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Isolation and in Vitro Translation of Zein Messenger Ribonucleic Acid†

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ABSTRACT: Zein messenger RNA was isolated from membrane-bound polyribosomes of developing maize kernels by oligo(dT)-cellulose chromatography. Translation of the mRNA in vitro yielded protein similar to native zein in amino acid content, ethanol solubility, and mobility on sodium dodecyl sulfate-polyacrylamide gels. The zein mRNA sedimented as a homogeneous peak on sucrose gradients and contained a poly(A)-rich region based upon hybridization to [^{3}H]poly(U). The mRNA had an apparent molecular weight of 540 000 on agarose-acrylamide gels. It synthesized both 21 800 and 19 000 molecular weight zein components in the wheat-germ cell-free protein synthesis system. The possibility of a polycistronic mRNA or two mRNAs of similar molecular weight is considered.

Zein, the principle storage protein in maize endosperm, is an ethanol soluble protein localized in structures called protein bodies (Wolf et al., 1967). We reported that this protein is synthesized primarily by membrane-bound polyribosomes in the developing endosperm (Larkins and Dalby, 1975), and similar results were obtained using polysomes associated with a protein body fraction (Burr and Burr, 1976).

Mutation at the *opaque*-2 locus causes a substantial reduction in zein levels and results in an increased proportion of nutritionally superior nonzein protein (Mertz et al., 1964). In addition to the reduction in total zein, the *opaque*-2 mutant is deficient in one of the major zein proteins, Z-1 (Lee et al., 1976). We reported that certain unique size classes of membrane-bound polyribosomes present in normal maize were absent in the *opaque*-2 mutant, and the Z-1 component was not synthesized in vitro by membrane-bound polyribosomes of *opaque*-2 (Jones et al., 1976). These results suggested there may be differences in the amount and type of mRNA associated with the membrane-bound polysomes of the mutant and (or) differences in the rate of mRNA translation.

We now report the successful isolation and in vitro translation of an mRNA which synthesized a protein similar to native zein. This is the first report of the purification of an mRNA coding for a seed storage protein.

Materials and Methods

Materials. [^{3}H]Poly(U)[1] (7.8 mCi/mmol) was obtained from Schwarz/Mann; [^{14}C]leucine (324 and 150 mCi/mmol), [^{14}C]lysine (150 mCi/mmol), [^{14}C]proline (125 mCi/mmol), [^{14}C]glutamic acid (125 mCi/mmol), and [^{14}C]glutamine (52 mCi/mmol) from Amersham/Searle. Ribonuclease-free sucrose and diethyl pyrocarbonate were from Sigma Chemical Co. Creatine phosphate kinase was from Calbiochem. Oligo(dT)-cellulose (type 7) was from P-L Biochemicals Inc. Omnifluor, Protosol, and Aquasol were from New England Nuclear. Hard red wheat, cv. *Centurk,* was a gift from Dr. Don M. Huber, Purdue University.

Polysome Isolation. Kernels from the maize inbred line W22 were frozen in liquid nitrogen 22 days after pollination and stored at −80 °C (Larkins et al., 1976). Groups of 30 kernels were ground in 25 ml of buffer A (200 mM Tris-HCl, pH 8.5, 200 mM sucrose, 50 mM $MgCl_2$, 60 mM KCl, and 1 mM dithiothreitol) with a chilled mortar and pestle. The extract was strained through four layers of cheesecloth and centrifuged at 500*g* for 5 min. The supernatant fraction was centrifuged at 37 000*g* for 10 min to pellet the membrane-bound polyribosomes. The 37 000*g* pellet was suspended in 5 ml of buffer A containing 1% Triton X-100 with a glass tissue homogenizer and centrifuged at 37 000*g* for 10 min. The supernatant fraction was layered over 4 ml of 2 M sucrose in buffer B (40 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, and 20 mM KCl) and the polysomes were pelleted by centrifuging for 3 h at 229 000*g* in the 65 rotor of a Beckman L2-65 ultracentrifuge. The polysome pellet was suspended in buffer B, layered on 150–600 mg/ml sucrose gradients, and centrifuged for 40 min at 189 000*g* in a Beckman SW 50.1 rotor. Gradients were scanned at 254 nm with an ISCO Model UA-5 absorbance monitor.

Isolation of Poly(A)-Containing RNA from Polyribosomes. All solutions were autoclaved and glassware was washed with 0.1% diethyl pyrocarbonate and boiled briefly to destroy ribonuclease activity.

