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Translation of the mRNA of the Maize Transcriptional Activator *Opaque-2* Is Inhibited by Upstream Open Reading Frames Present in the Leader Sequence

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The protein encoded by the *Opaque-2* (*O2*) gene is a transcription factor, translated from an mRNA that possesses an unusually long 5′ leader sequence containing three upstream open reading frames (uORFs). The efficiency of translation of *O2* mRNA has been tested in vivo by a transient assay in which the level of activation of the b32 promoter, a natural target of O2 protein, is measured. We show that uORF-less *O2* alleles possess a higher transactivation value than the wild-type allele and that the reduction in transactivation due to the uORFs is a *cis*-dominant effect. The data presented indicate that both uORF1 and uORF2 are involved in the reducing effect and suggest that both are likely to be translated.

INTRODUCTION

The *Opaque-2* (*O2*) gene of maize regulates the synthesis of the 22-kD or α-class of zeins, the major storage proteins of maize endosperm (Motto et al., 1989). In homozygous *o2* mutants, in addition to the 22-kD zeins, other endosperm-specific nonzein proteins are present at reduced levels, one of which is a 32-kD, albumin class cytosolic protein, designated b32 (Soave et al., 1981; Di Fonzo et al., 1988). The *O2* gene has been isolated via transposon tagging (Schmidt et al., 1987; Motto et al., 1988). Sequence analysis of the corresponding cDNA reveals homology with the leucine zipper class of *trans*-acting factors (Hartings et al., 1989; Schmidt et al., 1990), and the *O2* protein behaves as a sequence-specific DNA binding protein interacting with the promoter region of the b32 gene and activating transcription from this promoter in vivo (Lohmer et al., 1991).

The leader sequence of *O2* mRNA is unusually long and contains three short upstream open reading frames (uORFs; see Figure 1A); these are features shared with the mRNA of a number of other transcription factors and regulatory proteins (for example, general control of yeast amino acid biosynthesis activator [*GCN-4*], carbamoyl-phosphate synthetase A small subunit [*CPA-1*], a lymphocyte-specific protein kinase p56 [*lck*], and octopine synthase element binding factor-1 [OCSBF-1]; Mueller and Hinnebusch, 1986; Werner et al., 1987; Marth et al., 1988; Singh et al., 1989). Most eukaryotic mRNAs (95%) contain no upstream AUG codons (Kozak, 1987a), and the introduction of a single AUG into the 5′ leader of an mRNA can reduce the expression of the downstream coding sequences (Kozak, 1984). It has been proposed that ribosomes (or their 43S subunits) must bind at the 5′ end of the transcript and traverse the entire leader to gain access to the AUG start codon (the scanning model of translation; Kozak, 1978, 1989a). When an uORF is present, initiation of translation occurs preferentially at the uORF start codon and reduces initiation at downstream start codons, apparently because internal reinitiation is an inefficient process (Kozak, 1984; Liu et al.,1984).

A regulatory gene that possesses uORFs is *GCN-4*, which controls transcription of several amino acid biosynthetic genes in yeast (Hinnebusch, 1988, 1990). *GCN-4* expression is translationally controlled, coupled to the availability of amino acids, and this control depends on the presence of four short ORFs in the mRNA leader (Hinnebusch, 1988). Removal of the four short uORFs from the *GCN-4* transcript, either by deletion or point mutations in the four AUG start codons, results in high-level unregulated expression of *GCN-4*, independent of amino acid availability (Mueller and Hinnebusch, 1986). Thus, the four *GCN-4* uORFs constitute a translational control element that allows ribosomes to reach the start codon of the major ORF only when cells are deprived of an amino acid.

In this study, we investigated the influence of the three uORFs present in *O2* mRNA on its translation. We demonstrated that these uORFs reduce the activity of *O2* as a transcriptional activator. This inhibiting effect is *cis* dominant and requires at least the presence of uORF1 and uORF2.

RESULTS

Figure 1A shows schematically the ORFs present in the *O2* mRNA. Within the leader region, three short uORFs of three,

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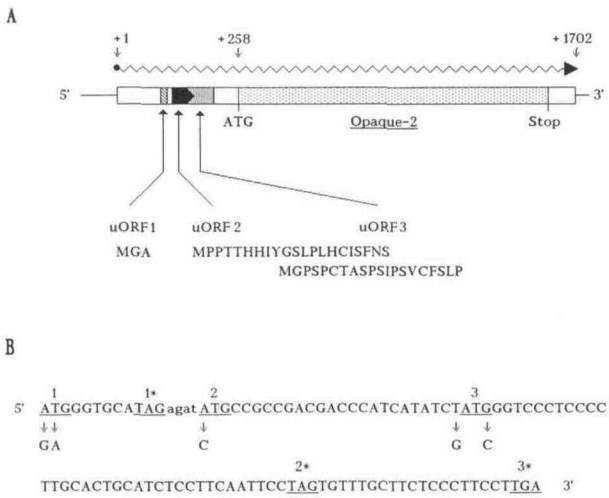

Figure 1. Schematic View of the *O2* Leader Region and the Point Mutations Introduced in the Upstream AUG Codons.

