STUDY OF ZEIN PROTEIN BODY FORMATION IN A HETEROLOGOUS SYSTEM (XENOPUS LAEVIS OOCYTE)

by

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(ABSTRACT)

Most seed storage proteins accumulate in protein bodies which are derived from the vacuole. Zeins, the major corn storage proteins, however, are retained in the endoplasmic reticulum (ER) and their protein bodies are derived from the ER. There are circumstantial and preliminary data indicating that 27K zein, the proline-rich zein, may span the ER membrane. This potential transmembrane feature is considered very significant to understand the mechanism for zeins' ER retention. The transmembrane feature may retain the 27K zein in the ER where it could serve as an anchor for other classes of zein through specific protein interactions. In this study, a heterologous system (Xenopus laevis oocytes) was used to investigate the potential transmembrane domain of 27K zein. This study utilized physical assays of proteolytic digestion (protease K) and chemical modification (biotinylation) on isolated protein vesicles from Xenopus oocytes injected with in vitro transcribed 27K zein mRNA. In addition, the transmembrane features were analyzed by monitoring the protein's mobility in the lumen of the ER by pulse-chase experiments. The results showed that the possibility of 27K zein as a transmembrane protein was consistently refuted in this study. The 27K zein protein was not affected by the protease K treatment or biotinylation. Moreover, 27K zein and total zeins moved freely in the lumen of the ER similar to a secretory protein (ovalbumin),
totally different from an ER membrane protein (a mutant transmembrane hemagglutinin envelope protein). The free movement, within the ER lumen, of total zeins under conditions where zein aggregates should form necessitates a reevaluation of the mechanisms responsible for zein polypeptides' ER retention and protein body formation. This study, therefore, concludes that 27K zein is not a protein body nucleating factor by virtue of an ER transmembrane feature or association with the ER membrane and that the significance of zein solubility should be reconsidered to explain the zeins' ER retention leading to protein body formation in the ER.
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INTRODUCTION

In plants, the need for organic molecules is autotrophically satisfied as long as photosynthetic activity is sustained. Inorganic nutrients, on the other hand, must be obtained from the external environments through absorbing organs: root hair and foliage (Salisbury and Ross, 1986). During germination, however, the embryo cannot be sufficiently nourished with the external source of inorganic nutrients due to the immaturity of the root and leaf systems. In developing seeds, large amounts of starch and oil are produced and stored as carbon sources while proteins are stored as sources of nitrogen, sulfur, and amino acids for young seedlings. These proteins are termed seed storage proteins which account for 10 to 25% of the dry weight of seeds while vegetative tissue consists of less than 5% protein.

Maize is one of the major cereal crops in the world. It is easily grown in many types of soil and produces high yields. Since its introduction from the New World, maize has become a major part of the human diet either directly or indirectly through animal feed. However, the nutritional quality of maize is considered poor mainly because of an imbalance with respect to the amino acid content. Corn is virtually devoid of two amino acids: lysine and tryptophan, essential for human and monogastric animal nutrition (Nelson, 1969). This deficiency results from the lack of these two amino acids in zeins, the major storage proteins, which constitute at least 50% of the total protein in maize endosperm. Consequently, zein has been the major target of many conventional breeding programs to improve the nutritional quality of maize seed.

With the emergence of genetic engineering, maize has been a major subject of renewed attention. Recent advance in genetic engineering allows many plant species, including maize, to be transformed with foreign genetic material. Many studies show that
maize cells are competent for genetic engineering and zein genes are successfully expressed in various heterologous systems. However, those studies report that gene products from the foreign genes result in undesirable forms: minimal (non-detectable) accumulation (Wallace et al., 1990), degradation during protein targeting and deposition in the vacuole (Hoffman et al., 1987, 1988; Ohtani et al., 1991), or unusable forms of the product. These reports emphasize that, for plant genetic engineering to be successful, the biological mechanism leading to protein targeting and accumulation must be further understood.

The protein body in the maize seed is believed to assemble in a unique fashion in comparison to other plants including the majority of cereal crops. In the developing seeds of barley, oat, and wheat, the storage proteins are accumulated in the central vacuole. Zeins, on the other hand, are retained in the lumen of the endoplasmic reticulum (ER) where they accumulate into protein bodies (Khoz and Wolf, 1970; Larkins and Hurkman, 1978). The mechanism accounting for zein protein body formation is not well characterized. Kelly (1985) suggests that transit sequences within zeins may cause zeins to form an aggregate which is excluded from the intracellular transport. Various heterologous studies also suggest that interaction among zeins may play a role in the zeins' ER retention and protein body formation. When *Xenopus* oocytes are injected with zein mRNAs, zein protein bodies are assembled with the identical density and physical characteristics as native ones (Hurkman et al., 1981). The significance of the zein-zein interaction was also supported by results from transgenic plant studies. In a tobacco plant, transformed with 15K zein gene, the 15K zein was targeted into the central vacuole rather than deposited in ER (Hoffman et al, 1987). This observation implies that zeins can be included in the default transport in the absence of the zein-zein interaction.
Recent studies suggest that one class of zein (27,000 M_r zein or 27K zein) may play a structural role in protein body formation. Ludevid et al. (1984) observed a significant morphological change with protein bodies when 27K zein was extracted from protein bodies by a reducing agent. An ultrastructure that 27K zein may form was even visualized by immunogold electron microscopy (Ludevid et al., 1984; Lending et al., 1988; Lending and Larkins, 1989). According to those observations, the majority of 27K zein is localized near the periphery of protein bodies while portions of 27K zein were shown to project toward the interior of the protein bodies (Lending and Larkins, 1989). The timing of 27K zein synthesis is consistent with its proposed role as an internal scaffold of the protein bodies; the protein is synthesized along with its putative cooperating proteins (β-zein and 16K γ-zein) before the major class of zein (α-zeins) is made and embedded into the matrix of β− and γ−zein proteins (Larkins et al., 1990).

In a developing maize endosperm, protein bodies vary greatly in density and size during protein body formation. Lending et al. (1988) suggest that this variation may correspond with varied amounts of 27K zein which may play a regulatory role in determining density and size of protein bodies (Lending and Larkins, 1989; Lopes and Larkins, 1991). This suggestion was in agreement with observations made from Xenopus laevis oocytes. When mRNAs for β and γ-zein were coinjected in addition to α-zein into oocytes, the density of subsequent protein bodies was shown to increase (Wallace et al., 1990). The potential regulatory role of 27K zein is further supported by the substantial phenotypic change of quality protein maize (QPM, see literature review section 4). In this mutant, the chalky, soft kernel of opaque-2 maize is converted into that of normal (glassy) maize. This distinction is believed to result from the genetic factor (modifier) which increases the content of 27K zein. Analysis of reciprocal F1 hybrids also reveals that the modifier genes are also functional by increasing 27K zein synthesis in normal and flouncy-2
backgrounds, suggesting that the effect of the modifier on 27K zein gene expression occurs independently of the opaque-2 mutation (Lopes and Larkins, 1991).

It is highly conceivable that the additional content of 27K zein converts the opaque phenotype by cross linking zein classes tighter within the protein bodies in QPM endosperm. However, recent investigations have indicated that protein bodies in the QPM do not undergo any morphological change even with the increased presence of 27K zein (Geetha et al., 1991). These observations imply that the vitreous phenotype may be an increased interaction between 27K zeins at the boundary of protein bodies (Geetha et al., 1991). As a result, protein bodies in the QPM endosperm may be densely packed in comparison to its counterpart.

27K zein shows unusual solubility. 27K zein requires a reducing agent (e.g., 2-mercaptaethanol, dithiothreitol) to be extracted by 70% ethanol from zein protein bodies whereas β-zein is readily extracted from the protein bodies in the absence of reducing agents even though it has more disulfide linkages than any other zeins. This unusual requirement of 27K zein is suggestive of its physical association with the membrane of the protein bodies. EM studies (Ludevid et al., 1984; Lending et al., 1988) using immunogold labeling show that 27K zeins are primarily localized to the periphery of the protein bodies although a fraction is still detectable in the interior. The proximity of the 27K zein to the membrane is also strongly supported by a fast disappearance of 27K zein, to the exclusion of other zeins, during germination (Torrent et al., 1989; Mohammad and Esen, 1990). 27K zein's primary structure reveals that 27K zein may even span the membrane of the protein body. The protein has a stretch of 37 hydrophobic amino acids between arginine (a basic amino acid) and glutamic acid (an acidic amino acid). Johnson (1989) showed that 27K zein was the only fraction, in isolated intact protein bodies, affected by proteolytic digestion and chemical modification. However, only a minuscule fraction of the 27K zein
was affected. Together, these investigations strongly suggest that 27K zein may be a transmembrane protein. The potential transmembrane feature of 27K zein could play a significant role in zein proteins' ER retention and, consequently, in the protein body formation since 27K zein can serve as an anchor for the other classes of zeins since 27K zein has been shown to form specific zein-zein interactions with all other classes of zein polypeptides (Johnson, 1989).

*Xenopus laevis* oocytes produce and store proteins mainly for the future use at embryonic stages (Lane, 1983 and 1989; Dumont 1978; Mohun et al. 1981), analogous to seeds which produce and store proteins required for germination. *Xenopus* oocytes show an efficiency and fidelity in translation, processing, and subcellular localization of zein. When *Xenopus laevis* oocytes are injected with native zein mRNAs, the oocytes system has been found to translate the message correctly, to process zein proteins (Larkins et al., 1979), and to package the zein into vesicles with the density identical to native zein protein bodies (Hurkman et al., 1981). These aspects facilitate studies regarding structure, biochemistry, physiology, and biological properties of zein proteins.

This study used the *Xenopus laevis* oocyte system to investigate whether 27K zein is a transmembrane protein which may explain zeins' ER retention and protein body formation in the ER. 27K zein mRNAs were prepared *in vitro* from a cDNA construct of 27K zein using SP6 RNA polymerase. Following injection of the *in vitro* transcribed 27K zein mRNA into the oocytes, 27K zein's potential transmembrane features were investigated focusing on the following specific questions:

1. Is 27K zein affected by proteolytic digestion of isolated intact protein bodies from oocytes?
2. Is 27K zein biotinylated in the isolated intact protein bodies?
3. Is 27K zein free to move in the lumen of the ER when movement of the protein is analyzed by pulse-chase experiments?

