Mapping of the DNA Binding Domain of the Copper-responsive Transcription Factor Mac1 from *Saccharomyces cerevisiae* *

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Mac1 from *Saccharomyces cerevisiae* activates transcription of genes, including *CTR1* in copper-deficient cells. N-terminal fusions of Mac1 with the herpes simplex VP16 activation domain were used to show that residues 1–159 in Mac1 constitute the minimal DNA binding domain. Mac1-(1–159) purified from *Escherichia coli* contains two bound Zn(II) ions. Electrophoretic mobility shift assays showed direct and specific binding by Mac1-(1–159) to a DNA duplex containing the copper-responsive element TTTGCTCA. The DNA binding affinity of Mac1-(1–159) for a duplex containing a single promoter element or an inverted repeat was 5 nM for the 1:1 complex. The N-terminal 40-residue segment of Mac1 is homologous to the DNA binding zinc module found in the copper-activated transcription factors Ace1 and Amt1. A *MAC1* mutation yielding a Cys → Tyr substitution at the first candidate zinc ligand position relative to Ace1 resulted in a loss of *in vivo* function. Two TTTGCTCA promoter elements are necessary for efficient Mac1-mediated transcriptional activation. The elements appear to function synergistically. Increasing the number of elements yields more than additive enhancements in *CTR1* expression.

Copper is an essential cofactor for a variety of proteins and is required for normal cellular functions (1, 2). In enzymes, copper ions participate in catalytic functions or fulfill structural roles (3). Abnormally low levels of copper can impair the function of many copper-dependent enzymes. However, abnormally high levels of copper can be toxic. The toxicity of copper may arise, in part, from cellular damage caused by reactive oxygen intermediates. Copper and iron ions can catalyze the formation of highly reactive hydroxyl radicals through the Fenton reaction (4). Hydroxyl radicals can react with biomolecules, resulting in polypeptide bond cleavage as well as DNA base and sugar oxidation (5–7). Homeostatic mechanisms exist in all cells to regulate the cellular concentration of copper ions, maintaining copper balance and minimizing the deleterious effects of excess copper ions.

The budding yeast *Saccharomyces cerevisiae* has been used as a model system to study the mechanisms of copper detoxification, transport, and distribution. The mechanism of high affinity copper uptake in *S. cerevisiae* has been determined and is predominantly in the form of Cu(II). Cu(II) ions are reduced to Cu(I) prior to transport into *S. cerevisiae* cells (10, 11). Cu(II) reduction is mediated at the cell surface by the NADPH-dependent ferric and cupric reductases Fre1 and Fre2 (10–12). Cu(I) is then transported across the plasma membrane by high affinity copper transporters Ctr1 and Ctr3 (9, 13).

Under copper starvation *FRE1, CTR1, CTR3*, and *FRE7* (a homologue of *FRE1*) are highly expressed. However, in the presence of elevated concentrations of copper these genes are down-regulated (10, 14–16). Copper-dependent inhibition of expression occurs at the level of transcription and is mediated by Mac1 (metal binding activator) (15–17). The role of Mac1 in the expression of *FRE1* and *CTR1* has been studied in cells with mutations in the *MAC1* locus (10, 15–17). Expression of *FRE1* and *CTR1* is not copper-inhibited in cells containing a semidominant gain of function *MAC1* mutation, resulting in a His to Gln substitution at residue 279 designated MAC1<sup>His<sub>279Gln</sub></sup> (17). *MAC1<sup>His<sub>279Gln</sub></sup>* cells exhibit a copper-hypersensitive phenotype, since copper uptake is unregulated (17). In contrast, a frameshift mutation in *MAC1*, designated *mac1<sup>-1</sup>* resulted in substantial loss of both Cu(II) and Fe(III) reduction as well as uptake (17). The observed phenotypes of *mac1<sup>-1</sup>* cells include respiratory deficiency and sensitivity to a myriad of stresses. The phenotypes are reversed upon addition of exogenous copper salts.

