 cells transformed with the single copy plasmid pRSMac1(HA) (10), the level of the phosphorylated Mac1-HAp was found to be very low and hardly detectable (Fig. 1*B*). To enhance immunological detection, the plasmid pRSMac1(3HA) was constructed as described under "Experimental Procedures" in which three copies of the HA epitope were inserted in Mac1p between the Gly-222 and Ala-223 residues. The Mac1-3HAp was functional in the activation of *CTR1* and *CTR3* transcription, judging from the growth on nonfermentable carbon source yeast medium containing the non-fermentable carbon source ethanol plate (Fig. 1*D*). As shown in Fig. 1*B*, in SLY2 cells harboring pRSMac1(3HA) plasmid, two specific protein bands were detected with one band significantly more abundant than the other, corresponding to nonphosphorylated and phosphorylated Mac1-3HAp, respectively. We then carried out metabolic labeling with ^{32}P-inorganic phosphate and subsequent immunoprecipitation. In Fig. 1*C*, a ^{32}P-radioactive protein of the Mac1-3HAp molecular weight was detected from ^{32}P metabolically labeled cells expressing Mac1-3HAp but not from the ^{32}P-labeled control cells. These results together have demonstrated that phosphorylation of Mac1p also occurs at a physiological level.

The Exogenous Copper Ions Disrupt the DNA Binding of Mac1p—Based on the findings described above, we speculate that our prior failure to detect the DNA binding activity of Mac1p in the yeast extracts is possibly due to the observed very low abundance of the phosphorylated Mac1p. We then conducted electrophoretic mobility shift assays to determine