In addition, the role of zeins' insolubility in ER retention and protein body formation was tested by measurements of total zeins' ability to move inside the lumen of the ER under conditions where aggregation is expected to occur.
LITERATURE REVIEW

1. Zeins: Major Storage Protein of Maize

Proteins of maize endosperm can be classified into four groups under the solubility scheme proposed by Osborne (1908 and 1924) and modified by Landry and Moureaux (1970): albumin (4%), globulin (2%), prolamin (55%), and glutelin (39%). Albumins represent proteins soluble in water, and globulins in saline solutions. Most of prolamins are soluble in alcohol while some require disulfide-bond reducing agents. Glutelin is soluble in dilute alkali and detergent solution. Zeins belong to the group of prolamins, extracted in 70% ethanol (or 55% isopropanol), and serve as the functional storage protein in maize.

Zeins are different from many plant storage proteins in terms of amino acid composition. Zeins contain high content of hydrophobic amino acids such that zeins are soluble only in aqueous solutions containing polar organic solvents. Amino acid analyses reveal that zeins are notably rich in alanine, glutamine, leucine, phenylalanine, and proline; however, zeins are virtually devoid of two essential amino acids, lysine and tryptophan, like other cereal prolamins (Gianazza, 1977; Paulis et al., 1969; Landry and Moureaux, 1970). This imbalance limits the quality of maize proteins for human and animal diet in many regions of the world.

The poor nutritional quality of maize might be partially improved by its mutants. Some maize mutants (Opaque-2 and 7) exhibit relatively higher content of the two essential amino acids compared to the wild type. The main reason for the better composition is due to the reduced total amount of zeins in the mutant endosperms. With less lysine (tryptophane)-poor zein, the relative amounts of the lysine (tryptophane)-richer, non-zein proteins increase. These mutants, however, are not widely accepted by many
farmers because of their overall lower yields and susceptibility to insect and fungus due to their soft, floury kernel which dry out more slowly than normal kernels.

2. Zein Components.

Zein proteins are classified into six groups of polypeptides of 27, 22, 19, 16, 15, and 10,000 according to the apparent molecular weight on SDS-polyacrylamide gel. On the basis of their apparent sizes, they are subsequently called as 27K, 22K, 19K, 16K, 15K, and 10K zeins, respectively. When zeins are separated by isoelectric focusing (IEF) between pH 5 and 9, approximately 28 components can be detected (Vitale et al., 1980; Burr and Burr, 1981). Charge heterogeneity is evident among zein polypeptides of 22 and 19K on IEF gels. The charge heterogeneity apparently originates from the differences in amino acid sequence which implies the expression of similar but not identical structural zein genes (Gianazza et al., 1977; Viotti et al., 1978). Proteins in other zein classes, however, exhibit little charge heterogeneity and probably represent a single or two polypeptides.

Zeins are also categorized on the basis of apparent differential solubility in aqueous ethanol (with or without reducing agents) into four structurally different classes: α-, β-, γ-, and δ-zeins (Esen, 1987 and 1990; Thomson and Larkins, 1989). This solubility classification corresponds to the information on the primary structures of zeins (e.g., Pedersen et al., 1982; Marks et al., 1985; Pedersen et al., 1986). Under this solubility classification system (later modified according to the sequence information), α-zeins corresponds to polypeptides of 22K and 19K zein and β-zein to 15K zein. Gamma-zein is made up of 27K and 16K zeins; alternatively, 27K zein is named as γ-zein$_1$ and 16K zein as γ-zein$_2$ (Esen, 1990). The 10K zein has been assigned into a fourth category, δ-zein.
a. α-zein

The alpha zeins are polypeptides with apparent molecular weights of 19,000 and 22,000. Alpha-zeins are extracted in 50-90% alcohol without reducing agents and account for at least 60% of the total zein fraction depending on maize variety. Zeins of the 19,000 size class exhibit high level of sequence identity (80 to 85%), whereas zeins of the 22,000 size class only show 65% identity. Mature proteins are composed of 210 to 245 amino acid residues.

Despite the difference in size, these two components of α-zein share structural similarity. According to amino acid sequence analysis and circular dichroism spectral data, both classes of α-zeins have been proposed to consist of α-helices (50-60 %), β-structure (1-5%), and random coil (40-50%) (Pedersen et al., 1982; Argos et al., 1982). A structural model also suggests that the α-helices are held together in an anti-parallel fashion and joined at their ends by a short glutamine rich turn region (Argos et al., 1982). In the endosperm cell, α–zeins constitute core regions surrounded by a shell of β– and γ– zeins within the protein bodies (Lending et al., 1988; Lending and Larkins, 1989). Lending and Larkins (1989) postulate that the α–zeins penetrate the protein mass of β– and γ–zein to reach the core areas at the later stage of protein body development.

b. β-zein

Beta zein consists of the 15K zein which accounts for 5% of the total zeins. The β-zein has 160 amino acid residues in its mature protein and relatively high content of methionine (10.8% in terms of mole %). This content of methionine is exceptionally high (at least 10 times) compared to other classes of zeins. Circular dichroism spectral data indicate that this protein has 44% β–structure, 39% random coils, and 17% α-helices
(Pedersen et al., 1986). In protein bodies, β-zeins are located at the periphery along with γ-zein (Lending et al., 1988; Lending and Larkins, 1989).

c. γ-zein

Gamma-zein accounts for approximately 20% of total zein: 27K (5-10%) and 16K (10-15%). Zeins requiring a reducing agent for extraction was first discovered from a heterogeneous group of polypeptides extracted in alcohol containing reducing agent by Moureaux and Landry (1968) and, independently, by Paulis and his colleagues (1968). Since the original definition of zein was limited to the portion of protein extractable from corn meal with 60-70% alcohol, much controversy and confusion was directed over whether this group of polypeptide should be classified as zein. Subsequently, this group of proteins, especially 27K γ-zein, was referred to by a number of ambiguous names: alcohol-soluble reduced glutelin (ASG) or reduced soluble protein (RSP) along with glutelin-1, zein-2, and zein-like. Amino acid sequence analyses, however, revealed that the ASG essentially consists of zein polypeptides according to the conventional classifications (Gianazza et al., 1976).

The amino acid sequences of γ-zeins differ from other zeins. The 27K γ-zein contains a hexapeptide repeat, P-P-P-V-H-L, eight times (Esen et al., 1982; Wang and Esen, 1986; Prat et al., 1985) while 16K γ-zein carries the sequence 3.5 times (Prat et al., 1987). These hexapeptide motifs have also been found in major storage proteins of other cereals: gliadin (of wheat; Scheets et al., 1985; Sugiyama et al., 1986), hordein (of barley; Shrewry et al., 1985a), γ-Kafirin (of sorghum; DeBarros et al., 1991), and γ-Coixin (of Coix; Leite et al., 1991). In addition, the 27K zein contains a nearly perfect stretch of 26 amino acids with the repeating sequence Pro-X.
Separation of 27K zein from the ASG was first reported by Paulis and Wall (1977). Based on the solubility in water, they fractionated the ASG into two groups, water-soluble and water-insoluble, and analyzed the fractions in terms of electrophoretic pattern and amino acid composition. The water-insoluble portion showed similar pattern and composition to those of zein; on the other hand, the water-soluble portion exhibited little similarity. A systematic separation study was made using cation exchange chromatography by Esen et al. (1982). When the ASG was eluted with buffer containing alcohol and 2-mercaptoethanol, polypeptides of 19-20,000 (later, determined as \( \alpha \)-zein) were eluted first and followed by those of 14-16,000 (then determined as \( \beta \)-zein) while late eluates exclusively contained a polypeptide of 27,000 which is very rich in proline. This proline-rich protein was concluded to be the same protein as the water-soluble ASG (Paulis and Wall, 1977) and the reduced soluble protein (RSP) by Wilson (1981).

27K zein is visualized as a diffuse band with an apparent molecular weight of 27,000 on SDS-polyacrylamide gel. This apparent molecular weight deviates from the true size of 27K zein. DNA sequence analysis reveals that 27K zein is made up of 204 amino acids whose calculated molecular mass is approximately 22,000. This discrepancy is believed largely due to its unusual abundance in basic amino acids (e.g., histidine and arginine) overall, and proline at the N-terminus. As a secondary structure, \( \gamma \)-zein is proposed to have an extended rod conformation (Thompson and Larkins, 1989). Within the protein bodies, \( \gamma \)-zeins are located near the boundary and surround the core region of \( \alpha \)-zein (Ludevid et al., 1984; Lending et al., 1988; Lending and Larkins, 1989).

d. \( \delta \)-zein

Delta zein accounts for 5% or less of the total zein in maize and has the apparent molecular weight of 10,000 if determined by SDS-PAGE. The protein is solubilized in
70% ethanol without reducing agent similar to α-zein. Deduced from the cDNA sequence, δ-zein is thought to have 129 amino acid residues with the calculated molecular mass of 14.4 Kd (Kirihara et al., 1988). Like the β-type, the δ-zein is very rich in methionine and cysteine; however, no information is available on its secondary or tertiary structure to date. In addition, a recent EM study (Stetler and Esen, 1992) using immunogold labeling reveals that δ-zein is evenly dispersed within the protein body.

3. Characterization of Zein Genes

Zein genes exhibit unique characteristics based on sequence analysis of zein clones, both cDNA and genomic DNA. Intervening sequences have not been found in any of the genomic clones (Pedersen et al., 1982; Messing et al., 1983). Many of the genes cloned are pseudogenes; however, approximately half of the 50-75 gene copies estimated using cDNA probes can be actually expressed and identified by IEF (Pedersen et al., 1980; Hagen and Rubenstein, 1981; Wilson and Larkins, 1982; Marks et al., 1985). Very different from α-zeins, other classes of zeins are encoded by a single gene or two which correspond to the number of proteins detected by IEF analysis. Any extensive homology has not been found among the functional zein genes except for 19K and 22K zeins, members of α-zein genes, which show 60 to 65% homology when zein cDNAs have been used in cross-hybridization studies to group closely related zein sequences (Marks et al., 1985b).

Genes encoding zein polypeptides have been located on the chromosomes by genetic linkage group analysis, and in situ hybridization. The genes encoding α-zeins are located on chromosomes 4, 7, and 10. Especially on the short arms of chromosomes 4 and 7, the α-zein genes are present in clusters of 7 and 8, respectively (Thompson and Larkins, 1989). Beta-zein has been located on chromosome 6 and γ-zein on chromosome 7 by
RFLP mapping (Murray et al., 1988). The short arm of the chromosome 7 is also shown to harbor a single gene encoding δ-zein (Thompson and Larkins, 1989).