(A) The wavy line represents *O2* mRNA and the numbers indicate the distance in nucleotides from the 5′ end. The putative gene products of the uORFs are given in the one-letter amino acid code.
(B) The sequence of the leader region containing all three uORFs, with asterisks marking the corresponding stop codons. Point mutations shown by arrows are those introduced in the transient expression constructs (see Figure 3) as follows: at the uORF1, ATG→GAG, to give *uORF1**; *at the uORF2*, ATG→CTG, *to give uORF2**; *and at uORF3*, TATG→GATC to give uORF3*.

21, and 20 codons precede the major ORF. uORF1 is in frame with the major ORF, whereas uORF2 and uORF3 are both out of frame with the major ORF and overlap each other. Figure 1B shows the sequence containing the three uORFs. Arrows indicate base changes introduced by mutagenesis to remove the start codons.

Size of the Gene Product of *Opaque-2*

Before the uORFs could be functionally analyzed, we needed to establish that the predicted uORFs were indeed upstream of the major ORF rather than part of it. An *O2* construct lacking the leader region has been shown to encode an *O2* gene product capable of transcriptional activation (Lohmer et al., 1991), and all of the components necessary for such a transcription factor, e.g., DNA binding domain, nuclear localization domain, and activation domain, have been located in the predicted major ORF (S. Lohmer and R.D. Thompson, manuscript in preparation). Whereas these data indicate that the leader region with the uORFs is not necessary for the function of *O2*, a translation initiation at uORF1, which is in frame with the predicted major ORF, cannot be ruled out. This event would, however, require a read-through at the stop codon of uORF1. The stop signal of uORF1 is the amber codon UAG, the stop codon for which most suppressor tRNA species have been isolated (Hatfield et al., 1990). UAG has been found in the ORF of a C-hordein gene from barley (Entwistle, 1988) and of a 22-kD zein gene from maize (Wandelt and Felix, 1989), suggesting that an amber suppressor tRNA species is present in endosperm cells of cereals. Indeed, UAG suppression has been demonstrated in barley endosperm extracts (Entwistle et al., 1991).

Read-through of the uORF1 stop codon would add 50 additional amino acid residues to the major ORF of *O2*. Therefore, a molecular weight comparison of the protein products of a leaderless and a leader-containing *O2* mRNA should indicate whether *O2* translation starts at the AUG of uORF1 or at the AUG of the major ORF.

An anti-*O2* antiserum was raised against an affinity-purified, *O2* glutathione-S-transferase (GST) fusion protein (see Methods) and was checked for specificity via immunoblot analysis. Nuclear extracts from maize wild type and *o2* mutant plants were separated by electrophoresis on SDS–polyacrylamide gels and electroblotted onto nitrocellulose membranes. As shown in Figure 2A, a 66-kD band was detected in wild-type extracts (lane

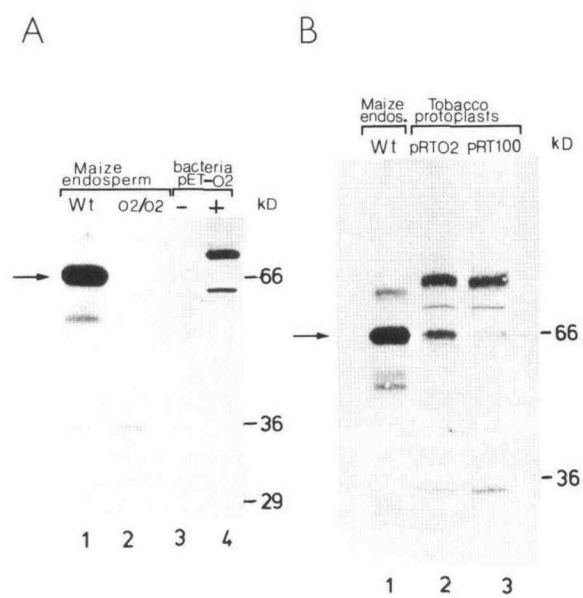

Figure 2. Molecular Mass Determination of *O2* via Protein Gel Blot Analysis.

(A) In lanes 1 and 2, nuclear extracts from the wild type (WT) and *o2* mutant endosperm cells, respectively, were loaded on a 7.5% SDS gel, whereas in lanes 3 and 4, bacterial extracts either from noninduced or induced cells, containing pET02, were loaded. After electrophoresis, the proteins were blotted onto nitrocellulose and incubated with an *O2*-specific polyclonal antiserum.
(B) Lane 1 contains nuclear extracts from wild-type maize endopserm cells. Lanes 2 and 3 contain protein extracts from protoplasts, which were transfected with the *O2* expression plasmid pRTO2 or with the vector pRT100.
The arrows indicate the position of *O2*; molecular mass markers are given at the right.

