Homeo Domain of the Yeast Repressor α2 Is a Sequence-Specific DNA-Binding Domain But Is Not Sufficient for Repression

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The α2 protein, the product of the *MATα2* gene, is a regulator of cell type in the yeast *Saccharomyces cerevisiae*. It represses transcription of a group of cell type–specific genes by binding to an operator located upstream of each target gene. Fifteen in-frame deletions within the coding region of the *MATα2* gene were constructed. The deletion alleles were examined for phenotypes conferred in vivo, and the encoded mutant proteins were assayed for ability to bind specifically to the operator in vitro. This analysis has revealed that the sequence-specific DNA-binding domain of α2 is located within a region of 68 amino acids. This region of α2 has significant homology with the homeo domain, a conserved sequence found in the products of several *Drosophila* homeotic and segmentation genes. In addition, there is a class of mutant α2 proteins that binds tightly and specifically to the operator in vitro, but fails to repress transcription in vivo.

THE α2 PROTEIN, THE PRODUCT OF THE *MATα2* GENE, IS an important regulator of cell type of the yeast *Saccharomyces cerevisiae* (*1*). In an α cell, α2 represses transcription of a class of cell type–specific genes called a-specific genes. In an **a** cell, the a-specific genes are expressed because the α2 protein is absent. An **a** cell can mate with an α cell to form a third cell type, the **a**/α diploid. In an **a**/α cell, α2 acts in combination with the **a**1 protein, the product of the *MAT***a***1* gene, to repress transcription of a class of genes called haploid-specific genes. The α2 repressor is a sequence-specific DNA-binding protein. It recognizes, with high specificity and affinity, a 32–base pair sequence found upstream of each a-specific gene (*2*).

The α2 repressor is a member of a large group of proteins related by homology to the homeo domain. The homeo domain is a conserved sequence of approximately 60 amino acids first found in the homeotic and segmentation gene products of *Drosophila* (*3*). Homology to the homeo domain was subsequently found in the yeast proteins α2 and **a**1 (*4, 5*) and in the inferred products of genes isolated from other invertebrates and several vertebrates. Because the homeo domain is so remarkably conserved and because it is found in proteins known to determine cell identity, it is thought to play an important role in regulating development.

To define the region of the α2 protein which binds operator DNA, we assayed 15 deletion-bearing mutants of α2 for DNA-binding activity. Our results indicate that the specific DNA-binding domain of α2 lies within a region that shares homology with the homeo domain. Our results further indicate that specific DNA binding is not sufficient for repression in vivo.

Construction of *MATα2* deletions. To identify the DNA-binding domain of the α2 protein, we first constructed in-frame deletions within the *MATα2* portion of a *MATα2-lacZ* hybrid gene (*6*) (Fig. 1). The *MATα2-lacZ* gene fusion into which deletions were introduced produces a hybrid protein containing all 210 amino acids of α2 fused to the amino terminus of enzymatically active *Escherichia coli* β-galactosidase, the product of the *lacZ* gene. This hybrid gene provides α2 function in vivo (*7*). The functional β-galactosidase "tag" facilitated detection of, and may also confer stability (*8*) on, the various deletion-bearing fused forms of α2. The deletions comprise three sets, (i) a series of deletions originating at the 5′ end of *MATα2* coding sequence extending toward the 3′ end, (ii) a series of overlapping deletions extending from the 3′ *MATα2-lacZ* fusion joint toward the 5′ end, and (iii) two internal deletions (Fig. 2).

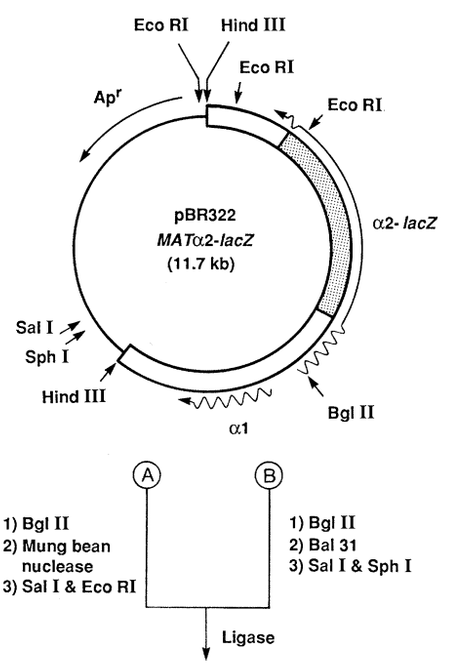

Fig. 1. Scheme for construction of *MATα2* deletions 4–62, 2–135, 4–140, 4–152, 4–179, and 2–200 (*6*). The open bar is *MATα* DNA. The shaded bar is *lacZ* DNA. The wavy lines represent *MATα1* and *MATα2* transcripts.

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Our nomenclature convention is to refer to a deletion by the amino acids that are missing; for example, deletion 160–188 removes amino acids 160 through 188. The same designation is used in referring both to the allele containing the corresponding deletion and to the encoded mutant α2 protein.

