In Vitro Uptake and Processing of Prezein and Other Maize Preproteins by Maize Membranes

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ABSTRACT A cell-free, mRNA-dependent system has been developed for the translation and processing of zein preproteins. A rough endoplasmic reticulum (RER)-enriched fraction, isolated by sucrose density gradients, can be treated with micrococcal nuclease to destroy endogenous messages. When these membranes are added to a wheat germ protein-synthesizing system together with zein mRNA, synthesis and processing of the polypeptides to the mature products takes place. The RER fraction from the endosperm has a different protein composition than that prepared from either the shoot or nucellar tissue and processes prezein more efficiently. The cleavage of the preproteins appears to be a cotranslational step as the completed preprotein chains cannot be processed, although they can be taken up to a limited extent. This small uptake, or absorption, of unprocessed zein seems to be an artifact and may be related to the unusual solubility properties of zein. Finally a sodium dodecyl sulfate (SDS)-urea polyacrylamide gel system has been developed which is particularly suited for the separation of low molecular weight proteins (<10,000 daltons). Using this method, we examined the products of in vitro zein processing and detected no presequence polypeptides. This suggests that the zein cleavage proteinase is probably an exopeptidase.

Zein is the major class of storage proteins found in the endosperm of maize. The protein is made predominantly in tissue adjacent to the aleurone and diminishes towards the central carbohydrate (floury) core. Zein mRNA is translated by polyribosomes attached to the outer surface of those endoplasmic reticulum cisternae that subsequently enlarge to form vesiculate protein bodies 1–2 μm in diameter (3, 12). When poly(A)+ RNA prepared from developing protein bodies is translated in the wheat germ cell-free system, zein polypeptides are produced that are respectively 1,000 and 2,000 daltons larger (4, 13) than the authentic zein chain classes of 19,000 and 22,500 daltons (4, 10, 13, 15). These additions apparently are at the amino termini (5) and are therefore presequences similar to those reported for many types of secretory proteins (28). Zein, however, is not transported out of the protein bodies once it has been synthesized but remains in its site of deposition until germination and early seedling growth. At that time, the zein is digested in situ, releasing products to be used by the growing embryo.

A description of the native transport system and the processing of the zein preproteins is contingent on a message-dependent system reconstituted from maize endosperm membranes. Comparable systems have already been described for bacteria (7), animal rough endoplasmic reticulum (RER) (23), mitochondria (14, 21), and chloroplasts (9). We describe here the development and employment of a homologous system from maize which will translate zein mRNA and process the preproteins made there to their mature form.

MATERIALS AND METHODS

Maize Tissue

Illinois High Protein (IHP), 1975 Selection, was used as the source for all material unless otherwise stated. All tissues were either used immediately or frozen in liquid nitrogen and stored at −85°C. Endosperms were dissected from kernels 15–18 d after pollination. Nucellar tissue (tissue within the pericarp that consists largely of the nucellus, but also includes the egg sac) was dissected from unfertilized ears 12 d after silk emergence. Shoot axes were separated by hand from 3-d-old seedlings that had been germinated sterilely in the dark at 30°C.

Zein Protein and Poly(A) + RNAs

Zein proteins were extracted from dissected endosperms of kernels at 15–18 d after pollination and at maturity (3). Protein body poly(A)+ RNA was prepared as described previously (4). Maize poly(A)+ RNAs for the small subunit of ribulose-1,5-bisphosphate carboxylase and for polypeptides 15 and 16 of the chlorophyll a/b protein complex were provided by R. Broglie and N.-H. Chua (Rockefeller University).

Isolation of Membranes

Routinely, 10–20 g of tissue were ground with a VirTis homogenizer (VirTis Co., Inc., Gardiner, N. Y.) in ice cold PB buffer (50 mM Tricine, pH 8.0, 100

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mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 2 mM adenosine-2',3'-monophosphoric acid) containing 20% sucrose (wt/vol), using 1 g tissue/ml grind buffer (3). The homogenate was filtered through two layers of cheesecloth over one layer of Miracloth and centrifuged at 3,000 *g* for 10 min. The supernate was layered onto gradients in Beckman SW41 nitrocellulose tubes (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) which consisted of an 8-ml linear gradient of 35–70% sucrose (wt/vol) in PB buffer, overlayered by a 1.5-ml pad of 22.2% sucrose (wt/vol) in the same buffer. Gradients were centrifuged at 35,000 rpm for 2 h at O°C.

Membrane bands were collected with a silanized Pasteur pipette which had been bent near the tip. The membranes were diluted with 2 vol of 10 mM HEPES, pH 7.6, 2 mM $MgCl_2$ and centrifuged at 100,000 *g* for 1 h. The pellets were taken up in 20% glycerol (vol/vol), 5 mM HEPES, pH 7.6, 1 mM calcium acetate, to 50 A_{260} U/ml and stored at −85°. Membrane preparations retain their activity for months.

