The Plant Cell, Vol. 5, 227–236, February 1993 © 1993 American Society of Plant Physiologists

OHP1: A Maize Basic Domain/Leucine Zipper Protein That Interacts with Opaque2

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***OPAQUE2* (*O2*) is a regulatory gene that predominantly affects the expression of the 22-kD class of zein storage protein genes at the level of transcription. The *O2* gene encodes a polypeptide belonging to the basic domain/leucine zipper (bZIP) class of transcriptional regulatory proteins. Our prior analyses have demonstrated that the O2 protein binds 22-kD zein gene promoters as a homodimer in vitro and have also suggested that O2 may bind as a heterodimer in vivo. To identify cDNAs encoding other bZIP motifs that might interact with O2, a portion encoding the bZIP motif from an O2 cDNA was used to screen an endosperm cDNA library. Sequence analysis of one isolated recombinant phage indicated the presence of a bZIP motif similar to O2. The protein product of this partial cDNA, designated OHP1, can bind the O2 target site both as a homodimer and in a heterodimeric complex with O2. Whole genome DNA gel blot analysis of maize recombinant inbreds revealed two strongly hybridizing restriction fragments, neither of which mapped close to any locus known to affect zein expression. RNA gel blot analysis revealed an ~1.7-kb transcript that is expressed in all organs examined except the female flower and is also expressed in endosperms homozygous for *o2* and other mutations that affect zein expression (*opaque7*, *floury2*, and *Defective endosperm b-30*). Based on these results and previously reported data, we propose models to accommodate OHP1 in the regulation of zein gene expression by O2.**

INTRODUCTION

The major class of seed storage protein in the maize endosperm is zein. Using SDS-PAGE, zein can be separated into polypeptides of distinct size classes: 27, 22, 19, 16, and 10 kD (Esen, 1986). These different size classes are encoded by distinct classes of structural genes that belong to a multigene family (Hagen and Rubenstein, 1981; Burr et al., 1982; Marks and Larkins, 1982). The expression of this multigene family is coordinately regulated during seed development (Marks et al., 1985). Regulation of zein gene expression is controlled by several different loci: *OPAQUE2* (*O2*), *FLOURY2* (*FL2*), *OPAQUE7* (*O7*), and *DEFECTIVE ENDOSPERM B-30* (*DEB30*) being among the more important (reviewed in Motto et al., 1989). The effect of mutations in these regulatory genes on the expression of specific classes of zein genes has been characterized (Di Fonzo et al., 1980; Burr and Burr, 1982; Soave and Salamini, 1984).

One of the more severe effects in zein gene expression arises from mutations in *O2* (for review, see Schmidt, 1993). Seeds homozygous for *o2* mutations show a severe decrease in the expression of the 22-kD class of zeins (Burr and Burr, 1982; Langridge et al., 1982). This effect is due to a decrease in transcription from these 22-kD zein genes (Kodrzycki et al., 1989). The *O2* gene has been cloned (Schmidt et al., 1987; Motto et al., 1988), and the O2 protein has been shown to bind to the promoter of 22-kD zein genes (Schmidt et al., 1990; Aukerman et al., 1991). This binding is sequence specific, and, when this O2 target sequence is placed upstream of a minimal promoter, it confers O2-specific control on reporter gene expression using various reporter genes (Schmidt et al., 1992; Ueda et al., 1992).

O2 is a member of the basic domain/leucine zipper (bZIP) class of transcriptional regulatory proteins (Hartings et al., 1989; Schmidt et al., 1990). The bZIP motif is a bipartite DNA binding motif consisting of a highly conserved stretch of basic amino acid residues followed by a heptameric repeat of leucine or other small hydrophobic residues (Kouzarides and Ziff, 1988; Turner and Tjian, 1989). This heptameric repeat was originally called the leucine zipper (Landschulz et al., 1988), and is responsible for dimerization through the formation of a coiled coil structure (O'Shea et al., 1989). This leucine zipper domain determines the ability of different bZIP proteins to interact. Some bZIP proteins can form functional homodimers and can interact with other bZIP proteins to form functional heterodimeric complexes (i.e., the *jun* family). Other bZIP proteins can form only functional homodimers (*GCN4*), whereas some form only functional heterodimers (the *fos* family) (for review see Abel and Maniatis, 1989). These different combinations of bZIP proteins can mediate different patterns of expression of the genes under their control (Chiu et al., 1989; Schutte et al., 1989).

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Our analysis of a mutant *o2* allele, *o2-676*, suggests that other maize bZIP proteins may mediate the interaction of O2 with the zein promoter (Aukerman et al., 1991). The o2-676 protein contains an arginine-to-lysine mutation in its basic domain that prevents the protein from interacting with the O2 target site in vitro. In vivo, however, the o2-676 phenotype is intermediate between that of an O2 (wild-type) plant and an o2 null plant with respect to both the levels of zein protein accumulation and 22-kD zein mRNA. The expression of the 22-kD zeins is greater in *o2-676* mutants than in null mutants, even though the bacterially expressed o2-676 protein behaves as a null mutant with respect to in vitro DNA binding (Aukerman et al., 1991). Based on these observations, we suggest that O2 may form a heterodimer with another bZIP protein in the endosperm. Heterodimer formation of o2-676 with another bZIP protein that binds the O2 target site might partially compensate for the o2-676 defect and result in the observed intermediate zein levels. Mobility shift assays using the O2 target site and proteins isolated from kernel nuclei indicate that another protein(s) besides O2 can recognize the O2 target site and may form heterodimers with O2 (Schmidt et al., 1992).