Sequence-specific DNA binding. The 15 deletion mutants of α2 were assayed for ability to bind tightly and specifically to an operator, with the use of the gene fusions shown in Fig. 2 (*9*). The mutant hybrid genes were introduced into a *MAT* deletion strain on a high copy number plasmid, and a crude extract was prepared from each resulting strain. The α2-LacZ hybrid protein in each of these crude extracts was assayed for specific DNA binding as diagramed in Fig. 3A and as follows. Two ^{32}P-labeled DNA fragments were added to a sample of each extract. One of these DNA fragments (65 bp) contains a synthetic operator that functions in vivo and binds α2 in vitro (*2*). The other DNA fragment (85 bp) lacks an α2 binding site and therefore serves as a negative control fragment. After a short incubation period, fixed *Staphylococcus aureus* cells coupled to a monoclonal antibody to β-galactosidase (anti-β-galactosidase) were added to each DNA-binding reaction (*10*). After another short incubation, the *S. aureus* cells were removed by centrifugation. Any labeled DNA that was precipitated was extracted from the sedimented cells and displayed on a polyacrylamide gel (Fig. 3B). Wild-type α2-LacZ hybrid protein selectively precipitates the DNA fragment bearing the synthetic operator (Fig. 3B, lane 5). The extract of a strain producing 4–210 mutant protein served as a negative control (Fig. 3B, lane 6); this strain produces a fusion protein consisting of only the first three amino acids of α2 fused to β-galactosidase. As indicated by the results, extracts from this strain show no specific operator binding.

Five of the 15 mutant α2 proteins retain sequence-specific DNA-binding activity. These mutant proteins are the first four members of the series missing amino-terminal residues (3–6, 3–20, 4–62, and 2–135) (Fig. 3B, lanes 20, 21, 22, and 14, respectively) and the mutant α2 missing the carboxyl-terminal seven amino acids (204–210; Fig. 3B, lane 15). The remaining ten mutant α2 proteins showed no detectable DNA binding.

Control experiments with the following results were performed to confirm the observed sequence-specific DNA binding. First, *S. aureus* cells lacking the anti-β-galactosidase fail to preferentially precipitate the operator fragment from a yeast extract containing the wild-type α2-LacZ hybrid protein (Fig. 3B, lane 4). Second, an unlabeled synthetic 32-bp operator competes for binding, whereas an unlabeled synthetic fragment of the same size from the yeast *GAL* promoter region does not compete (Fig. 3B, lanes 26 and 27).

These experiments allow us to assign the sequence-specific DNA-binding domain of α2 to the region between amino acids 135 and 204 (the protein consists of 210 amino acids total). This conclusion follows from the observation that mutant proteins 2–135 and 204–210 retain specific operator-binding activity. Consistent with this conclusion is the finding that mutants 141–152 and 160–188 do not bind operator.

Phenotypes conferred by *MATα2* deletions. The deletion alleles were next examined for phenotypes conferred in vivo. To determine whether the *MATα2* deletion alleles provide α2 function, we performed a complementation analysis. An α cell defective for α2 is unable to mate because it simultaneously expresses both α- and **a**-specific functions. Plasmid-borne *MATα2-lacZ* alleles were introduced into a strain containing a chromosomal deletion of the *MAT* locus and assayed for ability to restore α mating (*11*). This analysis could be done with the deletions in the hybrid gene since, as described above, the wild-type *MATα2-lacZ* gene, containing all of the *MATα2* coding sequence, provides functional α2 (*7*). All *MATα2-lacZ* deletion alleles shown in Fig. 2, with the exception of 204–210, fail to complement, as determined by a mating assay. The *MATα2Δ204–210-lacZ* allele is able to complement the chromosomal *MAT* deletion and restore α mating. A nonfusion *MATα2* gene containing deletion 3–6, 3–20, 141–152, or 160–188 (see below) also fails to complement the chromosomal *MAT* deletion, as determined by the mating assay, indicating that the lack of complementation is not due to interference by β-galactosidase. Therefore, none of the deletion alleles, with the exception of 204–210, provide α2 function.

As a direct test of the ability of the deletion alleles to provide α2 repression, repression of specific target genes by the products of the alleles containing smaller deletions was examined (*12*). Deletions 3–6, 3–20, 141–152, and 160–188 were recombined into an otherwise wild-type *MATα2* gene in the single copy number plasmid YCp50 and introduced into appropriate strains containing a deletion of the chromosomal *MAT* locus and a fusion of *lacZ* to the target gene *MFa2* or *HO*. Repression of the target genes by the products of the introduced plasmid-borne *MATα2* alleles could be assessed by determining the β-galactosidase activity of these strains. By examining repression of both *MFa2* and *HO*, we were able to

