Analysis of the Complexity and Frequency of Zein Genes in the Maize Genome†

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ABSTRACT: DNAs were synthesized by using zein mRNAs from normal and *opaque*-2 versions of the maize inbred W64A. The hybridization of zein mRNAs and cDNAs was more complex than that of ovalbumin mRNA and cDNA which was used as a standard. The hybridization of zein mRNAs and cDNAs occurred over 2.5 decades of R_0t and was best described by two kinetic components based on computer analysis using a least-squares procedure to analyze the data. Compared to the ovalbumin standard, the two components of the reaction involving mRNAs from the normal inbred had complexities of 200 and 3950 nucleotides. The complexity of these components was 338 and 3330 nucleotides for the reaction involving mRNAs from the *opaque*-2 mutant. When the hybrid formation of normal mRNA and cDNA was determined by hydroxylapatite chromatography rather than by S_1 nuclease digestion, only one kinetic component was observed with a complexity of 270 nucleotides. By hybrid-arrested translation of normal mRNA with cDNA prepared from *opaque*-2 mRNA it was demonstrated that a portion of the reduced sequence complexity in the mutant was due to a reduction in the mRNAs for the 22000 molecular weight prezein proteins. By hybridizing the cDNAs to maize DNA we determined that the zein genes were present in 1–5 copies/haploid genome.

The storage proteins of maize seed consist of several alcohol-soluble proteins (prolamines) that are collectively known as zein. These proteins are coded for by developmentally regulated genes that are active between 10 and 40 days after endosperm formation (Jones et al., 1977a). The most abundant zein proteins have molecular weights of 22000 and 19000, but there are also small amounts of 15000 and 10000 molecular weight proteins (Lee et al., 1976; Gianazza et al., 1977). When analyzed by isoelectric focusing, these proteins show charge heterogeneity (Righetti et al., 1977), suggesting that several polypeptides exist within each of these molecular weight groups. At least part of this heterogeneity has a genetic basis (Soave et al., 1978). Several endosperm mutations have been identified that suppress the synthesis of these proteins both quantitatively (Mertz et al., 1964; Nelson et al., 1965) and qualitatively (Jones et al., 1977a; Ma & Nelson, 1975), although the mechanism of these mutations is not understood. In *opaque*-2 mutants, zein synthesis is reduced by 60% compared to the normal genotype, and the 22000 molecular weight component is nearly absent (Jones et al., 1977b). In *opaque*-7 mutants, the 19000 molecular weight component is disproportionately reduced (Ma & Nelson, 1975).

mRNAs that direct the synthesis of zein proteins in vitro have been isolated from polysomes of rough endoplasmic reticulum membranes from developing endosperm (Larkins et al., 1976; Burr et al., 1978; Melcher, 1979). The proteins synthesized in cell-free systems by zein mRNAs have many properties of native proteins, except that they are synthesized as preproteins (Burr et al., 1978; Larkins & Hurkman, 1978). Polysomes (Jones et al., 1977b) or mRNAs (Larkins et al., 1979) from the *opaque*-2 mutant do not synthesize detectable amounts of the 22000 molecular weight zein component, suggesting that the level of these mRNAs is reduced in the mutant.

A number of questions remain to be answered regarding the number and sequence complexity of zein mRNAs, as well as the factors reducing zein synthesis in mutant endosperms. In this communication we report the synthesis of DNAs complementary to zein mRNAs and the analysis of their hybridization kinetics. We also report an analysis of frequency of zein genes in the maize genome based upon the hybridization of these cDNAs to the genomic DNA.

Materials and Methods

Isolation of Polysomal mRNAs. Developing maize seeds from the inbred lines W64A and W64A *opaque*-2 were harvested 22 days after pollination. After being frozen in liquid nitrogen, seeds were stored at −80 °C. Membrane-bound polysomes and poly(A) RNAs were isolated as described by Larkins & Hurkman (1978), except that poly(A) RNA was hybridized to oligo(dT)–cellulose 4 times. Zein-synthesizing mRNAs were isolated by separating the poly(A) RNA into 0.4-mL fractions on linear log sucrose density gradients. The peak fraction sedimenting at 13 S was precipitated with 2.5 volumes of 95% ethanol.

Oviduct polysomes were isolated as described by Bloom & Anderson (1978). Poly(A) RNA was recovered by the procedures used for zein mRNAs, and ovalbumin mRNA was isolated by two cycles of gradient centrifugation. This mRNA preparation was determined to be 93% pure based on immunoprecipitation of cell-free translation products with rabbit antiovalbumin antibodies (Palmiter et al., 1971).

Molecular weights of mRNAs were determined by electrophoresis in 1.8% agarose gels containing 2.2 M formaldehyde or 20 mM methylmercury hydroxide (Lehrach et al., 1977).

Synthesis of [^3H]cDNA. Zein and ovalbumin cDNAs were synthesized as described by Bloom & Anderson (1978). Reaction mixtures of 50 or 100 μL contained 50 mM Tris-HCl, pH 8.3 at 42 °C, 10 mM $MgCl_2$, 140 mM KCl, 30 mM 2-mercaptoethanol, 10–50 μg/mL oligo(dT)$_{12-18}$, 500 μM of each unlabeled deoxynucleotide triphosphate, 25 μM [^{3}H]-dCTP or [^{3}H]dCTP and [^{3}H]dATP (ICN, 25 and 10 Ci/mmol, respectively), 50–100 μg/mL mRNA, and 10 units of AMV[1] reverse transcriptase per μg of RNA. Under these

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conditions, cDNAs of ~350 nucleotides were synthesized. Greater than 90% of the cDNA hybridized to the mRNAs, and 3–7% was resistant to S_1 nuclease. Essentially full-length cDNAs were synthesized by raising the concentration of all four deoxynucleotide triphosphates to 500 μM.

