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Opaque-2 Is a Transcriptional Activator That Recognizes a Specific Target Site in 22-kD Zein Genes

Robert J. Schmidt,[1] Mariena Ketudat, Milo J. Aukerman, and Gisela Hoschek

Department of Biology, 0116, and Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92093

***opaque-2* (*o2*) is a regulatory locus in maize that plays an essential role in controlling the expression of genes encoding the 22-kD zein proteins. Through DNase I footprinting and DNA binding analyses, we have identified the binding site for the O2 protein (O2) in the promoter of 22-kD zein genes. The sequence in the 22-kD zein gene promoter that is recognized by O2 is similar to the target site recognized by other "basic/leucine zipper" (bZIP) proteins in that it contains an ACGT core that is necessary for DNA binding. The site is located in the −300 region relative to the translation start and lies about 20 bp downstream of the highly conserved zein gene sequence motif known as the "prolamin box." Employing gel mobility shift assays, we used O2 antibodies and nuclear extracts from an *o2* null mutant to demonstrate that the O2 protein in maize endosperm nuclei recognizes the target site in the zein gene promoter. Mobility shift assays using nuclear proteins from an *o2* null mutant indicated that other endosperm proteins in addition to O2 can bind the O2 target site and that O2 may be associated with one of these proteins. We also demonstrated that in yeast cells the O2 protein can activate expression of a *lacZ* gene containing a multimer of the O2 target sequence as part of its promoter, thus confirming its role as a transcriptional activator. A computer-assisted search indicated that the O2 target site is not present in the promoters of zein genes other than those of the 22-kD class. These data suggest a likely explanation at the molecular level for the differential effect of *o2* mutations on expression of certain members of the zein gene family.**

INTRODUCTION

Zeins are the prolamin class of seed storage proteins that accumulate to high levels in mature seeds of maize (Mertz et al., 1964; Murphy and Dalby, 1971; Lee et al., 1976). They represent a mixture of polypeptides encoded by a large gene family (Gianazza et al., 1976; Viotti et al., 1979; Hagen and Rubenstein, 1981; Burr et al., 1982). Fractionation by SDS-PAGE reveals five classes of proteins with apparent sizes of 27, 22, 19, 16, and 10 kD. The 22- and 19-kD size classes constitute 75 to 86% of the total zein fraction (Esen, 1987). Zein genomic clones and cDNAs encoding proteins of the 22- and 19-kD size classes have been isolated and sequenced. Although clearly related, 22-kD zein genes are distinct from 19-kD zein genes (Burr et al., 1982; Marks et al., 1985; Heidecker and Messing, 1986; Rubenstein and Geraghty, 1986).

Several mutations are known to affect the accumulation of 22- and 19-kD zein proteins (reviewed in Motto et al., 1989). One of these mutations, *opaque-2* (*o2*), causes a severe reduction in the levels of zein gene transcripts encoding polypeptides of the 22-kD size class (Pedersen et al., 1980; Burr and Burr, 1982; Langridge et al., 1982). This reduction in zein protein accumulation causes the affected kernels to take on the characteristic "opaque" appearance that distinguishes this phenotype from the normally translucent, vitreous endosperm of wild-type seed. An *o2* null allele can reduce total zein protein accumulation by 60 to 80% depending on inbred background (Aukerman et al., 1991). This effect is at least in part due to a lower rate of zein gene transcription, especially for the 22-kD zein class (Kodrzycki et al., 1989). These data are consistent with the idea of *o2* being a regulatory locus that encodes a transcriptional activator of zein gene expression.

The *O2* gene has been cloned (Schmidt et al., 1987; Motto et al., 1988). The subsequent isolation and sequencing of the O2 cDNA (Hartings et al., 1989; Schmidt et al., 1990) demonstrated that the product of the *o2* locus contains a leucine-zipper DNA binding motif (Landschulz et al., 1988). This eukaryotic DNA binding motif consists of a heptameric repeat of leucines (the "zipper"), which are responsible for dimer formation, adjacent to a cluster of positively charged amino acids (the "basic motif"), which are responsible for sequence-specific recognition of the target DNA (Neuberg et al., 1989; Turner and Tjian, 1989). This combination of a basic domain followed by the leucine repeat has been termed the bZIP domain (Vinson et al., 1989).

We have previously shown that O2 is capable of binding to the promoter of 22-kD zein genes (Schmidt et al., 1990) and that the bZIP domain in O2 mediates this binding (Aukerman et al., 1991). Here we report the initial characterization of the

[1] To whom correspondence should be addressed.

sequence bound by O2 in vitro and show that this sequence is recognized by the O2 protein extracted from nuclei of endosperm cells. This sequence is not present in promoters of the closely related 19-kD zein genes, nor in the promoters of more distantly related zein genes. This provides an explanation for the specific effect that *o2* mutations have on the expression of 22-kD zein genes. In addition, by expressing O2 in yeast and showing that it can activate transcription of a reporter gene containing a multimer of the O2 target site, we show that the O2/zein target site interaction represents an operational regulatory unit.

RESULTS

DNA Binding by O2 on 22-kD Zein Gene Promoters

We have shown previously that the O2 protein, expressed as a β-galactosidase–O2 fusion (β-gal::O2) in *Escherichia coli*, binds to a Sau3AI restriction fragment spanning the −300 region of the promoter element in 22-kD zein genes (Schmidt et al., 1990; Aukerman et al., 1991). This restriction fragment was cloned from pZ-1B (*22Z-1*) and pZ-2BS (*22Z-2*) (Aukerman et al., 1991) and sequenced. At the same time, the corresponding sequence was obtained for two additional 22-kD zein genes, *22Z-3* and *22Z-4*, that we have also cloned (see Methods). As shown in Figure 1, a restriction fragment in plasmid clones from *22Z-3* and *22Z-4*, as well as *22Z-1* and *22Z-2*, contain an O2 binding site. Like *22Z-1* and *22Z-2*, clone *22Z-4* has a 200-bp Sau3AI restriction fragment that binds O2. In *22Z-3*, on the other hand, O2 binds to a restriction fragment of about 2 kb. By restriction mapping and DNA sequence analysis, we determined that *22Z-3* is missing a Sau3AI restriction site that is present in each of the other zein genes. The loss of this site in *22Z-3* causes the O2 target site to be located on a 2-kb fragment instead of a 200-bp fragment. Figure 2 provides a sequence comparison of the Sau3AI fragments for each of the four 22-kD zein genes.

Among these four 22-kD zein gene clones, only *22Z-1* had two binding sites for O2. Sequence analysis indicated that this second site, about 1 kb upstream of the translation start in *22Z-1* (Schmidt et al., 1990), was part of nearly 400 bp of sequence having no homology to zein gene promoters (G. Hoschek and R. Schmidt, unpublished data). Because this portion of the *22Z-1* promoter bears no similarity to zein gene sequences, the presence of an O2 target site in this portion of the *22Z-1* promoter is unlikely to have any significance in zein gene expression.

