Cooperative DNA Binding and Sequence Discrimination by the Opaque2 bZIP Factor

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The maize Opaque2 (O2) protein is a basic leucine zipper transcription factor that controls the expression of distinct classes of endosperm genes through the recognition of different cis-acting elements in their promoters. The O2 target region in the promoter of the α-coxin gene was analyzed in detail and shown to comprise two closely adjacent binding sites, named O2u and O2d, which are related in sequence to the GCN4 binding site. Quantitative DNase footprint analysis indicated that O2 binding to α-coxin target sites is best described by a cooperative model. Transient expression assays showed that the two adjacent sites act synergistically. This synergy is mediated in part by cooperative DNA binding. In tobacco protoplasts, O2 binding at the O2u site is more important for enhancer activity than is binding at the O2d site, suggesting that the architecture of the O2-DNA complex is important for interaction with the transcriptional machinery.

INTRODUCTION

The major class of seed storage proteins in maize, Coix, and sorghum is a group of prolamin proteins called zeins, coxins, and kafirins, respectively. Based on their solubility, prolamin proteins can be grouped into four classes, α, β, γ, and δ, that can be separated further into polypeptides of distinct molecular mass by using SDS-PAGE (Esen, 1986; DeRose et al., 1989; Leite et al., 1990; Ottoboni et al., 1990).

Several mutations are known to affect the level of zeins in the maize endosperm (Motto et al., 1989). One of these mutants, opaque2 (o2), affects a DNA binding protein belonging to the basic leucine zipper (bZIP) class of transcriptional activators (Hartings et al., 1989; Schmidt et al., 1990). Coix and sorghum contain homologous O2 genes (Ottoboni et al., 1993; Pirovano et al., 1994; Vettore et al., 1998), indicating that this regulatory locus is conserved among these species.

O2 has been shown to activate the transcription of several maize and Coix endosperm genes through binding to significantly different cis-acting elements located in their promoters (Lohmer et al., 1991; Schmidt et al., 1992; Yunes et al., 1994b; Cord Neto et al., 1995; Maddaloni et al., 1996; Muth et al., 1996). Functional O2 binding sites have also been found in the seed-specific genes of pea lectin (de Pater et al., 1994), wheat prolamin (Holdsworth et al., 1995), and French bean phytohemagglutinin (Chern et al., 1996).

No consistent consensus sequence has been observed when O2 target sites are aligned. Although the mechanism whereby a protein recognizes such diverse sequences is not yet understood, it is possible to infer that specificity for the O2-DNA interaction has not been maximized during evolution.

In a previous report, we showed that the O2 binding sequence in the promoter of the 25-kD α-coxin gene spans ~30 nucleotides and that the palindrome GACATGTC was the most important determinant of effective O2 binding (Yunes et al., 1994b). However, the large size of this 30-nucleotide sequence led us to question the existence of an adjacent O2 binding site. Indeed, based on sequence similarity, we identified a sequence (TGACTA) at the 5′ side of the protected site that resembles the GCN4 binding site (Hill et al., 1986). The relevance of this GCN4-like motif was investigated using site-directed mutagenesis. A single mutation converting the TGACTA motif to TAACTA was sufficient to abolish O2 binding (Yunes et al., 1994a).

In plants, numerous examples exist in which gene regulation is mediated by DNA target sites bearing two neighboring DNA motifs that are related in sequence. The characterization of putative discriminator factors showed that for some of these targets, the same protein is able to bind both motifs (Tokuhisa et al., 1990; Fromm et al., 1991; Foley et al., 1993; Zhang et al., 1993; Feldbrügge et al., 1994; Feltkamp et al., 1994; Lam and Lam, 1995; Chern et al., 1996; Lu et al., 1996).

In this study, we show that O2 binding at the α-coxin promoter requires the previously identified palindrome (Yunes et al., 1994b) and an adjacent motif; both are related in sequence to the GCN4 binding site. Using gel mobility shift assays and footprint titration experiments, we demonstrate...
that the binding of O2 to the α-coixin promoter occurs via cooperative binding of two O2 dimers to the two adjacent sites. Analysis of the wild-type and mutant sites in transient expression assays provided interesting insights into the overall architecture of the functional nucleoprotein complex.

**RESULTS**

**Two O2 Homodimers Simultaneously Bind to the α-Coixin O2 Target Region**

The large size of the O2-footprinted sequence in the α-coixin α-3B promoter and the presence of putative bZIP target sequences in each half of this region (Yunes et al., 1994a, 1994b) led us to ask whether the O2 protein binds only to a single half of this region or simultaneously to both halves.

To address this question, an N-terminal deleted Coix O2-homologous protein containing 63 residues of the N-terminal region, the bZIP domain, and the C-terminal extension was expressed in Escherichia coli and partially purified by heat treatment according to Iwasa et al. (1993). Binding of this O2 protein to an oligonucleotide probe, oligo-Cx, containing the α-coixin O2-footprinted region plus five flanking nucleotides and a restriction enzyme site at each end (Figure 1A), was studied using standard electrophoretic mobility shift assays. As shown in Figure 1B, incubation of oligo-Cx with an increasing amount of O2 protein resulted in two retarded bands. At higher concentrations of protein, the lower complex shifted to the upper complex. The simplest interpretation of this result is that the lower retarded band results from the binding of one O2 homodimer to the oligonucleotide, whereas the upper band resulted from binding of a second O2 homodimer. At the highest concentration of protein, a fraction of the lower complex still remained. This probably reflected dissociation of the upper ligand complex during electrophoresis or while loading the samples onto the gel (Cann, 1989).