The size of cDNAs was determined by alkaline sucrose gradient (4–20% w/w) centrifugation (Keller & Taylor, 1977). Sheared DNA fragments sized on alkaline agarose gels were used as molecular weight standards.

Isolation of Maize DNA. Etiolated (6 day) corn shoots were homogenized in 5 volumes of an ice-cold buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 15 mM 2-mercaptoethanol, and 0.5 M sucrose. The homogenate was filtered through four layers of cheesecloth and Miracloth, and a crude nuclei pellet was isolated by centrifugation at 10000 rpm in a Sorvall GSA rotor for 30 min. The pellets were immediately extracted at 60 °C for 10 min with 5% $NaDodSO_4$ in 50 mM Tris–citrate, pH 7.4. After centrifugation and reextraction of the insoluble material, the combined supernatants were precipitated with ethanol. The precipitate was dissolved in 50 mM Tris–citrate, pH 7.4, containing 1 M NaCl and digested with 100 μg/mL proteinase K (Beckman) for 1 h at 37 °C. Insoluble material was removed by centrifugation, and the supernatant was deproteinized with chloroform–isoamyl alcohol (24:1 v/v) and ethanol precipitated. The DNA precipitate was dissolved in 1 × SSC, treated with DNase-free RNase A (100 μg/mL) for 1 h at 37 °C, deproteinized as before, and precipitated. The DNA was then dissolved in 10 mM Tris-HCl, pH 7.4, 15 mM NaCl, and 5 mM EDTA and adjusted to 1.70 g of CsCl per mL. It was banded by equilibrium density centrifugation in a Beckman SW41 rotor at 28000 rpm for 60 h, and the major DNA band at a density of 1.702 g/mL was dialyzed against the same buffer. The DNA was dissolved in 5 mM EDTA (pH 7.0) at a concentration of 1 mg/mL.

Nick Translation Labeling of DNA. In vitro labeling of DNA was performed by the modified nick translation procedure of Braun et al. (1978). Reaction mixtures of 100 μL contained 20 μM of all four deoxynucleotide triphosphates including [^{3}H]dCTP (ICN 25 Ci/mmol) or [^{32}P]dCTP (Amersham, 400 Ci/mmol), 70 mM $MgCl_2$, 70 mM potassium phosphate buffer, pH 7.4, 1 μg of DNA, and 0.07 or 0.027 unit of DNase I (Calbiochem) for maize and *Bacillus subtilus* DNA (PL), respectively. After 15 min at 37 °C, the DNase was inactivated by incubation at 70 °C for 10 min, cooled, and made up to 10 mM with 2-mercaptoethanol. Five units of *Escherichia coli* DNA polymerase I (Boehringer Mannheim) was added, and the reaction was incubated at 16 °C for 2 h. The reaction was stopped by incubation at 70 °C for 10 min, and labeled DNA was separated from precursors by chromatography on Sephadex G-100. This procedure yielded single-stranded DNA of 300 nucleotides as determined by alkaline sucrose gradient centrifugation. The specific radioactivities were 8×10^6 and 1.4×10^7 cpm/μg for maize and *B. subtilis* DNAs, respectively. A foldback of 5–8% was subtracted from the total counts per minute in hybridization reactions.

DNA Shearing and Fragment Length Determination. The DNA was sheared in 5 mM EDTA by sonication of six 20-s bursts (40% of max, Branson microtip) with intermittent cooling. This resulted in an average single-strand length of 300 nucleotides as determined by electrophoresis in 1.8% alkaline agarose gels (McDonell et al., 1977). Gels were stained with ethidium bromide (20 μg/mL) in 5 mM EDTA for 10 min and photographed. DNA fragments obtained from a micrococcal nuclease digestion of rat liver were used as markers (Bloom & Anderson, 1978).

RNA Hybridization with cDNA. All hybridizations were in 4-mL polyallomer Falcon tubes, and reactions were overlaid with 15 drops of mineral oil. The reaction mixtures of 20 μL contained 0.3 M NaCl, 2 mM EDTA, 0.1% $NaDodSO_4$, 20 mM Tris-HCl, pH 7.0 at 68 °C, cDNA, and excess mRNA containing 10 μg/mL yeast RNA. Routinely, 1000 cpm of [^{3}H]cDNAs (50 or 90 pg, 350 nucleotides) and 1–480 ng of mRNAs were used in each reaction. R_0t values were corrected to ER_0t by the salt factor 2.3 (Britten et al., 1974). Hybridization mixtures were premixed before pipetting, and reactions were started after heating to 106 °C for 5 min in water–glycerol. After incubation at 68 °C for 1 min to 24 h, reactions were stopped by freezing in liquid nitrogen. The extent of hybrid formation was determined by S_1 nuclease digestion (Bloom & Anderson, 1978). S_1-resistant duplexes were Cl_3AcOH precipitated in the presence of 300 μg of calf thymus DNA on GF/A filters (Whatman), and radioactivity was determined by liquid scintillation spectroscopy in Omnifluor (New England Nuclear).

Hybrid formation was also determined by chromatography on hydroxylapatite (DNA grade, Bio-Rad). One thousand counts per minute of [^{3}H]cDNA (800 nucleotides, 166 pg) synthesized from normal mRNA was hybridized to normal mRNA as described. The reactions were stopped by the addition of 4 mL of 0.12 M sodium phosphate (pH 7.0), 0.3 M NaCl, and 1% formaldehyde at 60 °C. Each sample was then passed over a 1-mL column of hydroxylapatite with 50 μg of *E. coli* DNA bound and equilibrated in 0.12 M phosphate buffer containing 0.3 M NaCl at 60 °C. The single-stranded fraction was further washed off with three 4-mL washes. The cDNA–mRNA hybrids were eluted at 60 °C with four 2-mL washes of 0.4 M sodium phosphate (pH 7.0) and 0.3 M NaCl. The flow through, all washes, and the dry hydroxylapatite were made to 4 mL with water, and the radioactivity was determined in 8 mL of Aquasol (New England Nuclear) by liquid scintillation spectroscopy.