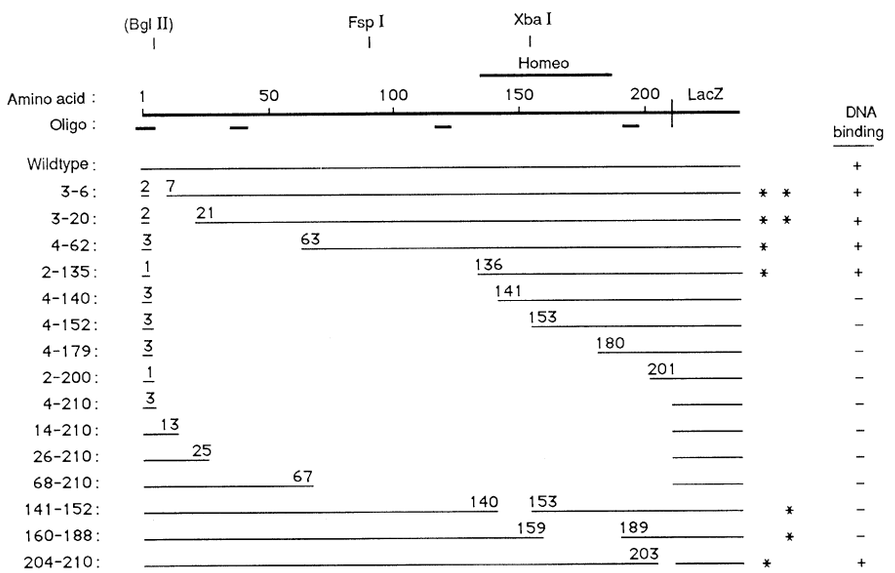

Fig. 2. Properties of *MATα2* deletions. All the deletions are nested in the coding sequence (*28*) of the *MATα2* portion of a *MATα2-lacZ* hybrid gene. The wild-type *MATα2-lacZ* hybrid contains all of the *MATα2* coding sequence, coding for 210 amino acids, fused to *E. coli lacZ*. The positions of the deletions are denoted by the number of the *MATα2* codon flanking each deletion end point. Codon number one is the initiation codon. The asterisks (*) indicate the deletions that confer a dominant phenotype. The asterisks in the left-hand column indicate the deletions that trans-inactivate α2. The asterisks in the right-hand column indicate the deletions that trans-inactivate **a**1-α2. The DNA binding refers to sequence-specific DNA binding by the encoded proteins. The region of α2 exhibiting homology with the homeotic gene products of *Drosophila* comprises amino acids 136 through 188 (*4, 5, 21*). The Bgl II site in *MATα2*, created by oligonucleotide-directed mutagenesis, is in parenthesis because it is not a naturally occurring site. The *lacZ* gene, coding for 1016 amino acid β-galactosidase (LacZ), is not drawn to scale.

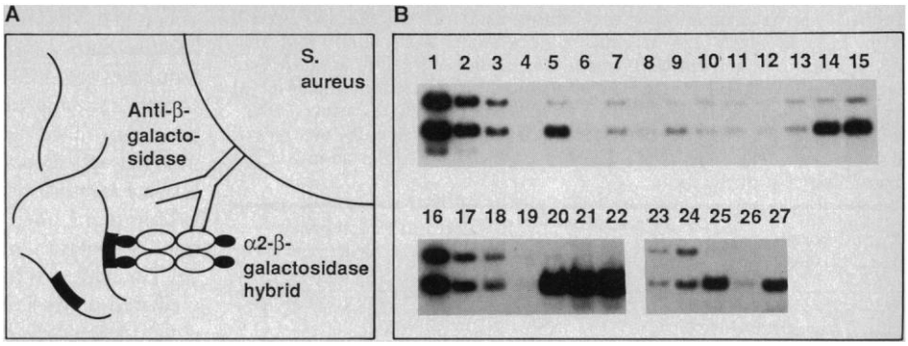

Fig. 3. Specific binding of wild-type and mutant α2-LacZ hybrid proteins to a synthetic operator. (**A**) Schematic representation of the DNA-binding assay. (**B**) Results of DNA-binding assays as follows. Lanes 1 to 15 show the results of one set of experiments. Lanes 1, 2, and 3 show 10, 3, and 1 percent, respectively, of the labeled DNA input in each binding reaction. Lane 4 shows the result of a control experiment identical to that of lane 5 with the exception that anti-β-galactosidase was omitted. Lanes 5 through 15 display the DNA bound by various α2-LacZ hybrid proteins. The correspondence between lane number and hybrid protein, wild type, or deletion mutant, is as follows: lane 5, wild type; lane 6, mutant 4–210; lane 7, mutant 2–200; lane 8, mutant 4–179; lane 9, mutant 4–140; lane 10, mutant 4–152; lane 11, mutant 141–152; lane 12, mutant 160–188; lane 13, mutant 4–140 (repeat); lane 14, mutant 2–135; lane 15, mutant 204–210. Lanes 16 to 22 show the results of a second set of experiments: lanes 16, 17, and 18 show 3, 1, and 0.3 percent of the input DNA, respectively. Lanes 19 to 22 show the DNA bound by the indicated deletion mutant: lane 19, mutant 4–210; lane 20, mutant 3–6; lane 21, mutant 3–20; and lane 22, mutant 4–62. Mutants 14–210, 26–210, and 68–210 fail to bind DNA. Lanes 23 to 27 show the results of a competition experiment. Lanes 23 and 24 show 0.3 and 1 percent of the input DNA. Lane 25 shows the DNA bound by the wild-type α2-LacZ hybrid protein (conditions are the same as for the experiment of lane 5. Lane 26 shows the result when the same reaction is carried out in a 20-fold molar excess of synthetic operator DNA. Lane 27 shows the result when the same binding reaction is carried out in a 20-fold molar excess of synthetic DNA of the same length but of a different sequence. (The sequence derives from the yeast *GAL1-GAL10* promoter region.)

determine whether the mutant alleles could provide repression by α2 alone and repression by α2 in combination with a1. The a-specific gene *MFa2*, coding for an a-factor mating pheromone, is repressed by α2 (*13*); the haploid-specific gene *HO*, encoding a function required for cell type switching, is repressed by a1-α2 (*14*). The *mfa2-lacZ* and *ho-lacZ* fusion strains containing the mutant alleles showed similar β-galactosidase activity to that observed with control strains lacking a *MATα2* gene or carrying a *MATα2* gene containing an early amber mutation (Table 1). Therefore, in agreement with the results obtained with the mating assay, none of the four deletion alleles examined provide α2 repression. Furthermore, the deletion alleles are defective for both α2 and a1-α2 repression (*15*).