1) and was absent from *o2* mutant plants (lane 2). The absence of the *O2* gene product in *o2* mutant plants is consistent with the phenotype reported previously by Schmidt et al. (1987) and Motto et al. (1988). Additional faint bands in lanes 1 of Figures 2A and 2B may also represent proteins related to O2, as they were not seen using preimmune serum.

This result was confirmed by expressing in *Escherichia coli* the *O2* cDNA lacking the leader inserted into the Φ T7-derived expression vector pET (Studier and Moffat, 1986). In this case *O2* was expressed as a hybrid protein, in which the first 14 amino acids were derived from the T7 phage gene Φ 10, thus increasing the predicted molecular weight of the O2 protein by ~2 kD. Expression of the leaderless construct in pETO2 did give rise to an O2 polypeptide migrating slightly more slowly than the native O2 polypeptide (Figure 2A, lane 4), confirming that the synthesis of the O2 protein is not initiated at any of the uORFs.

A second leaderless *O2* construct was prepared by inserting the major *O2* ORF into the plant expression vector pRT100 (Töpfer et al., 1987) under the control of the cauliflower mosaic virus (CaMV) 35S promoter, thereby allowing the transient expression of *O2* in plant cells. The resulting plasmid pRTO2, which encodes a hybrid protein with the first seven amino acids derived from the polylinker sequence of pRT100 (Figure 3A), was transfected into tobacco mesophyll protoplasts. After 18 hr, proteins were extracted and an immunoblot analysis with the *O2*-specific antibodies was performed. As shown in Figure 2B, the *O2* leaderless plasmid pRTO2 expresses a protein of identical mobility to that of the O2 protein from maize endosperm. Two additional high molecular weight proteins were detected in protoplasts by O2 antiserum and by preimmune serum and presumably represent nonspecific cross-reaction. Therefore, in vivo, a read-through of uORF1 or a combined read-through plus a frameshift of uORF2 or uORF3 can be ruled out as these would have given rise to a larger product in maize endosperm than that derived from pRTO2.

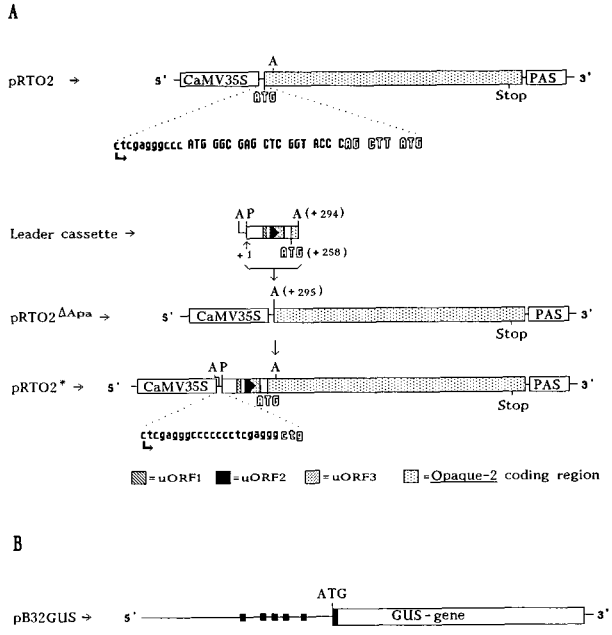

Figure 3. Constructs Used for Transient Gene Expression.

(A) In pRTO2, the leaderless *O2* cDNA is expressed via a transcriptional fusion with the CaMV 35S promoter. The construction of pRTO2 and its derivative pRTO2$^{\Delta Apa}$ is described more fully in Methods. The mutagenized O2 leader sequence cassettes were inserted into the ApaI site of pRTO2$^{\Delta Apa}$ to give the expression constructs listed in Figure 4, including ORF^{WT}. These constructs are collectively represented here as pRTO2*. The numbers indicate the distance in nucleotides from the 5′ end. A, ApaI; P, PstI; CaMV35S, the 35S promoter of cauliflower mosaic virus; PAS, polyadenylation signal. The sequence is that of the transcriptional fusion point between the CaMV 35S promoter and the *O2* gene. Capital letters represent the 5′ end of *O2*, whereas the small letters symbolize 35S sequences. The transcription start point, which is indicated by the arrow, is located 11 nucleotides upstream of the original *O2* transcription start point, thereby ensuring that all constructs used in this work possess the same transcription start point.

(B) Schematic view of the reporter plasmid pb32GUS (Lohmer et al., 1991). The b32 promoter was fused to the GUS coding sequence downstream of the b32 translation start, creating a translational fusion. The small black boxes indicate the binding sites of O2 protein (for details, see Lohmer et al., 1991).

uORFs Decrease the Expression of *Opaque-2*

The effect of the uORFs on expression levels of the *O2* gene product was investigated in vivo by transient gene expression in tobacco protoplasts. In this system, it has been shown that the O2 protein derived from expression of a transfected plasmid is able to transactivate the expression of the β-glucuronidase (GUS) reporter gene under the control of the promoter region of the b32 gene (Lohmer et al., 1991).

