A homeo domain protein lacking specific side chains of helix 3 can still bind DNA and direct transcriptional repression

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A series of mutations in the homeo domain of the yeast α2 protein were constructed to test, both in vivo and in vitro, predictions based on the α2–DNA cocrystal structure described by Wolberger et al. [1991]. The effects of the mutations were observed in three different contexts using authentic target DNA sequences: α2 binding alone to specific DNA, α2 binding cooperatively with MCM1 to specific DNA, and α2 binding cooperatively with α1 to specific DNA. As expected, changes in the amino acid residues that contact DNA in the X-ray structure severely compromised the ability of α2 to bind DNA alone and to bind DNA cooperatively with MCM1. In contrast, many of these same mutations, including a triple change that altered all the “recognition” residues of helix 3, had little or no effect on the cooperative binding of α2 and α1 to specific DNA, as determined both in vivo and in vitro. These results show that the ability of a homeo domain protein to correctly select and repress target genes does not necessarily depend on the residues commonly implicated in sequence-specific DNA binding.

[Key Words: Homeo domain proteins; DNA-binding specificity; combinatorial control]

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Based on the X-ray and NMR structures, we constructed a series of point mutations predicted to damage the DNA-binding affinity of α2 and tested the effects of the mutations in vitro and in vivo. Although most of the changes that we examined destroyed the ability of α2 to work alone and in combination with MCM1, to our surprise, most of the mutant α2s still functioned in combination with α1. We conclude that many of the specific changes that we examined destroyed the ability of α2 to X-ray structure are not required for DNA binding of α2 in conjunction with α1.

Results

Point mutations in the homeo domain of α2 reduce the level of α2/MCM1 repression in vivo

As described in Materials and methods, we developed a scheme to construct site-specific mutations in the α2 gene, to introduce these mutations into yeast for in vivo repression analysis, and to introduce the mutations into bacterial expression vectors to provide a source of pure protein for DNA-binding experiments.

The α2 protein is 210 residues in length and contains two stable domains [Sauer et al. 1988]. The homeo domain is part of the carboxy-terminal domain, and NMR and crystallography experiments have shown that this region forms three α-helices that pack into a tight bundle [Phillips et al. 1991; Wolberger et al. 1991]. A sketch of the α2 homeo domain bound to its DNA site is shown in Figure 1. The first helix [residues 138–150 of the intact protein] and the second helix [residues 159–169] pack against each other in a roughly antiparallel fashion, and both of these pack against the third helix, which lies across them. The third helix [residues 173–189], frequently termed the “recognition helix,” lies in the major groove of the DNA and makes three base-specific contacts and a series of sugar-phosphate backbone contacts. An extended region at the beginning of the α2 homeo domain [the amino-terminal arm], which lies in the minor groove of the DNA, appears to make at least one base-specific contact. Immediately following the carboxyl terminus of the homeo domain is a 20-amino-acid tail that does not contact DNA but is required for interaction with α1 [Strathern et al. 1988; Mak and Johnson 1993].

A list of the point mutations that we constructed in the α2 homeo domain and their effects on α2/MCM1-mediated repression are shown in Figure 2. For these experiments, the mutant α2 proteins were expressed from the α2 promoter carried on a CEN plasmid, and their activities were monitored using an asg operator reporter construct that was integrated into the yeast genome. In most cases, the results of mutations in the homeo domain agree well with predictions made from the crystal structure. For example, Arg-173, Gln-175, Lys-186, and Lys-188 all make contacts with the DNA phosphate backbone, and point mutations in any of these residues have a large deleterious effect on the level of α2/MCM1-mediated repression.

