

Three Mutations in *Zea mays* Affecting Zein Accumulation: A Comparison of Zein Polypeptides, In Vitro Synthesis and Processing, mRNA Levels, and Genomic Organization

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ABSTRACT We studied three mutations, *opaque-2* (*o2*), *opaque-7* (*o7*), and *floury-2* (*fl2*), each of which causes a depression in zein synthesis. We examined the processing efficiencies of the rough endoplasmic reticulum membranes in vitro, the levels of RNA transcription using cloned zein probes, and the genomic organization of the zein sequences as possible sites for the genetic defects. The results obtained indicate that the steps in prezein translation and processing occurring on the protein body membranes are not accountable for the lowered zein content in any of the mutations. The *o2* mutation that typically shows a paucity of 22.5-kdalton zein polypeptides was found to have a concomitant reduction in a particular subgroup of mRNAs coding for this size class. Southern analyses suggest that the *o2* mutation is not the result of a large deletion of tandem-linked zein genes.

There are three well-studied, nonlethal mutations known in maize that cause large changes in the relative proportions of the storage proteins. These are *opaque-2* (*o2*), *opaque-7* (*o7*), and *floury-2* (*fl2*). Their most notable effect is a profound reduction in the prolamine fraction of maize, called zein (32, 33, 36).

Zeins are the major storage proteins of the maize endosperm. They are actually a very large, heterogeneous family of related polypeptides that together comprise more than half of all the extractable proteins found in the seed at maturity. The zein family can be separated into two subclasses with molecular weights of ~19,000–21,000 and 22,000–23,000 (5, 16, 27, 33). More than 20 zein polypeptides have been estimated to exist on the basis of isoelectric focussing (IEF) (41) and two-dimensional IEF:SDS PAGE (9, 10, 17). Each maize strain apparently has a characteristic two-dimensional IEF:SDS PAGE pattern for its zeins. Partial amino-terminal sequences for the total zein fraction (2) and for zein proteins that had been separated into heavy (10, 26) and light (10) polypeptides indicated that the members of each class were closely related to each other and that the two size classes also shared some sequence homology.

Zein mRNA preparations have been purified and characterized by several laboratories (6, 47, 50). A number of cDNA clones of zein mRNAs have been constructed (4, 7, 39, 49) and one has been sequenced (15). Zein cDNA clones have been used to examine the extent of the heterogeneity in the zein family (7, 8, 10, 18, 38, 39). The results of such studies have shown that the zein proteins are encoded by a large number of structurally related sequences that can be divided into subfamilies. Of the 20 cDNA clones we analyzed, we recognized two

subfamilies that coded for 22.5-kdalton zeins and three subfamilies for the 19-kdalton class (7). Other multigene families have previously been described for chorion (42, 45), globin (25), insulin (3), tubulin (14, 21), and vitellogenin (48). Recently, genomic clones containing zein sequences have been reported (28, 51).

Zein proteins are synthesized by polyribosomes associated with endoplasmic reticulum (ER) cisternae (5, 26) in certain tissues of the endosperm (13). During development of the kernel, which takes ~45 d, zein accretions form in peripheral regions in the lumen of the rough ER (RER) cisternae. These ultimately develop into 1–2- μ m vesicular protein bodies (13, 23). Zeins are made as preproteins with 1–2-kdalton presequences (6, 26). We had earlier developed a cell-free, mRNA-dependent system from maize endosperm RER that correctly synthesizes, processes, and accumulates zein polypeptides (9). RER preparations from other maize tissues were not so efficient in processing (9). The zein presequences are apparently removed by an exopeptidase, perhaps in conjunction with an endopeptidase as has been postulated for proinsulin (22). There are indications that other modifications besides the presequence cleavage are involved in prezein processing (8, 10).