We then examined whether any of the *MATα2* deletion alleles confer a dominant Matα2$^-$ phenotype. We first tested all 15 deletions for trans-dominance to α2 repression when in a *MATα2-lacZ* hybrid gene, on the high copy number plasmid YEp13, using an α-factor halo assay (*16*). Dominant alleles which inactivate α2 repression in trans cause loss of an α-factor halo normally produced by α cells (*17*). Of the 15 deletions, five (3–6, 3–20, 4–62, 2–135, and 204–210) cause loss of α-factor halo production. Accordingly, these five deletions are trans-dominant to α2 repression. The remaining ten deletions, having no effect on α-factor halo production, are recessive.

To test more directly the trans-dominance to α2 repression, we examined the ability of selected mutant *MATα2* alleles to trans-inactivate α2 repression of the target gene *MFa2* (*18*). Deletions 3–6, 3–20, 4–62, 2–135, 4–140, 141–152, and 160–188 were recombined into a nonfusion *MATα2* gene on the high copy number plasmid YEp13. In this form, these deletions affect α-factor halo production in a manner similar to that observed when they are in a hybrid gene (as mentioned above). As the direct test of trans-dominance, a strain containing a *mfa2-lacZ* gene fusion and a chromosomal wild-type *MATα2* gene was transformed with the high copy number plasmids containing the mutant *MATα2* alleles, and the resultant strains were assayed for β-galactosidase activity. The β-galactosidase activity produced by these strains is an indication of the derepression of *MFa2* as a result of interference with endogenous α2 by the product of an introduced *MATα2* allele. As was predicted by the results of the α-factor halo test, alleles 3–6, 3–20, 4–62, and 2–135 trans-inactivate α2, resulting in 31, 28, 43, and 11 percent levels of full derepression of *MFa2* expression, respectively, compared to a basal level of approximately 5 percent (Table 2). Also in agreement with the results of the α-factor halo test, alleles 4–140, 141–152, and 160–188 do not cause trans-inactivation of α2.

The mutant alleles were also tested for trans-dominance to a1-α2 repression. To test for trans-dominance to a1-α2 repression, we first examined α-factor halo production by an a/α diploid containing the mutant alleles on a high copy number plasmid (*16*). Dominant alleles that trans-inactivate a1-α2 repression elicit α-factor production in an a/α diploid (*19*). According to the α-factor halo test, of the 15 deletion alleles, 11 are recessive, and 4 (3–6, 3–20, 141–152, and 160–188) are trans-dominant to a1-α2 repression.

As a more direct test of the trans-dominance to a1-α2 repression, we examined the ability of selected alleles to trans-inactivate repression of the target gene *HO* (*18*). As described above, the *HO* gene is repressed by the combined action of a1 and α2. Alleles 3–6, 3–20, 141–152, and 160–188 trans-inactivate a1–α2, resulting in 34, 21, 11, and 4.9 percent levels of full derepression of *HO* (as a *ho-lac* fusion) expression, respectively, compared to a basal level of <1 percent (Table 2). The alleles 4–62, 2–135, and 4–140 do not trans-inactivate a1-α2 repression of *HO*. These results are consistent with the α-factor halo assay results described above.

The homeo domain of α2 is a specific DNA-binding domain. The α2 repressor, the product of the *MATα2* gene, regulates cell type in the yeast *Saccharomyces cerevisiae* by binding to an operator upstream of target genes and thereby turning off transcription of these genes. The binding of α2 to its operator is tight and highly specific (*2*). Our results allow us to assign the specific operator-binding domain of α2 to a 68–amino acid region of the protein (210 amino acids total) lying between amino acids 135 and 204. Our results further suggest that this 68–amino acid region can fold and function independently, as has been shown for the DNA-binding domain of a number of prokaryotic proteins (*20*). These conclusions follow from the observation that deletions which remove either the first two-thirds of the protein (2–135) or the carboxyl-terminal seven amino acids (204–210) fail to disrupt specific operator-binding ability. We also note that a deletion that extends only five amino acids further into the protein (4–140) from the amino terminus results in a loss of specific DNA binding.

The 68-amino-acid sequence of α2, which we have identified as necessary and sufficient for DNA binding, shows considerable homology with the homeo domain (*4, 5*). The homology between

Table 1. Complementation of a chromosomal *MAT* deletion by YCp50-borne *MATα2* alleles (*12*). The allele numbers refer to the amino acids that are missing in the mutant α2 proteins. The ability of the indicated *MATα2* alleles to provide α2 and a1-α2 repression is reflected in the level of repression of *mfa2-lacZ* and *ho-lacZ* fusions, respectively, as assayed by β-galactosidase activity (*27*). All β-galactosidase activity values are averages of at least four independent assays. In all cases, the standard deviation was less than 15 percent of the mean.

