THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 274, No. 1, Issue of January 1, pp. 218–226, 1999
Printed in U.S.A.

Evidence for (Mac1p)$_2$·DNA Ternary Complex Formation in Mac1p-dependent Transactivation at the *CTR1* Promoter*

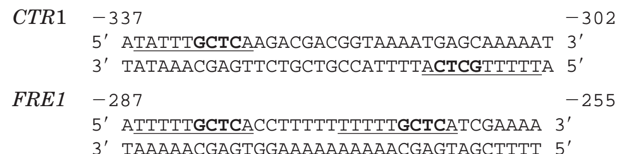
(Received for publication, August 19, 1998, and in revised form, September 25, 1998)

Atul Joshi, Mihaela Serpe, and Daniel J. Kosman‡

From the Department of Biochemistry, School of Medicine and Biomedical Sciences, State University of New York, Buffalo, New York 14214

The Mac1 protein in *Saccharomyces cerevisiae* is required for the expression *CTR1* and *FRE1*, which, respectively, encode the copper permease and metal reductase that participate in copper uptake. Mac1p binds to a core GCTC sequence present as a repeated unit in the promoters of both genes. We show here that Mac1p DNA binding required an intact N-terminal protein domain that includes a likely zinc finger motif. This binding was enhanced by the presence of a TATTT sequence immediately 5′ to the core GCTC, in contrast to a TTTTT one. This increased binding was demonstrated clearly *in vitro* in electrophoretic mobility shift assays that showed Mac1p·DNA complex formation to a single TATTTGCTC element but not to a TTTTTGCTC one. Furthermore, the fraction of Mac1p in a ternary (Mac1p)$_2$·DNA complex in comparison to a binary Mac1p·DNA complex increased when the DNA included two TATTTGCTC elements. A similar increase in ternary complex formation was demonstrated upon homologous mutation of the *FRE1* Mac1p-dependent promoter element. The *in vivo* importance of this ternary complex formation at the *CTR1* promoter was indicated by the stronger trans-activity of this promoter mutated to contain two TATTT elements and the attenuated activity of a mutant promoter containing two TTTTT elements that *in vitro* supported only a weak ternary complex signal in the shift assay. The stronger binding to TATTT appeared due to a more favorable protein contact with adenine in comparison to thymine at this position. An *in vivo* two-hybrid analysis demonstrated a Mac1p-Mac1p protein-protein interaction. This Mac1p-Mac1p interaction may promote (Mac1p)$_2$·DNA ternary complex formation at Mac1p-responsive upstream activating sequences.

The Mac1 protein is a 46-kDa polypeptide that is essential for the copper-regulated expression of high affinity copper uptake activity in the budding yeast *Saccharomyces cerevisiae* (1–7).[1] Several features of the regulatory activity of Mac1p have been described. For example, *in vitro* EMSA[2] has demonstrated that Mac1p binds to a sequence in upstream activating regions of two genes whose expression is known to be Mac1p-dependent, *CTR1* and *FRE1* (4). These two genes encode a high affinity copper permease (9) and metal reductase (10), respectively. The latter activity is required for both copper and iron uptake in yeast, that is reduction of medium Cu(II) and Fe(III) to the lower valent species is an essential first step of the accumulation of both metal ions (2, 11). The sequence element associated with Mac1p binding in these two loci is shown in Scheme I. Inspection of these two fragments in *CTR1* (9) and *FRE1* (10) and in other activating sequences thought to be associated with Mac1p regulation (3, 7, 12) indicates that the core binding site is given by GCTC (shown in boldface). For example, Mac1p- and copper-dependent protection of this region in one of these other loci, *CTR3*, which encodes a second copper permease not expressed in all yeast strains, has been demonstrated by *in vivo* DNA footprint analysis (7).



SCHEME I

In addition to these studies on Mac1p DNA binding activity, use of Mac1p fusions to heterologous DNA binding domains (DBD), *e.g.* that from Gal4 (5) and from the LexA protein (6), has provided evidence that Mac1p also has an inherent transactivation activity that is copper-dependent. The copper dependence of DNA binding and transactivation activity is negative, that is both activities are expressed in copper-deficient cells but are suppressed in copper-replete ones. The down-regulation of Mac1p activity occurs between 1 nM and 1 μM; at [copper] medium $\geq$1 μM little expression from the *CTR1* promoter is observed (5, 9).

As shown in Scheme I, the core GCTC-binding site in the *CTR1* promoter is found within a nearly perfect palindrome; this element in the *FRE1* locus is a direct repeat (sequences underlined in Scheme I). *CTR3* also contains an inverted repeat which, however, is separated by 44 base pairs. That both GCTC sites are essential for transactivation by Mac1p has been indicated by previous studies that demonstrated loss of such activity in reporter promoter constructs derived from the *FRE1* (10), *CTR1* (4), and *CTR3* (7) loci which contained only a single GCTC-containing element. The presence of this repeated motif suggested to us the possibility of Mac1p binding to both sites simultaneously if not cooperatively.

Thus, the objective of the work described herein was to delineate *in vitro* the gross structural features of the Mac1p·DNA complex that had been demonstrated by EMSA (4) and suggested by *in vivo* DNA footprint analysis (7) and to correlate these features with promoter activity *in vivo*. We first tested the hypothesis that the N-terminal domain of Mac1p, which contains a CCHC zinc finger motif (1, 13) and which is

* This work was supported by National Institutes of Health Grant GM46787 (to D. J. K.). 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.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, 140 Farber Hall, 3435 Main St., Buffalo, NY 14214. Tel.: 716-829-2842; Fax: 716-829-2661; E-mail: camkos@acsu.buffalo.edu.

[1] M. Serpe and D. J. Kosman, submitted for publication.

[2] The abbreviations used are: EMSA, electrophoretic mobility shift assay; DBD, DNA binding domain; PCR, polymerase chain reaction; ORF, open reading frame; WT, wild type.

This paper is available on line at http://www.jbc.org

homologous to the N-terminal regions of two other yeast trans-factors, Ace1p (14–16) and Amt1p (17–20), was essential to the binding of Mac1p to DNA. We next evaluated the possible formation of ternary complexes, that is (Mac1p)$_2$·DNA species, and determined what sequence characteristics in the DNA promoted such complex formation. We show a correlation between the *in vivo* activity of a promoter sequence and its ability to support formation of a (Mac1p)$_2$·DNA ternary complex *in vitro*. Finally, we present data from a two-hybrid analysis that demonstrate that in this assay, at the least, a Mac1p-Mac1p interaction can be demonstrated. The data indicate that Mac1p can and does bind at both sites in the *CTR1* promoter simultaneously and that formation of the resulting ternary complex correlates to downstream transcriptional activity.

EXPERIMENTAL PROCEDURES

Strains of S. cerevisiae and Growth Media—Three yeast strains were used in this study. For the one- and two-hybrid analyses, strain SFY526 (obtained from CLONTECH) was used as host (*MAT*a *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3, 112 can*^{r} *gal4-542 gal80-538 URA3::GAL1-lac*Z). For the promoter activity studies using *CTR1* promoter-*lac*Z reporter plasmids, DEY1457 was used as host (*MAT*α *ade6 can1 his3 leu2 trp1 ura3*). This strain was obtained from David Eide (21). For test of the complementing activity of mutant forms of Mac1p (ΔZFMac1p and ZF*Mac1p), DEY1457(*mac1*Δ) was used as host (DEY1457 *mac1::TRP1*) (4). 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 transformed strains (1.67 g/liter yeast nitrogen base without amino acids, 2% glucose *plus* the appropriate drop-out (otherwise complete)) mixture of amino acids; for growth of copper-depleted cultures, a completely synthetic, Chelex-treated medium was used that was prepared as described previously (2). 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. This medium was also used with glycerol replacing glucose as carbon source to test for the respiration competence of DEY1457 (*mac1*Δ) when performing functional tests of mutant forms of Mac1p. This is a standard test for *mac1*Δ-complementing clones that takes advantage of the iron (and, consequently, respiratory) deficiency in this background (1).

