An arginine to lysine substitution in the bZIP domain of an *opaque-2* mutant in maize abolishes specific DNA binding

Milo J. Aukerman,¹ Robert J. Schmidt,^{1,2} Benjamin Burr,³ and Frances A. Burr³

¹Department of Biology, C-016, and Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92093 USA; ³Biology Department, Brookhaven National Laboratory, Upton, New York 11973 USA

The *opaque-2* (*o2*) locus in maize encodes a transcription factor involved in the regulation of zein storage proteins. We have shown previously that the O2 protein contains a leucine zipper domain that binds to promoters of 22-kD zein genes. In this paper we characterize an EMS-induced *o2* allele, *o2-676*, that causes a 50% reduction in zein. We have found that the *o2-676* mutant protein does not show specific recognition of zein promoter fragments because of the substitution of a lysine residue for an arginine residue within the bZIP domain of *o2-676*. This particular arginine is conserved within the bZIP domains of all mammalian, fungal, and plant DNA binding proteins of this class. The correlation between this mutation in *o2* and the altered pattern of zein expression strongly suggests that O2 regulates transcription of certain members of the zein multigene family through direct interaction with the zein promoters and not through the transcriptional activation of some other regulator of zein gene expression.

[Key Words: Transcription factor; *Zea mays*; seed storage protein; bZIP domain; DNA binding; zein genes]

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Maize plants carrying an *opaque-2* (*o2*) mutation have seeds with an opaque, rather than glassy, appearance that is attributable to a dramatic reduction in the zein storage proteins. Zeins can be resolved by isoelectric focus (IEF)-SDS two-dimensional gels into two major size classes of 19 and 22 kD (Gianazza et al. 1976; Hagen and Rubenstein 1980). Zein genomic and cDNA clones encoding proteins of both the 19- and 22-kD classes have been isolated and sequenced. Although they are clearly related, zein genes expressing proteins of the 22-kD size class are distinct from those expressing members of the 19-kD size class (Burr et al. 1982; for review, see Heidecker and Messing 1986; Rubenstein and Geraghty 1986). Plants homozygous for *o2* typically show a 50–70% reduction in zein (Mertz et al. 1964; Nelson 1979), especially in the polypeptides of the 22-kD zein class (Soave et al. 1976; Burr and Burr 1982b). This reduction appears to result from a lower rate of transcription of zein genes, particularly those of the 22-kD class (Kordzycki et al. 1989).

The O2 gene has been cloned by transposon tagging (Schmidt et al. 1987; Motto et al. 1988). The sequence of the cDNA (Hartings et al. 1989; Schmidt et al. 1990) suggests that the O2 gene encodes a polypeptide containing a "leucine zipper" dimerization/DNA-binding (bZIP) domain characteristic of a class of mammalian and fungal transcription factors (Landschulz et al. 1988a). This

bZIP domain consists of a heptameric repeat of leucines with an adjacent, amino-terminal cluster of basic amino acids. The leucine repeat is proposed to function as a dimerization interface, while the highly conserved basic region directly contacts a DNA target site (Landschulz et al. 1988a). Our previous paper additionally showed that the bZIP domain of the O2 protein could bind specifically to the promoter region of a 22-kD zein gene (Schmidt et al. 1990).

Here we characterize an ethylmethane sulfonate (EMS)-induced *opaque-2* mutant allele, *o2-676*, for its effect on zein expression, and for the ability of its gene product to bind to zein promoters. Unlike the wild-type O2 protein, the *o2-676* protein does not display specific DNA binding in our in vitro assay. We have identified a single amino acid change within the basic domain of the *o2-676* gene product, arginine to lysine, which is responsible for this phenotype. This particular arginine is completely conserved in all bZIP proteins thus far isolated and is also found in the corresponding position of the basic domain of many helix-loop-helix and Myc-related proteins. Conservative amino acid substitutions at an asparagine residue near this invariant arginine in the basic domain also abolished the DNA-binding activity of the O2 protein. These experiments indicate that both amino acids play an essential role in the DNA-binding function of the O2 protein and that even conservative changes cannot be tolerated at these positions in the bZIP domain.

²Corresponding author.

Results

The o2-676 and o2-R alleles have different effects on zein expression

The standard *o2-R* mutant allele typically causes a reduction of polypeptides in the 22-kD class of zeins. We identified a novel opaque mutation from among 150 EMS-generated opaque mutants by screening for effects on zein synthesis by SDS–polyacrylamide gel electrophoresis. The new mutation had the opposite effect to that of the *o2-R* mutation; that is, it decreased the 19-kD zeins to a greater extent than the 22-kD zeins. From allelism tests between the new mutation and all known loci giving an opaque phenotype it was shown to be a recessive allele of the *o2* locus and given the designation *o2-676*. To ensure that the *o2-676* phenotype was not due to genetic background effects, the *o2-676* allele and the *o2-R* allele were backcrossed for six generations to three standard maize inbreds, W22, Oh43, and R802 (Fig. 1; Table 1). Table 1 shows the results of analyses of zein content in seed from three different inbred lines homozygous for the normal, the *o2-676*, or the *o2-R* allele. In each of the inbred backgrounds, zein protein levels in the *o2-676* mutant are reduced by 30–60% relative to wild type, whereas zein levels are decreased by 60–80% in the *o2-R* allele.