The complexes were stable in the presence of 2 ng of salmon sperm DNA per μL that was included in the binding reaction as a nonspecific competitor. In addition, formation of the two complexes was sequence specific because binding was clearly inhibited by the addition of unlabeled oligo-Cx (Figure 1C, lanes 3 to 8) but not by a 50-fold molar excess of the 63-mer Oct-1 oligonucleotide (Pharmacia band-shift kit) (Figure 1C, lane 10). No binding was detected when the same assays were performed using a protein extract from a control E. coli strain carrying the pET vector without the O2 coding sequence (data not shown).

To confirm this interpretation, we studied binding of the O2 protein by using several oligonucleotides (Figure 2A), which were identical to oligo-Cx, except for specific base substitutions in the downstream site, upstream site, or in both. When we used mutant oligonucleotides with at least one intact putative binding site, the mobility shift assay resulted in only a single retarded complex (Figure 2B, lanes 2 to 7). This was observed at an O2 protein concentration that, for the wild-type oligonucleotide (oligo-Cx), was sufficient to shift almost all of the probe to the second retarded complex (cf. Figure 2B, lanes 3 and 7, with Figure 1B, lane 10). O2 protein binding to the downstream site appeared to be stronger than that to the upstream site. As expected, the O2 protein was unable to bind to the double-site mutant oligonucleotide (Figure 2B, lanes 8 to 10).

These data are consistent with the suggestion that the O2-footprinted region in the α-coixin promoter indeed covers two adjacent binding sites at which two O2 homodimers can bind simultaneously.

**O2 Binds Cooperatively to Two Adjacent GCN4-like Sites**

The upstream and downstream sites were named O2u and O2d, respectively. As shown in Figure 3A, both sites possess homologous GCN4-like binding motifs. By dividing the footprinted region in the middle and considering the 5’-TGAC-3’ GCN4-like motif as a half site for an O2 dimer, the boundaries of each of the sites basically could be determined (Figure 3B). The O2d site includes the palindromic sequence GACATGTC previously shown to be the most important requirement for effective O2 binding (Yunes et al., 1994b). The complementary strand of O2d covers the sequence GATGACATGT, which is very similar to a GCN4 binding site (Arndt and Fink, 1986; Hill et al., 1986) and is identical to the consensus O2 binding sequence described for the b-32 gene (Lohmer et al., 1991). The O2u site shares a lesser degree of similarity with known O2 binding sequences.

A series of mutants generated for the two sites (Figure 3C) was analyzed for the mutants’ ability to bind the Coix O2 protein. In the m1 mutant, the TGAC motif of the O2u site was modified to TAAC. In the m2 mutation, the palindromic sequence GATGACATGT was disrupted, but the b-32-type O2 target site of the complementary strand, which matches as the consensus GATGACPuTGpu (Lohmer et al., 1991), was preserved. Note that the m2 mutation results in a C residue being replaced by a T residue in the complementary strand, thus preserving the consensus pyrimidine (Py). This situation was reversed in the m4 mutant, in which the palindrome was preserved but the b-32 complementary motif was disrupted. The m3 mutant was a combination of the m1 and m2 mutations.

An integral Coix O2 protein was expressed in E. coli, partially purified by heat treatment, and used in the footprint assays of Figures 4 to 6. The footprinted region obtained with the Coix O2 protein (Figure 4) was identical to that previously reported for the maize O2 protein (Yunes et al., 1994b).

All of the mutations decreased the binding efficiency, because a higher concentration of O2 protein was necessary...
to obtain complete DNase I protection (Figure 4). The m2 mutation was far less detrimental than was m4, indicating that the most important motif of O2d site was the GCN4-like sequence rather than the GACATGTC palindrome. Similarly, the decreased binding efficiency observed with the m1 mutant indicated that the GCN4-like motif in the O2u site was also required for accurate binding, as was previously reported (Yunes et al., 1994a).

In the normal promoter, the O2u and O2d sites were uniformly occupied at increasing amounts of protein. In contrast, in the mutant templates, the fractional protection of the mutated site at each of the protein concentrations was less than that observed for the adjacent nonmutated site. This was quite evident with m1 (Figure 4) but was also confirmed for the other mutants by densitometry of the footprint autoradiographs (data not shown).

Complete protection of the nonmutated sites in the mutant templates required higher O2 concentrations than were required for the wild-type promoter. Because the mutation of one or two nucleotides at a given site is unlikely to affect intrinsic binding to the adjacent site, the above results are a good indication of a cooperative interaction in which the occupancy of one site facilitates that of another and a mutation at one site decreases the overall binding affinity. This would explain why the highly detrimental effect of mutation m3 cannot be explained simply as the sum of the effects caused by mutations m1 and m2.

**Assessment of Cooperativity by Footprint Titration**

A quantitative DNase I footprint assay was used to demonstrate that O2 binding to the O2u and O2d sites occurred in conjunction with a cooperative mechanism. In a DNase I footprint experiment conducted over a suitable range of protein concentrations, the profiles of fractional protection at individual sites can be measured simultaneously and used to resolve not only the intrinsic binding constants but also those for cooperative interactions between the various sites (Brenowitz et al., 1986; Seena et al., 1986).

Individual binding equations for the O2u and O2d sites were constructed according to a statistical-thermodynamic
Using a more extensive protein dilution series than that shown in Figure 4, we were able to determine the fractional protection of the O2u and O2d sites for the normal DNA template. A subset of bands in each of the sites was used for optical density measurement (Figure 4). An if/then logic program was written to associate each data set with the proper individual-site binding equation, and nonlinear least-squares estimations were used (see Methods) to determine the best-fit parameters that yielded the minimum variance.

