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

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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 TATTTT sequence immediately 5' to the core GCTC, in contrast to a TTTTTT one. This increased binding was demonstrated clearly in vitro in electrophoretic mobility shift assays that showed Mac1p-DNA complex formation to a single TATTTTGC element but not to a TTTTTTGCCTT 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 TATTTTGC elements. A similar increase in ternary complex formation was demonstrated upon homologous mutation of the FRE1 Mac1p-dependent promoter element. The in vitro importance of this ternary complex formation at the CTR1 promoter was indicated by the stronger trans-activity of this promoter mutated to contain two TATTTT elements and the attenuated activity of a mutant promoter containing two TTTTTT elements that in vitro supported only a weak ternary complex signal in the shift assay. The stronger binding to TATTTT 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). Several features of the regulatory activity of Mac1p have been described. For example, in vitro EMSA 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).

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 trans-activation 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 ≥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

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

tween 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 simulta- 

neously and that formation of the 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 SY526 (obtained from CLONTech) was used as host (MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3, 112 can1 gal4-l42 gal80-538 URA3::GAL1-lacZ) for the promoter activity studies using CTR1 pro- 
moter-lacZ reporter plasmids, DEY1457 was used as host (MATa ade6 his1 leu2 trpl ura3). This strain was obtained from David Eide (21). For the test of the complementing activity of mutant forms of Mac1p (ΔZFMac1p and ZF+Mac1p), DEY1457(mac1Δ) 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 (other- 

wise complete)) mixture of amino acids; for growth of copper-depleted cultures, a completely synthetic, Chelex-treated medium was used that had been programmed with either vector alone (typically pGEMZf+) or with vector containing either wild type or mutated Mac1p-encoding sequences. Binding reactions were performed by pre- 

incubation 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 

in the EMSA figures; for binding mixture resolved on SDS-polyacrylamide gel electrophoresis, and the dried gel developed by autoradiography. In all cases, a single Mac1p 

variant was observed in the dried gel developed by autoradiography. In all cases, a single Mac1p 

was used to direct the use of InStat (Graph- 

Pad, San Diego, CA).

RESULTS

Mac1p N-terminal Zinc Finger Element Is Required for DNA Binding—Two mutant forms of Mac1p were tested to demon- 

strate 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 all the 6 histidine/cystein acid deletion in the 

n-terminal zinc finger domain, referred to as Mac1pN-term. The other was a C23S/H25N double mutant designated ZF+Mac1p; Cys-23 and His-25 are two of the five con- 

served 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 1 and Table I). Binding of wild type Mac1p to this probe
Idenification of \((\text{Mac1p})_2\)-DNA Ternary Complex

| Lanes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|-------|---|---|---|---|---|---|---|---|---|
| WGE-Mac1p | - | - | - | + | - | - | - | - | - |
| WGE-ZFMac1p | - | - | - | + | - | - | - | - | - |
| WT | - | - | - | - | - | - | - | + | + |

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), ZFMac1p (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 (ZFMac1p, lane 6; ZF*Mac1p, lane 8). Furthermore, neither mutant construct was capable of complementing a deletion in MAC1 when expressed in a Δmac1-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, \((\text{Mac1p})_2\)-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 R5 and R3, respectively). In probes R5 and R3, 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 R3, Fig. 2, lane 6) in comparison to mutation of the 3’ core (as in R5, 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’-TATTTGGCTC and the downstream one is 5’-GAGCAAAAA (5’-TTTTTTGGCTC on the opposite strand). The data above suggested the possibility that the presence of the TTTT 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 TTTTTGGCTC site cannot support formation of a detectable binary complex (Fig. 2, R5, probe, lane 6), whereas in the context of an intact upstream TATTTT-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’-TTTTTTGGCTC element in R5 (to which Mac1p binds weakly if at all, Fig. 2, lane 6) to 5’-TATTTGGCTC (again, reading on the opposite strand). The binding of Mac1p to this mutant, designated R5-3’T-A, would be expected to increase in comparison to binding to R5 itself. This prediction was tested by direct binding with R5-3’T-A 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 R5-3’T-A oligonucleotide (stronger competitor as shown in Fig. 3, lanes 6–9) than for R5 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 TATT or TTTT, followed by GCTC. The TATTTT-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 T5 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. 4A. Specifically, the symmetrical probe containing repeated TTTTTGGCTC elements (Fig. 4A, probe 5’-AT, lane 6) supported 75% ternary complex formation compared with WT probe (WT, lane 3), whereas the symmetrical probe containing TATTGGCTC (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. 4B) yielded a pattern in complete agreement with the direct binding data shown in Fig. 4A. Thus, the T5-containing direct repeat (5’-AT, lanes 3 and 4) was a poorer competitor than the TAT-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. 4A, lane 3, for this control). In summary, the data in Figs. 2–4 indicate that Mac1p binds more strongly to the sequence TATTGGCTC than to TTTTTGGCTC, and this better binding appears to stabilize what is interpreted to be a ternary (Mac1p)_2-DNA complex.
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.

