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Zein synthesis in maize endosperm by polyribosomes attached to protein bodies

(protein synthesis/prolamine/zein message)

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ABSTRACT **The protein bodies in maize endosperm are the sites of zein deposition. They are single membrane-bound vesicles with polysomes associated with the exterior surface of the membrane. These protein bodies were isolated by sucrose density gradients and characterized by electron microscopy and polyacrylamide gel electrophoresis. Polyribosomes dissociated from the surface of the membrane by detergent treatment were placed into an amino-acid incorporating system. Based on alcohol solubility, amino-acid composition, and molecular weight distribution, the product synthesized appeared to be largely, or entirely, zein. This suggests the existence of components which are specific for the synthesis of zein at the protein body membrane surface.**

Zein is the prolamine from maize endosperm. It is very hydrophobic and requires aqueous alcohols, a high concentration of urea, high pH, detergents, or a variety of nonaqueous organic solvents for solubilization (1, 2). Zein is deficient in basic or acidic amino acids but rich in amides, leucine, proline, and alanine (3). It may comprise as much as 50% of the endosperm protein of normal maize (4). Duvick (5) first proposed that protein bodies might be the repository for zein and further evidence for this was provided by Wolf *et al.* (6) and Christianson *et al.* (7). These protein bodies evidently develop from cisternae of the endoplasmic reticulum (8).

Morton and Raison (9) isolated protein bodies from wheat endosperm and found they incorporated ^{14}C-labeled amino acids. However, incorporation of radioactivity using dialyzed protein bodies was independent of added high-speed supernatant factors or ATP. In attempting to repeat their observations with protein bodies from maize endosperm, Wilson (10) concluded that incorporation in the absence of high-speed supernatant was probably the result of bacterial contamination. Nevertheless, the polyribosomes seen on the surface of the protein bodies in maize (Fig. 1) suggested to us that the protein bodies might be the specific site of synthesis, as well as deposition, of zein. We thought it plausible that a highly hydrophobic protein would be synthesized where it was stored rather than elsewhere in the cytoplasm.

Maize endosperm is a rich source of ribonuclease (11) and we have preliminary evidence indicating that some of this activity is associated with the protein bodies. Acid proteinases are also known to be associated with protein bodies in related genera (12). In addition, there is the likelihood that bacteria sediment with the protein bodies in sucrose gradients. For these reasons we decided not to use intact protein bodies for *in vitro* amino-acid incorporation studies. Rather we treated the isolated protein bodies with detergent to release the polysomes, then used the free polysomes to see if they would participate in zein synthesis.

MATERIALS AND METHODS

Electron Microscopy. Pieces of freshly harvested, 22-day postfertilization kernels of the *Zea mays* cross W64A × 182E were fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2% sucrose. Fixation was for 3 hr at 4° followed by 3 more hours at room temperature. The tissue was rinsed for 2–3 hr with several changes of 0.1 M cacodylate buffer containing decreasing amounts of sucrose. Postfixation with OsO_4 was carried out overnight at 4°. The tissue was subsequently dehydrated in a graded ethanol series, transferred to propylene oxide, and then embedded in Spurr's epoxy resin mixture (13). Sections were poststained with uranyl acetate, then lead citrate, and examined with a Hitachi HU-11C electron microscope.

Protein Body Isolation. All material was obtained from the single cross hybrid WF9 × B37 (Illinois Foundation Seeds), whose protein bodies are morphologically indistinguishable from those of W64A × 182E. Ears were harvested 15 days after controlled self-pollination and kernels were quick-frozen on dry ice. Pericarp and embryos were removed from the endosperm and the endosperms were added to 1 to 2 volumes of Tricine buffer [50 mM *N-tris*(hydroxymethyl)methylglycine, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, adjusted to pH 8.0 with KOH]