Figure 5 shows the titration data for each site together with the resolved individual-site binding isotherms. The two sites titrated at identical protein concentrations, and the binding transition for each site occurred over \(-1.5\) log units of O2 protein. The steepness of the transition was consistent with a cooperative binding mechanism. Because of the numerical correlation between the estimated parameters, unequivocal determination of all three free energies—\(\Delta G_u\), \(\Delta G_d\), and \(\Delta G_{ud}\)—was not possible from these data. Noncooperative binding, however, could be distinguished from cooperative binding by nonlinear least-squares fitting techniques (Brenowitz et al., 1986). This was done by fixing the cooper-
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The results of the analysis of several assumed values for cooperative free energy are shown in Figure 5 and Table 1. Comparison of the binding data with results of an analysis based on the assumption of noncooperative binding (Figure 5, dotted curve) indicates that there is a systematic deviation of the data from a noncooperative binding model. A better agreement of the data with the best-fit curve was observed for a cooperative model (Figure 5). Improvement of the fit to a cooperative model was also indicated by the values of the square root of the variance for the cooperative and noncooperative models. Models that included a negative cooperative free energy consistently yielded better values for the square root of the variance than did models that assumed a value of zero or greater for $\Delta G_{ud}$ (Table 1). The determination of absolute equilibrium binding constants requires that the protein concentration be known. Because the dimerization constant and the fraction of active O2 molecules in the protein preparation used in the footprint assays were not known, all of the calculated intrinsic binding and cooperative free energies were based on the total O2 concentration and can only be viewed as relative free energy terms (Brenowitz et al., 1989).

O2 Binding at Adjacent Sites Shows Differential Sequence Specificity and a Strict Spacing Constraint

From the preceding results, it is clear that the O2u and O2d sites work in a cooperative manner. To determine whether O2 binding to each of the sites requires these sites to be closely allocated, we analyzed four different mutant templates bearing separated or detached O2u and O2d sites.

In mutant m5 (Figure 3C), 10 nucleotides encompassing one helical turn were inserted between O2u and O2d, thus maintaining the sites in the same phase of the DNA helix. The boundaries of the intervening sequence mimicked the pre-existing nucleotides, thus preserving the natural flanking nucleotides for each site. In mutant m6 (Figure 3C), 14 nucleotides for the mutant template gels, the brackets show the same block of bands as in the normal gel and facilitate location of the O2u and O2d sites. The asterisks indicate the positions of mutated nucleotides. The concentrations of the integral Coix O2 protein in each lane are as follows: lanes 2, 9, 16, 23, and 30, no addition; lanes 3, 10, 17, 24, and 31, 18.7 nM; lanes 4, 11, 18, 25, and 32, 37.5 nM; lanes 5, 12, 19, 26, and 33, 56.2 nM; lanes 6, 13, 20, 27, and 34, 74.9 nM; and lanes 7, 14, 21, 28, and 35, 93.7 nM. Lanes 1, 8, 15, 22, and 29 show the G+A sequencing ladder. Note that the G+A sequencing ladder should be read as C+T when referring to the sequence shown at upper left.
were inserted, thus locating O2u and O2d sites on opposite faces of the DNA helix.

Reproducible DNase I footprints recorded at the O2u and O2d sites of the m5 mutant demonstrated once more that the single O2-footprinted sequence in the α-coixin promoter resulted from the presence of two adjacent binding sites (Figure 6A). The protection of O2u and O2d in mutant m5 was less complete and required a higher O2 concentration than that with the wild-type promoter. In addition, a greater degree of protection was observed at the O2d site compared with O2u. This finding indicated that the spacing between the two binding sites was critical and that the O2 protein had a higher affinity for the O2d site. The latter result was in keeping with data obtained from the mobility shift assay using mutant oligonucleotides. However, it must be interpreted with caution because we cannot determine definitively whether the 10-bp insertion significantly altered the primary recognition sequence at the individual sites, particularly the O2u site.

In the m6 mutant template, O2 still bound the O2d site but only very weakly protected the O2u site, as detected by visual inspection (Figure 6A). The sequences flanking the O2u and O2d sites in the m5 and m6 mutants were identically altered by the intervening sequence. Thus, the loss of binding to the O2u site in m6 cannot be attributed to a further alteration of the primary recognition sequence at this site but probably reflects the lack of cooperativity caused by the increased distance between the two sites or by the opposite stereospecific alignment.

Footprint analyses were also conducted with templates containing detached O2u and O2d sites. The O2u or O2d sites were deleted from clone m5, giving rise to m7 and m8, respectively (Figure 3C). The detached O2d site bound O2, whereas no clear protection was observed for the detached O2u site (Figure 6A). However, using scanning densitometry (data not shown) and a gel retardation assay (Figure 6B), we were able to demonstrate that O2 specifically recognized the detached O2u site, although with weaker affinity. This may not be the case for the wild-type site in which the O2u site sequence is in its normal environment. Indeed, m1 and m4 mutants clearly show the importance of the O2u site in the overall binding affinity and suggest that O2 binds the O2u site of the normal promoter with a higher affinity than that observed for the m5, m6, and m8 mutants. This interaction probably requires much greater sequence specificity than that with the O2d site, thus explaining why any variation in the recognition or flanking DNA sequence severely reduces O2 binding at the O2u site.

**Transient Expression Assay**

To determine the biological consequences of the different mutations, we conducted transient expression assays in tobacco mesophyll protoplasts by using reporter plasmids expressing the β-glucuronidase (GUS) gene under the control of either the normal 285-bp α-coixin promoter fragment or the mutant versions m1 to m7 (Figure 7A). The Coix O2 protein expression vector, pRTO2Coix (Figure 7A), was used in the cotransfection experiments. The pDH51 vector, a derivative consisting of the luc gene encoding firefly luciferase (LUC) under the control of the constitutive cauliflower mosaic virus 35S promoter, was included as an internal control to assess the efficiency of protoplast transformation.