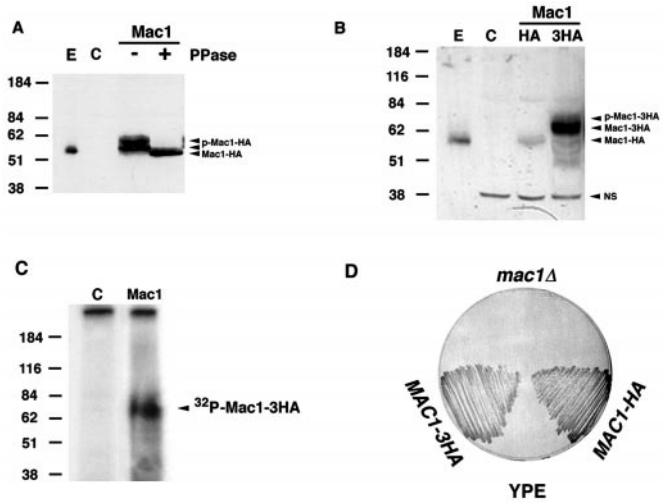

FIG. 1. **Mac1p undergoes posttranslational phosphorylation modification in *S. cerevisiae*.** *A,* protein phosphatase treatment of Mac1p overexpressed in yeast. Whole cell extracts were prepared using the glass beads disruption method from SLY2 (*mac1*Δ) harboring either control vector pGPD423 or pGPD-Mac1HA(opt) plasmid. The extracts were both treated and left untreated with λ- protein phosphatase at 30 °C for 1 h and then precipitated using trichloroacetic acid and analyzed by immunoblotting with anti-HA antibody Y11 (1:500 dilution) and goat anti-rabbit IgG conjugated to horse radish peroxidase (1:1000 dilution). *PPase*, λ-protein phosphatase; *Lane* −, not treated with PPase; *Lane* +, treated with PPase. For *A, B,* and *C*, the positions of the molecular weight standards are indicated to the *left*, and on the *right*, the phosphorylated and nonphosphorylated Mac1 proteins are marked with an *arrowhead* and Mac1-HA, Mac1-3HA, or p-Mac1-HA and p-Mac1-3HA, respectively. *Lane E,* the Mac1-HAp expressed in *E. coli*; *lane C*, control cells; *Mac1*, cells expressing Mac1p. *B,* detection of phosphorylated Mac1p at its physiological level by immunoblotting. SLY2 cells harboring pRSMac1(3HA) or pRSMac1(HA) or control vector pRS313 plasmid were grown in copper-depleted medium. Yeast whole cell extracts were prepared, and Mac1-HAp and Mac1-3HA were detected by immunoblotting as described above. *C, in vivo* ^{32}P-labeling and immunoprecipitation of Mac1p. SLY2 cells transformed with pRSMac1(3HA) or control plasmid pRS313 were grown in copper-depleted medium in the presence of ^{32}P-inorganic phosphate, and whole cell protein extracts were prepared as described under "Experimental Procedures." Immunoprecipitation was carried out with anti-HA antibody 12CA5 and protein A-Sepharose beads. Immunocomplexes were analyzed on an 8.5% SDS-polyacrylamide gel. *D,* Mac1-3HA is functional in the activation of *CTR1* and *CTR3* transcription. Transformants of SLY2 with the vector pRS313 (*mac1*Δ), pRSMac1(HA) (*MAC1-HA*), or pRSMac1(3HA) (*MAC1-HA*) were streaked onto yeast medium containing a nonfermentable carbon source (*YPE*). Cells were incubated for 48 h and photographed.

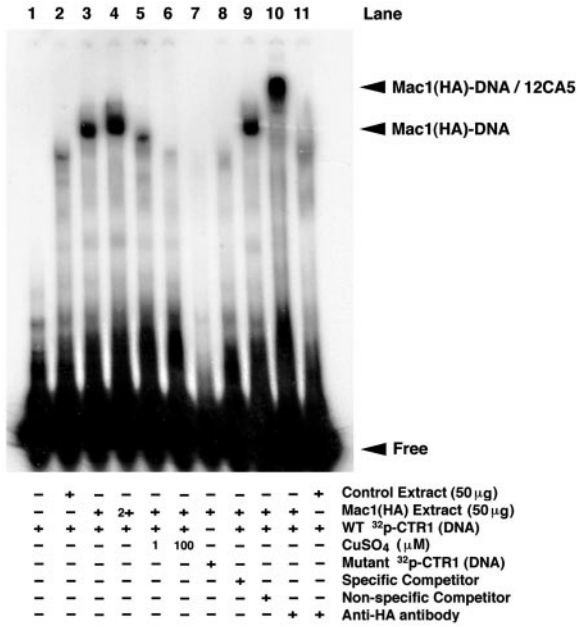

FIG. 2. **Exogenous copper ions disrupt the DNA binding of Mac1p.** Electrophoretic mobility shift assays using ^{32}P-labeled *CTR1* promoter fragments containing the CuREs or the mutant are shown (9). Whole cell extracts were prepared from either control cells (SLY2 transformed with the vector plasmid pGPD423) or Mac1-HAp expressing cells (SLY2 containing pGPD-Mac1(opt)HA) and used in binding reactions in the presence of poly[dI-dC]·poly[dI-dC]. $CuSO_4$ was added to final concentrations of 1 μM (*lane 4*) and 100 μM (*lane 5*). The mAb 12CA5 was incorporated into reactions (*lanes 10* and *11*), and the reactions were incubated at 4 °C for 20 min. DNA binding reaction mixtures were electrophoretically fractionated on a 5% native polyacrylamide gel. *Free*, free probe DNA; *Mac1(HA)-DNA*, the Mac1(HA)-CTR1 complex; *Mac1(HA)-DNA/12CA5*, the Mac1(HA)-CTR1 supershifted complex by the mAb 12CA5.