This assay permits all structural interactions that might exist between the *O2* leader sequence and the coding region of the *O2* mRNA to be retained, in contrast to assays in which the *O2* leader was to be fused directly to a reporter sequence.

Protoplasts prepared from tobacco SR1 leaves were cotransfected with circular forms of *O2* expression plasmids carrying the *O2* cDNA or derivatives thereof, as shown in Figure 3A, in the presence of the reporter plasmid pb32GUS (Figure 3B; Lohmer et al., 1991). The effect of mutations within the leader on expression was studied by assaying GUS activity. GUS activity was found to increase linearly for at least 24 hr and the ratio of GUS activities for different mutants remained constant (data not shown).

All of the *O2* expression plasmids were constructed so that they contained the same external transcription start site, which is located in the CaMV 35S promoter (Figure 3A), to ensure that all constructs exhibited the same transcription frequency for *O2*. Variations in transcript levels between constructs were controlled by total RNA slot blot hybridizations using an *O2* leader probe and did not exceed 5%, thus ruling out effects

of the uORF mutagenesis on RNA processing or stability (data not shown). Under these conditions, the observed variations in the levels of activation of the reporter plasmid pb32GUS depended only on the accumulation of the O2 protein as translated from mRNAs transcribed from the various *O2* expression plasmids.

The effects of the uORFs on the expression of *O2* were investigated by introducing point mutations in the uORFs via site-directed mutagenesis. Using pRTO2 (also referred to as $ORF^{\Delta L}$), in which the *O2* cDNA minus the leader is expressed from the CaMV 35S promoter (Figures 2B and 3A), the derivative $pRTO2^{\Delta Apa}$ was prepared which lacks the codons for the first 12 amino acids of O2. Into this plasmid were inserted the mutagenized leader sequences as a cassette, containing at the 3′ end the 12 missing codons, thereby leaving unchanged the major ORF of the *O2* gene (Figure 3A) and avoiding any unwanted mutations of the major *O2* ORF that might arise while generating each construct.

As a first step in the leader analysis, we compared the transactivation produced by an *O2* expression plasmid containing the complete leader of the wild-type mRNA (ORF^{WT}) with that of a plasmid lacking the leader (pRTO2, $ORF^{\Delta L}$). The transactivation values of these *O2* constructs (Figure 4A) demonstrated that the presence of the leader sequence in *O2* mRNA decreases the transactivation of O2 by fivefold. To test if the uORFs are involved in this suppression effect, the AUG start codons of all three uORFs were mutated to missense codons, via point mutations, to give rise to the expression plasmid ORF^{1*2*3*}. The nucleotide changes introduced and their relative positions in the leader are shown in Figure 1B. The effect of the mutations introduced on predicted secondary structure of the leader was checked using the GCG Programmes and found to have negligible influence on overall ΔG values. As seen in Figure 4A, the construct ORF^{1*2*3*} behaves similarly to $ORF^{\Delta L}$ in the cotransfection assay, demonstrating that it is the presence of the uORFs and not possible secondary structure effects of the leader which reduces the transactivation level seen.

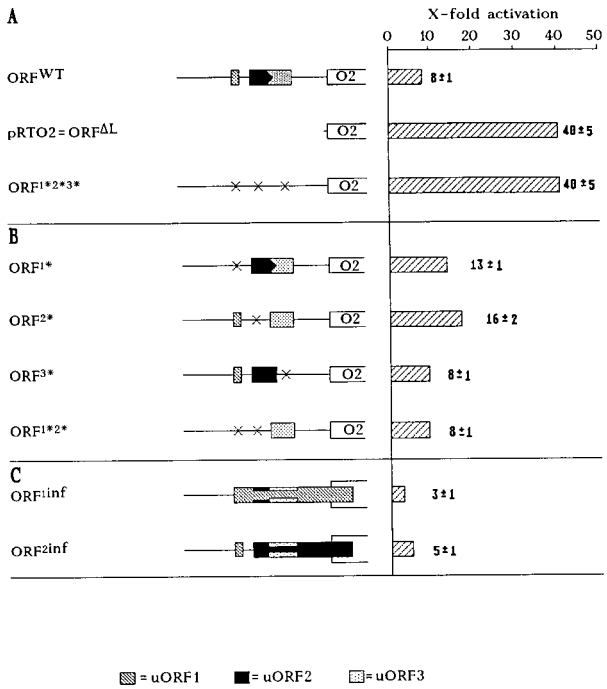

Figure 4. Effects of the uORFs on the Transactivation Potential of *O2*.

(A) and **(B)** Effects of the leader and single uORF mutations on the transactivation potential of *O2*.

(C) ORF interference assay.