All treatments were assayed in each of the protoplast preparations. GUS and LUC activities were determined, and the relative expression of GUS to LUC in each sample was calculated. Figure 7B shows the relative average expression of the various templates alone and after cotransfection with the O2 effector vector. A clear difference in the responses of the different constructs was observed upon cotransfection with the pRTO2Coix effector plasmid.

The activity of each construct transfected without O2 corresponds to the changes in activity observed in the presence of the O2 protein (Figure 7B). This background activity was most likely due to activity promoted by other bZIP proteins in tobacco cells that interacted through the same sites as did the O2 protein. Consequently, the data could not be
presented in terms of the fold activation of each promoter in response to O2, because in such a way, differences between transactivation of constructs would be masked.

The m1 mutation caused ~45% loss of transactivation activity, whereas m2 caused only a very small, nonsignificant (P > 0.05) decrease in activity (4%) compared with that of wild-type (normal) activity. The reduction in transactivation associated with the m3 mutant (which contained both the m1 and m2 mutations) was higher than the simple sum of the effects caused by m1 and m2 (Figure 7B). This suggests the existence of synergism between O2u and O2d in transcription from the normal promoter. Cooperative O2 binding is likely to contribute to transcriptional synergism by providing a more stable coupling at the enhancers and increasing the affinity of the protein for relatively weak binding sites.

The m4 mutant produced a nonsignificant (P > 0.05) increase (~6%) in the level of O2 transcriptional activation compared with that of wild-type (normal) activity (Figure 7B). On the other hand, this mutation significantly decreased the O2 binding affinity (Figure 4), suggesting that the in vitro binding conditions were more stringent than those in vivo. This may also be the case for m2 and m3. Based on the footprint profile of m4 (Figure 4), an in vivo transactivation activity similar to that of m1 mutant was expected. The differences in transactivation between m1 and m4 probably derive from differences in the O2 loading of the individual O2u and O2d binding sites. Although the amount of O2 protein necessary to obtain a complete footprint in m1 and m4 templates was similar (Figure 4), in m1, the O2d site was the principal site occupied, whereas in m4, it was the O2u site. Thus, the transient expression results indicated that at least in tobacco protoplasts, the O2u site was more important in enhancing activity than was the O2d site.

The m5 mutation caused a loss of transactivation activity similar to that seen with the m3 mutation (~64%). The m6 mutation resulted in an ~37% decrease in activity (similar to

![Figure 6. Binding of the Coix O2-Homologous Protein to Different Mutant Templates Bearing Separated or Detached O2u and O2d Sites.](image)

(A) DNase I protection experiment. The DNA templates m5 to m8 are indicated above each gel (for their sequences, refer to Figure 3). For explanations of the brackets at right, see the legend to Figure 4. The concentrations of the integral Coix O2 protein added to each lane are as follows: Lanes 2, 9, 16, and 23, no addition; lanes 3 and 10, 30.9 nM; lanes 4 and 11, 93.7 nM; lanes 5 and 12, 156.4 nM; lanes 6 and 13, 218.3 nM; and lanes 7 and 14, 281.0 nM. Lane 17, 4.7 nM; lane 18, 14.1 nM; lane 19, 37.5 nM; lane 20, 56.2 nM; and lane 21, 74.9 nM. Lane 24, 56.2 nM; lane 25, 234.2 nM; lane 26, 374.7 nM; and lane 27, 515.2 nM. Lanes 1, 8, 15, and 22 show the G+A sequencing ladder. For m5, m6, and m8, the G+A sequencing ladder should be read as C+T when referring to the sequence shown in Figure 3.

(B) Mobility shift assay using the same labeled fragments (m7 and m8) as given in (A) and increasing N-terminal truncated Coix O2 protein amounts: lanes 1 and 6, 0.25 ng; lanes 2 and 7, 0.5 ng; lanes 3 and 8, 2.5 ng; and lanes 4 and 9, 5 ng. In lane 5, no protein was added. The position of the free probe (fp) and the protein-DNA complex (arrow) are shown. The diamond indicates the position of a band corresponding to the vector plasmid, resulting from incomplete purification of the inserted fragment.
mutation m1). The m5 mutant was less detrimental to the in vitro binding of O2 than were m3 and m6, indicating that the functional properties of a protein–DNA interaction do not always correlate with binding affinities. In the case of m5, the interaction between two O2 dimers bound at the O2u and O2d sites probably precluded the formation of a domain competent to activate transcription or effective interaction with the transcription complex, perhaps through steric constraints. The m7 mutant retained poor transactivation activity (Figure 7B); this is consistent with the considerations discussed above concerning the importance of the O2u site.

**DISCUSSION**

Two linked sites, O2u and O2d, are responsible for O2 binding and transactivation of the α-coixin gene promoter. These sites show a considerable degree of sequence divergence. Whereas O2d is almost identical to the B1 and B4 binding sites of the b-32 gene (Lohmer et al., 1991), O2u has little homology to any O2 binding site described thus far. Nevertheless, both sites contain a TGAC motif, which is also part of the CREB/ATF and GCN4/AP1 binding sites. Two linked O2 binding sites are also present in the promoter of the 22-kD α-zein gene. The stronger TCCACG-TAGA O2 target region in the promoter of the 22-kD α-zein gene is flanked by a weakly footprinted sequence comprising the domain TGCATGTC (Schmidt et al., 1992). Recently, this site, called Z2, was found to be important for O2 transactivation (Muth et al., 1996).

Two closely spaced plant bZIP binding sites have been reported for the octopine synthase (ocs) elements. These elements occur in a group of extensively studied cis-acting sequences present in the promoters of some Agrobacterium and caulimovirus genes (Bouchez et al., 1989). A considerable degree of sequence divergence occurs among ocs elements, and only rarely does an ocs element contain two identical adjacent motifs (Bouchez et al., 1989; Fox et al., 1992; Ulmasov et al., 1994). As shown in Figure 8, the double O2 binding sites of the α-coixin and α-zein promoters share sequence similarities with ocs elements.