whether or not the Mac1p DNA binding was affected by copper ions. To conduct this experiment, we used yeast whole cell extracts, prepared from the SLY2 containing the pGPD-Mac1HA(opt) plasmid and ^{32}P-labeled wild type *CTR1* promoter DNA fragment, and a mutant fragment in which both CuREs are mutated (9). The yeast cells were grown in copper-deficient medium, and extracts were prepared as described above. As shown in Fig. 2, a shifted complex was detected in the reactions between ^{32}P-labeled wild type *CTR1* promoter DNA and the extract prepared from cells expressing Mac1-HA (*lanes 3* and *4*) but not from control cells (*lane 2*). The addition of $CuSO_4$ at both 1 μM and 100 μM concentrations (*lane 5* and *6*) disrupted the complex. In the reaction containing the mutant *CTR1* promoter DNA, no such complex was detected (*lane 7*), confirming previous genetic analysis and *in vivo* footprinting that the CuREs serve as the copper-responsive elements in the *CTR1* and *CTR3* promoters (9). Inclusion of the anti-HA antibody in the reaction (*lane 10*) caused a supershift of the complex, indicating that Mac1-HAp is present in the complex. Competition reactions (*lanes 8* and *9*) show that Mac1-HA formed a specific complex with the wild type *CTR1* promoter DNA. Our data then demonstrate that the Mac1p DNA binding is copper ion-responsive, suggesting that the copper ion-triggered disruption of Mac1p DNA binding probably results in the transcriptional inactivation of *CTR1* and *CTR3*.

The Phosphorylation and Cu^+ Coordination of Mac1p Are Required to Bind the CuRE in the CTR1 Promoter—We next characterized the effect of Mac1p phosphorylation on its DNA binding by performing the electrophoretic mobility shift assays using the same yeast extracts, both treated and not treated with protein phosphatase (as shown in Fig. 3). Consistent with the data in Fig. 2, the Mac1-HAp-containing complex was detected (*lanes 3* and *4*), and the complex was disrupted by copper (*lanes 5* and *6*) and by the isoelectronic Ag^+ as well (*lane 7*). Ag^+ represses the *CTR1* and *CTR3* transcription in a manner indistinguishable to Cu^+ (9). These data further substantiate our finding that Mac1p DNA binding is copper ion-responsive. Also shown in the Fig. 3, the addition of BCS (*lane 8*), the Cu^+-specific chelator, disrupted the complex, but EDTA (*lane 9*), a mainly divalent metal ion chelator, had no effect. These results indicate that the Mac1p DNA binding is also Cu^+ coordination-dependent, much like the Ace1p and Amt1p, suggesting that the DNA-binding active Mac1p is likely to be Cu^+-bound even under copper-limiting conditions. In *lane 10*, the phosphatase treatment almost completely abolished the DNA binding activity of the Mac1-HAp in the extract sample showing that the phosphorylation of Mac1p is required to bind the *CTR1* promoter. Interestingly, the addition of the phosphatase inhibitors (*lane 11*) did not prevent copper ions from disrupting Mac1-HA DNA binding *in vitro*, implying that copper disrupts the DNA binding of Mac1p probably through a mechanism