Because sequence comparison of a number of bZIP proteins from plants revealed a striking conservation among their basic domains (see Weisshaar et al., 1991), a DNA fragment encoding the basic domain and a portion of the leucine zipper of *O2* was used as a probe to screen an endosperm cDNA library to identify cDNA clones encoding bZIP motifs. Using reduced stringency hybridization techniques, a novel partial cDNA clone was isolated that encodes a bZIP protein which we have designated as O2 heterodimerizing protein 1 (OHP1). The OHP1 protein is able to bind to the O2 target site in the promoter of 22-kD zein genes both as a homodimer (OHP1/OHP1) and as a heterodimer with O2 (OHP1/O2). A second cDNA has also been identified that encodes a second bZIP protein, OHP2, which is 99% identical with OHP1 within the bZIP motif.

RESULTS

Isolation of a cDNA from Maize Endosperm That Encodes a bZIP Protein

An endosperm cDNA library (Aukerman et al., 1991) was screened with a 279-bp StuI-SalI fragment of the O2 cDNA that encodes the basic domain and the first five of seven hydrophobic residues which comprise the leucine zipper of O2. A total of 120,000 phage plaques were screened on duplicate filters under reduced stringency conditions. Six clones that appeared on both of the duplicate filters were isolated and purified. All six of these clones displayed reduced hybridization signals when hybridized at 65°C, as compared to hybridization at 50°C. To ensure that these were not merely unusual isolates of the mutant o2 cDNA (o2-676) present in this library, all six positive clones were screened with a 3′-specific O2 probe. Three of the isolated clones hybridized to this probe. DNA samples from the three phage isolates that did not hybridize to the 3′ *O2* probe were restricted with EcoRI and analyzed on agarose gels.

A 0.9-kb EcoRI fragment from λ clone 44 selectively hybridized to the O2 StuI-SalI probe under reduced stringency conditions. It was subcloned into pBluescript II KS+ and sequenced. The nucleotide and predicted amino acid sequence of the 0.9-kb EcoRI fragment, hereafter referred to as 44M, is shown in Figure 1A. Within this deduced polypeptide is a bZIP motif consisting of a highly basic region followed by a heptameric repeat of hydrophobic residues (L, L, L, V, A, L, L). We have designated this protein OHP1. Using the 3′ 540-bp of the 44M probe, a potentially full-length OHP1 cDNA was isolated in a second screening of the endosperm cDNA library. The sequence of the longest cDNA isolated is shown in Figure 1A. Note that the first ATG codon is located within the context of an optimal plant initiation sequence (Lutcke et al., 1987). Also isolated in the second screening of the endosperm cDNA library is a second cDNA that encodes a bZIP protein highly similar to OHP1. This second protein has been designated OHP2. The sequence of the OHP2 cDNA is shown in Figure 1B.

Two OHP Genes Are Present in the Genome

Gel blot analysis of restriction enzyme digests of maize genomic DNA hybridized with 44M at moderate stringency revealed two strongly hybridizing restriction fragments using a number of different restriction enzymes (data not shown). As shown in Figure 2, by following the inheritance of HindIII-generated restriction fragment length polymorphisms among a population of recombinant inbreds (see Burr and Burr, 1991), the two restriction fragments were mapped to position 46 on chromosome 1L and position 19 on chromosome 5S. At high stringency, 44M hybridized only to the gene on 1L (data not shown), indicating that the *OHP1* gene is located on 1L. Similar analysis of the recombinant inbreds using an internal EcoRV-PstI fragment from the OHP2 cDNA determined that the OHP2 cDNA was the product of the 5S gene. Neither of these positions on 1L or 5S closely matches the map positions of any known morphological mutants affecting endosperm development.

Sequence Comparisons of OHP1, OHP2, and O2

A comparison of the deduced amino acid sequences of OHP1 and OHP2 with O2 shows strong identity through the portion encoding the bZIP motif, as shown in Figure 3, but little similarity outside of this region. The OHP1 and O2 sequences have 76% identity over the 75 amino acids that comprise the bZIP motifs. The identity at the nucleotide level is also 76% over the 225 nucleotides that encode the bZIP motifs. The OHP1