Poly(A)-containing mRNA was isolated by a modification of the procedure of Krystosek et al. (1975). Membrane-bound polyribosome pellets were dissolved in 10 mM Tris-HCl, pH

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[1] Abbreviations used: poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

7.5, containing 0.5 M NaCl and 0.5% sodium dodecyl sulfate. Aliquots of 10 ml (20–25 A_{260} units/ml) were mixed with a 0.5 g of oligo(dT)-cellulose in a 15-ml Millipore Pyrex funnel with a Gelman GA-6 filter placed over the fritted-glass filter support. After washing with 20 ml of sodium dodecyl sulfate buffer, a second aliquot of 10 ml was added. This was repeated until the mRNA from 700 to 800 A_{260} units of polysomes was processed.

The oligo(dT)-cellulose was washed with buffer minus sodium dodecyl sulfate until there was no visable evidence of detergent. Poly(A)-containing RNA was eluted with 10 mM Tris-HCl pH 7.5, at 50 °C. Eluted RNA was adjusted to 0.3 M KCl, chilled to 4 °C, and adsorbed and eluted two successive times. Approximately 4% of the total A_{260} units applied was initially bound, but only 1.3% remained after the third binding cycle. Poly(A) RNA was adjusted to 0.2 M potassium acetate and precipitated overnight at −20 °C after adding 2 volumes of absolute ethanol. In some instances 1 A_{260}/ml tRNA was added to facilitate precipitation. After pelleting by centrifugation at 10 000*g* for 20 min, the RNA was dissolved in sterile distilled H_2O and frozen in small aliquots at −80 °C.

Translation of mRNA in the Wheat-Germ Cell-Free System. A standard cell-free amino acid incorporating system (Marcu and Dudock, 1974) was prepared from wheat germ isolated by the method of Marcus (1974). The complete system in a final volume of 50 μl contained: 15 μl of wheat-germ supernatant (1 A_{260} unit), 20 mM Hepes, pH 7.4, 2 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 40 μg/ml creatine phosphokinase, 8 mM creatine phosphate, 2.5 mM magnesium acetate, 86 mM KCl, 0.25 μCi of [^{14}C]leucine, 25 μM of 19 unlabeled amino acids, and variable amounts of messenger RNA. Mixtures were incubated for 60 min at 27 °C. The hot 5% trichloroacetic acid insoluble protein was removed by filtration with Whatman GF/A filters, dried under a heat lamp, and counted in Omnifluor-toluene in a Beckman LS-100 scintillation counter with a ^{14}C efficiency of 80%. Hot 70% ethanol-soluble protein was prepared from the reaction mixtures by the procedure of Dalby (1974).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Hot 5% trichloroacetic acid insoluble protein from the protein synthetic reaction mixtures was isolated by centrifugation and washed three times with cold 5% trichloroacetic acid. Each sample was dissolved in 0.1 ml of 50 mM Tris-HCl, pH 6.9, containing 0.5% sodium dodecyl sulfate, 1% 2-mercaptoethanol, with or without carrier zein. Samples were dialyzed against 250 ml of buffer for 2 days with three changes of buffer, and electrophoresis was done on 15% sodium dodecyl sulfate gels as previously described (Larkins and Dalby, 1975). Gels were cut into 1-mm slices and radioactivity was determined after solubilizing the slices in Omnifluor-toluene containing 3% Protosol. Zein was purified from mature kernels of the normal maize inbred W22 and used as a standard (Lee et al., 1976).

Analysis of RNA. Poly(A) RNA was centrifuged on linear log gradients (Brakke and Van Pelt, 1970) formed by layering 1.0, 1.75, 1.06, 0.68, 0.44, and 0.37 ml of 325, 270, 210, 160, 100, and 0 mg/ml sucrose, respectively, in 10 mM Hepes, pH 7.5, 100 mM KCl, with or without 1 mM EDTA (Haines et al., 1974). Gradients equilibrated overnight at 4 °C prior to use. RNA was dissolved in gradient buffer, heated to 60 °C for 30 min, chilled to 4 °C, and centrifuged for 5 h at 189 000*g* in a Beckman SW50.1 rotor. Gradients were scanned photometrically at 254 nm and 0.25-ml fractions collected. RNA was precipited by adding an equal volume of gradient buffer, 0.5 A_{260} unit of tRNA, and two volumes of absolute ethanol.