Despite the variation in structural genes, zein genes share several common features in their flanking regions. The putative TATA box (TATAA) and CAAT consensus sequence, typical of eukaryotic promoters, are found within the 180 nucleotides (-180) from the 5' transcription initiation site (Pedersen, 1982; Boronat et al., 1986; Pedersen et al., 1986). A highly conserved sequence (TGTAAG) is present at approximate positions between -310 and -340 except 15K zein whose equivalent sequence resides at -200. This sequence is found upstream of cereal storage proteins of wheat and barley (e.g., B-hordein, α-gliadin, LMW-glutelin, and HMW-glutelin) and known as '330 element' that is similar to the 'core' element of the SV 40 enhancer (GTTGAAAG) and has been termed as a prolamin box. This presence of a highly conserved sequence common to such a divergent range of species suggests it plays a regulatory role. An opaque-2 binding box or a rudimentary α2 binding box is also present in all known zein genes. The α2 box is located around -291 (Schmidt et al., 1992; Ueda et al., 1992) and -146 for β-zein (K. Pedersen, personal communication). The proximity of the opaque-2 binding site to the prolamin box might indicate that the proteins, bound to the two sites, cooperate to enhance the level of zein expression (Schmidt et al., 1992).

Zein genomic clones contain consensus polyadenylation sequences at the 3' end of zein genes, typical of those reported for other eukaryotic genes (Pedersen et al., 1982, 1986; Messing et al., 1983). Although some of the cDNA clones analyzed have polyadenylation signals identical to those of mammalian genes (AATAAA), three variants (AATAAT, AATAAG, AATGAA) have been reported. S1 nuclease mapping of the 3' flanking regions indicates that all of these sequences are utilized. While there appears to be
a preference for specific sequences among gene classes or subfamilies, their polyadenylation signals are too diverse to draw conclusions concerning preferential usage.

4. Mutations Affecting Zein Synthesis and Accumulation

Synthesis and accumulation of zein, as well as other plant storage protein, are highly coordinated during seed development. This complex type of coordination implies the involvement of various regulatory mechanisms, corresponding to the state of seed development, in the transcription of the structural genes, in the translation efficiency, and in the rate of zein accumulation. The expression of a zein gene is governed by various loci regulating the initiation and rate of zein synthesis and accumulation; a variety of mutant alleles for these loci have been identified.

Mutations have been reported which affect the initiation of zein synthesis (Manzocchi et al., 1980). Such mutants influence the integrity of endosperm and overall content of zein (Motto et al., 1989). They result in defective endosperms and reduced amount of total zein in maize seeds. Most of the mutants reported of this category preferentially delay the onset of 22K zein synthesis. All of the defective endosperm (de)-mutants, for example, delay the zein synthesis by at least 10 days compared to wild type. On the other hand, Mucronate (Mc) apparently affects all classes of zein to the same extent.

A number of mutations have been identified which influence the rate of zein accumulation in the endosperm of developing maize seeds. Mutations in this category include two groups. One group of mutations (e.g., o2 and o7) affect preferentially certain fractions of zein: 19K and 22K zein, respectively; whereas, opaque-6 (o6) and floury-2 (fl2), for example, alter protein accumulation of all α-zeins.
Opaque-2 is a recessive mutant which produces a soft, blunt and opaque kernel in comparison to the wild type which exhibits a hard, horny, and translucent kernel. This mutant is referred to as a high-lysine corn because of its relatively higher percentage of lysine than their normal counterparts. Protein content in o2 plants, however, is reduced between 5 and 26% relative to wild type, depending on genetic backgrounds (Popovic et al., 1974). The cause of relative high content of lysine in o2 seed is the result of a substantial reduction of zein which is devoid of lysine (Tsai and Dalby, 1974). In o2 endosperm, the synthesis of zein is delayed, reduced, and terminated at 30 DAP, approximately 15 days earlier than normal corn (Dalby and Tsai, 1974; Jones et al., 1977). Genetic crosses reveal that the o2 allele interacts additively with o7, o6, de-b30, and Mc while epistatically with fl2.

When zein was synthesized from isolated membrane-bound polysomes of o2 mutants, the 22,000 size class was virtually absent (Jones et al., 1977b) while other classes of zeins are present corresponding to that of normal counterparts. In a related study, Jones and his colleagues (1977a) concluded that the reduced synthesis of zein in the o2 mutant is the result of significant (50%) reduction of membrane-bound polyribosome in comparison to the normal counterpart. Unusual high levels of ribonuclease activity observed in o2 (two or more times that of normal) indicates that zein mRNA may have a fast turnover rate in the o2 mutant due to the increased level of ribonuclease (Jones et al., 1977b; Tsai et al., 1978). Considering the high variability of ribonuclease levels among different inbred lines and nearly exclusive influence on 22K zeins, the effect of ribonuclease on the phenotypes is not considered to be directly related to reduced zein synthesis. Rather, Pedersen et al. (1980) demonstrated that the 22K zein is negatively regulated at the transcriptional level in o2 endosperm where the level of corresponding mRNA is exclusively reduced 100 fold compared to normal maize.
The O2 locus is now known to reside on the short arm of chromosome 7 and to encode a trans-acting transcriptional factor which activates the synthesis of 22K zeins whose loci are located on chromosome 4 (Schmidt et al., 1987; Motto et al., 1989). Difficulty in the identification of the gene and protein misled some investigators to conclude that O2 protein may act through a soluble cytoplasmic factor. The b-32 protein was a candidate for the postulated mediating factor in the transcriptional activation by O2 (Soave et al., 1981; Motto et al., 1988). However, sequence information from cDNA and genomic DNA reveals that the O2 protein has a leucine zipper motif which is common in animal proto-oncogene binding factors (fos, jun, and myc) and the yeast transcriptional activator (GCN 4) (Harting et al., 1989). In addition, the same study also showed that the O2 protein has a motif similar to a metal binding protein.

The mutation opaque-7 affects primarily the 19,000 size class inhibiting the zein synthesis by 80% (DiFonzo et al., 1979). The genetic factor involving in O7 phenotype is located on the long arm of chromosome 10 (Motto et al., 1985). This mutation also reduces starch content in the endosperm and the O7 locus is believed to encode a genetic factor involved in various regulatory pathways. In terms of non-protein nitrogen content, however, o7 endosperm retains at least three times more than the normal counterpart. This high level of non-protein nitrogen is also seen in sh4 (a starch modifying mutant; see below).

Mutations affecting zein synthesis of all classes to the same extent have been described. Opaque-6 affects zein accumulation up to 88.5%, which is the most severe inhibition known to date. Floury-2 reduces (approximately 35%) both the 19,000 and 22,000 size classes (Jones, 1978). Protein bodies in fl2 endosperm appear irregular and the ultrastructures of the zein protein bodies are highly unorganized (Lending et al., 1988). Concomitant with the inhibition in zein synthesis and deposition, b-70 (a soluble protein
with molecular weight of 70,000) is overproduced and found in abundance within the
floury-2 protein bodies. The basis for the overproduction of the b-70 protein and
deformation of the protein bodies is not known; however, the overexpression of b-70 is
suggested as a cellular response to the activity of the factor encoded by the O7 locus
(Boston et al., 1991).

Overexpression of b-70 is also recognized in other zein regulatory mutant: de-B30
and Mc (Boston et al., 1991). Through biochemical characterization of b-70, Fontes et al.
(1991) suggest that the protein resemble the heat-shock protein 70 (hsp-70) family and
may be a plant homologue of the mammalian immunoglobulin heavy chain binding protein
(BiP). BiP has been implicated as a molecular chaperon that mediates proper protein
folding and sorting of proteins by binding to the nascent proteins in the ER compartment.
As with BiP, b-70 is shown to have post-translational modifications and ER retention
signal near the C-terminal. B-70 accumulates in the ER and protein bodies corresponding
to its mRNA level and the severity of the mutation affecting zein synthesis and deposition
(Boston et al., 1991). This observation led the investigators to suggest that b-70 may play
a role as a molecular chaperon in facilitating zein retention in the ER and its accumulation
in the ER-derived protein bodies following overexpression induced by abnormal proteins
in the mutants.

Starch mutants also negatively affect zein synthesis. Such mutants include two
groups: starch-deficient mutants and starch-modifying mutants. The former refers to those
which reduce starch biosynthesis: shrunken-1 (sh1), shrunken-2 (sh2), shrunken-4 (sh4),
brittle-1 (bt1), brittle-2 (bt2) and sugary-1 (su1). On the basis of weight at maturity,
homozygous sh4 mutants accumulate zeins approximately 50% of wild type levels,
comparable to that found in homozygous o2, o7, or fl2, mutants (Tsai and Dalby, 1974).
The percentage may be inaccurate since the figure fails to consider the smaller mass
resulting from reduced amount of starch in \textit{sh4} endosperms. Starch-modifying mutants affect the normal ratio of amyllose to amylopectin such as \textit{amylose-extender (ae)}, \textit{dull (du)}, \textit{sugary-2 (su2)} and \textit{waxy (wx)}.

Interrelationships among loci involving zein synthesis have been reported. The loci for \textit{o2} and \textit{o7} interact in an additive fashion. In the case of double mutation, protein synthesis of the two \textit{\alpha}-zein classes (19K and 22K zein) are inhibited additively (Motto et al., 1988). This additive inhibition implies that the two loci are involved in zein gene regulation independent of each other. The \textit{fl2} locus also interacts with \textit{o2} and \textit{o7}, in an epistatic fashion for a further decrease in zein accumulation. This effect appears to be cumulative (Tsai et al., 1978).

Starch mutants also accompany a significant reduction in zein accumulation when associated with \textit{o2}, \textit{o7}, or \textit{fl2} genotypes. Combined with the \textit{o2} mutation, most starch-deficient mutants drastically reduce zein accumulation, especially \textit{\alpha}-zein, in a synergistic fashion. When some starch mutations (\textit{sh2} and \textit{sh4}) interact with \textit{fl2}, however, the level of zein synthesis appear to be less affected. If zein level is determined based on a per kernel or endosperm basis, the level of zeins in this mutant is only 10\% of that found in normal endosperms, suggesting that \textit{sh4} influences zein synthesis to a different degree than does \textit{o2}, \textit{o7} or \textit{fl2} (Tsai and Dalby, 1974). It is not known whether this effect is related to observed abnormalities in phosphorylases (Tsai and Nelson, 1969), a reduced level of pyridoxal phosphate (Burr and Nelson, 1973), or a pleitropic gene effect of the \textit{sh4} locus on starch and zein synthesis in this mutant (Tsai and Dalby, 1974). Of the mutant alleles, \textit{o2} and \textit{o7} are completely recessive (Tsai and Dalby, 1974).