The *o2* locus has long been proposed to be a regulatory locus for zein synthesis (35). Depending on the particular maize strain background, there is generally a partial to nearly complete suppression of the 22.5-kdalton class of zein polypeptides in the *o2* mutants as analyzed by one-dimensional SDS PAGE (27, 34, 43). In two-dimensional IEF:SDS PAGE, a few minor species of the 19-kdalton class have also been reported to be diminished (17). Such major, class-specific effects have not

been observed for *o7* and *fl2* (27, 34), although the overall content of zeins is substantially reduced. Because zeins are synthesized as preproteins on a particular type of RER membrane, any mutation affecting their site-specific synthesis, transport, or processing would be expected to interfere with zein accumulation (5, 20). A decreased zein content could also be brought about by a lower level of zein mRNA transcription. The three mutants—*o2*, *o7*, and *fl2*—were therefore examined for differences in protein processing, transcription, and genomic organization in an attempt to learn which steps in zein biosynthesis were being adversely affected.

MATERIALS AND METHODS

Maize Strains

The following maize inbreds and their mutant counterparts were used in this study: Illinois High Protein (1977 Selection); W22+, W22 *o2*, W22 *o7*, W22 *fl2*; W64A+, W64A *o2*; R802+, R802 *o2*. The plants were self-pollinated and the ears were harvested 18–20 d after pollination. Endosperm tissue was dissected from the kernels into liquid nitrogen and stored at -85°C .

Zein cDNA Clones

From 20 characterized cDNA zein clones, four clones, representing four different zein subfamilies, were selected for use. The details of their construction and characterization are contained in another paper (7).

TABLE I
Zein cDNA Clones

Zein class	Clone	Restriction endonuclease sites characteristic of cloned insert	Size of insert
			bp
22.5	B14	<i>Hpa</i> II	605
	B36	<i>Hha</i> I, <i>Hae</i> II, <i>Hpa</i> II	295
19	B37	<i>Hae</i> III*	415
	B59	<i>Hpa</i> II, <i>Hae</i> II, <i>Hae</i> III, <i>Hha</i> I	800

bp, base pair.

* Other members of this subfamily were also cleaved by *Pst* I.

Preparation of Materials

Procedures previously detailed were used for the preparation of authentic zeins (5), zein mRNAs (6), and maize DNA (7). RER-enriched fractions were prepared from endosperms stored at -85°C and were kept at -85°C until use (9, 11).

Methods

SDS PAGE was performed according to Anderson et al. (1), except that a linear 10–15% acrylamide gradient was used. Two-dimensional IEF:SDS PAGE (37) was carried out using ampholytes of pH 6–8 in the first dimension and 15% polyacrylamide:0.1% SDS in the second dimension.

The conditions for the *in vitro* translation and processing of prezeins have been published (9, 11). Each 25- μl reaction mixture contained 0.3 μg of poly(A)+ RNA and 5 μl of micrococcal nuclease-treated endosperm RER membranes at 100 OD₂₆₀ U/ml. The incubation was carried out for 90 min at 25°C , after which the reactions were treated with 0.04 $\mu\text{g}/\mu\text{l}$ proteinase K (EM Laboratories, Inc., Elmsford, NY) at 4°C for 45 min. 1 μl of a saturated solution of phenylmethylsulphonyl fluoride (PMSF) (Calbiochem-Behring Corp., La Jolla, CA) in 95% ethanol was added and the reaction was left on ice for 10 min. The membranes were pelleted by centrifugation and taken up in electrophoresis sample buffer. Gels for fluorography were treated with Enhance (New England Nuclear, Boston, MA) and exposed to Kodak X-Omat AR film at -85°C .

Poly(A)+ RNAs were denatured in dimethyl sulfoxide (DMSO):glyoxal according to McMaster and Carmichael (30) and electrophoresed in 1% agarose gels containing 10 mM Tris-HCl, 10 mM boric acid, 1 mM EDTA, pH 7 (Ray Zielinski, personal communication). Blotting to nitrocellulose and hybridization conditions were those of Thomas (46).

Maize DNA was digested with restriction endonucleases *Eco* RI and *Hind* III (New England Biolabs, Beverly, MA), using the protocol suggested by the

supplier, and electrophoresed in 0.4% neutral agarose gels (29). Modified Southern transfer (44) and hybridization were performed by the method of Klessig et al. (24). Recombinant plasmids used as probes were labeled with ^{32}P by nick-translation (40).