Plasmid Construction—All PCR-generated fragments, mutations, and reading frames in the plasmid constructs described below were confirmed by dideoxy sequencing using the T7 Sequenase 2.0 kit from Amersham Life Sciences, Inc. The DNA sequence for the Mac1p ORF was derived from the plasmid MAC16 that was obtained by inserting a *MAC1 Sac*I (−162) to *Hin*dIII(+1997) fragment (1) into the complementary restriction sites in vector pRS316 (22). The ORF for ΔZFMac1p (lacking residues 1–41) was constructed using the endogenous *Nco*I site (+121) in *MAC1* which includes the codon for Met-42. The *Nco*I (filled in)/*Sal*I fragment from MAC16 was ligated into the *Sma*I/*Sal*I sites in pGEM3Zf(+) (Promega) generating pGEM-ΔZF for *in vitro* synthesis of ΔZFMac1p and into the bait pGBT9 (giving pGB-ΔZF) and catch pGAD424 (giving pGAD-ZF) vectors for one- and two-hybrid analyses (23). To construct whole Mac1p ORF fusions, the endogenous *Nco*I (+121) site in MAC16 was destroyed by PCR mutagenesis, and a new *Nco*I site was engineered at the +1 nucleotide (translation start). This modified *Nco*I(−1)/*Pst*I(+210) fragment from MAC16 was exchanged with the *Nco*I(+121)/*Pst*I(+210) fragment in pGB-ΔZF to generate pGB-MAC, comprising the whole Mac1p ORF. The recombinant *Nco*I(−1)/*Sal*I(+1927) 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 (C23S/H25N) was engineered by two rounds of PCR mutagenesis using pGEM-MAC as the template. The *Nco*I(−1)/*Hin*dIII(+1927) fragment from pGEM-ZF*MAC was filled-in and subcloned into the *Sma*I sites in pGBT9 and pGAD424 to make plasmids pGB-ZF*MAC and pGAD-ZF*MAC, respectively.

ΔZF-Mac1p and ZF*Mac1p were functionally tested by complementation in DEY1457Δ*mac1* in the following manner. The respective ORFs from pGEM-ΔZF and pGEM-ZF*MAC were subcloned into vector pADH; the resulting recombinants were subsequently transformed into the Mac1p *minus* strain and the transformants tested for respiration competence as described above. pADH was constructed from pGAD424 by replacing the *Hin*dIII fragment in this vector that contained all sequences from *GAL4* by a multiple cloning site containing an *Nco*I site. Thus, pADH contained the yeast 2μ replication origin, the promoter and terminator from *ADH1*, and *LEU2* for plasmid selection (23).

The wild type *CTR1* promoter-*lac*Z fusion reporter plasmid was obtained from Andrew Dancis (see Ref. 9). The two promoter mutants, C5′AT-*lac*Z and C3′TA-*lac*Z, were derived from the wild type by site-directed mutagenesis using the Quick-Change kit from Stratagene. For the former plasmid, the complementary primers used were 5′-GCAAATCATGGGATTTTTGCTCAAGAC and 5′-GTCTTGAGCAAAAATCCCATGATTTGC (A to T transition underlined), whereas for the latter the primers used were 5′-CGGTAAAATGAGCAAATATGGCACGATCC and 5′-GGATCGTGCCATATTTGCTCATTTTACCG (T to A transition underlined).

EMSAs—The double-stranded oligonucleotides used in the EMSAs are given in Table I. A typical binding reaction contained 2–10 fmol of radiolabeled probe, 0.5 μg of salmon sperm DNA, 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, 0.6 mM dithiothreitol, and competitor DNA when present (14). This 15-μl binding mixture, in addition, contained 3–5 μl of a wheat germ extract transcription-translation reaction (Promega) that had been programmed with either vector alone (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 with or without competitor DNA; 2–10 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 6.0% polyacrylamide gel at 4 °C. The gel was dried and exposed to a PhosphorImager screen and to Kodak Biomax™ 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 figures herein were imaged directly from these digitized intensities. In Figs. 2–6, the relative amount of a given complex with respect to the control is shown using a *bar graph*; the control was the radioactivity associated with the faster migrating complex formed with the WT *CTR1* promoter probe which we have assumed represents a binary Mac1p·DNA complex. Note that in some experiments either $CuCl_2$ (up to 100 μM) or bathocuproine disulfonate (100 μM) were added to determine a potential effect of free [copper] on the EMSA results. None of these treatments had a specific effect as has been reported (4).

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 [^{35}S]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 by appropriate molecular weight. Based on quantitation of the film by image analysis (Bio-Rad Gel Doc system) equivalent amounts of protein were produced from all templates.

β-Galactosidase Assays—Yeast transformants were grown in the appropriate Chelex-treated/copper-supplemented defined medium for at least 5 doublings until the cultures reached mid-log phase (O.D.$_{660\ nm}$ = 1.5–2.0). Samples (2 × 10^7 cells) were assayed in triplicate for β-galactosidase activity which was expressed in Miller units in the standard fashion (24). The data in Fig. 7 are from three independent experiments (samples in triplicate), and statistical significance of differences in mean values was determined with the use of InStat (GraphPad, San Diego, CA).

RESULTS

Mac1p N-terminal Zinc Finger Element Is Required for DNA Binding—Two mutant forms of Mac1p were tested to demonstrate that DNA binding was due at least in part to the N-terminal portion of Mac1p (1) that was homologous to the zinc finger, DNA-binding domain in both Ace1p (15, 16) and Amt1p (17). One mutant had an N-terminal 41-amino acid deletion (ΔZFMac1p). The other was a C23S/H25N double mutant designated ZF*Mac1p; Cys-23 and His-25 are two of the five conserved residues in the zinc finger-like motif found in the three trans-factors. These two constructs, as well as wild type Mac1p, were then tested by EMSA as shown in Fig. 1 using the wild type *CTR1* Mac1p-specific promoter element as probe (see Scheme I and Table I). Binding of wild type Mac1p to this probe

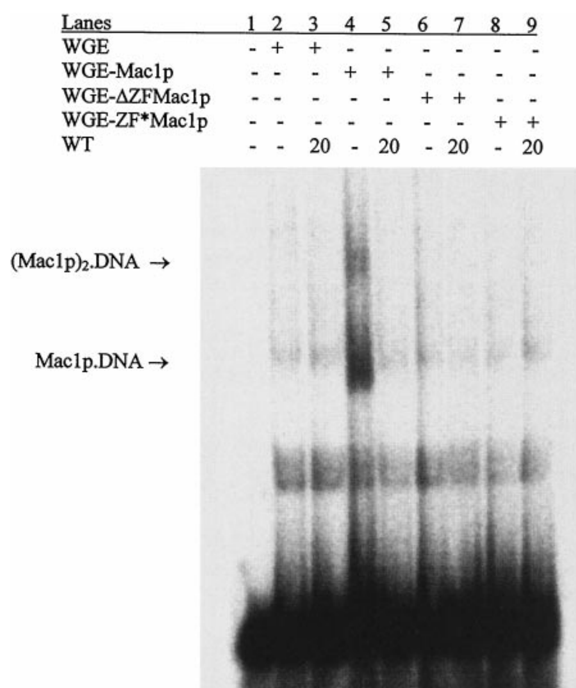


FIG. 1. **N-terminal zinc finger like domain of Mac1p is involved in DNA binding.** Binding reactions were performed with 10 fmol of radiolabeled wild type probe derived from the *CTR1* promoter (see Table I for probes). Wild type Mac1p (*lane 4*), ΔZFMac1p (*lane 6*), and ZF*Mac1p (*lane 8*) were produced *in vitro* using the coupled wheat germ extract system (*WGE*, negative control, *lanes 2* and *3*) and added to the binding reaction as indicated in the figure. When present, unlabeled wild type oligonucleotide derived from the *CTR1* promoter (*WT*) was added at a concentration 20-fold greater than the probe (*lanes 3, 5, 7,* and *9*). The image is obtained from a Bio-Rad PhosphorImager as described under "Experimental Procedures."

was clearly evident (*lane 4*). In contrast, neither mutant protein yielded a detectable level of protein-DNA complex in this assay (ΔZFMac1p, *lane 6*; ZF*Mac1p, *lane 8*). Furthermore, neither mutant construct was capable of complementing a deletion in *MAC1* when expressed in a Δ*mac1*-containing background (see "Experimental Procedures" and data not shown). These results most reasonably suggest that a major determinant of functional Mac1p binding to DNA resides in the Ace1p/Amt1p homology element in the N-terminal domain of the protein.