All the mutations described have been those affecting \textit{\alpha}-zeins. There is only one known case of mutations exclusive to non-\textit{\alpha}-zeins. The floury phenotype of \textit{opaque-2} is modified in certain genetic backgrounds to where some regions of the endosperm are
more glassy than others. The genes involving in this modification are called opaque-2 modifier and the carrier of the factors is named as quality protein maize (QPM). Biochemical analysis reveals that amino acid composition of QPM is very similar to that of o2 (Paez et. al., 1969; Ortega and Bates, 1983). Although it is not understood how the opaque-2 modifier conditions the floury phenotype, recent studies indicate that the acting mechanism is achieved through an overexpression of a certain zein fraction: γ-zein, the proline-rich protein, located at the boundary of the protein bodies (Lopes and Larkins, 1991; Wallace et al., 1990; Paiva et al., 1991). In addition, analysis of reciprocal F1 hybrids also reveals that the modifier genes are also effective in a semidominant manner by increasing 27K zein synthesis in normal and floury-2 backgrounds, suggesting that the modifier affects the expression of 27K zein independent of the opaque-2 mutation (Lopes and Larkins, 1991; Geetha et al., 1991).

5. Accumulation of Storage Proteins into Protein Bodies in Maize and Other Plant Species.

a. Maize

Zeins are synthesized between 10 and 45 DAP (days after pollination). Under the light microscope, protein bodies are first observed as early as 12 DAP. At this stage, protein bodies are few in number and less than 0.5 micrometer in diameter. By 16 DAP, the number and size of protein bodies have increased rapidly with a diameter of approximately 0.75 micrometer. At 25 DAP protein bodies are more numerous with sizes ranging up to 1.25 micrometer while most are in the range of 0.75 micrometer in diameter. Protein bodies of 45 DAP seem to have reached the maturation stage where staining pattern is much different from earlier stage; protein bodies are 1 micrometer or more in
diameter and often contain a narrow, irregular, darkly staining band which has projections into the interior.

The origin of protein bodies in maize seed was controversial; that is, whether the protein bodies are derived from vacuoles or plastids. However, studies using electron microscopy (EM) and biochemical techniques (Larkins and Hurkman, 1978) reveal that the maize protein bodies originate from endoplasmic reticulum (ER). Khoo and Wolf (1970) could detect ribosomes on the surface of the protein bodies in electron micrographs. This observation was supported by a number of studies (Larkins et al., 1976; Burr and Burr, 1976; Larkins and Hurkman, 1978). They were also successful in isolating polyribosomes from a protein body containing fraction using differential centrifugation, linear sucrose gradients, and discontinuous sucrose gradients, respectively. Larkins and Hurkman (1978) also demonstrated that NADH-cytochrome C reductase, a marker enzyme for ER, exist on the surface of protein bodies. In addition, when polysomes were subjected to in vitro translation, the translation products were found to correspond to those from maize endosperm although the in vitro translation products were consistently 2,000 daltons larger than native zeins.

The various zeins are localized distinctively within the protein bodies. The heterogeneous localization is evident when the protein bodies are stained with lead citrate and uranyl acetate (Lending et al., 1988). Usually the periphery is stained darker than core region although many of the protein bodies are evenly stained. Differences in the compositions of the protein bodies are more obvious when the ultrastructures of the protein bodies were visualized with immunogold electron microscopy. The lightly stained core areas preferentially react with antisera against α-zein while the darkly stained regions primarily react with antisera against β-zein and γ-zeins (Ludevid et al., 1984; Lending et al., 1988). Lending et al. (1988) termed these α-zein staining areas locules. The protein of
the fourth zein class, δ-zein, is present homogeneously throughout the protein bodies (Esen and Stetler, 1992).

The relative amounts and distributions of these proteins vary widely, suggesting a more complex internal organization than originally reported (Lending et al., 1988; Lending and Larkins, 1989). Protein bodies of 50 DAP seeds stain differently than earlier stages; protein bodies 1 micrometer or more in diameter often contain a narrow, irregular, darkly staining band which has projections into the interior. This range of staining and immunogold labeling was interpreted to reflect differences in the spatial and/or temporal synthesis of zeins (Lending and Larkins, 1989). Identical conclusions were derived by Johnson (1989) from measurements of zein-zein interactions in vitro and in vivo. Based on changes in the zein composition of developing protein bodies, Lending and Larkins (1989) proposed a model for zein protein body formation. According to the model, β- and γ-zeins are synthesized and accumulated earlier in endosperm development and α-zeins subsequently penetrate the aggregate of β- and γ-zeins at later stages to complete the zein protein body formation.

To explain the zein's ER retention and protein body formation, interactions among zeins are predominantly used (Wallace et al., 1987, 1990; Wallace and Larkins, 1988; Lending and Larkins, 1989). Under this proposed mechanism, zeins assemble into insoluble aggregates in the lumen of the ER which are responsible for ER retention and the growing insoluble aggregates are enclosed by the ER membrane to develop into protein bodies.

This proposed model may be indirectly supported by observations from various heterologous studies. When only α-zein was expressed in petunia, the zein was mostly localized in the central vacuole (Wallace et al., 1990). Hoffman et al. (1987) reported a similar observation that β-zein are mostly found in the vacuole if expressed singularly in a
tobacco plant, implying that zein is included in the default pathway without interactions with other zein classes in the lumen of the ER. However, the proposed mechanism has significant shortcomings. First, the model is supported by few observations. No additional example is available to support this proposed mechanism to date regardless of plant or animal system. In addition, prolamin storage proteins, to which zeins belong, do not depend on ER retention mechanisms to make protein body formation in the other cereal endosperms. These shortcomings suggest that zeins' ER retention mechanism need to be further studied.

Mutations affecting storage protein accumulation have been utilized in the study of zein protein body formation. In most cases, those mutations result in an opaque phenotype and smaller protein bodies compared to normal counterpart (Mertz et al., 1964; Nelson et al., 1965; Motto et al., 1989). In opaque-2 endosperm, protein bodies exhibit a different morphology compared to normal endosperms. Opaque-2 protein bodies are smaller (0.1-0.3 μm) in diameter than their counterparts (1-2 μm). The small size of opaque-2 protein bodies apparently create air spaces around the starch grains in dried endosperms, which causes the typical opaque and floury phenotype of the mature endosperm (Robutti et al., 1974). Although mutant protein bodies are smaller due to reduced amount of α-zein, they show nearly identical densities as wild type protein bodies. On a SDS-polyacrylamide gel, the mutants show much less amount of α-zeins than the wild type while other classes of zeins show the equivalent of the wild type.

The mutations affecting zein synthesis may cause a disturbance in the ultrastructure of zein protein bodies. Unlike normal spherical protein bodies, floury-2 protein bodies exhibit irregular appearance and an altered arrangement of the storage proteins visualized by electron microscopy (Lending and Larkins, 1989; Fontes et al., 1991). In opaque-2 protein bodies, the locules of α-zein are significantly reduced in size and apparently retain
the size found in immature endosperm even to the end of mature stages (Lending and Larkins, 1989).

The floury phenotype of opaque-2 is modified in quality protein maize (QPM) endosperm, of which some regions are more glassy than others. Although it is not understood how the opaque-2 modifier converts the floury phenotype, recent studies indicate that the overexpression of γ-zein mediates the phenotypic conversion (Lopes and Larkins, 1991; Wallace et al., 1990; Paiva et al., 1991). Geetha et al. (1991) reported that the increased presence of γ-zein in the maize endosperm does not affect the morphology of protein bodies but affect the phenotype of endosperm. Based on this observation, they suggested that the γ-zein may protrude out of the membrane; then, the exposed portions are covalently linked between protein bodies. As a result, the covalent linkage could furnish a mechanism for cementing protein bodies around starch grains in the maize endosperm.

b. Sorghum (Sorghum bicolor L.) and Teosinte (Zea mays spp mexicana)

The major storage proteins of sorghum (Sorghum bicolor L.) are called kafrins that are soluble in alcohol (particularly tertiary butanol) often with addition of mercaptoethanol (Jones and Beckwith, 1970). SDS-PAGE of proteins extracted from isolated protein bodies demonstrated that kafrins were the major protein component (Paulis and Wall, 1979; Taylor et al., 1984). In terms of amino acid composition, kafrins are similar to zeins; rich in glutamine and proline but deficient in lysine (Guiragossian et al., 1978; Paulis and Wall, 1979). A high degree of homology in primary structure implies that kafrins are encoded by a small multigene family which may be closely related to the zeins (Bietz, 1982).
Kafirins are found to be accumulated into protein bodies of 0.4-2.0 micrometer in diameter (Seckinger and Wolf, 1973; Taylor et al., 1984). The origin of the protein bodies is not known, even though it is postulated to be from vacuoles unlike maize (DeBarros et al., 1991). Within the protein bodies, darkly staining inclusions which resemble the proteins of the surrounding matrix, were often found to be contiguous with the matrix. Taylor et al. (1984) suggested that the invaginations of the matrix may play a role in protein body degradation since the surrounding matrix proteins contain predominantly glutelins, some of which have been shown to possess proteolytic activity (Adams et al., 1976). Similar structures are also visible in electron micrographs of protein bodies from maize endosperm (Leding et al., 1988). Much attention has been paid to γ-kafirin in understanding the mechanism of protein body formation in sorghum and other cereal since its counterpart in maize (γ-zein) appears to play an important role in protein body formation and they share 70% homology.

Less is known about the storage proteins of the probable wild ancestor of maize, teosinte (Zea mays spp. mexicana). Based on SDS-PAGE patterns, amino acid composition of the storage proteins, and high level of DNA homology, the teosinte storage proteins may correctly be called zeins (Paulis and wall, 1977).

c. Wheat (Triticums)

The major storage proteins of wheat are prolaminins called gliadins. The gliadins show apparent molecular weights between 30 and 60 Kd on SDS-polyacrylamide gel and are classified into four groups of monomeric proteins (α, β, γ, and ω-gliadins) based on the mobility (i.e., α–gliadin, the fast moving) on acidic starch gels (Woychik, 1961). With accumulation of the data regarding biochemical similarity and chromosomesal location, the
four groups have been assigned into two groups: sulfur-poor (ω-gliadin) and sulfur-rich gliadins (α, β, and γ-gliadins) (Kreis et al., 1985).