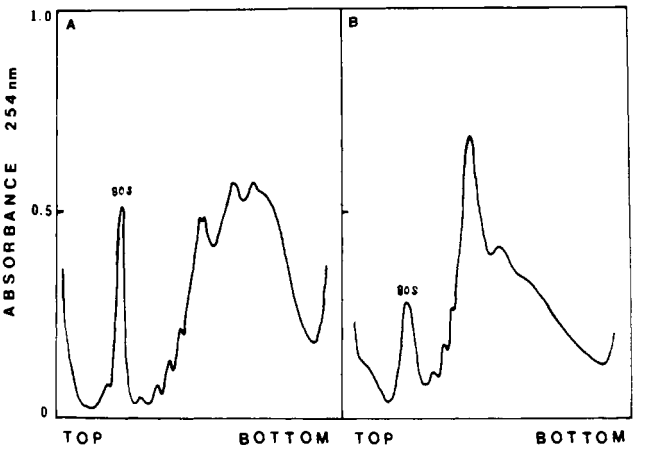

FIGURE 1: Membrane-bound polyribosomes isolated from fresh frozen or stored maize kernels. Polysomes were isolated from seven fresh frozen kernels (A) or eight frozen kernels stored for 6 months at −80 °C (B).

Samples were dissolved in distilled H_2O and assayed in the wheat-germ cell-free system.

The presence of poly(A) in the messenger fraction was tested by hybridization with [^{3}H]poly(U). The reaction mixture contained in a final volume of 0.25 ml: 62 μg of RNA, 0.25 μCi of [^{3}H]poly(U), 10 mM Hepes, pH 7.5, and 100 mM KCl (Haines et al., 1974). Samples were incubated for 20 min at 4 °C and centrifuged on sucrose gradients. Gradient fractions of 0.25 ml were diluted with 1 ml of H_2O and counted in 10 ml of Aquasol.

Gel electrophoresis of RNA was on 9-cm 2.4% polyacrylamide gels containing 0.5% agarose in 36 mM Tris, 30 mM NaH_2PO_4, and 1 mM EDTA, pH 7.2 (Roheim et al., 1974). Preelectrophoresis was for 90 min at 5 mA/gel; then 50 μl samples containing sucrose were layered and electrophoresis was performed at room temperature for 2.5 h at 6 mA/gel. Gels were soaked in distilled H_2O and scanned at 260 nm with a Gilford Model 2410 gel scanner.

Molecular weight of zein mRNA was estimated by comparing the migration of TMV RNA, 2.0×10^6 (Loening, 1969), BMV RNA, 1.0×10^6, 0.70×10^6, and 0.3×10^6 (Lane and Kaesberg, 1971), maize ribosomal RNA, 1.33×10^6 and 0.7×10^6, and *Escherichia coli* ribosomal RNA, 1.1×10^6 and 0.56×10^6 (Pring and Thornbury, 1975). Maize and *E. coli* ribosomal RNAs were isolated by phenol-chloroform extraction (Palmiter, 1974). TMV RNA was isolated by the procedure of Marcus (1974), and BMV RNA was a gift from Dr. A. O. Jackson, Purdue University.

Results

Isolation of Poly(A) RNA from Membrane-Bound Polyribosomes. Figure 1A depicts the membrane-bound polyribosomes isolated from fresh maize kernels, or frozen kernels stored at −80 °C for 3 months. We were unable to isolate the very large polysome size classes after storage for 6 months (Figure 1B), although the polysome profiles did not indicate extensive degradation by ribonuclease (Larkins et al., 1976). Since the polysomes did not appear degraded, we assumed the mRNA associated with them was intact and this material was subsequently used for mRNA isolation.

Poly(A)-containing RNA was isolated from membrane-bound polyribosomes solubilized in sodium dodecyl sulfate buffer. This procedure permitted the isolation of functional mRNA and avoided many of the problems associated with phenol extraction (Krystosek et al., 1975). The poly(A)-con-

TABLE I: Characteristics of Protein Synthesis in the Wheat-Germ Cell-Free System Directed by Exogenous mRNA.[a]

Components	[^{14}C]Leu Incorp/ 50 μl Reaction (cpm)
Complete system	
Tobacco mosaic virus RNA (9 μg)	162 000
Poly(A)-containing RNA from maize membrane-bound polyribosomes (1.7 μg)	143 000
Poly(A)-containing RNA from maize membrane-bound polyribosomes plus aurintricarboxylic acid (50 μM)	1 790
Deletions	
Minus poly(A) RNA	404
Minus wheat-germ supernatant	434
Minus unlabeled amino acids	61 700

[a] Composition of the cell-free reaction mixtures is described in Materials and Methods. Incorporation into hot acid-insoluble radioactivity was determined after 60-min incubation. Deletion of wheat-germ supernatant or 19 unlabeled amino acids was from reactions containing 2 μg of poly(A) RNA.