Identification of the O2 Binding Site in 22-kD Zein Genes

The sequence of the Sau3AI restriction fragments is very similar for all four genes (Figure 2) and encompasses a portion

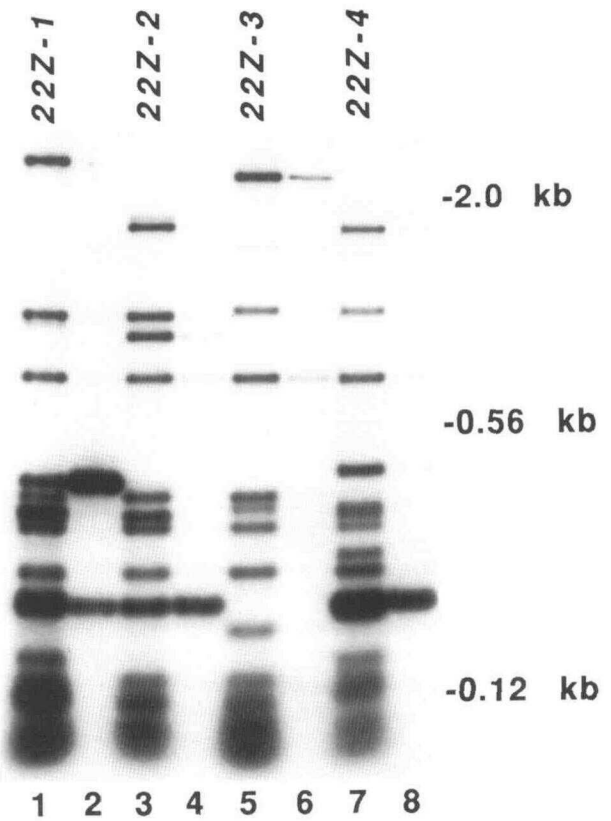

Figure 1. Selective Binding of O2 to Restriction Fragments from Four 22-kD Zein Genes.

Plasmids containing genomic clones from four different 22-kD zein genes, *22Z-1*, *22Z-2*, *22Z-3*, and *22Z-4*, were digested with Sau3AI, end labeled, and incubated with immunoselected β-gal::O2 fusion protein. Odd numbered lanes show the labeled restriction fragments that were incubated with the fusion protein. Even numbered lanes show the fragments that were specifically retained by the β-gal::O2 fusion. *Length markers are given at right.*

of the 22-kD zein promoter between −310 and −102 relative to the translation start in *22Z-4* (GenBank accession No. M86591). Using the β-gal::O2 fusion protein (Schmidt et al., 1990), we performed in vitro footprinting analyses to determine what portion of the zein promoter fragment was protected by the binding of O2 to the 200-bp Sau3AI fragments. Footprinting assays were performed on both strands, and a representative sample of three separate analyses is shown in Figure 3. Along the bottom strand, a single strong footprint is apparent spanning 18 nucleotides near one end of the Sau3AI restriction fragment from *22Z-2*. The binding of O2 to the upper strand produced a strong footprint spanning 14 nucleotides that overlapped the footprint on the bottom strand. In addition, a weaker footprint extending 5′ of the region of strongest footprinting was observed at the highest concentrations of O2 (Figure 3).

Because bZIP proteins have been observed to bind palindromic sequences (see Table 1), we looked for the presence

of such a sequence in the region of strong footprinting. The region of strongest protection encompasses the imperfect palindromic sequence 5′-TCCACGTAGA-3′. As shown in Table 1, this sequence is similar to the binding site sequences (target sites) recognized by several other plant bZIP proteins and suggested to us that this site was the target sequence for O2 in 22-kD zein gene promoters. A computer-assisted search was made to locate this target site in other zein gene promoters. If this represented the O2 target site, we anticipated that the site would not be present in zein genes other than those from 22-kD zein genes because the 22-kD class is the one most affected by *o2* mutations. Indeed, among the zein gene promoters listed with GenBank, we found this site to be present only in promoters from 22-kD zein genes.

Even though our search of the GenBank data base had not detected the O2 target site in zein genes other than those of the 22-kD class, we performed DNA binding analyses on two genes from the closely related 19-kD zein gene class. Our intent was to determine if some other sequence might be present that, although different from the O2 target site in 22-kD zein genes, might still interact strongly with the O2 protein. As shown in Figure 4, there is no convincing binding of O2 to the restriction fragments from the 19-kD zein genes *zE19* and *zE25* (Spena et al., 1982) under conditions where strong binding to zein promoter fragments in *22Z-1* are apparent. A 2-kb Sau3AI restriction fragment in p19A1, a clone of 22-kD zein gene *zA1* (Spena et al., 1982), is bound by the β-gal::O2 fusion protein. Sequence analysis, shown in Figure 5, indicates that *zA1* contains the O2 target site (A. Viotti, unpublished data),

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22Z-4   GATCATGCAT GTCATTCCAC GTAGATGAAA AGAATTCCTA TATAAATGCA
22Z-3   GATCATGCAT TTCATTCCAC GTAGAT-AAA AGAGTTCCTA TATAGAATGG
22Z-2   GATCATGCAT GTCATTCCAC GTAGATGAAA AGAATTCCTA TATAAAATGA
22Z-1   GATCATGCAT GTCATTCCAC GTAGAT-AAA AGAATTCCTA TATA-AATGA

22Z-4   CACCTTTTCT TGTAGGTAGT GGAAAGTATC TTTCCAGCAA AGACCATAGT
22Z-3   CACATTTTCT TGTAGGTAGT GGAAAGTATC TTTCCAGCAA ATACCATA-T
22Z-2   CACCTTTTCT TGTAGGTAGT GGAAAGTATC TTTCCAGCAA AGACCTTA-T
22Z-1   CACATTTTCT TGTAGGTAGT GGAAAGTATC TTTCCAGCAA AGAC-----T

22Z-4   AATCCGGATA AAGTTGATAA CTAAATGTCA AAATAGAGTA GATGCCATAT
22Z-3   A-TCCG-ATA AAGATGATAA CTAAATGTCG AAATCGAGTA GATGCCATAT
22Z-2   ---CCG-ATA AAGTTGATAA CTAAATGTCG AAATAGAGTA GATGCCATAT
22Z-1   AATCCG-ATA AAGTTGATAA CTAAATGTCG AAAAAGAGTA GATGCCATAT

22Z-4   CATCTATACC TTATCTGTTG TTTGGAGAAA --AGACTAA- TCCAAAAATA
22Z-3   CATCTATATC TTATCTGTTG TTTGGAAAAA -AAGACAAAA TCCAACAATA
22Z-2   CATCTATACC TTATCTGTTG TTTGGAAAA- --AGACTAAA TCCAAAAATA
22Z-1   CA-CTATACC TTATCTGTTG TTTGGAAAAA AAAGACTAAC TACAAAAA-A

22Z-4   TATATGA--- GATC
22Z-3   TATTCGA--- GACC
22Z-2   TATATATAAG GATC
22Z-1   TATATATGA- GATC
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Figure 2. Sequence Comparison of the Sau3AI Restriction Fragments Bound by O2.

The sequences of the O2-bound Sau3AI restriction fragments from four different 22-kD zein gene clones are shown beginning at the 5′ Sau3AI site. Sequences have been aligned to maximize similarities. The dashes represent nucleotides that are missing in one sequence relative to another. Relative to the initiation codon in *22Z-4*, the sequence runs from −310 to −102. Note that *22Z-3* is missing the 3′ Sau3AI site. The 10-bp palindrome that is recognized by O2 (see Figure 3) is indicated by a line over the sequence in *22Z-4*.