The thermal stability of cDNA–mRNA duplexes was determined by heating for 5 min in 0.18 M NaCl in hybridization buffer. The temperature of the reactions was increased in 5 °C intervals. After being frozen in liquid nitrogen, duplexes were assayed for sensitivity to S_1 nuclease.

DNA Reassociation and cDNA–DNA Hybridization. DNA reassociations were performed similarly to RNA-excess hybridizations in 0.6 M NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 7.0 at 68 °C, 15000 cpm of nick-transplanted tracer DNA, and 20 ng to 220 μg of sheared (300 nucleotide) maize DNA. The C_0t values were corrected to EC_0t by the salt factor 4.9 (Britten et al., 1974). S_1 nuclease digestion was done at 50 °C for 1 h. Sufficient calf thymus DNA was added to obtain a final DNA concentration of 300 μg, the DNA was precipitated with 5% Cl_3AcOH GF/C filters (Whatman) and solubilized in 0.75 mL of NCS solubilizer (Amersham), and radioactivity was determined by scintillation spectroscopy in 10 mL of Omnifluor (New England Nuclear).

The reassociation of *B. subtilis* DNA was monitored as an internal standard. *B. subtilis* DNA (10000 cpm) labeled with ^{32}P by nick translation was reassociated with 1.4 μg of 300-nucleotide sheared DNA in the presence of the highest concentration of maize DNA.

Hybridization of zein cDNAs with total DNA was in reaction mixtures containing 500 cpm of cDNA (26 pg, 300

[1] Abbreviations used: AMV, avian myeloblastosis virus; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid.

nucleotides) and 220 μg of sheared total DNA (300 nucleotides).

Cell-Free Translation of mRNAs. A wheat germ cell-free translation system was prepared from wheat embryos by the method of Marcu & Dudock (1974). Translations of mRNA were in 50-μL reaction mixtures containing 0.25 μCi (350 Ci/mmol, Amersham) of [^{14}C]leucine for 1 h. Alternatively, zein mRNAs were translated in a mRNA-dependent cell-free system prepared from micrococcal nuclease treated rabbit reticulocyte lysate (Pelham & Jackson, 1976). Radioactive products were precipitated with 2 mL of acetone at −20 °C for 1 h and collected by centrifugation. Proteins were analyzed on 12.5% NaDodSO$_4$–polyacrylamide gels (Larkins & Hurkman, 1978); fluorography was essentially as described by Laskey & Mills (1975).

Hybrid-Arrested Cell-Free Translations. Hybridization of full-length *opaque*-2 cDNAs to normal zein mRNAs was carried out under similar conditions for the RNA-excess hybridization, except yeast RNA was omitted. Reaction mixtures of 50 μL contained 50 ng of normal zein mRNA and from 0 to 140 ng of *opaque*-2 cDNA. Following hybridization for 40 min, 19 μg of maize tRNA and 0.1 volume of 3 M potassium acetate were added and the samples were precipitated with 2.5 volumes of 95% ethanol in SW 50.1 polyallomer tubes. The precipitates were washed thoroughly with 70% ethanol to remove NaDodSO$_4$ and dissolved in 20 μL of water. Hybrids were melted in centrifuge tubes by heating to 100 °C for 2 min and quick cooled in ice water. Translations were in 50-μL reaction mixtures containing 0.25 μCi each of [^{14}C]glutamine (New England Nuclear, 250 mCi/mmol) and [^{14}C]leucine (354 mCi/mmol, Amersham) for 2 h in the wheat germ cell-free system. Radioactive products were analyzed as above.

Computer Analyses of Hybridization Kinetics. The kinetic parameters for RNA-excess hybridization, DNA reassociation, and cDNA–DNA hybridization were obtained with a computer program which uses a least-squares procedure to analyze pseudo-first-order and second-order reactions (Pearson et al., 1977) and was kindly made available by Dr. D. E. Graham, Purdue University.

Results

Analysis of Zein mRNA. Zein mRNAs from normal maize and *opaque*-2 mutant endosperms were purified from membrane-bound polysomes by oligo(dT)–cellulose chromatography and sucrose density gradient ultracentrifugation. The 13S peaks, which contained greater than 80% of the gradient RNA (Larkins et al., 1976), had apparent molecular weights of 365 000 ± 15 000 as determined by electrophoresis on 1.8% agarose gels containing formaldehyde or methylmercury (not shown). This value is in agreement with that determined by Burr et al. (1978) based on electrophoresis in formaldehyde gels and electron microscopic measurements.

The polypeptide products coded for by the zein mRNAs in both wheat germ and rabbit reticulocyte cell-free systems are shown in Figure 1. The zein mRNAs from normal maize coded predominately for the two major prezein polypeptides (parts B and D of Figure 1). A smaller amount of the minor zein component (M_r 15 000) was also synthesized. The pre-22 000 molecular weight components, however, were not detected in the translation products of *opaque*-2 mRNA (parts A and C of Figure 1), an observation which is in agreement with earlier reports (Larkins et al., 1979).