YCp50-borne *MATα2* allele	β-Galactosidase activity	
	mfa2-lacZ	*ho-lacZ*
Wild type	9	0.15
Amber	122	7.5
3–6	145	5.0
3–20	137	6.2
141–152	177	6.8
160–188	164	6.6
None	110	7.1

α2 and the *Drosophila* homeo domains is particularly evident over approximately 20 amino acids (amino acids 169 through 188 of α2). However, O'Farrell (*21*) has noted that this homology is more extensive if three amino acids of α2 are deleted. With this new alignment, the homology in α2 is expanded to 53 amino acids, extending from amino acid 136 to amino acid 188. Our convention is to refer to this 53–amino acid region as the homeo domain of α2. The region of α2 that we have shown is necessary and sufficient for DNA binding comprises amino acids 136 through 203. Desplan *et al.* (*22*) have shown that a 144–amino acid segment from the *Drosophila* engrailed protein which contains a homeo domain is capable of preferential DNA binding. Our results, combined with these, suggest that at least part of this highly conserved region serves as a sequence-specific DNA-binding domain in all proteins that contain it. Our results are consistent with the proposal of Laughon and Scott (*4*) that part of the homeo domain (amino acids 160 through 188 of α2) folds into a α-helix–turn–α-helix structure, which is known to make sequence-specific contacts with DNA for several prokaryotic DNA-binding proteins.

The DNA-binding domain of α2 is not sufficient for repression. Our deletion analysis of *MATα2* has also revealed a novel type of repressor mutant. Some of the mutant α2 proteins (3–6, 3–20, 4–62, and 2–135) have the ability to bind specifically to the operator in vitro, but fail to repress in vivo (Table 1). We can rule out two trivial explanations that would account for this unusual finding. (i) The mutant α2 proteins do not appear to be less stable or less abundant than wild-type α2. The operator-binding activity of the mutant extracts was equal to or, in some cases, greater than that of a wild-type extract. (ii) The mutant repressors do not appear to have significantly altered specificity or affinity of binding (Fig. 3). We can provide two possible explanations for the properties of the mutant repressors. (i) The mutant repressors could be defective in nuclear localization. According to this explanation, they cannot repress in vivo because they do not have proper access to the DNA to bind it. Evidence supporting this explanation is that all these mutants are altered in a region (amino acids 1 through 13) previously shown to contain a nuclear localization determinant (*7*). However, these mutant proteins are localized to the nucleus (*23*), as determined by indirect immunofluorescence. Therefore, should the repression defect be the result of improper localization, the localization must be altered in a parameter not measurable or detectable by indirect immunofluorescence, such as the rate of transport into the nucleus or subnuclear localization. (ii) A second explanation is that the defective repressors are "negative control" mutants. Wild-type α2 represses at a distance; these mutant α2 proteins might bind the operator in vivo but lack the ability to repress at a distance, possibly because they cannot interact with additional proteins or DNA sequences required for this action at a distance. Analogous positive control mutants of GAL4 and GCN4, two yeast transcriptional activators, have been described (*24*). These GAL4 and GCN4 mutants appear to bind to their recognition sequence but fail to activate transcription.

Dominant phenotypes. Some of the *MATα2* deletion alleles are trans-dominant when present in a high copy number plasmid (Fig. 2). The alleles 3–6 and 3–20 trans-inactivate both α2- and a1-α2-mediated repression. The alleles 4–62, 2–135, and 204–210 trans-inactivate only α2-mediated repression, and the alleles 141–152 and 160–188 trans-inactivate only a1-α2-mediated repression. In principle, this trans-dominance could be due either to inactivation by subunit mixing or to exclusion of wild-type α2 from the operator by bound mutant protein. However, the finding that the 204–210 allele complements a *MATα2* deletion but trans-inactivates wild-type α2 is particularly difficult to explain without invoking subunit mixing. The simplest explanation in this case is that a 204–210 homomultimer is functional whereas a heteromultimer, composed of wild-type and 204–210 subunits, is, for unknown reasons, not functional. Experiments with purified α2 indicate that α2 is indeed a multimer; in solution, purified α2 is a stable tetramer (*25*).

Although we cannot provide a detailed molecular explanation for the dominant phenotypes, two inferences can be made from the dominance studies. First, an α2 subunit contact site, a site required for multimer formation, is between amino acids 135 and 204. Regardless of whether the dominance is due to subunit mixing or exclusion from the operator (as discussed above), the mutant proteins that trans-inactivate α2 must form multimers (*26*). Therefore, on the basis of the deletions that eliminate the trans-inactivation of α2, a subunit interaction site can be assigned to a region between amino acids 135 and 204. Since this is also the region containing the homeo domain, the homeo domain of α2 (amino acids 136 through 188) appears to be both a DNA-binding domain

Table 2. Quantification of trans-dominance of YEp13-borne *MATα2* alleles to α2 and a1-α2 repression (*18, 27*). The allele numbers indicate the amino acids that are deleted in the mutant α2 proteins. The amber mutation is in the fifth codon of *MATα2*. All values are averages of at least four independent determinations. In all cases, the standard deviation was less than 20 percent of the mean.