The effect of the *o2-R* and *o2-676* alleles on zein expression can be better visualized by analyzing the zeins extracted from these mutants on two-dimensional IEF/

Table 1. Zein yields in different maize inbreds that were homozygous or heterozygous for the indicated alleles of *o2*

	Milligrams of zein per gram of meal		
	W22	OH43	R802
<i>O2</i>	51	45	47
<i>o2-676</i>	20	31	27
<i>o2-R</i>	10	11	20
		OH43	R802
<i>o2-676</i> / <i>o2-676</i> / <i>o2-R</i>		20	22
<i>o2-R</i> / <i>o2-R</i> / <i>o2-676</i>		24	13
<i>o2-676</i> / <i>o2-676</i> / <i>O2</i>		40	44

The top half shows yields from endosperms homozygous for the indicated alleles. The bottom half shows yields from heterozygous endosperms with the indicated triploid allelic combinations. Reciprocal crosses between *o2-676* and *o2-R* mutants in W22 were not performed. Values represent the average of at least three separate determinations.

SDS/polyacrylamide gels. Analyses of the three inbred lines produced similar results. Figure 2 shows the spectrum of zein polypeptides affected by the two different *o2* mutant alleles in the maize inbred lines W22 and R802. In endosperms homozygous for the *o2-R* allele, expression of the 22-kD zeins is dramatically reduced, while a few members of the 19-kD zeins are still present. The expression of these 19-kD zein polypeptides can occur in the absence of *O2* protein because the *o2-R* allele does not produce detectable *O2* mRNA (Schmidt et al. 1987). Unlike *o2-R*, the *o2-676* mutant expresses members of both zein classes (Fig. 2). Although the overall zein protein level is reduced in this mutant when compared to the wild-type allele (Table 1), the spectrum of proteins produced is more similar to that of the normal *O2* allele than it is to the *o2-R* allele.

To demonstrate that the effects of the *o2* and *o2-676* mutations are at the level of zein gene transcription, we performed RNA slot blot analyses of the zein transcript levels from each of the three *o2* homozygotes. As can be seen in Figure 3, transcript for the 22-kD class of zeins is reduced in both *o2* mutants relative to wild type. The reduction is more severe in *o2-R* than in *o2-676*. This result is in good agreement with what would be expected for the 22-kD zein transcript levels on the basis of the results of the zein yield data and the two-dimensional gel analysis; namely, that the effect of the *o2-676* mutation on 22-kD zein gene expression is not as severe as seen for the *o2-R* allele.

Because the levels of some of the 19-kD zein proteins appeared reduced relative to the 22-kD zeins in *o2-676*, we asked whether this was the consequence of reduced transcription in this class of zeins. The same slot blot was reprobed with two cDNA clones that recognize transcripts from two distinct subfamilies of 19-kD zein genes (subfamilies 2 and 3; Rubenstein and Geraghty 1986). One of these, B41, is more severely reduced in *o2-676* than in *o2-R*, whereas the expression of the other, B54, is

Figure 1. Kernel phenotypes of the three *o2* alleles in different inbred backgrounds. (Top) Seeds cut longitudinally in half show the phenotype of the indicated *o2* alleles after six generations of backcrossing to three standard inbreds, W22, OH43, and R802. The whitish, chalky endosperms in the *o2* mutants contrast with the hard, vitreous endosperms so conspicuously apparent on either side of the starchy crown of the wild-type kernels. A portion of the embryo is apparent in the middle to lower center of each kernel. (Bottom) The phenotypes obtained after reciprocal crosses between *o2-676* and *o2-R* mutants and between *o2-676* and the normal *O2*. The dosage of the *o2* alleles in the triploid endosperm is indicated. The normal endosperm phenotype is obtained when a single normal *O2* allele is present, indicating that the *o2-676* allele is not codominant.

Figure 2. Two-dimensional gel profiles of the 19- and 22-kD zein polypeptides from seed homozygous for *O2*, *o2-676*, or *o2-R*. The effects of these mutations are shown for two different inbred backgrounds, W22 and R802. The *o2-676* mutant allele does not reduce 22-kD zein protein levels as severely as the *o2-R* allele does. Zeins were alcohol-extracted from wild-type or mutant endosperms, and separated by charge in the first dimension (IEF), followed by molecular mass separation in the second dimension (SDS-PAGE).

more similar in the two mutants. These results show that the transcription of at least one member of the 19-kD zein gene family is more severely reduced in *o2-676* than in *o2-R*.

To determine whether the *o2-676* allele produced normal amounts of an *O2*-specific mRNA, an RNA blot hybridization analysis was performed. Using a 0.6-kb *EcoRI* fragment of the *O2* cDNA as a probe, we detected a prominent *O2* message in the endosperm of plants homozygous for the wild-type and *o2-676* alleles (Fig. 4). In endosperm homozygous for the *o2-R* allele, this message is completely absent (Fig. 4), confirming our previous analysis of this allele (Schmidt et al. 1987). The similar size and abundance of the *o2-676* and wild-type transcripts suggests that the phenotype of the *o2-676* mutation is not a consequence of reduced *o2* gene expression. Further characterization of the mutation focused directly on the *o2-676* gene product. An *o2-676* cDNA clone was isolated and used to generate mRNA for translation in a wheat germ translation system. The in vitro-generated *o2-676* polypeptide had a similar mobility on SDS-polyacrylamide gels to the normal *O2* protein (data not shown). These results suggested that the *o2-676* allele makes an *O2* protein in vivo that is defective in some function necessary for zein gene activation.

An arginine residue in the basic domain of the O2 protein is changed to lysine in o2-676

Previous work has established that the normal *O2* protein contains a bZIP domain that can bind to zein pro-

motors (Schmidt et al. 1990). It was thus possible that the defect in the *o2-676* protein was located within the bZIP domain. We investigated this possibility by sequencing a *StuI*-*SalI* restriction fragment of the *o2-676* cDNA (nucleotides 571–855 in *O2*; Schmidt et al. 1990) that encompasses the bZIP domain. Comparison of the *O2* and *o2-676* cDNA sequences revealed a G-to-A transition that changes an arginine to a lysine in the basic region of the *o2-676* bZIP domain (Fig. 5). Sequence alignment of the basic domains of several bZIP proteins reveals that most of the arginine and lysine residues in this region are not completely conserved and thus may be interchangeable at many positions. The particular arginine that is substituted in the *o2-676* allele is unique in that it is completely conserved in all bZIP proteins sequenced thus far (Fig. 5; Vinson et al. 1989). This arginine is also conserved within the corresponding basic domains of the helix-loop-helix and Myc-related protein family (Prendergast and Ziff 1989).