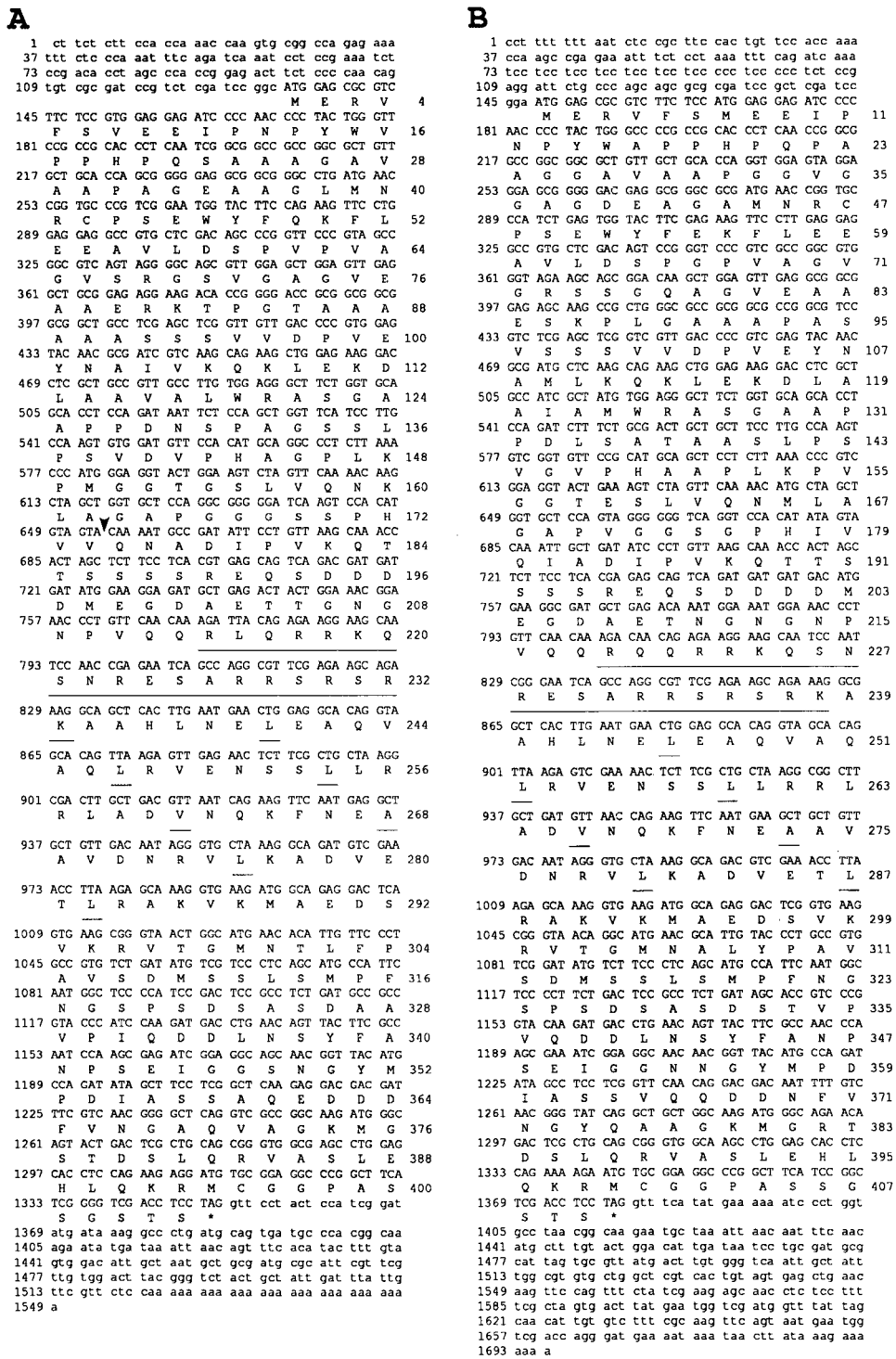

Figure 1. Nucleotide and Deduced Amino Acid Sequence of OHP1 and OHP2.

(A) The sequence of the longest OHP1 cDNA clone. The basic domain and the seven leucine heptad repeats are underlined. The arrow indicates the start of the partial cDNA clone designated 44M. Numbers along the left indicate nucleotide number; those along the right, amino acid residue number (GenBank accession number L00623).

(B) The sequence of the longest OHP2 cDNA clone (GenBank accession number L06478).

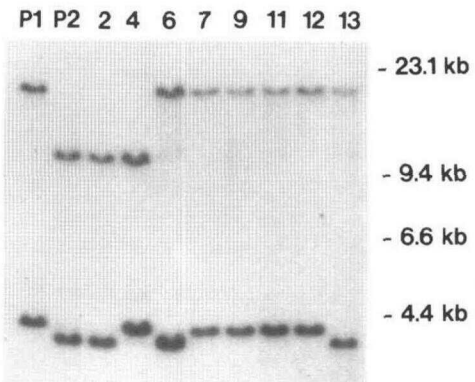

Figure 2. DNA Gel Blot Analysis Using 44M.

DNAs isolated from the two parental lines CM37 (P1) and T232 (P2) and the recombinant inbreds derived from these lines were digested with HindIII and analyzed by DNA gel blot analysis using probe 44M. Four restriction fragments that hybridize to the 44M probe are present within the population. A portion is shown of the film that demonstrates the independent assortment of the four fragments among the progeny. The two fragments map to different locations within the maize genome: one to chromosome 1L, the other to 5S. OHP1 is the product of the 1L gene and OHP2 is the product of the 5S gene. Numbers above the lanes indicate the designations given to the individual recombinant inbreds analyzed. Positions of the length markers are indicated at right.

and OHP2 proteins are 88% identical; within the bZIP motif this identity increases to 99%, as is summarized in Table 1. Excluding the bZIP motif, a GenBank data base search revealed no other protein containing significant homology to the OHP cDNAs, including the other maize bZIP proteins (Singh et al., 1990).