RESULTS

IEF:SDS PAGE of Zeins

Authentic zein samples prepared from mature endosperms of W22+, W22 *o2*, W22 *o7*, W22 *fl2*; W64A+, W64A *o2*; and R802+, R802 *o2* were compared by two-dimensional IEF:SDS PAGE. The results for W22+ and its corresponding mutants only are shown in Fig. 1. In W22 *o2*, there is a major suppression of most of the polypeptides of the 22.5-kdalton class. The same effect is observed in W64A *o2*, confirming the results of Hagen and Rubenstein (17), and for R802 *o2*. As had been previously noted for W64A *o2* and Oh43 *o2* (17), a few minor components of the 19-kdalton class also seem to be missing in W22 *o2* (Fig. 2) and in R802 *o2*. There are, however, also several new minor spots in the 22.5-kdalton class and an increase in some components of the 19-kdalton class (Figs. 1 and 2). The polypeptide changes in W22 *o7* and W22 *fl2* are more subtle. W22 *o7* has some spots absent from both molecular weight size classes, while W22 *fl2* seems to be missing

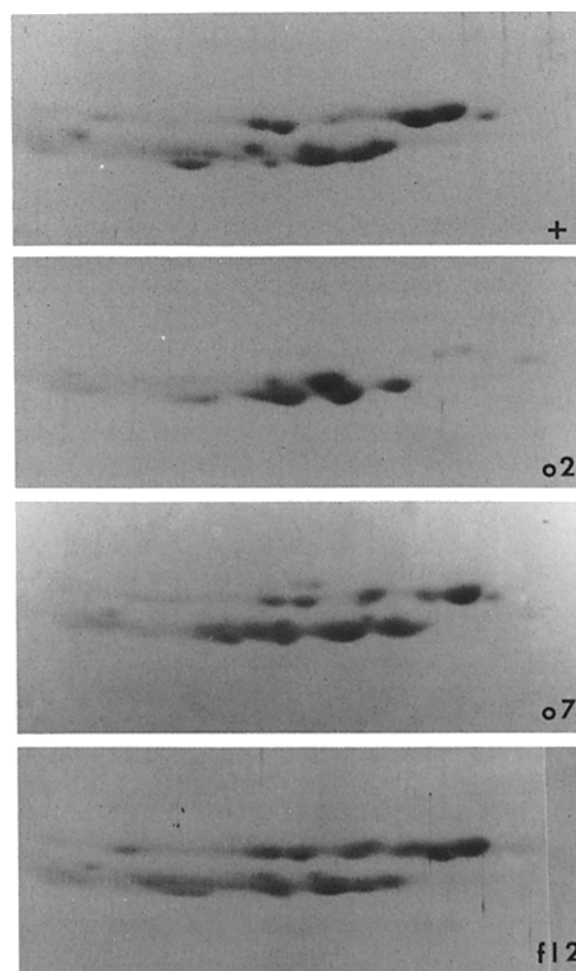


FIGURE 1 Two-dimensional IEF:SDS PAGE of W22 wild-type and mutant zeins. First dimension (horizontal): IEF, ampholytes of pH 6–8, cathode to the right. Second dimension (vertical): 15% polyacrylamide:0.1% SDS, anode at bottom. Gel stained with Coomassie Brilliant Blue.

spots from only the lower molecular weight size class. Many quantitative changes, either higher or lower, are noted in the remaining polypeptides in all three mutations. It should be recalled, nevertheless, that the total amount of zeins in the seed has been shown to be decreased in the case of each of the three mutations (32, 33, 36).

In Vitro Processing of Prezeins

Native (untreated) RER membranes from endosperms of wild-type W22, and the W22 mutant counterparts, were added to the wheat germ cell-free system under conditions which permitted processing of prezeins (9, 11). The results are depicted in Fig. 3. W22+ RER makes both the 19- and the 22.5-kdalton classes of zeins. Untreated membranes of W22 *o2* produce only the 19-kdalton class, while W22 *o7* has both major zein classes. Mostly 19-kdalton zeins are synthesized in W22 *fi2*, but there is also a minor band at ~25 kdaltons. Additional bands are present in *o2* and *o7* at 13 and 30 kdaltons. The lower molecular weight products in the basal region of the fluorogram are premature chain termination peptides that are frequently observed in cell-free translations of zein mRNAs. All the products detected are resistant to proteinase K digestion, indicating that they are inside the RER vesicles. Comparisons with translations carried out using only zein mRNAs, and no membranes, indicate that these zein oligopeptides have already lost their presequences (data not shown). Treatment of the membranes with micrococcal nuclease obliterates all synthetic activity (Fig. 3). However, when wild-type zein mRNAs are added to treated membranes, both zein classes are translated and correctly processed by all four