EM studies on the developing wheat endosperm reveal that protein body formations are first detected between 5 and 10 days after flowering (DAF) (Parker et al., 1982; Bechtel, 1982; Kim et al., 1988). At this stage one protein mass is contained within a single membrane; later, four or more bodies are shown in cluster within lipoprotein-bound protein bodies which are derived from vacuoles (Parker et al., 1982; Kim et al., 1988). These authors concluded that wheat storage proteins are synthesized on membrane-bound polysomes, translocated into the RER and were subsequently secreted internally into vacuoles, a variation of the mechanism by which casein is synthesized and secreted in mammary glands in the cytoplasm.

No agreement has been reached regarding the subcellular location of gliadin aggregation and subsequent protein body formation. Also, the role of the Golgi apparatus is still controversial toward the gliadin protein body formation. Using subcellular fractionation, Millin et al. (1982) demonstrated that the majority of the storage proteins are deposited into protein bodies surrounded by RER membrane. The role of the Golgi apparatus was challenged by the observation that it was absent 12 DAF at a time when storage proteins are actively synthesized (Briarty, 1978; Briarty et al., 1979). Campbell et al. (1981) actually proposed that a direct link exists between the RER and protein bodies which may minimize the need for the Golgi apparatus in storage protein deposition. On the other hand, the Golgi apparatus was thought to play a role in the condensation of the RER synthesized storage proteins (Buttrose, 1963). Like other cereals, however, the Golgi apparatus is present until at least 40 DAF and contains dense protease-degradable material (Campbell et al., 1981; Parker 1982; Bechtel and Gaines, 1982, Parker and Hawes, 1982; Kim et al., 1988).
These two lines of convincing evidence strongly suggest that there are two alternative pathways in storage protein aggregation and transport. In the developing wheat endosperm, some prolamsins may aggregate within RER and are directly transported to the vacuole as in intact protein bodies while others utilize the Golgi for protein concentration (Campbell et al., 1981; Parker, 1982; Bechtel, 1982). The suggested alternative pathways were supported in a heterologous study using Xenopus oocyte (Simon et al., 1990). The subcellular transport of wheat storage proteins occurs in two separate routes depending on the class of protein. In a study by Simon et al. (1990), the majority of the α-gliadin was retained within oocytes; whereas, most of γ-gliadin was secreted by way of the Golgi apparatus. This observation indicates that α-gliadin is stored in vesicles derived from the ER and γ-gliadin's subcellular route is through the Golgi apparatus.

Wheat storage proteins are deposited in a clearly different and complicated mechanism in comparison to zeins in maize. The major differences are the involvement of the Golgi apparatus in protein deposition and the final destination of the storage proteins. In addition, maize storage proteins accumulate in individual protein bodies while wheat storage proteins constitute a protein matrix (Bechtel et al., 1982b).

d. Barley (Hordeum vulgare)

Hordeins, prolamsins, are the functional storage protein in barley endosperm. As in wheat and maize, the storage protein can be classified according to the sulfur content: sulfur-rich and sulfur-poor hordein. Sulfur-rich hordein includes B hordein and D hordein. B hordein accounts for 80 to 90 % of the total prolamin fraction in barley endosperm with the molecular weight ranging from 35,000 to 46,000. D hordein has an apparent molecular weight of 100,500 on SDS-polyacrylamide gels; its actual molecular weight is only 56,000. This discrepancy is explained by the amount and distribution of proline residue in
the protein (Shewry and Miflin, 1983). Sulfur-poor prolamin of barley is termed C hordein which accounts for 10-20% of total hordein in barley endosperm. The molecular weights of the C hordeins vary among different cultivars between 54,000 and 60,000 with additional minor bands having molecular weight between 49,000 and 72,000. Another group of barley protein, identified as A hordein; are removed from the list of prolamin since it has uncharacteristic amino acid composition and does not accumulate in the protein bodies (Miflin and Shrewry, 1977; Saldeco et al., 1980).

Their protein body structure and formation have been studied by electron microscopy (Cameron-Mills, 1980; Cameron-Mills et al., 1980). These studies showed that hordeins are synthesized as early as 20 DAF. At this stage RER has just begun to proliferate in a few cells. Protein bodies were observed in both large and small vacuoles. Protein bodies in small vacuoles were associated with a fibrous material which was observed to fill the spaces between adjacent protein bodies. The fibrous material was often observed to be associated with small electron dense spheres whose function is unclear. Protein bodies within large vacuoles appeared as isolated deposits with no fibrous material apparent.

Cameron-Mills et al. (1980) have suggested that the fibrous material, which appears to be associated with regions of rapid protein accumulation, may be a transient structure composed of storage proteins identical to those in the homogenous, dense deposits within small vacuoles. Thus, the fibrous material would represent an intermediate structure in the condensation process of storage proteins during seed dehydration. One question put forward by the authors was: how these hydrophobic proteins remain soluble in the ER and during transport to their final destination. Their solubility may depend on low protein concentrations and extreme pHs which would increase their solubility in the aqueous environment of the ER. By reducing the alcohol concentration of a sample of
isolated hordeins, it was observed by EM that the proteins would aggregate into protein body-like formations suggesting that hordeins will naturally associate and condense into highly packed structures. Alternatively, Miflin and Shewry (1979) have suggested that protein bodies originate directly from the deposition of protein into the ER rather than into vacuoles. This view is supported by the finding that vacuolar marker enzymes were absent in protein body fractions of barley as well as in those of wheat and maize (Miflin et al., 1981). Later in seed development large, homogenous aggregates are formed as smaller deposits begin to fuse, however, the single, large protein matrix found in wheat endosperm cells does not occur in barley. The mechanisms are not known how the storage proteins are transported from the ER to the storage vacuoles. The Golgi apparatus has not been implicated for this role as was the case in wheat, however, further study of this question is necessary.

**e. Rye (Secales)**

As with wheat and barley, the storage proteins of rye have been divided into three classes according to relative amount of sulfur-containing amino acids and molecular weight. The sulfur-rich prolamins of rye are collectively called γ-secalins (Shewry et al., 1982), and characterized by their relatively high cystein composition as well as typical prolamin amino acid content. Gamma-secalins are subdivided into two groups by approximate molecular weights of 40,000 and 75,000, B secalin and 75,000 γ-secalin, respectively (Charbonnier et al., 1981). The B secalin exists only as monomers containing intramolecular disulfide bonds; however, the 75,000 γ-secalin exists as monomers and disulfide bond stabilized aggregates, similar to the γ-gliadins of wheat (Field et al., 1983; Shewry et al., 1983d). Minor differences exist in the amino acid compositions of these two groups: the 75,000 γ-secalin contains more glutamate plus glutamine and proline, and the
B-secalin contains more leucine and isoleucine (Shewry et al., 1982). Amino-terminal sequences of both groups resulted in a single sequence for each, with near perfect homology through the first 20 amino acids (Shewry et al., 1982).

Another class of sulfur-rich prolamin in rye is termed as the high molecular weight (HMW) secalins. These proteins were similar in size to HMW fractions of wheat and barley (Shewry et al., 1983d, 1984). In terms of amino acid composition, the HMW secalins has been shown to be similar to wheat glutenins and barley D hordeins.

The sulfur-poor prolamins of rye are called C secalins (Charbonnier et al., 1981) or ω-secalins (Kasarda et al., 1983). C secalins account for 10-20% of the total prolamin fractions (Shewry et al., 1983c) with molecular weights ranging from 48,000 to 53,000 on SDS-PAGE (Kasarda et al., 1983; Shewry et al., 1983d). C secalins are characterized by their high content of glutamate (glutamine), proline and phenylalanine (Kasarda et al., 1983). In these proteins, most (90%) of the glutamate have been replaced with glutamine as for wheat and barley (Shewry and Miflin, 1985). With respect to the N-terminal sequences, C secalins appears very similar as the ω-gliadins of wheat and the C hordeins of barley (Kasarda et al., 1983).

A detailed EM study of the accumulation of secalins into protein bodies has not been reported. The deposition of secalins, however, has been studied by SDS-PAGE (Shewry et al., 1983d). The 75,000 γ-secalins were observed to appear at 14 DAF and remained the dominant group of storage proteins during the course of seed maturation the protein although some fluctuations in the relative levels of the different secalins were detected.

Secalins are synthesized on membrane-bound polysomes. EM studies on developing rye endosperms suggests that the protein bodies are derived from the ER (Miflin et al., 1981; Miflin and Burgess, 1982). However, this suggestion is controversial.
as is the case with the origin of barley and wheat protein bodies (Cameron-Mills, 1980; Parker, 1982). Membrane surrounding the rye protein bodies appear incomplete. When protein bodies are isolated, they contain less B-secalins and the ω-secalins than found in endosperms, indicating that these proteins were exposed and partially solubilized during protein body isolation. In addition, the fact that proteinase K easily digests all secalins in protein bodies in the absence of detergent is indicative of the incompleteness of the membrane (Shewry et al., 1983).

f. Rice (*Oryza sativa* L.)

In rice (*Oryza sativa* L.) the major storage proteins are glutelins rather than prolamins. Glutelins comprise 80% of the total seed protein; whereas, prolamins account for less than 5% (Juliano, 1972). Glutenin is composed of two major components, α (acidic) and β (basic) subunits having molecular weights of 30,000-36,000 and 19,000-22,000, respectively (Yamagata et al., 1982). These subunits result from the processing of a signal sequence and the post-translational cleavage of a 57,000 Kd precursor (Yamagata et al., 1982; Luthe, 1983; Krishnan and Okita, 1986). In addition, IEF analysis indicates that α subunits include at least 12 classes and β subunits 9 classes (Wen and Luthe, 1985).

Storage proteins in rice begin to be synthesized and accumulate into protein bodies about seven DAF (Mitsda et al., 1967; del Rosario et al., 1968; Bechtel and Pomeranz, 1978). In the developing rice endosperm two types of protein bodies are evident under electron microscope (Tanaka et al., 1980). One type of protein body (Type I) is described to have a spherical shape with inner layers of prolamins whose molecular weight is 13,000. The other type (Type II) was evenly stained throughout the protein body despite their heterogeneous composition with globulins and glutelins. A third type of protein body was postulated (Bechtel and Juliano, 1980); however, its contents have not been investigated.
Proteins having molecular weights of 10,000, 13,000 and 16,000 which accumulate in Type I protein body were not post-translationally modified in pulse-chase experiments (Yamagata et al., 1982). These protein bodies have also been shown to have polysomes attached to their surrounding membrane indicating that they may originate from the RER as is in the case of maize (Bechtel and Juliano, 1980; Yamagata et al., 1982). Oparka and Harris (1982) have suggested that all of the protein body-containing vacuoles are initiated by dilation of the ER. On the basis of electron micrographs, a pair of studies have suggested that the Type II protein body is derived from the Golgi apparatus (Yamagata et al., 1982; Bechtel and Juliano, 1980). Yamagata and his colleagues also suggested that, due to the insolubility of the glutelin subunits, post-translational processing may occur only after the 57,000 proglutenin reaches the Type II protein body.