Amino acid substitutions in residues that make major groove base-specific contacts in the X-ray crystal structure, Ser-181, Asn-182, and Arg-185, also have large damaging effects on the level of repression. This result confirms that all of these residues play major roles in the recognition of the DNA. The Ser-181–Gln mutation is noteworthy in that this position has been studied extensively in other homeo domain proteins, and, for some cases, it has been proposed to be a major determinant of DNA-binding specificity [Hanes and Brent 1989; Treisman et al. 1989; Schier and Gehring 1993]. In the α2 X-ray structure, this side chain has been modeled to make an extended van der Waals contact with the third position in the major groove of the operator [Wolberger et al. 1991]. The Gln substitution, which inserts a larger, bulkier side chain in place of Ser-181, does not have as large deleterious effect on the level of repression as might be expected if this residue provides a critical determinant of DNA-binding specificity. In the engrailed homeo domain cocrystal structure, the Gln residue at the homologous position contacts the second position of the operator [Kissinger et al. 1990]. A substitution of this residue from Gln to Ala had only a small effect on its DNA-binding activity, suggesting that the Gln at this position may only play a small role in determining the engrailed DNA-binding affinity and specificity [Ades and Sauer 1994]. One explanation for the partial repression by the α2 Ser-181–Gln mutant is that the Gln side chain...
Figure 2. In vivo assays for transcriptional repression by α2/MCM1 and α1/α2. (A) Assay for α-specific gene repression by α2 mutants. Plasmid pAV115, a CEN LEU2 plasmid, containing the entire MATα locus with a specific site-directed mutation in the α2 gene (α2* here), was transformed into a matA strain containing an integrated copy of a derivative of pSJ3, an αsg reporter, at the URA3 locus on chromosome V. α2/MCM1-mediated repression was monitored by measuring the β-galactosidase produced from a CYC1-lacZ promoter that contains an α2/MCM1 operator site. The MCM1 protein was expressed from its endogenous gene on chromosome XIII. Nonrepressed conditions (tester strain transformed with pAV114, a blank control vector) produced an average 150 ± 10 units of β-galactosidase activity. Full repression of the reporter promoter (tester strain transformed with pAV115 containing a wild-type copy of α2), gave an average of 6 ± 1 units of β-galactosidase activity, giving ~22-fold repression of the reporter promoter. (B) Assay for haploid-specific gene repression of α2 mutants. α1/α2-mediated repression by the α2 mutants was measured by a similar strategy using a MATα tester strain that contains an integrated copy of the CYC1-lacZ promoter containing an hsg operator. The α1 protein was expressed from its endogenous gene at the MAT locus on chromosome III. The presence of wild-type α2 in this tester strain repressed lacZ expression from 30 units (pAV114) to 3 units (pAV115 wild-type α2), a 10-fold repression of the promoter construct. (C) The effects of site-directed mutations in α2 on α2/MCM1- and α1/α2-mediated repression. The percent activity of each mutant relative to wild-type levels is shown. The calculations are based on the average of at least three independent transformants of each mutant.

may be repositioned so that it contacts the second position of the operator instead of the third base pair.

The NMR and X-ray homeo domain structures show that the amino-terminal region of the homeo domain wraps partially around the DNA to make contacts in the minor groove (Kissinger et al. 1990; Otting et al. 1990; Wolberger et al. 1991; Billeter et al. 1993; Klemm et al. 1994). In the engrailed structure, Arg side chains at the third and fifth positions of the homeo domain contact thymines at two adjacent base pairs. Residues at the third and fifth positions of other homeo domains are mostly Arg, suggesting that these contacts with the DNA are conserved and are important in binding affinity and specificity. The α2 homeo domain contains two Arg residues at the fourth [Arg-132] and seventh [Arg-135] positions instead of the third and fifth positions as in other homeo domain proteins. In the α2 cocrystal structure, residues Arg-132-Thr-137 lie in the minor groove; but only the Arg-135 side chain is well defined and is modeled to contact two adjacent base pairs (Wolberger et al. 1991). The large deleterious effect of the Arg135-Ala mutation indicates that Arg-135 is important for repression and DNA binding. The other arginine in the amino-terminal arm, Arg-132, appears to have only a minor role. The lack of a second additional DNA contact made by the amino-terminal arm may suggest why the DNA-binding affinity of α2 is intrinsically relatively low and why it requires accessory proteins to bind DNA with high affinity and specificity.

Mutations in side chains that lie close to the DNA but that do not appear to make a direct contact, such as Lys-177 and Asn-178, reduce the level of repression 5- to 10-fold, suggesting that these side chains may also play a role in binding when α2 is bound in complex with MCM1. In the α2 cocrystal structure, Asn-178 lies in the major groove, but it does not appear to make any base-specific or phosphate-backbone contacts. One substitution at this position, Asn-178-Ile, introduces a larger side chain, and it is likely that it interferes with DNA binding. The other mutant, Asn-178-Ala, is harder to
reconcile with the crystal structure because the substitution effectively removes the side chain. It is possible that Asn-178 is involved in contacting the DNA through a water-mediated hydrogen bond (Gehring et al. 1994) and that a substitution of this side chain with alanine would remove this contact (C. Wolberger, pers. comm.).

Mutations in other solvent-exposed residues that are distal from the DNA, such as residues Asn-164 and Asn-167, which lie on the back side of the homeo domain in helix 2, have little or no effect on the level of repression and serve as convenient controls. Altogether, these results show that a2 side chains that make contacts with the DNA in the cocrystal structure are required for efficient repression with MCM1 in vivo.