TABLE II: Comparison of Amino Acid Incorporation Directed by Poly(A) RNA.[a]

Amino Acid	pmol of CCl_3COOH Insoluble	pmol of Ethanol Soluble
Gln	185	169
Leu	162	136
Pro	127	90
Lys	7	4
Glu	7	3

[a] Reaction mixtures of 100 μl contained 0.1 μCi of either [^{14}C]-leucine, [^{14}C]proline, [^{14}C]lysine, [^{14}C]glutamic acid, or 0.5 μCi of [^{14}C]glutamine and 3.6 μg of poly(A) RNA. After incubation for 60 min, 50 μl was spotted on Whatman 3 MM filter paper disks for determination of hot acid-insoluble radioactivity. The remaining 50 μl was adjusted to 70% ethanol by adding 0.12 ml of absolute ethanol. After extraction at 60 °C for 45 min, the mixtures were centrifuged to precipitate insoluble protein and two 50-μl aliquots were spotted on Whatman 3MM filter paper discs. The discs were passed through the zein wash procedure (Dalby, 1974) and 70% ethanol soluble radioactivity was determined. The values expressed were the average of triplicate assays, and samples were counted to 5% relative standard error.

taining RNA bound by oligo(dT)-cellulose in high salt buffer (10 mM Hepes, pH 7.5, and 500 mM NaCl) was contaminated with ribosomal RNA (e.g., Figure 5). Generally 2 to 3% of the total A_{260} units was recovered after two cycles of binding to oligo(dT)-cellulose. Binding in 500 mM NaCl at room temperature followed by two hybridizations in 300 mM KCl at 4 °C removed much of the contaminating ribosomal RNA. The yield of 250 kernels was 100 A_{260} units of membrane-bound polyribosomes, and 1.3 A_{260} units of RNA remained bound to oligo(dT)-cellulose after three cycles of hybridization.

Translation of Poly(A) RNA in the Wheat-Germ Cell-Free System. The translational properties of the poly(A)-containing RNA are described in Table I. Both TMV RNA and poly(A) RNA were active mRNAs. Protein synthesis required exogenous mRNA as well as the inititation reaction since the initiation inhibitor aurintricarboxylic acid (Marcus et al., 1970) prevented incorporation. Passing the wheat-germ supernatant over a Sephadex G-25 column (Marcu and Dudock, 1974; Roberts and Paterson, 1972) removed much of the endogenous amino acids, and incorporation was reduced nearly 60% in the absence of exogenous unlabeled amino acids.

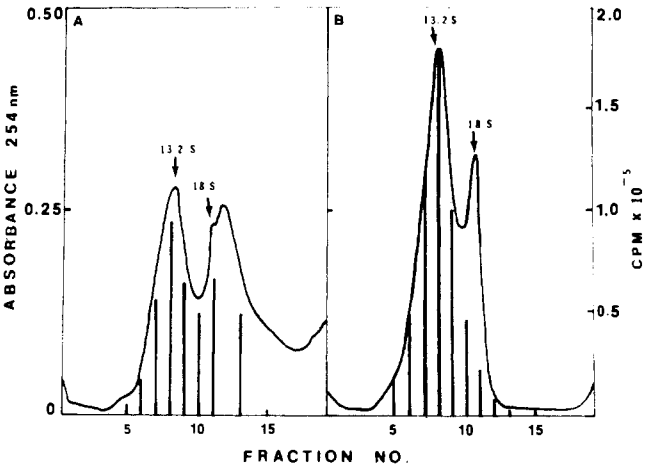

FIGURE 2: Sucrose gradient analysis of poly(A)-containing RNA isolated from membrane-bound polyribosomes. Poly(A)-containing RNA (50 μg in 0.25 ml of 10 mM Hepes, pH 7.5, 100 mM KCl, and 1 mM EDTA) was layered directly (A), or heated to 60 °C for 3 min, chilled, and then layered on linear log sucrose gradients (B). After centrifugation the optical density at 254 nm (—) was monitored with an ISCO UA-5 absorbance monitor. RNA was precipitated from 0.25-ml fractions and aliquots were tested for mRNA activity in the wheat-germ cell-free system (histogram).

The Mg^{2+} optimum was 2.5 mM which agreed with other reports, although K^+ optimum was 86 mM which was lower than reported (Marcu and Dudock, 1974; Roberts and Paterson, 1972). The poly(A) RNA was not dialyzed prior to translation and may have contained some K^+ after ethanol precipitation in the presence of potassium acetate.