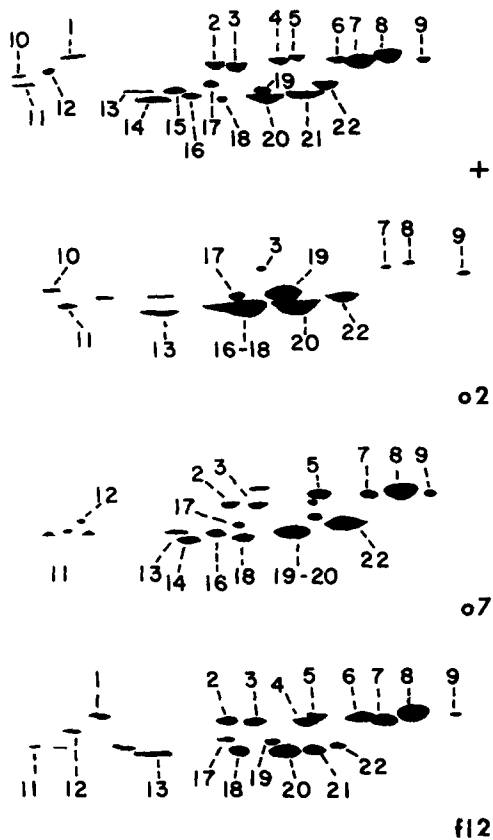


FIGURE 2 Diagram of gels shown in Fig. 1. The spots numbered in the mutants correspond in migration to the spots so numbered in the wild type.

strains (Fig. 3). Slight variations in synthetic efficiency were reflected in the quantities of zeins made by wild-type and mutant membranes. These were dependent on the endosperms used, the conditions of membrane isolation, and the particular cell-free synthesis. No consistent differences were noted that could be definitively attributed to genotype.

The relationship between the type of zein mRNA preparation employed and the classes of zein synthesized was examined by incubating treated membranes of all four strains with zein mRNAs prepared from either W22+ or W22 *o2*. As seen in Fig. 4, each kind of messenger preparation produced the same

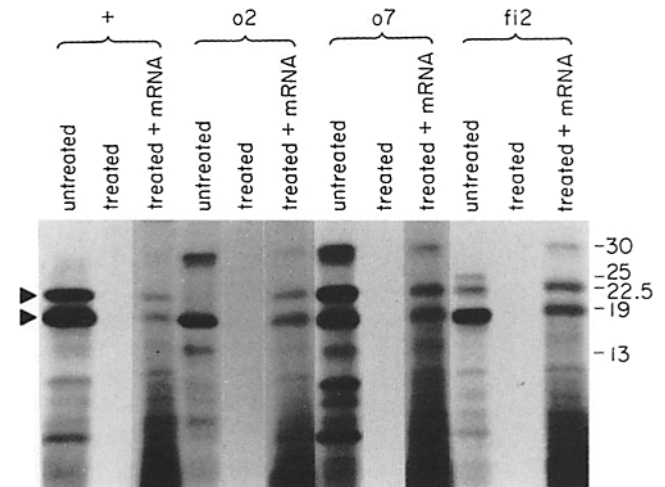


FIGURE 3 Fluorogram of a 10-15% linear polyacrylamide:0.1% SDS gel showing products of in vitro synthesis with W22 wild-type and mutant membranes. Each of the four sets of reactions shows from left to right. *Untreated*: endosperm RER, not treated with micrococcal nuclease (mature zein polypeptides synthesized). *Treated*: membranes treated with nuclease (no synthesis). *Treated + mRNA*: nuclease-treated membranes + wild-type zein mRNAs (mature zein polypeptides synthesized). Arrowheads indicate the positions of authentic zeins. Molecular weights, $\times 10^3$.

