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Operator-constitutive mutations in a DNA sequence recognized by a yeast homeodomain

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Homeodomain proteins regulate transcription in organisms as diverse as yeasts, mammals and plants, often effecting key decisions in development. Although homeodomains can selectively recognize certain DNA sequences, a question has arisen as to how specific this interaction is and how much it contributes to the ability of these proteins to properly select target genes in the cell. This question is particularly an issue in cases where the homeodomain proteins recognize DNA cooperatively with other DNA-binding proteins. In this paper, we examine the issue of DNA binding specificity for the homeodomain of the yeast α2 protein (which recognizes the a-specific gene operator cooperatively with the MCM1 protein) by examining both *in vivo* and *in vitro* the effects of point mutations in its recognition sequence. We found that most changes in the homeodomain recognition sequence produced only small effects on both homeodomain affinity as measured *in vitro* (with and without the helper protein MCM1) and operator function as determined *in vivo*. This tolerance for operator mutations illustrates in a systematic way the modest DNA-binding specificity of the α2 homeodomain and contrasts with the behavior of many of the bacterial and phage repressors where single point mutations in the operator can have dramatic effects on affinity. This tolerance for different sequences may arise from the fact that most of the interactions made between the α2 homeodomain and the DNA occur through long amino acid side chains; we suggest that these side chains can reconfigure in order to create surfaces complementary to many different DNA sequences. The relaxed DNA-binding specificity of homeodomain proteins such as α2 may be an important feature that permits new regulatory circuits to evolve rapidly from existing components.

Key words: cooperative DNA binding/DNA–protein interaction/homeodomain/*Saccharomyces cerevisiae*/transcriptional repression

Introduction

Over the past decade, studies of development in many organisms have revealed that important decisions are often carried out by proteins that regulate transcription. Although the transcriptional regulatory circuitry can differ from one organism to the next, the components that comprise these circuits are often conserved across large phylogenetic distances. A striking example is the homeodomain, a 60 amino acid DNA-binding domain found in yeasts, nematodes, flies, mammals and plants [for reviews, see Scott *et al.* (1989) and Wüthrich and Gehring (1992)]. Structural studies of three homeodomains (Antennapedia and Engrailed from *Drosophila melanogaster* and α2 from *Saccharomyces cerevisiae*) have shown that, despite considerable differences in amino acid sequences, the three domains have nearly identical backbone structures (Quian *et al.*, 1989; Billeter *et al.*, 1990; Kissinger *et al.*, 1990; Otting *et al.*, 1990; Phillips *et al.*, 1991; Wolberger *et al.*, 1991). Moreover, the three homeodomains all interact with DNA in fundamentally similar ways, even though they display distinct sequence preferences [see above references and Affolter *et al.* (1990), Desplan *et al.* (1988) and Sauer *et al.* (1988)].

One of the roles of the yeast α2 protein is to repress transcription of a group of genes called the **a**-specific genes (for reviews, see Herskowitz, 1989; Sprague, 1990; Dolan and Fields, 1991; Johnson, 1992). Because α2 is present in both α cells and **a**/α cells, but absent in **a** cells, this group of genes is expressed only in **a** cells. The **a**-specific gene operator (*asg* operator), the DNA sequence through which α2 exerts its repressive effects, was first identified biochemically through direct DNA-binding experiments utilizing a purified α2-β-galactosidase fusion protein (Johnson and Herskowitz, 1985). Although α2 shows a distinct preference for the *asg* operator over other DNA sequences, this discrimination is apparently not of sufficient strength to allow α2, on its own, to occupy the *asg* operator in the cell. Rather, α2 also relies on protein–protein interactions with a second sequence-specific DNA-binding protein, MCM1, a member of the serum response factor (SRF) group of proteins (Keleher *et al.*, 1988, 1989; Passmore *et al.*, 1989; Ammerer, 1990; for review of the SRF proteins, see Treisman, 1992). A model showing these two proteins interacting on the *asg* operator is shown in Figure 1 (Smith and Johnson, 1992; Vershon and Johnson, 1993; for review, see Johnson, 1992). MCM1—even in the absence of α2—occupies the operator *in vivo* (Keleher *et al.*, 1992) and since MCM1 interacts with α2, the question arises as to how important and how specific are the homeodomain–DNA interactions. To address this question, we examined the effects of point mutations in an *asg* operator (that from upstream of the *STE6* gene) on the binding of the α2 homeodomain *in vitro* and on the function of the operator *in vivo*.

The interaction of α2 with the *asg* operator provides a number of particular advantages for understanding a homeodomain–DNA interaction in a biological setting. (i) The operator utilized in these experiments is naturally occurring and is known to control expression of a target gene for α2, *STE6* (Johnson and Herskowitz, 1985). (ii) This

regulatory circuit is relatively simple and permits the quantitative analysis of the operator mutations *in vivo*. (iii) The co-factor (MCM1) with which α2 binds the operator is known, and both proteins have been purified to apparent homogeneity (Keleher *et al.*, 1988; Sauer *et al.*, 1988; Ammerer, 1990). Moreover, the interaction between these proteins has been well characterized (Vershon and Johnson, 1993). (iv) Although MCM1 and α2 bind the operator cooperatively, α2, at elevated concentrations *in vitro*, will bind the operator on its own (Keleher *et al.*, 1988; Smith and Johnson, 1992). We therefore were able to test the effect of operator mutations on the affinity of α2 in both the presence and the absence of its co-factor. (v) The structure of the α2 homeodomain bound to a variant of the *asg* operator is known (Phillips *et al.*, 1991; Wolberger *et al.*, 1991), allowing a correlation of the genetics and biochemistry with structural features of the interaction.