Electron Microscopy

Samples of the bands from sucrose density gradients were fixed in 5% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.4, postfixed in 1% OsO_4 in the same buffer, dehydrated in an ethanol series, and embedded in Spurr's resin, "firm" formulation. Sections of the material were poststained with 1% aqueous uranyl acetate and Reynolds lead citrate.

Preparation of Treated Membranes

Before in vitro synthesis, membrane preparations were treated with *Streptococcus aureus* (micrococcal) nuclease (P-L Biochemicals, Inc., Milwaukee, Wis.) at 10–15 U/ml for 10 min at 20°C. The membranes were brought to 4 mM EGTA and centrifuged at 100,000 *g* for 30 min. The pellets were resuspended in 20% glycerol (vol/vol), 3 mM HEPES, pH 7.6, 1 mM magnesium acetate to 100 A_{260} U/ml and kept on ice.

In Vitro Protein Synthesis

In vitro protein synthesis was performed essentially as previously described (4). A typical reaction mixture of 25 μl included 0.3 μg of poly(A)+ RNA, 20 μm [^{3}H]leucine, and 4–5 μl of treated membranes at 100 A_{260} U/ml. Incubation was carried out at 25°C for 90 min. Reaction mixtures containing membranes were posttreated with 0.04 μg/μl proteinase K (EM Laboratories, Inc., Elmsford, N. Y.) at 4°C for 45 min. Protease action was terminated by adding 1 μl of a saturated solution of phenylmethylsulphonylfluoride (PMSF) in 95% ethanol. After being cooled on ice for 10 min, the reaction mixtures were centrifuged and the membrane pellets taken up in sample buffer for polyacrylamide gel electrophoresis (PAGE) (1). Incorporation of label into trichloroacetic acid–precipitable counts was determined as described in an earlier paper (3).

For the posttranslation uptake studies, the synthesis reaction mixture with poly(A)+ RNA was treated with micrococcal nuclease (as just described) after the 90-min translation period. 4–5 μl of micrococcal nuclease-treated membranes were added and incubation was continued for 60 min at 25°C. Membranes were then treated with proteinase K and dissolved in gel sample buffer.

PAGE

Products of the in vitro synthesis and processing experiments were electrophoresed in 15% polyacrylamide:0.1% SDS gels (1), two-dimensional isoelectric focusing (IEF):SDS polyacrylamide gels, using pH 6–8 ampholytes (LKB Instruments, Inc., Rockville, Md.) (17), or 12.5% polyacrylamide:0.1% SDS:8 M urea slab gels. The method for the urea gels was based on a procedure originally developed for tube gels by Swank and Munkres (25), but which, unfortunately, cannot be adapted directly for slab gel use. The gel composition we settled on finally is the following: running gel = 12.5% acrylamide, 0.11% bisacrylamide, 8 M urea, 0.34 M Tris-phosphoric acid, pH 8.0, 0.1% SDS, 0.0005% *N,N,N',N'*-tetramethylethylenediamine (TEMED), 0.05% NH_4perSO_4; stacking gel = 5.0% acrylamide, 0.07% bisacrylamide, 8 M urea, 0.125 M Tris-phosphoric acid, pH 6.8, 0.1% SDS, 0.0005% TEMED, 0.05% NH_4perSO_4. The solutions are warmed in a 50°C water bath to dissolve the urea. Degassing is unnecessary and polymerization generally occurs in <5 min. The sample preparation buffer is 0.02 M Tris-phosphoric acid, pH 6.8, 8 M urea, 0.008% bromophenol blue, 0.1% SDS, 1% 2-mercaptoethanol. Samples are boiled for three min before use. The electrophoresis buffer is 0.1 N phosphoric acid adjusted to pH 6.8 with solid Tris and contains 0.1% SDS. Electrophoresis is done at 50 V, room temperature, until the tracking dye is 1 cm from the bottom edge (or, in our gels, 8.5 cm from the origin). Length of the run is usually about 15 h. Low molecular weight standards used are the cyanogen bromide fragments of cytochrome *c* and myoglobin. Gels for fluorography were treated with Enhance (New England Nuclear, Boston, Mass.) after destaining and exposed to Kodak X-Omat R film at −85°.

RESULTS AND DISCUSSION

Gradient Analysis

After centrifugation, the sucrose density gradients of endosperm homogenates have the banding pattern seen in Fig. 1 *a*. Band *A* consists of mostly free polyribosomes; there are also smooth membrane vesicles and a few small protein bodies and mitochondrial fragments (Fig. 1 *b*). Band *B* has larger RER and smooth membrane vesicles, fewer free polyribosomes, and an occasional small protein body or mitochondrion (Fig. 1 *c*). Band *C* contains predominantly mitochondria; but it also has some RER and small protein bodies (Fig. 1 *d*). Band *D* has about an equal proportion of small to medium-sized protein bodies and RER (possibly RER connected to protein bodies), and also contains some smooth vesicles and a few mitochondria (Fig. 1 *e*). Band *E* is almost entirely protein bodies (Fig. 1 *f*).