The mechanism of copper attenuation of Mac1 function appears to involve copper repression of both the DNA binding function and transactivation (16, 18). Fusions of the Gal4 DNA binding domain with the Mac1 transactivation domain revealed copper attenuation of transcriptional activation of a *GAL1/lacZ* reporter gene (18). A repeated Mac1-responsive element consisting of at least two repeats of the sequence TTTGCTC(T/G)A(G) has been identified in 5′ sequences of *CTR1, CTR3, FRE1*, and *FRE7* (15, 16, 19). The presence of two repeats of this sequence appears to be critical for high level expression of Mac1-regulated genes (16, 19). *In vivo* DNA footprinting has shown a loss of protection of the TTTGCTCG repeats in the promoter of *CTR3* upon addition of copper salts (16). The loss of protection in the *in vivo* DNA footprint suggests the loss of DNA binding in response to copper treatment. Whether the loss of protection of this sequence is due to the loss of DNA binding activity or nuclear export is unclear.

In this study the DNA binding domain of Mac1 was mapped to within the N-terminal 159 residues. Mac1-(1–159) was purified from *E. coli* as either a His<sub>6</sub> tag or a glutathione S-transferase fusion and contained two bound Zn(II) ions. The purified zinc-Mac1-(1–159) complex was capable of specific and high affinity binding to a single TTTGCTCA element. Sequential binding of the Mac1-(1–159) protein to a DNA probe containing two TTTGCTCA elements was observed, suggesting that Mac1-(1–159) is a monomer. The requirement for at least two TTTGCTC(T/G)A(G) elements in the promoters of Mac1-
regulated genes appears to be due to synergism between the Mac1 binding sites.

MATERIALS AND METHODS

MAC1-VP16 Activation Domain Fusions—A minimal activation domain (AD) from the herpes simplex virus-1 VP16 protein (VP16 AD) was amplified by PCR with vector pSJ193 (20) as a template creating 5′ BamHI, NotI, and ClaI sites and a 3′ HindIII site. The PCR product was digested with BamHI/HindIII and subcloned into vector pTV102-U (21), which contains the ADH1 promoter and terminator separated by a multiple cloning site, creating vector pVT-VP16. Vector pVT-VP16 contains ClaI and NotI sites before the sequences for the VP16 AD. The N terminus of Mac1 was amplified by PCR, generating common 5′ and 3′ ClaI and NotI sites and four different 3′ ends with 2–3 codons in each. The PCR products contained sequences coding for the N-terminal 194, 159, 125, or 101 amino acids. The MAC1 PCR products were digested with BamHI/NotI and subcloned into pVT-VP16 generating vectors Mac1-(1–194)/VP16, Mac1-(1–159)/VP16, Mac1-(1–125)/VP16, and Mac1-(1–101)/VP16.

Modified CTR1/λac Reporter Plasmids—CTR1 promoter/λac hybrid genes containing either one, two, three, or four TTTGCTCA elements were constructed in a derivative of the ΔUAS vector pNB404 with a LEU2 selectable marker (pNB404L) (22). CTR1 promoter sequences were amplified by PCR and subcloned into pNB404L to generate vectors pC1 sequences –323 to –299 (relative to the translation start site) and pC2 and –539 to –299. A promoter containing three TTTGCTCA elements (pC3) was generated by PCR mutagenesis using a modified primer containing a copy of sequences –339 to –325 5′ to the repeated TTTGCTCA element in pC2. The spacing between the second and third TTTGCTCA elements was 12 nucleotides, which is similar to that seen in the CTR1 promoter of 14 nucleotides. pC4 containing four TTTGCTCA elements was constructed in a similar manner to pC3. The spacing between the third and fourth TTTGCTCA elements was 12 nucleotides.

Mac1−Myc Epitope Fusions—Six copies of the Myc epitope from vector CS2+MT (23) were excised as a BamHI/StuI fragment and subcloned into pHolly (generously supplied by Richard Palmiter) a pBluescript derivative with a small insert creating sites for NotI, NdeI, and StuI, to pick up a stop codon. The Myc epitope was excised as a BamHI/StuI fragment and cloned into the yeast expression vector pYeF2 generating pYeF2-Myc. The MAC1 truncations were excised from the VP16 AD fusions as BamHI/ClaI fragments and subcloned into pYeF2-Myc-generating vectors Mac1-(1–194)/Myc, Mac1-(1–159)/Myc, Mac1-(1–125)/Myc, and Mac1-(1–101)/Myc for galactose-inducible expression in yeast.