**Table 1. Relative Microscopic Gibbs Free Energies of the Interaction of O2 with the Two Sites of the Normal α-Coixin Promoter**

<table>
<thead>
<tr>
<th>Cooperative Energy ΔGud</th>
<th>Intrinsic Binding Energies</th>
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<td></td>
<td>Upstream Site ΔGu</td>
<td>Downstream Site ΔGd</td>
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<td>−11.2 ± 0.1</td>
<td>−11.2 ± 0.1</td>
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<tr>
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<td>−10.8 ± 0.1</td>
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<tr>
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The relative binding free energies (in kilocalories per mole, with 67% confidence intervals) were estimated from the analysis of titration data obtained from footprints of the normal DNA template. ΔGud values were fixed at the values shown, and estimates of the two intrinsic free energy terms for binding of the O2 protein to each site were determined.

bSquare root of the variance of the fitted curves.

**O2 Binds Cooperatively to the Adjacent Target Sites**

By using footprint titration experiments with normal or mutant DNA templates, we were able to show that O2 protein binds cooperatively to the adjacent O2u and O2d sites. The GCN4 protein also seems to bind cooperatively to very close repeated sequences in promoters of the his3 and his4 genes (Arndt and Fink, 1986). Recently, preliminary data suggested that TGA2 and TGA5, two Arabidopsis bZIP factors, are able to bind cooperatively to the two TGACG motifs in the as-1 site, an ocs element found in the cauliflower mosaic virus 35S promoter (Lam and Lam, 1995).

The footprint titration result for the normal DNA template was quantified by measuring the fractional saturation of the individual sites. The data were analyzed by nonlinear regression to calculate the free energy components involved in the O2-DNA interaction. This analysis showed that O2 binds to the O2u and O2d sites in a cooperative manner, thus definitively demonstrating the cooperative binding of a bZIP transcriptional activator.

Although it was possible to show that O2 binds cooperatively to both sites, the binding data from the normal DNA template alone did not allow unequivocal resolution of all three binding free energies. To resolve this difficulty, it is necessary to analyze simultaneously the quantitative footprint data from “reduced valency” mutant templates (Koblan et al., 1992; Senear and Bolen, 1992).

We did not assess whether the cooperativity derives from direct protein–protein interactions or whether it is the product of an altered DNA conformation induced by O2 binding. In the case of the O2u and O2d sites, the spacing and/or stereospecific alignment between the binding sites was critical for cooperativity. This situation may vary, depending on the binding sites. In cases in which global protein folding transitions are coupled to DNA binding, it is likely that the final protein conformation will be a function of the DNA sequence (Spolar and Record, 1994). Thus, the folding of residues to create regions of the protein responsible for protein–protein interactions depends on the DNA sequence. A similar rationale can be envisioned if one considers cooperativity to be the product of a DNA conformational change. In this case, different sequence-dependent DNA distortions are induced by protein binding, giving rise to DNA structures that may or may not facilitate O2 binding at an adjacent site. It seems plausible that the sequence and distance of the
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O2u and O2d sites have evolved in concert to achieve best O2 binding affinity and correct interaction with the transcriptional machinery (see below).

Evolutionary Significance and Biological Role for Cooperativity of O2

To date, O2 is the only plant bZIP factor to which a regulatory function has been assigned. Surprisingly, there is considerable variability in the functional O2 binding sites thus far identified (Lohmer et al., 1991; Schmidt et al., 1992; Yunes et al., 1994b; Cord Neto et al., 1995; Maddaloni et al., 1996; Muth et al., 1996). This implies that specificity has not been maximized during evolution, which is in accordance with the statistical-mechanical selection theory of Berg and von Hippel (1987). This theory states that the recognizer protein is not designed for maximum specificity because of an evolutionary requirement for flexibility.

Weaker discrimination factors permit a fine tuning or modulation of binding to different specific sites. This may represent a means by which the O2 protein can control the expression of different classes of endosperm genes (Kodrzycki et al., 1989; Yunes et al., 1994a; Cord Neto et al., 1995; Maddaloni et al., 1996).

To reduce the competitive binding to pseudosites in the genome, a tendency toward larger DNA sites is required. An effective increase in site size, without the undesirable enlargement of the discrimination factor, is achieved by dimerization or cooperative binding of the same protein to two neighboring binding sites so that the recognition sequence consists of the two sites together (Berg and von Hippel, 1987). In physiological terms, cooperative binding has the advantage of conferring a higher sensitivity to the small increases in abundance or activity of O2 and, hence, maximizing the response.

Figure 7. Transactivation of Normal and Mutant a-Coixin Promoters by the Coix O2-Homologous Protein in Tobacco Protoplasts.

(A) Schematic representation of chimeric reporter and effector constructs used to analyze the interaction between the O2 protein and the a-coixin promoter. P285a-CGUS, GUS gene under the control of the normal a-coixin promoter. P285m1GUS to P285m7GUS, GUS gene under the control of the mutant m1 to m7 promoters described in Figure 3. The promoter is represented as a black arrowhead, and the O2u and O2d sites are represented as open ovals. pRTO2Coix is the Coix O2 cDNA under the control of the cauliflower mosaic virus 35S promoter (arrowhead). The translated sequence of the O2 cDNA is represented as a box. Restriction enzyme sites are A, AccI; Ap, Apal; B, BamHI; E, EcoRI; H3, HindIII; K, KpnI; N, NcoI; P, PstI; S, SalI; Sm, SmaI; Sp, SpII; X, XbaI; Xh, Xhol. The internal control pDHLUC, which consists of LUC under the control of the 35S promoter, is not shown.