The o2-676 protein cannot bind specifically to zein promoter fragments

We have recently demonstrated that the *O2* protein can bind to 22-kD zein gene promoter fragments in vitro and that the bZIP domain alone was sufficient for this interaction (Schmidt et al. 1990). Because the arginine-to-lysine change in the mutant *o2-676* protein is in the bZIP region, it seemed likely that this mutation affected the ability of the protein to recognize zein promoters. We tested this idea by constructing a fusion of the bZIP mo-

Figure 3. Analysis of zein transcripts in the *O2*, *o2-676*, and *o2-R* mutants. An autoradiogram of slot blots of total RNA from R802 endosperms homozygous for the indicated *o2* alleles. Approximately equal amounts of mRNA are present for each genotype, as indicated by probing with *pvu55* (*top*). This probe (Burr and Burr 1982a) is a fragment of the maize sucrose synthetase gene, an abundant enzyme involved in starch biosynthesis. The first two subjacent panels show the same blot reprobed with the zein cDNA probes B14 and B41. (*Bottom*) A separate blot that was loaded with the same amounts of RNA as at *top* and probed with B54. The B14 probe is a cDNA clone encoding a 22-kD zein; both B41 and B54 probes are cDNAs belonging to the 19-kD zein class (Burr et al. 1982). Hybridizations were performed at high stringency to minimize hybridization between classes. The relative concentrations of RNA per slot are indicated at *top left*.

tif of the *o2-676* cDNA with the *lacZ* gene (Rüther and Müller-Hill 1983), and assaying the resultant fusion protein for DNA-binding activity.

Upon induction with isopropylthiogalactoside (IPTG), *Escherichia coli* cells containing a *lacZ/o2-676* bZIP fusion construct expressed a protein with an apparent molecular mass of 130 kD that was detectable by SDS-PAGE (Fig. 6A, lane 4a). A protein consisting of the wild-type bZIP region fused to β -gal displayed a similar mobility (Fig. 6A, lane 3a). These two proteins are referred to as the *o2-676* bZIP and *O2* bZIP fusion proteins, respectively. Another fusion of almost the entire coding sequence of the *O2* cDNA to *lacZ* produces a polypeptide, termed the *O2* fusion protein, of at least 162 kD (Fig. 6A, lane 2a). To assay for DNA binding, each protein was immunoprecipitated out of crude bacterial extracts with a monoclonal antibody against β -gal, and then incubated with labeled zein DNA. Specifically bound zein fragments were then recovered and analyzed (Schmidt et al. 1990).

As shown in Figure 6B, the *O2* fusion protein binds to

two labeled *Sau3AI* fragments of pZ-1B, a zein genomic clone that specifies a 22-kD zein protein. When the same *O2* fusion protein is incubated with another 22-kD zein genomic clone, pZ-2BS, a single 200-bp fragment is bound (Fig. 6B). Sequence analysis shows that this fragment is identical to the 200-bp fragment of pZ-1B that is bound by the fusion protein (data not shown). Both 200-bp sequences can be mapped to a region ~300 nucleotides upstream of the zein-coding sequences, within the promoter regions of their respective genomic maps (Fig. 6C). Although *O2* binds a 500-bp element farther upstream in pZ-1B, this site is not present in pZ-2BS (Fig. 6C; G. Hoschek and R.J. Schmidt, unpubl.) nor in the sequence of any other published 22-kD zein gene promoter (Spena et al. 1982; Langridge and Feix 1983; Kridl et al. 1984; Brown et al. 1986). When the *O2* bZIP fusion protein is incubated with either pZ-1B or pZ-2BS fragments, a similar pattern of binding is seen (Fig. 6B, lanes 3).

Whereas the wild-type fusion proteins consistently show specific binding to the zein promoter fragments in our *in vitro* assay, the *o2-676* bZIP fusion protein shows no significant binding (Fig. 6B, lanes 4). The inability to detect DNA binding by the *o2-676* fusion protein in our *in vitro*-binding assay is not due to insufficient amounts of the *o2-676* bZIP protein in the assay; there was more *o2-676* bZIP fusion protein utilized for the DNA-binding assay than there was of the *O2* bZIP fusion protein (Fig. 6A, lanes 3b and 4b).

The observed correlation between loss of DNA binding and substitution at the invariant arginine in the *o2-676* protein implies that this residue is essential for the DNA-binding activity of the *O2* protein. If the substitution of lysine at the invariant arginine position is the only relevant change with respect to the DNA-binding phenotype, then changing this single residue back to arginine should restore wild-type binding. Site-directed

Figure 4. Northern blot analysis of *O2*-specific transcripts. Poly(A)⁺ RNA was extracted at 22 DAP from OH43 endosperms that were homozygous for the *O2*, *o2-R*, or *o2-676* alleles. Approximately 2.5 μ g of poly(A)⁺-enriched RNA was loaded in each lane. The *O2* probe was a 0.6-kb *EcoRI* fragment of the *O2* cDNA (Schmidt et al. 1990). The blot was stripped and rehybridized with *pvu55*, the sucrose synthetase probe, as a loading control.