The time course of incorporation using poly(A) RNA was linear throughout 50 min of incubation, and there was active incorporation for up to 90 min.

Comparison of Incorporation Using Different Labeled Amino Acids. Zein is noted for its unique amino acid composition. The protein is rich in glutamic acid (thought to be glutamine), leucine, alanine, and proline, but nearly devoid of the essential amino acids lysine and tryptophan (Murphy and Dalby, 1971). From a comparison of [^{14}C]glutamine, [^{14}C]-leucine, [^{14}C]proline, [^{14}C]lysine, and [^{14}C]glutamic acid incorporation, directed by poly(A) RNA from the membrane-bound polyribosomes, the protein synthesized in vitro was similar to native zein (Table II). The protein had a high content of glutamine, leucine, and proline, but contained little lysine or glutamic acid. The amino acids were primarily incorporated into ethanol-soluble protein, and the mole fraction ratios of the various amino acids agreed with the values reported for zein (Murphy and Dalby, 1971).

Analysis of Zein mRNA on Sucrose Gradients. Ovalbumin and hemoglobin mRNAs form aggregates which can be dissociated by heating and rapid cooling prior to sucrose gradient analysis (Haines et al., 1974; Kabat, 1972). Zein mRNA behaved similarly. Two peaks of mRNA activity were recovered from sucrose gradients when the poly(A) RNA was not heated prior to centrifugation (Figure 2A). The first sedimented at 13.2 S and the second at approximately 23 S. When heated to 60 °C for 3 min, and then rapidly cooled (Figure 2B), only one peak of mRNA activity resulted. The mRNA peak sedi-

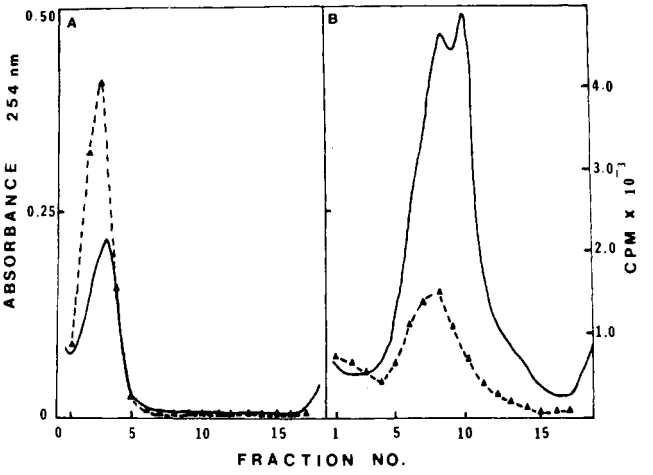

FIGURE 3: Hybridization of poly(A) RNA with [^{3}H]poly(U). [^{3}H]-Poly(U) (A) or an equal amount of [^{3}H]poly(U) plus 60 μg of poly(A) RNA (B) were incubated at 4 °C for 20 min in 10 mM Hepes, pH 7.5, containing 100 mM KCl. Samples of 0.25 ml were centrifuged on linear log sucrose gradients. After centrifugation the optical density at 254 nm (—) was monitored and the [^{3}H]radioactivity (▲) determined in 0.25-ml fractions.

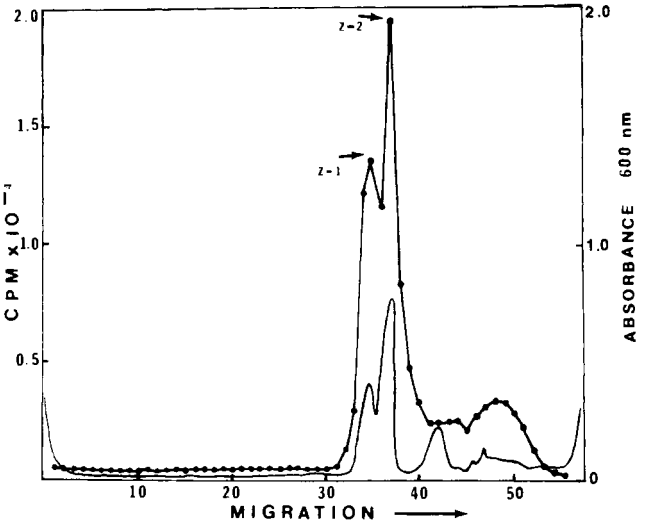

FIGURE 4: Sodium dodecyl sulfate gel analysis of the protein synthesized in vitro by 13.2S poly(A) RNA. Messenger RNA sedimenting at 13.2 S on a linear log sucrose gradient was translated in the wheat-germ cell-free system. The hot 5% trichloroacetic acid insoluble protein was dialyzed against 50 mM Tris-HCl, pH 6.9, 0.5% sodium dodecyl sulfate, and 1% 2-mercaptoethanol. Electrophoresis was as described in Materials and Methods. The [^{14}C]leucine radioactivity (●) was determined in 1-mm gel slices. The zein standard (—) was stained with Coomassie blue and scanned at 600 nm.