Mac1 Myc fusions were transformed into yeast strain YJJ1 (generously provided by Stefan Jentsch). YJJ1 (mac1-1) has a frameshift in the MAC1 gene, resulting in a loss of function of MAC1. Yeast cultures were grown in synthetic complete medium lacking uracil with either 30 M CuSO4 added. All cultures had 2% galactose added as a carbon source. The cells were harvested by centrifugation and resuspended in lysis buffer (10 mM Tris-HCl, pH 8, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and lysed with glass beads. The lysed cells were centrifuged and frozen at −70 °C. Cells were resuspended in the same buffer and lysed by vortexing with glass beads. β-Galactosidase activities were assayed using o-nitrophenyl β-D-galactopyranoside as a substrate. The absorbance at 420 nm was recorded, and protein concentrations were determined by the method of Bradford (24). Each sample was assayed in triplicate.

Western Blot Analysis—Mac1-(1–194)/Myc, Mac1-(1–159)/Myc, Mac1-(1–125)/Myc, and Mac1-(1–101)/Myc in pYeF2 were grown in low copper synthetic complete medium lacking uracil to an A600 of 1.0 in medium containing 2% raffinose in place of dextrose. Protein expression was induced by the addition of galactose to 2%. Cells were grown for an additional 6 h. The cells were harvested by centrifugation, washed with water, resuspended in lysis buffer, and lysed by vortexing with glass beads (3 cycles) and filtered. Sodium dodecyl gel electrophoresis was carried out on a 12% gel. Proteins were transferred to nitrocellulose and the blots incubated with the 9E10 anti-c-Myc (Boehringer Mannheim) in blocking buffer (10% non-fat dry milk, phosphate-buffered saline, and 0.1% Tween 20). Detection by enhanced chemiluminescence was performed after incubation with horseradish peroxidase-conjugated secondary antibody.

Electrophoretic Mobility Shift Assay—Yeast extracts from cells expressing Mac1-(1–194)/Myc, Mac1-(1–159)/Myc, Mac1-(1–125)/Myc, and Mac1-(1–101)/Myc as well as cells harboring the starting plasmid were tested for binding to an oligonucleotide containing the Mac1 binding site. E. coli extracts from cells expressing Mac1-(1–194)/HA, Mac1-(1–159)/HA, and Mac1-(1–125)/HA were also tested for binding to this duplex. Mac1-(1–159) purified from E. coli was tested for binding to oligonucleotides containing the two TGGTCTCA copper-responsive sites in CTR1 or mutant duplexes. The oligonucleotides were end labeled with γ32P]ATP, boiled for 3 min, and slowly cooled to anneal. Protein extracts or purified Mac1-(1–159) was added to a 26-nucleotide duplex containing a half-site with one copy of the repeated TGGTCTCA element to give a final concentration of 0.4 mM to induce production of the protein. Thirty min after induction, 1 mM ZnSO4 was added, and the cells were grown for an additional 3 h. The cells were collected by centrifugation and washed once in 250 μl ice-cold sonication buffer (20 mM NaH2PO4 pH 7.5, 300 mM NaCl, 1% glycerol, and 1 mM EDTA) and resuspended in 250 μl ice-cold sonication buffer. The lysate was centrifuged at 4 °C for 30 min at 100,000 × g, and the supernatant was collected and filtered through a 0.45-μm filter. The Mac1-(1–159)His tag protein was purified using nickel-nitrilotriacetic acid agarose (Qiagen). The extract was loaded onto the nickel-nitrilotriacetic acid agarose column and washed with 10 column volumes of sonication buffer. The Mac1-(1–159)/His tag protein was eluted with a 0–500 mM imidazole linear gradient in sonication buffer. The Mac1-(1–159)-GST fusion protein was purified using glutathione-Sepharose (Amersham Pharmacia Biotech). Triton X-100 was added to 1%, and the extract was loaded onto the glutathione-Sepharose column and washed with 10 column volumes of sonication buffer. The Mac1-(1–159)-GST fusion protein was eluted with 20 mM glutathione in 50 mM Tris-HCl, pH 8. The eluted proteins were monitored by SDS-polyacrylamide gel electrophoresis with a single band at the correct molecular weight for each fusion was observed.