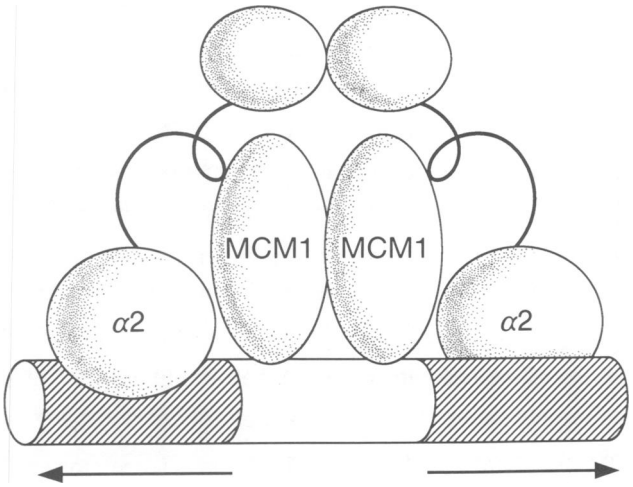
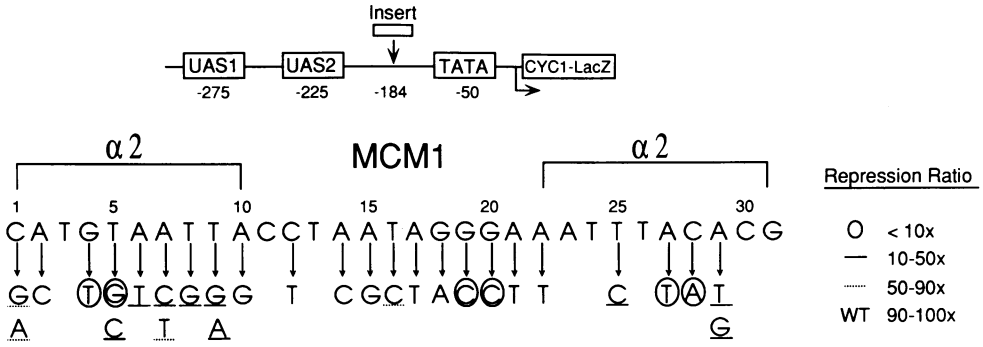


Fig. 1. Model for MCM1 and α2 bound to the **a**-specific gene operator (see Keleher *et al.*, 1988; Vershon and Johnson, 1993).

Results

Isolation of operator mutations and determination of their strengths in vivo

The base sequence of the *STE6* operator is given in Figure 2. As shown in Figure 1, a dimer of MCM1 binds to the center of the operator while a dimer of α2 occupies the flanks of the operator. The two proteins bind cooperatively and correct arrangement and spacing of the MCM1/α2 recognition sequences are required for this cooperative binding (see Smith and Johnson, 1992). Deletion of the MCM1 recognition sequences or its substitution with 7 bp of different sequence results in a complete loss of repression *in vivo* (Keleher *et al.*, 1988), as does the deletion of one or both α2 half-sites (data not shown). These results indicate that there are no functional redundancies within the operator, and that single point mutations can be analyzed *in vivo* in a meaningful way. Since the α2 homeodomain, when removed



Position	Mutation	Repression ratio	% Operator occupancy
WT		100	99
1	C → G	83	99
1	C → A	65	98
2	A → C	>100	>99
4	G → T	5.1	80
5	T → G	5.3	81
5	T → C	18	94
6	A → T	14	93
7	A → C	23	96
7	A → T	70	99
8	T → G	18	94
9	T → G	20	95
9	T → A	35	97
10	A → G	91	99
12	C → T	>100	>99

Position	Mutation	Repression ratio	% Operator occupancy
WT		100	99
14	A → C	>100	>99
15	A → G	>100	>99
16	T → C	58	98
17	A → T	>100	>99
18	G → A	>100	>99
19	G → C	9.2	90
20	G → C	3.4	71
21	A → T	>100	>99
22	A → T	>100	>99
25	T → C	19	95
27	A → T	1.9	47
28	C → A	3.4	71
29	A → T	13	92
29	A → G	20	95

Fig. 2. Operator mutations and their effect on repression *in vivo*. The wild-type (WT) sequence of the *STE6* operator is indicated. The areas of α2 contact, indicated by brackets, are based on DNase I and hydroxyl radical protection data (Sauer *et al.*, 1988), crystallographic evidence (Wolberger *et al.*, 1991), and this work. The point mutations examined in this paper are indicated below the wild-type sequence. The ability of each operator to repress transcription was determined by inserting the mutant operator into the *CYC1* promoter (see diagram above the sequence) and measuring β-galactosidase enzyme activity in otherwise isogenic **a** and α cells. The data are expressed as repression ratios, the ratio of β-galactosidase activity in **a** cells to that in α cells. For each mutant operator, three independent transformants were isolated and each transformant was assayed in duplicate. The results are summarized directly below the sequence of the *asg* operator, and repression ratios are given in the table.

from the dimerization domain, binds independently to the two half-sites of the operator (Smith and Johnson, 1992), these simple point mutations could be easily analyzed and interpreted biochemically, as well.

A variety of point mutations in the 31 base *STE6* operator were created using a random mutagenesis scheme (see Materials and methods). Single substitutions at 18 different positions were obtained after sequencing over 100 independently derived operators. In addition, we synthesized *ad hoc* a number of specifically altered operators to complement those obtained from the random mutagenesis scheme. Figure 2 shows the wild-type operator sequence and the single point mutations, 28 in all, that were subsequently analyzed.

The ability of these mutant operators to bring about repression *in vivo* was tested by inserting them upstream of a *CYC1−lacZ* gene fusion. The operators were inserted between the UAS and TATA sequences, a position in which the wild-type operator gives ~100-fold repression in α cells (which contain α2) when compared with **a** cells (which lack α2). The degree of repression conferred by each mutant operator was determined by measuring the β-galactosidase levels in both **a** and α cells; the results (Figure 2) are expressed in terms of repression ratios, that is, the ratio of the level in **a** cells divided by the level in α cells. Thus, a wild-type operator gives a repression ratio of approximately 100; a *CYC1* construct lacking an operator would give a repression ratio of 1. As explained in more detail below, we have also converted the measured repression ratios to an estimate of the occupancy of the operator by α2 *in vivo*. These values are also given in Figure 2.

Our results can be summarized as follows: six point mutations had relatively strong effects, reducing the repression ratios to <10% of wild-type; two of these mutations lay in the MCM1 recognition sequence and four in the homeodomain recognition sequence. Thirteen mutations showed weak effects, and nine changes had no observable effect. Eight positions in the operator were not tested. We note that the sequence of the operator has twofold symmetry, that it functions when inserted into the *CYC1* promoter in either orientation (Johnson and Herskowitz, 1985), and that the effects of a point mutation in one half are roughly equivalent to the effect of the corresponding mutation in the other half. If the twofold symmetry of the operator is invoked, every position was effectively tested. We assumed that the phenotype of these operator mutations results from a reduced affinity for α2, MCM1, or both. In order to relate the effects *in vivo* to changes in affinity, we turned to biochemical experiments using purified proteins.