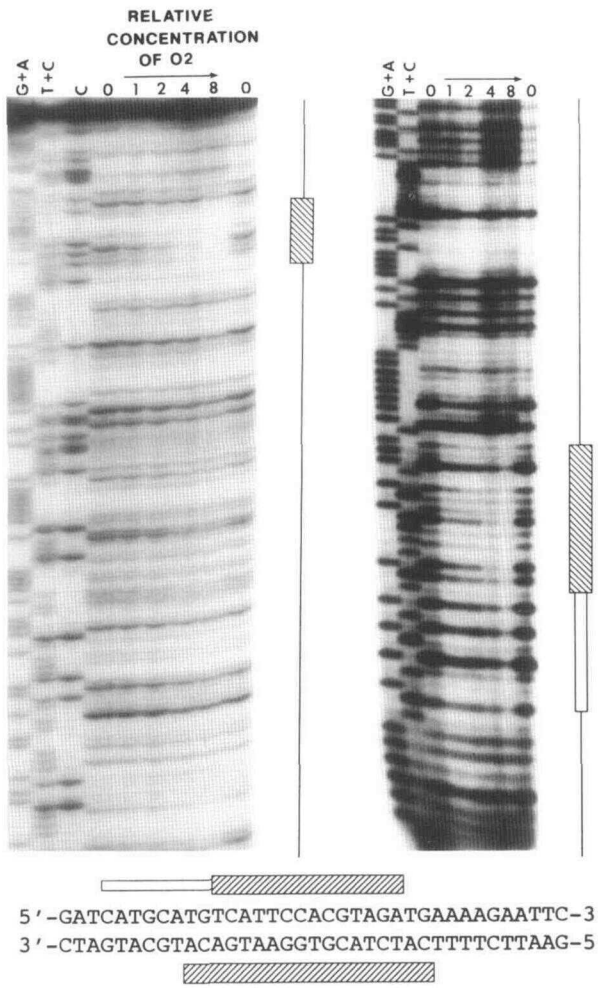

Figure 3. In Vitro Footprinting of the O2 Fusion Protein on the 22-kD Zein Promoter.

The Sau3AI restriction fragment from *22Z-2* that was retained by the β-gal::O2 fusion in the DNA binding assays was used for the footprinting. The first three lanes in the left panel and the first two lanes in the right panel are Maxam and Gilbert sequencing ladders. Labeled zein promoter fragment was incubated with DNase I without prior incubation with β-gal::O2 (lanes marked 0) or with increasing amounts of the fusion protein (lanes 1, 2, 4, and 8). The left panel shows the footprint analysis performed on the bottom strand, whereas the right panel shows the footprint along the top strand. The hatched box represents the region of strongest footprinting and the unhatched box indicates a region of weak footprinting. A portion of the sequence, −310 to −274, is shown along the bottom of the two panels. The protected nucleotides are indicated by the hatched and open boxes along each DNA strand.

but, like our clone of *22Z-3* described earlier, *zA1* is missing one of the conserved Sau3AI restriction sites. These results confirm that whereas O2 can recognize a target site in 22-kD zein gene promoters, no apparent binding site exists in the promoter from these 19-kD zein genes.

Table 1. DNA Sequences Recognized by Plant bZIP Proteins

bZIP Protein	Target Sequence	Promoter	Reference
O2	5′-TCCACGTAGA-3′	Maize 22-kD Zein	This paper
CPRF-1	5′-TCCACGTGGC-3′	Parsley Chalcone Synthase	Wiesshaar et al. (1991)
EmBP-1	5′-GACACGTGGC-3′	Wheat ABA-responsive	Guiltinan et al. (1990)
GBF-1	5′-TCCACGTGGC-3′	Arabidopsis *rbcS-1A*	Schindler et al. (1992)
HBP-1	5′-GCCACGTCAC-3′	Wheat Histone	Tabata et al. (1989)
OCSBF-1	5′-CTTACGT$^{A\,A\,G}_{C\,T\,G}$-3′	Octopine Synthase	Singh et al. (1990)
TAF-1	5′-GGTACGTGGC-3′	Rice ABA-responsive	Oeda et al. (1991)
TGA1a	5′-CTGACGTAAG-3′	35S Cauliflower Mosaic Virus	Katagiri et al. (1989)

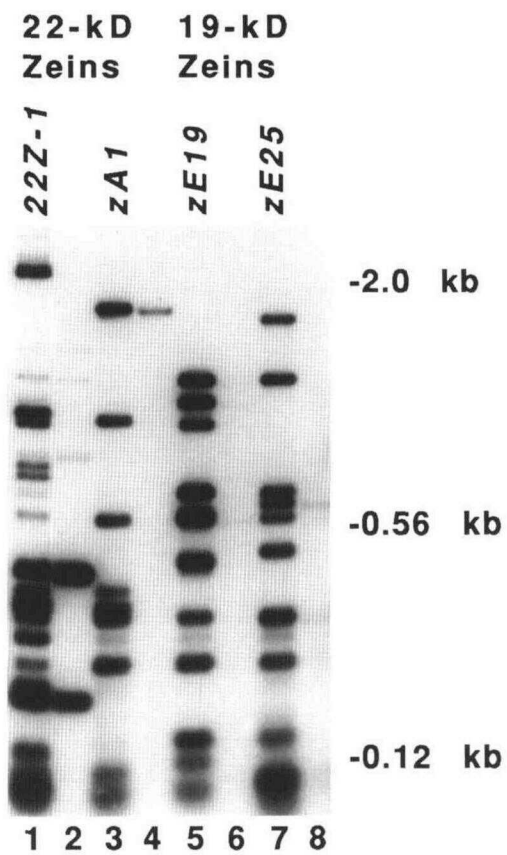

Figure 4. Selective Binding of O2 to 22-kD Zein Gene Promoter Fragments but Not 19-kD Zein Gene Fragments.

Plasmids containing genomic clones from two different 22-kD zein genes (*22Z-1* and *zA1*) and plasmids containing genomic clones from two 19-kD zein genes (*zE19* and *zE25*) were digested with Sau3AI, end labeled, and incubated with immunoselected β-gal::O2 fusion protein. Odd numbered lanes show the labeled restriction fragments that were incubated with the fusion protein. Even numbered lanes show the fragments that were specifically retained by the β-gal::O2 fusion. Length markers are given at right.

Among 22-kD zein genes listed in GenBank, only two contained a perfect match to the O2 target site. As shown in Figure 5, the genes *pML2* (Wandelt and Feix, 1989) and *zA1* (Spena et al., 1982) both have the O2 target site in the same position as found in our own 22-kD zein gene clones. However, the 22-kD zein genes *pML1* (Langridge and Feix, 1983; Wandelt and Feix, 1989) and *Z7* (Kridl et al., 1982) each differ from the TCCACGTAGA target site by a single nucleotide change in the ACGT core of the imperfect palindrome (Figure 5). All four of these previously characterized zein genes contain one or more in-frame stop codons in their respective coding sequences. Based on the presence of these in-frame stop codons, each of these previously characterized 22-kD zein genes is thought to be a pseudogene. The presence of a large family of pseudogenes is characteristic of the zein gene family (Heidecker and Messing, 1986; Rubenstein and Geraghty, 1986). It appears that at least two of these genes (*pML1* and *Z7*), in addition to containing mutations in the coding sequence, have sustained mutations in the O2 target site. We have completed the sequencing of *22Z-4* (GenBank accession No. M86591) and find that, unlike any previously described 22-kD zein genes, *22Z-4* has a full-length open reading frame that specifies a complete 22-kD zein (G. Hoschek and R. Schmidt, unpublished data).