Sucrose density gradients of shoot or nucellus tissue homogenates show only two visual bands—one of which corresponds to the position of band B of the endosperm gradients and the other to the position of band C. In these gradients, the nuclei and plastids remaining in the homogenate after the initial low speed centrifugation step are recovered in the pellet of the gradient.

Membrane Treatment

Dosage of micrococcal nuclease from 0 to 75 U/ml showed that 15 U/ml was sufficient for complete inhibition of endogenous messages (data not shown). In the membrane-supplemented syntheses, band B gave the best processing activity (dpm/μl A_{260} U treated membranes), so this fraction was used in all subsequent processing experiments. Although bands *D* and *E* were also able to process zein, the proportion of zein: membrane proteins is considerably higher, and this probably accounts for the observed depression of processing. On the other hand, preparations from band *A* are probably less efficient because there are fewer RER vesicles.

In Vitro Translation and Processing

Fig. 2 shows the in vitro translation products of (*a*) the untreated RER membranes from developing endosperm, (*b*) the RER after treatment with micrococcal nuclease, (*c*) the treated membranes incubated with zein mRNA, and (*d*) the products of zein mRNA alone. The proof that these are actually zein preproteins which are being translated by zein mRNAs has been adequately established by us and by other investigators in previous papers (3, 4, 13, 27).

When untreated RER was put into the wheat germ cell-free system, major bands were observed at positions corresponding to the sizes of the mature authentic zein classes, M_r = 19,000 and 22,500 daltons (Fig. 2 *A*). In some cases the bands resolved as doublets, as was the case for the upper band here. This particular translation also showed a light band at 20,000 daltons which probably represents unprocessed 19,000-dalton zein polypeptides. Micrococcal nuclease treatment effectively abolished translation (Fig. 2 *B*). (Electron microscopic examination, however, revealed that polysome structures were still present after nuclease digestion, and gel electrophoresis indicated that there had been no discernible degradation of the rRNAs. Evidently, only the mRNAs are affected by the nuclease treat-

ment. As the RER membranes cannot be considered to be "stripped" of their polyribosomes, they will instead simply be termed "treated.") Incubation of zein mRNA with treated RER showed that zein polypeptides were being synthesized and processed to their mature sizes (Fig. 2 *C*). A small amount of unprocessed zein was again sometimes noted. For compar-