The zein mRNAs from normal maize and *opaque*-2 were used as templates for the synthesis of cDNAs by AMV reverse transcriptase. Analysis of the cDNA products by alkaline

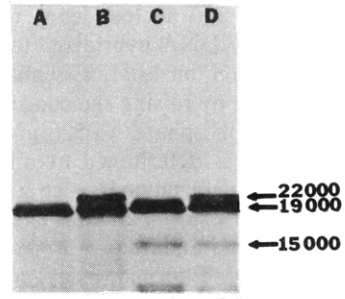

FIGURE 1: Translation of zein mRNAs. One microgram of normal or *opaque*-2 mRNA was translated in cell-free systems from wheat germ and rabbit reticulocytes in the presence of 0.25 μCi of [^{14}C]-leucine. NaDodSO$_4$–polyacrylamide gel electrophoresis and fluorography were carried out as described under Materials and Methods. (Lane A) *opaque*-2 mRNAs in wheat germ; (lane B) normal mRNAs in wheat germ; (lane C) *opaque*-2 mRNAs in reticulocyte; (lane D) normal mRNAs in reticulocyte.

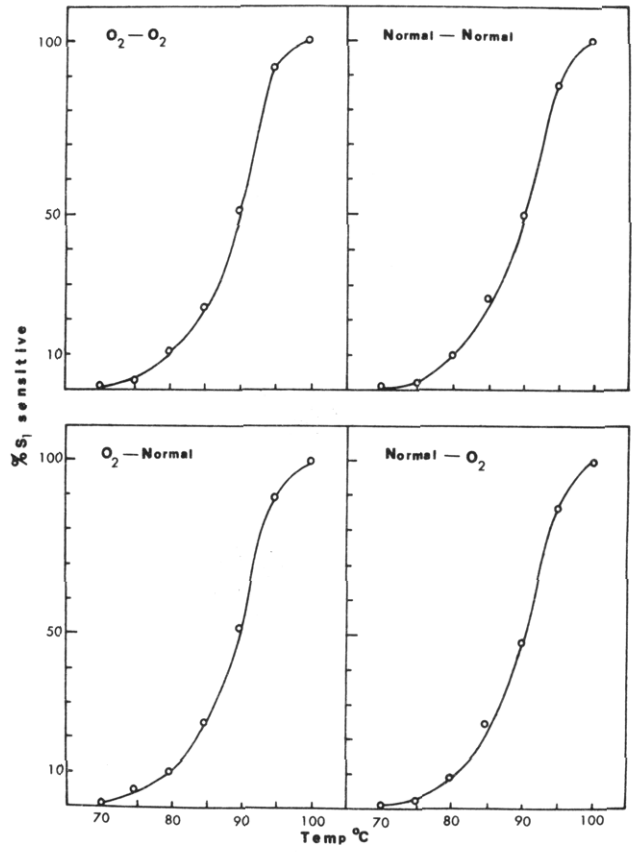

FIGURE 2: Melting curves of cDNA–mRNA duplexes. mRNAs and cDNAs were hybridized to an R_0t of 1.02 (12.0 for normal cDNAs–opaque-2 mRNAs), after which the concentration of NaCl was diluted to 0.18 M. Aliquots were heated for 5 min at the indicated temperatures, and the S_1-insensitive radioactivity was determined.

sucrose gradient centrifugation revealed molecular weights corresponding to 350 nucleotides with low concentrations of labeled nucleotide (25 μM) or essentially full-length copies when higher nucleotide concentrations (500 μM) were used in the synthesis. These cDNAs were hybridized to zein mRNAs from both normal and *opaque*-2 zein mRNA. The melting temperatures in 0.18 M NaCl of all four possible combinations of cDNAs (350 nucleotides) and mRNAs were 90 ± 1.5 °C, and each sample exhibited a symmetrical melting profile with no apparent discontinuities (Figure 2). Essentially the same profiles and T_m values of 90 °C were obtained when hybrids with full-length cDNAs or when cDNA–mRNA templates from the cDNA syntheses were melted (data not