Although all subsequent analyses were performed with OHP1 (44M), the high degree of similarity between the bZIP motifs of OHP1 and OHP2 makes it likely that OHP2 will have similar properties to OHP1 with respect to O2 and the O2 target site (see below).

OHP1 Is Expressed in Numerous Organs and in All Opaque Mutants Tested

RNA gel blot analysis of poly(A)$^+$ RNA probed with a 540-bp 3′ fragment of 44M indicated that an ~1.7-kb transcript is expressed in endosperms, roots, shoots, leaves, and embryos of a W22 inbred, as shown in Figure 4A. No transcript could be found in poly(A)$^+$ RNA isolated from the female flowers (data not shown). The same size transcript is also expressed in endosperms that were homozygous for the *o2*, *o7*, *fl2*, and *Deb30* mutations (Figure 4B). The pattern of expression is unchanged when RNA gel blot analyses are performed at either high or moderate stringency, suggesting that the gel blot analyses reflect the expression of the OHP1 gene.

OHP1 Can Bind the O2 Target Site as a Homodimer and a Heterodimer

The presence of a bZIP motif in 44M suggested that OHP1 would bind to DNA either as a homodimer or as a heterodimer with another bZIP protein. We had previously postulated that O2 would bind the 22-kD zein gene promoter as a heterodimer with another bZIP protein (Aukerman et al., 1991; Schmidt et al., 1992). Because both O2 and OHP1 are expressed in maize endosperm, we asked whether OHP1 and O2 could form heterodimers that bind the target site in the 22-kD zein gene promoter. The O2 and 44M cDNAs were transcribed and translated in vitro, and the protein products were then assayed for their ability to bind to the O2 target site (Schmidt et al., 1992) using electrophoretic mobility shift assays. (Because the original 44M cDNA was incomplete, an initiation codon was introduced into the fragment as described in Methods.) As shown in Figure 5A, both O2 and OHP1 can bind to the O2 target site when translated separately. A mutation in the ACGT core of the O2 target site prevents both proteins from binding and confirms that these interactions are sequence specific (Figure 5A). It is unclear why the OHP1/DNA complex migrates as two separate bands in this assay. On SDS–polyacrylamide gels, the OHP1 translation product migrates as a doublet (Figure 5B, far right lane). It is likely that more than one of these polypeptides is able to bind the DNA probe, which would explain the presence of two bands in the mobility shift assay.

When OHP1 and O2 are cotranslated and assayed for binding to the O2 target site, a complex of intermediate size appears when using the wild-type binding site but not the mutant binding site (Figure 5A). This intermediate complex indicates that OHP1 and O2 are capable of forming heterodimers which also recognize the same target site. The formation of such heterodimeric complexes, however, is not dependent on the two

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                BASIC DOMAIN                          LEUCINE HEPTAD REPEAT
                                        1 *     2     * 3     * 4       5*      6       7
O2      ..RvrkRKeSNRESARRSRyRKAAHLkE LEdQVAQ LkaENSc LLRRiAa lNQKyNd AnVDNRV LrADmET LRAKVKM..
OHP1    ..RlqrRKqSNRESARRSRsRKAAHLnE LEaQVAQ LrvENSs LLRRlAd vNQKfNe AaVDNRV LkADvET LRAKVKM..
OHP2    ..RqqrRKqSNRESARRSRsRKAAHLnE LEaQVAQ LrvENSs LLRRlAd vNQKfNe AaVDNRV LkADvET LRAKVKM..
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Figure 3. Comparison of the bZIP Motifs of OHP1, OHP2, and O2.

Uppercase letters indicate that the amino acid residues are identical in all three motifs. Asterisks indicate the four nonconserved changes within the leucine zippers of OHP1, OHP2, and O2.

Table 1. Comparison of O2 with OHP1 and OHP2

Protein	Gene Map Position	Amino Acid Residues	Predicted Mol. Wt. ($\times 10^{-3}$)	% Identity with O2 bZIP Domain	% Identity with OHP1	% Identity with OHP1 bZIP Domain
O2	7S	437	47.1	–[a]	19	76
OHP1	1L	405	42.3	76	–	–
OHP2	5S	410	42.9	76	88	99

[a] –, 100%.

proteins being cotranslated. When the two proteins are translated independently and then the translation products are mixed, the OHP1/O2 heterodimeric complex is observed in the binding assay (Figure 5A). The formation of the heterodimer upon mixing is temperature dependent; when the two proteins are preincubated at low temperatures (0°C) and then assayed under normal conditions, the amount of heterodimer obtained is very low. As the temperature of preincubation increases (25 and 37°C), the amount of heterodimer formation increases.

Analysis of a number of bZIP proteins isolated from other systems has demonstrated that the heterodimeric complex between two bZIP proteins may have a significantly different affinity for a binding site than either protein as a homodimeric complex. The most well-known example of this involves the mammalian oncoproteins Fos and Jun. The Fos/Jun heterodimer has a greater affinity for the TRE than either the Jun/Jun homodimer, which binds weakly, or the Fos/Fos homodimer, which does not appear to bind at all (Halazonetis et al., 1988; Nakabeppu et al., 1988; Zerial et al., 1989). We determined whether the OHP1/O2 heterodimer had a significantly greater affinity for the O2 target site than did either of the homodimers by observing the effects that decreasing the amounts of labeled target site DNA had on the formation of protein–DNA complexes, as analyzed by electrophoretic mobility shift assays. As shown in Figure 6, all of these species appear to bind the target site with similar affinities. This suggests that, in vivo, if O2 and OHP1 are expressed at equal levels, the O2/OHP1 complex would be twice as likely to be bound as either homodimer based on the stoichiometry above.