Operator mutations with reduced affinities for the α2 homeodomain

First, we tested the affinities of a selected group of mutant operators for the isolated α2 homeodomain. We utilized a purified fragment of α2 (amino acids 136−210) identical to that analyzed crystallographically (Wolberger *et al.*, 1991) and by NMR (Phillips *et al.*, 1991). In contrast to the dimeric full-length α2, this homeodomain fragment is a monomer in solution, even at concentrations as high as 1 mM (Phillips *et al.*, 1991), and binds to the operator in two steps, as illustrated by the gel mobility shift experiment of Figure 3. At low concentrations (lanes 15−13 of Figure 3A), a single shifted band is observed and corresponds to an α2 homeodomain fragment bound to a single half-site of the operator. In fact, this species is a roughly equimolar mixture of operators, some with the left half-site occupied and some with the right half-site occupied, indicating that the affinity of the homeodomain for the two half-sites is the same [data not shown; see Smith and Johnson (1992) for a detailed description]. At higher concentrations (panel A), both halves of the operator are filled, resulting in a second mobility shift (lanes 12−5 of Figure 3A). Finally, at the highest homeodomain concentrations used in this experiment, the binding appears non-specific, as judged by the heterogeneity of the shifted species (lanes 4−1 of Figure 3A). This experiment was repeated using an operator carrying the strongest mutation (27A → T), which showed a repression ratio of 2. Occupancy of the undamaged half-site is seen at the expected concentration (providing an internal control); however, occupancy of the mutant half-site is not observed (Figure 3B). In fact, the homeodomain fragment appears to bind non-specifically to the fragment before it clearly occupies the damaged half-site. From this experiment, we can conclude that the 27A → T mutation reduces the affinity of the α2 homeodomain for that half-site by a factor of at least 30.

Similar experiments were carried out for the point mutants 28C → A and 29A → T. These mutant operators still provided a reasonable degree of repression *in vivo* (4-fold and 20-fold residual repression, respectively). The experiment shown in Figure 3D and E revealed proportionate decreases in affinity caused by these mutations. That is, the

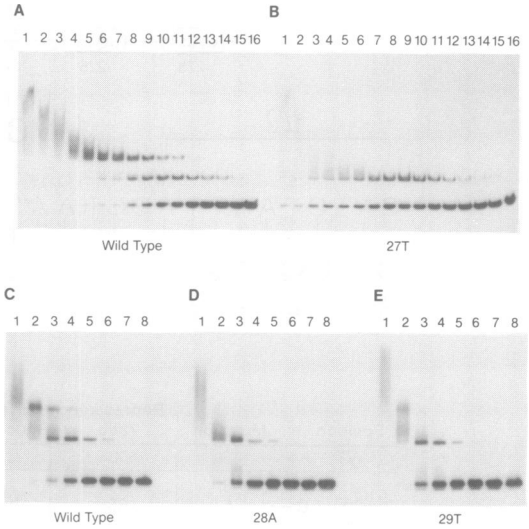


Fig. 3. Interaction between the α2 homeodomain and various mutant operators. A 71 bp ^{32}P-labeled DNA fragment containing either a wild-type site ('WT', panels **A** and **C**), the 27A → T operator (panel **B**), the 28C → A operator (panel **D**) or the 29T → A operator (panel **E**) was incubated with purified α2 homeodomain fragment, electrophoresed through a native gel and displayed by autoradiography. The concentration of homeodomain fragment for each lane in panels A and B is as follows: lane 1, 2.0×10^{-6} M; lane 2, 1.3×10^{-6} M; lane 3, 8.8×10^{-7} M; lane 4, 5.9×10^{-7} M; lane 5, 3.9×10^{-7} M; lane 6, 2.6×10^{-7} M; lane 7, 1.8×10^{-7} M; lane 8, 1.2×10^{-7} M; lane 9, 7.8×10^{-8} M; lane 10, 5.2×10^{-8} M; lane 11, 3.5×10^{-8} M; lane 12, 2.3×10^{-8} M; lane 13, 1.5×10^{-8} M; lane 14, 1.0×10^{-8} M; lane 15, 6.8×10^{-9} M. Lane 16 in each panel contains no α2 protein. The concentrations of α2 for panels C, D and E are: lane 1, 9.8×10^{-5} M; lane 2, 4.9×10^{-5} M; lane 3, 2.4×10^{-5} M; lane 4, 1.2×10^{-5} M; lane 5, 6×10^{-6} M; lane 6, 3.0×10^{-6} M; lane 7, 1.5×10^{-6} M. Lane 8 in panels C−E contains no α2 protein.

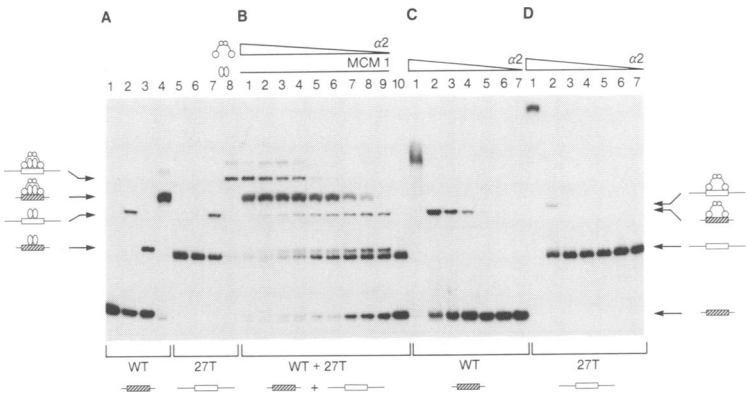


Fig. 4. Interaction of intact α2 and an MCM1 fragment with the 27A → T operator. Panels **C** and **D** show the binding of intact α2 alone to a fragment bearing the wild-type (WT) operator (71 bp, panel C) or the mutant 27A → T operator (155 bp, panel D). The concentration of α2 in each lane is as follows: lane 1, 6.8×10^{-7} M; lane 2, 2.3×10^{-7} M; lane 3, 6.8×10^{-8} M; lane 4, 2.3×10^{-8} M; lane 5, 6.8×10^{-9} M; lane 6, 2.3×10^{-9} M; lane 7 in each panel contains no α2 protein. Panel **B** shows an experiment in which MCM1 concentration is held constant at 2.9×10^{-7} M, and α2 is varied from 6.8×10^{-8} M (lane 1) to 6.8×10^{-12} M (lane 9). In this panel, the mutant and wild-type operators were mixed to more easily detect differences in binding affinities. The concentration of α2 is as follows: lane 1, 6.8×10^{-8} M; lane 2, 2.3×10^{-8} M; lane 3, 6.8×10^{-9} M; lane 4, 2.3×10^{-9} M; lane 5, 6.8×10^{-10} M; lane 6, 2.3×10^{-10} M; lane 7, 6.8×10^{-11} M; lane 8, 2.3×10^{-11} M; lane 9, 6.8×10^{-12} M and lane 10 contains no α2 protein. Cartoons at the top of panel B and the bottom of each panel indicate protein and DNA respectively added to each reaction. Cartoons at the left and right of the figure represent the protein−DNA complex thought to be present in each band. The lanes in panel **A** are controls to enable identification of the different species in panels B−D. Lanes 1−4 show the wild-type operator and lanes 5−8 show the mutant operator with no protein (lanes 1 and 5), 6.8×10^{-8} M α2 (lanes 2 and 6), 2.9×10^{-7} M MCM1 (lanes 3 and 7) or a combination of 6.8×10^{-8} M α2 and 2.9×10^{-7} M MCM1 (lanes 4 and 8).