Figure 5. Comparison of the basic domains in O2 and o2-676 with several other bZIP proteins. The invariant arginine in the basic region is indicated with an asterisk, with the lysine substitution in o2-676 designated in boldface. Only the first leucine (boldface) of the leucine heptad repeat is shown. The deduced amino acid sequence of the leucine heptad repeat (not shown) in o2-676 is identical to the sequence in O2 (Schmidt et al. 1990). The bZIP consensus is based on the consensus shown in Vinson et al. (1989). The letter B is used to designate positions in the basic region where either arginine or lysine is the predominant residue. The asparagine (N) and arginine (R) in the consensus sequence are completely conserved in all bZIP proteins described thus far and are shown boxed. Sources for sequences: (O2) Schmidt et al. (1990); (Fos) Van Straaten et al. (1983); (FRA1) Cohen and Curran (1988); (JunB) Ryder et al. (1988); (c-Jun) Angel et al. (1988); (GCN4) Hinnebusch (1984); (CPC1) Paluh et al. (1988); (CREB) Gonzalez et al. (1989); (C/EBP) Landschulz et al. (1988b); (TGA1a) Katagiri et al. (1989); (HBP1) Tabata et al. (1989).

o2-676	. . . RVRKRKES	N	R	E	S	A	R	R	S	*	K	Y	R	K	A	A	H	L	K	E	¹ L	E	D	Q	. . .
O2	. . . RVRKRKES	N	R	E	S	A	R	R	S	R	Y	R	K	A	A	H	L	K	E	L	E	D	Q	. . .	
FOS	. . . KRRIRRRER	N	K	M	A	A	A	K	C	R	N	R	R	R	E	L	T	D	T	L	Q	A	E	. . .	
FRA1	. . . RRRVRRER	N	K	L	A	A	A	K	C	R	N	R	R	K	E	L	T	D	F	L	Q	A	E	. . .	
JUNB	. . . KVERKRLRN	N	R	L	A	A	T	K	C	R	K	R	K	L	E	R	I	A	R	L	E	D	K	. . .	
c-JUN	. . . KAERKMRN	N	R	I	A	A	S	K	C	R	K	R	K	L	E	R	I	A	R	L	E	E	K	. . .	
GCN4	. . . PAALKRAR	N	T	E	A	A	R	S	R	A	R	K	L	Q	R	M	K	Q	L	E	D	K	. . .		
CPC1	. . . VVAMKRARN	N	T	L	A	A	R	K	S	R	E	R	K	A	Q	R	L	E	E	L	E	A	K	. . .	
CREB	. . . KREVRLMK	N	R	E	A	A	R	E	C	R	R	K	K	K	E	Y	V	K	C	L	E	N	R	. . .	
C/EBP	. . . EYRVRRER	N	N	I	A	V	R	K	S	R	D	K	A	Q	R	N	V	E	T	Q	Q	K	. . .		
TGA1a	. . . KVLRLRLAQ	N	R	E	A	A	R	K	S	R	L	R	K	K	A	Y	V	Q	Q	L	E	N	S	. . .	
HBP1	. . . KKQKRKLS	N	R	E	S	A	R	R	S	R	L	R	K	Q	A	E	C	E	E	L	G	Q	R	. . .	
bZIP	. . . - - - - BB - B	N	R	-	A	A	B	-	R	-	B	-	-	-	-	-	-	-	-	L	-	-	. . .		
Consensus																									

mutagenesis was used to reintroduce the invariant arginine codon at the position of the substituted lysine codon in the o2-676 cDNA. In-frame fusions to the *lacZ* gene were generated for the mutagenized template (o2-676 K-R) and also for the original o2-676 cDNA. On induction in *E. coli*, these two constructs both produced fusion proteins of a similar size (>162 kD) to the O2 fusion protein described earlier (data not shown).

The original o2-676 and o2-676 K-R fusion proteins were tested for DNA binding by the immunoprecipitation assay. As indicated in Figure 7, the o2-676 fusion protein does not bind detectably to the 200-bp zein target fragment; however, the o2-676 K-R fusion protein does bind to the target fragment. Comparison of the intensity of the radioactive band retained by o2-676 K-R to that retained by the wild-type O2 fusion protein suggests that the affinities of these two proteins for the target fragment are similar, if not identical (Fig. 7). Thus, the reintroduction of the invariant arginine back into the o2-676 protein results in a complete restoration of wild-type DNA binding.

Mutations at an invariant asparagine in the basic domain of O2 also abolish DNA binding

Although the overall structure of the bZIP domain is conserved in proteins from a wide variety of organisms, there are only two residues within the domain that are completely conserved throughout the whole bZIP family. The arginine that is substituted in the o2-676 protein represents one of these; the other is an asparagine residue located 8 amino acids amino-terminal to the invariant arginine (Fig. 5; Vinson et al. 1989). We reasoned that, similar to the invariant arginine, the invariant asparagine may play a critical role in DNA binding. To test this idea, we constructed three separate site-directed substitutions of varying severity (asparagine to glu-

tamine, N-Q; asparagine to alanine, N-A; and asparagine to aspartic acid, N-D) at the asparagine codon in the wild-type cDNA. Fusions to *lacZ* were constructed as described previously, and the resultant fusion proteins were tested for DNA-binding activity.

As shown in Figure 7, none of the three proteins substituted at the invariant asparagine bind in any detectable fashion to the zein target DNA. Even a somewhat conservative change, asparagine to glutamine, is unable to bind, indicating a stringent requirement for an asparagine at this position to maintain DNA-binding activity.

Can the o2-676 protein show weak association with the zein promoter under less stringent conditions?