**g. Oat (Avena sativa)**

The major storage proteins of oat (Avena sativa) are globulins rather than prolamins unlike most other cereals (Peterson, 1978). The globulin fraction represents 50-80% of the total seed protein in oat (Walburg and Larkins, 1986) whereas the prolamin, avenin, account for less than 15%. Avenin proteins have structural homology to the gliadins of wheat, the B-hordeins of barley, and γ-secalin of rye. Oat globulins exist as in hexamers having molecular weights of 60,000-65,000. In turn, each subunit is composed of an acidic and basic polypeptide of 36,000-40,000 and 20,000-23,000, respectively (Brinegar and Peterson, 1982a; Walburg and Larkins, 1983). In vitro and in vivo labeling studies on the subunits of 60,000 also reveals that the subunits are synthesized as precursors harboring one basic and one acidic domain which is post-translationally cleaved to yield the two polypeptides linked by a disulfide bond (Brinegar and Peterson, 1982a; Matlashewski et al., 1982; Walburg and Larkins, 1983).
Protein body development begins seven DAF and continues through 16 DAF by light and electron microscopic studies (Saigo et al., 1983). This observation is consistent with the finding that in vivo protein synthesis sustains between nine DAF and continues through 19 DAF (Peterson and Brinegar, 1983). Electron micrographs also revealed that protein bodies were formed within vacuoles like barley (Cameron-Mills and von Wettstein, 1980) and wheat (Parker, 1981; 1982). For the mechanism of protein body formation in oat endosperm, Bechtel and Gaines (1982) have suggested that the Golgi apparatus may be involved in storage protein transport and in concentrating the strage protein, based on the results that protease-digestible vesicles may originate from the Golgi apparatus in oat and several other cereals. An additional mechanism was proposed from an EM study on protein deposition in the subaleurone cells. Since the Golgi apparatus was rarely present and has no apparent role in protein transport from the RER to the storage vacuoles, Saigo et al. (1983) suggested that the RER may be directly linked to the vacuole in oat endosperm and that the prolamin aggregate within RER and transported to vacuole, thus, bypassing the Golgi apparatus. The same study, however, did not negate the presence of the Golgi-mediated pathway for storage protein transport in the oat endosperm.

h. French Bean (Phaseolus vulgaris)

Phaseolins are the major storage protein (globulin) in French bean seeds. They account for 36-46% of the total seed protein (Ersland et al., 1983). Phaseolin has an approximate molecular weight of 600,000 as a tetramer whose subunits are arranged in a tetrahedral conformation (Sun et al., 1974; Tulloch and Blagrove, 1985).

Phaseolins begin to accumulate in the cotyledons from approximately 14 DAF. Their accumulation culminates between 16 and 28 DAF (Sun et al., 1978). These observations are in agreement with the timing of RER proliferation (Bollini and
Phaseolin is synthesized on membrane-bound polysomes, and translocated to the lumen of the ER. Following removal of signal peptides and glycosylation, the newly synthesized phaseolin is transported through the Golgi apparatus for the modification on the oligosaccharide chain and then deposited into protein bodies derived from the vacuoles. A heterologous study using *Xenopus* oocyte reveals that the newly synthesized phaseolins need oligomerize to exit from the ER to the vacuolar protein bodies. When the oligomerization was minimized by injecting low amount of phaseolin mRNA, majority of phaseolins was retained in the endoplasmic reticulum as monomers (Ceriotti et al., 1991). If oocytes, on the other hand, were injected with sufficient (30 fold) mRNA, most of the phaseolin was shown to be transported out of the ER compartment.

**i. Field Bean (*Vicia faba* L.)**

Viciolin is the globulin storage protein in the field bean (*Vicia faba*) and separated into several components having molecular weights of 43,00-55,000 on SDS-polyacrylamide gel (Bailey and Boulter, 1972; Derbyshire et al., 1976). Viciolin is synthesized and processed on the RER as most storage proteins; however, is not post-translationally cleaved (Weber et al., 1981).

Viciolin begins to synthesize in *V. faba* immediately after the cell expansion stage of seed development (Millerd et al., 1971; Müntz et al., 1981). In addition, the timing of viciolin accumulation is highly correlate with those of the endoreplication of nuclear DNA, accumulation of polyadenylated RNAs, and expansion of the ER membranes (Ersland et al., 1983; Payne et al., 1971; Püchel et al., 1979).
j. Pea (*Pisum sativum*)

The storage proteins of pea (*Pisum sativum*) are also globulins which include three classes of proteins: legumin, vicilin and convicilin (Ersland et al., 1983). As with field bean, oats and rice, legumin consists of six subunits having an approximate molecular weight of 60,000 (Casey et al., 1980). Vicilins consist of three heterogeneous subunits which have a molecular weight of 50,000 (Thomson et al., 1978; Gatehouse et al., 1981). Convicilin consists of three or four subunits with molecular weight of approximately 70,000 (Casey and Sanger, 1980; Croy et al., 1980). Convicilin has been shown to be immunologically related to vicilin; however, it contains no vicilin subunits (Croy et al., 1980).

Storage protein synthesis in pea begins at the same stage of development as in *V. faba* (Millerd and Spencer, 1984). All three groups of proteins are synthesized on membrane-bound polysomes, translocated into the lumen of the ER and deposited into protein bodies in the cotyledons (Ersland et al., 1983). Legumin is synthesized as a preproprotein which is post-translationally cleaved to yield an α and β subunit (Croy et al., 1980; Spencer and Higgins, 1980). Subunits of vicilin and convicilin are also believed to derive from large precursors which are cleaved post-translationally (Gatehouse et al., 1981).

k. Soybean (*Glycine max*)

In soybean (*Glycine max*), the major storage protein is glycinin, a globulin, which is extracted with dilute saline. It accounts for 13 to 18% of the dry weight of the mature seed and approximately 40% of the total protein content (Hughes and Murphy, 1983). Glycinin homologues are ubiquitously found in seeds of monocot and dicots; they are collectively termed as 11s globulins or legumin-like proteins.
Glycinin is isolated from the mature seeds as a hexamer having an apparent molecular weight of 320,000. Assembly of the hexamer appears to involve two steps. In the ER of cotyledon, monomeric subunits initially put together into trimers referred to as proglycin with 8-9S (or 180 Kd). The proglycin is believed to transport to vacuole via the Golgi apparatus (Dickinson et al., 1989). The assembly of 11S hexamer is completed in the storage vacuole.

The removal of the transient peptide by proteolytic processing appears very important for the glycinins to be assembled and subsequently packaged. The first cleavage involves the removal of a signal peptide which is responsible for guiding the protein into the lumen of the ER. The second processing takes place within the central vacuole. In this case, the posttranslational processing is believed to serve as a signal for the 11S subunits to assemble into a higher order in the protein bodies. This removal of transient peptides brings about two polypeptide chains; namely, 40 Kd acidic polypeptide and 20Kd basic polypeptide (Tumer et al., 1981 and 1982; Barton et al., 1982). The existence of the precursor harboring the two polypeptides was confirmed in a heterologous system, Xenopus oocyte (Tumer and Richter, 1982). When the oocytes were injected with glycinin poly (A)+ mRNA, signal peptide was removed from the conglycinin while the two domains were not cleaved from each other (Tumer and Richter, 1982). The site of cleavage, between asparagine and glycine, is highly conserved during the evolution (Simon et al., 1985; Chlan et al., 1986; Takaiwa et al., 1986; Walburg and Larkins, 1986).

6. Zein Expression and Accumulation in Heterologous Systems

With recent advances in transformation, various studies have been performed to understand factors affecting zein gene regulation and zein polypeptides' accumulation. A maize regeneration system has been devised very recently (Rhodes et al., 1988); however,
lower frequency of transformation and regeneration is still the limiting factor in generating transgenic maize plants. Therefore, the majority of the studies have depended on heterologous systems: *E. coli*, yeast, other higher plants, and animal system.

Zein genes, especially 27K zein, was shown to be expressed in *E. coli*. The zein expression, however, resulted in cell death (Wang and Esen, 1985). When genes corresponding to 20 and 22 K zeins are introduced into the yeast chromosomes, the genes are shown to be successfully transcribed (Langridge et al., 1984; Corragio et al., 1988); however, zein polypeptides have not been detected.

A number of studies have been performed in heterologous higher plants. Golsbough et al. (1986) initially showed the expression of a 19K and 15K zein gene in transgenic sunflower callus following transformation with *Agrobacterium tumefaciens*. Although the transformed tissues produced transcripts of sizes corresponding to those in maize endosperm, they failed to produce any detectable zein.

Tissue-specific expression of the zein genes was reported from transgenic studies using petunia and tobacco plants (Hoffman et al., 1987; Rousseli et al., 1988; Schernthaner et al., 1988; Wallace et al., 1990). In all studies, zein genes were predominantly expressed in the endosperm. In addition, the zein was mostly localized in the central vacuole when only α-zein was expressed in petunia (Wallace et al., 1990). Hoffman et al. (1987) reported a similar observation that β-zeins are mostly found in the vacuole if expressed alone in a tobacco plant. This observation implies that zeins can be included in the default pathway unless zeins interact one another in the lumen of the ER.

The significance of the zein-zein interaction in zein polypeptides' ER retention and zein protein body formation was studied in *Xenopus* oocytes.
7. *Xenopus* oocytes as in vivo Translation System to Study Zein Protein Body Formation

Since the pioneering work of Gourdon and his colleagues (1971), *Xenopus* oocytes have been extensively used as an in vivo system to study transcription, replication, assembly, partition, and translation of injected molecules. Also, the oocyte system has been shown to transport a multitude of foreign animal and plant proteins into correct subcellular location, regardless whether the proteins reside in the ER or are transported via the Golgi apparatus, indicating that the general process of protein transport has been highly conserved during evolution. *Xenopus* oocytes have advantages especially when biological properties of a protein are studied in vivo since it produces biologically active proteins following mRNA injection. The oocytes are very efficient translation system (at least thirty-fold and up to 1,000 times, better than in vitro systems: wheat germ extract and reticulocyte lysate). In the oocytes, the translation products resemble native product since the oocyte can process the signal peptide and modify (phosphorylate and glycosylate, if specified by the amino acid sequence) the protein as in its native cell. Its large size (1.0-1.2 mm in diameter at stages V-VI) allows a study concerning synthesis and subsequent translocation of secretory protein based on spatial distinction.