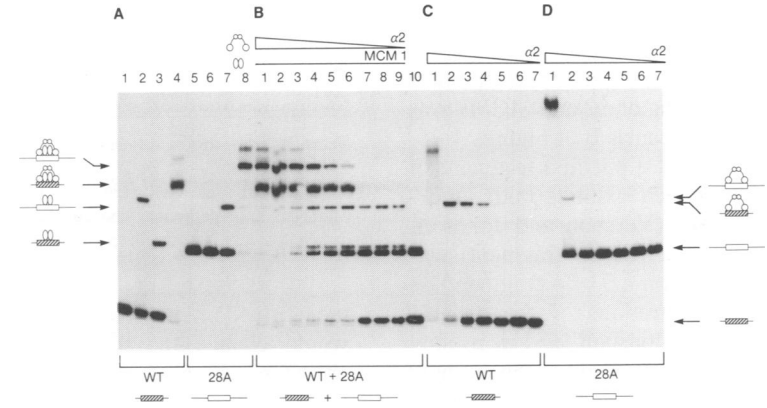


Fig. 5. Interaction of intact α2 and an MCM1 fragment with the 28C → A operator. The experiment described in Figure 3 was repeated, with the 28C → A mutant operator. The organization and concentrations of protein and cartoon representations of shifted species are the same as indicated in Figure 4 for all lanes.

second half-site in both mutant operators was filled, but at concentrations significantly higher than those needed to fill the second half-site in a wild-type operator. We estimate that the 28C → A and 29A → T mutations reduce the affinity of the homeodomain to the corresponding half-site by factors of ~20-fold and ~5-fold, respectively. The binding of the isolated homeodomain of α2 to the operators thus produced a hierarchy of affinities, wild-type > 29T > 28A > 27T, that parallels the effects observed *in vivo*. In these experiments, the K_D of the isolated homeodomain for a wild-type half site was $\sim 10^{-7}$ M (see also Sauer *et al.*, 1988).

We performed similar DNA-binding experiments with the following additional operator mutations: 1C → G, 4G → T, 5T → G, 6A → T, 7A → C, 8T → G and 9T → G. We found that 4G → T and 5T → G reduced the affinity for the α2 homeodomain by ~10-fold, whereas the remaining mutations analyzed showed effects ranging from 1.5-fold to 5-fold (not shown). We estimate that these DNA-binding experiments are accurate to a factor of ~2, and it was therefore difficult to establish a meaningful hierarchy of binding *in vitro* with these weaker operator mutations. Taken as a whole, the effects of the mutations *in vitro* parallel the effects observed *in vivo*. A quantitative comparison of these two types of measurements is given in the Discussion.

Effects of operator mutations in the presence of MCM1

α2 does not exist in the cell as an isolated homeodomain and we therefore examined the effect of three operator mutations (those analyzed in Figure 3) on the recognition by intact α2 which binds the operator as a dimer. The experiments of Figures 4−6, panels C and D show that the 27A → T, 28C → A and 29A → T mutations reduce the affinity of intact α2 dimers for the operator by factors of 30, 10 and 3 respectively when compared with that of the wild-type operator. These values are consistent with the idea that the dimer recognizes an operator with one healthy and

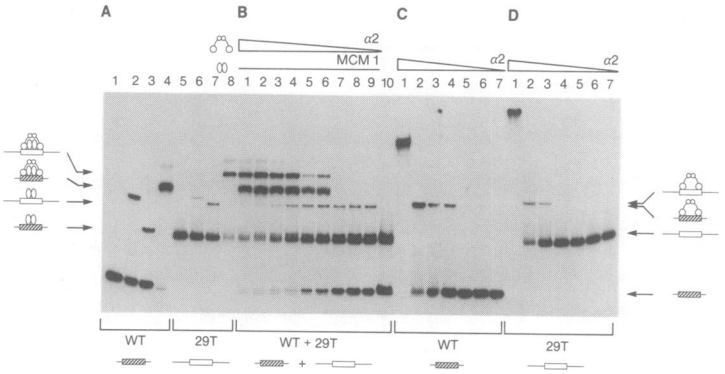


Fig. 6. Interaction of intact α2 and an MCM1 fragment with the 29A → T mutant operator. The experiment described in Figure 3 was repeated using the 29A → T mutant operator. The organization and concentrations of protein and cartoon representations of shifted species are the same as indicated in Figure 4 for all lanes.

one damaged half-site. The intact α2 dimers fill the mutant operators in a single step and the affinities of the dimers for the mutant operators are higher than those of the homeodomain fragment, a result that derives from the favorable effects of coupling two DNA-binding domains (see Sauer *et al.*, 1988).

Finally, α2 requires interaction with the MCM1 protein in order to occupy the *asg* operator *in vivo*. The two proteins bind cooperatively to the operator, presumably by making a direct protein−protein contact (see Figure 1; see Vershon and Johnson, 1993). The effect of the same three operator mutations on the cooperative binding of α2 and MCM1 was tested in the experiments shown in panels B of Figures 4−6. For these experiments, we utilized a purified fragment of MCM1 (amino acids 1−96) which exhibits both DNA-binding and interaction with α2 (Vershon and Johnson, 1993). The mutant operator (on a 155 bp fragment) was mixed with a wild-type operator (on a 71 bp fragment) as an internal control. The concentrations of the MCM1 fragment and the DNA were held constant, and increasing amounts of α2 were added. Again, these experiments show that the 27A → T mutation shows the most pronounced effect, reducing the affinity of α2/MCM1 for the operator by a factor of ~50. Although these effects are difficult to quantify, it can be estimated that 28C → A and 29A → T reduce α2/MCM1 affinity by a factor of approximately 10 and 3, respectively.

We conclude from this set of experiments that these three operator mutations reduce the affinity of the α2 homeodomain proportional to their effects *in vivo* (see Discussion for a more quantitative assessment). Furthermore, the experiments show that the decrease in affinity caused by each operator mutation is roughly the same for the isolated monomeric homeodomain, the intact α2 dimer and the α2 dimer in combination with MCM1. This result suggests that the binding of the homeodomain is relatively independent in all three contexts.