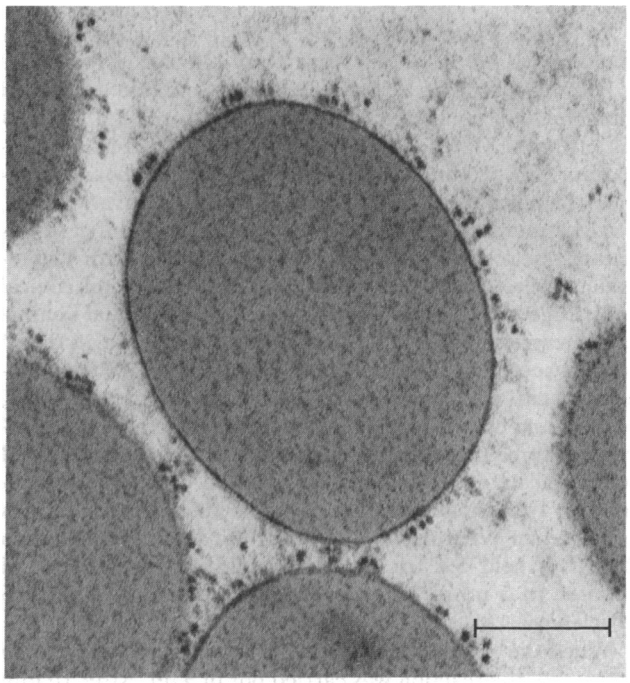

FIG. 1. Protein bodies in a cell four layers below the subaleuron in the lateral horny portion of the endosperm. The protein bodies are vesicles delimited by a single unit membrane and contain a matrix which is principally zein. Polyribosomes are associated with the outer surface of the membrane. Bar = 0.5 μm.

containing 30% (weight/volume) sucrose, 2 mM mixed 2′ and 3′ isomers of adenylic acid (14), and 7 mM diethylpyrocarbonate at 0°. The endosperms were ground for about 3 min at the slow-speed setting in a Virtis 45 homogenizer, filtered through one layer of coarse muslin, and centrifuged at 75 × *g* for 5 min. Two to 3 ml of supernatant were placed on top of 38 ml of 35 to 70% (weight/volume) linear sucrose gradients in Tricine buffer and centrifuged at 25,000 rpm for 25 min in a Beckman SW27 rotor. Fractions containing the protein bodies were pooled and stored at −15°.

RNA was determined by the "modified procedure" of Fleck and Munro (15) using the extinction $E^{1\%}_{260} = 312$ (16). Protein concentration was estimated by the biuret method using bovine serum albumin as a standard.

Preparation of Polysomes. Protein bodies were thawed and dissolved in chilled Tricine buffer with 2 mM 2′- and 3′-AMP and 2% Brij 58. After 20–30 min on ice, they were centrifuged for 10 min at 10,000 × *g* to pellet the protein bodies. The supernatants were layered on top of 2.5 ml of 2 M sucrose in Tricine buffer and centrifuged at 42,000 rpm in a Beckman type 50 rotor for 4–5 hr. The resulting supernatant was removed by suction and the tubes were rinsed briefly with ice water and dried with Kleenex. The pellets were resuspended in 50 mM Tris·HCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, pH 7.5 (17). Polysomes from shoots were prepared from 3-day-old dark-grown WF9 × B37 plumules (3–10 mm long) ground in a Potter–Elvehjem homogenizer in Tricine buffer with 2 mM 2′- and 3′-AMP and 2% Brij 58 and were directly layered over 2 M sucrose.

Polyacrylamide Gel Electrophoresis. Electrophoretic analysis in sodium dodecyl sulfate–polyacrylamide gels was performed using the method of Fairbanks *et al.* (18) adjusted to 10% acrylamide. To measure the distribution of radioactivity the gels were frozen, cut into 1 mm sections, digested in perchloric acid and hydrogen peroxide, and counted in 6 g/liter of 2,5-diphenyloxazole in toluene with 33% Triton X-100 (19).