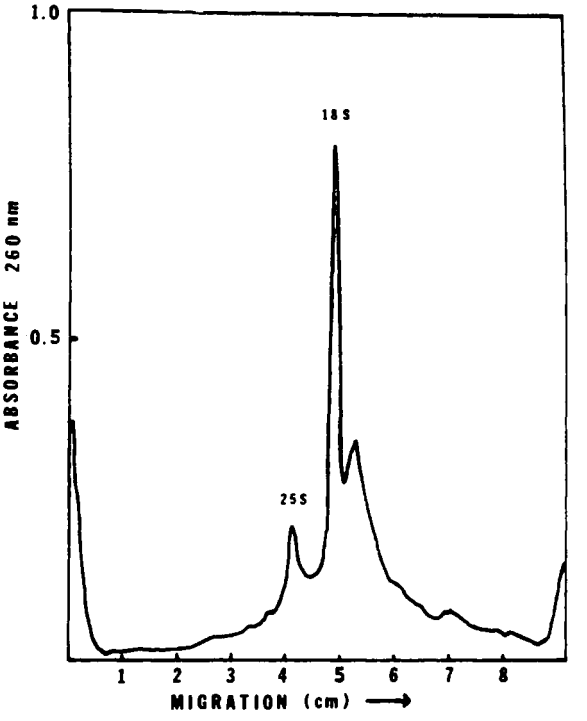

FIGURE 5: Determination of zein mRNA molecular weight by polyacrylamide gel electrophoresis. Twenty micrograms of RNA was dissolved in electrophoresis buffer and electrophoresis was on 2.4% polyacrylamide gels as described in Materials and Methods. Gels were placed in distilled H_2O for 60 min before scanning at 260 nm (—).

menting at 23 S appeared to be an aggregate of mRNA and 18 S ribosomal RNA, similar to that reported for hemoglobin mRNA (Kabat, 1972).

Since the mRNA was isolated by hydridization to oligo(dT)-cellulose, we assumed that it contained a poly(A)-rich region. To confirm this, the RNA was hybridized to [^{3}H]-poly(U) and analyzed on sucrose gradients (Figure 3). In the absence of mRNA, [^{3}H]poly(U) sedimented near the top of the gradient (Figure 3A). A mixture of [^{3}H]poly(U) plus 60 μg of the RNA bound by oligo(dT)-cellulose revealed one major peak of ^{3}H activity which cosedimented with the 13.2S mRNA peak.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Analysis of the Protein Synthesized by the 13.2S mRNA Messenger RNA that sedimented at 13.2 S on sucrose gradients was translated in the wheat-germ cell-free system. The hot 5% trichloroacetic acid insoluble protein synthesized in vitro was solubilized in sodium dodecyl sulfate buffer and analyzed by gel electrophoresis. The majority of the radioactivity comigrated with the two principle zein components, Z-1 and Z-2 (Figure 4). Smaller molecular weight radioactive material was also present which may correspond to the lower molecular weight zein components or nascent chains of the larger zein components. Attempts to remove nascent polypeptides by centrifuging the reaction mixtures after incubation resulted in pelleting of the labeled protein.

Estimation of Molecular Weight of Zein mRNA. Electrophoretic analysis of zein mRNA isolated after two cycles of hybridization in high salt buffer indicated contamination with ribosomal RNA (Figure 5). In addition to two peaks of ribosomal RNA, there was an additional RNA peak which corresponded to zein mRNA. By comparison of the migration of zein mRNA with RNAs of known molecular weight, we estimated the molecular weight of the zein mRNA to be 540 000. This corresponded to an mRNA of 16 S, which was larger than the value obtained on sucrose gradients.