Point mutations in the homeo domain of α2 do not reduce the level of α1/α2 repression in vivo

The effect of the α2 mutations on α1/α2-mediated repression was tested in a similar way using an integrated hsg-operator reporter, with α1 being supplied from a chromosomal copy of MATA (Fig. 2B). We found that most of the α2 mutants that completely destroy α2/MCM1-mediated repression (above) have little or no effect on α1/α2-mediated repression. Even amino acid substitutions with large damaging effects on α2/MCM1-mediated repression and predicted by the crystal structure to affect key protein-DNA contacts (e.g., Lys-188–Ile or Arg-185–Met) function at nearly wild-type levels with α1.

As a control for this experiment, we constructed a point mutation in α2 [Leu-196→Ser] that was shown previously to abolish α1/α2-mediated repression of haploid-specific genes but not α2/MCM1-mediated repression of the α-specific genes (Strathern et al. 1988). Residue Leu-196 lies in the carboxy-terminal tail region adjacent to the homeo domain, and deletion analysis of the α2 protein has established that this tail region is important for cooperative DNA binding between α1 and α2 (Mak and Johnson 1993). In accordance with the previous analysis of this mutant, it retains the ability to repress transcription with MCM1 at roughly wild-type levels but fails to repress transcription with the α1 protein (Fig. 2). This result serves as a control and indicates that the observed repression of the α1/α2 operator site by the α2 mutants that we constructed is not the result of a failure of our assays to detect a loss of repression.

One possible interpretation of these results is that the levels of α1 and α2 proteins in the cell are sufficiently high to enable them to bind to the α1/α2 site, even though the protein–DNA interactions have been substantially weakened. Mutations in a single side chain that contacts the DNA might therefore have a small but undetectable effect on the level of repression. To test this model, a triple mutant—Ser-181-Ala/Asn-182-Ala/Arg-185-Ala, referred to as H3-3A—that removes all three side chains that make base-specific contacts in the major groove of the DNA was constructed. As expected, this mutant was completely defective in repressing transcription with MCM1, however, it still showed significant levels of repression with α1 (Fig. 2C). We conclude from these results that when in a complex with α1, these residues are not crucial for repression in the cell.

Integration of α2 mutants into the chromosomal MAT locus

In the experiments described above, the α2 protein was expressed from its endogenous promoter on a low copy CEN plasmid. To rule out complications resulting from plasmid loss or overexpression, we constructed stable chromosomal copies of the α2 mutations at the MAT locus (see Materials and methods). α Cells carrying the mutant α2 [mata2 cells] were tested for repression of an asg reporter, and MATA/mata2 cells were tested for repression of an hsg reporter. The results are shown in Figure 3 and, in general, agree with those utilizing CEN plasmids. A couple of the mutants, such as Ser-181–Ile, and Arg-185–Met, show a larger effect on α1/α2-mediated repression than in the experiments utilizing CEN plasmids, and the amino-terminal arm mutant [Arg-135–Ala] also appears to partially affect α1/α2-mediated repression. However, the most striking result is still exemplified by the phenotype of the triple mutant H3-3A: complete disruption of α2/MCM1-mediated repression but wild-type levels of α1/α2-mediated repression.

Mutations in the α2 homeo domain destroy repression of endogenous α-specific genes but not endogenous haploid-specific genes

Although it seemed unlikely, it was formally possible that the reporters used in the experiments described above misrepresent the behavior of authentic α-specific and haploid-specific genes. To rule out this possibility, we tested the effects of the mutations on the repression of endogenous α- and haploid-specific genes using several bioassays. In general, these tests agree well with those utilizing the reporter constructs; a brief description of them follows (see also Materials and methods).

α Strains, each bearing a different mutant α2 integrated at the MAT locus [i.e., mata2 strains], were assayed for their mating type (by testing their ability to mate with either MATA or MATA tester strains) and for their production of α- and α-mating factor (by the formation of halos on appropriate tester strains that are growth sensitive to mating factor) (Sprague 1991). In α cells, a strong mutation in the homeo domain, such as the triple mutant H3-3A, exhibits the same phenotype as a deletion of the α2 gene (Table 1). This mutant fails to repress α-specific genes, such as STE6 and BAR1, which allows the production of α-factor and the degradation of α-factor, respectively. Thus, a mata2 strain carrying the H3-3A allele exhibits an α halo, whereas a wild-type α strain exhibits an α halo. This strain also fails to mate with either α or α cells, a phenotype characteristic of a mata2 deletion strain, because both α-specific and α-specific genes are expressed (Strathern et al. 1981).