(B) Relative GUS/LUC expression of the different constructs in transiently transformed tobacco protoplasts. Ten micrograms of the reporter vectors P285a-CGUS (normal) or P285m1GUS to P285m7GUS (m1 to m7) plus 10 μg of the pDHLUC internal control was transfected alone (white bars) or together with a twofold molar excess of the effector plasmid pRTO2Coix (black bars). For each transfected protoplast sample, the activity of GUS was divided by that of LUC to obtain a relative expression. The histogram represents the mean ± SD of the expression from two protoplast preparations. Lowercase letters over the bars represent significant differences (P < 0.05) between activities as determined by analysis of variance and the Tuckey HSD test, using the Statistica program (StatSoft, Tulsa, OK). The total number of replicas per treatment was six and nine for the protoplasts transfected with templates alone and those cotransfected with O2, respectively.
Comparison of the O2 Double Binding Sites with the ocs Elements

Homologous O2u and O2d site sequences are outlined by dotted boxes and indicated by open boxes, respectively. The homologous 5\'-TGAC-3\' motifs are indicated by arrows. Note that sequence divergence observed both within the ocs elements and between ocs elements and the O2 binding sites are comparable. The sources are the 22-kD \(\alpha\)-zein (Muth et al., 1996), ocs, nos, mas b, mas e (Fox et al., 1992), ags, and cauliflower mosaic virus 35S (Bouchez et al., 1989).

The Two Adjacent Binding Sites and Transcriptional Activation

Transient expression assays in tobacco protoplasts revealed that a mutation in either of the adjacent sites or in both sites simultaneously affected the transactivation by O2 in a manner that cannot be explained simply by an additive mechanism. Thus, the O2u and O2d sites may act synergistically to achieve a high level of \(\alpha\)-coixin gene expression.

Recently, Muth et al. (1996) demonstrated that the maize O2 protein binds to three sites in the \(\alpha\)-zein promoter and that one of these sites, which has the lowest affinity of all three, confers no detectable O2-dependent promoter activation by itself but significantly increases activation in combination with any of the other two sites. Similarly, a synergistic effect on transcription was observed when both binding motifs of ocs elements were occupied (Bouchez et al., 1989; Singh et al., 1989; Ellis et al., 1993; Kim et al., 1991; Emami and Carey, 1992).

The cooperative binding of two or more activators to DNA can, in principle, generate a synergistic effect on transcription because the activator's affinity for DNA increases, allowing a template bearing multiple sites to become saturated at a lower concentration of an activator than would a template bearing a single site. Thus, it is likely that cooperative binding of O2 to the linked sites contributes to the observed synergistic effect on transcription. However, cooperative O2 binding is unlikely to be the only mechanism contributing to a synergistic action. Some templates have significantly different transcriptional activity but require similar amounts of O2 for binding saturation of the whole target region (cf. m1 and m4). This suggests that global binding affinity may not always be a definitive indicator of transcriptional activity. In the context of our assay, O2 binding at the O2u site was more important for enhancing activity, which is in keeping with the finding that the upstream site of the ocs element was also reported to be more important for activity (Singh et al., 1989). Therefore, each of the O2u and O2d sites might have different roles in determining the arrangement of the nucleoprotein complex and the subsequent interaction with the transcriptional machinery. In this regard, we suggest that the O2 molecules that cooperatively bind to the 10-bp separated sites of m5 may form an unusual protein (or DNA) structure that precludes formation of or hinders a domain able to activate transcription, as reported for the thyroid hormone receptor (Glass et al., 1988) and the \(\lambda\) repressor (Hochschild and Ptashne, 1988).

METHODS

Construction of Plasmids Containing Mutant O2 Target Sequences

The P285u-C clone (Yunes et al., 1994b), containing the EcoRI-BamHI 285-bp \(\alpha\)-coixin promoter fragment spanning the Opaque2 (O2) target sequence, served as the template for the site-specific mutagenesis performed by asymmetric polymerase chain reaction (PCR; Perrin and Gilliland, 1990), using the M13 universal and reverse primers and one of the following mutant primers: O2c-m1, 5\'-TGACAAAAATTAAGACAGAGC-3'; O2c-m2, 5\'-TGGGACTACGACCATCATCCTAG-3'; O2c-m3, 5\'-TGACAAAAATTAAGACAGACGACTCATCCTAG-3'; O2c-m4, 5\'-GAGACATGTCGCTCTAGCTTA-3'; and O2c-m5, 5\'-AAATTGACTAGGAGACATCGAGACATGTCAT-
CTC-3'. Note that primer O2c-m5 creates an XhoI site (underlined). The amplified fragments were subsequently digested with EcoRI and BamHI and inserted into the pBluescript KS+ vector (Stratagene, La Jolla, CA), resulting in the clones P285m1, P285m2, P285m3, P285m4, and P285m5, respectively. The P285m6 mutant was obtained by subcloning the 285-bp EcoRI-BamHI fragment of P285m6GUS (see below) into the KS vector. A detached O2d site, P285m7, was obtained by digesting plasmid P285m7GUS (see below) with HindIII and NcoI, recovering the 175-bp fragment, filling the recessed ends with the Klenow fragment of DNA polymerase I, and cloning into the Smal site of the pBluescript KS+ vector. A clone with an isolated O2u site, P285m8, was obtained by subcloning the 200-bp EcoRI-NcoI fragment of P285m8GUS (see below) in place of the 285-bp EcoRI-NcoI fragment of P285w. All of the clones were verified by sequencing.