Overproduction of α2 can partially suppress the operator mutations

Although the lowered affinities of the mutant operators for the α2 homeodomain can account for the effects observed *in vivo*, a second hypothesis can be raised. According to this, the concentration of α2 in the cell would be sufficiently high for α2 to occupy the mutant operators efficiently; however, the homeodomain would make aberrant DNA contacts that would change the precise configuration of α2 on the operator, leading to a loss of effective repression. Indeed, it has been proposed that MCM1 can exist in at least two distinct conformations depending upon the DNA sequence to which it is bound (Tan and Richmond, 1990). We distinguished between these possibilities for α2 by the following experiment: an increase in the intracellular concentration of α2 should at least partially correct a simple DNA affinity defect, but should not influence a defect in precise positioning of α2 or in a DNA-induced conformational change of α2. The three point mutations analyzed in detail above (27A → T, 28C → A and 29A → T) were introduced into both wild-type α cells and into α cells that also carried a high-copy plasmid that overexpressed α2 ~10-fold. We found that overproduction of α2 significantly suppressed all three operator constitutive mutations (Table I), arguing that the effect of the operator mutation was due simply to a lowered affinity for the α2 homeodomain.

Table I. Overproduction of α2 *in vivo* can suppress at least some operator mutations

Operator	Expression level			Repression ratio	
	a cells	α cells	α cells + α2	**a**/α	**a**/α + α2
Wild-type	1190	14	3.4	83	350
No site	650	230	220	2.8	2.9
27T	950	330	100	2.8	9.8
28A	910	190	29	4.8	31
29T	1040	33	14	31	73

The *CYC1* constructs described in Figure 1 carrying wild-type, 27A → T, 28C → A or 29A → T mutations were introduced into **a** cells, α cells and α cells carrying a plasmid that overproduces α2 (see Materials and methods). The values in the table represent units of β-galactosidase activity determined in duplicate for three independent transformations of each strain.

Operator mutations with reduced affinities for MCM1

Our initial studies of the collection of operator mutations (Figure 2) revealed that two mutations located in the center of the operator (i.e. 19G → C and 20G → C) produced a strong operator-constitutive phenotype. Since this portion of the operator can be deleted with no loss in binding affinity for purified α2 protein (Sauer *et al.*, 1988), it seemed likely

that these mutations would affect the interaction with MCM1. To test this idea, we compared the affinities of a purified fragment of MCM1 (amino acids 1–96) for these two mutant operators. In addition, we created a third operator mutant which bore both 19G → C and 20G → C as well as the two symmetrically located changes 11C → G and 12C → G. *In vivo*, this quadruple mutant operator gave a repression ratio of 2.8, as compared with 3.4 for 20G → C and 9.2 for 19G → C. The experiment shown in Figure 7 shows that all three mutant operators had severely reduced affinities for the MCM1 fragment, with the quadruple mutant showing the most pronounced effect. We estimate that the quadruple mutant is reduced in affinity by a factor of at least 100 and the single mutants by factors of ~30. For these experiments, a wild-type operator (on a larger DNA fragment) was included as an internal control. This analysis shows that operators compromised in their affinities for MCM1 show a strong operator-constitutive phenotype *in vivo*. Consistent with this idea, a neighboring mutation (17A → T, Figure 2) that had no effect on phenotype *in vivo* showed no decrease in MCM1 binding (not shown).

Discussion

In this paper, we have determined the effects of point mutations in a DNA sequence recognized by a homeodomain, that of the yeast α2 protein. This recognition sequence is part of the **a**-specific gene operator (*asg* operator) which directs the transcriptional repression of genes to which it is linked. The effects of these operator mutations were studied in two ways. First, all the mutant operators were examined *in vivo* for the extent to which they allow constitutive expression under conditions where the wild-type operator directs very tight (100-fold) repression. Second, for a subset of the mutations, we measured the affinity *in vitro* for the α2 homeodomain, the intact α2 dimer, and the intact α2 dimer binding with MCM1. We first consider the extent to which the measurements taken *in vivo* correlate with the biochemistry.

Correlation between results obtained in vivo and in vitro

With a few basic assumptions it is possible to estimate from the repression ratios obtained in Figure 2 the relative affinities of the mutant operators for the α2 homeodomain *in vivo*. Binding of α2 to the operator can be described by the relation

$$\text{operator occupancy} = \frac{1}{1 + K_D/[\alpha 2]}$$

where [α2] is the concentration of free α2. The first assumption is that the various test promoter constructs used in the measurements of Figure 2 do not perturb the concentration of free α2 in the cell, an assumption that seems justified since introduction of a wild-type *asg* operator on a multicopy plasmid does not significantly derepress the chromosomal **a**-specific genes (not shown). The second assumption is that the expression ratio measured *in vivo* directly reflects the fraction of operators that are occupied. For the wild-type operator, the repression ratio is 100. The assumption is that, in a population of cells observed instantaneously, 99% of the operators are occupied and 1% are free. For the 29A → T mutant, for example, which exhibits a repression ratio of 13, we would calculate an operator occupancy of 92%. The calculated operator occupancies for all the operator mutations are given in Figure 2. The assumption that the repression ratios are proportional to the operator occupancies (and do not, for example, reflect differences in the efficiency of the repression once the operator is filled) is consistent with the experiment of Table I which shows that the repression ratios (and by assumption, the operator occupancies) improve when the intracellular concentration of α2 is raised.

Since the repression ratios for the wild-type and mutant operators were measured for a single concentration of α2, that in an α cell, the relative K_Ds can be estimated by rearranging the above equation:

$$\frac{K_D}{[\alpha 2]} = \frac{1 - \text{operator occupancy}}{\text{operator occupancy}}.$$

If the K_D for the wild-type operator is arbitrarily set at 1, then that for 29A → T is calculated to be 9, that of 28C → A to be 40, and that of 27A → T to be 100.

These estimates can be compared with the affinity changes obtained by the direct α2 homeodomain binding experiments

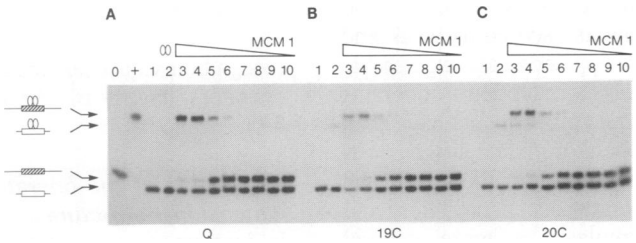


Fig. 7. Binding of MCM1 to selected mutant operators. Fragments bearing mutant operators Q ('quadruple'), 19G → C and 20G → C were tested for their ability to bind purified $MCM1_{1-96}$ protein (Vershon and Johnson, 1993). The sequence and phenotype of each point mutant is summarized in Figure 2. The quadruple mutant, Q, is described in the text. Each mutant was isolated as a 71 bp fragment and was mixed with an 86 bp fragment bearing the wild-type operator. For each set, lane 0 shows the WT operator with no protein added and lane + shows the wild-type operator and 2.9×10^{-7} M MCM1. For each panel, lane 1 shows only the mutant operator with no added protein and lane 2 shows the mutant operator with 2.9×10^{-7} M MCM1. For each panel, lanes 3–9 show the effect of adding increasing amounts of MCM1 to a mixture of the wild-type operator and a mutant operator. The concentration of MCM1 is as follows: lane 3, 2.9×10^{-7} M; lane 4, 9.8×10^{-8} M; lane 5, 2.9×10^{-8} M; lane 6, 9.8×10^{-9} M; lane 7, 2.9×10^{-9} M; lane 8, 9.8×10^{-10} M; lane 9, 2.9×10^{-10} M and lane 10, 9.8×10^{-11} M. Cartoons to the left of panel A represent the DNA or protein–DNA complex thought to be present in each band.