A Single Base Substitution in the O2 Target Site Inhibits DNA Binding

Because even single base changes can dramatically affect the ability of bZIP proteins to recognize their binding sites (Oeda et al., 1991; Tabata et al., 1991), it seemed likely that O2 would not bind as well to the altered O2 target sequence in *pML1* or *Z7* if, indeed, the TCCACGTAGA sequence represented the O2 target in 22-kD zein promoters. To test this, we performed O2 DNA binding assays on plasmids that contained either the 37-bp Sau3AI-EcoRI fragment from *22Z-4* (Figure 5) or an otherwise identical plasmid that contained the corresponding sequence from *pML1* (Figure 5). These plasmids were designated pZ-4SE and pZ-4SEm, respectively. As constructed (see

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                    O2 Target Sequence
  -310                    ┌──┐            -274
5'-GATCATGCATGTCATTCCACGTAGAT-AAAAGAATTC-3' 22Z-1
5'-GATCATGCATGTCATTCCACGTAGATGAAAAGAATTC-3' 22Z-2
5'-GATCATGCATTTCATTCCACGTAGAT-AAAAGAGTTC-3' 22Z-3
5'-GATCATGCATGTCATTCCACGTAGATGAAAAGAATTC-3' 22Z-4
5'-GATCATGCATGTCATTCCACATAGATTAAAAAAAATC-3' pML1
5'-GATCATCGATTTCATTCCACGTAGATAAAAAGAGTTC-3' pML2
5'-GATGATGCATGTCA-TCCAAGTATATGAAAATAATTC-3' Z7
5'-GATCATGCATCTCATTCCACGTAGATAAAAAGAGTTC-3' zA1
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Figure 5. Comparison of the O2 Target Site and Neighboring Sequence in 22-kD Zein Genes.

The O2 target site is present in all but two of the known 22-kD zein genes. The 22-kD zein genes *pML1* and *Z7* are missing the ACGT core (boxed) of the O2 target site. The sequences are numbered relative to the initiation codon in *22Z-4*.

Methods), the pZ-4SEm sequence differs from pZ-4SE by two nucleotides, only one of which is located within the O2 target site. The two plasmids were used in DNA binding assays to determine the ability of O2 to recognize the altered target site sequence. As shown in Figure 6, the O2 protein binds to a single restriction fragment in pZ-4SE, but not to the corresponding fragment in pZ-4SEm that contained the mutated O2 target site from *pML1*. This result demonstrates that (1) even outside of the context of the zein promoter, this 37-bp Sau3AI-EcoRI fragment is sufficient by itself to confer O2 binding and (2) that the ACGT core within the partially palindromic sequence TCCACGTAGA is important for O2 recognition. Binding to the mutant sequence can be detected upon overexposure of the autoradiogram (data not shown), indicating that this change does not abolish O2 binding entirely.

The O2 Protein in Endosperm Binds the O2 Target Site

The results of our in vitro footprinting and DNA binding had defined a sequence motif that was specifically recognized by the β-gal::O2 fusion protein. If this binding site represents the site recognized by the O2 protein, then mobility shift assays with the O2 target sequence should detect the O2 protein made in endosperm cells. To test this, the O2 target site fragment from pZ-4SE was end labeled and incubated with nuclear extracts from endosperm tissue. DNA–protein complexes were then separated from unbound fragments by electrophoresis on native polyacrylamide gels and detected by autoradiography. As shown in Figure 7A, a mobility shift is detected when a nuclear extract from wild-type endosperm is incubated with the fragment containing the O2 target site. Binding to the O2 target site is severely reduced when the sequence contains the mutated ACGT core found in pZ-4SEm (Figure 7A). This demonstrates that the proteins in the nuclear extract that bind to the target site are specifically recognizing the ACGT sequence motif. Although not well resolved on short gel runs, the mobility shift is comprised of three separate complexes that can be better visualized with longer electrophoresis times (Figure 7C). To address whether any of these complexes contain the O2 protein, we preincubated the wild-type extract with antisera raised against the O2 protein (Varagona et al., 1991) and then assayed for binding to the target site. As shown in Figure 7B, the O2 immune sera partially inhibits binding, whereas preimmune sera has no effect. The longer electrophoresis run (Figure 7C) indicates that treatment with the O2 antisera predominantly inhibits the uppermost complex, although the middle complex is also somewhat reduced. This result strongly suggests that O2 is present in at least one of the complexes bound by the target site probe.

As a second approach to demonstrate the presence of O2 in one or more of the complexes, we performed the mobility shift assay using nuclear extracts from an *o2* null mutant (Aukerman et al., 1991). Interestingly, partial binding is obtained from the *o2* extract (Figure 7A), suggesting that there are proteins other than O2 in endosperm nuclei that can bind to the O2 target site. As expected, this *o2* null binding activity is unaffected by either preimmune or immune sera to O2 (Figure

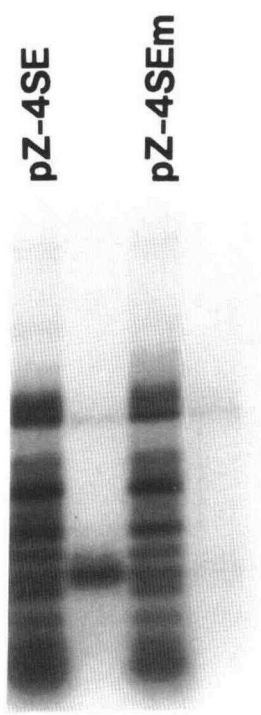


Figure 6. Binding of O2 to the Wild-Type and Mutant Target Sites.

Plasmids pZ-4SE and pZ-4SEm were digested with Sau3AI and HinfI, end labeled, and incubated with the immunoselected β-gal::O2 fusion protein. The lane under each plasmid designation shows the labeled restriction fragments that were incubated with the fusion protein. Adjacent lanes show the fragments that were specifically retained by the β-gal::O2 fusion. Plasmid pZ-4SEm contains a G-to-A change in the ACGT core of the O2 target site.

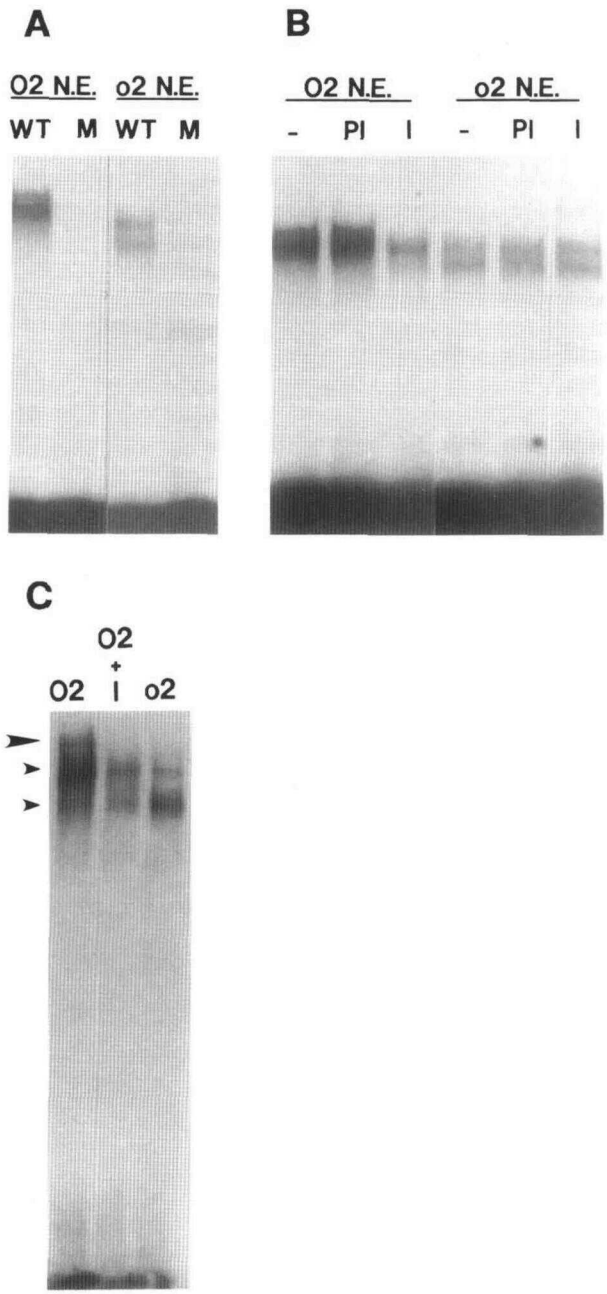


Figure 7. Mobility Shift Assay with Nuclear Extracts from Maize Endosperm Cells.