To test the effects of the α2 mutations on α1/α2-mediated repression of haploid-specific genes, we examined
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MATα/mata2 strains for their production of α- or α-factor and for their sporulation efficiency (Table 2). In these strains the a2 mutations that we constructed show two types of halo phenotypes that agree well with the results from the reporter constructs. One set of mutants, H3-3A, Ser-181–Gln, and Lys-188–Ile, produce small α halos on the tester strain. The presence of an α halo shows that these mutants fail to repress transcription of the a-specific genes [see above]. The small halos indicate that only a low level of the α-factor is being expressed. The most plausible explanation for the decrease in mating-factor expression is that these a2 mutants are working in combination with the a1 protein to repress transcription of the haploid-specific gene STE12, which is required for full expression of the a-specific genes (Fields and Herskowitz 1985). This result agrees well with the results of the hsg reporters that show full repression in strains carrying these a2 mutations. The other set of mutants, exemplified by Ser-181–Ile, Arg-185–Met and Arg-135–Ala, produce a large α halo on the tester strains. All of these mutants show slightly reduced levels of repression of the hsg reporter; therefore, the most likely explanation for the larger halos is that these strains not only fail to repress a-specific genes but also allow sufficient expression of STE12 for a-specific gene activation.

We also examined the ability of the MATa/mata2 mutant strains to sporulate. We found that strains containing the wild-type a2, H3-3A, Ser-181–Gln, or Lys-188–Ile mutants are able to sporulate efficiently, showing that a1/a2-mediated repression is functional in all of these strains. However, strains that show a slight reduction in their ability to repress the hsg reporter fail to sporulate. The most likely explanation for this phenotype is that these mutants fail to completely repress the haploid-specific RME1 gene, a repressor of the meiotic pathway (Mitchell and Herskowitz 1986).

In conclusion, the tests for mating, mating pheromone production, and sporulation are in excellent agreement with the assays utilizing reporter constructs and provide an independent confirmation of the results.

α2 homeo domain mutants fail to bind DNA on their own in vitro but still bind cooperatively with a1

To examine directly the effects of the α2 point substitutions on DNA binding, the mutant proteins were expressed in Escherichia coli, purified to >90% homogeneity, and assayed by electrophoretic mobility shift assays (Kelche et al. 1988; Sauer et al. 1988; Goutte and Johnson 1993). Wild-type intact α2 protein was compared with the same concentration of H3-3A, Arg-135–Ala, Lys-188–Ile, and Leu-196–Ser mutant proteins in binding to the hsg operator on their own and cooperatively with MCM1 (Fig. 4A,B). The DNA-binding affinity of the H3-3A mutant protein appears to be at least 100-fold lower than wild-type protein, a result expected because the mutant protein lacks all of the base-specific contacts that the homeo domain makes in the major groove of the DNA. Single amino acid substitutions in these three positions also significantly reduce the level...
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Table 1. Mating-type and mating-factor production in α2 mutant strains

| Strain   | Genotype          | Repression of hsg reporter (%) | Halo* | Mating with MATa strain | Mating with MATα strain |
|----------|-------------------|-------------------------------|-------|------------------------|------------------------|
| 246.1.1  | MATa              | 100                           | −     | +                      | +                      |
| EG123    | MATa              | 0                             | +     | −                      | −                      |
| 23a182   | Δα mata2          | 0                             | +     | −                      | −                      |
| YJ08     | wild type         | 100                           | −     | +                      | +                      |
| YJ05     | R135A             | 0                             | +     | −                      | −                      |
| YJ03     | S181I             | 1                             | +     | −                      | −                      |
| YJ07     | S181Q             | 2                             | +     | −                      | −                      |
| YJ02     | R185M             | 0                             | +     | −                      | −                      |
| YJ06     | K188I             | 2                             | +     | −                      | −                      |
| YJ01     | H3-3A             | 0                             | +     | −                      | −                      |
| YJ04     | L196S             | 100                           | −     | +                      | +                      |

* (+) The strain forms a halo on the appropriate pheromone-sensitive tester strain.
* (+/-) A halo is formed on the tester strain, but it is significantly smaller than a normal halo.

of binding to the site on their own (data not shown). The affinity of Arg-135-Ala appears to be reduced by ~50-fold; this mutation removes the base-specific contact that the amino-terminal arm of the homeo domain makes in the minor groove of the DNA. The affinity of Lys-188-Ile, a mutant in a side chain that makes phosphate-backbone contact in the cocrystal structure, is also reduced by ~50-fold. These results indicate that the reduced level of repression in vivo is the result of the failure of α2 to bind to the operator site with wild-type affinity.