Each uORF construct was cotransfected with the reporter plasmid pb32GUS. The GUS values obtained are given as fold activation in comparison with a transfection of pb32GUS alone. Crosses symbolize the substitution of the AUG start codon of the uORFs by missense codons (for mutations introduced, see Figure 1B). Each transactivation value given is the mean of at least 10 independent assays and the error value is the mean deviation over 10 assays.

uORFs Act in *cis* and Not in *trans* to Reduce *O2* Translation

To determine whether putative products of the *O2* uORFs act in *trans* to decrease the transactivation potential of *O2* or if the inhibitory effect of the uORFs is *cis* dominant, a complementation analysis between a plasmid possessing the uORFs (ORF^{WT}) and one lacking the uORFs (ORF^{1*2*3*}) was conducted, as shown in Figure 5. If the inhibitory effect of the uORFs is mediated in *trans*, then the presence of ORF^{WT} should reduce the transactivation value of ORF^{1*2*3*} to a value similar to that for ORF^{WT} (eightfold). If the inhibitory effect of the uORFs is *cis* dominant, then the expression of ORF^{1*2*3*} would not be influenced by the presence of ORF^{WT}. In the complementation mix, equimolar amounts of both expression plasmids were transfected; their molar sum was the same as for the control transfections to take into account the possibility that the uptake of DNA by protoplasts might not be proportional to the amount of DNA supplied. Therefore, in the case of a *cis* effect, GUS activity should be half of the sum of the transactivation values of ORF^{1*2*3*} (40-fold) and ORF^{WT} (eightfold), which would be 24-fold (eightfold + 40-fold ÷ 2).

From 10 independent complementation assays, an average transactivation value of 26 was obtained for the complementation mix. This supports the conclusion that the inhibitory effect of the uORFs is *cis* dominant and indicates that the putative peptide products of the uORFs per se are not mediating the inhibitory effect of the uORFs.

uORF1 and uORF2 Are Primarily Responsible for the Reduction of *O2* Translation

To determine if all of the uORFs, or a particular combination of them, are involved in repression of translation, site-directed mutagenesis of uORF1, uORF2, and uORF3 to introduce point

mutations in each of the start codons was undertaken, generating the constructs ORF^{1*}, ORF^{2*}, and ORF^{3*}, where asterisks denote the mutated ORF. Eliminating the start codon of uORF1 (ORF^{1*}) increases the transactivation value of *O2* (Figure 4B). The same effect was also observed for ORF^{2*}, whereas a missense mutation in the start codon of uORF3 (ORF^{3*}) did not influence the transactivation potential of *O2* (Figure 4B). However, when the start codons of uORF1 and uORF2 were knocked out together (ORF^{1*2*}), then the transactivation activity of the *O2* derivative was similar to that of ORF^{WT}, in contrast to the single uORF mutation. This suggests that uORF3, which under normal conditions is embedded in uORF2, inhibits translation in the absence of uORF1 and uORF2.

Is uORF1 Recognized by the Ribosomal Complex?

The scanning model for translation initiation (Kozak, 1989a) postulates binding of the 43S ribosomal subunit complex at the 5′ end of mRNA, followed by scanning of the mRNA for an AUG codon present in a favorable context for initiation of translation. How efficiently the first AUG encountered by the 43S subunit is recognized depends on the nucleotide sequence of the surrounding region and on its distance from the cap site. Mutation of the uORF1 start codon increases the translation of the major *O2* ORF; thus, uORF1 is likely to be recognized by the 43S initiation complex and translated. Because uORF1 encodes only a tripeptide, generating antibodies against this peptide for its direct detection is not practical, and alternative experiments were necessary to establish whether uORF1 is indeed recognized by the 43S translation initiation complex.

If the ATG of uORF1 is used as a start codon by the ribosomes, then extension of uORF1 to overlap it with the major ORF out of frame should further reduce the translation of the major ORF. Most ribosomes scanning such a modified mRNA would be expected to initiate translation of the new mutant ORF at uORF1 and; therefore, would be unavailable for initiation at the main ORF's AUG.

This ORF interference assay was carried out by changing the stop codon of uORF1 from UAG to UUG. Because uORF1 and the major ORF are in frame, a second mutagenesis reaction was carried out to introduce an additional nucleotide and to generate a frameshift (+1). In the resulting construct, termed ORF^{1inf}, uORF1 is extended from 3 to 104 amino acids with a new stop codon located 165 nucleotides downstream from the start codon of the major ORF. ORF^{1inf} transactivates at a much lower level than ORF^{WT} (Figure 4C). From 10 independent experiments, the transactivation potential of ORF^{1inf} was threefold as compared to eightfold for ORF^{WT}; therefore, the interference resulted in more than a 50% reduction of the transactivation observed.

From this result, three conclusions can be drawn for the behavior of the ribosomes during scanning of the leader region: (1) uORF1 is recognized and translated; (2) a fraction of ribosomes normally reach the major ORF after translating ORF1 (reinitiation); and (3) not all ribosomes that translate the major ORF have translated uORF1 first; otherwise the capacity of ORF^{1inf} to activate the b32 promoter would have decreased to zero, assuming that no internal initiation or resumed scanning occurs.