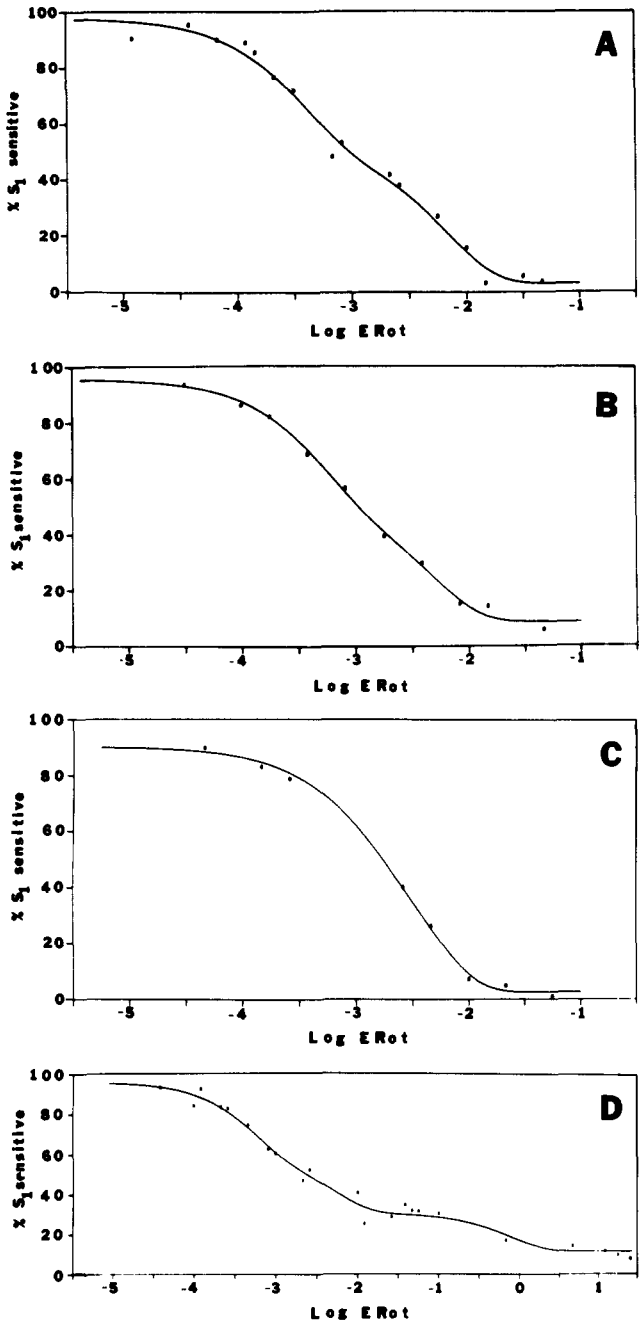

FIGURE 3: Hybridization of cDNAs and mRNAs. [^{3}H]cDNA (1000 cpm, 350 nucleotides) prepared with zein mRNAs from both normal and *opaque*-2 was hybridized in mRNA excess with both mRNAs. The hybridization curves represent the best least-squares fit for pseudo-first-order reactions with two components (three components for normal cDNA and *opaque*-2 mRNA). (A) Normal cDNA–normal mRNA, root mean square error = 3.3%; (B) *opaque*-2 cDNA–*opaque*-2 mRNA, root mean square error = 1.9%; (C) *opaque*-2 cDNA–normal mRNA, root mean square error =2.4%; (D) normal cDNA–*opaque*-2 mRNA, root mean square error = 4.1%. Rate constants are described in Table I, and the kinetic complexities were determined relative to the rate constant of 350-nucleotide ovalbumin cDNA–mRNA hybridizations.

shown). Little mispairing of mRNAs and cDNAs therefore occurred under these hybridization conditions.

The cDNAs from normal and *opaque*-2 mRNAs were hybridized to normal and *opaque*-2 mRNAs under conditions of RNA excess (Figure 3) to determine whether the absence of the higher molecular weight zein component in *opaque*-2 mRNA translation products (parts A and C of Figure 1) was a reflection of the deficiency of specific zein mRNA sequences.

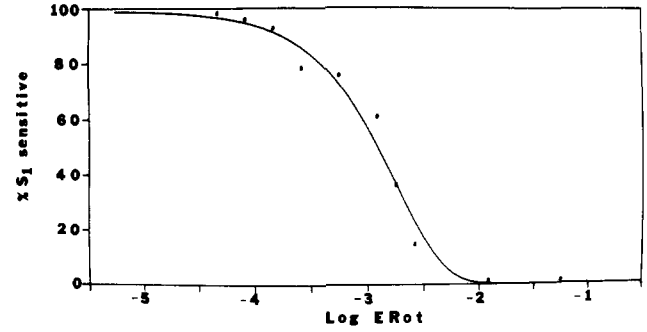

FIGURE 4: Hybridization of ovalbumin cDNA and mRNA. Excess ovalbumin mRNA was hybridized with [^{3}H]cDNA (1000 cpm, 350 nucleotides) as a kinetic standard. The least-squares fit for this single component is shown, and the rate constant of 563 M^{-1} s^{-1} was calculated. The root mean square error of this fit was 3.7%.

The hybridization of ovalbumin mRNA with its cDNA (Figure 4) was used as a kinetic standard. The ovalbumin reaction occurred within 1.5 decades of ER_0t, as would be expected for a pseudo-first-order reaction involving one component (Galau et al., 1974). All of the hybridizations involving zein cDNAs and mRNAs encompassed at least 2.5 decades of ER_0t, indicating a heterogeneous population of mRNAs. The analyses shown in parts A–C of Figure 3 are free computer-fit solutions for two pseudo-first-order components. The hybridization between *opaque*-2 mRNA and normal cDNA (Figure 3D) was best fitted by three components. While these are not the only possible solutions, they are the simplest solutions that fit the data. The kinetic parameters of these solutions are shown in Table I. Components 1 and 2 constitute nearly equal mass fractions of the mRNA population. In the reaction of normal mRNAs with both normal and *opaque*-2 cDNAs the first component had a complexity equivalent to one-fourth of the average zein mRNA complexity and the second component was 4 times that of the average zein mRNA. In the case of *opaque*-2 mRNA, components 1 and 2 had complexities of one-third and 3 times that of the average mRNA.

Greater than 90% of the cDNA annealed to the homologous (parts A and B of Figure 3) and heterologous (parts C and D of Figure 3) mRNAs, indicating that at least 90% of the normal and *opaque*-2 mRNA sequences were common. When mRNA from normal endosperm reacted with homologous cDNA or the cDNA synthesized with *opaque*-2 mRNA, the reactions were complete at an ER_0t of 10^{-2} (parts A and C of Figure 3). Similarly, the hybridization of *opaque*-2 mutant mRNA with its homologous cDNA was complete at the same ER_0t (Figure 3B). However, only 60% of the normal cDNA reacted with *opaque*-2 mRNA by an ER_0t of 10^{-2}, with the remainder of the reaction requiring an additional 3 decades of ER_0t for completion (Figure 3D). These observations indicate that 30–40% of the sequences represented in the normal zein mRNA are substantially reduced (about 200-fold) in the *opaque*-2 mRNA.