(A) Autoradiograph shows the band shift from incubating nuclear extracts isolated from wild-type (O2 N.E.) or *o2* null (o2 N.E.) endosperms with the labeled O2 binding site probe from pZ-4SE (WT) or the mutant binding site probe from pZ-4SEm (M).

(B) Wild-type (O2 N.E.) or *o2* null (o2 N.E.) nuclear extracts were incubated with preimmune sera (PI) or immune sera against O2 (I), or left untreated (–) prior to performing the binding assay with the labeled O2 binding site probe from pZ-4SE.

(C) Binding reactions with nuclear extracts and labeled probe from pZ-4SE were subjected to longer electrophoresis time. The complexes formed in the wild-type extract (O2) were resolved into three bands (arrowheads). The uppermost complex, indicated by the larger arrowhead, is completely abolished by preincubation of the extract with immune sera against O2 (O2 + I). This complex is also absent in a binding reaction performed with an *o2* null extract (o2).

7B). Increased resolution of the *o2* null binding activity indicates that its pattern resembles that of wild-type extract treated with O2 sera; i.e., the uppermost band is absent, and the middle band is reduced relative to wild type (Figure 7C). Thus, by two independent criteria, we have demonstrated that the O2 protein in endosperm nuclei recognizes the O2 target sequence in zein gene promoters that was identified by experiments performed with the β-gal::O2 fusion protein from *E. coli*.

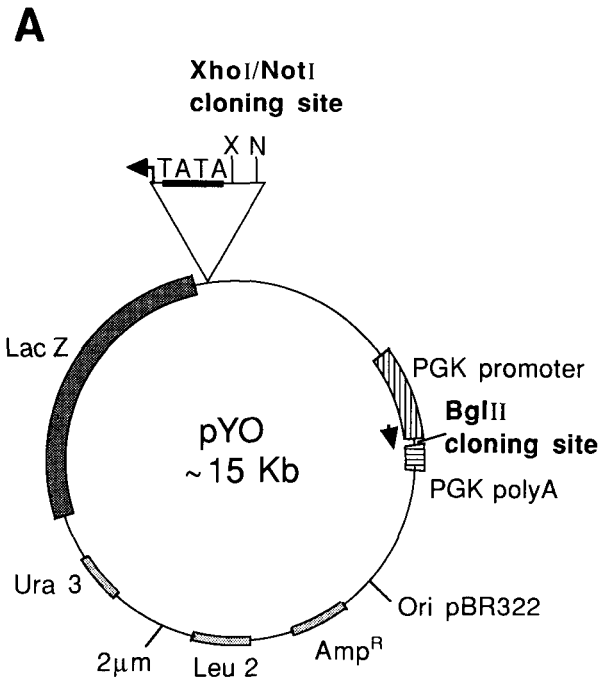
O2 Activates Transcription from a Promoter Containing the O2 Target Site

We next asked if O2 could activate expression of a reporter gene that contained the O2 binding site. To perform these analyses, we used a derivative of a yeast/bacteria shuttle vector (Metzger et al., 1988) to generate a construct that expresses O2 under the control of the phosphoglycerate kinase promoter and that contains the O2 target site upstream of a minimal promoter driving the *E. coli lacZ* gene (Figure 8A). Because spacing between the target site and other promoter elements can be important to transcriptional activation, we made a multimer of the O2 target site to increase the likelihood that a suitable phasing would be presented to the transcriptional machinery. Shown in Figure 8B are the results from several analyses on different constructs. With plasmids lacking either O2 or the O2 target site, only background levels of β-gal activity could be detected. However, in constructs that express O2 and also contain a pentamer of the O2 target site, levels of β-gal activity increased as much as 600-fold over background (Figure 8B). Highest levels of *lacZ* gene expression were obtained from those constructs containing a deletion of the untranslated leader region in the O2 cDNA. A plasmid containing the O2 cDNA in inverse orientation produced only background levels of β-gal activity (Figure 8B). These results demonstrate that O2 is a transcriptional activator and that it can activate transcription of a gene containing the *O2* target site as part of its promoter.

DISCUSSION

The Binding Site for O2 Is Only Found in 22-kD Zein Genes

Mutations in *o2* have long been known to cause severe reductions in the accumulation of zein proteins (Mertz et al., 1964).



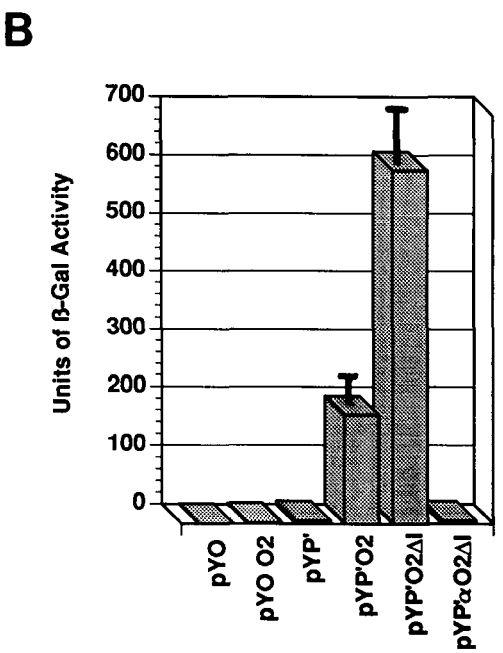


Figure 8. Transcriptional Activation by O2 in Yeast.

(A) Diagram of the yeast/bacteria shuttle vector used to study O2-mediated activation of transcription. The O2 cDNA was inserted into the unique BglII site situated between the yeast phosphoglycerate kinase (PGK) promoter and polyadenylation signal. A multimer of the O2 target site was inserted directionally into the XhoI-NotI cloning site upstream of the *E. coli lacZ* gene. The *lacZ* reporter gene is under the control of the *GAL1* minimal promoter that has been deleted for the GAL4 upstream activating sites.

(B) β-Galactosidase gene expression activated by O2. Yeast cells, transformed with the indicated vectors, were grown to midlog phase and assayed for *lacZ* expression. pYO is the plasmid backbone alone. pYO O2 expresses O2 but is lacking the O2 target site. pYP′ contains a pentamer of the O2 target site but lacks O2. pYP′O2 has the pentamer of the O2 target site and expresses O2. pYP′O2ΔI has the pentamer of the O2 target site and expresses O2 from an O2 cDNA containing a deletion in the untranslated leader. pYP′αO2ΔI has the O2ΔI sequence inserted in the opposite orientation from that in pYP′O2ΔI.