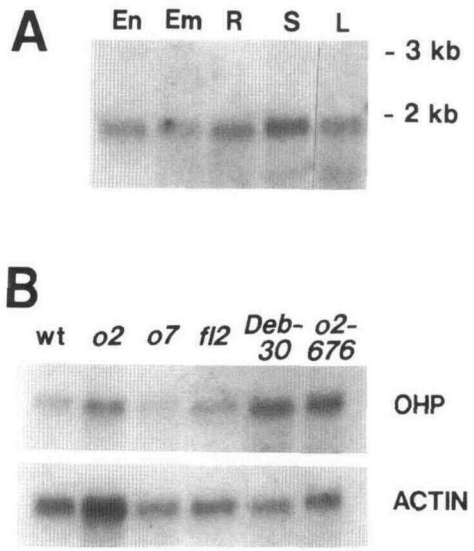

Figure 4. OHP1 Expression in the Organs of the Maize Plant and in Several Mutant Endosperms.

(A) Poly(A)$^+$ mRNAs from W22 endosperm (En), embryo (Em), roots (R), shoots (S), and leaves (L) were analyzed by RNA gel blot analysis using the 3′ 540-bp fragment of 44M. An ~1.7-kb transcript is present in all organs. Positions of the length markers are indicated at right. **(B)** Poly(A)$^+$ mRNAs from endosperms homozygous for mutations known to affect zein expression were analyzed by RNA gel blot analysis using the same probe as in **(A)**. Again, an ~1.7-kb transcript is present in all of these mutants. The actin probe is shown as a loading control. wt, wild type.

DISCUSSION

A new cDNA clone has been isolated that encodes a maize bZIP protein capable of interacting with another known transcriptional regulatory protein, O2. The protein product of this cDNA clone, OHP1, can bind to the O2 target site in the promoters of 22-kD zein genes as a homodimer and as a heterodimer with O2. These results and the observation that both genes are expressed at the same time in the maize endosperm may indicate that OHP1 plays a role in the regulation of 22-kD zein gene expression.

DNA gel blot analysis using a partial cDNA clone of OHP1 (44M) as a probe revealed that two independent loci (one on the 1L chromosome, the other on the 5S) contain sequence of sufficiently high similarity to be recognized by this probe at moderate stringency (Figure 2). These genes are not located at loci that have been identified by characterized mutations, and thus this mapping information provides no indication of the physiological role(s) of the protein products of these genes. Increasing the stringency of the hybridization indicated that the OHP1 cDNA is the product of the gene on chromosome 1L (data not shown). A cDNA product of the 5S gene was

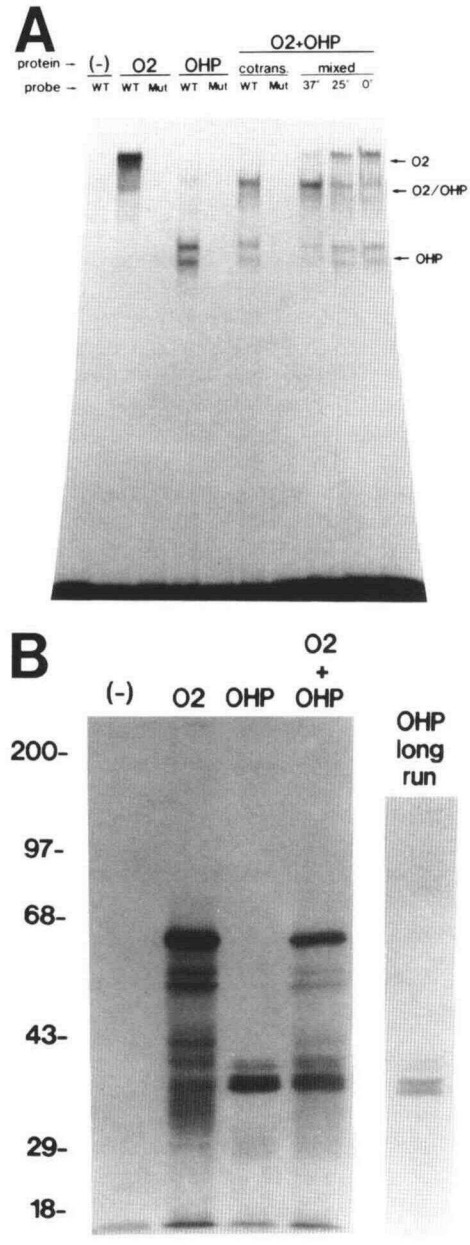

Figure 5. Electrophoretic Mobility Shift Assays with O2 and OHP1 Translated in Vitro.