Normal zein mRNA (50 ng) was hybridized with up to a threefold excess of full-length *opaque*-2 cDNAs and the reactants were translated in a wheat germ cell-free protein-synthesizing system (Figures 5 and 6) to determine if the RNA sequences which are at a reduced concentration in *opaque*-2 zein mRNA correspond to those sequences which code for the 22 000 molecular weight zein component. A comparison of the products from a direct translation of mRNA (Figure 5, lanes 1 and 8) with those from a reaction mixture containing no cDNA (Figure 5, lane 2) indicated that mRNA recovery and translatability were not deleteriously affected as a result

Table I: Analysis of Zein mRNA Complexity[a]

cDNA–mRNA	component	fraction of mRNA	K_{obsd} (M^{-1} s^{-1})	K_{pure} (M^{-1} s^{-1})[b]	complexity (nucleotides)[c]	no. of 1100-nucleotide sequences[d]
normal–normal	1	0.45	2640	5580	200	0.2
	2	0.50	150	285	3950	4
opaque-2–*opaque*-2	1	0.42	1610	3330	340	0.3
	2	0.45	175	338	3330	3
opaque-2–normal	1	0.22	1130	4460	250	0.3
	2	0.65	234	313	3600	4
normal–*opaque*-2	1	0.36	1810	4260	260	0.3
	2	0.30	188	533	2110	2
	3	0.19	1.2	5.6	203×10^3	200

[a] Kinetic parameters from Figure 3. [b] $K_{pure} = K_{obsd}$/fraction of mRNAs normalized to 100% reactability. [c] $C = K_{ov}C_{ov}/K_{pure}$; K_{ov} = 563 M^{-1} s^{-1}. The cDNAs were 350 nucleotides in all cases to avoid effects of length. [d] The average complexity of the mRNAs was calculated to be 1100 based on electrophoresis in formaldehyde-containing agarose gels.

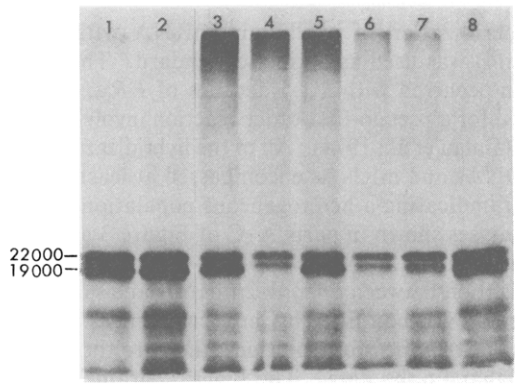

FIGURE 5: Hybrid-arrested cell-free translation of zein mRNAs. Varying amounts of full-length *opaque*-2 cDNAs were hybridized with 50 ng of normal zein mRNAs as described under Materials and Methods. (Lanes 1 and 8) Nonhybridized mRNAs; (lane 2) no cDNA (mock hybridization); (lane 3) 103 ng of cDNA melted out; (lane 4) 103 ng of cDNA hybrid arrested; (lane 5) 51 ng of cDNA melted out; (lane 6) 51 ng of cDNA hybrid arrested; (lane 7) 20 ng of cDNA hybrid arrested. Markers indicate the position of 19 000 and 22 000 prezein molecular weight components.

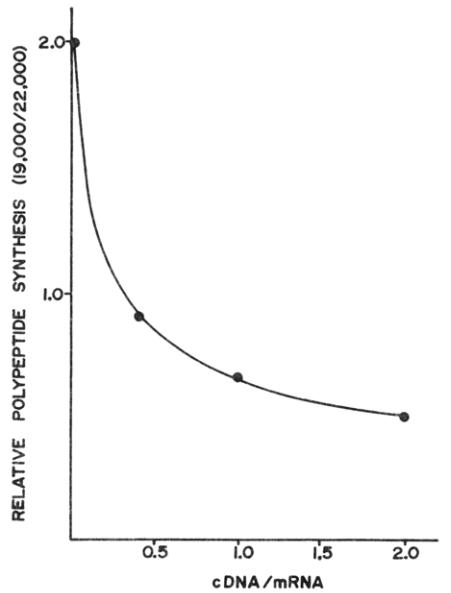

FIGURE 6: Relative polypeptide synthesis. Lanes from the hybrid-arrested translations (Figure 5) were scanned in a densitometer, and the amounts of the pre-19 000 molecular weight component relative to the pre-22 000 molecular weight component were graphed as a function of the mass ratio of cDNA to mRNA.

of the hybridization. The relative labeling of the major zein components was similar in these reactions, although there was a slightly larger proportion of smaller molecular weight material present in the hybrid-arrested translation. All of the major zein components were present in both reactions.

A comparison of reactions that were hybrid-arrested with *opaque*-2 cDNA (Figure 5, lanes 4, 6, and 7) with those where the hybrids were melted out prior to translation (Figure 5, lanes 3 and 5) showed that synthesis of the 19 000 and 15 000 molecular weight precursors was preferentially inhibited by *opaque*-2 cDNA. Although the synthesis of these components was never completely blocked, there was a proportional decrease in products with increasing cDNA based on densitometer analysis of the fluorograph (Figure 6). These results demonstrated that mRNAs for the larger molecular weight zein component were substantially reduced in mRNA from the *opaque*-2 mutant.

To further address the question of the low complexity component of the cDNA-mRNA hyridizations, we analyzed hybrid formation by hydroxylapatite chromatography rather than S_1 digestion. When essentially full-length cDNAs (800 nucleotides) synthesized from normal mRNAs were hybridized to normal mRNAs, only one component was observed (Figure 7) with a rate constant of 3130 M^{-1} s^{-1} equivalent to a complexity of 270 nucleotides (without any rate correction due to change in tracer length but normalized to 100% reactivity). This one component thus has a kinetic complexity approaching that of the rapid reacting component observed by S_1 digestion.