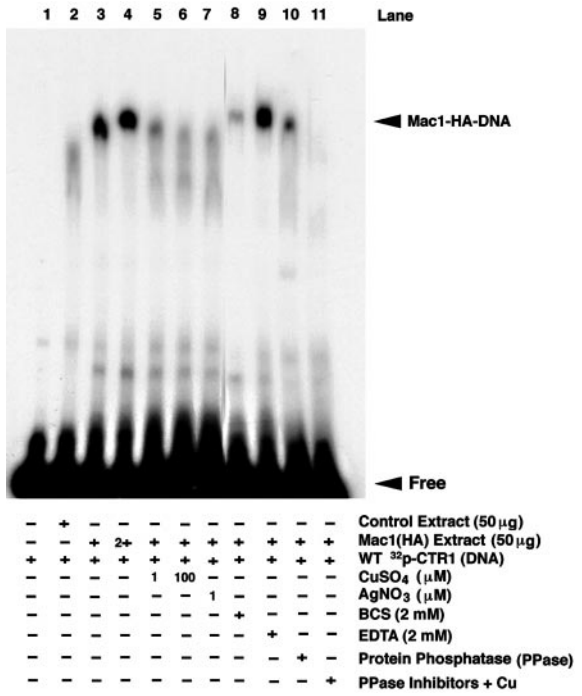

FIG. 3. **Phosphorylation and Cu^+ coordination of Mac1p are required for binding *CTR1* promoter DNA.** Electrophoretic mobility shift assays using ^{32}P-labeled *CTR1* promoter fragments and phosphatase-treated protein extracts are shown. The same whole cell extracts were used, and the reactions were carried out as described in the Fig. 2 in the presence of poly[dI-dC]·poly[dI-dC]. $CuSO_4$ (1 μM), $AgNO_3$ (1 μM), BCS (2 mM), and EDTA (2 mM) were added to the final concentrations as shown at the *bottom* of the figure. The extract was treated with calf intestinal phosphatase for 30 min at 37 °C, both in the presence and absence of the phosphatase inhibitors (40). DNA binding reaction mixtures were electrophoretically fractionated on a 5% native polyacrylamide gel. *Free*, free probe DNA; *Mac1-HA-DNA*, the Mac1(HA)-CTR1 complex.

other than dephosphorylation. These results together show that the two, phosphorylation and Cu^+ coordination, are both required for the Mac1p to bind the CuREs in the promoters of *CTR1* and *CTR3*.

DISCUSSION

In this report, we have demonstrated that Mac1p undergoes phosphorylation modification in yeast cells but not in bacteria and that the phosphorylation is required for Mac1p to bind to its target gene, the *CTR1* promoter (and presumably the *CTR3* promoter as well). The nonphosphorylated Mac1p failed to bind DNA at all. A previous report, however, has shown that the separated DNA-binding domain is able to bind the *CTR1* promoter (15). Our lab has also found that the purified DNA-binding domain of Mac1p is able to bind *CTR1* promoter eventhough the DNA-binding domain protein is not phosphorylated (data not shown). Taken together, these findings suggest that the activity of the DNA-binding domain is inhibited in the nonphosphorylated Mac1p and that the inhibition is abolished upon phosphorylation of Mac1p. Consistent with this notion, two earlier studies (17, 18) have reported that there are probably intermolecular interactions between the DNA-binding and activation domains of Mac1p and that the activation domain negatively may regulate the activity of the DNA-binding domain (19). Therefore, in Mac1p, the activation domain regulates the activity of the DNA-binding domain possibly in a phosphorylation-dependent fashion. Supporting this mechanism, our preliminary studies indicate that the phosphorylation occurs within the activation domain of Mac1p, not in the DNA-binding domain.[2] The activation domain of Mac1p clearly plays an active role in the regulation of *CTR1* and *CTR3* transcription in response to changes in copper ion concentrations. The phosphorylation of proteins is a general mechanism for cells to respond to extracellular signaling (25). This report indicates that regulation of copper ion uptake most likely involves a phosphorylation-based signal transduction mechanism. A recent study (26) has also indicated that Aft1p, the iron-sensing factor, undergoes possible phosphorylation modification. Therefore, the phosphorylation modification may represent a new yet common mechanism by which cells sense metal ions.