(A) Assays using the wild-type O2 target site (WT) as a probe and assays using the mutant target site (Mut) are shown. Lanes labeled O2 contain wheat germ lysate programmed with O2 RNA, whereas OHP lanes contain lysate programmed with OHP1 RNA. The lane designated (–) is a reaction performed with unprogrammed lysate. O2 and OHP1 RNAs were cotranslated (cotrans.) to allow heterodimer formation prior to assaying for DNA binding. Alternatively, the RNAs were translated separately, and then the two proteins were mixed together and incubated for 10 min at the three temperatures indicated (37, 25, and 0°C) before assaying. Indicated at right are the positions of the O2/O2 homodimer, the OHP1/OHP1 homodimer, and the O2/OHP1 heterodimer.

isolated in a second screening of the endosperm cDNA library and has been designated OHP2. A number of different restriction fragment length polymorphism probes have been identified that, like 44M, map to both 1L and 5S (Burr and Burr, 1991).

RNA gel blot analyses revealed that the *OHP1* gene encodes an mRNA of ~1.7 kb. With the exception of the floral organs, this transcript is expressed in all organs examined (Figure 4A). Probing poly(A)$^+$ mRNA from root, shoot, leaf, endosperm, and embryo tissue with the 3′ 540-bp fragment of 44M produced an apparent single band ~1.7 kb in length under conditions that allowed hybridization only to the 1L sequence as assayed on DNA gel blots. In contrast, female flower tissue did not express this transcript.

OHP1 appears to be expressed at normal levels in the endosperm tissue isolated from several mutants of maize known to affect zein expression, that is, *fl2*, *o2*, *o7*, and *Deb30*

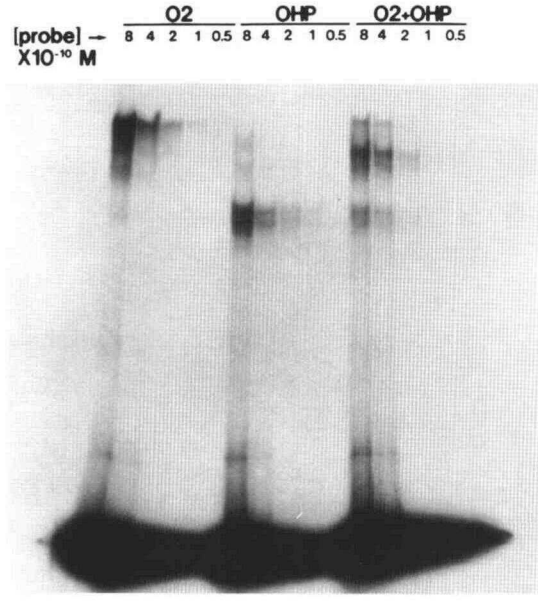

Figure 6. Comparison of DNA Binding Affinities of O2/O2, OHP1/OHP1, and O2/OHP1.

Lysates containing equivalent molar amounts of either O2, OHP1, or an equimolar mixture of O2 and OHP1 were incubated with decreasing concentrations of wild-type target site probe (0.8 to 0.05 nM), and then assayed by mobility shift. The O2/OHP1 heterodimer appears to have roughly the same affinity for the probe as that of O2 alone or OHP1 alone.

(B) Aliquots of lysates programmed with the indicated RNAs were labeled during the translation reaction with ^{35}S-methionine and separated by electrophoresis on an 8% SDS–polyacrylamide gel. Size markers are given in kilodaltons at left. The OHP1 translation product migrates as a doublet, as shown in the longer electrophoresis run (far-right lane).

(Figure 4B). Although the hybridization conditions used in these analyses were sufficiently stringent that the probe was specific for the 1L gene (as assayed on DNA blots), it is formally possible that these analyses reflect the expression of both *OHP1* and *OHP2*. Further analysis of the expression of both of these transcripts is in progress.

Although DNA gel blot analysis under moderate stringency revealed hybridization to two distinct loci, RNA gel blot analysis at moderate stringency revealed only one transcript that hybridizes to the OHP1 probe. This suggests that the transcription products of both the *OHP1* and *OHP2* genes are similar in size and cannot be distinguished on these RNA gel blots. Isolation and characterization of a cDNA product from the 5S gene (*OHP2*) supports this supposition, as the OHP1 and OHP2 cDNA clones differ in size by only 150 bp.

The OHP1 and OHP2 proteins are 76% identical to O2 within the bZIP motif. Although the high degree of identity between the basic domains of these three bZIP proteins is not unique (Weisshaar et al., 1991), the similarity of their leucine zipper domains is unusual. Most bZIP proteins have leucine zippers composed of three to five heptameric repeats of hydrophobic residues, but OHP1, OHP2, and O2 contain seven such heptameric repeats. Although OHP1 and OHP2 have a valine residue at the fourth hydrophobic position (where O2 has a leucine), all have an alanine residue at the fifth hydrophobic position (Figure 3). Conservation of the residues within the leucine zippers is not appreciable among most bZIP proteins; however, a comparison of the leucine zippers of OHP1 and OHP2 with O2 reveals a high identity (74%) and a very high similarity (93%).