Analysis of Zein Gene Frequency in the Maize Genome. The reassociation of maize DNA revealed three kinetic components which corresponded to the repetitive, middle repetitive, and single-copy components of the genome (Figure 8A, curve a analyzed by a modified second-order rate equation to fit S_1 kinetics). The most rapidly reassociating component accounted for 38% of the DNA mass ($C_0t_{1/2} = 4.5 \times 10^{-3}$ M s) and the middle repetitive component accounted for 44% of the DNA mass ($C_0t_{1/2}$ = 8.9 M s), while the single-copy component accounted for 19% of the DNA mass ($C_0t_{1/2}$ = 2320 M s). Because high DNA concentrations (8–10 mg/mL) were necessary in the EC_0t range of the single-copy component, we monitored the reassociation of *B. subtilis* DNA in the presence of maize DNA (Figure 8B) to ensure that an accurate rate of maize DNA reassociation was measured. The rate of reassociation of *B. subtilis* DNA in the presence of maize DNA was retarded by a factor of 1.5 compared to its reassociation alone, indicating that elements such as viscosity retarded the reassociation of the maize DNA. This factor was used to correct the $C_0t_{1/2}$ value of the maize single-copy component from 2320 to 1590 M s. By comparing the corrected rate of the maize single-copy component with the rate of the *B. subtilis* reassociation and the complexity of the *B.*

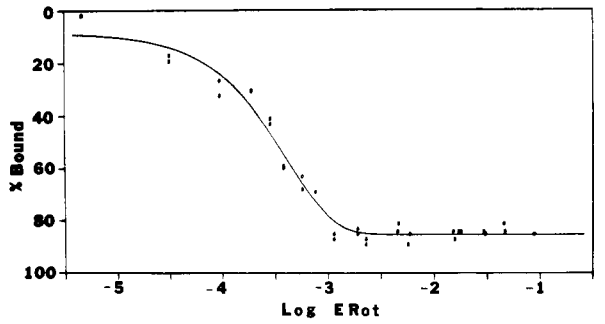

FIGURE 7: Hybridization of normal cDNAs with normal mRNAs. [^{3}H]cDNAs (1000 cpm, 800 nucleotides) were hybridized to excess mRNA, and hybrid formation was determined by chromatography on hydroxylapatite (see Materials and Methods). The curve shows the best least-squares fit to a pseudo-first-order reaction with one component. This solution has a rate constant of 3150 M^{-1} s^{-1}. (No correction for cDNA length has been made.) This solution has a root mean square error of 4.0%.

subtilis genome, we calculated a kinetic complexity of 5×10^9 nucleotide pairs for the maize genome. This corresponded to 5.3 pg of DNA per haploid genome, which is in good agreement with a value of 5.05 pg determined by chemical means (Phillips et al., 1974).

The hybridization of zein cDNAs with the genomic DNA is shown in curve b of Figure 8A. This hybridization fits an S_1-modified second-order reaction with a $C_0t_{1/2}$ of 1050 M s. Approximately 60% of the input cDNA became resistant to S_1 nuclease, which would be expected with moderate DNA excess. The rate of the cDNA–DNA hybridization was 2.2 times faster than the reassociation of the single-copy component of the maize genome. The same rate constants were observed whether *opaque*-2 or normal cDNAs were used and more important whether the hybridization was performed at normal stringency (0.6 M NaCl, 68 °C) or at reduced stringency (0.6 M NaCl, 58 °C) (data not shown). These results indicate that there are possibly two but probably no more than five copies of each zein gene present in the maize genome.

Discussion

In the endosperm mutant *opaque*-2, the nutritional quality of the seed protein is enhanced by a reduction in the low lysine-containing zein proteins (Mertz et al., 1964). The specific factors responsible for the reduced synthesis of these proteins are unknown, but they may involve effects on mRNA transcription. As shown in Figure 1, the mRNA directing the synthesis of the 22 000 molecular weight zein component was apparently absent in *opaque*-2. The hybridization study shown in Figure 3D also suggests that 30–40% of the zein mRNA sequences were substantially reduced in the mutant, and based on the hybrid-arrested translation (Figures 5 and 6), it is evident that these sequences correspond to those which code for the 22 000 molecular weight zein component. However, the total sequence complexity of the zein mRNAs in the mutant was similar to that in normal maize, because *opaque*-2 mRNA reacted to the same extent as normal mRNA with the cDNA from normal maize. The apparent absence of the 22 000 molecular weight product in the translation assays of *opaque*-2 mRNA is therefore probably a reflection of a reduction rather than the absence of these mRNA sequences.

Although the zein proteins reveal relatively few bands on $NaDodSO_4$ gels (Lee et al., 1976; Gianazza et al., 1977) and are very similar in terms of amino acid composition (Lee et al., 1976; Gianazza et al., 1977) and tryptic–chymotryptic peptide maps (Fraij & Melcher, 1978), the hybridization of zein mRNAs and cDNA demonstrates a substantial sequence complexity among these proteins. The best computer fit of the data (Figure 3, Table I) indicated two different kinetic components. The second component of the reaction for normal mRNA had sufficient complexity for four 1100-nucleotide sequences, while that for *opaque*-2 mRNA was equivalent to three. The first component of both reactions had a complexity between 200 and 340 nucleotides and may represent internal repeats and sequence homology among heterologous mRNA species.