Characterization of Mac1p·DNA Binary and (Mac1p)$_2$·DNA Ternary Complexes at the CTR1 Promoter—The repeated nature of the core GCTC element suggested to us the possible formation of a transcriptionally active protein-DNA complex, (Mac1p)$_2$·DNA. We tested this model by first establishing the presence of such a complex by EMSA and then by delineating the sequence features of the DNA that promoted its formation.

In fact, two mass species were apparent in an EMSA in which the wild type *CTR1* sequence (WT, Table I) was used as probe (Fig. 2, *lane 3*; see also Fig. 1, wild type Mac1p, *lane 4*). The more slowly migrating of these two complexes (*shaded bar*, quantitation below) was present with an abundance of 50% that of the faster migrating species (*solid bar*, quantitation below). We infer that the more slowly migrating species is larger in mass and arises from separate protein molecules binding to the two core elements. This inference is derived from the disappearance of this slowly migrating species when either core element was mutated indicating that two elements were required for the formation of this complex (*lanes 6* and *9*, probes $R_{5'}$ and $R_{3'}$, respectively). In probes $R_{5'}$ and $R_{3'}$, the core sequences in either the 5′ or 3′ element, respectively, were randomized (Table I). However, the data indicated that these two sites were not equivalent since the fraction of the probe found in what we will refer to as a binary Mac1p complex (the smaller, faster migrating species) was essentially absent if the 5′ core sequence was mutated (in $R_{5'}$, Fig. 2, *lane 6*) in comparison to mutation of the 3′ core (as in $R_{3'}$, Fig. 2, *lane 9*). This result suggested that a binary complex at the 5′ element was more stable than one at the 3′ sequence.

The primary structural difference between these two core elements resides in the sequence immediately 5′ to both. The upstream element is 5′-TATTTGCTC and the downstream one is 5′-GAGCAAAAA (5′-TTTTTGCTC on the opposite strand). The data above suggested the possibility that the presence of the TTTTT sequence in the latter 3′ element dampened Mac1p binding to the core or, alternatively, the A in the former 5′ element was preferentially recognized by Mac1p. Furthermore, the EMSA indicated that binding of Mac1p to the apparently weaker 3′ element in the wild type promoter, as must occur in a ternary complex, was linked to Mac1p binding to the apparently stronger 5′ element. This latter conclusion derives from the fact that a single TTTTTGCTC site cannot support formation of a detectable binary complex (Fig. 2, $R_{5'}$ probe, *lane 6*), whereas in the context of an intact upstream TATTT-containing element, ternary complex formation does occur which must involve Mac1p binding to the 3′ element (*WT* probe, *lane 3*).

One test of this conclusion was to convert the 3′-TTTTT-GCTC element in $R_{5'}$ (to which Mac1p binds weakly if at all, Fig. 2, *lane 6*) to 5′-TATTTGCTC (again, reading on the opposite strand). The binding of Mac1p to this mutant, designated $R_{5'}$-3′-TA, would be expected to increase in comparison to binding to $R_{5'}$ itself. This prediction was tested by direct binding with $R_{5'}$-3′-TA as probe (data not shown; see however, Fig. 6, *lane 3*) and also by the more quantitative competition assay. Thus, unlabeled mutant oligonucleotides were used as competitor DNA of the Mac1p binding to the WT *CTR1* promoter element as probe (Fig. 3, control, *lane 3*). Based on these competition data as in Fig. 3 we estimate that Mac1p has a 3–4-fold greater affinity for the $R_{5'}$-3′-TA oligonucleotide (stronger competitor as shown in Fig. 3, *lanes 6–9*) than for $R_{5'}$ itself (Fig. 3, *lanes 4–6*; also, see quantitation).

A second test of this conclusion was to use as probe mutated wild type double-stranded oligonucleotides in which both elements contained either TATTT or TTTTT, followed by GCTC. The TATTT-containing repeat would be expected to support both stronger binding overall and, importantly, a larger fraction of complex in what we propose is (Mac1p)$_2$·DNA. In contrast, the mutant that had T_5 at both sites would be expected to have weaker binding overall and little or no ternary complex formation. Both of these predictions were confirmed by experiment as demonstrated by the EMSA shown in Fig. 4*A*. Specifically, the symmetrical probe containing repeated TTTTT-GCTC elements (Fig. 4*A*, probe *5′-AT*, *lane 6*) supported 75% ternary complex formation compared with WT probe (*WT*, *lane 3*), whereas the symmetrical probe containing TATTTGCTC (probe *5′-TA*, *lane 9*) supported 150% of this complex compared with WT (*shaded bars*, quantitation). Furthermore, competition experiments in which wild type was used as probe and these two mutant oligonucleotides were used as competitors (see Fig. 4*B*) yielded a pattern in complete agreement with the direct binding data shown in Fig. 4*A*. Thus, the T_5-containing direct repeat (*5′-AT, lanes 3* and *4*) was a poorer competitor than the TAT_3-containing species (*3′-TA*, *lanes 5* and *6*; data in bar graph presented as a percent of values for the WT probe alone, see Fig. 4*A*, *lane 3*, for this control). In summary, the data in Figs. 2–4 indicate that Mac1p binds more strongly to the sequence TATTTGCTC than to TTTTTGCTC, and this better binding appears to stabilize what is interpreted to be a ternary (Mac1p)$_2$·DNA complex.

Table I
Double-stranded oligonucleotides used in mobility shift assays
Only the top strand is shown, 5′ → 3′. The base substitutions are underlined.

Source	Designation	Sequence
CTR1	WT	GATCCGATATTTGCTCAAGACGACGGTAAAATGAGCAAAAATCG
	$R_{5'}$	GATCCGATAGTCATCTTAGACGACGGTAAAATGAGCAAAAATCG
	$R_{3'}$	GATCCGATATTTGCTCAAGACGACGGTAAAAAAGATGACAATCG
	$R_{5'}$-3′-TA[a] (R$_{5'}$-A40T)[b]	GATCCGATAGTCATCTTAGACGACGGTAAAATGAGCAAATATCG
	5′-AT	GATCCGATTTTTGCTCAAGACGACGGTAAAATGAGCAAAAATCG
	3′-TA[a]	GATCCGATATTTGCTCAAGACGACGGTAAAATGAGCAAATATCG
	$R_{5'}$-A39T[a]	GATCCGATAGTCATCTTAGACGACGGTAAAATGAGCAATAATCG
	$R_{5'}$-A41T[a]	GATCCGATAGTCATCTTAGACGACGGTAAAATGAGCAAAATTCG
FRE1	WT	CTGATATTTTTGCTCACCTTTTTTTTTTGCTCATCGAAA
	3′-TA	CTGATATTTTTGCTCACCTTTTTTATTTGCTCATCGAAA

[a] A → T transition on the top strand (shown) results in a T → A transition in the Mac1p-binding site on the bottom strand.
[b] $R_{5'}$-A40T is the alternate name for $R_{5'}$-3′-TA.