***In Vitro* Protein Synthesis.** A high-speed supernatant fraction was prepared (20) from 3-day-old dark-grown WF9 × B37 shoots. The complete reaction mixture (modified from ref. 17) contained 50 mM Tris·HCl, pH 7.6, 13 mM KCl, 10 mM $MgCl_2$, 2.7 mM disodium ATP, 0.5 mM monosodium GTP, 6.1 mM tricyclohexylamine phospho*enol*pyruvate, 13 units/ml of rabbit skeletal muscle pyruvate kinase, 5 mM 2-mercaptoethanol, 0.2 mg of protein per ml of supernatant fraction, polysomes, and tritiated amino acid. The reactions were carried out at 36° in 0.5 ml in 12 ml conical centrifuge tubes. They were stopped with an equal volume of the corresponding 0.1 M unlabeled amino acid in 0.2 M sodium acetate, pH 4.0, followed with 1 ml of 10% trichloroacetic acid. The tubes were heated to 90° for 30 min, chilled, and centrifuged. The pellets were successively resuspended three times in 0.1 M unlabeled amino acid in 0.2 M sodium acetate, pH 4.0, and precipitated with an equal volume of 10% trichloroacetic acid. The final precipitate was collected on Whatman GF/C glass fiber filters, washed with 25 ml of cold 5% trichloroacetic acid, dried at 80°, and counted in 5 ml of toluene-based scintillation fluid at 23% efficiency.

When the product of amino-acid incorporation was to be examined, the reaction was carried out in 5 ml. After 10 min at 36°, 0.1 mmol of unlabeled L-leucine was added in 0.5 ml and the reaction was continued for an additional 10 min. The reaction was stopped by adding absolute ethanol to a final concentration of 70% (vol/vol). After 5 min at 4° the mixture was centrifuged at 17,000 × *g* for 10 min. To the resultant supernatant was added approximately 50 μg of ^{14}C-labeled zein. After lyophilization the residue was taken up in 2 ml of 0.1 M L-leucine in 0.3 M NaOH and heated at 37° for 30 min. It was then precipitated with a final concentration of 1.2 M perchloric acid, chilled, and centrifuged. The pellet was successively resuspended three times in alkaline 0.1 M L-leucine and precipitated with 1 M perchlorate. The pellet was finally washed with ethanol:ether (1:1) and then dissolved (18) for gel electrophoresis.

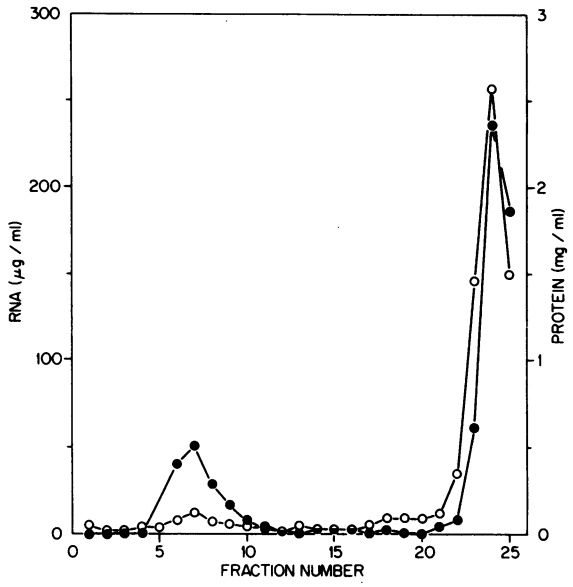

FIG. 2. Estimation of protein and RNA in sucrose gradient fractions. ●, protein; ○, RNA. The lower peak coincides with the opaque white zone seen in gradient after centrifugation. Sedimentation was from right to left.

Source of Materials. Unless otherwise stated zein was prepared (21) from 22-day-old endosperm of WF9 × B37. ^{14}C-Labeled zein was prepared by reducing a 1% solution of zein in 8 M urea, 0.5 M Tris·HCl, pH 8.0, with 5 mM dithiothreitol and then alkylating with a 10% excess over sulfhydryls of [^{14}C]iodoacetic acid. Tritiated amino acids were purchased from Amersham-Searle. Except for Brij 58 (Atlas), disodium ATP (Schwarz/Mann), streptomycin sulfate (Pfizer), and soybean trypsin inhibitor (Gallard-Schlesinger), all biochemicals were obtained from Sigma. Materials for gel electrophoresis came from Bio-Rad.