β-Galactosidase Assays—Plasmids Mac1 pVT102-U, Mac1-(1–194)/VP16, Mac1-(1–159)/VP16, Mac1-(1–125)/VP16, Mac1-(1–101)/VP16, and pVT102-U were cotransformed with a CTR1/λac reporter construct containing an inverted repeat of the TTTGCTCA element into yeast strain YJJ1. Plasmids pC1, pC2, pC3, and pC4 were cotransformed into YJJ1 with Mac1 pVT102-U. Yeast transformants were grown in low copper synthetic complete medium with 2% dextrose lacking uracil and leucine. The cells were grown to an A600 of 1.0 with the addition of either 30 μM or 100 μM CuSO4 added to the growth media. The cells were pelleted and washed with Z-buffer (85 mM NaH2PO4, 45 mM Na2HPO4, 10 mM KCl, 5 mM β-mercaptoethanol, and 1 mM MgSO4) and frozen at −70 °C. Cells were resuspended in the same buffer and lysed by vortexing with glass beads. β-Galactosidase activities were assayed using o-nitrophenyl β-D-galactopyranoside as a substrate. The absorbance at 420 nm was recorded, and protein concentrations were determined by the method of Bradford (24). Each sample was assayed in triplicate.

The abbreviations used are: AD, activation domain; PCR, polymerase chain reaction; BCS, bathocuproine sulfonate; HA, hemagglutinin; GST, glutathione S-transferase.
pair probe containing the inverted repeat of the TTTGCTCA sequence (cat ggg ata TTT GCT Caa gag ggc gta aaa atG AGC AAA aat ggc acg). The protein and DNA were incubated for 15 min at 25 °C prior to electrophoresis of the protein-DNA complexes on 6% polyacrylamide gels and were visualized by autoradiography.

RESULTS

MAC1-VP16 Activation Domain Fusions—Many fungal transcription factors have distinct and separable DNA binding domains and transactivation domains (26, 27). Thus, one strategy to map the DNA binding domain of Mac1 is to evaluate the in vivo function of Mac1 truncates fused with a heterologous activation domain. The candidate DNA binding domain of Mac1 was expected to be located near the N-terminal end of the protein as the N-terminal 40 residues are homologous to the conserved DNA binding zinc module in Ace1 and Amt1 (17, 28, 29). The minimal DNA binding domain of Ace1 consists of two independent modules, the N-terminal 40-residue zinc motif and an adjacent domain stabilized by a polycopper cluster (28, 30). Only the N-terminal zinc module is conserved in Mac1. If Mac1 resembles Ace1 in having two independent domains participating in DNA binding, one would expect the minimal DNA binding domain of Mac1 to consist of a region greater than just the conserved N-terminal zinc module.

The minimal AD from the herpes simplex virus-1 VP16 has been used extensively as a heterologous activator (31–34). Fusion of the N-terminal 194 residues of Mac1 to residues 417–491 of VP16 generated a functional transcription factor. Transformation of the MAC1-(codons 1–194)/VP16 fusion reversed the methionine auxotrophy of mac1-1 cells (14, 17) (Fig. 1). Cotransformation with both the MAC1-(1–194)/VP16 hybrid and a CTR1/lacZ reporter fusion resulted in lacZ expression (Fig. 2). The Mac1/VP16 fusion gave greater CTR1/lacZ expression than did an episomal full-length Mac1 containing its own activator. The VP16 AD is a strong activator and may be more potent than the Mac1 AD. The Mac1-(1–194)/VP16 AD fusion was not modulated by the addition of CuSO4 as is the full-length Mac1. The lack of copper modulation was expected as the candidate copper binding motif resides in the C-terminal activation domain (18). To further narrow the DNA binding domain of Mac1, three other C-terminal truncations were constructed, terminating at residues 159, 125, and 101. The activity of the Mac1-(1–159)/VP16 AD fusion was identical to that of Mac1-(1–194)/VP16. However, truncation to residue 125 markedly reduced the activity of the CTR1/lacZ reporter gene, and truncation to residue 101 essentially eliminated activity (Fig. 2).

Yeast extracts from mac1-1 cells expressing Mac1-(1–194)/Myc were tested for binding to a DNA duplex containing a single TTTGCTCA element (Fig. 3A). Addition of Mac1-(1–194)/Myc to the probe resulted in the appearance of a gel-retarded complex. The addition of a monoclonal anti c-Myc antibody resulted in a supershift, demonstrating that the gel-retarded complex contained Mac1-(1–194)/Myc (Fig. 3A). The Mac1-DNA complex was observed in both copper-deficient (+BCS) and copper-replete cells (Fig. 3B). Yeast extracts from cells expressing Mac1-(1–159)/Myc, Mac1-(1–125)/Myc, and Mac1-(1–101)/Myc were compared with Mac1-(1–194)/Myc for binding to the DNA duplex. Mac1-(1–194)/Myc and Mac1-(1–159)/Myc gave gel shifts of approximately equal intensity, whereas the shift due to Mac1-(1–125)/Myc was less intense, and no shift was observed for Mac1-(1–101)/Myc (Fig. 4A). Western analysis of these extracts demonstrated that Mac1-(1–194)/Myc, Mac1-(1–159)/Myc, and Mac1-(1–125)/Myc were all expressed at the same level, whereas the expression of Mac1-(1–101)/Myc was substantially less (Fig. 4B). These results suggested that the minimal DNA binding domain was within the N-terminal 159 residues.