The o2-676 protein shows no affinity for the zein promoter fragment in vitro, yet the o2-676 mutation does not behave as a null allele in vivo (Table 1; Fig. 2), as would be expected if the o2-676 mutant protein is incapable of binding to its target site. This implies that the o2-676 protein is partially functional in vivo. One explanation, then, for the partial functionality of the o2-676 allele is that the o2-676 protein binds weakly to zein promoters in vivo but that the conditions of our in vitro binding assay are too restrictive to detect this weak association. To detect weaker interactions of the o2-676 fusion protein with the zein promoter fragment, we varied a parameter of the in vitro DNA-binding assay that is known to affect the capacity of proteins to bind to nucleic acid; namely, the concentration of NaCl (Desplan et al. 1985; Laughon et al. 1988). As NaCl in the binding reaction is lowered from 200 mM, the wild-type fusion protein binds nonspecifically to many fragments, but the o2-676 protein is unable to bind even nonspecifically at 20 mM NaCl (Fig. 8). In addition, no specific interaction of o2-676 with the zein promoter fragment was detected under conditions of decreasing amounts of nonspecific

Figure 6. The DNA-binding domain in wild-type O2 can bind to zein promoters, whereas the corresponding domain in o2-676 cannot. (A) Bacterial expression and immunoprecipitation of the fusion proteins. *E. coli* that contain the plasmid expressing β -galactosidase (β -gal) alone (lanes 1), the β -gal/O2 fusion construct (lanes 2), the β -gal/O2 bZIP fusion construct (lanes 3), or the β -gal/o2-676 bZIP fusion construct (lanes 4) were induced with IPTG and harvested 2–3 hr later for analysis. (Lanes a) Crude whole cell lysates; (lanes b) proteins obtained by immunoprecipitation with antibodies to β -galactosidase and affinity to protein A–Sepharose. An amount of protein equivalent to that present in lanes b was used in the DNA-binding assays shown in B. The proteins generated by the bZIP fusions are indicated by the arrowhead. Molecular mass markers are indicated at left. (B) DNA-binding assays. Binding reactions utilized zein genomic subclones pZ-1B or pZ-2BS (C), or a control vector containing 1.5 kb of nonzein maize DNA. Shown are results of the assay with immunoprecipitates from β -gal alone (lanes 1), the O2 fusion protein (lanes 2), the O2 bZIP fusion protein (lanes 3), or the o2-676 bZIP fusion protein (lanes 4). The first unnumbered lane in each set shows the labeled *Sau*3AI digests of the respective plasmids used in the binding assay. Relative size markers are indicated at right. A subset of the data reported here, namely the binding of pZ-1B by the O2 and O2 bZIP fusion proteins, was reported previously in Schmidt et al. (1990). (C) Restriction map of the 14-kb *Xho*I genomic clone [Schmidt et al. 1990], containing a tandem arrangement of two 22-kD zein genes, designated Z1 and Z2. Subclones of fragments containing each of these genes, pZ-1B and pZ-2BS, are indicated below the map with arrows. Sites bound by O2 are shown as shaded ovals. The zein-coding sequences are indicated as hatched boxes, with an arrow above showing the direction of transcription. The labels B, E, H, and X represent restriction sites for *Bam*HI, *Eco*RI, *Hind*III, and *Xho*I, respectively.

carrier DNA (data not shown), even in combination with low (20 mM) NaCl. Nonspecific binding, a property associated with all DNA-binding proteins, was not observed with o2-676 unless the nonspecific carrier DNA was lowered to 1 μ g/ml and NaCl concentrations were reduced to 20 mM.

Discussion

Site-directed mutagenesis experiments on the basic regions of the mammalian bZIP proteins Fos, Jun, C/EBP, and CREB have delineated important residues in this domain for DNA-binding activity. In most cases, blocks of two or more residues were substituted or deleted, which reduced or eliminated DNA-binding activity [Kouzarides and Ziff 1988; Gentz et al. 1989; Landschulz et al. 1989; Neuberg et al. 1989; Turner and Tjian 1989; Dwarki et al. 1990]. Substitutions at individual amino acids in the basic region have generally been of an extreme nature [Gentz et al. 1989; Neuberg et al. 1989; Dwarki et al. 1990; Ransone et al. 1990]. For example, basic residues have been replaced by uncharged or acidic residues, producing alterations in DNA binding of variable severity. One possible interpretation of these results is that the

overall distribution of positive charge within the basic domain is an important component of DNA binding by bZIP proteins. Nevertheless, it is unclear from these previous studies which specific residues within the basic domain are truly critical for the recognition of DNA target sites by bZIP proteins, since all single-site substitutions that abolished function in these studies were non-conservative changes. We have identified two amino acids in the basic region of the bZIP protein Opaque-2 that appear to be essential to its DNA-binding activity. Conservative amino acid substitutions at either the invariant arginine or the invariant asparagine result in a complete loss of detectable DNA binding by the O2 protein. Because of the complete conservation of these residues within the bZIP protein family, it is quite likely that they play a key role in the general DNA-binding properties of the bZIP motif.