Discussion

Rapid freezing with liquid nitrogen was a prerequisite for isolating large size classes of membrane-bound polyribosomes which synthesize zein (Larkins et al., 1976). However, after prolonged storage the amount of these large size classes was reduced (Figure 1). This did not appear to result from ribonuclease activity since the proportion of small polyribosome size classes (dimers, trimers, etc.) did not increase. Therefore the reduced amount of large polyribosomes may have resulted from release of large polyribosomes from the endoplasmic reticulum or dissociation of ribosomes from mRNA. There was no detectable increase of large polyribosome size classes in the

free polyribosomes (data not shown), suggesting the second explanation may be valid. The fact that mRNA isolated from these polysomes was functional in cell-free protein synthesis (Table I, Figure 2) confirms that the mRNA was not degraded.

Because zein is the principle protein synthesized by membrane-bound polyribosomes in developing maize kernels (Larkins and Dalby, 1975), we assumed that these polyribosomes were enriched with zein mRNA. Since there have been several reports of mRNAs containing poly(A)-rich regions in both animal (Haines et al., 1974; Krystosek et al., 1975) and plant cells (Higgins et al., 1973; Verma et al., 1975), we attempted to purify zein mRNA by oligo(dT)-cellulose chromatography. Isolation of poly(A) RNA from polysomes dissolved in sodium dodecyl sulfate buffer (Krysotsek et al., 1975) was a simple and convenient method to purify the mRNA.

The mRNA coding for zein synthesis appeared to contain a poly(A)-rich region similar to other eukaryotic mRNAs since it was isolated by oligo(dT)-cellulose chromatography and hybridized with [^{3}H]poly(U) (Figure 3). It appeared to be the principle poly(A)-rich mRNA associated with the membrane-bound polyribosomes. The poly(A) RNA revealed one major absorbance peak on sucrose gradients which coincided with the mRNA activity (Figure 2B). A corresponding RNA peak was obtained on agarose–acrylamide gels (Figure 5).

We utilized the wheat-germ cell-free system to translate the mRNA because this system has little endogenous mRNA activity and translates many exogenous mRNAs with fidelity (Roberts and Paterson, 1972; Verma et al., 1975). The optimal pH, salt, mRNA, and ribosome concentrations were similar to those reported by others for this system (Marcu and Dudock, 1974; Roberts and Paterson, 1972).

Our results confirm that zein was the principle protein synthesized in the cell-free system since the majority of the radioactivity was incorporated into ethanol-soluble protein (Table II) with an amino acid content similar to native zein (Murphy and Dalby, 1971). We analyzed the hot acid-insoluble radioactive protein on sodium dodecyl sulfate gels (Figure 4) rather than only the 70% ethanol soluble protein (Burr and Burr, 1976) to obtain the total protein synthesized in vitro. The majority of the radioactivity migrated with the two major zein proteins whether or not carrier zein was present. We were unable to determine whether the smaller molecular weight radioactive protein corresponded to the smaller zein proteins or nascent polypeptides of Z-1 and Z-2 since centrifuging the reaction mixtures pelleted all radioactive protein with the ribosomes. This was probably due to the poor solubility of the hydrophobic protein. We hope to further characterize the product by immunology and protease mapping.

Poly(A) RNA revealed a homogeneous peak of messenger activity on sucrose gradients sedimenting at 13.2 S. This corresponded to a molecular weight of 350 000 using the relationship of Spirin (1963). On polyacrylamide gels (Figure 5) it had an apparent molecular weight of 540 000, or 16 S. We also analyzed the mRNA under denaturing conditions on formamide gels (data not shown), and the molecular weight increased slightly. Although there was variation in the molecular weight estimated by sucrose gradient centrifugation and electrophoretic mobility, such variation has been reported for viral RNAs (Jackson and Brakke, 1973).

Since the mRNA synthesized the 21 800 and 19 000 molecular weight zein components, it may code for both proteins. The major zein proteins have a combined molecular weight of 41 000, and from the amino acid mole fraction values (Murphy and Dalby, 1971) contain approximately 317 amino acids. A messenger RNA of 540 000 molecular weight that contained a polyadenylated 3′ terminus of 200 nucleotides would contain sufficient bases to code for both of the major zein proteins. This would explain the relationship between the large size of the membrane-bound polyribosomes and the small molecular weight of the zein proteins (Larkins et al., 1976).

An alternative explanation, that there are two mRNAs, must also be considered. Z-1 and Z-2 differ by only 2800 molecular weight, and two mRNAs of similar size could exist. Since approximately 50% of the nucleotides of the ovalbumin mRNA are not translated into protein (Shapiro and Schimke, (1975), this may be a valid explanation. If there were two mRNAs of similar size, however, only about 33% of the mRNA would be translated into storage protein.