Our finding that copper ions affect the DNA binding of Mac1p represents important progress in the understanding of the mechanism by which Mac1p functions in the regulation of *CTR1* and *CTR3* transcription. The sequence homology among Mac1p, Ace1p, and Amt1p within their NH_2-terminal DNA-binding domain suggests that these factors may function similarly in sensing copper ions (7). A recent study (27) has reported that Cuf1, a critical factor in the regulation of copper and iron uptake in *Schizosaccharomyces pombe*, also shares this homology. It is a known fact that for the Ace1p and Amt1p to activate the transcription of MT genes, both proteins have to bind copper ions first and then become DNA-binding active (28, 29). This mechanism is clearly suited to assure that this detoxifying response is only activated in cells exposed to toxic levels of copper ions, not under physiological conditions because MTs inhibit the copper acquisition by other copper-dependent enzymes, such as Cu,Zn-SOD (30). Therefore, the Ace1p and Amt1p are sensors for the toxic level of copper ions. Our studies have shown that the Mac1p DNA binding is also Cu^+ coordination-dependent, eventhough the deficiency of copper ions triggers the Mac1p to activate the transcription of *CTR1* and *CTR3*. Equally important, we have also demonstrated that the exogenous copper ion and isoelectronic Ag^+ disrupt the Cu^+ coordination-dependent DNA binding of Mac1p. Our results are seemingly contradictory, yet they unmistakably indicate that the Mac1p is able to sense two different copper levels, reflecting the very nature of copper in living organisms, essential yet toxic depending on its concentrations. We speculate that the dependence of Mac1p DNA binding on Cu^+ coordination probably represents the ability of Mac1p to sense the physiological copper level, whereas the copper disruption of Mac1p DNA binding may exemplify its ability to sense the toxic level of copper ions. Mac1p contains two clustered metal ion binding motifs of typical C*X*C and C*XX*C nature located within the DNA-binding and the activation domains, respectively (7). The DNA-binding and the activation domains are the possible structural basis for the sensing of the two copper levels by Mac1p. An important question is how Mac1p can detect these two different copper levels? And equally important, under the copper-limiting condition, why is the Mac1p able to bind copper ions and activate the *CTR1* and *CTR3* transcription but not the Ace1p, eventhough Mac1p and Ace1p share an almost identical Cu^+ binding motif within their DNA-binding domains? One plausible model is that the copper ion binding motifs within the DNA-binding and activation domains of Mac1p may have different affinities to copper ions and that Mac1p DNA-binding domain has much higher affinity to copper ions than does the Ace1p DNA-binding domain. Biochemical studies currently underway in our laboratory will shed light on this possible mechanism.

The transcription of *CTR1* and *CTR3* has been shown previ-

[2] Z. Zhu, M. Crooks, M. Depaz, and J. Heredia, manuscript in preparation.

ously to be extremely sensitive to increases in exogenous copper ion (9). Our data have provided a framework for understanding how Mac1p may activate the transcription under copper-limiting conditions. However, the mechanism by which the increase in copper ion concentrations triggers the inactivation of *CTR1* and *CTR3* transcription is still poorly understood. We have reported here that copper triggers the repression of *CTR1* and *CTR3* transcription possibly by disrupting the DNA-binding of Mac1p. Because the phosphatase inhibitors did not prevent copper from disrupting Mac1p-binding DNA (Fig. 3), this suggests that copper disrupts Mac1p DNA binding potentially by a means other than triggering dephosphorylation of Mac1p (10).[2] The new features of how Mac1p functions as reported in this work, particularly the ability to sense the two different levels of copper ions, are consistent with its regulatory role in copper ion transport. Further studies are warranted to determine the precise molecular mechanism by which cells differentiate the physiological from toxic levels of copper ions. This is particularly true because Menkes' and Wilson diseases are caused by copper ion deficiency and excess copper ion toxicity, respectively (for review see Refs. 31 and 32).

Acknowledgments—We thank other members in the Zhu lab for critically reading the manuscript and for valuable suggestions.