Their effect on zein accumulation is most noticeable among members of the 22-kD class, whereas the closely related 19-kD zeins and other zein size classes are not as severely affected (reviewed in Motto et al., 1989). This correlates with the greater reduction in the 22-kD zein mRNAs relative to other zein mRNAs (Burr et al., 1982; Langridge et al., 1982). Kodrzycki et al. (1989) showed that these reductions are due to a decreased rate of 22-kD zein transcription in the *o2* mutant. Here we have shown that there is a specific sequence in the promoter of 22-kD zein genes to which O2 can bind, and that, on the basis of DNA binding analyses and computer-assisted searches of the GenBank data base, this sequence is not present in promoters of the closely related 19-kD zein genes or in the promoters of 27- and 10-kD zein genes.

The O2 footprint observed in DNase I protection assays encompasses the imperfect palindromic sequence TCCACGTAGA. We have shown that a mutation within the ACGT core of this sequence interferes with O2 binding. Because many bZIP proteins recognize target sites containing short palindromic sequences having an ACGT core, it is likely that the 10-bp imperfect palindrome is the recognition sequence for O2 in the 22-kD zein gene. In this study, we have not determined the importance to O2 binding of nucleotides outside of the ACGT core. However, in Ueda et al. (1992), we show that two nucleotides flanking the ACGT core are also critical to high affinity binding by the O2 protein and to the ability of O2 to activate transcription in maize endosperm cells from a promoter containing the O2 target site.

As shown in Figure 9, the O2 target site lies about 20 nucleotides 3′ of the −330 consensus sequence, a 15-bp sequence that is conserved in all zein genes (Brown et al., 1986). This highly conserved sequence is sometimes referred to as the "prolamin box" due to its presence at about the same location in the promoters of prolamin genes from other cereals. The significance of this sequence to zein gene expression is not known. A protein from endosperm nuclei will bind to zein gene promoters and protect this site from DNase I digestion (Maier et al., 1987). The close proximity of the O2 target site to the prolamin box might indicate that the proteins that bind in this region, namely O2 and the prolamin box binding protein, may work in concert to promote high levels of 22-kD zein gene expression. Examples exist (Liu and Green, 1990) where cooperation between transcription factors on eukaryotic promoters leads to elevated levels of gene expression. One possibility is that the prolamin box, which is present in all zein

Figure 9. Comparison of the −300 Regions of 22- and 19-kD Zein Gene Promoters from *22Z-4* and *zE19*.

The −330 consensus sequence (prolamin box) that is absolutely conserved in both 22- and 19-kD zein genes is indicated. The O2 target sequence in the 22-kD zein gene is also indicated.

genes, provides the endosperm-specific component to zein gene expression. The O2 target site, found only in 22-kD zein genes, would provide a mechanism for the specific enhancement in expression of this class alone. The potential interaction between the proteins that bind these two sites should be amenable to study using the particle gun to introduce reporter gene constructs under the control of the zein promoter into endosperm tissue. The usefulness of the particle gun in dissecting the interaction of regulatory genes on a promoter has already been demonstrated in the case of the maize *C1* and *R* regulatory genes on the *Bronze-1* promoter/reporter constructs (Goff et al., 1990, 1991; Roth et al., 1991).

The O2 Binding Site Sequence in 22-kD Zein Genes Is Different from the Binding Sites in the *b-32* Gene

O2 plays a role in regulating the expression of another endosperm protein, b-32 (Soave et al., 1981; Di Fonzo et al., 1986; Manzocchi et al., 1986). The *b-32* gene encodes a water-soluble 32-kD albumin that accumulates in the cytosol during endosperm development (Soave et al., 1981; Di Fonzo et al., 1986). In *o2* mutants, the level of b-32 mRNA is reduced to 5 to 10% of wild-type levels. DNase I footprinting onto the promoter of the *b-32* gene using a glutathione-*S*-transferase::O2 fusion protein identified a series of five putative O2 binding sites having a consensus sequence of GATGAPyPuTGPu (Lohmer et al., 1991). Surprisingly, neither the consensus sequence nor any of the individual sites to which O2 is reported to bind in the *b-32* promoter corresponds to the O2 binding site that we have identified in 22-kD zein genes. Although plant bZIP proteins can recognize sites that differ slightly from the preferred target site (Oeda et al., 1991; Tabata et al., 1991), strong binding has not been observed to sites that fail to contain an ACGT core. In our own in vitro DNA binding assays, we found that O2 can recognize the target sites bound by the histone binding protein (HBP-1) and the G-box binding factor (GBF-1), although not as strongly as it does the O2 target site in the 22-kD zein promoter (G. Hoscheck and R. Schmidt, unpublished data). Each of these target sites recognized by O2 contains the ACGT core common to the recognition sequence of many plant bZIP proteins (Table 1). This ACGT core is critical to O2 binding because we have demonstrated that O2 does not bind strongly to a mutant site where ACGT has been replaced by ACAT (Figure 6). Target site recognition by other plant bZIP proteins also is known to be affected by mutations in the ACGT core (Guiltinan et al., 1990; Oeda et al., 1991; Tabata et al., 1991). Surprisingly, only one of the five sites (box 5) in the *b-32* promoter contains a sequence having the ACGT core. This sequence, GTTGACGTGA, is still strikingly different from the site in 22-kD zein genes.

On the basis of similarity in their consensus (GATGAPyPuTGPu) to the sequence (GATGATGCATG) adjacent to the prolamin box of a 22-kD zein gene, Lohmer et al. (1991) suggested that O2 recognizes this site in 22-kD zein promoters. As can be seen in Figure 5 this precise sequence is only present in the pseudogene, *Z7*, and not in other 22-kD zein genes. However, a similar sequence (GATCATGCATG), at the corresponding position, is present in each of the other 22-kD zein genes. We have shown that this site is clearly not the preferred recognition site in 22-kD zein genes. The Sau3AI restriction fragment that we used for DNA footprinting and DNA binding contains this region of the zein promoter, yet O2 does not strongly footprint over this sequence. Also, although this sequence was present in both pZ-4SE and pZ-4SEm, O2 bound strongly only to the fragment from pZ-4SE. The corresponding fragment in pZ-4SEm, to which O2 did not bind, contained a change in the ACGT core of the TCCACGTAGA target sequence but had the GATCATGCATG sequence intact.

The observed discrepancy in target sites identified by Lohmer et al. (1991) and us may reflect physiologically significant differences in the interaction of O2 on the promoter of *b-32* versus 22-kD zein genes. Alternatively, it may be a function of the conditions used for the in vitro DNA binding analyses. Our two groups have expressed O2 as fusions to different proteins and performed different types of DNA binding assays under different conditions. Because all of these DNA binding studies are based on analyses performed in vitro using O2 fusion proteins produced in *E. coli*, it is important to demonstrate that the putative target site functions to bind the O2 protein made in endosperm cells. Using the target site from the 22-kD zein gene promoters, we have shown, through gel shift analyses, that the O2 protein from isolated endosperm cell nuclei binds to this site (Figure 7). The putative O2 target sites in the *b-32* promoter have not been shown to bind the O2 protein in maize endosperm.

O2 May Form Heterodimers with Other Proteins

Our mobility shift assays indicate that O2 is not the only protein in endosperm nuclei that can bind to the O2 target site. In extracts from wild-type nuclei, at least three different complexes are observed (Figure 7C). Only the upper complex is completely eliminated by preincubation with O2 antibodies, and it is this same upper complex that is completely absent in *o2* nuclear extracts (Figure 7C). The O2 antibodies also appear to reduce the signal from the middle of the three complexes, suggesting that the antibodies recognize similar epitopes in these proteins or that O2 is also a part of this complex. One possibility is that in maize endosperm cells O2 interacts with the zein promoter as a heterodimer with another bZIP protein. A subset of the shifted complexes seen with wild-type extracts may represent a combination of O2 homodimers and heterodimeric complexes between O2 and other proteins. Previously, we have presented indirect evidence in support of O2 interacting with the zein promoter in vivo as a heterodimeric complex (Aukerman et al., 1991). A subset of the wild-type binding activity must be independent of O2 because some of the complexes are still present in the *o2* null extract (Figure 7).