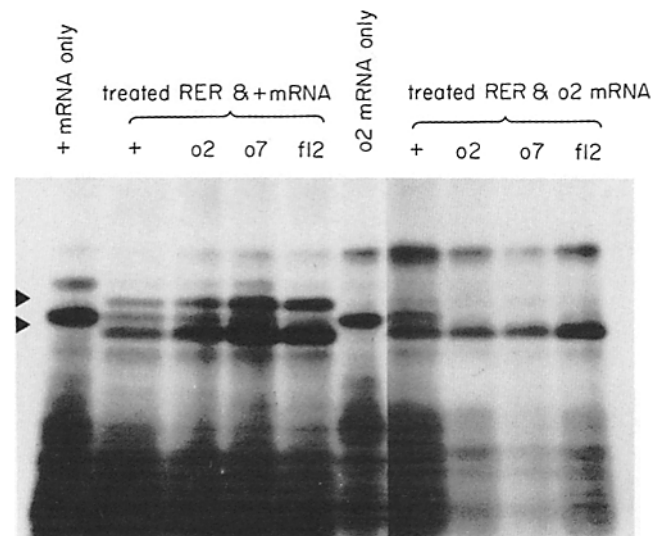


FIGURE 4 Fluorogram of in vitro synthesis products of W22+ and W22 *o2* poly(A)+ RNAs. The first and sixth lanes show the prezeins produced by zein mRNAs alone; the products are larger than mature zeins. The other reactions containing treated membranes make mature-sized zeins characteristic of either (+) or *o2* mRNAs. All four types of membranes are capable of processing. Arrowheads indicate the positions of authentic zeins.

characteristic zeins with all four types of membranes. With wild-type mRNAs both zein classes were synthesized and processed (Fig. 4, left), while with *o2* mRNAs only the 19-kdalton class was detected. We know from other work (9) that the band between the 19- and 22.5-kdalton bands seen in some of the lanes represents some unprocessed 19-kdalton zein that is taken up by the membranes and is an artifact of the *in vitro* system.

Transcription Studies

The *in vitro* synthesis and processing experiments suggested that the suppression of zeins in *o2* might be due to the absence of specific mRNAs coding for 22.5-kdalton zeins. The mRNA preparations of normal and mutant endosperms were tested by filter hybridization with cloned zein cDNA probes. Poly(A)+ RNAs of the four strains were electrophoresed in agarose gels under denaturing conditions and transferred to nitrocellulose filters. Filters of replicate gels were hybridized with nick-translated probes prepared from zein clones B14, B36, B37, and B59. B14 and B36 are clones for two different subfamilies of the 22.5-kdalton zein class; B37 and B59 are clones for two distinct subfamilies of the 19-kdalton class (see Materials and Methods).

The results of the filter hybridizations are presented in Fig. 5. Denatured zein mRNAs have been previously estimated to be 1.1 to 1.2 kilobase pair (kbp) in length by gel electrophoresis and electron microscopy (6). Each of the probes employed recognized a major set of complementary sequences in this size range in the poly(A)+ RNAs from all four strains, except for probe B14. Only an extremely weak signal is detected when W22 *o2* is hybridized with B14. Initially, it seemed as though no homologous sequences were present, but re-exposure of the filter for a longer time showed that a faint band was present. B14 has been classified as a 22.5-kdalton clone (7), which is the class of peptides that is suppressed in *o2*. On the other hand, B36, the other probe for the 22.5-kdalton class, gave a good signal in *o2*. B37 and B59, both 19-kdalton clones, hybridized to all the strains, except that the response was rather weak for *o7*. There were variations in signal strength between the four strains with a given probe although equivalent amounts of poly(A)+ RNAs were loaded into each sample well and all samples were electrophoresed in the same gel.