REFERENCES

1. Linder, M. C. (1991) *Biochemistry of Copper*, Plenum Press, New York
2. Karin, K. D., and Tyeklar, Z. (1993) *Bioinorganic Chemistry of Copper*, Chapman and Hall, New York
3. Halliwell, B., and Gutteridge, J. M. C. (1990) *Methods Enzymol.* **186,** 1–85
4. Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J., and Klausner, R. D. (1994) *Cell* **76,** 393–402
5. Knight, S. A. B., Labbé, S., Kwon, L. F., Kosman, D. J., and Thiele, D. J. (1996) *Genes Dev.* **10,** 1917–1929
6. Eng Ooi, C., Rabinovich, E., Dancis, A., Bonifacino, J. S., and Klausner, R. D. (1996) *EMBO J.* **15,** 3515–3523
7. Jungmann, J., Reins, H.-A., Lee, J., Romeo, A., Hassett, R. Kosman, D., and Jentsch, S. (1993) *EMBO J.* **12,** 5051–5056
8. Yamaguchi-Iwai, Y., Serpe, M., Haile, D., Yang, W., Kosman, D. J., Klausner, R. D., and Dancis, A. (1997) *J. Biol. Chem.* **272,** 17711–17718
9. Labbé, S., Zhu, Z., and Thiele, D. J. (1997) *J. Biol. Chem.* **272,** 15951–15958
10. Zhu, Z., Labbé, S., and Thiele, D. J. (1998) *J. Biol. Chem.* **273,** 1277–1280
11. Thiele, D. J. (1988) *Mol. Cell. Biol.* **8,** 2745–2752
12. Welch, J., Fogel, S., Buchman, C., and Karin, M. (1989) *EMBO J.* **8,** 255–260
13. Szczypka, M., and Thiele, D. J. (1989) *Mol. Cell. Biol.* **9,** 421–429
14. Thiele, D. J. (1992) *Nucleic Acids Res.* **20,** 1183–1191
15. Jensen, L. T., Posewitz, M. C., Srinivasan, C., and Winge, D. R. (1998) *J. Biol. Chem.* **273,** 23805–23811
16. Thorvaldsen, J. L., Sewell, A. K., Tanner, A. M., Peltier, J. M., Pickering, I. J., George, G. N., and Winge, D. R. (1994) *Biochemistry* **33,** 9566–9577
17. Jensen, L. T., and Winge, D. R. (1998) *EMBO J.* **17,** 5400–5408
18. Graden, J. A., and Winge, D. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5550–5555
19. Serpe, M, Joshi, A., and Kosman, D. J. (1999) *J. Biol. Chem.* **274,** 29211–29219
20. Mumberg, D., Müller, R., and Funk, M. (1995) *Gene* (*Amst.*) **156,** 119–122
21. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York
22. Liu, X. D., and Thiele, D. J. (1996) *Genes Dev.* **10,** 592–603
23. Zhu, Z., and Thiele, D. J. (1996) *Cell* **87,** 459–470
24. Lu, P-J., Zhou, X. Z., Shen, M. H., and Lu, K. P. (1999) *Science* **283,** 325–328
25. Bhalla, U. S., and Iyengar, R. (1999) *Science* **283,** 381–387
26. Casaa, C., Aldea, M., Espinet, C., Gallego, C., Gil, R., and Herrero, E. (1997) *Yeast* **13,** 621–637
27. Labbé, S., Peña, M. M. O., Fernandes, A. R., and Thiele, D. J. (1999) *J. Biol. Chem.* **51,** 36252–36260
28. Buchman, C., Skroch, P., Welch, J., Fogel, E., and Karin, M. (1989) *Mol. Cell. Biol.* **9,** 4091–4095
29. Evans, C., Engelke, D., and Thiele, D. J. (1990) *Mol. Cell. Biol.* **10,** 426–429
30. Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C., and O'Halloran, T. V. (1999) *Science* **284,** 805–808
31. Harris, Z. L., and Gitlin, J. D. (1996) *Am. J. Clin. Nutr.* **63,** 836S–841S
32. Vulpe, C. D., and Packman, S. (1995) *Annu. Rev. Nutr.* **15,** 293–322
33. Peña, M. M. O., Puig, S., and Thiele, D. J. (2000) *J. Biol. Chem.* **275,** 33244–33251