RESULTS AND DISCUSSION

Protein bodies are found predominantly in the horny endosperm a few layers below the subaleuron (5). As shown in Figs. 1 and 3, they are spherical to irregularly shaped vesicles bounded by a single membrane and filled with a homogeneous matrix. The particles attached to the membranes have a diameter of 250 Å and occur in clusters or whorls. Their size, configuration, and association with membranes of apparent endoplasmic reticulum origin is consistent with our supposition that they are polyribosomes.

In our sucrose density gradients there is only one optically scattering band, which sediments to a density of 1.26 g/ml (Fig. 2). Analysis of the gradient reveals that zein is found only in this band. A typical distribution is shown in Fig. 3. There is little evidence of damage caused by the isolation procedure such as hypertonicity effects, leakage of contents, or detachment of polysomes (Fig. 3, inset).

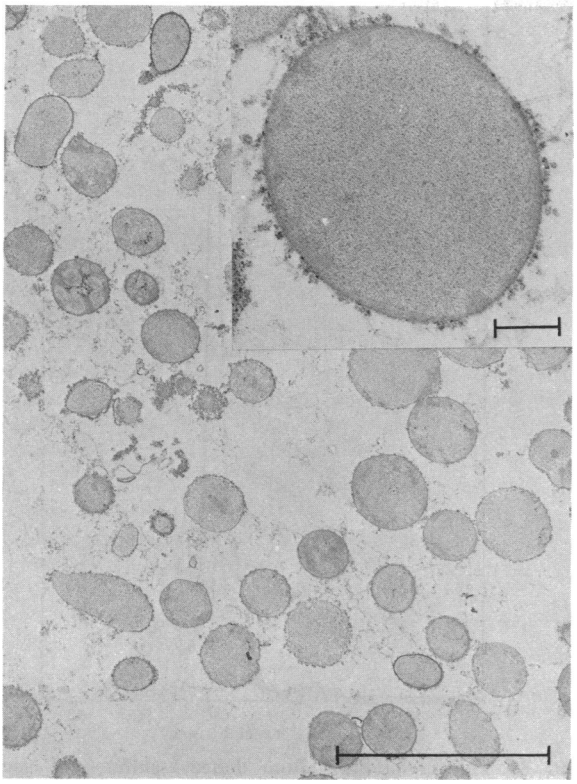

FIG. 3. Protein bodies isolated in a sucrose density gradient. They average 1.5 μm in diameter. While there is some debris, the fraction appears to be free of other organelles. Bar = 5 μm. Inset: Note the retention of polysomes on the membrane surface. Bar = 0.25 μm.

As reported by Misra *et al.* (22) zein gives two closely migrating bands when electrophoresed on 10% polyacrylamide–0.1% sodium dodecyl sulfate gels. Comparison of the migration of zein polypeptides with proteins of known molecular weight indicates that they have molecular weights of 22,500 and 19,000 (Fig. 4). These two bands are seen when zein is obtained from 15 day, 22 day, and mature endosperm. Furthermore, material extracted into 60%, 70%, and 90% ethanol gave both bands. Protein bodies can be dissolved in sodium dodecyl sulfate. Electrophoretic comparison on polyacrylamide gels of the protein body fraction with zein extracted from developing endosperm and the 70% ethanol-extractable material from the isolated protein bodies indicates that zein is the major component (Fig. 5).

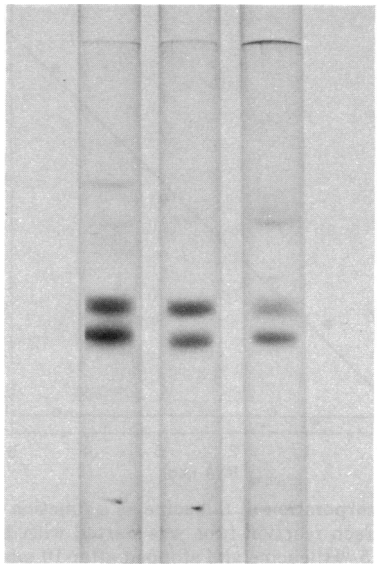

FIG. 5. Polyacrylamide–sodium dodecyl sulfate gel electrophoresis. Left to right: Protein bodies from sucrose gradient, zein isolated from 22-day-old endosperm, zein isolated from protein body fraction. Migration was from top to bottom.