Activity of Mac1 Mutants—The N-terminal 194 residues of Mac1 contain the 40-residue candidate zinc module conserved in Ace1 and two downstream Cys-Cys sequences. To determine whether the conserved zinc module was important for function, a mutation in codon 11 was engineered, resulting in a Cys3→Tyr substitution. A corresponding Cys3→Tyr mutation in Ace1 abolished in vivo function and attenuated DNA binding activity. The mutation was engineered in the full-length MAC1 open reading frame, and the mutant gene was transfected into mac1-1 cells to test for function. Cells harboring a mutant Mac1 with the Cys3→Tyr substitution were unable to activate expression of CTR1/lacZ (Fig. 5).

Two Cys-Cys sequence motifs exist in Mac1. To determine whether the Cys-Cys motifs were important in the function of Mac1, double Cys-to-Ser substitutions were engineered in full-length Mac1 at residues 69 and 70 and 172 and 173. These mutant Mac1 molecules were active in vivo and yielded wild-
type copper regulation of $\text{CTR1}/\text{lacZ}$ expression (Fig. 5). The presence of the same double substitutions in $\text{Mac1-(1–194)}/\text{HA}$ from $E.\text{coli}$ extracts did not impair the ability of $\text{Mac1-(1–194)}$ to bind to the half-site duplex containing a single TTTGCTCA element (data not shown). These experiments suggest that the two Cys-Cys pairs are not important for the formation of the $\text{Mac1}-z$ DNA complex.

Purification of $\text{Mac1-(1–159)}$—$\text{Mac1-(1–159)}$ was expressed in $E.\text{coli}$ as either a C-terminal His$_6$ tag or as a GST fusion. The $\text{Mac1-(1–159)}/\text{His}$ tag protein was affinity-purified using nickel-nitrilotriacetic acid-agarose, and the $\text{Mac1-(1–159)}/\text{GST}$ fusion protein was affinity-purified using glutathione-Sepharose. The purified fusion proteins showed single stained bands on SDS-polyacrylamide gel electrophoresis consistent with the calculated molecular mass of 20 kDa for $\text{Mac1-(1–159)}/\text{His}$ tag and 45 kDa for $\text{Mac1-(1–159)}/\text{GST}$ (data not shown). Each fusion had the correct amino acid composition by amino acid analysis. Metal analysis by atomic absorption spectroscopy revealed that each $\text{Mac1-(1–159)}$ fusion protein bound two zinc ions. A similar zinc binding stoichiometry for the two fusion proteins suggests that zinc binding is not occurring within the poly(His) tag.

Purified $\text{Mac1-(1–159)}$ was tested for its ability to bind to DNA duplexes containing either the copper-responsive element (TTTGCTCA) or two mutant duplexes with changes in the element as well as a longer duplex containing the inverted repeat of the TTTGCTCA element separated by 14 nucleotides derived from the $\text{CTR1}$ promoter. The addition of $\text{Mac1-(1–159)}$ to the half-site duplex containing the wild-type sequence resulted in the appearance of a gel-retarded complex (Fig. 6). No complex was observed in gel shift assays with mutant DNA duplexes containing sequences GGGGCTCA or TTTGAAAA (Fig. 6). The DNA binding affinities of $\text{Mac1-(1–159)}$ for each TTTGCTCA site within the $\text{CTR1}$ promoter were near 5 nM (Fig. 7, A and B). A 5 nM concentration of $\text{Mac1-(1–159)}$ resulted in 50% of the DNA probe being present as a protein-DNA complex. Sequential binding of $\text{Mac1}$ was observed to a DNA duplex containing the inverted repeat. The affinity for occupancy of the first site was similar to the isolated half-site at 4 nM. The binding affinity of the second $\text{Mac1-(1–159)}$ molecule was approximately 11 nM (Fig. 7C).