*Xenopus laevis* oocytes produce and store proteins mainly for future use at embryonic stages (Lane, 1983 and 1989; Dumont 1978; Mohun et al. 1981). In this respect, *Xenopus* oocytes resemble plant seeds which produce and store proteins required for germination. Moreover, the oocyte shows efficiency and fidelity in translation, processing, and subcellular localization of zein. When *Xenopus laevis* oocytes were injected with native zein mRNAs, the oocyte system has been found to translate the message correctly, process zein proteins (Larkins et al., 1979), and package the zein into vesicles with the density identical to that of native zein protein bodies (Hurkman et al., 1981). *Xenopus* oocytes also translate in vitro transcribed zein mRNA; correctly process
the signal peptides; and accumulate subsequent zein polypeptides in dense protein bodies comparable to native zein protein bodies (Wallace et al., 1988). These aspects facilitate studies regarding structure, biochemistry, physiology, and biological properties of zein proteins.

Significant progress is being made in the transformation of maize plants. However, routine regeneration of fertile transgenic maize plants is not anticipated in the near future. In the absence of a workable regeneration system for maize, the use of *Xenopus* oocytes may yield applicable information concerning translation of zein mRNA and its protein body formation.

Even though zein can be studied directly in the developing endosperm, *Xenopus* oocyte still is an attractive system. By using this system, the transport of individual zein polypeptide can be studied independent of other classes of zein polypeptides, which is not possible in developing maize endosperm. Taken together, *Xenopus* oocyte system would be one of the best *in vivo* system available to solve the questions regarding different aspects of zein protein body formation such as a potential transmembrane feature of 27K zein which may lead to the other zeins' ER retention or whether zein aggregates are retained in the ER by insolubility.
MATERIALS AND METHODS

A. Preparation of mRNA

1. Native mRNAs

a. Zein mRNA

Maize inbred line W64A+ was planted in the field and hand-pollinated. Developing seeds were harvested at various days after pollination (DAP), frozen in liquid nitrogen, and stored at -80°C. Membrane-bound polysomes were isolated from the maize seeds 18 DAP according to Larkins and Hurkman (1978) with minor modifications.

Zein poly (A)+ mRNA was isolated from the membrane-bound polysomes according to a modification of Larkins and Hurkman (1978). After polysome pellets were melted by adding prewarmed (37°C) 2X binding buffer (see appendix 1 for the composition of all buffers used in the poly (A)+ mRNA isolation), the polysome solution was incubated at 37°C for 20 minutes with proteinase K (0.1 mg/ml). The polysomes were subjected to binding with oligo dT cellulose (New England Biolab) in a 30 ml Corex tube for 30 minutes by inverting or tipping intermittently at room temperature. At the end of binding, the oligo dT cellulose was collected by centrifugation and washed sequentially with 1X buffer, wash buffer, KCl buffer, and Low KCl buffer. Washing with wash buffer was continued until no foam, from SDS, was visible. The oligo dT cellulose was collected as aforementioned by centrifugation. Poly (A)+ mRNA was eluted by warming the cellulose at 60°C for 60 seconds in 4, 4, 2, 2 ml of elution buffer and the absorbance at 260 (A$_{260}$) was read immediately after each elution. To eliminate rRNA binding to the poly (A)+ mRNA, all of the eluates containing a significant A$_{260}$ reading were combined into a Corex tube, heated at 60°C for 90 seconds, chilled in ice water, and KCl was added to 0.5 M. Steps involving binding, washing, and elution was repeated until RNA recovery
reached at least 80% of the $A_{260}$ bound in the previous cycle except the poly (A)+ mRNAs were eluted by adding 3, 3, 1, 1 ml of elution buffer instead of 4, 4, 2, 2 ml. Then, the eluates containing significant $A_{260}$ were subjected to the ethanol precipitation by adding 5 M potassium acetate and 95% ethanol at 0.1 and 2.5 volumes of the eluates. After centrifugation at 180,000g for 1 hour, the mRNA pellet was dissolved in sterile distilled water and stored at -80°C until needed.

b. Chicken egg white mRNA

Chicken ovalbumin mRNA was isolated basically as described for the zein polysome and mRNA isolation. The magnum portion of oviduct was obtained from Rhode Island Red hens. Tissue was collected by scraping the inner wall of the oviduct by a sterile microscope slide and homogenized in five times excess (v/w) of buffer A (200 mM Tris-HCl, pH 8.5, 200 mM Sucrose, 60 mM KCl, 50 mM MgCl$_2$, 1 mM DTT) using a Polytron homogenizer. After homogenates were centrifuged at 500g at 4°C for 5 minutes to remove nuclei and cell debris, the supernatant was collected and subjected to centrifugation at 37,000g to obtain membranes. The combined high-speed supernatants were directly layered on 4 ml sucrose pads and polysomes were pelleted by centrifugation at 120,000g for five hours (Beckman Ti 50.2 rotor). The membrane fractions underwent additional homogenization in a Buffer A containing 1% Triton X-100 to release membrane-bound polysomes and the membranes were removed by centrifugation as before. Ovalbumin poly(A)+ RNA was isolated by the identical procedure as for zein.

2. Synthetic mRNAs

Synthetic mRNAs of 27K zein and a mutant hemagglutinin (HA$_{env}$) were prepared using SP6 polymerase from DNA templates of pSP627K and pSP6HA$_{env}$. The
former plasmid harbors full length of 27K zein cDNA with the SP6 promoter and a poly(dA)-poly (dT) homopolymer; whereas, the latter contains the DNA sequence of hemagglutinin whose signal peptide and first two amino acids have been substituted with the signal and six amino acids of the Rous sarcoma virus envelope protein (Gething et al., 1986). Once plasmid DNAs were amplified and isolated by a large scale preparation (Maniatis et al., 1985), they were linearized using either Hind III (for pSP627K) or Sac I (for pSP6 HAenv). The restriction enzymes were added at 1.5 units per μg of the stock DNA added. Autoclaved distilled water was added to bring up the reaction volume to 250 μl, which was incubated at 37°C for 3 hours. This incubation was extended overnight. Linearization of the plasmid DNA was verified on a 0.8% agarose gel.

The linearized plasmid DNA was purified by three cycles of phenol extraction. The DNA was precipitated by adding 2.5 volumes of ethanol and 0.1 volume of potassium acetate. This precipitation was performed three times to remove any trace of phenol from the DNA. In vitro transcription was initiated by adding SP6 RNA polymerase and nucleotides (see Appendix 2 for components and concentrations). CAP (m7GpppG) was added at 10 times molar excess of GTP to ensure the effective capping of the RNA transcript for the initial 45 minute incubation, after which GTP was supplemented to the concentration equivalent of CAP. Efficiency of the reaction was assessed according to the incorporation of 3H-UTP (40 Ci/mmole, 0.5 μCi per μl of reaction) into the transcript by TCA precipitation procedure.

When in vitro transcription was completed, the DNA templates were removed by incubating for 15 minutes at 37 °C with RNase-free DNase I (Boehringer Mannheim) at 20 - 50 μg/ml (or 1 unit per μg of DNA added). The RNA transcript was purified by phenol extraction and ethanol precipitation.
B. Preparation of oocytes and Microinjection

1. Removal of ovary

Ovary was manually stripped from an adult *Xenopus laevis* (*Xenopus I*: Ann Arbor, MI) after the frog was chilled (anaesthetized) on a bed of ice for 20 minutes. Once a small piece of ovary was removed from a 1 cm wide incision with the ovary tied, two layers of membrane (muscle and skin) were sutured separately. The excised ovary was divided into pieces of 30-50 oocytes, washed in OR2 (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 5.0 mM HEPES, 3.8 mM NaOH, pH 7.8, 1.0 mM Na₂HPO₄), and incubated in a fresh OR2 at 14°C overnight until defolliculation and injection.

2. Defolliculation

Defolliculation procedure included the removal of tissue layers surrounding oocytes which are resilient and which keep the injection needle from penetrating into the oocytes. The membranes were removed either manually or chemically with collagenase (Type I, *Boehringer Mannheim*). Manual defolliculation was done with two pairs of fine-tipped forceps (Dumont No. 4, *Fisher*) under a dissecting microscope at 12X magnification. When more than twenty oocytes were needed, ovary was treated with collagenase (1 mg/ml) in OR2 for two hours or until at least one fourth of oocytes were released from a piece of ovary. Further separation of oocytes was done by gently agitating the piece of ovary in fresh OR2 with fire-polished Pasteur pipettes. Healthy stage V-VI oocytes were collected in a petri dish containing fresh OR2 and placed on ice until microinjection.
3. Microinjection needle construction

Injection needles were prepared by drawing out a borosilicate glass micropipette (for 10 μl, Fisher) with a commercial injection needle puller (Narishige). To generate an angled tip, the pulled pipette was pinched off diagonally at approximately 0.5 cm from the tapered end with a pair of flame-sterilized watch maker forceps. Graduation of the needle was first done by marking scales on the needle. Later experiments, however, utilized a piece of transparency on which scales of 0.05 mm, equivalent to 25 nl, were delineated by a graphic software. Graduation was easily completed by attaching a piece of transparency to the needle by a strip of parafilm. This new procedure allowed the used needle, which may retain isotope, to be discarded freely after injection.

C. Proteinase K Digestion of Protein Bodies from Oocytes

1. Proteinase K digestion

To determine whether 27K zein spans the ER membrane, protein bodies from oocytes were treated with proteinase K according to Johnson (1989). The protein bodies were prepared according to Wallace et al. (1988). Forty oocytes were injected with 25 ng of in vitro transcribed 27K zein mRNA in a 50 nl injection volume and incubated for 24 hours at 14°C. The oocytes were homogenized in an oocyte homogenization buffer (30 μl per oocyte; 20mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM MgCl₂, 0.3 M NaCl, 2 mM EDTA, 10% sucrose) by passing them through a pipette tip five times. Aliquots of the homogenates were incubated with proteinase K (0, 0.5, 1.0 mg/ml) on ice 30 minutes either in the presence or absence of 1% Triton X-100. Incubations were terminated by adding phenyl methylsulfonyl fluoride (PMSF) to a concentration of 1.0 mg/ml. Proteins were extracted at 60°C for 10 minutes in 70% ethanol containing 5% 2-mercaptoethanol

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and 1% Triton X-100 during agitation (Vortex). The insoluble portion was removed by centrifuging at 10,000g for 5 minutes whereas the supernatant was collected and dried to completeness in a vacuum desiccator. The dried remnant was resuspended in 2X sample buffer, electrophoresed on SDS containing polyacrylamide gels and subjected to Western blotting.