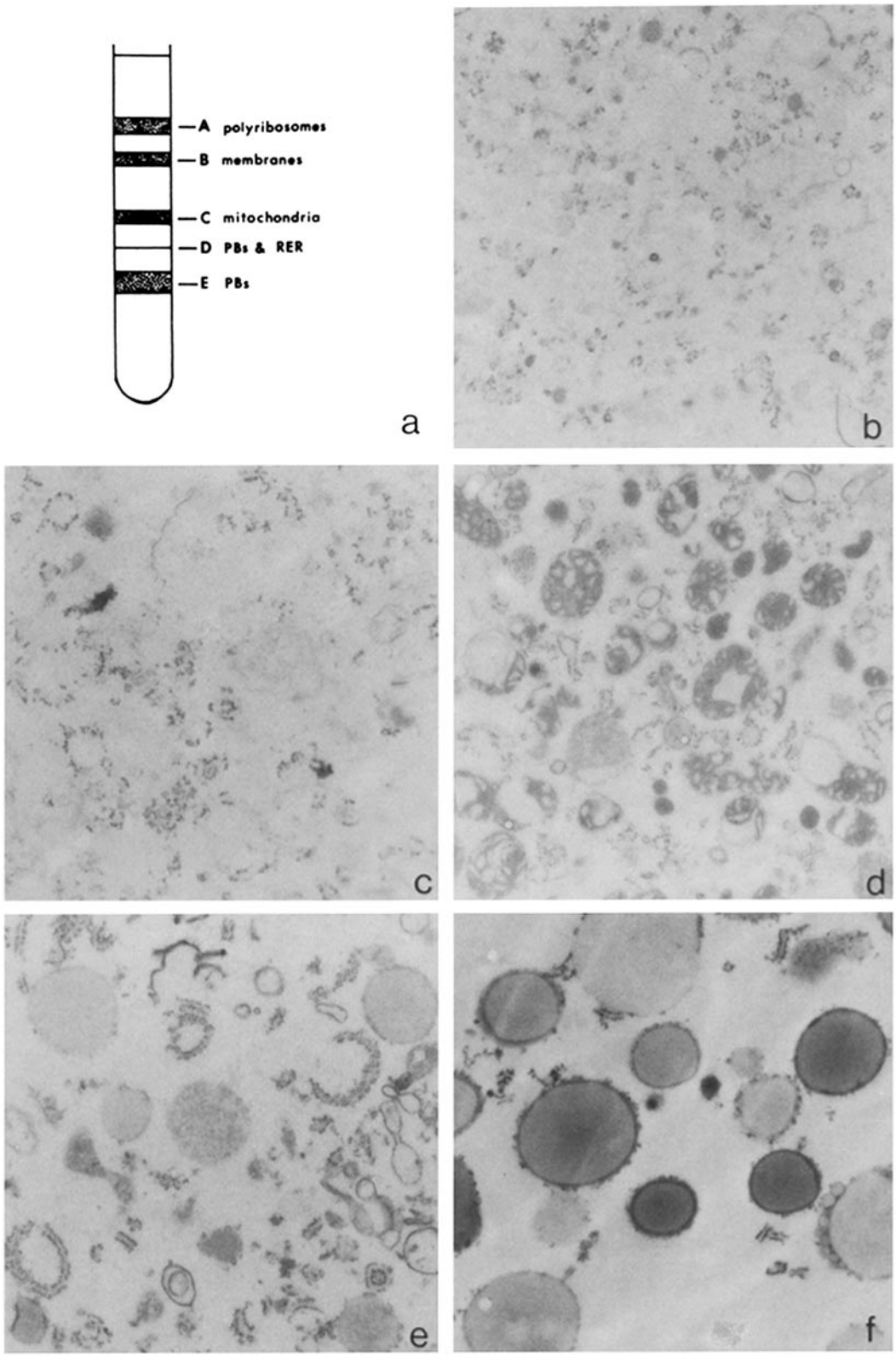

FIGURE 1 (*a*) Diagram of the bands seen in the sucrose density gradients of endosperm homogenates after centrifugation. Each band is identified as to its major component(s). (*b–f*) Electron micrographs of samples prepared from each of the bands shown in *a*: (*b*) band *A* = free polyribosomes, small protein bodies, and small smooth membrane fragments; (*c*) band *B* = free and bound polyribosomes, smooth and rough membranes; (*d*) band *C* = mostly mitochondria, also smooth and rough membranes; (*e*) band *D* = protein bodies and rough membranes; (*f*) band *E* = almost exclusively protein bodies. All micrographs, × 18,800.

ative purposes the last track (Fig. 2 *D*) contains the unprocessed zein bands produced by the translation of zein mRNA alone. These have molecular weights of 20,000 and 24,500 daltons, respectively, 1,000 and 2,000 daltons larger than the authentic zein classes of 19,000 and 22,500 daltons.

Uptake and processing in vitro were inefficient processes as compared to messenger RNA translation alone. With [^{3}H]-leucine, which gives the best incorporation, it may be reduced to 10% of a synthesis with only poly(A)+ RNA. Shields and Blobel (23) had also observed inhibition when translating proinsulin mRNA in the wheat germ cell-free system supplemented with dog pancreas microsomes. They attributed the inhibition to unknown factors of the wheat germ extract, as there was no inhibition noted when reticulate lysate was used. Despite this drawback, we have decided to continue to use the maize RER with the wheat germ extract to keep with a more homologous system that might more closely approximate the in vivo situation.

In a previous article we had summed up our evidence for the glucosylation of zein polypeptides (5). We have not yet determined whether this occurs in the reconstituted system. Hagen and Rubenstein (11) have analyzed zein from mature kernels of six inbred lines of maize by two-dimensional IEF–SDS PAGE. Each line was found to give a different but characteristic pattern. The line used in our studies, IHP, was one of those examined. Our two-dimensional patterns for developing (Fig. 3) and mature IHP zeins were identical but differed from that published by Hagen and Rubenstein most notably at two positions: there is an additional major spot in our gels (Fig. 3 *b*, single-headed arrow), and the spot closest to the anode of M_r = 19,000 (Fig. 3 *b*, double-headed arrow) is replaced in their pattern by a spot at approximately the same isoelectric point but of M_r = 22,500. The relative intensities of the respective spots are also different. These differences, however, probably reflect the fact that IHP is not an inbred line of maize and that we used the 1975 Selection, whereas Hagen and Rubenstein used the 1971 Selection.

The processed and unprocessed zeins made in vitro were examined on two-dimensional IEF-SDS polyacrylamide gels and compared with unlabeled authentic zeins electrophoresed in the same gel. There was good agreement between the spots for the processed chains (Fig. 4 *a*) and those for authentic zein (Fig. 4 *b*), indicating that correct processing must be occurring in vitro. There was one additional spot (Fig. 4 *a*, arrow), however, which probably represented some unprocessed 24,500-dalton zein. Also, a few minor spots were missing. Their absence might be due to their mRNAs being present in substantially smaller amounts or being outcompeted by the other zein messages in vitro.

RER-enriched fractions were also prepared from young shoots and nucellar tissue to see whether endosperm RER was specialized for the processing of zein preproteins. SDS PAGE analysis showed that whereas protein bands in the shoot and the nucellar tissue RER preparations were similar, the patterns were quite different from that of the endosperm RER (Fig. 5). Likewise, both the shoot and the nucellus RER appeared to process zein in vitro much less efficiently than the endosperm RER (Fig. 6). Shoot and nucellus preparations showed about the same degree of processing. The differences in protein composition and zein processing efficiencies in endosperm vs. shoot and nucellus could mean that the former has a type of RER particularly adapted for zein processing, but this remains to be more rigorously examined.