The *FRE1* promoter provides a natural test of this model. The EMSA shown in Fig. 5 demonstrates that little complex formation at the *FRE1* promoter element was observed (Fig. 5, *FRE1-WT* probe, *lane 6*; compare with binding to the *CTR1-WT* probe, *lane 3*). Competition by this *FRE1* element of Mac1p binding to the wild type *CTR1* 44-mer showed that, indeed, Mac1p has a weaker affinity for the former sequence (data not shown). To show that this weaker binding was likely due to the absence of even one TATTT-containing site in the WT *FRE1* probe, a mutant *FRE1* element was used in which the 3′ site contained the requisite T to A transition. Consistent with our model, while the wild type *FRE1* element gave only a weak signal in the EMSA (Fig. 5, *lane 6*), the mutant probe supported the formation of both binary and ternary complexes (Fig. 5, *FRE1*-3′-TA probe, *lane 9*). Although the stability of these complexes appeared less than with the wild type *CTR1* element (which also has one each of the two types of T-rich sequences), the relative amount of the two complexes at the mutant *FRE1* oligonucleotide was similar to what was observed with the *CTR1* one (see quantitation). Importantly, the fact that a single TATTT at the *FRE1* promoter converted this element from one that supported only a very weak interaction overall to one that actually could support ternary complex formation suggests a model in which formation of the ternary complex could be cooperative, *i.e.* that it might involve a thermodynamically important Mac1p-Mac1p protein-protein interaction linked to Mac1p binding at a TATTT-containing site.[3]

Origin of the Stronger Binding of Mac1p to TATTTGCTC—One simple explanation for the difference in Mac1p binding to the TATTTGCTC sequence is that a specific protein-DNA interaction could be more favorable with A in comparison to T at this −4 position (relative to the G in the core sequence). To test this possibility, three probes were constructed based on the random 5′ sequence ($R_{5'}$, Fig. 2) in which one of the thymines in the wild type 3′ element was replaced by adenine. Thus, this series consisted of oligonucleotides containing 5′-TTTTTGCTC ($R_{5'}$), 5′-ATTTTGCTC ($R_{5'}$-A41T), 5′-TATTTGCTC ($R_{5'}$-A40T; this is identical to $R_{5'}$-3′-TA used as competitor in Fig. 3), and 5′-TTATTGCTC ($R_{5'}$-A39T; sequences given for bottom strand, Scheme I). If the specific placement of the A were important to Mac1p binding, Mac1p binding to only one of these probes would be observed, presumably to $R_{5'}$-A40T as indicated by the competition data in Fig. 3. As the EMSA results in Fig. 6 show, Mac1p appeared to make a more favorable contact with A in comparison to T at this −4 position in as much as binding to the single, 3′-GCTC in $R_{5'}$ was observed only with the probe containing 5′-TATTTGCTC on the bottom strand (Fig. 6, *$R_{5'}$-A40T*, *lane 3*). In terms of binding affinity for Mac1p, $R_{5'}$-A40T should be equivalent to $R_{3'}$, since both mutant oligonucleotides have only one TATTTGCTC site. Comparison of the data for $R_{5'}$-40T (Fig. 6, *lane 3*) and $R_{3'}$ binding (Fig. 2, *lane 9)* shows this to be the case.

(Mac1p)$_2$·DNA Complex Formation in Vitro Correlates to Stronger Promoter Activity in Vivo—To test whether the ternary complex formation indicated by the EMSA data might be functionally important, the WT *CTR1* promoter, in a fusion to the *lac*Z gene, was mutated to contain either two TTTTT or two TATTT elements. The transcriptional activity of the wild type and two mutant promoters was then quantitated by standard β-galactosidase assay. To demonstrate also the relative dependence of the activity of these promoter constructs on the copper concentration in the medium, cultures were grown in a copper-depleted medium (estimated 0.5 nM residual copper, "Experimental Procedures") or in the presence of added copper. The results of these measurements are shown in Fig. 7; they demonstrate a positive correlation between transcriptional activity *in vivo* and the fraction of Mac1p in (Mac1p)$_2$·DNA *in vitro*. Specifically, while the 5′-AT mutant promoter (Fig. 7, *squares*) supported only 65% the expression seen from WT *CTR1* (Fig. 7, *circles*), the 3′-TA mutant supported 115% of this expression (Fig. 7, *triangles*). These differences can be compared with those described for the EMSA results (Fig. 4*A*) in which the symmetrical T_5-containing 5′-AT and symmetrical TAT_3-containing 3′-TA probes supported 75 and 150% (Mac1p)$_2$·DNA formation, respectively (in comparison to the *WT CTR1* probe, Fig. 4*A*, *lane 3*).

The mutant promoters were equivalently down-regulated by copper indicating their essential dependence on Mac1p function. However, the dependence of this down-regulation on $[copper]_{medium}$ was shifted to higher values for the more active promoter element, that is in our model, the greater the fraction of ternary protein-DNA complex. From the data in Fig. 7, one can estimate that the $[copper]_{medium}$ that resulted in the half-maximal change in trans-activity for the WT *CTR1* promoter was ~20 nM (Fig. 7, *circles*); for the 5′-AT (*triangles*) and 3′-TA (*squares*) mutant *CTR1* promoter sequences it was 10 and 30 nM $CuCl_2$, respectively. This pattern would be consistent with a differential Mac1p-DNA interaction in the three promoter constructs. The fact that a single Mac1p-binding site is incapable of supporting transcription *in vivo* has been demonstrated previously (4, 7, 12). Thus, the quantitative data shown in Fig. 7

[3] A double *FRE1* promoter mutant was not constructed (both sites containing TATTT) since in making such a mutation at the 5′ site, one would be constructing a perfect TATA element due to the flanking sequence. This would likely be target for the transcription factors present in the *in vitro* transcription/translation mix used to generate the Mac1p for these experiments giving rise to a confusing Mac1p-independent mobility shift(s).

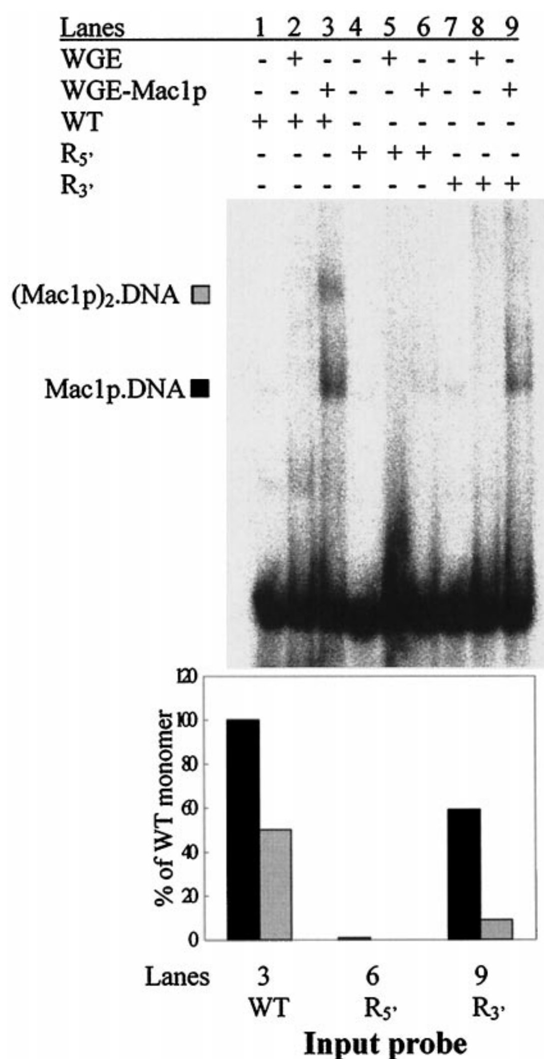


FIG. 2. **Formation of binary and ternary Mac1p-DNA complexes on the *CTR1* promoter.** Binding reactions were performed with 2 fmol of the probe DNA as described under "Experimental Procedures." Radiolabeled wild type (*WT*, *lane 3*) or mutated $R_{5'}$ (*lane 6*) or $R_{3'}$ (*lane 9*) double-stranded oligonucleotides derived from the *CTR1* promoter (Table I) were incubated with either 5 μl of coupled wheat germ extract alone (*WGE*, negative control) or with 5 μl of wheat germ extract containing wild type Mac1p (*WGE-Mac1p*). The binding reactions were electrophoretically resolved on a 6.0% gel, and the gel was dried and exposed to a PhosphorImager screen. The resulting bands were quantitated using Molecular Analyst, and the digitized data were plotted as bar graphs with respect to the control, the counts associated with the faster migrating complex formed with the WT *CTR1* probe. The data shown are representative of three experiments; although the absolute amount of complex varied, the relative amounts of the complexes formed (as given by percent of wild type monomer species) varied by less than 5%.