in vitro (1, 5, 20 and >30; see Figure 3). Although the relative values obtained by the two methods (one *in vivo*, one *in vitro*) are only approximate and were obtained under different conditions, they agree reasonably well and indicate that, from the repression ratios measured *in vivo*, approximate k_ds can be obtained. Similar types of analyses have been carried out for prokaryotic repressors [see, for example, Jobe *et al.* (1974), Ebright (1986), Betz *et al.* (1986) and Lehming *et al.* (1987) for studies on the *E.coli lac* repressor].

Most of the operator mutations have relatively small effects on homeodomain recognition

What do the operator mutations reveal about the specificity of the homeodomain–operator interaction? The positions of most of the operator mutations examined in this study and their effects measured *in vivo* are summarized in Figure 8B along with the α2 homeodomain operator structure as determined by Wolberger *et al.* (1991). For this figure, operator mutations taken from both operator half-sites are superimposed on a single half-site. We felt that this superimposition was justified by the facts that (i) the two half-sites have nearly the same sequence (Figure 8A) and (ii) crystallographic analysis indicated that the α2 homeodomain was similarly positioned on both half-sites (Wolberger *et al.*, 1991). As indicated in the figure, contacts with base pairs are made by the side chains of helix 3 which are positioned in the major groove, and by an amino-terminal arm which lies in the minor groove.

The results displayed in Figure 8 reveal several features of the homeodomain–operator interaction. First, the majority of changes examined have only modest effects on the extent of operator occupancy *in vivo*. Most of the operator mutations scored as having 'intermediate' or 'weak' effects reduce the occupancy of the operator *in vivo* from 99% (for wild-type) to a minimum of 90%. These changes, according to the calculations discussed above and supported by our biochemical experiments, would be produced by changes in the affinity of the α2 homeodomain for the operator *in vivo* of <10-fold.

We note that these small changes in operator occupancy do exhibit a measurable effect *in vivo* because the repression by α2/MCM1 on a wild-type operator is extremely tight. Consequently, small reductions in the extent of occupancy produce measurable changes in the level of transcription. If, for example, α2/MCM1 activated transcription instead of repressing it, the operator mutants scored as weak and intermediate would probably have appeared indistinguishable from the wild-type *in vivo*; that is, a reduction in operator occupancy from 99% to 90%, would probably not have been detectable. Hence, the fact that operator mutant analysis was carried out on an efficient repression system allowed for the detection of small changes in operator occupancy. Berg and von Hippel (1988) have formulated a more general description of this point.

Although most of the changes summarized in Figure 8 produced only small effects on the extent of operator occupancy *in vivo*, changes at every position did have at least some damaging effect. This result suggests that homeodomain recognition sequences of the wild-type operator have been optimized for the highest affinity. Consistent with this idea is the fact that 10 naturally occurring α2 homeodomain

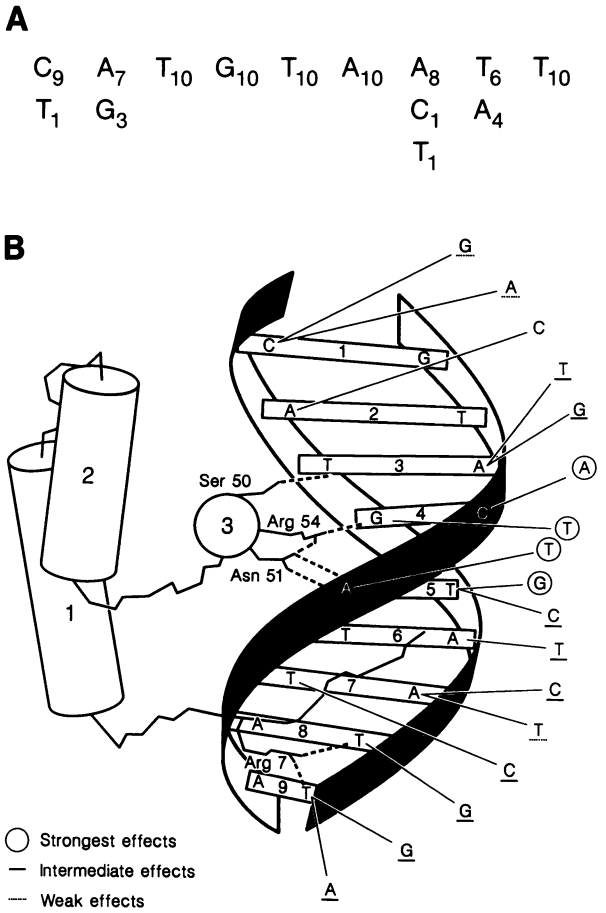


Fig. 8. Summary of the effects of operator mutations. Panel **A** shows the consensus α2 homeodomain recognition sequences derived from the five known **a**-specific gene operators, each of which has two homeodomain recognition sequences arranged with dyad symmetry (Johnson and Herskowitz, 1985; Miller *et al.*, 1985). Panel **B** depicts a model for the α2 homeodomain DNA complex deduced crystallographically by Wolberger *et al.* (1991). This diagram shows a single homeodomain interacting with a consensus **a**-specific gene operator half-site. Base substitutions that were made at the *STE6* operator are indicated and their effects on repression are summarized (see Figure 1). Since the two *STE6* half-sites are nearly identical (and the left half matches the *asg* consensus perfectly), substitutions made in both half-sites have been superimposed for this figure. As described in the text, the mutations scored as 'strongest' reduce the affinity of the homeodomain by factors greater than 10, as measured both *in vivo* and *in vitro*. Those marked as 'intermediate' reduce the affinity from between 10-fold and 2-fold and those marked as weak have a <2-fold effect on affinity.

recognition sequences (from the five different **a**-specific gene operators) have nearly the same DNA sequences (see Figure 8A).