The processing of maize α-amylase by endosperm RER was recently examined to see how it might compare with that of zein. α-Amylase is known to be made *de novo* in the aleurone during germination and secreted into the internal cells of the endosperm, where it digests the starch (8). Although amylase represents another endosperm protein, it is produced during a completely separate period in development than zein and is made by another tissue. (There are no zein protein bodies in the maize aleurone, and no zein can be detected by SDS PAGE of preparations from isolated aleurone tissue.) Recently, in barley and in wheat, α-amylase has been shown to be synthesized as a larger precursor: barley precursor = 45,000 daltons; barley processed subunit = 42,000 daltons (16); wheat precursor = 43,500 daltons; and wheat processed subunit = 42,000 dal-

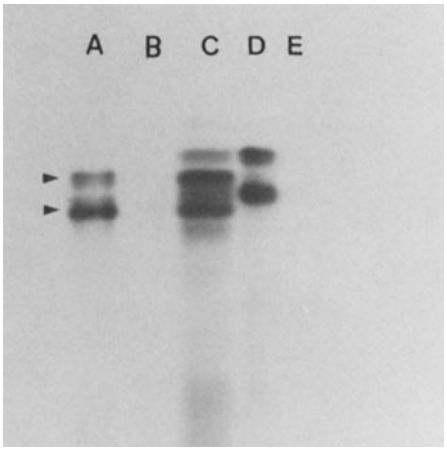

FIGURE 2 Fluorogram of SDS PAGE showing prezeins and processed zeins made in vitro: (*A*) untreated membranes; (*B*) membranes after treatment with micrococcal nuclease; (*C*) treated membranes incubated with zein mRNA; (*D*) zein mRNA only; and (*E*) synthesis mixture with no additions. Arrowheads at left indicate positions in the gel of authentic zein which were determined by Coomassie Blue staining.

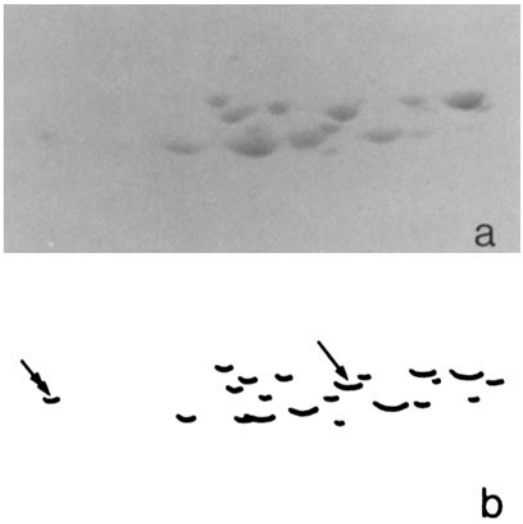

FIGURE 3 Two-dimensional electrophoresis of zein from 16- to 18-d postpollination IHP endosperms; first dimension = IEF in the horizontal direction, anode at left; second dimension = 15% PA: 0.1% SDS in the vertical direction, anode at bottom: (*a*) Coomassie Blue-stained gel; and (*b*) diagram of *a*, particularly to indicate positions of the minor spots. Single- and doubleheaded arrows indicate differences with respect to the previously published pattern for mature IHP zein (11).

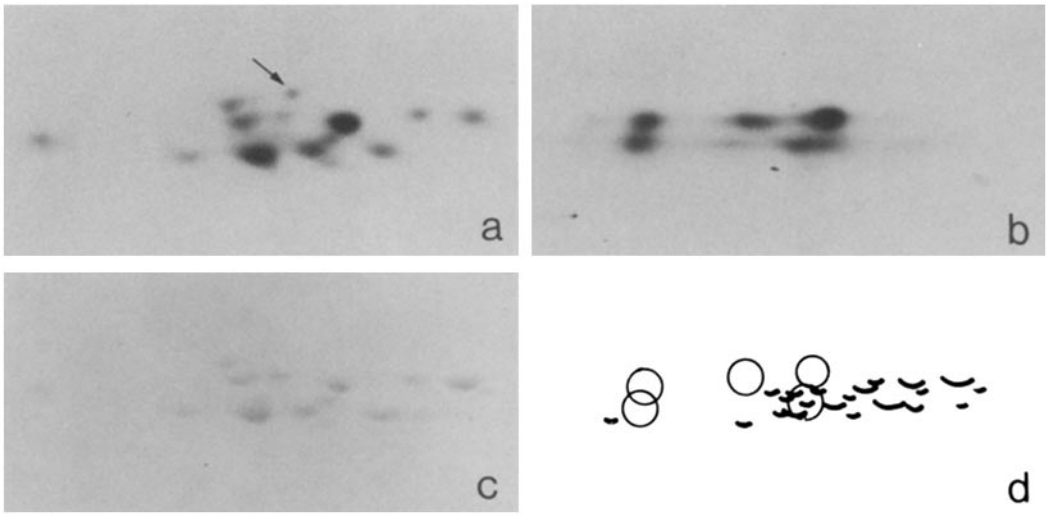

FIGURE 4 Two-dimensional electrophoresis of processed and unprocessed zein synthesized in vitro: (*a*) fluorogram of processed zein produced by zein mRNA in the presence of treated endosperm RER; (*b*) same gel used for *a* stained with Coomassie Blue to show positions of authentic zein markers (the spots in *a* coincided perfectly with the authentic zein positions of *b* except for the spot indicated by the arrow, which is probably some unprocessed zein); (*c*) fluorogram of unprocessed products made by zein mRNA alone; and (*d*) diagram showing relationship of unprocessed zein spots seen in *c*, indicated by circles, to authentic zein that was electrophoresed in the same gel.