The broadness of the band being detected is probably due to the heterogeneous sizes of the zein mRNAs, in terms of having

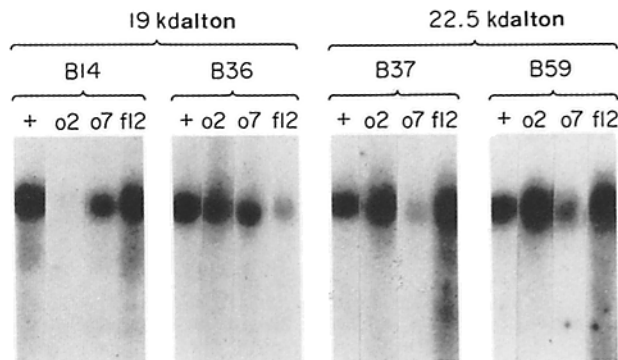


FIGURE 5 Autoradiogram showing the results of wild-type and mutant poly(A)+ RNAs denatured in DMSO:glyoxal, electrophoresed in a neutral agarose gel, blotted to nitrocellulose, and hybridized with four nick-translated cDNA probes. B14 and B36: cDNA clones of 22.5-kdalton zeins. B37 and B59: cDNA clones of 19-kdalton zeins. Each channel contained 2 μ g poly(A)+ RNA.

both different size classes (50) and partially degraded 3' poly(A) termini (6, 8). There is some hybridization with lower molecular weight products, particularly with W22 *fl2* RNA. It is not known whether this is due to degradation either *in vivo* or as a result of preparation.

Genome Organization

The RNA filter hybridizations showed that a certain class of mRNA transcripts was severely reduced in the *o2* mutant. The B14 clone that was homologous to this class of messages in *o2* enabled us to examine *o2* DNA for deletions, or other types of structural alterations, in the sequence(s) coding for that class. This part of the study was carried out using *o2* in the W64A inbred background rather than in W22, as *o2* had arisen as a spontaneous mutation in the W64A line. Moreover, preliminary experiments had already indicated to us that W22+ and W22 *o2* showed some restriction fragment polymorphism.

DNAs from wild-type W64A and W64A *o2* were digested with *Eco* RI, *Hind* III, and *Bgl* I. The digests were electrophoresed on neutral agarose gels and transferred to nitrocellulose. Recombinant DNA from clone B14 was labeled by nick-translocation and used as a hybridization probe (24). No differences in the restriction fragment patterns were observed when (+) or *o2* DNAs were digested with either *Bgl* II or *Hind* III (Fig. 6).

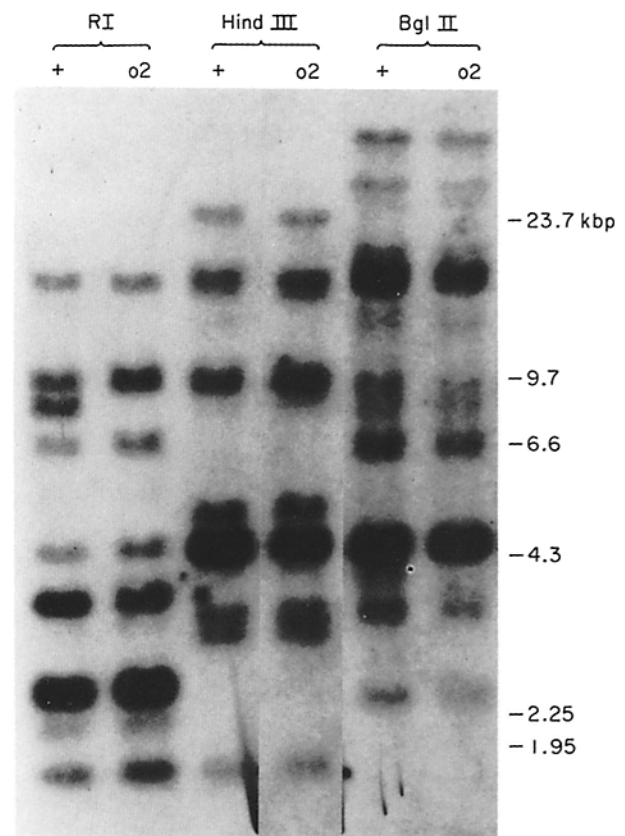


FIGURE 6 Southern transfer hybridization of wild-type and mutant DNAs. DNAs from W64A+ and W64A *o2* were digested with restriction endonucleases *Eco* RI, *Hind* III, and *Bgl* II and electrophoresed on a 0.4% neutral agarose gel. The probe used was nick-translated DNA from the cDNA clone B14. The only difference noted in the restriction patterns was observed with *Eco* RI: the third band from the origin seen in (+) is missing in *o2* and is seemingly replaced by another band that comigrates with the band just above. Markers used were *Hind* III digested λ DNA.