**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 (R5, 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'-TTATTTGCTC (R5), 5'-ATTTTTGCTC (R5-A41T), 5'-TATTTTTGCTC (R5-A40T; this is identical to R5-3'-TA used as competitor in Fig. 3), and 5'-TTATTTGCTC (R5-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 R5-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 R5 was observed only with the probe containing 5'-TATTTGCTC on the bottom strand (Fig. 6, R5-A40T, lane 3). In terms of binding affinity for Mac1p, R5-A40T should be equivalent to R5, since both mutant oligonucleotides have only one TATTTGCTC site. Comparison of the data for R5-A40T (Fig. 6, lane 3) and R5 binding (Fig. 2, lane 9) shows this to be the case.

(Mac1p)2DNA 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 lacZ 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)2DNA in vitro. Specifically, while the 5'-AT mutant promoter (Fig. 7, squares) supported only 65% of 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. 4A) in which the symmetrical T5-containing 5'-AT and symmetrical T5-containing 3'-TA probes supported 75 and 150% (Mac1p)2DNA formation, respectively (in comparison to the WT CTR1 probe, Fig. 4A, 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 CuCl2, 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

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**TABLE I**

**Double-stranded oligonucleotides used in mobility shift assays**

| Source | Designation | Sequence |
|--------|-------------|----------|
| CTR1   | WT          | GGTTCTTGTCGTCAGACGACGGGTTAAAATTCAGCAAATTCGG |
|        | R5          | GGTTCTTGTCGTCAGACGACGGGTTAAATTCAGCAAATTCGG |
|        | R5-3'-TA    | GGTTCTTGTCGTCAGACGACGGGTTAAAATTCAGCAAATTCGG |
|        | (R5-A40T)   | GGTTCTTGTCGTCAGACGACGGGTTAAAATTCAGCAAATTCGG |
|        | 5'-AT       | GGTTCTTGTCGTCAGACGACGGGTTAAAATTCAGCAAATTCGG |
|        | 3'-TA       | GGTTCTTGTCGTCAGACGACGGGTTAAAATTCAGCAAATTCGG |
|        | R5-A39'T    | GGTTCTTGTCGTCAGACGACGGGTTAAAATTCAGCAAATTCGG |
|        | R5-A41'T    | GGTTCTTGTCGTCAGACGACGGGTTAAAATTCAGCAAATTCGG |
| FRE1   | WT          | CTGATTATTTTGCTCAGCTTTTTTTTTTTTGCTCATGAAA |
|        | 3'-TA       | CTGATTATTTTGCTCAGCTTTTTTTTTTTTGCTCATGAAA |

*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* R5-A40T is the alternate name for R5-3'-TA.

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3 A double FRE1 promoter mutant was not constructed (both sites containing TATT) 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).
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 that occurs when cells are exposed to \([\text{copper]}\) medium \(>10 \, \mu\text{M}\) 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, \((\text{Mac1p})_2\) 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 \textit{in vivo} two-hybrid genetic approach (23). To do so, fusions of wild type Mac1p, the \(\Delta ZF\)Mac1p 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 \textit{in vivo} interaction by the standard two-hybrid assay in cells grown in both the absence and presence of copper. 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%.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{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 \(\mu\)l of coupled wheat germ extract alone (WGE, negative control) or with 5 \(\mu\)l 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%.

\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{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.

\end{figure}
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). 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). 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).
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’-3’ sequence, specifically, binding is stronger to TATTTGCTC than to TTTTTGCTC; 4. Mac1p appears to make a specific and favorable contact with adenine at the 24 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

**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.** TA transitions were made in the 3’-T$_5$ region of the R$_3$ CTR1 probe used in Fig. 2 (lane 1) to yield core 3’-binding sites of 5’-ATTTTGCTC (R$_3$-A1T, lane 2), 5’-TATTTGCTC (R$_3$-A40T, lane 3, equivalent to R$_3$-3’-TA, Fig. 3), and 5’-TTATTGCTC (R$_3$-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-lacZ 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 $\beta$-galactosidase activities which are presented in the standard fashion (Miller units). The bars indicate $\pm$ 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).

TATTG 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
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 transcription activation 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) (14), 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, TTTCGGCTG (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), TTTCGGTC, 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 formed, and, furthermore, that the formation of both, particularly the more slowly migrating one, appears driven by a stronger Mac1p binding to the 5’ element, TATTTCGGTC. 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 TATTTCGGTC-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 invariant thymine, or T(T/C)XXGCTG (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 TATTTC is more consistent with the ternary complex model. With

### Table II

Two-hybrid analysis of the Mac1p-Mac1p interaction

| Media additions | Mac1p/ Mac1p | Mac1p ΔZFMac1p | ΔZFΔMac1p/ Mac1p | ΔZFΔMac1p ΔZFMac1p | ΔZFMac1p ΔZFMac1p | ZFΔMac1p ΔZFMac1p |
|-----------------|--------------|----------------|------------------|--------------------|--------------------|-------------------|
| None            | 0.32         | 2.94           | 4.42             | 15.39              | 0.51               |
| (TA of bait)    | (0.04)       | (0.35)         | (0.05)           |                    |                    |
| CuCl₂, 10 μM   | 0.38         | 3.65           | 3.73             | 14.80              | 0.37               |
| (TA of bait)    | (0.02)       | (0.03)         | (0.02)           |                    |                    |
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 intramolecular 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 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 (cooper-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) 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 [copper]_{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) 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 verification. 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 Mac1p 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.

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