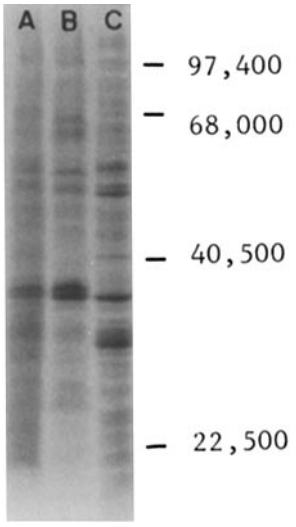

FIGURE 5 Samples of the band *B* fractions prepared from (*A*) shoot, (*B*) nucellus, and (*C*) endosperm electrophoresed on a 7.5–15% PA: 0.1% SDS gel. Molecular weight standards indicated at right. The patterns for the shoot and nucellus membranes are very similar, and both are distinctly different from the endosperm membranes.

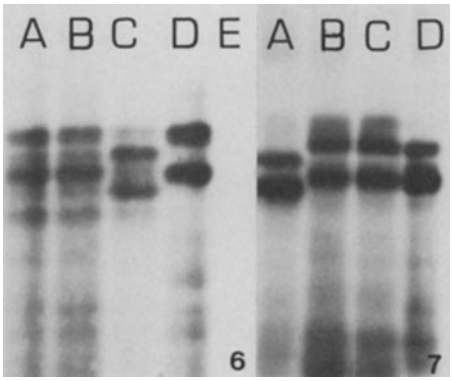

FIGURE 6 Cotranslational processing by RER membranes prepared from different tissues: zein poly(A)+ RNA translated in the presence of nuclease-treated membranes from (*A*) shoot, (*B*) nucellus, and (*C*) endosperm compared with (*D*) synthesis with zein poly(A)+ RNA alone and (*E*) reaction mixture with no additions. Processing is seen only in C.

FIGURE 7 Posttranslational uptake by membranes: zein synthesized in vitro was incubated in the presence of treated membranes. After synthesis, the reactions were digested with proteinase K and the products electrophoresed on SDS PAGE. (*A*) Zein made by co-translational processing is compared with unprocessed zein seen in posttranslational uptake using nuclease-treated membranes from (*B*) shoot, (*C*) nucellus, and (*D*) endosperm. RER from all three tissues can take up some zein posttranslationally, and to about the same extent, but does not process it.

tons (18). In maize we found that amylase was similarly made as a preprotein of 48,000 daltons, which was cotranslationally processed by endosperm RER to a 44,000-dalton protein (data not shown). As with zein, the preamylase was cleaved more efficiently by endosperm RER than by shoot RER. Again, there was also a little uptake of unprocessed chains. This evidence suggests that the RER of the inner endosperm is not substantially different from that of the aleurone but supports the notion that it is unlike RER in other, more distantly-related, tissues. Identification of the maize protein as α-amylase was via precipitation of the in vitro products with rabbit anti-barley α-amylase IgG which had been provided by J. Varner (Washington University).

To find out when processing was occurring, treated membranes were added under conditions allowing posttranslational uptake: zein mRNA was translated in the wheat germ cell-free system and, after the synthesis period, micrococcal nuclease was added to destroy messenger activity. The reaction mixtures were supplemented with nuclease-treated membranes from endosperm, shoot, or nucellus and allowed to incubate for an hour before treatment with proteinase K. As seen in Fig. 7, there was uptake, but no processing, by all three types of RER preparations. The uptake was small and equivalent for all

three. It should be emphasized that the posttranslational uptake of prezein was much less than the cotranslational uptake of zein, and a greater volume of the reaction mixture had to be electrophoresed for the fluorograms. The integrity of the unprocessed zein chains indicates that cleavage of the presequence must normally be a cotranslational event. Therefore the unprocessed, proteinase K-resistant zein polypeptides that are sometimes noted in cotranslational uptake and processing experiments must be entering the membranes by an abnormal route or in an unfavorable configuration which does not allow cleavage. It is not known whether these unprocessed preproteins are transported into the lumen of the RER vesicles or whether they are confined to the membrane layers. Sevarino and Poyton (21) have reported that the precursor to yeast cytochrome *c* oxidase subunit II can be co- or posttranslationally processed in vitro. Although normally being cotranslationally processed, the precursor accumulated in the mitochondrial membranes in the presence of aurintricarboxylic acid. Removal of the drug allowed posttranslational processing to proceed.