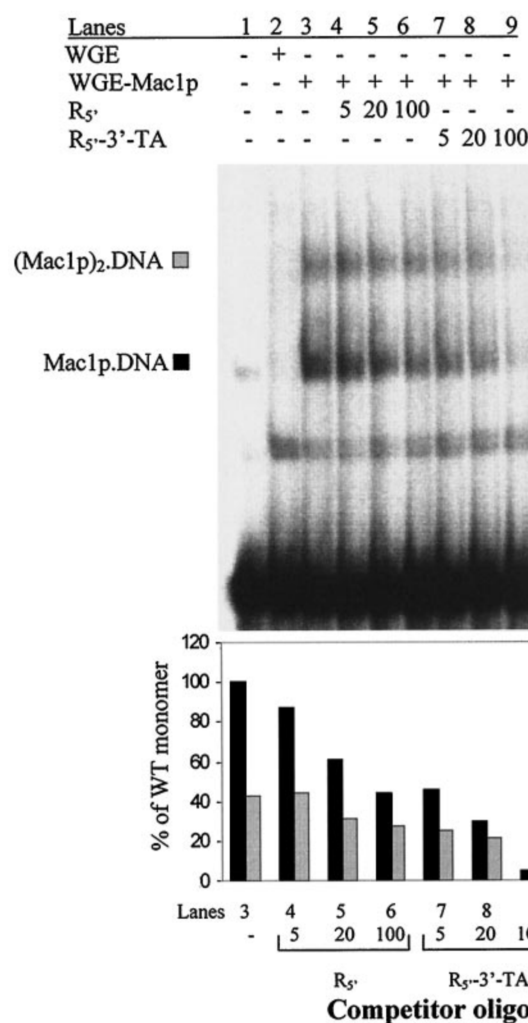


FIG. 3. **Dependence of Mac1p binding on DNA sequences 5′-TATTT *versus* 5′-TTTTT.** Binary (Mac1p·DNA) and ternary [(Mac1p)$_2$·DNA] complexes formed on the WT *CTR1* probe (*lane 3*) were competed by 5-, 20-, and 100-fold excess of $R_{5'}$ (*lanes 4–6*) or $R_{5'}$-TA (*lanes 7–9*) oligonucleotides. Binding reactions were analyzed and the data presented as described above.

are consistent with a model in which a (more stable) ternary Mac1p-containing DNA complex at the *CTR1* promoter is active in the subsequent up-regulation of the expression of this gene. Furthermore, they indicate that the structural difference(s) between TATTT and TTTTT that modulate the stability of this complex *in vitro* apply *in vivo* as well. It is important to note that the copper-dependent down-regulation of Mac1p trans-activity that occurs between 1 and 100 nM (as in Fig. 7) is entirely independent of the turnover of Mac1 protein that occurs when cells are exposed to $[\text{copper}]_{\text{medium}} > 10$ μM or under conditions of incipient copper toxicity (25).

A Mac1p-Mac1p Interaction Can Be Demonstrated by a Two-hybrid Analysis—We inferred above that formation of what we suggest is a ternary, (Mac1p)$_2$·DNA complex might involve a thermodynamic contribution due to a protein-protein interaction between two Mac1p molecules. However, the data do not exclude an alternative model in which two Mac1p molecules bind to the DNA completely independently of one another. We have been unable to produce and isolate Mac1p that is active in the DNA binding experiments necessary to distinguish between these two possibilities. Therefore, we chose to demonstrate a possible Mac1p-Mac1p interaction independent of DNA binding by the *in vivo* two-hybrid genetic approach (23).[1] To do so, fusions of wild type Mac1p, the ΔZFMac1p truncation, and the mutated zinc finger Mac1p, ZF*Mac1p, were made to both the Gal4 DNA-binding (DBD) and transactivation domains. These fusions were tested for *in vivo* interaction by the standard two-hybrid assay in cells grown in both the absence and presence of copper. The results of these several assays are summarized in Table II. Also included in this table (in parentheses) are representative values for the Mac1p-Gal4 DBD fusions alone in a one-hybrid assay. These values describe the inherent transactivation activity of Mac1p and are needed for comparative purposes in as much as this Mac1p trans-activity is copper-dependent (5, 6).[1]

The data in Table II supported two basic conclusions. First, irrespective of the strength of the Mac1p-Mac1p interaction indicated by a given fusion pair, the interaction was essentially

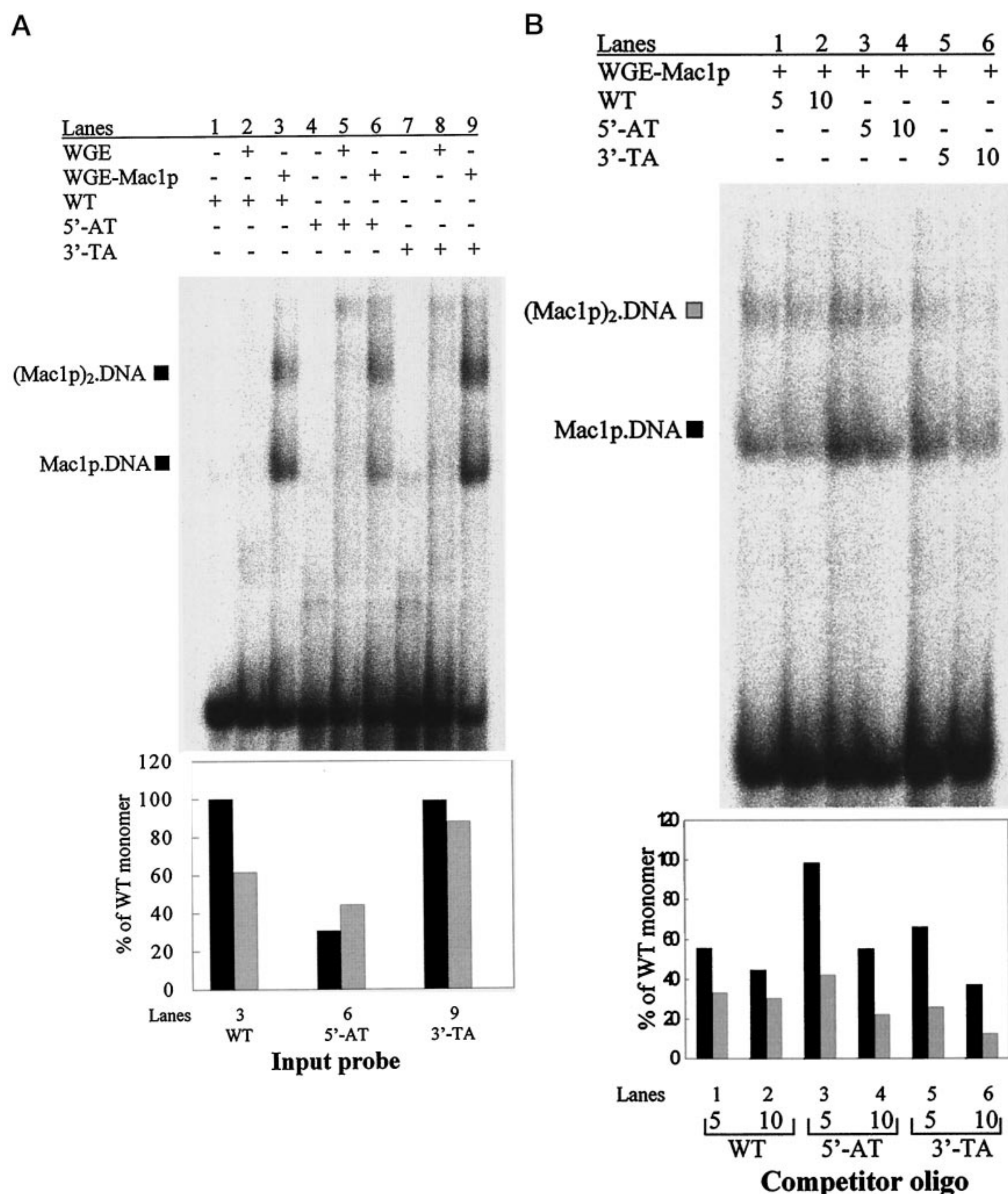


FIG. 4. **Distribution of binary and ternary complexes on wild type and mutant *CTR1* probes.** Binding reactions were performed with 2 fmol of probe DNA as described under "Experimental Procedures." *A,* WT (*lane 3*) or mutant 5′-AT (*lane 6*) or mutant 3′-TA (*lane 9*) oligonucleotides derived from the WT *CTR1* probe (Table I) were added to the binding reactions as probe DNA and analyzed as above. *B,* WT *CTR1* oligonucleotide was used as probe DNA. Competition experiments were carried out using 5- and 10-fold excess of WT (*lanes 1* and *2*) or 5′-AT (*lanes 3* and *4*) or 3′-TA (*lanes 5* and *6*) mutant oligonucleotides derived from the WT *CTR1* probe. Analysis of the PhosphorImages was as described.