Synergism between $\text{Mac1}$ Binding Sites—Repeated TTTGCTCA elements do not appear to be necessary for high affinity DNA binding based on the electrophoretic mobility shift assay data. Since the $\text{Mac1-(1–194)/VP16}$ fusion was able to stimulate transcription to a higher level than wild-type $\text{Mac1}$, we tested whether the fusion was active on a promoter with a single TTTGCTCA element. The fusion stimulated transcrip-
tion of lacZ from a promoter containing a single element 100-fold compared with a promoter lacking a TTTGCTCA element, but lacZ expression was an additional 4-fold greater with two TTTGCTCA promoter elements compared with one element (data not shown). The 4-fold difference in activity between one and two elements with Mac1-(1–194)/VP16 is suggestive of synergism between the sites. The four known Mac1-regulated genes in yeast require two TTTGC(T/G)C(A/G) elements for Mac1 responsiveness.

To determine whether the requirement for two elements was due to synergism between the Mac1 binding sites, promoter/lacZ hybrid genes containing either one, two, three, or four TTTGCTCA elements were engineered and tested for activity in cells containing an intact Mac1 (Fig. 8). Compared with the control vector containing no upstream activation sequences, low level activation is seen with a single TTTGCTCA element. However, a promoter/lacZ fusion containing two elements yielded approximately 8-fold greater lacZ expression than that for a single element. The presence of three elements increased expression nearly 5-fold more than the expression level of the promoter/lacZ fusion with two elements. The promoter fusion containing four elements was about 2-fold higher than the promoter fusion containing three elements. The increase in expression in each case is more than additive, suggesting that the requirement for two TTTGCTCA elements is due to synergism between the sites.

**DISCUSSION**

The N-terminal 40 residues of Mac1 are highly homologous with a corresponding segment in the copper-activated Ace1 transcription factor from *S. cerevisiae* (17). The conserved region is one of two domains forming the DNA binding segment of Ace1 (28, 30). The homologous 40-residue domain in Ace1 and Amt1 (the Ace1 ortholog in *Candida glabrata* (36)) was shown to comprise a nonclassical zinc module (28, 37). The high level of homology between Mac1 and Ace1 in this region suggested that the DNA binding domain of Mac1 would contain at least the N-terminal 40 residues. Mac1/VP16 fusions were used to map the DNA binding domain of Mac1. Fusions containing Mac1-(1–194) and Mac1-(1–159) were identical in their ability to express a CTR1/lacZ reporter gene. Further truncations yielding Mac1-(1–125)/VP16 and Mac1-(1–101)/VP16 were substantially less active in vivo and in DNA binding in vitro, suggesting that these Mac1 polypeptides do not contain the full DNA binding domain. The minimal DNA binding domain of Mac1 appears to consist of the N-terminal 159 residues.
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DNA containing the inverted repeat was the same as with either DNA half-site, suggesting that the two sites are independent with no cooperativity in binding. If binding cooperativity occurred between the two sites, the DNA binding affinity of Mac1-(1–159) to the duplex containing the palindromic element would be expected to be greater for the 2:1 complex than the additive affinity for duplexes containing a single element.

Repeated TTTGCTCA elements are not necessary for high affinity DNA binding based on the electrophoretic mobility shift assay data. However, two elements are necessary for high level in vivo transcriptional activation of genes regulated by Mac1. The requirement for two Mac1 binding sites appears to be due to synergism between the sites. This is seen with both the Mac1/VP16 fusion and intact Mac1. Increasing the number of TTTGCTCA elements in promoter/lacZ fusions from one to four showed a more than additive effect on lacZ expression levels consistent with synergism. Synergistic activation through multiple sites is well established with a number of yeast factors, including Gal4 (35, 40, 41). Increasing the number of Gal4 sites gave much more than additive effects in promoting transcription (35). Furthermore, the observed synergism in Gal4 is dependent on the strength of the activator (35). A Gal4 fusion with a strong activator such as the VP16 AD resulted in activation from a single site, whereas a fusion with a weak activator did not. We observe a similar effect using the VP16 activator. Using the weak activator Gal4 fusion, Carey et al. (35) observed a 66-fold stimulation in transcription by increasing the number of Gal4 sites from 1 to 5. This is compared with a 75-fold enhancement in transcription by increasing the number of Mac1 sites from 1 to 4.

Synergism in Gal4 and other transcriptional activators has been suggested to be a consequence of interaction of each of the bound activators with some component or components of the transcriptional machinery rather than with each other (35, 40). Synergism between the Mac1 molecules bound to multiple sites may be important for high level expression of Mac1-regulated genes.

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