The lack of processing in the posttranslational uptake experiments suggested that the inclusion of prezeins in the membranes was an artifact of the isolated RER. To support this idea, and to learn more about the characteristics of the posttranslational uptake, we examined the energy requirements and the uptake of other preproteins by membranes.

In our in vitro synthesis, pyruvate kinase is used as the NTP-generating enzyme. The enzyme is readily inhibited by Ca^{++}, and its effect on protein synthesis is marked, even at low concentrations (Table I). When Ca^{++} was added to the posttranslation reaction mixture along with treated membranes, no difference was noted (Fig. 8), indicating, as anticipated, that the uptake of unprocessed zein polypeptides is not dependent on NTPs and probably represents passive absorption. This has been substantiated by other experiments in which treated membranes were incubated with in vitro synthesized prezein in the presence of alkaline phosphatase. As before, a little uptake of unprocessed polypeptides was observed.

The latitude of posttranslational absorption was tested by using preproteins normally destined for another organelle, specifically the chloroplast. Polypeptides 15 and 16 of the chlorophyll a/b complex and the small subunit of ribulose-1,5-bisphosphate (RuBP) carboxylase are proteins that are encoded by the nuclear genome and are made in the cytoplasm as larger precursors. When they are transported into the chloroplast, there is a posttranslational cleavage of the presequence, yielding the final authentic protein (9, 20). Maize poly(A)+ RNAs enriched for polypeptides 15 and 16 and for the small subunit of RuBP carboxylase were translated in vitro in the wheat germ cell-free system. After 90 min of synthesis, treated endosperm RER was added and incubation continued for 1 h. The fluorograms showed that there was only a very faint band at the position corresponding to the small subunit and no uptake at all for the chlorophyll a/b polypeptides (Fig. 9). Unlike the prezeins, therefore, the absorption of these proteins seems negligible. (The polypeptides analyzed were those resistant to proteinase K digestion. One reviewer suggested that perhaps the chloroplast proteins were not taken up because they were degraded during the posttranslational incubation period by the treated membranes. To discount this, synthesis and posttranslational uptake was repeated omitting the proteinase K treatment. Bands were seen at the expected positions for the chloroplast polypeptides.) The posttranslational uptake of prezein appears to be a rather exceptional artifact and might be related to zein's unique chemical properties: zein is extremely hydrophobic and is soluble only in aqueous organic solvents (e.g. 70% ethanol); it is heavily amidated and lacks significant net charge (19, 24). These characteristics would make zein more compatible than most other typical proteins to the environment of the membranes.

The technique described for SDS–urea polyacrylamide gels is capable of resolving proteins in the range of 30,000 to <1,000 daltons (upper limit not determined), but is especially useful for separating polypeptides below M_r = 10,000 (Figs. 10 and 11). Good linearity between 10,000 and 2,000 daltons is repeatedly observed. The bands after Coomassie Blue staining are, however, much thicker than those seen in the usual SDS polyacrylamide gels. The urea is washed out during staining and destaining and thereby poses no problem for drying the gel. The method differs from that of Swank and Munkres (25) in the relative proportions of the components used and from

TABLE I

Inhibition of Zein Synthesis in the Wheat Germ Cell-free System by Calcium Acetate

Calcium acetate	[^{3}H]leucine
mM	*dpm/μl*
0	30,000
2	3,200
4	1,000
6	235
10	145
20	130

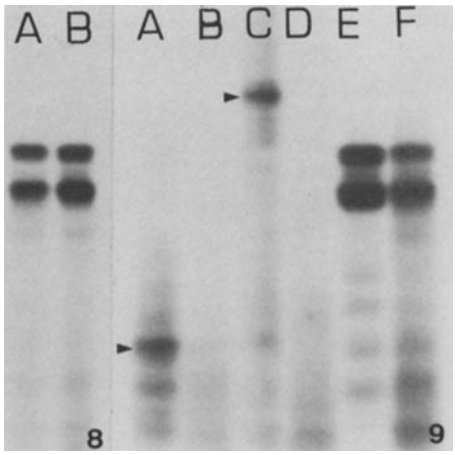

FIGURE 8 Ca^{++} can be used to inhibit pyruvate kinase, the ATP-generating enzyme in the synthesis reaction. Zein mRNA was translated in the wheat germ system and terminated by micrococcal nuclease. Nuclease-treated membranes were added to reactions, both without calcium acetate (*A*) and with it (*B*), and incubated for an additional 60 min before proteinase K treatment. There is no effect of Ca^{++} on the posttranslational uptake of prezein, indicating that this uptake is not dependent on energy.

FIGURE 9 Posttranslational uptake by endosperm RER of the chloroplast preproteins for the small subunit of RuBP carboxylase and for polypeptides 15 and 16 of the chlorophyll a/b complex compared to that of prezein: (*A*) in vitro translation of poly(A) + RNA enriched for the small subunit (arrowhead), (*B*) posttranslational uptake of *A*; (*C*) in vitro translation of poly(A)+ RNA enriched for polypeptides 15 and 16 (arrowhead); (*D*) posttranslational uptake of *C*; (*E*) in vitro translation of zein mRNA; and (*F*) posttranslational uptake of prezein. Only prezein is taken up to any significant extent.

that published more recently by Bethesda Research Laboratories (2) in the choice of buffer, the acrylamide: bisacrylamide ratio, and the concentration of urea.