Overproduction of the Coix (Coix lacryma-jobi) O2-Homologous Protein in Escherichia coli

The incomplete O2 cdna was excised from clone pCO2-1 (Vettore et al., 1998) by digestion with BamHI and XhoI. The XhoI-recessed end was partially filled with dTTP and dCTP by using the Klenow fragment, and the resulting fragment was cloned into the BamHI site of the pET3d (Studier et al., 1990), giving rise to the vector pETO2Cx.

The entire Coix O2 coding sequence was amplified from ATG to position +382 by using clone KS02-212/374 (see below) as the template, together with the M13 reverse primer and the oligonucleotide O2ATG (5'-CAATCGATCATATGACATGACAAAATTGACATGGAGACATTTCCTCTCTAGCTTA-3') and 3'-ACTCGTTTTATAATATCCCTGTACAGTAGTAGATCAGATTTAAGG-5'; and oligo-UD, 5'-GCTCTAAGTGAACAAAATTAATTTAGGAGACATTTCCTCTCTAGCTTA-3' and 3'-ACTCGTTTTATAATATCCCTGTACAGTAGTAGATCAGATTTAAGG-5'.

These oligonucleotides, at a concentration of 50 μg/mL, were annealed in buffered 150 mM NaCl by heating at 65°C for 5 min and gradually cooling to room temperature. For radiolabeling, 250 ng of annealed oligonucleotides was extended with the Klenow fragment in a mixture containing 50 mM Tris-HCl, 10 mM MgCl2, pH 7.6, α-32P-dATP, and the remaining unlabeled deoxynucleotide triphosphates.

The DNA for the mobility shift assays using the insert fragments of P285m7 and P285m8 was prepared as for the footprint assays (see below). Protein-DNA complexes were formed by mixing different amounts of proteins with 2 μg of sonicated salmon sperm DNA per mL in 50 μL of binding buffer (10 mM Hepes-KOH, pH 7.9, 50 mM KCl, 1 mM EDTA, 10 mM DTT, 2 mg/mL BSA, and 10% glycerol). After a 5-min preincubation at 25°C, the labeled probe (10,000 cpm) was added, and the mixture was incubated for 20 min at 25°C. At the end of this incubation period, the mixture was fractionated on 5% acrylamide gels (30:1, polyacrylamide-bisacrylamide) in 0.25 × TBE (50 mM Tris, 42 mM boric acid, and 0.5 mM EDTA) at 12°C.

DNase I Footprint Titrations

Preparation of DNA

P285w–C or P285m1 to P285m8 plasmid DNA was digested with EcoRI and NotI, and the binding site-containing fragments were isolated using agarose gel electrophoresis. After quantitation of DNA, 300 ng of each fragment was labeled by a fill-in reaction of the 3'-recessed EcoRI end using the Klenow fragment and α-32P-dATP plus unlabeled deoxynucleotide triphosphates. The labeled fragments were purified by PAGE, followed by standard "crush-and-soak" procedures. The molar concentration of DNA was measured based on the starting amount of DNA and on the counts recovered. The estimated concentration of the specific DNA fragment in each experiment was used to calculate the free protein ligand concentration during data analysis (see below).

DNase I Footprinting

Reactions were performed at 25°C in a mixture containing 12.5 mM Hepes-KOH, pH 7.5, 50 mM KCl, 0.5 mM DTT, 1% glycerol, 0.05% Nonidet P-40, 1 μg of poly(dI-dC), and 20,000 to 60,000 cpm of end-labeled DNA in a total volume of 50 or 200 μL. The reactions were gently mixed every 2 to 3 min for 35 min. MgCl2 and CaCl2 were then added to final concentrations of 1 and 0.5 mM, respectively. After 1 min of incubation, 0.1 unit of DNase I (high-performance liquid chromatography pure; Pharmacia, Uppsala, Sweden) was added, and after mixing, the reaction was incubated for another minute. The reaction was stopped by the addition of DNase I stop solution to yield final concentrations of 137 mM sodium acetate, pH 5.2, 22.8 mM boric acid, and 0.5 mM EDTA. The G + A sequencing ladder was prepared using a Sure Track Footprinting Kit (Sigma) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assays

DNA probes were prepared from the following synthetic pairs of oligonucleotides: oligo-Cx, 5'-GCTCTAGATGACAAAATTGACTAGAGACCATCTCATCTCTAGCTTA-3' and 3'-ACTCGTTTTATAATATCCCTGTACAGTAGTAGATCAGATTTAAGG-5'; oligo-Dm, 5'-GCTCTAAGTGAACAAAATTAATTTAGGAGACATTTCCTCTCTAGCTTA-3' and 3'-ACTCGTTTTATAATATCCCTGTACAGTAGTAGATCAGATTTAAGG-5'; oligo-Um, 5'-GCTCTAGATGACAAAATTGACTAGAGACCATCTCATCTCTAGCTTA-3' and 3'-ACTCGTTTTATAATATCCCTGTACAGTAGTAGATCAGATTTAAGG-5'.

These oligonucleotides were annealed in buffered 150 mM NaCl by heating at 65°C for 5 min and gradually cooling to room temperature. The G + A sequencing ladder was prepared using a Sure Track Footprinting Kit (Sigma) according to the manufacturer's instructions.
recommendations. Autoradiography of the dried gels was done at −70°C, using prefashed x-ray film and an intensifying screen.

**Data Analysis**

Two-dimensional optical scans of the footprint autoradiographs were obtained using a Bio-Rad GS-700 imaging densitometer set for a spatial resolution of 56 μm (450 dots per inch) and a pixel depth of 12 (12-bit precision). The optical density was processed using the Molecular Analyst/PC software package (Bio-Rad). The fractional protection of the sites 02u (pu) and 02d (pd) was determined by the method of Brenowitz et al. (1986).