Based upon the similarity of the leucine zippers of O2 and OHP1, it is not unexpected that OHP1 forms heterodimers with O2 (O2/OHP1), as well as stable homodimers (OHP1/OHP1) (Figure 5A). These homo- and heterodimeric complexes are specific for the O2 target site in 22-kD zein genes, as evidenced by gel shift analysis using mutant DNA probes which failed to produce a shifted complex (Figure 5A). Not surprisingly, OHP2 is also capable of binding to the O2 target site as a homodimer (data not shown). Although other maize bZIP proteins have been identified (Singh et al., 1990), their possible expression in endosperm was not examined. Likewise, their ability to form stable complexes with O2 that can bind the O2 target site in vitro has not been investigated.

Although we originally hypothesized that the o2-676 protein, which is incapable of binding to the O2 target site in vitro, might heterodimerize in vivo with another bZIP protein and bind weakly to the O2 target site, partially restoring expression of the 22-kD zeins, we have been unable to demonstrate heterodimer formation of OHP1 with o2-676 in vitro (data not shown). This may indicate that (1) OHP1 is not the hypothetical bZIP protein that was proposed to form heterodimers with o2-676 and stabilize the binding of o2-676 to the zein promoter (Aukerman et al., 1991); (2) other maize proteins not present within the gel shift assays are required to stabilize the heterodimer association with the DNA in vivo (see Lamb and McKnight, 1991); or (3) the explanation for o2-676 activity in vivo depends on a mechanism other than stabilization through heterodimer formation or protein–protein contacts.

Because both OHP1 and O2 are expressed in the endosperm and the dimeric OHP1/OHP1, OHP1/O2, and O2/O2 complexes all bind to the O2 target site, regulation of 22-kD zein gene expression may be mediated by one (or more) of these different dimeric complexes through binding the O2 target site and interacting with the general transcriptional machinery and/or other regulatory proteins that bind to the promoter of the 22-kD zein genes.

It is clear that heterodimer formation can have profound effects on the affinities of DNA binding by bZIP or helix-loop-helix proteins (see Lamb and McKnight, 1991). This is exemplified by the Fos/Jun heterodimer, which has been shown to bind its target site much better than either protein alone. Likewise, the helix-loop-helix heterodimers between MyoD, E12, and E47 (Murre et. al., 1989) and between Myc and Max (Blackwood and Eisenmann, 1991) bind their target sequence with higher affinities than either homodimeric complex. These precedents impelled us to test whether the heterodimer between O2 and OHP1 behaved in a similar manner. The data indicate, however, that the O2/OHP1 heterodimer binds no better to the O2 target sequence in the 22-kD zein promoter than does either homodimer alone (Figure 6). This result provides no clue as to the function of OHP1 in vivo. We know that heterodimer formation of O2 with OHP1 is not essential for O2 to activate transcription, because O2 can readily perform this function when expressed in a heterologous system in the absence of other maize factors (Schmidt et al., 1992). It remains a formal possibility, however, that the O2/OHP1 heterodimer possesses novel regulatory properties in vivo that distinguish it from either the O2 or OHP1 homodimers. This property could be either synergistic (coexpression of O2 with OHP1 might result in an enhancement of O2-activated transcription through the O2 target site) or antagonistic (coexpression of O2 with OHP1 may reduce the expression of the 22-kD zein genes).

If the OHP1 homodimer were a transcriptional activator with similar levels of expression and activity to the O2 homodimer, the effects of the loss of O2 in *o2* mutants would not be expected to produce a phenotype because OHP1 and O2 would be functionally redundant. The fact that mutations in *O2* lead to a decrease in 22-kD zein gene expression in spite of the fact that OHP1 is still expressed raises the following possibilities: (1) OHP1 can activate transcription of 22-kD zein genes only when complexed as a heterodimer with O2; (2) expression of OHP1 protein is significantly less than O2, and this level of OHP1 is incapable of activating zein gene expression; or (3) unlike O2 and most bZIP proteins characterized to date, OHP1 is not a transcriptional activator but rather a repressor (Cowell et al., 1992; Karpinski et al., 1992), and the role of O2 as an endosperm-specific factor is to relieve this repression. Finally, it is also a possiblity that the *OHP* gene products, either alone or in association with O2, could regulate genes other than the 22-kD zeins.

METHODS

Library Screening

An endosperm cDNA library constructed from an R802 inbred homozygous for the *o2-676* mutation (Aukerman et al., 1991) was screened with a 279-bp StuI-SalI fragment of the O2 cDNA (Schmidt et al., 1990) under reduced stringency conditions. Approximately 40,000 plaque-forming units were plated on 15-cm-diameter Petri plates. Duplicate lifts were taken from each plate using Amersham Hybond-N filters. The filters were denatured in 0.5 M NaOH, 1.5 M NaCl, 2 mM EDTA for 30 sec followed by neutralization in 0.5 M Tris-HCl, pH 7.0, 3.0 M NaCl, 2 mM EDTA for 30 sec. The filters were then rinsed briefly in 2 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), cross-linked for 1 min on a 310-nm UV light box, and baked under vacuum at 80°C for 2 hr. Filters were prehybridized in 5 × SSPE/5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.5% SDS, 25 μg/mL salmon sperm DNA at 65°C overnight. The filters were hybridized at 50°C for 3 days in fresh prehybridization solution to which 1 to 3 × 10^6 cpm/mL of denatured random primer–labeled probe had been added. The filters were washed in 5 × SSPE once at room temperature, three times for 30 min each in 5 × SSPE, 0.1% SDS at 45°C, and then in 5 × SSPE at room temperature. Filters were wrapped in plastic wrap and exposed to film overnight. To obtain full-length cDNAs for the *OHP1* gene, the same cDNA library was screened as given above at 65°C using the 3′ 540-bp fragment of the partial OHP1 cDNA clone 44M.