The wild-type and mutant α2 proteins were also tested for cooperative binding with α1 to the hsg operator (Fig. 4C). The binding of the H3-3A, Arg-135-Ala, and Lys-188-Ile mutant proteins are not detectably reduced from that of the wild-type protein. As a control, Leu-196-Ser is reduced at least 200-fold in binding to the hsg operator with α1. Thus, the results obtained in vitro are consistent with our in vivo observations and confirm that the "base-specific" contacts between α2 and DNA are not crucial for the ability of α2 to act in combination with α1.

Table 2. Mating-factor production and sporulation efficiency in diploid α2 mutant strains

| Strain     | Genotype          | Repression of hsg reporter (%) | Halo* | Sporulation efficiency (%) |
|------------|-------------------|-------------------------------|-------|---------------------------|
| EG123 × 246.1.1 | a/a              | 100                           | −     | +                         | 38 |
| EG123 × 23a182  | a/Δα mata2       | 3                             | +     | −                         | <0.1 |
| Y18        | a/WT              | 100                           | −     | −                         | 39 |
| Y15        | a/R135A           | 35                            | +     | −                         | <0.1 |
| Y13        | a/S181I           | 20                            | +     | −                         | <0.1 |
| Y17        | a/S181Q           | 100                           | +/-   | −                         | 11 |
| Y12        | a/R185M           | 45                            | +     | −                         | <0.1 |
| Y16        | a/K188I           | 100                            | +/-   | −                         | 16 |
| Y11        | a/H3-3A           | 100                            | +/-   | −                         | 20 |
| Y14        | a/L196S           | 2                             | −     | +                         | <0.1 |

* (+) That particular strain forms a halo on the appropriate pheromone-sensitive tester strain; (+/-) A halo is formed on the tester strain, but it is significantly smaller than a normal halo.

Discussion

The homeo domain protein α2, a transcriptional repressor in yeast, acts in combination with two additional proteins: With MCM1, α2 turns off the a-specific genes; with α1, α2 turns off the haploid-specific genes. The former combination occurs in α and α/α cells, and the latter occurs in the a/α cell only.

The X-ray structure of the α2 homeo domain complexed with a variant of the a-specific gene operator has been described (Wolberger et al. 1991), and we constructed a series of point mutations in the α2 homeo domain designed to impair base-specific and phosphate-backbone contacts without destabilizing the homeo domain structure. The analysis of these mutations leads to a surprising observation: Although nearly all of the mutations impaired the specific DNA binding of the α2 homeo domain on its own and impaired the combined action of α2 and MCM1, many of these same mutations had little or no effect on the cooperative binding of α2 and α1 to DNA. Moreover, many of these mutant α2s worked as efficiently in vivo as did wild-type α2 in re-
Figure 4. Mutations in the α2 homeo domain have reduced binding affinity to the asg operator with and without MCM1 but have nearly wild-type binding affinity to the hsg operator in combination with α1. (A) Mobility retardation assay of the asg operator produced by decreasing concentrations of wild-type α2 (lanes 2–6), L196S (lanes 7–11), K188I (lanes 12–16), H3-3A (lanes 17–21), and R135A (lanes 22–26) mutant proteins in the absence of MCM1. The concentrations of α2 proteins are varied by fivefold dilutions from 4 × 10^{-7} M (lanes 2, 7, 12, 17, 22) to 6.4 × 10^{-10} M (lanes 6, 11, 16, 21, 26). Free DNA is shown in lane 1. (B) Mobility retardation assay to the asg operator produced by decreasing concentrations of wild-type α2 (lanes 3–7), L196S (lanes 8–12), K188I (lanes 13–17), H3-3A (lanes 18–22), and R135A (lanes 23–27) mutant proteins in the presence of a constant amount of MCM1 are shown. The concentrations of α2 proteins are varied by fivefold dilutions from 1.6 × 10^{-8} M (lanes 3, 8, 13, 18, 23) to 2.5 × 10^{-11} M (lanes 7, 12, 17, 22, 27). Note that lane 4 in A has the same concentration as lane 3 in B. Lane 2 shows the shift produced by MCM1 in the absence of α2, and lane 1 shows the free DNA. (C) Mobility retardation assay to the hsg operator produced by decreasing concentrations of wild-type α2 (lanes 2–6), L196S (lanes 7–11), K188I (lanes 12–16), H3-3A (lanes 17–21), and R135A (lanes 22–26) α2 protein in the presence of a constant amount of α1. The concentrations of α2 proteins are varied by fivefold dilutions from 4 × 10^{-7} M (lanes 2, 7, 12, 17, 22) to 6.4 × 10^{-10} M (lanes 6, 11, 16, 21, 26). The shifted band at the highest concentration of L196S (lane 7) is slightly higher than a normal α1/α2 shift and migrates at a position observed by a dimer of α2 binding alone to the site.

pressing transcription of the haploid genes in combination with α1. Thus, when taken in combination with MCM1, the homeo domain mutations had the predicted effect; when taken with α1, the mutations were largely silent. How can this “split phenotype” be explained? We consider four possibilities.