An ORF interference assay between uORF2 and the major ORF was also carried out to determine if overlapping uORF2 with the major *O2* ORF influences the level of expression of the O2 protein. For constructing the plasmid ORF^{2inf}, only stop codon alterations were necessary because uORF2 is out of frame with the major ORF. In the construction of ORF^{2inf}, the stop codon of uORF2 was altered from UAG to GAG, and in a second mutagenesis, a stop codon located between uORF2 and the major ORF was changed from UGA to CGA. As shown in Figure 4C, ORF^{2inf} is also able to interfere with the translation of the *O2* major ORF, giving a reduction in the transactivation level as compared to wild type.

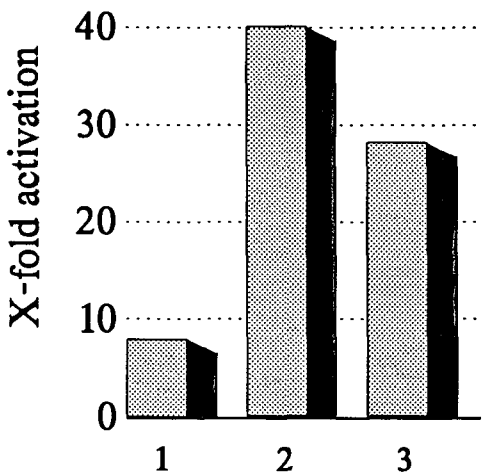

Figure 5. Complementation Analysis Showing the Effect of the uORFs on the *O2* Transactivation Potential in *trans*.

The GUS activities obtained are represented as fold transactivation exerted by *O2* on the b32 promoter. Bar 1 represents protoplasts that were transfected with 2 μg of ORF^{WT} and 5 μg of pb32GUS. Bar 2 represents protoplasts that were transfected with 2 μg of ORF^{1*2*3*} and 5 μg of pb32GUS. Bar 3 is the complementation mix and shows protoplasts that were transfected with 1 μg ORF^{WT}, 1 μg ORF^{1*2*3*}, and 5 μg pb32GUS.

DISCUSSION

In this report, the effect of the uORF present in the mRNA of the maize regulatory gene *O2* on translation of the major ORF was analyzed. The experiments were designed to address three issues: whether the predicted uORFs are indeed upstream of the major ORF rather than forming part of it, whether the uORFs have an effect on the expression of *O2*, and how the uORFs exert their effect on translation.

From Figure 2, it is apparent that the molecular weight of O2 protein derived from a leaderless mRNA is the same as

that of the native O2 protein; this result would not be expected if the translation of *O2* were to start at one of the uORFs. Therefore, the uORFs are not part of the major ORF.

Data pertaining to whether the uORFs affect expression of *O2* or translation are discussed in terms of the scanning hypothesis for eukaryotic translation (Kozak, 1978, 1989a). Kozak proposed that the initiation complex assembles at the 5′ end of the mRNA and scans in the 3′ direction until an AUG codon is encountered, whereupon translation begins. Insertion of an additional AUG codon into the mRNA leader generally reduces translation of downstream coding sequences (Kozak, 1987b), even when a stop codon is located in frame directly downstream of the new AUG codon. The fact that such an in-frame stop codon does not abolish the inhibitory effect of an upstream AUG suggests that reinitiation of ribosomes at an internal start site is usually inefficient (Sherman and Stewart, 1982; Johansen et al., 1984; Kozak, 1984; Liu et al., 1984), even if exceptions to this rule occur (Thach, 1992).

The effect of the uORFs present in the leader of the *O2* mRNA has been assayed via expression of *O2* in tobacco protoplasts. The *O2* mRNA leader reduces the ability of *O2* constructs to transactivate their natural target promoter b32 by a factor of five in this system (as obtained by the comparison of the transactivation values of the leaderless *O2* derivative pRTO2 [40-fold]) and the wild-type derivative ORF^{WT} (eightfold). Evidence that the uORFs are responsible for this effect has been obtained from the expression of the *O2* construct ORF^{1*2*3*}, in which each of the uORF's start codons was changed to a missense codon. This *O2* construct lacking the uORFs, which differs from the wild-type allele by only five nucleotide changes, shows the same high transactivation value as that of the leaderless *O2* allele pRTO2, indicating that the uORFs reduce the transactivation potential of ORF^{WT}. The uORFs presumably reduce translation of the major *O2* ORF via dissociation of ribosomes at their stop codons. This means that when all of the uORFs have been eliminated, all ribosomes which initiate scanning at the 5′ end of *O2* mRNA should reach the major ORF and translate it. Because the transactivation values of $ORF^{\Delta L}$ and ORF^{1*2*3*} are identical, it can be inferred that a similar number of ribosomes translates the *O2* ORF of the leaderless *O2* allele as the *O2* ORF of ORF^{1*2*3*} (allele with the mutated uORFs). In ORF^{WT}, however, most of the ribosomes that bind probably dissociate at uORF stop codons while scanning the leader sequence. Further experiments are necessary to quantify this effect.