Host cell type	YEp13-borne *MATα2* allele	% of full derepression
	Effect on α2 repression of a*-specific gene MFα2*	
a	None	100
α	3–6	31
α	3–20	28
α	4–62	43
α	2–135	11
α	4–140	4.7
α	141–152	5.5
α	160–188	6.4
α	Amber	4.9
α	None	4.8
	Effect on a1*-α2 repression of haploid-specific gene HO*	
a/Δ	None	100
a/α	3–6	34
a/α	3–20	21
a/α	4–62	<1
a/α	2–135	<1
a/α	4–140	<1
a/α	141–152	11
a/α	160–188	4.9
a/α	Amber	<1
a/α	None	<1

and a subunit contact site. Interestingly, the homeo domain of the *Drosophila* engrailed protein also appears to be both a DNA-binding domain (*22*) and a subunit contact site (*21*). Such a proximity of a subunit contact site and a DNA-binding domain has been observed in several prokaryotic repressors (*20*).

A second inference that can be made from the dominance studies is that the **a**1 and α2 proteins interact directly to repress haploid-specific genes. This inference follows from the findings that two mutant α2 proteins, 141–152 and 160–188, trans-inactivate **a**1–α2, yet do not trans-inactivate α2, and do not bind operator DNA. A direct interaction between **a**1 and α2 is the simplest interpretation of these findings. On the basis of the deletions that eliminate the trans-inactivation of **a**1-α2, we propose that an **a**1-α2 interaction site lies between amino acids 20 and 141.

Our results suggest the following simple model for the functional organization of α2. The α2 protein consists of at least three functional regions: a region at the amino terminus required for repression or transport to the nucleus, a region in the middle required for interaction with **a**1, and a region at the carboxyl terminus required for sequence-specific DNA binding and for contact between α2 subunits.