The in vivo-generated substitution of lysine for arginine in the o2-676 protein is a very conservative change, yet it results in complete loss of DNA binding in vitro. One might expect that basic residues such as arginine and lysine would be interchangeable to some degree in the basic region since the overall charge of the region would be maintained. Indeed, all of the other basic

Figure 7. DNA-binding analysis of site-directed mutants of O2 and o2-676. The binding assay utilized plasmid pZ2-P2, which consists of a Bluescript KS vector containing the O2-binding site within a 200-bp *Sau3AI* fragment of the Z2 zein promoter (see Fig. 6C). The flanking lanes show the labeled *Sau3AI* fragments of pZ2-P2 DNA used in the binding reaction. The inner lanes are results of incubating pZ2-P2 fragments with one of several β -gal fusion proteins: β -gal alone, O2 fusion containing substitutions at the invariant asparagine (N-Q, N-A, N-D), wild-type O2 fusion (WT), o2-676 fusion (o2-676), o2-676 lysine to arginine fusion (o2-676 K-R). Approximately equal amounts of each fusion protein were used in the DNA-binding assay. Relative size markers are indicated at right. The upper fragment of the two fragments retained by wild-type and o2-676 K-R arises from a mobility shift of the 200-bp zein target fragment due to incomplete phenol extraction of the proteins bound to it.

amino acids within the basic domain are incompletely conserved among the bZIP proteins, suggesting either that any protein–DNA contacts made by these residues can be accomplished by either arginine or lysine or that these residues are functioning separately in each different bZIP protein to make sequence-specific contacts with the respective DNA target site of each bZIP protein. Systematic substitutions of arginine for lysine and vice versa have not been performed on any bZIP protein to determine the effects of such subtle changes.

The invariant arginine may make a contribution to nonspecific binding. The wild-type O2 protein binds nonspecifically to DNA as the salt concentration is lowered, whereas the o2-676 protein does not bind at all under these conditions (Fig. 8). Clearly, the arginine-to-lysine substitution in o2-676 has not only affected the protein's ability to specifically bind to its target sequence but the mutation reduced dramatically the nonspecific DNA-binding activity that the wild-type O2 protein, like most DNA binding proteins, has as a component of its total activity. This result, and the fact that the substituted arginine is completely conserved among the bZIP proteins, suggests that this residue is performing a more general role in DNA binding than the other basic residues in the bZIP domain and that it may make a large contribution to the nonspecific DNA binding component in particular.

Subtle changes at the invariant asparagine in the basic

region of O2 resulted in complete loss of binding to the target sequence, suggesting that this residue, like the invariant arginine, is essential for DNA-binding activity. Recently, Vinson and colleagues (1989) have proposed that the invariant asparagine functions to angulate the basic regions of each helical bZIP domain in the dimeric protein, thus allowing the two DNA-binding domains to wrap around the major groove of the DNA double helix. The formation of this "scissors-grip" structure presumably provides added stability to the protein–DNA complex. Other amino acids besides asparagine at this position presumably would not angulate the helical basic domain correctly, and thus stable binding would not occur (Vinson et al. 1989).

Because the o2-676 protein does not bind DNA in vitro, the substitution of lysine for the invariant arginine in the o2-676 gene product is most likely the lesion that gives rise to the reduced zein gene expression observed for o2-676 in vivo. The restoration of the wild-type sequence by site-specific mutagenesis in o2-676, leading to the restoration of binding activity, argues that the G-to-A transition in the bZIP domain of o2-676 is the only significant change with regard to DNA binding. However, we cannot rule out other sequence changes in o2-676 that may have occurred outside of the bZIP domain, but within some other unknown domain that is critical for zein activation.

Although mutations at o2 primarily impact the expression of the 22-kD class of zeins, some members of the 19-kD zein class are affected as well in the o2-R and o2-676 mutants (Fig. 2). Particularly surprising is that some of the 19-kD zeins appear to be affected more severely by the o2-676 mutation than are some of the 22-kD zeins. This is especially noticeable in R802 and, to a lesser extent, in W22. For at least one member of the 19-kD zeins this effect is observable at the level of gene transcription (Fig. 3). In o2-676 endosperms the levels of B41 transcript are affected more severely than they are in

Figure 8. The effects of decreasing NaCl concentrations on the DNA binding of O2 and o2-676 fusion proteins. Numbers above each pair of lanes refer to the millimolar concentration of NaCl used in the binding assay. Fragments retained by the o2-676 fusion protein or the O2 fusion protein are shown in lanes a and b, respectively. The flanking lanes show the labeled fragments from a *Sau3AI* digest of pZ2-P2 (legend to Fig. 7). Twice as much total DNA was loaded in the left flanking lane, compared with the right. Relative size markers are indicated at right.

o2-R endosperms. However, B41 may be atypical of the 19-kD zeins (Rubenstein and Geraghty 1986) and may not be reflective of this class as a whole. On the basis of a comparison of cDNA sequence information, Rubenstein and Geraghty (1986) have placed B41 in a small subfamily of 19-kD zeins consisting of one other member, B59. When another zein cDNA encoding a 19-kD zein protein (B54) is used as a probe, no significant difference is observed in the levels of transcript present in the *o2-676* and *o2-R* endosperms (Fig. 3). Also, three other 19-kD zein probes, B20, B36, and A30 (Burr et al. 1982), showed little or no difference in the levels of transcript between *o2-R* and *o2-676*, even though both genotypes showed a reduction in transcript levels relative to wild type (data not shown). The exact role that O2 may play in the expression of the 19-kD class is not clear. We have been unable to demonstrate binding of O2 to 19-kD zein gene promoters (belonging to the same subfamily of 19-kD zeins as B54) in our in vitro-binding assays (G. Hoscheck and R.J. Schmidt, unpubl.).

The inability of the *o2-676* protein to bind 22-kD zein gene promoters in vitro suggests that it also should not be able to bind zein promoters in vivo. If this were the case, we would expect the expression pattern of the zeins in the *o2-676* mutant to be very similar to that seen in the *o2-R* allele. This is not the case; members of the 22-kD zein class are expressed in *o2-676*, whereas they are completely absent in the *o2* null (see Fig. 2). The reason for this apparent inconsistency between the in vivo and in vitro observations is not clear. It is possible that our in vitro assay is not sensitive enough to detect weak but selective interactions of the *o2-676* protein with the zein target fragment. These interactions may be sufficient in vivo to partially activate the zein genes. It is also possible that the *o2-676* protein-DNA complex is somehow stabilized in vivo by other maize proteins not present in our in vitro assay.