In contrast to vertebrates and invertebrates, the region surrounding an AUG in plants has been proposed to have less influence on the efficiency of translation initiation (Lütcke et al., 1987), although preference for a purine at −3 with respect to the AUG was observed (Kozak, 1989b). To check the possibility that uORF1 and uORF2 might not normally be recognized by scanning ribosomes due to a suboptimal start codon context, the sequences around the start codons of uORF1 and uORF2 were modified to resemble that of the major ORF, which must be recognized by the ribosomes. These constructs, however, showed the same transactivation potential as ORF^{WT} (data not shown), thus supporting the hypothesis that uORF1 and uORF2 are normally recognized by scanning ribosomes.

Further evidence that at least ORF1 is recognized by the initiation complex was provided by the ORF interference assay (Figure 4C), in which an out-of-frame translation initiated at uORF1 and running into the major *O2* ORF reduced, but did not completely eliminate, *O2* activity in the protoplast assay. We conclude that the reduction in *O2* activity arises because ribosomes that have initiated at uORF1 are unable to translate the major *O2* ORF, assuming that backscanning is negligible (Johansen et al., 1984; Peabody and Berg, 1986; Sedman et al., 1989). The remaining *O2* activity seen is presumably derived either from reinitiation (Kozak, 1989a) or imperfect recognition (bypass) of uORF1. This might be exacerbated in the wild-type allele in which uORF1 is only three residues long (Kozak, 1987b).

The complementation assay, as shown in Figure 5, demonstrated that the suppressing effect of the uORFs is not exerted in *trans*, from which we infer that the polypeptides encoded by the uORFs play no active role in negatively controlling the translation of the *O2* major ORF. This result resembles the cases of *GCN-4* and *CPA-1*, in which the effect of the uORFS is also *cis* dominant with the peculiarity that in *CPA-1* the sequence information of the single uORF is essential for suppression.

It is interesting to note that a small proportion of cellular mRNAs, including several protooncogenes (<5%; Kozak, 1987a) contain uORFs in their leader regions. Several such mRNAs are encoded by protooncogenes (Kozak, 1987a), in which uORF deletions may cause oncogenic conversion (Marth et al., 1988). It is quite possible that transcriptional regulation for such genes is not sufficient for a fine control of their expression and at the translational level a particular cellular status may be better sensored.

Two nonviral uORF-containing genes that have been analyzed in detail are *GCN-4* and *CPA-1*, both involved in the regulation of amino acid biosynthesis in yeast. They are primarily regulated at the translational level through the inhibitory effect of their uORFs (Mueller and Hinnebusch, 1986; Werner et al., 1987). In *GCN-4*, the first and fourth of four uORFs act positively and negatively, respectively, in regulating the translation of the *GCN-4* protein such that it is blocked under conditions of adequate amino acid supply and only synthesized when amino acids are scarce.

It remains to be established whether the uORF-mediated inhibition of *O2* expression observed in our assay represents an in vivo modulation of expression in response to some environmental or developmental signals, as is the case for *GCN-4*. During synthesis of zeins, which are under the control of *O2*, there is an increase in the accepting activity of the tRNA for glutamine, alanine, and leucine, the most abundant amino acids in zeins (Viotti et al., 1978). Because the translation rate of the uORFs is dependent on the concentration of

loaded tRNAs, such changes may modulate the efficiency of translation of *O2* and thereby influence the level of zein gene transcription.

METHODS

Production and Purification of O2-Specific Antibodies

After introduction of $pGEXO2_{55}$ into *Escherichia coli* BL-21 by transformation, the $GSTO2_{55}$ fusion protein was isolated, as described by Lohmer et al. (1991). Following affinity purification on a glutathione column, the highly purified O2 fusion protein was subjected to preparative SDS-PAGE (Laemmli, 1970). The corresponding band was visualized by incubating the gel with ice-cold 0.25 M KCl (Nelles and Bamburg, 1976) and cut out. After electroelution of the O2 fusion protein from the gel slices (2 hr, 250 V) in dialysis tubes (Elgin, 1975), the fusion protein was injected as a 1:1 mixture with complete Freund's adjuvant into rabbits. The rabbits were injected at four subcutaneous and two intramuscular sites, boosted after 4 weeks, and bled 1 week after boosting.

Purified glutathione-S-transferase (GST) was dialyzed against 0.1 M Hepes, pH 7.5, and used to prepare an affinity column (Affigel 10; Bio-Rad) according to the manufacturer's protocol. Ten milliliters of antiserum was diluted 1:1 with PBS and loaded onto the GST-coupled affinity column to eliminate those bodies reacting against GST. The flow through was collected and extensively preadsorbed by an *E. coli* protein extract to eliminate those antibodies recognizing *E. coli* proteins. The supernatant containing the O2-specific antiserum was stored frozen in small portions at −70°C. The specificity of the purified antiserum was checked via protein gel blot analysis of O2 protein–expressing *E. coli* extracts and control strains.