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6. Deletions 4–210, 14–210, 26–210, and 68–210 were constructed as described (*7*). Deletions 3–6, 3–20, 141–152, 160–188, and 204–210 were constructed by oligonucleotide-directed mutagenesis (*29*). Deletions 4–62, 2–135, 4–140, 4–152, 4–179, and 2–200 were constructed as follows (Fig. 1). A Bgl II site early in *MATα2* (Fig. 2) was generated by oligonucleotide-directed mutagenesis. The 5′ end of the Bgl II site on the coding strand is the third position of the third codon. Unidirectional deletions originating at this Bgl II site and extending toward the *MATα2-lacZ* fusion joint were obtained by treating two separate pools, A and B, of the plasmid shown in Fig. 1 as indicated. Conditions for enzyme reactions were as described (*30*). Deletions with appropriate end points within *MATα2* were identified by the pattern of hybridization with oligonucleotides. Bacterial colonies derived from a transformation with the ligation products (Fig. 1) were transferred to nitrocellulose filters and sequentially probed with different ^{32}P-labeled oligonucleotides. The different oligonucleotides hybridize to the regions of *MATα2* shown in Fig. 2. Colony hybridizations were performed as described (*30*). Before each hybridization, the radioactivity on a filter from a previous probing with a different oligonucleotide was stripped off by washing the filter at a nonpermissive temperature in 0.9*M* NaCl, 0.09*M* sodium citrate, *p*H 7.0 (6× SSC). Deletion end points were mapped to finer resolution by determining whether the Fsp I and Xba I sites (Fig. 2) were present. Appropriate *MATα2-lacZ* alleles were transferred from pBR322 to the yeast plasmid YEp13, by the Hind III sites flanking the yeast DNA (Fig. 1), and introduced into yeast strain HR125-5dα (*7*). The yeast transformants were tested for blue colony color on the β-galactosidase indicator 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG); colonies that gave positive results (blue colony color) on XG medium were assumed to contain in-frame *MATα2* deletions. The DNA sequences of the regions of *MATα2* assumed to contain in-frame deletions were determined by the dideoxy method, and were found to indeed contain in-frame deletions. The oligonucleotide that hybridizes closest to a particular deletion whose sequence was being determined was used as a primer. The sequences of 4–179 and 2–200 were determined with the use of a universal primer which hybridizes to the early portion of *lacZ*.
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9. Strain HR125-5dalf [*Δ(MAT)::CAN1-14 trp1 leu2-3,112 ura3-52 his3 his4*] was transformed with high copy number plasmids bearing each of the deletion mutations shown in Fig. 2. From each strain, a crude extract (the 13,000*g* supernatant) was prepared as described (*2*), except that a Mini-bead beater was substituted for the Vortexer. Specific DNA binding by a wild-type or deletion-bearing α2-LacZ hybrid protein in a crude extract was assayed as described (*2*) and as outlined in Fig. 3A. To a 20-μl sample of each extract, two ^{32}P-labeled DNA fragments were added, each to a concentration of approximately 10^{-10}*M*. The smaller fragment (total length, 65 bp) contains the 32-bp synthetic α2 binding site described (*2*). The larger fragment (85 bp) lacks an α2 binding site and serves as an internal negative control to monitor nonspecific DNA binding. After a 30-minute incubation period at 0°C, fixed *S. aureus* cells to which anti-β-galactosidase had been coupled were added. After a 1-hour incubation period, the *S. aureus*–antibody–hybrid protein complexes were removed by centrifugation and washed. The DNA that precipitated with a complex was isolated and subsequently visualized by electrophoresis and autoradiography.
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11. Complementation of a chromosomal *MAT* deletion in strain HR125-5dalf [*Δ(MAT)::CAN1-14 trp1 leu2-3,112 ura3-52 his3 his4*] by YEp13- or YCp50-borne *MATα2-lacZ* and *MATα2* deletion alleles was assayed by a patch-mating assay (*31*). The *Δ(MAT)::CAN1-14* allele is a deletion of the entire *MAT* locus with an insertion of the *CAN1* gene at the deletion site, constructed in vitro and generously provided by P. Siliciano and K. Tatchell. All plasmid-borne *MATα2* and *MATα2-lacZ* alleles described in this article include the entire *MATα* locus, inserted into YCp50 or YEp13 by the Hind III sites flanking this locus (Fig. 1).
12. Selected *MATα2* alleles were tested directly for ability to provide repression of specific target genes. Repression by α2 alone was tested by introducing the YCp50-borne alleles into a *MAT* deletion strain containing a fusion of *lacZ* to the α2-regulated gene *MFa2*. Repression by **a**1-α2 was tested in a similar manner with a diploid strain containing a *MATa1* gene, coding for **a**1, and an *ho-lacZ* fusion. The ability of the introduced *MATα2* alleles to provide α2 and **a**1-α2 repression is reflected in the β-galactosidase activity (*27*) of these *mfa2-lacZ* and *ho-lacZ* fusion strains, respectively (Table 1). The *mfa2-lacZ* fusion strain was MH64-3c [*Δ(MAT)::CAN1-14 mfa2-lacZ trp1 leu2 ura3 his4*]. The *mfa2-lacZ* fusion was derived from strain SM1179, provided by S. Michaelis. The *ho-lacZ* fusion strain was MH81 [*MATa/Δ(MAT)::CAN1-14 ho-lacZ/ho-lacZ leu2/leu2 ura3/ura3 his4/his4 met/met*]. The *ho-lacZ* fusion (*32*) was derived from strain 1998, provided by I. Herskowitz. The *Δ(MAT)::CAN1-14* allele in strains MH64-3c and MH81 was derived from crosses with strain MH52-3c [*Δ(MAT)::CAN1-14 trp1 leu2-3,112 ura3-52 his4 rme1*].
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15. Porter and Smith reported that amino acid substitutions in the region of α2, which Laughon and Scott (*4*) proposed folds into an α-helix–turn–α-helix and which exhibits homeo domain homology (*4, 5*) (amino acids 160 through 188), eliminate repressor activity [S. Porter and M. Smith, *Nature (London)* **320**, 766 (1986)]. Tatchell *et al.* have shown that changes in α2 (as a result of linker insertions in *MATα2*) in regions other than the homeo domain also eliminate repressor activity [K. Tatchell, K. A. Nasmyth, B. D. Hall, C. Astell, M. Smith, *Cell* **27**, 25 (1981).
16. Trans-dominance of *MATα2-lacZ* and *MATα2* alleles was assayed by an α-factor halo test. Patches of HR125-5d cells (*7*) of α and **a**/α cell type, containing a wild-type chromosomal *MATα2* gene and a YEp13-borne allele to be tested for trans-dominance, were replica-plated onto medium that had been previously spread with cells, XMB4-12b (*31*), sensitive to α-factor. After appropriate incubation, strains that produce α-factor are surrounded by a halo, a zone in which growth of the α-factor sensitive strain is inhibited.
17. A normal α cell produces α-factor, a pheromone that arrests growth of **a** cells as a first step in the mating process. The production of α-factor can be detected by a zone of growth inhibition, a halo, caused by the secretion of α-factor on a lawn of sensitive **a** cells. An α cell defective for α2 does not produce an α-factor halo because, although it produces α-factor, it also produces an extracellular protease, presumably the product of the *BAR1* gene, that degrades α-factor (*31, 33*). Transcription of the *BAR1* gene is normally repressed by α2 (*34*).
18. The trans-dominance of selected alleles was quantified by introducing these *MATα2* alleles, present in YEp13, into (i) the α strain SM1196, which contains a chromosomal fusion of *lacZ* to the α2-regulated gene *MFa2* and into (ii) the **a**/α strain MH60, which contains a chromosomal fusion of *lacZ* to the **a**1-α2–regulated gene *HO*. The β-galactosidase activity (*27*) of these strains correlates with the derepression of the *mfa2-lacZ* and *ho-lacZ* genes because of trans-inactivation of endogenous α2 and **a**1-α2, respectively, by the introduced plasmid-borne *MATα2* alleles (Table 2). The α *mfa2-lacZ* fusion strain was SM1196 (*MATα mfa2-lacZ trp1 leu2 ura3 his4 can1*). The levels of *mfa2-lacZ* derepression obtained with SM1196 are expressed as percentages of the fully derepressed level of *mfa2-lacZ* of isogenic **a** strain SM1179 (*MATa mfa2-lacZ trp1 leu2 ura3 his4 can1*). Strains SM1179 and SM1196 were constructed and provided by S. Michaelis. The **a**/α *ho-lacZ* fusion strain was MH60 (*MATa/MATα ho-lacZ/ho-lacZ leu2/leu2 ura3-52/ura3-52 his/his met/met can1/can1 HMRα/HMRα*). The levels of derepression of *ho-lacZ* obtained with MH60 are expressed as percentages of the fully derepressed level of *ho-lacZ* of **a**/Δ strain MH81 (see above).
19. Diploids do not normally make α-factor because **a**1-α2 represses several genes—*MATα1* (S. Fields, personal communication), *STE4* (L. Bell and V. MacKay, personal communication), *STE5* (R. Freedman and J. Thorner, personal communication), and *STE12* (S. Fields, personal communication)—that encode activators of α-factor gene transcription. Dominant alleles, which trans-inactivate **a**1-α2 repression, consequently elicit α-factor production in a diploid.
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26. If subunit mixing is the cause of the dominance, then the mutant subunits must clearly form multimers. If, on the other hand, exclusion from the operator is the cause of the dominance, the mutant α2 must still be capable of forming multimers (subunit contacts) since α2 must be at least a dimer to bind operator specifically (*2*).