Yeast Is a Useful Heterologous System for Evaluating Transactivation by O2

Through our in vitro DNA binding analyses, we had previously shown that O2 was capable of binding to 22-kD zein gene promoters in the absence of other maize proteins. From those experiments, we concluded that O2 could bind its target site as a homodimer. Left unresolved was the question of whether O2 was capable of activating transcription upon binding as a homodimer or if transcriptional activation would require a heterodimeric complex between O2 and another bZIP protein. In this study, we have demonstrated that O2 can activate gene transcription in the absence of other maize proteins. When O2 is expressed in yeast, it can activate the transcription of the *lacZ* gene containing a multimer of the O2 target site as a component of its promoter. Furthermore, in Ueda et al. (1992), we show that in maize endosperm cells O2 is capable of activating transcription from a single binding site when that site is in the context of the *22Z-4* promoter.

The domain in the O2 protein responsible for transactivation has not been identified. We anticipate that by using a combination of the yeast and endosperm suspension cell culture systems we will be able to quickly define the O2 activation domain as well as other important functional domains of this protein. In addition, the possibility exists for utilizing yeast as an efficient system to study the details of the interaction of O2 with its target site as well as its interaction with other bZIP proteins and the RNA polymerase II complex.

METHODS

Bacterial Plasmids

Plasmids pZ-1B and pZ-2BS are clones of *22Z-1* and *22Z-2* (Z1 and Z2; Aukerman et al., 1991) that were derived from a single 14-kb XhoI genomic restriction fragment (Schmidt et al., 1990). Clones of *22Z-3* and *22Z-4* were derived from a separate 14-kb XhoI genomic restriction fragment that was isolated at the same time as was the XhoI restriction fragment containing *22Z-1* and *22Z-2*. Subclones from the original genomic clones were generated in pBluescript KS+ (Stratagene). Plasmid pZ-3BS contains a 3-kb BamHI-SalI fragment that begins 1.1 kb 5′ of the *22Z-3* coding sequence. Plasmid pZ-4B contains a 4-kb BamHI fragment that begins 1 kb 5′ of the *22Z-4* coding sequence. In addition to encompassing the P2 promoter region from *22Z-4*, clone pZ-4B extends into the P1 promoter region of *22Z-3*. Plasmid clones pUE2 and pUE10 were a gift from A. Viotti. These clones were derived from a genomic restriction fragment containing a tandem duplication of 19-kD zein genes *zE19* and *zE25* (Spena et al., 1982). Each of the subclones was generated in the plasmid vector pUC8. Clone pUE2 is a 4.1-kb BamHI fragment that spans the entire intragenic region between *zE19* and *zE25* and therefore includes the *zE25* promoter. Plasmid pUE10 is a 4-kb EcoRI-BglII fragment that begins 2 kb 5′ of the *zE19* coding sequence. Plasmid p19A1 is a subclone of the *o2*-dependent 22-kD zein gene, *zA1* (Spena et al., 1982). Clone p19A1 is a 2-kb BamHI-EcoRI subclone in pUC19 that includes nearly 900 bp upstream of the translation start in *zA1*.

DNA Binding Assays

All DNA binding assays utilized β-galactosidase–O2 fusion proteins (β-gal::O2) that were expressed in *Escherichia coli* and partially purified by immunoselection with antibodies to β-galactosidase, as described previously (Schmidt et al., 1990). To assay for DNA binding, 15 μL of protein A–Sepharose beads containing the β-gal::O2 immunocomplex was resuspended in 40 μL of DNA binding buffer (150 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM Tris-HCl plus 0.1 mg/mL salmon sperm DNA) at pH 7.8. Approximately 40 ng of ^{32}P-labeled Sau3AI digests of plasmid DNA containing the zein promoter fragments was incubated with the O2 fusion protein for 30 min at room temperature with intermittent agitation. Washes, processing, and agarose gel electrophoresis of the retained fragment were performed as described previously (Schmidt et al., 1990). Plasmid pZ-4SE is a pUC18 derivative that contains a 37-bp Sau3AI-EcoRI restriction fragment from *22Z-4* (Figure 5) that contains the O2 target site, TCCACGTAGA. To generate plasmid pZ-4SEm, an oligonucleotide was made to the 37-bp *pML1* sequence (Figure 5), except that an EcoRI restriction site was generated at the 3′ end. Mutually primed synthesis was then used to generate a double-stranded fragment. A 32-bp NsiI-EcoRI restriction fragment was isolated and substituted for the NsiI-EcoRI fragment from *22Z-4* in pZ-4SE. The presence of the correct sequence was confirmed by sequencing. Plasmid pZ-4SEm has two differences relative to the sequence in pZ-4SE, one within the O2 target site (TCCACATAGA) and another two nucleotides 3′ of the target site (Figure 5). The change outside of the target site was considered insignificant because it lies outside of the O2-protected nucleotides seen with the DNase I footprinting, and the nucleotides at this position are not conserved among the other 22-kD zein genes that bind O2 (Figure 5). Both plasmid DNAs were digested

with a combination of Sau3AI and HinfI prior to end labeling. Sau3AI alone was not used because this would have generated a 2-kb restriction fragment containing the O2 target site, which is not retained as well by the immunoselected β-gal::O2 fusion. All digested DNAs were end labeled with α-^{32}P by using the Klenow fragment of DNA polymerase I.

DNase I Footprinting

The 200-bp Sau3AI restriction fragment from *22Z-2* that contains the O2 target site (Figure 2) was cloned into the BamHI site of pBluescript KS+. An XbaI-HindIII fragment containing the 200-bp Sau3AI sequence and flanking polylinker sites was isolated from agarose gels by Elutip purification (Schleicher & Schuell) after 5′ end labeling on one or the other strand with T4 polynucleotide kinase and γ-^{32}P-ATP. For each reaction, 150,000 cpm (about 20 fmol) of end-labeled DNA was incubated with 2, 4, 8, or 16 μL of protein A–Sepharose beads containing the immunoprecipitated β-gal::O2 fusion in a total volume of 50 μL of binding buffer containing 12.5 mM Hepes, pH 7.5, 50 mM KCl, 10% glycerol, 0.05% Nonidet P-40, and 0.5 mM DTT. Reactions were gently mixed every 2 to 3 min for 15 min at 4°C, followed by 1 min without mixing at 25°C. An equal volume of 10 mM $MgCl_2$, and 5 mM $CaCl_2$ was added to the reaction and, after mixing, was allowed to incubate another minute at 25°C. Two microliters of a freshly diluted stock (10 ng/μL) of DNase I (Pharmacia LKB Biotechnology Inc., 3.3 units/μg) was added, the contents were mixed by vortexing, and the reaction was stopped after 1 min by the addition of 90 μL of stop buffer (20 mM EDTA, 1% SDS, 0.3 M NaCl, 250 μg/mL tRNA) followed by vortexing. Each sample was extracted with 100 μL of phenol, followed by extraction with 100 μL of a 24:1 $CHCl_3$/isoamyl alcohol mixture. The DNA was ethanol precipitated from the aqueous phase, and the labeled fragments were separated by electrophoresis on an 8% sequencing gel. The same end-labeled DNA was treated with the chemical cleavage reactions (Maxam and Gilbert, 1980) including modifications (Eckert, 1987) and run on lanes adjacent to the DNase footprinting reactions.