DNA Gel Blot Analysis and Mapping

Conditions for DNA gel blot hybridizations were as previously reported (Evola et al., 1986), except that the hybridizations were performed at either 42°C in 50% formamide (moderate stringency) or 55°C in 50% formamide (high stringency). The genome map locations of the isolated clones were obtained by DNA gel blot analysis using the maize recombinant inbreds CM37 and T232 (see Burr and Burr, 1991), and the 0.9-kb EcoRI fragment (44M) and an internal EcoRV-PstI fragment from OHP2 as probes. Computer analysis of the results was furnished by Ben Burr, Brookhaven National Laboratory, Upton, NY.

RNA Gel Blot Analysis

RNA from endosperm harvested 22 days after pollination was isolated from the W22 inbred homozygous for the wild type or the *opaque2* (*o2*), *floury2* (*fl2*), *opaque7* (*o7*), or *o2-676* mutation. *Defective endosperm B-30* (*Deb30*) was in the B37 background. RNA was also isolated from roots, shoots, leaves, embryos, and female flowers of the W22 inbred. All RNAs were poly(A)$^+$-selected using oligo(dT) columns.

Poly(A)$^+$ RNA samples (1.5 μg each) were separated by electrophoresis on a 1% agarose gel containing 7.4% formaldehyde and 1 × Mops solution (20 mM 3-(*N*-morpholino)propanesulfonic acid [Mops], 5 mM sodium acetate, 1 mM EDTA). The gel was stained in a solution of 0.125 μg/mL acridine orange, equilibrated in 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 30 min followed by 20 × SSC for 30 min, and then transferred overnight, using standard blotting techniques (Sambrook et al., 1989), to Amersham Hybond-N or nitrocellulose membranes. Conditions for prehybridization and hybridization were as described previously (Schmidt et al., 1987), except that hybridizations were performed at either 42°C in 50% formamide (moderate stringency) or 55°C in 50% formamide (high stringency). The random primer–labeled probes used in the RNA gel blot analyses were the 540-bp 3′ fragment from 44M and a 3.5-kb BamHI-EcoRI fragment of the maize actin gene (kindly provided by K. Cone, University of Missouri, Columbia). Filters were washed four times in 0.1 × SSC, 0.1% SDS at 55°C, dried, and exposed to film with screens.

Gel Shift Analysis

An initiation codon was introduced into the partial OHP1 cDNA reading frame by the following method. The 0.9-kb EcoRI fragment from λ clone 44 (44M) was subcloned into the EcoRI site in pBluescript II KS+ (Stratagene). This plasmid was digested with SmaI to create a blunt end at the 5′ end of the partial OHP1 reading frame. NcoI linkers were ligated to the ends of the restricted DNA to create an in-frame ATG initiation codon. The resulting construct was cleaved with NcoI, and then religated to circularize the plasmid. The resulting OHP1 plasmid was cleaved at the 3′ end with HindIII, and the O2 cDNA in pT7/T3-18 was cleaved at the 3′ end with XbaI.

Each linearized template was transcribed in vitro with T7 RNA polymerase, and an aliquot of the transcription product was used to program a wheat germ lysate translation system (Promega), according to the manufacturer's specifications. Part of each translation reaction was labeled with ^{35}S-methionine, and analyzed on 8% SDS–polyacrylamide gels. The unlabeled translated proteins were assayed for DNA binding by equilibrating 8 μL of programmed lysate for 5 min at room temperature in a mobility shift buffer (10 mM Hepes, pH 7.8, 50 mM KCl, 1 mM EDTA, 10 mM DTT, 2 mg/mL BSA, 100 μg/mL salmon sperm DNA, 10% glycerol). Labeled O2 binding site DNA (0.5 ng) was added, and the reaction (25 μL final volume) was incubated at room temperature for 20 min and loaded onto a 4% polyacrylamide gel cast in 0.25 × Tris-borate-EDTA. Electrophoresis was performed for 2 to 3 hr at 30 mA, followed by drying of the gel and autoradiography.

The probes used in this experiment were identical to those described in Schmidt et al. (1992) and were labeled by the fill-in reaction using the Klenow fragment of DNA polymerase I and ^{32}P-dATP. Identical reaction conditions were used in the probe titration experiment (Figure 6), except that final probe concentrations were adjusted to those indicated in the figure, and lysate concentrations were adjusted so that equimolar amounts of O2, OHP1, and O2/OHP1 were incubated with the labeled probe.

ACKNOWLEDGMENTS

We thank Dr. Martin Yanofsky for technical assistance in obtaining the OHP1 cDNA and Dr. John Walker for providing the maize actin probe. This research was supported by grants to R.J.S. from the National Institutes of Health (GM41286) and the McKnight Foundation and to L.D.P. by the Howard Hughes Medical Institute Predoctoral Program.

Received October 8, 1992; accepted December 4, 1992.

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