The RNA eluted from the protein bodies after detergent treatment and pelleted through 2 M sucrose has not been characterized by centrifugation. However, it was isolated under conditions which should have excluded unbound proteins, ribosomal monomers, and membrane-bound polysomes (23). There is ample functional evidence that it is polysomal.

When the polysomes obtained from the protein bodies are used as the particulate component in the amino-acid incorporation system of Mans and Novelli (20), they support the incorporation of L-leucine (as well as L-proline and L-tyrosine, results not shown). The magnitude of incorporation is

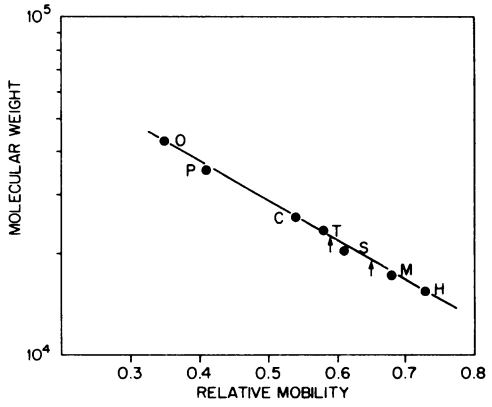

FIG. 4. Determination of the molecular weights of zein (arrows) on 10% polyacrylamide–sodium dodecyl sulfate gels by comparison with the relative mobilities of proteins of known molecular weight. O, ovalbumin, egg white; P, pepsin, hog stomach mucosa; C, α-chymotrypsinogen-A, bovine pancreas; T, trypsin, bovine pancreas; S, trypsin inhibitor, soybean (Kunitz); M, myoglobin, equine skeletal muscle; H, hemoglobin, bovine.

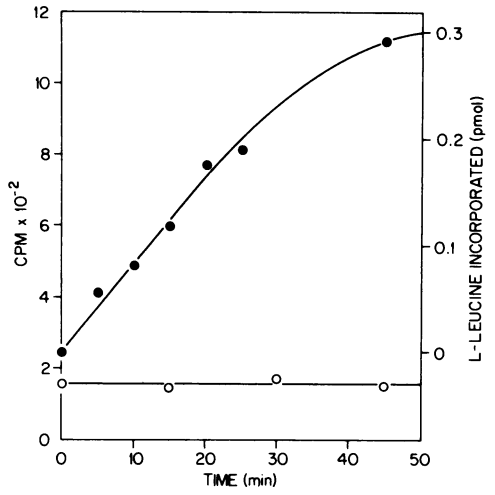

FIG. 6. Time course of incorporation. Each reaction was started with 10 nmol of 5.8 μCi/nmol L-[*4,5*-^{3}H]leucine. ●, tubes received 1.1 μg of polysomal RNA from protein bodies. O, the otherwise complete reaction mixture received no particulate RNA.

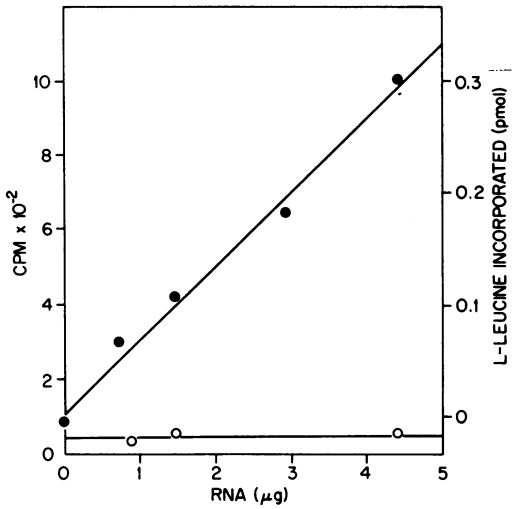

FIG. 7. Incorporation of L-leucine as a function of particulate RNA added. Each reaction tube was started with 10 nmol of 5.8 μCi/nmol L-[4,5-^{3}H]leucine and stopped after 10 min. ●, tubes received the complete reaction mixture. ○, no high-speed supernatant was added.