In theory, the simplest explanation would be that the α2 homeo domain does not contact DNA in the α1/α2-DNA complex. However, an extensive set of protein-DNA cross-linking experiments and chemical protection experiments has shown that the α2 homeo domain does contact DNA when bound with α1 (Goutte and Johnson 1993, 1994). Moreover, genetic and biochemical experiments have shown that the homeo domain of α2 is required for the DNA-binding activity of α1/α2 (Porter and Smith 1986; Hall and Johnson 1987; Goutte and Johnson 1988).

A second possible explanation for the differential effects of the mutations is that α2 may contact DNA in two distinct ways: one in the α2/DNA and α2/MCM1/DNA complexes and the other in the α1/α2/DNA complex. According to this explanation, the point mutations would compromise the former but not the latter mode of DNA binding. Although it is difficult to rule out small differences in orientation, several lines of experiments suggest that α2 contacts DNA in roughly the same way in the three different complexes. First, chemical protection experiments show that the homeo domain of α2 makes a similar set of DNA contacts whether or not it is complexed with α1 (Goutte and Johnson 1994). Second, if the orientation of the α2 homeo domain was dramatically shifted in the α1/α2 complex, one would expect other surface-exposed residues of the homeo domain to contact DNA. However, we have shown that mutations of residues on the surface of helix 2 or the beginning of helix 3 had no effect on α1/α2 activity [Fig. 2C]. Thus, it seems unlikely that α2 has two drastically different ways of contacting DNA.
A third possibility is based on a model where α1 and α2 make equal contributions to the DNA-binding affinity and specificity of the heterodimer. According to this model, only half of the heterodimer-DNA contacts would be compromised by the α2 mutations, because α1 is still wild type. In contrast, because α2 binds as a dimer with MCM1, the α2 mutations would be expected to have a much more deleterious effect (roughly the square of the heterodimer affinity decrease). Although this argument must be true in principle, we feel that it cannot adequately explain the wild-type phenotype of the α2 mutants taken in combination with α1. The binding of a dimeric protein (whether from prokaryotes or eukaryotes) to DNA is typically disrupted by the elimination of one set of monomer-DNA contacts. This applies to the binding of an α2 dimer with MCM1: The removal of one set of specific α2-DNA contacts (by mutation of one-half of the operator) severely disrupts α2/MCM1 repression in vivo (Smith and Johnson 1994). Thus, it seems unlikely that an α1/α2 heterodimer could accommodate a loss of half (or nearly half) of its specific DNA contacts and still function in the cell.

A fourth possibility, and the one we favor, is that α1 provides the majority of the DNA-binding specificity for the heterodimer and that the constraints on α2-DNA recognition are therefore loosened. Two additional observations are consistent with this idea: [1] When the known hsg operators are compared, the α1 halves of the site are conserved much more than are the α2 halves [Miller et al. 1985; Goutte and Johnson 1994]. [2] Under a specific set of biochemical conditions, the specific/nonspecific DNA-binding ratio is ~30 for the α2 dimer but ~30,000-fold for the α1/α2 heterodimer, again suggesting that α1 provides a large fraction of the specificity. Because under the same conditions α1 does not bind DNA alone, we were unable to directly determine the α1 specificity ratio. Therefore, we propose that in the α1/α2 DNA complex, α1 is providing the majority of the specific DNA contacts and the α2/DNA interface can tolerate changes in the residues that contact DNA without significantly compromising the ability of the heterodimer to recognize specific DNA in the cell. According to this model, the main role of α2 in the heterodimer would be to provide protein-protein contacts that convert α1 from a weak to a tight binding form.