An if/then logic was used to assign the data points to the correct equation described below. Energetic parameters for the interaction of the O2 protein with the α-coixin binding sites, as well as the end points for each transition curve, were estimated by nonlinear least-squares analysis, using the NLIN procedure of the SAS program (SAS Institute Inc., Cary, NC). NLIN was specified to use the multivariate secant (DUD) iterative method and the G4SINGULAR option. The latter specifies that a g4 or Moore–Penrose inverse be used in parameter estimation if the jacobian is (or becomes) of less than full rank. As convergence criteria, NLIN was specified to run the iterative process until the decrease in square sum error was 10−6 and the maximum change among the parameters was 10−5. The reliability of the parameter estimation process was evaluated by the cross-correlation coefficient, using a critical limit of −0.96. To evaluate the uniqueness of the estimated parameters, we performed multiple least-squares parameter estimations with a variety of different starting values such that all of them converged to the same set of parameter estimates.

The individual binding equations for the sites 02u and 02d, constructed according to a statistical-thermodynamic model (Ackers et al., 1982, 1983, 1990), the putative site for transcription initiation in the Coix O2 genomic clone (Vettoire et al., 1998), the sequence spanning positions +382 to +1406 was amplified by PCR and inserted into the Accl sites of pBluescript KS+, giving rise to clone KSO2+382/1406. The PCR primers used were the reverse sequence primer and the oligonucleotide O2C382 (5'-TTCCGACAGGTCGACCTCGGCCCTTC-3'), which creates an Accl restriction site (underlined) at position +382 without changing the amino acid sequence.

By comparison with the maize O2 cDNA clone 6-1 (Schmidt et al., 1990), the putative site for transcription initiation in the Coix O2 genomic clone (Vettoire et al., 1998) was identified at position −212. The Coix O2 sequence from positions −212 to +382 was amplified and inserted into the EcoRI and KpnI sites of pBluescript KS+, giving rise to clone KSO2-212/374. The oligonucleotides for the PCR were O2C-

**Construction of Plasmids for the Transient Expression Assay**

All sequence position numbers refer to the ATG initiation codon. The reporter vectors P285m1GUS, P285m2GUS, P285m3GUS, P285m4GUS, and P285m5GUS were constructed by excision of the 285-bp (295 bp for m5) EcoRI-NcoI fragment from the counterpart clones P285m1, P285m2, P285m3, P285m4, and P285m5 and insertion into the P285m-CGUS vector (Yunes et al., 1994b), which was digested with the same enzymes, thus replacing the normal 285-bp α-coixin promoter fragment. Vector P285m7GUS was obtained by digesting P285m5GUS with EcoRI and Xhol to delete a 100-bp fragment spanning the O2u site, followed by filling the ends with the Klenow fragment and recircularizing with T4 DNA ligase. Vector P285m8GUS was obtained by digesting P285m5GUS with Xhol and Apal to delete a 100-bp fragment spanning the O2d site, followed by making blunt ends with T4 DNA polymerase and recircularizing with T4 DNA ligase. Because the putative TATA box was also deleted, P285m8GUS was not assayed in the protoplast transfections.

pRTO2Coix is an effector vector containing the Coix O2-homologous sequence spanning positions −212 to 1406, with the latter being under control of the cauliflower mosaic virus 35S promoter of pRT101 (Töpfer et al., 1987). From the incomplete Coix O2 cDNA clone pCO2-1 (Vettore et al., 1998), the sequence spanning positions +382 to +1406 was amplified by PCR and inserted into the Accl and KpnI sites of pBluescript KS+, giving rise to clone KSO2+382/1406. The PCR primers used were the reverse sequence primer and the oligonucleotide O2C382 (5'-GCCAGGTCGACCTCGGCCCTTC-3'), which creates an Accl restriction site (underlined) at position +382 without changing the amino acid sequence.

**Transient Expression Assays in Tobacco Mesophyll Protoplasts**

Transient expression assays with tobacco mesophyll protoplasts were performed essentially as described previously (Yunes et al., 1994b). Ten micrograms of the reporter vectors with either 20 μg of the pRTO2Coix effector vector or 20 μg of sonicated salmon sperm DNA plus 10 μg of the pDH1uc internal control was used in the transformation of 4 × 105 protoplasts. The treatments were analyzed in a complete block design in which each block corresponded to a protoplast preparation. A total of 16 treatments were included in each of two different protoplast preparations. In the first protoplast
preparation, three repetitions of each of the treatments were assayed. In the second protoplast preparation, treatments without O2 transactivation were assayed in triplicate, whereas treatments with O2 transactivation were assayed using six replicates. Twenty-four hours after transformation, protoplasts were collected, and the protein was extracted in 300 μL of 1 × lysis buffer (Leckie et al., 1994). For the measurement of β-glucuronidase (GUS) activity, we assayed 50 μL of extract in 200 μL of 1 mM 4-methylumbelliferyl β-D-glucuronide dissolved in GUS extraction buffer (Jefferson, 1987). One hundred microliters of the reaction was withdrawn at 30 and 60 min and added to 900 μL of stop buffer (0.2 M Na2CO3) to terminate the reaction. Fluorescence was measured with a fluorescence spectrophotometer (model F-2000; Hitachi, Tokyo, Japan). For luciferase (LUC) activity, 5 μL of extract was added to 100 μL of a LUC assay reagent (E1483; Promega, Madison, WI), and luminescence was immediately measured for a 20-sec interval by using a luminometer (model TD-20/20; Turner Designs, Sunnyvale, CA).

To calculate the expression of the test GUS construct relative to the expression of the internal control LUC construct (pDHLUC) for each sample, we divided GUS activity (fluorescence per 30 min per 50 μL of extract) by LUC activity (luminescence per 20 sec per 5 μL of extract). For each construct, the average relative expression (±SD) was calculated from the total number of samples.

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