linear with time for approximately 20 min (Fig. 6) and is linearly dependent upon the amount of polysomal RNA added (Fig. 7). Since the inadvertent inclusion of bacterial contaminants into protein-synthesizing systems derived from protein bodies has been a problem (10), it is important to note in Fig. 7 that protein body polysomes do not support amino-acid incorporation in the absence of added high-speed supernatant factors. The rate of incorporation of the complete mixture is equal to, or greater than, the rates reported by Mans and Novelli (20).

When polysomes obtained from protein bodies were used in the *in vitro* amino-acid incorporation system, protein synthesis was 50% inhibited by 0.065 mM puromycin and 0.165 mM streptomycin. Polysomes from shoots were similarly inhibited with 0.096 mM puromycin and 0.185 mM streptomycin. Polysomes from both sources were equally insensitive to chloramphenicol (7% inhibited at 0.46 mM) and cycloheximide (8% at 0.2 mM).

Since we expected that the polysomes contained the message and nascent chains of zein, it was important to characterize the product of *in vitro* incorporation. Table 1 summarizes an experiment showing that lysine and tryptophan were not incorporated when polysomes were obtained from protein bodies but that lysine and tryptophan incorporation

Table 1. Incorporation of amino acids using polysomes from different sources

	Rate (pmol/min·mg RNA)	
	Polysomes from protein bodies	Polysomes from shoots
L-Leucine	10.5	6.2
L-Lysine	<0.11	1.2
L-Tryptophan	<0.03	0.7

Hot-trichloroacetic-acid-insoluble radioactivity was measured in duplicate after 0 and 10 min incubation. Each reaction tube contained 10 nmol of ^{3}H-labeled amino acid. Specific activities were, respectively, 5.8, 0.225, and 1.0 μCi/nmol for leucine, lysine, and tryptophan. Each reaction time was started with 7.3 μg of particulate RNA from protein bodies or 9.1 μg from shoots.

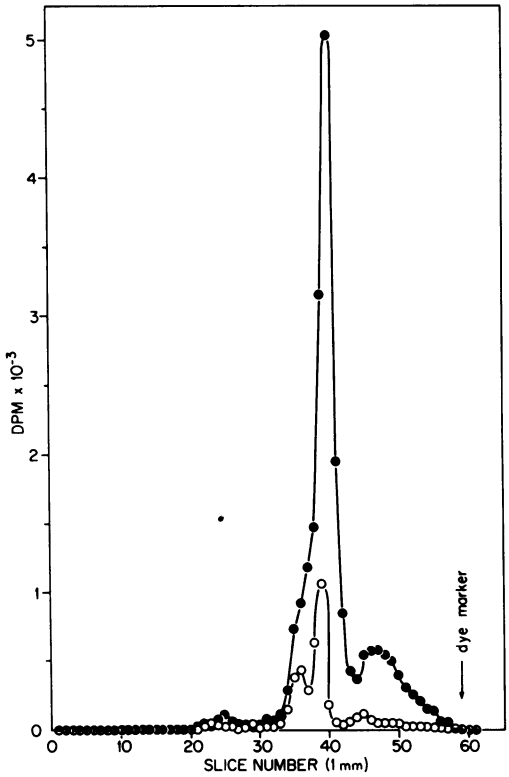

FIG. 8. Polyacrylamide–sodium dodecyl sulfate gel electrophoresis of 70% ethanol-soluble product of *in vitro* amino-acid incorporation. Reaction mixture in a total volume of 5 ml was started with 32 μg of particulate RNA from protein bodies and 10 nmol of 58 μCi/nmol L-[4,5-^{3}H]leucine. After 10 min at 30° a 10,000-fold excess of unlabeled L-leucine was added. The reaction was stopped after an additional 10 min with 70% ethanol and approximately 50 μg of ^{14}C-alkylated zein was added to the resultant supernatants. ●, ^{3}H; ○, ^{14}C. Migration was from left to right.