Correlation of the operator mutants with the three-dimensional structure

In contrast to most of the operator mutants, changes at two positions, 4 and 5, produced reasonably strong effects, decreasing the affinity of the homeodomain by at least a factor of 10 as determined both *in vivo* and *in vitro*. As shown in Figure 8, the A of base pair 5 is contacted by Asn51 of α2. A similar contact is made between the Engrailed homeodomain and DNA (Kissinger *et al.*, 1990), leading Wolberger *et al.* (1991) to propose that this Asn51–A5 interaction is a common feature of homeodomain–DNA

interactions. The fact that our strongest operator mutation, 27A → T, disrupts this interaction is consistent with this idea. However, if the intracellular concentration of α2 is raised by ~10-fold, the 27A → T operator is significantly occupied by α2 (Table I). Thus, it appears as though the α2 homeodomain can still occupy this mutant operator *in vivo* despite the loss of this key contact. The biochemical experiment shown in Figure 4 also shows that the α2 at elevated concentration can efficiently occupy this operator. It is possible that the entire α2 homeodomain has shifted its register on this operator to allow for an alternative Asn51−A interaction. However, since the interaction between α2 and MCM1 restricts the positioning of the α2 homeodomain on the DNA (Smith and Johnson, 1992), we regard this possibility as unlikely. Base pair 4 of the operator is contacted by Arg54, and changes in this base pair also significantly disrupt the homeodomain−DNA interaction. Thus, our analysis points to bases 4 and 5 as key determinants in the recognition of DNA by the α2 homeodomain. Consistent with this view, a change of Arg54 to alanine or a change of Asn51 to alanine also severely reduces the affinity of the homeodomain for the operator (A.K.Vershon and A.D.Johnson, in preparation).

We next consider the interaction between Ser50 and base pair 3. Ser50 is the ninth position of helix 3, and genetic and biochemical experiments have demonstrated the importance of this residue as a specificity determinant in the *Drosophila* Paired, Fushi terazu and Bicoid homeodomains (Hanes and Brent, 1989; Treisman *et al.*, 1989). In the case of α2, this interaction contributes only slightly to the overall affinity of the homeodomain−DNA interaction. Changes of base pair 3 result in a decrease in operator occupancy (in an α cell) from 99% (for wild-type) to 95% (for 29A → G) and 93% (for 29A → T). Consistent with the relatively small magnitude of these changes, a change of Ser50 to glutamine does not significantly disrupt the interaction between α2 and its operator (A.D.Johnson and A.K.Vershon, in preparation). We conclude that the interaction between the ninth position of helix 3 and the operator is relatively unimportant for the binding of the α2 protein. In the X-ray derived structure, Ser50 appears to contact base pair 3, although the bond distance appears too great to be accounted for by a simple hydrogen bond (Wolberger *et al.*, 1991).

Next, we consider the effects of changing the bases (6−9) contacted in the minor groove by the amino-terminal arm of the homeodomain. All of the operator mutants lying within these positions show weak or intermediate phenotypes, indicating that the contacts made by the arm are base-specific. Removal of the amino-terminal arm of the Antennapedia homeodomain reduces its affinity *in vitro* significantly (Affolter *et al.*, 1990) and a change of Arg7 of α2 to glycine has a similar effect on α2, as determined both *in vivo* and *in vitro* (A.K.Vershon and A.D.Johnson, in preparation).

Mutations of base pair 1 have small, but reproducible, effects on the interaction of the α2 homeodomain with the operator. Although the bases of position 1 do not appear to be contacted directly by the α2 homeodomain in the X-ray structure, contacts are made to the phosphodiester backbone at base pair 1, and it is possible that the effect may be due to the dependence on the precise configuration of the backbone on the base sequence.

Based on the results of this mutagenesis study, the strength of the α2 homeodomain−DNA interaction appears to depend upon a DNA sequence that spans nine base pairs. The base identities at positions 4 and 5 appear to have the most significant affect on the affinity, although no single position appears critical *in vivo*. As discussed above, even the strongest operator mutant (27A → T) appears to be efficiently occupied *in vivo* by simply raising the intracellular concentration of α2 by ~10-fold.

Homeodomain proteins in vivo

Finally, we turn to two issues that seem, at least on the surface, surprising. First, a comparison of the five known *asg* operators show a very high conservation of the homeodomain recognition sequences. However, as we have shown here, most deviations from this sequence show only modest biological effects. As alluded to above, it seems likely that the operators have maintained nearly identical sequences to ensure that the genes that they control are very tightly repressed in the appropriate cell types. One requirement for tight repression is a nearly complete occupancy of the operator, estimated in this case to be >99%. Why is such a high degree of repression necessary? The **a**-specific genes, the targets of α2/MCM1 repression, encode proteins required specifically by **a** cells to mate. They include the structural genes for **a**-factor and the structural gene for a receptor on the surface of **a** cells which is activated by binding of α-factor (for reviews, see Herskowitz, 1989; Sprague, 1990; Doland and Fields, 1991). In α and **a**/α cells (where α2/MCM1 repression functions) the **a**-specific genes are not needed; moreover, their inappropriate expression can compromise the ability of α cells to function properly (see for example, Bender and Sprague, 1989). Thus it would seem that a very efficient repression system is necessary for proper functioning of α cells. If α2 activated genes (instead of repressing them) small decreases in operator occupancy would have little consequence (see above) and we would predict that the consensus sequence would be much less striking (see Berg and von Hippel, 1988).

A second issue arises from comparison of an α2 dimer with the bacterial *lac* and the bacteriophage λ and 434 repressors. Although all four proteins are members of the helix−turn−helix superfamily and all bind as dimers to a twofold symmetric DNA operator, the bacterial repressors bind their sequences more tightly than does an α2 dimer and they show a higher ability to discriminate their recognition sequences from other DNA. A manifestation of this second point is the pronounced effects of operator mutations on the binding of the bacterial and phage repressors (see, for examples, Jobe *et al.*, 1974; Betz *et al.*, 1986; Ebright, 1986; Lehming *et al.*, 1987 for effects on *lac* repressor). In contrast, most changes in bases contacted by α2 produce only small defects in the affinity of an α2 dimer for its operator. Other homeodomain proteins seem to show similar effects. For example, Desplan *et al.* (1985) identified many engrailed binding sites on λ DNA where, in similar experiments, Maniatis *et al.* (1973) found only a small number of λ repressor binding sites, those of two operators O_R and O_L. More recently, Affolter *et al.* (1990), Ekker *et al.* (1991), Florence *et al.* (1991) and Percival-Smith *et al.* (1992) showed that the Ultrabithorax and Fushi tarazu homeodomains could efficiently recognize a number of

different sequences. Moreover, Schier and Gehring (1993) have demonstrated that multiple mutations in a high-affinity Fushi tarazu binding site only partially compromise the ability of Fushi tarazu to occupy the site in flies. It seems plausible that homeodomain proteins, have evolved to be able to interact reasonably well with many different DNA sequences, whereas the bacterial and phage repressors are much less forgiving. For the case of α2, we know that the modest binding specificity is at least in part compensated by interactions with other DNA-binding proteins such as MCM1.