Another explanation to account for the levels of zein gene expression observed in vivo with the failure to detect DNA binding in vitro is to suppose that *o2-676* (and O2) binds to zein promoters as a heterodimer with another bZIP protein in vivo. The mammalian bZIP protein Jun, for example, can bind to its target site as a homodimer but binds this site with a higher affinity as a heterodimer with another bZIP protein, Fos (Halazonetis et al. 1988; Rauscher et al. 1988; Smeal et al. 1989). Recently, other examples of bZIP protein heterodimers have been elucidated (Hai et al. 1989; Benbrook and Jones 1990). In the *o2-676* mutant, this heterodimer formation might partially compensate for the DNA-binding defect of the *o2-676* protein by providing one bZIP motif that retains the invariant arginine residue. This *o2-676*-bZIP-X heterodimer might be able to associate weakly with the zein target site and thus facilitate the partial activation of the zein genes that we observe in vivo for *o2-676*. It is known that other loci regulate zein gene expression, among them *opaque-7* and *floury-2*. Although the gene products of these loci have not been isolated, they may encode proteins that form heterodimers with O2. Heterodimer formation of the *o2-676*

product with one or more proteins might account for both the presence of some 22-kD zeins and the reduction in at least some members of the 19-kD class.

We have demonstrated here and elsewhere (Schmidt et al. 1990) that O2 binds to the promoter of 22-kD zein genes. The arginine to lysine mutation in the bZIP domain of *o2-676* affects recognition of the zein target sequence by the mutant protein and results in differential expression of members of the zein multigene family. This argues strongly in favor of a model in which O2 regulates zein gene transcription by direct interaction with zein gene promoters and not by the induction of the synthesis of an intermediary regulatory molecule, as has been suggested by others (Soave et al. 1981; Di Fonzo et al. 1988).

Materials and methods

Mutant isolations

The *o2-R* allele occurred as a spontaneous mutation in maize inbred W64A (W.R. Singleton and D.F. Jones, unpubl.). This allele, backcrossed into maize inbred W22, was characterized previously as a null allele based on the absence of any detectable O2 message by Northern analysis (Schmidt et al. 1987). We screened a large collection of M2 ears from EMS mutagenesis at the University of Missouri that were provided by M.G. Neuffer (Neuffer and Sheridan 1980). We selected 138 nonlethal opaque mutants for further examination. An additional 12 opaque mutants generated at Brookhaven were also screened. Seed from each segregating population of opaque mutants were analyzed for zein protein by one-dimensional SDS-PAGE. In one mutant from the Missouri material, 676, the 19-kD zeins appeared to be affected more significantly than the 22-kD zeins.

Zein protein analyses

Mature kernels were soaked in water for 10 min to loosen pericarp from the endosperm. After removal of the pericarp and embryo, endosperms were first ground in an electric coffee mill and then pulverized to a fine powder with mortar and pestle. The ground endosperm meal was sifted through a 250- μ m screen. The portion passing through the screen was stored in a desiccator under vacuum for 24 hr prior to extracting zein proteins. For each of at least three independent determinations, 0.4 or 0.5 grams of meal was extracted with 5 ml of 70% ETOH for 0.5 hr with frequent vortexing. The contents were then centrifuged at 15,000g for 15 min at 4°C. The supernatant was transferred carefully into dialysis tubing and dialyzed for 15 hr against several changes of deionized water. The contents of the dialysis bag, now containing precipitated zein proteins, was lyophilized to remove the water. Following lyophilization the zein protein was weighed and stored at 4°C. Two-dimensional IEF/SDS/PAGE (O'Farrell 1975) was performed using ampholytes of pH 6–8 in the first dimension and 15% polyacrylamide/0.1% SDS in the second dimension. Gels from the first dimension were equilibrated for a minimum of 1 hr in a solution of 62.5 mM Tris (pH 6.8), 10% glycerol, 5% β -mercaptoethanol, and 0.004% bromophenol blue before mounting on the second-dimension SDS gel. First-dimension tube gels were loaded with 50 μ g of zein proteins extracted from kernels homozygous for the normal O2 allele, and 25 μ g of zein protein from kernels homozygous for the *o2* mutant alleles. Because there are fewer zein proteins present in the mutants, it was

necessary to use less protein to prevent overloading of the gel and loss of resolution.

Fusion proteins

Construction of the β -gal/O2 and the β -gal/O2 bZIP fusion proteins was described previously (Schmidt et al. 1990). The β -gal/o2-676 fusion construct was generated by digesting the o2-676 cDNA in Bluescript SK with *Apal*, which cuts at nucleotide +38 in O2 (Schmidt et al. 1990), and *XbaI*, which cuts in the 3'-polylinker sequence, gel-purifying the 1.4-kb *Apal/XbaI* fragment and blunt-ending with the Klenow fragment of DNA polymerase I. *HindIII* linkers were ligated to the ends, and the fragment was cloned into the *HindIII* site of the *lacZ* bacterial expression vector pUR288 (Rüther and Müller-Hill 1983). The resultant β -gal/o2-676 fusion protein, like the β -gal/O2 fusion described previously (Schmidt et al. 1990), contains all but the first 13 amino acids of the o2-676 protein. The β -gal/o2-676 bZIP construct was made in a similar fashion, except that a *StuI/SalI* digest was used to isolate an internal 0.3-kb fragment of the o2-676 cDNA (nucleotides 571–855 of O2) that was cloned into pUR288. Because the o2-676 cDNA contained a polymorphism that destroyed the *PstI* site present in the O2 cDNA (nucleotide 566), we were unable to fuse the o2-676 fragment to *lacZ* at the same codon as was done for the O2 bZIP construct (Schmidt et al. 1990). The *StuI* site lies 4 bp to the 3' side of the *PstI* site; thus, the o2-676 bZIP construct presumably would be lacking one codon at the β -gal/o2-676 junction, relative to the O2 bZIP construct. Upon sequencing the junction between *lacZ* and o2-676 in the o2-676 bZIP fusion construct, we discovered a 9-bp deletion that removed an additional three amino acids of the o2-676 moiety, such that the o2-676 bZIP protein is shortened by four amino acids relative to the O2 bZIP fusion construct. This deletion lies 33 residues amino-terminal of the actual bZIP domain. Since the o2-676 fusion protein, which obviously contains these four amino acids, does not bind the zein target sequence, we conclude that the deletion of these four residues in the o2-676 bZIP fusion is not responsible for the DNA-binding defect observed for the o2-676 bZIP protein.