If the membrane presequence peptidase were an endoprotease, then cotranslational cleavage of the zein preproteins should produce the mature zein proteins and two polypeptide classes of 1,000 and 2,000 daltons. When either the supernate or the 100,000 *g* membrane pellet was electrophoresed on the low molecular weight SDS-urea polyacrylamide gels, nothing could be detected in these size classes (Fig. 12). This was true whether the label used was [^{3}H]leucine or [^{35}S]methionine. Purified protein bodies likewise show no polypeptides in this region. It would seem probable, therefore, that the cleavage enzyme is an exopeptidase. The possibility of an endopeptidase coupled with a highly active exopeptidase, however, cannot be excluded.

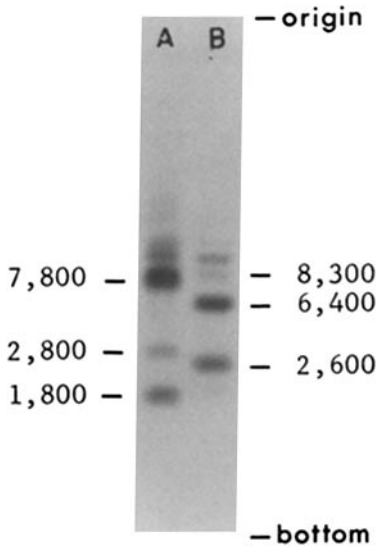

FIGURE 10 Cyanogen bromide cleavage fragments of (*A*) cytochrome *c* and (*B*) myoglobin electrophoresed on a 12.5% PA:0.1% SDS:8 M urea gel stained with Coomassie Blue. Molecular weights indicated to the sides.

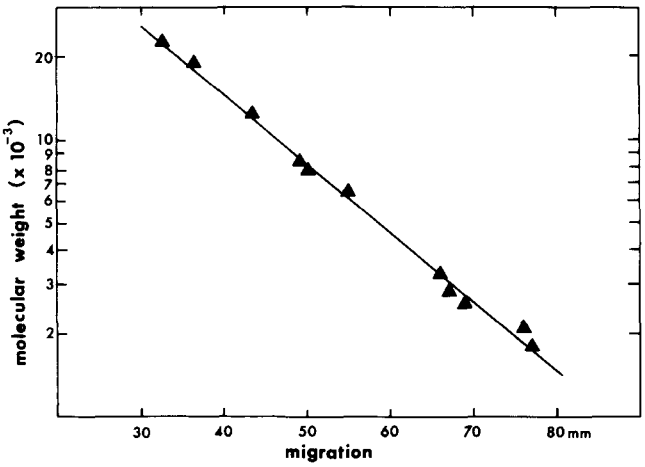

FIGURE 11 Plot of the migration of standards electrophoresed on a low molecular weight resolving 12.5% PA:0.1% SDS:8 M urea gel. Molecular weights indicated in parentheses: insulin A (2,300), insulin B (3,460); cyanogen bromide cleavage fragments of cytochrome *c* (7,760, 2,780, 1,810); uncleaved cytochrome *c* (12,300); cyanogen bromide cleavage fragments of myoglobin (8,270, 6,420, 2,550); zein (22,500, 19,000).

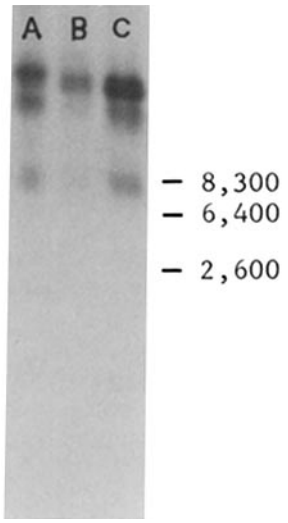

FIGURE 12 Fluorogram of in vitro translation products electrophoresed on a 12.5% PA:0.1% SDS:8 M urea gel for the possible zein presequences: (*A*) zein mRNA only (unprocessed); (*B*) supernate of zein mRNA translated in the presence of treated membranes after 100,000 *g* centrifugation; and (*C*) pellet of *A*.

The in vitro method we have developed will allow further study of the uptake and processing of prezein under conditions which simulate the native system. Undoubtedly, the technique can also be adapted for developing homologous systems in other plants to examine processing of other proteins.

The large array of zeins and the fact that they are synthesized as preproteins has meant that cDNA clones of zein messages (6, 26) could not be assigned to specific zein proteins. But, by using endosperm poly(A)+ RNA which hybridized to the cloned sequences in the translation-processing system, the precise identification of each clone should be possible.

ADDENDUM

Since this paper was written, leucine has been shown to be present in the protein presequence by sequence analysis of a zein cDNA clone. I. R. Rubenstein, University of Minnesota, personal communication.

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