independent of medium copper (compare values in first and third rows, all columns). This was in contrast to the results from the one-hybrid analysis that showed that the inherent trans-activity of Mac1p was consistently 7–10-fold greater in copper-deficient cells in comparison to copper-replete ones. This copper dependence is illustrated best by the transcriptional activity due to the ΔZFMac1p fusion (Table II, third column, compare second and fourth rows).[4] Second, a Mac1p-Mac1p interaction was increased ~10-fold upon removal of the N-terminal sequences suggested above to have DNA binding activity as in the ΔZF truncation (Table II, compare first to subsequent columns). This fold increase paralleled the increase in the trans-activity of this truncated Mac1p noted directly above (Table II, third column).[1] The effect of N-terminal deletion was geometric, that is this domain in each Mac1p fusion contributed equally in energy terms to the masking of the strong Mac1p-Mac1p interaction indicated for the ΔZFMac1p fusion pair (compare columns 1, 2, and 4, first and third rows). Significantly, increase in neither trans-activity nor Mac1p-Mac1p interaction was observed when a fusion of the C23S/H25N double mutant was used (ZF*Mac1p, last column), a mutant which lacks DNA binding activity (Fig. 1) but which still has an N-terminal domain. Interaction between two mol-

[4] Full-length Mac1p (or the mutant ZF*Mac1p) has very little trans-activity in a one-hybrid fusion. This activity resides in the C-terminal half of the molecule and is expressed in the one-hybrid assay only when N-terminal Mac1p truncations, such as ΔZFMac1p, are fused to the Gal4 DBD (Table II).[1]

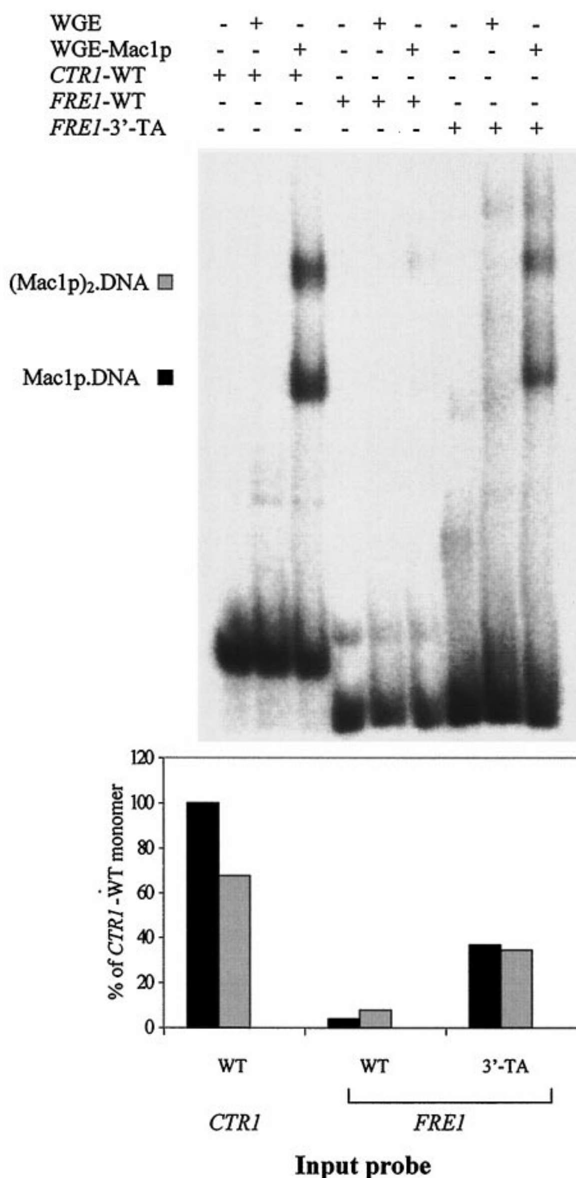


FIG. 5. **Interaction of Mac1p with wild type and mutant *FRE1* promoter elements.** WT *CTR1* (CTR1-WT, 2 fmol, *lane 3*), WT *FRE1* (FRE1-WT, 4 fmol, *lane 6*), or mutated *FRE1* (*FRE1*-$R_{3'}$-TA, 4 fmol, *lane 9*) oligonucleotides (Table I) were used as probes in this EMSA. The binding mixtures were analyzed and quantitated as described.

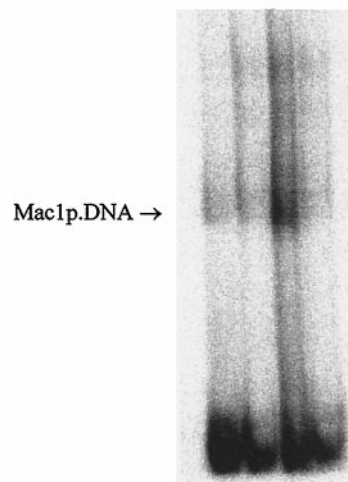


FIG. 6. **Interaction of Mac1p with adenine *versus* thymine in the T-rich sequence in the *CTR1* promoter.** T/A transitions were made in the 3′-T_5 region of the $R_{5'}$ *CTR1* probe used in Fig. 2 (*lane 1*) to yield core 3′-binding sites of 5′-ATTTTGCTC ($R_{5'}$-A41T, *lane 2*), 5′-TATTTGCTC ($R_{5'}$-A40T, *lane 3*, equivalent to $R_{5'}$-3′-TA, Fig. 3), and 5′-TTATTGCTC ($R_{5'}$-A39T, *lane 4*). Each of these oligonucleotides were then used as probe. The binding mixtures were analyzed and quantitated as described.

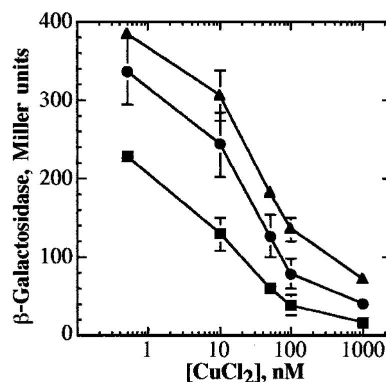


FIG. 7. **Dependence of *CTR1* promoter activity on DNA sequences 5′-TATTT *versus* 5′-TTTTT.** The effect of putative (Mac1p)$_2$·DNA ternary complex formation was assessed *in vivo* using *CTR1* promoter-*lac*Z fusions in strain DEY1457. The fusions included WT (*circles*, *middle trace*) and 5′-AT (*triangles*, *upper trace*) and 3′-TA (*squares*, *lower trace*) mutant *CTR1* promoter sequences. Mid-log yeast cultures grown in copper-depleted media supplemented with either none, or 10, 50, 100, or 1000 nM $CuCl_2$ were used to measure β-galactosidase activities which are presented in the standard fashion (Miller units). The bars indicate ±S.D. ($n = 3$ separate experiments, samples in triplicate; the absence of visible bars indicates the S.D. was 8 Miller units or less). At each [copper], the values for the three promoters were different from one another at $p < 0.001$ or better. The [copper] in the medium with no added copper was estimated to be 0.5 nM since this level was below the detectable limit of flameless atomic absorption spectrophotometry. This estimate was based on the residual [copper] in the individual components used to make up the medium and the affinity of Chelex for Cu(II).

ecules of this mutant form was identical to that between the wild type protein, *i.e.* it was weak, giving a value only slightly above the blank.