The complexity of the zein mRNA–cDNA hybridization is analogous to that of trout testis protamine mRNA (Sakai

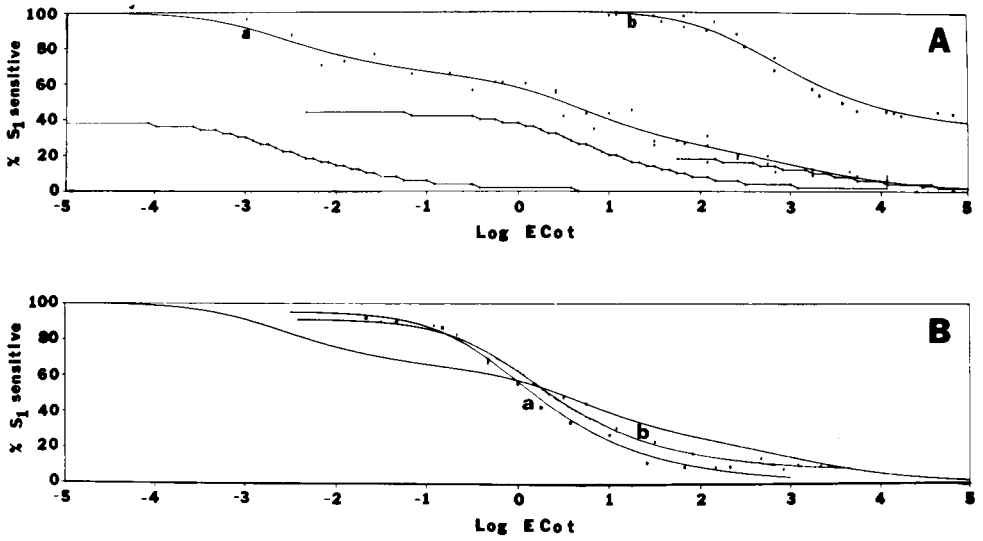

FIGURE 8: Analysis of reassociation kinetics of maize DNA and kinetics of cDNA–DNA hybridization (300-nucleotide sheared fragments and cDNAs were used). (A) The curve through the data points (a) represents the best least-squares solution using three second-order components. In the DNA reassociation, 38% reacted as repetitive DNA with a rate constant k = 860 M^{-1} s^{-1} and $C_0t_{1/2}$ of 4.5×10^{-3} M s, 44% as middle repetitive DNA with a rate constant k = 0.43 M^{-1} s^{-1} and $C_0t_{1/2}$ = 8.9 M s, and 19% as a single-copy DNA with a rate constant $k = 1.7 \times 10^{-3}$ M^{-1} s^{-1}. However, after correction for rate retardation (see B), the single-copy component had a rate constant of $k = 2.4 \times 10^{-3}$ M^{-1} s^{-1} and $C_0t_{1/2}$ = 1590 M s. The root mean square error for this solution was 5.1%. The zein cDNA–DNA hybridization, curve b, was in 10–12 mg/mL DNA (see Materials and Methods). The rate constant without correction for retardation was 3.7×10^{-3} M^{-1} s^{-1} or $C_0t_{1/2}$ = 1050 M s. The root mean square error of this solution was 2.9%. (B) Reassociation of ^{32}P-labeled *B. subtilis* DNA. *B. subtilis* DNA was reassociated by itself (a) or in the high EC_0t range of the maize DNA reassociation (b). The reassociation rate constants were 1.6 and 2.4 M^{-1} s^{-1} in the presence and absence of maize DNA, respectively.

et al., 1978) and feather keratin mRNA (Powell et al., 1976). In these systems it has been shown that several mRNA sequences are present, and depending on the conditions used for nucleic acid hybridization, cross-hybridization of mRNAs and cDNAs may occur. The hybridization kinetics as analyzed by hydroxylapatite chromatography (Figure 7) showed only the low complexity component and thus indicate the existence of internal sequence repeats in the mRNAs or regions of complete homology among different mRNAs. The results of the hybrid-arrested translation and melting profiles indicate that the stringency of our hybridization conditions was such that little cross-hybridization between the mRNAs for the 19 000 and 22 000 molecular weight components occurred. However, this does not exclude the possibility of cross-hybridization between mRNAs and cDNAs specifying different 19 000 or 22 000 molecular weight zein components.

We must point out that the computer fits of the hybridization reactions are not the only solutions for the data. The rates of the homologous reactions (parts A and B of Figure 3) can be forced on the heterologous reactions (parts C and D of Figure 3) without a marked change in the root mean square error. It is also possible to fit the data to a single component, although this changed the root mean square error from 3.3 to 7.6% for the normal mRNA–cDNA reaction (Figure 3A). All of the reactions were, however, more complex than the ovalbumin mRNA–cDNA standard which clearly reacted as a single component (Figure 4).

The frequency of zein genes was determined by hybridization of zein cDNAs to the maize DNA. Because of the large size of the maize genome, only a moderate driver sequence excess could be obtained even with cDNA labeled by two nucleotides. However, this ratio was enhanced by the complexity of the cDNA population. The cDNA hybridized to the genomic DNA at a rate of 2.2 times faster than the single-copy component of the DNA. Based on this result, we concluded that there are possibly two copies of each zein gene in the genome and probably no more than five. We are reluctant to extrapolate further from these data because of the moderate excess of driver DNA. As reported by Muto (1977), there are inherent errors in extrapolating gene copy numbers from moderate DNA-excess hybridizations. When we applied his formula for determining gene frequency by moderate DNA-excess hybridization, we also calculated a gene copy number of two. Based on this result, we believe our determination of the gene frequency to be reasonably accurate. However, with a slight to moderate DNA excess this number could even be an overestimation. This would also be the case if there were cross-reaction between portions of different genes.

Although large amounts of storage proteins are synthesized in the developing endosperm during a reasonably short period of time, these proteins are coded by what would be considered single-copy rather than repetitive genes. In this respect, the zein genes in maize are similar to a variety of well characterized genes in both animals [reviewed in Dawid & Wahli (1979)] and plants (Cashmore, 1979), in that sequence amplification is not requisite for the accumulation of large quantities of specific mRNAs. Whether the accumulation of zein-specific mRNAs in maize endosperm results from elevated mRNA transcription or enhanced mRNA stability is currently under investigation.

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