27. β-galactosidase activity was assayed as described by J. H. Miller [*Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972)]. Cells to be assayed for β-galactosidase activity were cultured in the appropriate medium adjusted to *p*H 7.0, 0.1*M* potassium phosphate, and permeabilized with chloroform and sodium dodecyl sulfate.
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35. We thank S. Michaelis and I. Herskowitz for strains; C. Keleher and E. Fodor for DNA fragments; P. O'Farrell, J. Theis, S. Fields, V. MacKay, L. Bell, T. Manney, R. Freedman, and J. Thorner for unpublished results; B. Sauer, P. Sternberg, S. Poole, P. O'Farrell, and S. Fried for comments on the manuscript, and H. Boyer for making this work possible. Supported by NIH grant GM35284 (M.N.H.) and by U.C. research grants (A.D.J.).

13 April 1987; accepted 6 July 1987

Structure of the Nucleotide Activation Switch in Glycogen Phosphorylase a

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Adenosine monophosphate is required for the activation of glycogen phosphorylase b and for release of the inhibition of phosphorylase a by glucose. Two molecules of adenosine monophosphate (AMP) bind to symmetry related sites at the subunit interface of the phosphorylase dimer. Adenosine triphosphate (ATP) binds to the same site, but does not promote catalytic activity. The structure of glucose-inhibited phosphorylase a bound to AMP and also of the complex formed with glucose and ATP is described. Crystallographic refinement of these complexes reveals that structural changes are associated with AMP but not ATP binding. The origin of these effects can be traced to different effector binding modes exhibited by AMP and ATP, respectively. The conformational changes associated with AMP binding traverse multiple paths in the enzyme and link the effector and catalytic sites.

GLYCOGEN PHOSPHORYLASE (GP) MOBILIZES GLUCOSE-1-phosphate (G1P) in eukaryotes and is regulated by two parallel mechanisms. The primary control, common to all of the tissue specific isozymes, occurs through phosphorylation of the GP dimer by a specific kinase (*1*), which is in turn regulated via extracellular neural and hormonal signals. A second, intracellular control is exerted through the cooperative binding of adenosine monophosphate (AMP) to the unphosphorylated enzyme phosphorylase b (GPb). The mechanism and efficiency of AMP activation varies among the tissue isozymes (*2*). Binding of AMP to one subunit of muscle GPb both promotes the binding of AMP to the second subunit (*3*) and activates the dimer for substrate binding and catalysis. GPb is inactive in the absence of AMP (*4*) and is 80 percent as active as the phosphorylated enzyme, phosphorylase a (GPa) (*5*) in its presence.

Phosphorylase a functions efficiently in the absence of AMP, although AMP activates at low substrate concentrations (*6*) and releases the enzyme from glucose inhibition (*7*). AMP binds to GPa

Table 1. Crystallographic data and refinement results.

Crystal data							
Ligand	No. of crystals	Reflections*		R_m†	Cell (Å)‡		rms§ $\Delta F/F$ (%)
		Total	Accepted		a	c	
AMP	14	16,203	12,442	0.030	128.3	117.0	14.0
ATP	14	16,398	12,615	0.022	128.2	117.0	6.0

Refinement results			
Ligand	R_{cryst}‖	Δ bond¶ (Å)	Δ angle (Å)
AMP	0.12	0.02	0.04
ATP	0.11	0.02	0.04

*Reflections were accepted for inclusion in the data set if $I_{hkl}/\sigma_I > 3.0$, where I_{hkl} is the measured intensity and σ_I is the standard error. †A set of common scaling reflections is measured for each crystal (data set); the agreement among sets is given by the merging index:

$$R_{merge} = \{\Sigma_{hkl}\Sigma_n((I^n_{hkl} - I_{hkl})I_{hkl})\}/N_{hkl}$$

where I^n_{hkl} are the intensities of scaling reflections for the nth crystal and I_{hkl} is the mean intensity. The summation is taken over the N measurements of (hkl). ‡Cell constants for the parent GP crystals containing glucose at the catalytic site are $a = b = 128.4$ Å and $c = 116.4$ Å. §The root mean square (rms) difference in observed scattering amplitude with respect to parent crystals. ‖The crystallographic R factor is a measure of agreement between observed ($|F(obs)_{hkl}|$) structure factor amplitudes and amplitudes ($|F(calc)_{hkl}|$) calculated from the atomic model:

$$R_{cryst} = \Sigma_{hkl}[(||F(obs)_{hkl}| - |F(calc)_{hkl}||]/|F(obs)_{hkl}|$$

¶The mean deviation between bond distances (Δ bond) in the refined model and ideal bond distances, and the corresponding mean deviation between bond angle distances (Δ angle) and ideal angle distances.

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