Nucleotide Sequence Analysis

Clones of the zein promoter fragments were sequenced by the dideoxy nucleotide method employing the Sequenase enzyme (United States Biochemical Corp.) or modified T7 polymerase from Pharmacia LKB Biotechnology Inc. The sequence of the *22Z-4* clone required the prior generation of overlapping deletions using exonuclease III as described in the Unidirectional Deletion Kit (Pharmacia LKB Biotechnology Inc.).

Nuclear Extracts

Whole kernels from plants homozygous for either the wild-type *O2* or null *o2* allele were harvested at 15 to 18 days after pollination, quick-frozen in liquid nitrogen, and stored at −80°C. All subsequent extraction steps were performed at 0 to 4°C. To isolate nuclei, 20 g of frozen kernels were ground briefly (about 5 sec) in a precooled electric coffee mill, and the powder was transferred to a mortar containing 10 mL of HB (10 mM Pipes, pH 7.0, 10 mM NaCl, 10 mM $MgCl_2$, 250 mM sucrose, 1.0 M hexylene glycol, 20% [v/v] glycerol, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride [PMSF]). The thawed tissue was ground gently with a pestle for 1 to 2 min, and an additional 30 mL of HB was stirred into the extract for a final volume of 40 mL. The extract was filtered through one layer of Miracloth (Calbiochem), and the filtrate was transferred into 30-mL Corex tubes and underlaid with 5-mL cushions of a Percoll solution (81% [v/v] Percoll [Pharmacia LKB Biotechnology Inc.], 10 mM Pipes, pH 7.0, 10 mM NaCl, 3 mM $MgCl_2$, 250 mM sucrose, 0.5 M hexylene glycol, 5 mM 2-mercaptoethanol, 0.1 mM PMSF). Centrifugation was performed in a rotor (model No. JS13.1, Beckman Instruments) for 10 min at 2500*g*. The supernatant above the HB/Percoll interface was removed by aspiration, and the yellow layer containing nuclei and membranes was collected and transferred to a fresh tube. The nuclei were gently resuspended in 10 mL of 10 mM Hepes, pH 7.9, 25 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and centrifuged in a rotor (model No. JA20, Beckman Instruments) for 10 min at 3000*g*. After aspiration of the supernatant, the pellet was resuspended in 5 mL of 20 mM Hepes, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and sonicated to disrupt the nuclei. The lysate was mixed by rotation for 15 min at 4°C and then centrifuged in a rotor (model No. Ti70.1, Beckman Instruments) for 40 min at 100,000*g*. The supernatant was dialyzed for 4 hr against 10 mM Hepes, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 15% glycerol, with two 500-mL buffer changes. The extracts were concentrated twofold to threefold by centrifugation in Centricon-30 filter/concentrators (Amicon, Beverly, MA), and frozen at −80°C until needed.

Mobility Shift Assays

Plasmids pZ-4SE and pZ-4SEm were digested with HindIII and EcoRI, which cut in the pUC18 polylinker and thus released the 37-bp O2 target site fragments. The resultant fragments were end labeled with ^{32}P and the Klenow fragment, and gel purified away from the vector on 10% polyacrylamide gels. Eight micrograms of wild-type or *o2* mutant extract was incubated 5 min at room temperature in binding buffer (10 mM Hepes, pH 7.9, 50 mM KCl, 1 mM EDTA, 10 mM DTT, 2 mg/mL BSA, 100 μg/mL salmon testes DNA, 10% glycerol). Where indicated, 1 μL of preimmune or immune O2 sera (Varagona et al., 1991) was included in the incubation. End-labeled O2 target site fragment was then added to the above mixture to give a total reaction volume of 25 μL, and the reaction was incubated for 20 min at room temperature. Bromophenol blue dye was then added, and the samples were loaded onto a 4% 0.25 × TBE polyacrylamide gel. Electrophoresis was carried out in 0.25 × TBE at 4°C. The gel was dried onto Nytran membranes and exposed to autoradiographic film overnight.

Construction of Yeast Plasmids

The yeast/bacteria shuttle plasmids used in this study are all derivatives of pYO (a gift of C. Hart, Centre National de la Recherche Scientifique, Strasbourg, France). This plasmid was generated by deleting the estrogen responsive element (ERE) from upstream of the *lacZ* reporter gene and deleting the human estrogen receptor (hER) cDNA sequence from the plasmid pYERE/HER (Metzger et al., 1988). The plasmid pYP′ was generated by inserting a pentamer of the O2 binding site (described below) into the XhoI-NotI site upstream of the *lacZ* reporter gene in pYO. Insertion of the O2 cDNA in the sense orientation places O2 under the control of the yeast phosphoglycerate kinase constitutive promoter in pYO or pYP′ to generate pYO O2 and pYP′O2, respectively. Insertion of an O2 cDNA that has been deleted to −63 relative to the initiation codon (O2ΔI) into pYP′ generates pYP′O2ΔI. Plasmid pYP′αO2ΔI is identical to pYP′O2ΔI except that O2ΔI is in the antisense orientation. O2ΔI has all three upstream open reading frames

(Schmidt et al., 1990) deleted. All of the plasmids were transformed into yeast strain RH151-7B (*ura⁻ trp⁻ leu⁻*) as described previously (Treco, 1987). The transformants were plated onto synthetic complete media (SCM) (Treco, 1987) lacking uracil, then replated onto SCM lacking uracil and leucine. The transformants were grown from stationary phase to midlog phase with constant agitation at 30°C in SCM liquid media lacking uracil and leucine. β-Galactosidase activity was measured as previously described (Reynolds and Lundbald, 1987). During this period of growth, about 30% of the cells would lose their plasmid regardless of which plasmid they carried. Because we did not observe any differences in plasmid loss among transformants, β-galactosidase activities were not corrected for the number of cells containing the plasmid. Expression of every construct was examined with at least five independent transformants.

A pentamer of the O2 binding site was constructed in the following fashion: a dimer was created by digesting plasmid pZ-4SE with NsiI and EcoRI, isolating the fragment containing the O2 target site, treating with T4 DNA ligase, and cloning the resulting fragments into EcoRI-cut pBluescript KS+. Dimers were confirmed by DNA sequencing and used to generate trimers. A plasmid containing the dimer was digested with BamHI and NsiI to generate a fragment containing a single target site. An additional digestion was performed on the dimer-containing plasmid with HindIII and PstI to release a fragment containing the entire dimer. The monomer and dimer fragments were combined, treated with T4 DNA ligase to join the monomer and dimer at the common PstI-NsiI overhanging ends, and finally mixed and ligated into a pBluescript KS+ plasmid cut with BamHI and HindIII. The plasmid, now containing a trimer of the O2 target site, was used to generate the pentamer by repeating the procedure described above. This method is derived from that described previously (Eisenberg et al., 1990). The pentamer consists of five repeats of the sequence 5′-TGCATGTCATTCCACGTAGAGAAAAGAATTCT-3′ with four of the repeats in one orientation and one in the other orientation. Directional cloning into the pYO vector generates a pentamer binding site having four of the repeats oriented in the same direction (relative to the transcription start) as is the O2 binding site in the 22-kD zein gene promoter.

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