DISCUSSION

The experiments described here establish six new facts and/or inferences about the structure-function relationships pertaining to the Mac1p-DNA interaction. These are as follows: 1) Mac1p DNA binding activity requires at the least the CCHC Ace1p/Amt1p zinc finger homology domain; 2) a complex of Mac1p and its DNA target forms in an EMSA that is most reasonably described as a ternary, (Mac1p)$_2$·DNA species in addition to a Mac1p·DNA binary one; 3) Mac1p binding to any core GCTC element is modulated by the immediate 5′ sequence, specifically, binding is stronger to TATTTGCTC than to TTTTTGCTC; 4) Mac1p appears to make a specific and favorable contact with adenine at the −4 sequence position (tAttt) in comparison to a thymine (tTttt); 5) a mutated *CTR1* promoter that conforms to a perfect palindrome including the TATTT sequence supports a larger fraction of Mac1p in the ternary complex *in vitro* and a stronger Mac1p-dependent expression from the *CTR1* promoter *in vivo*; and 6) a Mac1p-Mac1p protein-protein interaction occurs that appears negatively modulated by the N-terminal domain of the protein that is essential to DNA binding. Based on these new facts and on

TABLE II
Two-hybrid analysis of the Mac1p-Mac1p interaction

	Mac1p[a] Mac1p[b]	Mac1p ΔZFMac1p	ΔZFMac1p Mac1p	ΔZFMac1p ΔZFMac1p	ZF*Mac1p ZF*Mac1p
Media additions			*β-galactosidase activity, Miller units*[c]		
None	0.32	2.94	4.42	15.39	0.51
(TA of bait)[d]	(0.04)		(0.35)		(0.05)
$CuCl_2$, 10 μM	0.38	3.65	3.73	14.80	0.37
(TA of bait)[d]	(0.02)		(0.03)		(0.02)

[a] Gal4 DBD fusion (bait).
[b] Gal4 TAD fusion (catch).
[c] All values were read against the blank value (vectors alone) which was 0.02 Miller units. The values shown are means from one experiment (n = 3 cell samples, S.E. ±4–8%) that was representative of 3 or 4 separate experiments for each transformant. For comparison, the positive control in the two-hybrid assay, p53 (bait)/SV40 T-antigen (catch), had a value of 39.35 Miller units. In the absence of added copper ("None"), the media used were estimated to have 0.5 nM residual copper.
[d] Values in parentheses are for the transactivity (TA) of the fusion protein bait in a one-hybrid assay at the [Cu] indicated. The transactivity of the Mac1p fusions resides in the C-terminal domain (residues 203–417) which gives a value of 21.21 Miller units (−Cu) when fused to the Gal4 DBD. At [Cu] = 1 μM, this value is only 0.35 units demonstrating its copper dependence.[1]

previously reported ones, we propose the following model of how the interactions of Mac1p with itself and with its DNA binding site are linked to the activation of gene expression at Mac1p-dependent genes.

The components of this model are as follows. (*a*) $(Mac1p)_2$·DNA is, at the least, a transcriptionally active species. Mac1p-dependent transactivation requires two Mac1p DNA-binding sites, as in the *FRE1* (10), *CTR1* (4), and *CTR3* promoters (7). A single core GCTC element cannot support Mac1p- and copper-dependent expression from these promoters. Our data indicate that this requirement is most reasonably explained by a model in which the Mac1p·DNA complex that is competent for transcriptional activation is a ternary one, $(Mac1p)_2$·DNA. (*b*) Mac1p can be in (minimally) two conformational states. The "resting" state is inactive with respect to any of the interactions necessary for Mac1p-dependent transactivation, *i.e.* DNA binding, Mac1p-Mac1p interaction, and recruitment of components of the pre-initiation complex. The "active" state can participate in each of these intermolecular interactions. (*c*) The interconversion between these two states is linked to these three interactions, that is Mac1p has the characteristics of a thermodynamically cooperative system: the *intermolecular* interactions in the active state (which include all interacting components) compensate for the loss of the *intramolecular* ones (in Mac1p) which stabilize the resting state. (*d*) The DNA-binding, zinc finger-containing N-terminal domain is the key element in this cooperativity in that in the resting, unbound (to DNA) Mac1p conformation this domain masks those other domains involved in either the Mac1p-Mac1p interaction or in the recruitment of other transcription factors. Reciprocally, in the resting state these other domains mask the N-terminal domain from the DNA.

That the DNA binding activity of Mac1p requires at the least the N-terminal domain including an intact CCHC motif is not surprising. Previous work with Ace1p (16) and Amt1p (19) suggested that in both of those systems the homologous N-terminal domains made important DNA contacts, as, for example, in the major groove in the case of Ace1p binding to the metal-responsive element in the *CUP1* promoter (16). However, in neither case are the core *cis* elements (GCTG in the case of the Ace1p-binding site) and the flanking regions, which also make protein contacts, repeated as they are in *CTR1* and *CTR3* (and *FRE1*, albeit as a direct rather than an inverted repeat). Furthermore, the C-terminal core cysteine-rich regions of Ace1p and Amt1p, which when bound to Cu(I) form what has been described as a "copper fist," also make specific DNA contacts (16, 19). Indeed, it is the copper fist domain in Ace1p that makes the major contacts with the Ace1p-core binding site, TTTCCGCTG (16). Structure-function relationships in Mac1p appear to be quite different. Current data are most consistent with a more strict demarcation of function between the N- and C-terminal domains in the protein; the C-terminal domain expresses all of the transactivation activity (and its copper dependence, much as in Ace1p and Amt1p), whereas the N terminus is required for DNA binding. Note also that both Ace1p and Amt1p are *positively* regulated by copper, the inverse of the behavior of Mac1p in response to copper level.

The presence of the inverted repeat in the *CTR1* (and *CTR3*) promoters (3, 7, 9), TTTGCTC, and the fact that promoter deletion analyses in the *CTR1* (4) and *CTR3* promoters (7) demonstrated the need for both repeats suggested to us that Mac1p might bind at both sites irrespective of the precise nature of the ternary complex. Our data are the first that support this possibility. Our EMSA results show clearly that two Mac1p-dependent complexes form and, furthermore, that the formation of both, particularly the more slowly migrating one, appears driven by a stronger Mac1p binding to the 5′ element, TATTTGCTC. Importantly, this stronger binding *in vitro* can be correlated with stronger promoter activity *in vivo* in support of our model that a $(Mac1p)_2$·DNA complex is at the least more active transcriptionally if not *the* active complex. However, our data do not prove this latter constraint, leaving open the question of whether both, or only the ternary complex, can support downstream transcription initiation.

The stronger Mac1p binding to the TATTT-containing motif appeared due to a more favorable interaction with A in comparison to T at this −4 position specifically. Of interest is that of the six Mac1p elements analyzed (two each in *CTR1*, *CTR3*, and *FRE1*), only two contain an adenine at this position, one in *CTR1* and one in *CTR3*. In contrast, the equivalent −4 position in the Ace1p and Amt1p core binding sites at various loci has an invarient thymine, or T(T/C)*XX*GCTG (8, 19). This conservation suggests that this specific nucleotide base-protein contact, which has been confirmed by experiment (16, 19), makes a more substantive contribution to the overall stability of the protein·DNA complex in the case of Ace1p and Amt1p than it does in the case of Mac1p. One explanation for this difference would be that in the case of Mac1p-DNA ternary complex formation, a Mac1p-Mac1p interaction provides an additional and significant driving force.

One caveat of our work is that we have not provided direct evidence for the presence of two Mac1p molecules in the more slowly migrating DNA complex. Although we have attributed this difference in mobility to a larger size, *i.e.* to a $(Mac1p)_2$·DNA complex, another possibility is that the more slowly migrating species is a conformer of the Mac1p·DNA binary complex. However, the appearance of this species in the binding of Mac1p to the *FRE1* promoter element upon mutation of only one of the core sequences to include an upstream TATTT is more consistent with a ternary complex model. With

this mutant oligonucleotide as probe, the distribution of Mac1p-DNA species was equivalent to what was observed with the wild type *CTR1* promoter, that is introducing one strong site led to the appearance of both mobility species. This can most simply be explained by a cooperative binding model in which the "good" site recruits Mac1p and that the resulting DNA-protein interaction stabilizes a second Mac1p binding (to an inherently weaker DNA-binding site) through a Mac1p-Mac1p interaction. However, while a cooperative dimer model is consistent with the EMSA results shown, it clearly remains to be tested more rigorously than it has been here.