Regardless of their precise molecular explanation, the results in this paper show that in one context (acting with MCM1) the α2 homeo domain is extremely sensitive to mutations that change residues responsible for sequence-specific binding. In another context (with α1), the α2 homeo domain appears buffered, that is, relatively insensitive to these same changes. Other studies of homeo domain proteins have also revealed, in some in vivo contexts, a relative insensitivity to mutations in the DNA-binding residues. Porter and Smith [1986] analyzed in vivo a series of point mutations in the α2 homeo domain. These studies were carried out before structural information for homeo domains was available, and, viewed in retrospect, many of these changes probably destabilized the homeo domain structure and thereby inactivated both α2/MCM1 and α1/α2 repression in vivo. However, several mutations (Asn-178-His, Lys-186-Asn, and Lys-188-Ile) showed a selective reduction in α2/MCM1 repression, a result that anticipated some of the results presented in this paper. Another of their mutations, Asn-182-Asp, compromised α2/MCM1 function as well as α1/α2 function in vivo, whereas a change of Asn-182 to Ala [Fig. 1C] inactivated only α2/MCM1 function. In the crystal structure, the Asn-182 side chain contacts another side chain in helix 3 as well as the DNA, and there are several possible reasons for the different phenotypes of these two mutations. Because the Asn-182-Asp protein is deficient in all functions of α2, it is possible that it is expressed poorly or is less stable than the wild type. A second possibility is that the change from Asn to Asp introduces an unfavorable negative charge into the DNA-protein interface that simply prevents the close approach of DNA and protein.

Experiments with Drosophila homeo domains have also revealed instances where mutations in DNA-binding residues do not necessarily eliminate biological functions. Schier and Gehring [1993] showed that a ftz to bcd altered specificity mutant, created by a Gln-50-Lys change in ftz, provides efficient rescue of parasegments 8 and 14 but poorer rescue of other structures. Other mutations that compromise the strength of the ftz–DNA interaction show similar effects [Furukubo-Tokunaga et al. 1992].

These observations have several implications for the general understanding of homeo domain proteins. First, the study of homeo domain–DNA interactions in isolation, although of undeniable importance, may not necessarily reflect how these proteins act in vivo. For example, there appears to be little correlation between the behavior of many of the mutant α2 proteins in binding DNA alone when compared with binding DNA with α1. In particular, the alteration of residues that, as seen to the X-ray structure, make specific contacts with DNA (Arg-135, Ser-181, Asn-182, and Arg-185) drastically reduces specific DNA binding by α2 alone but has little or no effect on the cooperative binding of α2 and α1 to DNA.

A second implication of these observations relates to the way homeo domain proteins may have evolved to participate in more than one regulatory circuit. It would seem that the specific DNA-binding residues of α2 are tailored for binding to asg operator with MCM1. On the basis of homologies in other fungi, it has been proposed that the α1/α2 circuit is of more ancient origin than the α2/MCM1 circuit [Johnson 1992]. These ideas suggest a model where the α1/α2 circuit could be maintained while allowing the α2 homeo domain to undergo changes in DNA-binding specificity. That is, features of the α1/α2 circuit “free up” the DNA-binding residues of the α2 homeo domain for testing out and perhaps adopting new circuits without compromising the existing circuit. As many homeo domain proteins from other organisms are known to participate in multiple circuits, perhaps situations where homeo domains are buffered against mutational changes will be common.
Materials and methods

Plasmids

Mutations in the α2 gene were constructed by site-directed oligonucleotide mutagenesis using the dUTP, ung system (Smith 1985) in plasmid pAV99, a pUC19-α2 vector with a single-stranded bacteriophage 1 origin of replication (Mak and Johnson 1993). Oligonucleotides, used in the mutagenesis or construction of DNA-binding sites, were synthesized at the Biotechnology Resource Center at University of California San Francisco (UCSF) or on an ABI 392 synthesizer at the Waksman Institute, Rutgers University. Isolates of pAV115, a CEN LEU2 yeast plasmid with a 4.3-kb HindIII fragment that contains the entire MATα locus and the specific site-directed mutations in the α2 gene, were constructed by cloning the 0.6-kb BglII-BamHI fragment from pAV99 containing the different mutations in the α2 gene. The construction of plasmids pAV99, pAV114 [a version of pAV115 without the MATα fragment], and pAV115 was described in Mak and Johnson (1993). Plasmids pAV71 and pAV73, reporter vectors with and without the STE6 α2/MCM1 DNA-binding site controlling expression of aCYC1-lacZ fusion, have been described elsewhere (Vershon et al. 1992). Plasmid pV1103, which was used as a reporter plasmid for α1α2-mediated repression, was constructed by inserting an oligonucleotide that contains a consensus α1/α2 binding site [CATACTATTATATCA] into the SalI site in the CYC1-lacZ fusion promoter of pAV73.