Although the reduced synthesis of zein in high-lysine mutants was reported over 10 years ago (Mertz et al., 1964; Nelson et al., 1965), the mechanism(s) by which zein synthesis is affected remains unknown. Isolation of zein mRNA and study of the reactions affecting its synthesis and translation should help define how these mutants alter maize storage proteins and increase the nutritional quality of the seed.

Acknowledgments

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Base Sequence Complexity of the Stable RNA Species of *Drosophila melanogaster*†

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ABSTRACT: The base sequence complexity of *Drosophila* transfer RNA (tRNA), 5S RNA, and 18S + 28S ribosomal RNA was determined by analyzing the kinetics of RNA–DNA hybridization on membrane filters. We find that *Drosophila* tRNA is made up from about 59 basic nucleotide sequences distinguishable by hybridization, suggesting that many of the 99 tRNA species resolved by reverse phase chromatography (RPC-5) are homogenic. In contrast 5S RNA was found to contain a single family of sequences. Either 18S ribosomal RNA (rRNA) alone, or 18S + 28S rRNA together, behaved kinetically as two sequence families, and the possible basis for this unexpected result is discussed.

Drosophila melanogaster is one of the few eukaryotes in which the transfer RNAs for all 20 amino acids have been thoroughly analyzed by reverse phase chromatography (RPC).[1] White et al. (1973a) have shown that *Drosophila* tRNA can be resolved on RPC-5 columns (Pearson et al., 1971) into 99 peaks. The pattern is somewhat more complex than that found in *Escherichia coli,* human, or mouse, where about 56 distinct components are resolved (Gallo and Pestka, 1970). White et al. (1973b) have evidence which suggests that several chromatographically distinct forms of isoaccepting tRNAs essentially have the same nucleotide sequence and are probably products of the same genes. The conversion of one form to another is believed to be mediated by a tRNA modifying enzyme which modifies a single nucleotide. These authors proposed the term "homogenic" to describe tRNAs presumably transcribed from the same genes which are chromatographically distinct as a result of different degrees of post-transcriptional modification.

These findings raise the possibility that other isoaccepting forms of tRNA might also be homogenic. In order to investigate this possibility, the kinetic hybridization technique of Birnstiel et al. (1972) was employed in this study to examine the sequence complexity of 4S RNA of *Drosophila melanogaster.* In this method, the time course of hybridization of a constant large excess of RNA with DNA immobilized on filters is followed at optimum rate temperature (t_{opt}). The kinetics of hybridization are pseudo-first-order with respect to RNA under these conditions, but conveniently can be expressed as a double-reciprocal plot (Bishop, 1969). The saturation value is calculated from the intercept of the linear curve at 1/time = 0. The time taken to reach half of the saturation value ($t_{1/2}$) can then be determined and the reaction expressed in terms of $C_r t_{1/2}$ where C_r is the molar concentration of ribonucleotides in solution. The value $C_r t_{1/2}$ has been shown to be a constant (Birnstiel et al., 1972), which is directly proportional to the kinetic or base sequence complexity of a given RNA. Within certain limits, $C_r t_{1/2}$ is independent of both the degree of fragmentation of the RNA and the amount of complementary DNA on the filter. By comparing the $C_r t_{1/2}$ of a class of RNA with that of a standard RNA of known complexity, it is possible to determine the number of families of gene transcripts which make up the RNA in question.

The $C_r t_{1/2}$ of *Drosophila* 4S RNA was measured and the kinetic complexity was determined relative to that of *Bacillus subtilis* 16S + 23S ribosomal RNA, which has been previously shown to be a kinetically homogeneous RNA (Birnstiel et al., 1972). The results suggest that *Drosophila* 4S RNA is composed of approximately 59 families of sequences coded for by about 590 genes. The complexity of *Drosophila* 5S RNA was also examined by the kinetic hybridization technique and found to be composed of a single family of sequences. *Drosophila* 18S + 28S RNA showed unexpected hybridization properties and a possible molecular basis for these results is discussed.

Materials and Methods

Isolation of RNA from Drosophila Larvae. Tritium-labeled RNA was prepared from 3rd instar larvae of *Drosophila melanogaster* according to Steffensen and Wimber (1971). Specific activities ranged from 2.5×10^4 to 1×10^5 cpm/μg.

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[1] Abbreviations used: rRNA and tRNA, ribosomal and transfer ribonucleic acids; RPC, reverse phase chromatography; EDTA, ethylenediaminetetraacetic acid; OAc, acetate; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; CIB, chromatin isolation buffer; TE buffer, 0.1 M EDTA–0.1 M Tris, pH 8.4; SSC, standard saline citrate.