Inspection of the available DNA–protein co-crystal structures suggests several possible explanations for the ability of the homeodomains to interact reasonably well with different DNA sequences. First, most of the contacts with DNA (to the bases and especially to the phosphodiester backbone) are made by relatively long side chains. Although some of these side chains appear constrained by a network of side chain–side chain interactions (see Wolberger *et al.*, 1991), others could, at least in principle, reconfigure to adapt to different DNA sequences. In contrast, the bacterial and phage repressors make some of their contacts with DNA through relatively short side chains and include interactions between the polypeptide backbone and the DNA backbone [for reviews, see Pabo and Sauer (1992) and Harrison (1992)]. For example, the 434 repressor dimer, O_L1 complex shows eight hydrogen bonds between peptide NH groups and oxygens of the DNA backbone. This extensive set of backbone contacts strictly positions the 434 repressor on the operator, tucking the helix–turn–helix unit into the major groove. It is possible that in this and related cases, a single base pair change would disrupt several protein–DNA contacts by preventing the close approach of the protein to DNA. For the homeodomains, it is possible that a base pair change could be accommodated by a reconfiguration of the long amino acid side chains. For example, some base pair changes might simply result in a loss of a contact without compromising neighboring contacts. Other base pair changes might be accommodated by a reconfiguration of several amino acid side chains to make an alternative set of contacts with DNA. We view these long side chains of α2 as providing a molecular 'cushion' which would allow the homeodomain to fit against different DNA sequences. A second factor that may contribute to the ability of α2 to accommodate to different sequences is the flexibility of the amino-terminal arm. In solution, this arm appears unstructured (Phillips *et al.*, 1991) and could presumably adopt different conformations on different DNA sequences. Finally, the serine in helix 3 (position 50) is located too far from the major groove to make a direct hydrogen bond with a base (Wolberger *et al.*, 1991); perhaps through water-mediated contacts, the serine side chain (which can serve as a hydrogen bond donor or acceptor) could favorably interact with several different bases. Since many of the structural and biological features of other homeodomain proteins (see Scott *et al.*, 1989; Wolberger *et al.*, 1991; Wüthrich and Gehring, 1993) resemble those of α2, some of these ideas may apply to other homeodomain proteins, as well.

We feel that this tolerance for different DNA sequences may be an important feature of at least some homeodomain proteins. As organisms evolve new regulatory circuits from existing components the ability to rapidly produce new, functional combinations of regulators would seem to be advantageous. The ability of regulators such as the homeodomain proteins to accommodate to different DNA sequences would facilitate the formation of new regulatory combinations. If the circuit was advantageous to the organism, it could slowly be tightened or 'fixed' by evolution of the target DNA sequences to those optimally recognized by the new regulatory combinations.

Materials and methods

Mutagenesis of the asg operator

The oligonucleotide GAGGTCGACATGTAATTACCTAATAGGGAA-ATTTACACGCTCGAGC was synthesized by Bruce Malcolm (University of California, Berkeley) using 90% of the base indicated and 3.3% of each of the other three bases. The 5′ end contains a *SalI* site and the 3′ end an *XhoI* site. The last eight bases of the oligonucleotide were designed as a palindrome to facilitate conversion to a double-stranded form through mutually primed synthesis. Hybridization and filling in yielded a pool of synthetic operator fragments, each one bearing two operators and a central *XhoI* site. Cleavage with *SalI* and *XhoI* gave individual operator duplexes—each with a 5′ *SalI* end and a 3′ *XhoI* end. In addition, a number of specifically altered operators were individually synthesized to complement those obtained from random mutagenesis.

Plasmid and strain constructions

Duplexed operators were cloned into the unique *SalI* site of pAV73 (pLGΔ312S with a *BglII* linker at the *SmaI* site) (see Keleher *et al.*, 1988). Recombinant plasmids were transformed into *E.coli* and identified by single-stranded sequencing with Sequenase. Plasmids bearing single point mutations were prepared from *E.coli*, and transformed into isogenic **a** and α cells. **a** cells are EG123 (*MAT***a** *trp1 leu2 ura3 his4*), and α cells are 246-1 (Tatchell *et al.*, 1981; Siliciano and Tatchell, 1984). Plasmid AJ166, a gift of Kelly Komachi, is CV13 with a 4.3 kb fragment bearing *MATα*. It was utilized to overproduce α2 in the experiment shown in Table I.

β-galactosidase assays

β-Galactosidase assays were performed as described by Miller (1972), except that the yeast cells were permeabilized with 0.05% chloroform and 0.0025% SDS. Cells were grown in glucose medium (2%) as described by Sherman *et al.* (1979). To avoid derepression of the *CYC1* promoter due to glucose deprivation, glucose was added to 2% to each culture 1 h before it was assayed.

Proteins

α2, a gift of Arkady Mak, was overproduced and purified from *E.coli* as described in Sauer *et al.* (1988). The α2 homeodomain fragment (amino acids 136–210), a gift of Andrew Vershon, was overproduced and purified from *E.coli*, as was fragment 1–96 of MCM1 (Vershon and Johnson, 1993).

Mobility shift assays

Gel mobility shift assays were performed in a buffer containing 20 mM Tris, pH 8, 0.1 mM EDTA, 50 mM NaCl, 10 mg/ml BSA (Fraction V), 5% glycerol and 10 ng/ml *E.coli* DNA cut with *HaeIII*. The appropriate end-labeled operator DNA was added (to ~0.5 nM) along with purified protein(s) at the concentrations given in the figure legends. The reactions were mixed and incubated at room temperature for 10 min for the experiments shown in Figure 3 and for 60 min for the experiments shown in Figures 4–7. All gel mobility shift experiments (except where specified below) were electrophoresed through 6%, 1 × TBE, native polyacrylamide gels at 200 V for 2 h. Gels were then dried and autoradiographed.

For Figures 3–7, operators were labeled with ^{32}P for use as probes by cutting with appropriate enzymes and filling in 5′ overhangs (4 bp in all cases) with the Klenow fragment of DNA polymerase I. In Figure 3, the wild-type and mutant operators were isolated from pAV73 (Vershon and Johnson, 1993) as 71 bp *MluI–BstNI* fragments. In Figures 4–6, the wild-type fragment was isolated as in Figure 3, and the mutant operator was isolated from pAV73 as 155 bp *MluI–BglII* fragments. In Figure 7, the wild-type operator was isolated from a pUC19 derivative (see Keleher *et al.*, 1988) as an 86 bp *EcoRI–HindIII* fragment and the mutant operators were prepared from pAV73 as described for Figure 3.

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