Protein Gel Blot Analysis

Immunoblots were prepared as described by Towbin et al. (1979). Cell or nuclear extracts were denatured in a boiling water bath for 5 min. Proteins were separated on 7.5% polyacrylamide gels (Laemmli, 1970). Protein transfer to nitrocellulose membrane (Schleicher & Schuell) was performed at a constant current of 300 mA for 4 hr. The nitrocellulose was stained with Ponceau red to establish that the blots contained approximately the same amount of protein in each lane. Immunoadsorption was performed with the affinity-purified, O2-specific antibodies, and the detection was carried out with the ECL Western Blotting Detection System from Amersham International.

Site-Directed Mutagenesis

To introduce the desired point mutations, the phosphoramidite method (Sayers et al., 1988) was used, as described by the manufacturer (Amersham). To carry out the mutagenesis reaction, a single-stranded DNA template was necessary. The leader region of *O2* was inserted as a 280-bp-long KpnI fragment into the KpnI site of M13mp19 (Yanisch-Perron et al., 1985), generating m13O2L, which was transformed into TG-1. Single-stranded DNA for the mutagenesis reaction was isolated, as described by Maniatis et al. (1982). Mutagenic primers were synthesized with an Applied Biosystems (Weiterstadt, Germany) 392-oligonucleotide synthesizer. The primers for generating upstream open reading frame $(uORF)^{1*}$, $uORF^{2*}$, and $uORF^{3*}$ were designed such that in each mutant a restriction site was created in the course of substituting each upstream AUG codon (XhoI, BglII, and PvuI, respectively), allowing rapid and reliable screening for mutant plasmids. In the case of $uORF^{1inf}$ and $uORF^{2inf}$, positive clones were identified via sequencing of single-stranded DNA of randomly picked plaques. The mutagenesis procedure was carried out with a site-directed mutagenesis kit from Amersham following the supplier's instructions.

Constructs for Transient Gene Expression

From positive clones of the mutagenesis reaction, double-standed DNA was prepared (Maniatis et al., 1982) and cut with ApaI, thereby releasing the mutagenized leader fragment, which contained 23 foreign nucleotides at its 5′ end and was terminated by codon 12 of the *O2* encoding region at its 3′ end (Figure 3A). Each of the mutagenized fragments was inserted into the ApaI site of the plasmid $pRTO2^{\Delta Apa}$ (Figure 3A), which was constructed as follows. *pRTO2* (see next section for this construction) was digested with ApaI and religated, resulting in a shortened *O2* coding region, starting at codon 13. Through insertion of the mutagenized leader fragment or of the wild-type leader fragment, the complete leader region and the *O2* coding region were restored, generating the expression plasmids ORF^{1*}, ORF^{2*}, ORF^{3*}, ORF^{1*2*3*}, ORF^{1imp}, ORF^{2imp}, ORF^{1inf}, ORF^{2inf}, and ORF^{WT}. In each case, the sequence of the inserted leader was determined by the dideoxy sequencing technique (Sanger et al., 1977), using a variety of synthetic oligonucleotides as primers.

Construction of pRTO2 was carried out in the following way. To remove the 280-bp-long leader sequence of *O2*, the plasmid pMM1a, containing the complete *O2* cDNA, was cut with ApaI, linearizing the plasmid at amino acid position 12. Then, a linker carrying the coding sequence for the 12 N-terminal amino acids and a HindIII restriction site in front of the coding sequence was fused to the ApaI site. The complete ORF of *O2* was released as a HindIII–BamHI double digest 1.6-kb fragment, which was made flush ended by treatment with the Klenow fragment of DNA Polymerase I. For generating pRTO2, this fragment was inserted into the filled-in HindIII restriction site of the plant expression vector pRT100 (Töpfer et al., 1987).

The construction of the reporter plasmid pGUSb32 is described elsewhere (Lohmer et al., 1991).

Transient Gene Expression in Tobacco Protoplasts and β-Glucuronidase (GUS) Assay

Five grams of young leaves of in vitro–cultured Nicotiana tabacum cv Petit Havana SR1 was digested under sterile conditions in 100 mL of P1 medium (449 mg Murashige-Skoog salts [Murashige and Skoog, 1962], vitamins, 10 mg myoinositol, 25 mg D-xylose, 13.6 g sucrose, 0.1 g naphthalene acetic acid, 0.02 mg kinetin, pH 5.6, 600 milliosmoles) in the presence of 1 g cellulase and 0.5 g Macerozyme (R-10; Serva, Heidelberg, Germany). Protoplasts were floated, washed, transfected, and incubated to test for transient expression, as described in Negrutiu et al. (1987), with the exception that the P1 medium was used instead of the K3 medium.

GUS enzyme activities in tobacco extracts were determined essentially as described by Jefferson (1987). Fluorescence was measured with a Perkin-Elmer Luminescence Spectrometer LS30 fluorometer.

A solution of 4 mM 4-methylumbelliferone (Sigma) in 0.2 M sodium carbonate was used to calibrate the fluorescence intensity.

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