was detected when an equivalent amount of polysomal RNA from shoots was used. Lysine and tryptophan are absent, or nearly absent, from zein (3). This experiment is critical because it applies to the total trichloroacetic-acid-precipitable incorporation, whereas the following experiment pertains only to those chains released from the polysomes into 70% ethanol, which selectively solubilizes zein. Using protein-body-derived polysomes, about 25% of the incorporated radioactive material was released into the supernatant after 10 min when the reaction mixture was adjusted to 70% ethanol. Insoluble material was removed by centrifugation. The supernatant was lyophilized and the residue was washed to remove radioactivity not incorporated into protein. Fig. 8 shows that the major portion of the incorporated [^{3}H]leucine migrated with the lower molecular weight peak of ^{14}C-labeled zein. There was a definite shoulder of the tritium peak repeatedly seen in the region of the higher molecular weight zein band and the major tritium peak migrated slightly faster than the major ^{14}C peak. We do not know if this represents premature release of nascent chains or if the apparent molecular weight of marker zein was increased by postsynthetic modification or alkylation. Nevertheless, there is good agreement between the migration of the two isotopes. No tritium peaks were observed in the 70% ethanol-soluble product when a similar experiment was conducted with polysomes derived from the shoot.

It is not known how many different zein polypeptides there are and if they are structurally related. The polyacrylamide-sodium dodecyl sulfate gels show two bands. The two

are accumulated in different amounts in genetically different lines (22). If, as seems likely, there is more than one polypeptide, they are probably not encoded by a single polycistronic messenger, as electron micrographs of *in situ* protein bodies show that many of the associated polysomes have eight ribosomal units. Using the relationship of Noll (23) for bacterial polysomes, this would indicate a messenger RNA of 660 nucleotides. Assuming the existence of a polyadenylate 3′ terminus of 200 nucleotides, this would be sufficient for a single protein of 19,000 daltons.

This work demonstrates that zein is synthesized at its site of deposition. It implies some specificity of the components associated with this compartmentalization. It is possible that nascent chains expressing the amino-terminal portion of newly synthesized zein are recognized by receptors on the protein body surface which bind the polysomes to the membrane and facilitate transport of the released chains into the organelles, essentially as proposed by Blobel and Sabatini (24). Alternatively there may be a class of ribosomes specifically bound to some of the endoplasmic reticulum cisternae which recognizes the zein message(s) (e.g., ref. 25). Assuming there are genetically defined steps which are specific to the synthesis of zein, blocks at any of these would interrupt its synthesis. There are at least three unlinked mutations in maize which lead to a reduction in zein but do not appear to alter it structurally. In the high lysine strain, *opaque-2*, protein bodies are reduced in number and size (26); there is a marked reduction in alcohol-soluble proteins and an increase in the relatively lysine- and tryptophan-rich albumins and globulins (21) and in free amino acids (27). Since zein synthesis is localized, the mutant could be deficient for a receptor, membrane component, or particular class of ribosomal RNA or protein. Alcohol-soluble prolamines are the major proteins of most cereal grains and have a similar amino-acid composition. It is not surprising that high lysine mutations in barley (28) and sorghum (29) also have a greatly reduced content of prolamine.

Our work indicates a means of isolating a specific class of messenger RNA(s) from a higher plant. Furthermore, this messenger or its complementary copies can be used as a probe in studying the regulation of synthesis of an agronomically important storage protein in normal maize and in nutritionally interesting mutant lines.

The initial electron microscopical observations leading to this work were made while we were associated with Prof. O. E. Nelson at the University of Wisconsin. His encouragement and advice have been key factors in the success of this project. We are grateful to Drs. J. S. Cook, R. J. Mans, L. C. Waters, and C. M. Wilson for helpful suggestions and careful reading of this manuscript. Research was sponsored by the U.S. Energy Research and Development Administration under contract with the Union Carbide Corp.

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