With *Eco* RI a single change was noted—the third band from the origin was W64A+ is missing in *o2* and is probably replaced by a larger fragment that comigrates with the band immediately above (Fig. 6).

DISCUSSION

Mutations that cause a reduction in zein content are of interest because they result in a more nutritional grain. This is because the zein proteins, which are deficient in lysine and tryptophan, typically comprise the majority of proteins found in the seed. Suppression of zeins is accompanied by a reciprocal rise in other storage proteins, particularly certain globulins (12) that have a more typical amino acid composition. In this study, we examined three such mutations, *o2*, *o7*, and *f12*, for possible defects which might account for the decrease in zein content. Wild-type and mutant lines were compared for qualitative differences in zein synthesis, prezein processing, zein mRNA transcription, and zein genomic organization.

The mutations, for the most part, were compared in the W22 background. It should be restated that *o2* originated as a mutation in another line (W64A) and was crossed into the W22 inbred. Likewise, *f12* arose in a noninbred stock and was similarly crossed into W22. The *o7* mutation, however, was identified in W22 (31). The W22 *o2* and W22 *f12* lines have been backcrossed to W22 for an unknown number of generations to make the material as isogenic as possible. Nevertheless, a certain level of background heterogeneity undoubtedly persists.

The *o2* mutation is the only one known to exhibit a strong suppression of largely one size class of zein polypeptides. This selective decrease in the 22.5-kdalton zein class is well-known from earlier electrophoretic studies and has been reported by a number of workers (27, 34, 43). The relative proportions of zeins do not seem to vary in either (+) or *o2* during endosperm development (41). In the case of *o7* or *f12*, only minor polypeptides are missing in two-dimensional gels, although there is a great reduction in total zein content. It therefore appears that in these latter two mutations the effect is a general inhibition of the synthesis of all zein classes. Either mutation provokes a differential response that is distinct from the wild type. The observation that the relative proportions of the polypeptides are seemingly independently altered may be significant.

The *in vitro* translation-processing experiments suggest that none of the mutations implicate the membrane-polysomal apparatus as being defective. The membranes from all three mutants functioned similarly when incubated with a given mRNA preparation and, in particular, were able to synthesize wild-type zein from (+) zein mRNAs. Moreover, no differences were found when RER fractions from wild-type and mutant strains were examined on SDS polyacrylamide gels (data not shown). The 30-kdalton band seen in some translations seems to be a nonzein mRNA product that copurifies with the zein messages in *o2* and *o7*. This polypeptide does not have a presequence (cf. Fig. 4), but is alcohol-soluble. The contaminants, however, do not detract from the essential point being made—that the mutant membranes process zein proteins as effectively as wild-type RER.

By the use of cloned zein probes, we have been able to demonstrate that the decrease of zein proteins in W22 *o2* is apparently the result of the suppression of transcription for a select subgroup of 22.5-kdalton zein genes. Jones et al. (20) had earlier reported that there was a marked reduction of the heavier class zeins in cell-free translations with *o2* polysomes.

Another consequence of the *o2* mutation is an increase (up to five times, depending on the particular inbred) in the level of ribonuclease (52). Nevertheless, given the structural similarities of the different zein classes (2, 7, 10) and the common intracellular localization of the mRNAs (5), it seems improbable that there would be a more rapid degradation of only a particular subgroup of zein messages. It is interesting to speculate on how the *o2* mutation could be causing selective suppression. Perhaps the genomic sequence encoding this subgroup of polypeptides have some structural similarity, or perhaps they are tightly linked and controlled by a *cis*-acting mechanism. At present, the map locations of the genes being affected is not known relative to *o2*, which is located on the short arm of chromosome 7. A deletion of tandem-linked genes can be discounted as the genomic filter hybridizations do not reveal any losses that could substantiate this. By contrast, in *Bombyx mori*, Southern analysis has shown that the *Gr^B* mutation that causes the suppression of several chorion proteins is apparently the result of a large deletion of a cluster of chorion genes (19). The zein sequences are not amplified in the endosperm during the time they are expressed (7). Therefore a mechanism that prevents their amplification, as in the case of the *ocelliless* mutation of *Drosophila* (45), can be also ruled out.

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