DNA-binding assay

Fusion proteins were expressed in *E. coli* and partially purified by immunoprecipitation with antibodies to β -galactosidase, essentially as described (Schmidt et al. 1990). An aliquot of the immunoprecipitate containing the bound fusion protein was boiled for 5 min in SDS loading buffer before electrophoresis on 7.5% polyacrylamide/0.1% SDS gels (Laemmli 1970). To assay for DNA binding, an amount of the immunoprecipitated fusion protein equivalent to that analyzed on the protein gel was resuspended in 40 μ l of DNA-binding buffer [150 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM Tris-HCl (pH 7.8)] plus 0.1 mg/ml salmon sperm DNA. Approximately 40 ng of a 32 P-labeled *Sau3AI* digest of plasmid DNA containing the 200-bp zein target fragment was then added. Incubation, washing, and processing of the retained fragments were performed as described previously (Schmidt et al. 1990). This assay is a modification of the original assay described by McKay (1981). For testing the effect of lowering NaCl concentration on DNA binding, the incubation and washing steps were carried out in binding buffers adjusted to the various NaCl concentrations indicated in Figure 8.

Site-directed mutagenesis

To create the o2-676 K-R fusion construct, the o2-676 fusion construct was digested with *HindIII*, and the 1.4-kb o2-676

cDNA fragment was isolated and inserted into the Bluescript SK vector (Stratagene). Next, 50 μ l of an overnight culture of XL1-Blue bacteria (Stratagene) containing this plasmid was infected with $\sim 10^{12}$ pfu of helper phage R408 and subsequently was diluted into 3 ml 2XYT (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl) + 0.1% glucose + 50 μ g/ml carbenicillin. Following 1–2 days of incubation, single-stranded DNA was purified according to the protocol described in the Amersham version 2 oligonucleotide-directed in vitro mutagenesis kit, except that PEG precipitations were carried out in 3.5 M NH_4Ac instead of 2.5 M NaCl. A 22-bp oligonucleotide specifying the K-R substitution was annealed to the single-stranded DNA template, and all subsequent mutagenesis steps were performed with the Amersham kit, according to the provided protocol. Mutant clones were screened by dideoxy sequencing, and a clone with the correct K-R codon substitution was digested with *HindIII* to shuttle the 1.4-kb o2-676 K-R fragment back into pUR288. The three mutants at the invariant asparagine, N-Q, N-A, and N-D, were generated in a similar fashion, except that the 1.4-kb O2 fragment was subcloned into the Bluescript vector instead of the o2-676 fragment. All three asparagine mutant constructs were sequenced from the *PstI* site to the *SalI* site (nucleotides 566–855 of O2) to ensure that no changes other than the desired one had occurred within the bZIP domain.

RNA analyses

Each slot of the RNA slot blots (Schleicher & Schuell Minifold II) was loaded with a sample of total RNA in 400 μ l of 13.5% formaldehyde, 7.5 $\times$ SSC, 5 μ g of salmon sperm DNA after incubating at 65°C for 15 min. Equivalent amounts of mRNA, as determined by hybridization to pvu55 (Burr and Burr 1982a), were loaded for each genotype. Following the application of RNA each sample was washed with 400 μ l of 10 $\times$ SSC and dried in a vacuum oven at 80°C. Hybridizations were performed at 55°C in 50% formamide as described previously (Schmidt et al. 1987). The o2 RNA blot analysis was performed as described in Schmidt et al. (1987), except that the O2 probe was a 0.6-kb *EcoRI* fragment from the 3' end of the O2 cDNA (Schmidt et al. 1990), and the probes were labeled by the random priming method (Feinberg and Vogelstein 1983).

Bacterial strains, cDNA, and DNA clones

E. coli strain JM109 was used to express all of the fusion proteins described. XL1-Blue (Stratagene) was used to generate single-stranded DNA templates for site-directed mutagenesis. The o2-676 cDNA was isolated from a library constructed from 22-day-old endosperms of inbred R802 that were homozygous for the o2-676 allele. The cDNA clones were identified by screening the library with O2-specific probes, as described in Schmidt et al. (1990) for the cloning of the normal allele of O2. Isolation of the zein genomic clone was described previously (Schmidt et al. 1990). Plasmids pZ-1B and pZ-2BS were generated by subcloning the 4.5-kb *BamHI* fragment and the 3.5-kb *BamHI-SalI* fragment, respectively, into Bluescript KS. These clones are indicated on the genomic map in Figure 6C; the *SalI* site, which is not indicated, lies adjacent to the 3' *HindIII* site. pZ2-P2 was generated by subcloning a 200-bp *Sau3AI* fragment of pZ-2BS that contains the O2-binding site into Bluescript KS. All sequencing was performed by the dideoxy method using the Sequenase enzyme (U.S. Biochemical).

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An arginine to lysine substitution in the bZIP domain of an opaque-2 mutant in maize abolishes specific DNA binding.

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