Yeast strains

Most of the strains used in this study are congenic with strain EG123 (tpi1 leu2 ura3 his4) and only vary at the MAT locus (Siliciano and Tatchell 1984). Wild-type MATα and MATα strains are EG123 and 246.1.1, and the wild-type MATα/MATα diploid used in these experiments is a product of a mating between these two strains. mata and mata2 cells are strains KT23a8 and 23a182 [Tatchell et al. 1981] and were used as controls in the CYC1-lacZ repression and biological assays. The control MATα/mata2 strain is from a mating of strains EG123×23a182. The construction of reporter strains containing integrated copies of CYC1-lacZ fusions with α2/MCM1 or α1/α2 DNA-binding sites by K. Komachi has been outlined in Mak and Johnson (1993).

Strain YV50 was constructed by integration at the MAT locus of a 4.3-kb HindIII MATα1, mata2::URA3 fragment in which the entire α2 gene has been replaced with URA3. This strain was used to integrate the α2 mutants into the MAT locus by the following procedure: Isolates of pAV115, containing different site-directed mutations in the α2 gene, were digested with HindIII, and the linear 4.3-kb MATα fragment was transformed into strain YV50 by the lithium acetate procedure (Ito et al. 1983). The transformed strains were grown on YEPD plates overnight at 30°C and then replated on SD+5-fluoroorotic acid (5-FOA) plates to confirm the loss of the α2 mutation in the resulting strains was verified by Southern blot for the presence of the α2 BamHI-BglII fragment and the MATα allele. Strains Y118 [wild-type MATα/MATα], Y111 [MATα/mata2 H3-3A], Y112 [MATα/mata2 Arg-185–Met], Y113 [MATα/mata2 Ser-181–Ile], Y114 [MATα/mata2 Leu-196–Ser], Y115 [MATα/mata2 Arg-135–Ala], Y116 [MATα/mata2 Lys-188–Ile], and Y117 [MATα/mata2 Ser-181–Gln] were constructed by the above procedure.

Protein purification

Full-length wild-type α2 [residues 1–210] was purified from strain SY903.1 containing plasmid pDS-1, a pUC19-α2 expression vector, by a modified version of the procedure in Sauer et al. (1988) as described in Mak and Johnson (1993). Mutant α2 proteins were purified by the same procedure from transformants of strain SY903.1 with pAV100 containing mutations in the α2 gene. Plasmid pAV100, a pUC19-α2 vector, was constructed by removing a 1.4-kb BamHI fragment from pAV99 that is 200 bp past the termination codon of the α2 gene. We have observed that removal of this fragment allows greater expression of the α2 protein. Purified mutant and wild-type α2 proteins were dialyzed into S + 500 buffer [50 mM Tris-HCl at pH 8.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 500 mM NaCl]. The concentrations of the purified proteins were determined by measuring the absorbance at 280 nm and using the theoretical extinction coefficient of OD 0.60 per mg/ml for full-length α2. The relative purities and concentrations of the different proteins were verified by Coomassie staining of SDS gels of the samples. α1 Protein was purified from bacteria transformed with a P77-α1 expression vector and was a gift from C. Goutte (UCSF, CA). The MCM1−α7 protein used in the DNA binding experiments was a fragment of the intact protein (residues 1–97) that contains the DNA-binding domain and was purified from bacteria that contain pAV154 as described in Vershon and Johnson (1993).

DNA-binding experiments

The labeled wild-type α2/MCM1 [from the STE6 promoter] and α1/α2 [from the MFA1 promoter] sites used in the gel mobility shift experiments were generated by isolating the 86- and 73-bp fragments from an EcoRI and HindIII restriction digest of pCK1 and pCG25, respectively [Goutte and Johnson 1988; Keleher et al. 1988], and filling the 5' overhangs with Klenow polymerase. Mobility retardation assays were performed in 20 mM Tris (pH 8.0), 1 mM EDTA, 5 mM MgCl2, 10 mM/m of BSA (fraction V), 5% glycerol, 0.1% NP-40, and 10 μg/ml of sheared salmon sperm DNA. Protein dilutions were made in 50 mM Tris (pH 8.0), 1 mM EDTA, 500 mM NaCl, 10 mM 2-mercaptoethanol, and 10 mg/ml of BSA. Five microliters of α2 and 5 μl of MCM1, α1, or protein dilution buffer were added to 40 μl of end-labeled operator fragment diluted in assay buffer, so that the final NaCl concentration was 100 mM. Reactions were incubated at room temperature for at least 1.5 hr and one-half of the reaction was loaded onto 6% polyacrylamide gel and electrophoresed at 200
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V for 2 hr. Gels were dried and scanned on a Molecular Dynamics PhosphorImager.

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