The two-hybrid results indicated that Mac1p can, in effect, self-associate. One aspect of these data was particularly significant, namely the potential protein-protein interaction was strongly suppressed by an intact N-terminal domain. Thus, the ΔZF construct that lacked Mac1p residues 1–40 supported a 10–40-fold stronger interaction in comparison to the fusions with Mac1p (1–417) as both bait and catch. We interpret this result to show that the N-terminal domain (whether wild type or mutated, *e.g.* as in ZF*Mac1p) sterically blocks self-association of Mac1p by an *intramolecular* contact with the region in Mac1p involved in the *intermolecular* protein-protein interaction. In our model, we postulate that this intramolecular interaction is replaced (energetically compensated for) by the intermolecular one the N-terminal domain has with the DNA. In the Mac1p·DNA complex, therefore, the DNA-bound Mac1p (conformationally equivalent to ΔZFMac1p) is active for binding to another molecule of Mac1p. The *apparent* strength of this latter interaction will be dependent on whether the second Mac1p is also active for binding (is bound to DNA, resulting in a ternary complex) or, in the two-hybrid case, whether the second Mac1p is intact or N-terminal deleted.

We interpret the one-hybrid data to indicate that the N-terminal domain also blocks the recruitment by Mac1p of components of the general transcription machinery (as it does the apparent Mac1p-Mac1p interaction) when Mac1p itself is not bound to DNA via its own N-terminal domain. Thus, the 10-fold increase in the (copper-dependent) expressed trans-activity in the N-terminal deleted construct in comparison to Mac1p-(1–417) was nicely consistent with the suggestion that the DNA-binding N-terminal domain masks elements in the C-terminal region involved in both types of protein-protein interaction.

The role of copper in the regulation of the inherent transcriptional activity of Mac1p was not clarified by the results presented here. Clearly, Mac1p, either by itself or as the TA domain in a DBD fusion, *e.g.* to Gal4 DBD (above and Ref. 5)[1] or to the LexA protein (6), is down-regulated by a copper concentration in the medium that is known to down-regulate the expression of *CTR1* (*cf.* Fig. 7). The $[\text{copper}]_{\text{medium}}$ that causes a 50% suppression of the transcriptional activity of Mac1p is ~20 nM. Furthermore, *in vivo* DNA footprinting at the *CTR3* promoter (Ref. 18, and at the *CTR1* promoter as well)[5] has demonstrated a Mac1p-dependent protection at the Mac1p-binding sites that is negatively modulated by copper. On the other hand, neither the Mac1p-Mac1p protein-protein interaction indicated by our two-hybrid data nor the *in vitro* DNA binding experiments employing a full-length Mac1p (EMSA) exhibited a copper dependence (described under "Experimental Procedures"). Whereas the latter results could be ascribed to the use of, for example, *in vitro* generated protein, the former result cannot be so easily disregarded. The fact that the same fusion exhibited a copper-dependent trans-activity in a one-hybrid assay but a copper-independent protein-protein interaction in a two-hybrid one most reasonably suggests that one *specific* protein-protein interaction, self-association, is, in fact, not modulated by the alteration in Mac1p that is caused by copper.

Clearly, the model that we propose here requires additional rigorous experimental evaluation. For example, one could predict differential protection of the two GCTC elements in the wild type *CTR1* promoter in a DNA footprinting experiment, whether *in vivo* or *in vitro*. The one published *in vivo* footprint, at the *CTR3* promoter, was relatively symmetric (7). However, additional footprinting experiments using altered Mac1p-binding sites as used here in the EMSA along with Mac1 protein that either has or does not have the potential to self-associate could provide new and important insight into the protein-protein and protein-DNA equilibria involved in the Mac1p-DNA interaction whether or not they specifically confirm the present model. These experiments are in progress.

Acknowledgments—We are indebted to Dennis Winge for communication of the promoter activity results that have been referenced herein prior to their publication. We thank Richard Hassett for the preparation of the copper-free medium used in this work and Annette Romeo for essential and diverse technical assistance. The plasmids received from David Eide and Andrew Dancis and used in this work are gratefully acknowledged.

[5] M. Serpe and D. Kosman, unpublished results presented at the Genetics Society of America Meeting, August 6–11, 1996, Madison, WI.

REFERENCES

1. Jungmann, J., Reins, H.-A., Lee, J., Romeo, A., Hassett, R., Kosman, D., and Jentsch, 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., Hassett, R., Kosman, D. J., and Thiele, D. J. (1996) *Genes Dev.* **10,** 1517–1529
4. Yamaguchi-Iwai, Y., Serpe, M., Haile, D., Yang, W., Kosman, D. J., Klausner, R., and Dancis, A. (1997) *J. Biol. Chem.* **272,** 17711–17718
5. Graden, J. A., and Winge, D. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5550–5555
6. Georgatsou, E., and Alexandraki, D. (1994) *Mol. Cell. Biol.* **14,** 3065–3073
7. Labbé, S., Zhu, Z., and Thiele, D. J. (1997) *J. Biol. Chem.* **272,** 15951–15958
8. Winge, D. R. (1998) *Prog. Nucleic Acids Res. Mol. Biol.* **58,** 165–195
9. Dancis, A., Haile, D., Yuan, D. S., and Klausner, R. D. (1994) *J. Biol. Chem.* **269,** 25660–25667
10. Dancis, A., Roman, D. G., Anderson, G. J., Hinnebusch, A. G., and Klausner, R. D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3869–3873
11. Kosman, D. J. (1993) in *Metal Ions in Fungi* (Winkelmann, G. and Winge, D. R., eds) pp. 1–38, Marcel Dekker, Inc., New York
12. Martins, L. J., Jensen, L. T., Simons, J. R., Keller, G. L., and Winge, D. R. (1998) *J. Biol. Chem.* **273,** 23716–23721
13. Schmidt, M. H., and Berg, J. M. (1992) in *Transcriptional Regulation* (McKnight, S. L. and Yamamoto, K. R., eds) Vol. 1, pp. 599–613, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Buchman, C., Skroch, P., Dixon, W., Tullius, T. D., and Karin, M. (1990) *Mol. Cell. Biol.* **10,** 4778–4787
15. Dameron, C. T., George, G. N., Arnold, P., Santhanagopalan, V., and Winge, D. R. (1993) *Biochemistry* **32,** 7294–7301
16. Dobi, A., Dameron, C. T., Hu, S., Hamer, D., and Winge, D. R. (1995) *J. Biol. Chem.* **270,** 10171–10178
17. Farrell, R. A., Thoraldsen, J. L., and Winge, D. R. (1996) *Biochemistry* **35,** 1571–1580
18. Graden, J. A., Posewitz, M. C., Simon, J. R., George, G. N., Pickering, I. J., and Winge, D. R. (1996) *Biochemistry* **35,** 14583–14589
19. Koch, K. A., and Thiele, D. J. (1996) *Mol. Cell. Biol.* **16,** 724–734
20. Zhu, Z., and Thiele, D. J. (1996) *Cell* **87,** 459–470
21. Dix, D. R., Bridgham, J. T., Broderius, M. A., Byersdorfer, C. A., and Eide, D. J. (1994) *J. Biol. Chem.* **269,** 26092–26099
22. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
23. Bartel, P. L., Chen, C.-T., Sternglanz, R., and Fields, S. (1993) in *Cellular Interactions in Development: A Practical Approach* (Hartley, D. A., ed) pp. 153–179, Oxford University Press, Oxford
24. Miller, J. H. (1972) *Experiments in Molecular Genetics*, pp. 352–355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Zhu, Z., Labbé, S., Peña, M., and Thiele, D. J. (1998) *J. Biol. Chem.* **273,** 1277–1280