2. Western blotting and immunological detection of 27K zein

Following SDS-PAGE, proteins were transferred to nitrocellulose (Schleicher & Schell, Keene, NH) electrophoretically at 14 V overnight in TGM buffer (20 mM Tris-HCl, pH 8, 150 mM Glycine, 20% methanol). Filters were blocked for 18 hours at 37°C with a Tris-buffered saline (TBS; 15 mM Tris-HCl, pH 7.4, 200mM NaCl) containing 1% ovalbumin in a heat sealable plastic bag.

Following blocking, filters were washed three times for 15 minutes with gentle shaking in fresh TBS containing 0.1% Tween 20. When the wash was completed, the nitrocellulose filter was blotted on 3MM paper, air-dried for 1 hour, and incubated in TBS containing 1:1000 diluted polyclonal antibody against γ-zein (gift from Dr. A. Esen) for 20 minutes. At the end of the incubation, the filter was washed three times in a fresh 1% Tween-20 TBS by gentle shaking and incubated in TBS containing 1:300 diluted protein A conjugated Horse Radish Peroxidase (HRP, BioRad). The filters were washed three times for 20 minutes in 0.5% Tween-20 TBS. Finally, the washed filter was developed in ECL (Enhanced Chemiluminescence, Amersham) reagents for 10 minutes in the dark. The nitrocellulose filter was covered with plastic wrap and exposed to Kodak X-ray film for 30 minutes.
D. Biotinylation of Protein Bodies

1. Vesicle isolation and Protein biotinylation

In order to determine whether the 27K zein is exposed from the membrane, protein bodies from oocytes were treated with biotinylation reagent according to Johnson (1989). Oocytes, injected with or without 27K zein mRNA (25 ng in a 50 nl injection sample per oocyte), were homogenized as described under Methods for proteinase K (section C.1) in a potassium phosphate buffer (30μl/oocyte; 100 mM NaCl, 2 mM MgCl₂, 70 mM KH₂PO₄, pH 7.4) containing 0.2 M sucrose. Protein bodies, then, were isolated according to the cushion method described by Colman (1984). Following homogenization, the homogenate was layered and centrifuged on 400 μl cushion of the potassium phosphate buffer containing 20% sucrose at 13,000g for 25 minutes in a 1.5 ml microcentrifuge tube to obtain a protein body fraction. At the end of centrifugation, the pellet (protein bodies and other vesicles) was resuspended in the fresh potassium phosphate buffer containing 0.2 M sucrose. Protein bodies, from injected and non-injected (control) oocytes, were incubated with biotin amidocaproate N-hydroxysuccinimide ester (BANHS) at the concentration of 4.5 mg/ml or 10 mM. BANHS was delivered dissolved in 10 μl of N,N-dimethylformamide. The incubations were maintained at room temperature for four hours, and quenched for 20 minutes by adding a 100 fold molar excess of lysine. Proteins were extracted by adding sufficient ethanol to achieve 70% (v/v) or urea to 30% (w/v) in the presence of 5% 2-mercaptoethanol and 1% Triton X-100. The extracted protein was dried in a vacuum desiccator, electrophoresed on 15% polyacrylamide gels containing SDS, and transferred to nitrocellulose filters as described in Methods for proteinase K digestion. Lanes were subjected either to biotinylation detection or to antibody detection of 27K zein
using avidin conjugated HRP or Protein A conjugated HRP, respectively (see next section or section C. 2).

2. Detection of biotinylated protein
   The filter was blocked for 18 hours at 37°C in TBS containing 1% ovalbumin. Following blocking, the filter was blotted with 3MM paper, air dried for 1 hour, and incubated with avidin labeled HRP (BioRad, 1:1,500 dilution) in buffer A (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl₂) for 20 minutes. The biotin-avidin complexes were visualized by impregnating the filters in ECL protein detection reagent as described in the proteinase K assay.

E. Pulse-Chase to monitor protein movement

1. Analysis of zein ³H-leucine quenching and mRNA diffusion
   The rate of ³H-leucine quenching during chase period was assessed by measuring ³H-leucine incorporation into zein following coinjection of native zein mRNA (50 ng) and ³H-leucine (0.5 μCi; 115 Ci/m mole) in a volume of 50 nl. The incorporation rate was measured for 15 hours while the oocytes were placed in OR2 containing 100X excess of cold leucine (8.7 mM) immediately after the injection. In parallel experiments, the incorporation was also measured for three hours during incubation in OR2 (free from the excess amount of the leucine); subsequently, they were placed in OR2 containing 100X leucine for twelve hours. At the indicated times, the oocytes were frozen on a bed of dry ice and stored at -80°C until the collection of oocytes was completed. Zein was extracted in 70% ethanol and counted in a scintillation spectrometer after precipitation on 3MM paper with TCA.
The diffusion of mRNA was assessed on the basis of the distribution of labeled zein by hemisphere. At various time points (0.5, 2, 4, 6, 8 hours) after native zein mRNA injection (50 ng), oocytes were injected with $^3$H-leucine (0.5 μCi, 115 Ci/m mole) and incubated for three hours in OR2. The oocytes then were frozen on a bed of dry ice and dissected along the equator into halves. The zein was extracted and counted as described in the previous section.

2. Pulse-chase

a. 27K zein

The movement of the 27K zein was analyzed by a pulse-chase assay according to Ceriotti and Colman (1988). 27K zein mRNA (25 ng) was coinjected with $^3$H-leucine (0.5 μCi) in a 50 nl injection per oocyte either into the vegetal or animal hemisphere. Typically, 50 oocytes were injected in an experiment. As a control for the specific injection, the distribution of $^3$H-leucine (and 27K zein mRNA) was determined immediately following injection as follows: five to eight oocytes were frozen by placing them on used X-ray film on a bed of dry ice and storing them at -80°C. When oocytes reached the consistency of butter during thawing, they were manually dissected by a chilled sterile scalpel along the equator which separates the vegetal and animal hemispheres. The dissected oocytes were pooled by hemisphere and the hemisphere specific injections were verified by counting in a scintillation spectrometer. The remaining oocytes were incubated in OR2 for three hours; then, half of the oocytes were manually dissected as aforementioned and pooled by hemisphere. The remaining half of oocytes were chased in OR2 containing 100 fold excess of non-radioactive leucine for 12 hours. At the end of the chase period, oocytes were dissected and pooled by half as aforementioned.
Since all labeled zein polypeptides are protected inside the ER membrane in the oocyte (this study; Larkins et al. 1979; Wallace et al. 1988), zein has been extracted directly from the pooled hemispheres without isolating the protein body vesicle first. The pooled halves were separately homogenized in 70% ethanol containing 5% 2-mercaptoethanol and proteins were extracted as described in the proteinase K digestion (section C. 2). Ten microliter of the zein extraction was spotted on 3MM and precipitated in 10% TCA, and radioactivity was measured in a scintillation spectrometer. The remaining zein was completely dried in a vacuum desiccator, electrophoresed on a 15% polyacrylamide gel containing SDS, and subjected to fluorography.

b. Mutant Envelope Hemagglutinin

As a transmembrane control, the movement of the $\text{HA}_{\text{env}}$ protein was analyzed as described below. Following coinjection of \textit{in vitro} transcribed $\text{HA}_{\text{env}}$ mRNA (5 ng) and $^{35}$S-methionine (0.4 µCi, 1170 Ci/m mole) in a 50 nl injection volume and incubation in OR2 medium for three hours for 12 hours, half of the oocytes were removed and stored in -80°C. The remainder of the oocytes were further incubated in OR2 containing 100 fold excess non-radioactive methionine. Those oocytes were dissected into halves, which were pooled separately similarly as described in the analysis of 27K zein movement. After the pooled halves were homogenized in a homogenization buffer (20 µl per oocyte half, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 20 mM Tris-HCl, pH 7.4), $\text{HA}_{\text{env}}$ were immunoprecipitated according to Colman (1984). The immunoprecipitation reactions was prepared by adding an equal volume of RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 7.4) into the oocyte homogenate. Then, antisera against $\text{HA}_{\text{env}}$ (gift from Dr. A. Colman, University of Birmingham, United Kingdom) was added at 4 µl per milliliter of the precipitation
reaction, which was subsequently incubated on ice for 1 hour. Following the addition of protein A-sepharose (Pharmacia) at 20 μl per milliliter of reaction, the precipitation reaction was maintained on ice with shaking for an additional 30 minutes. The precipitation mixtures were then centrifuged for 30 seconds in an Eppendorf microcentrifuge. The pellets were washed with 1.0 ml RIPA buffer containing 0.5 M NaCl. The pellets were then washed four more times, as follows: washes 1 and 2, 1.0 ml each with RIPA; washes 3 and 4, 1.0 ml each with TAB buffer (50 mM Tris-HCl, pH 7.5, 0.02 % sodium azide, 2 mM benzamidine). After the final wash, the pellets were resuspended in 50 μl TAB containing 5% DTT and 2% SDS, boiled for 3 minutes, and centrifuged once again. The resultant supernatants were electrophoresed and subjected to the fluorography procedure (see following section). The radioactive HAenv was quantitated after the band corresponding to that on the fluorography was sliced and counted by a liquid scintillation spectrometer.

c. Ovalbumin

In control experiments with ovalbumin, a secretory protein, oocytes were injected with 10 ng of ovalbumin mRNA and 0.4 μCi 35S-methionine (1170 Ci/mmmole) in a 50 nl injection and incubated in OR2 medium for three hours. Half of the oocytes were then collected and stored at -80°C while the rest of oocytes were further incubated in OR2 containing 100 fold excess non-radioactive methionine for twelve hours. Those oocytes were dissected and pooled by hemispheres as described in the HAenv experiment. Following homogenization in homogenization buffer, ovalbumin was immunoprecipitated similarly as in the HAenv experiment by the addition of affinity-purified polyclonal antibodies against ovalbumin (Sigma) at 10 μl (12 μg) per milliliter of precipitation volume.
3 Fluorography

After electrophoresis on SDS-gel containing 15% polyacrylamide, the gel was fixed in a 5.3% acetic acid for 1 hour and subjected to the fluorography procedure described by Lasky and Mills (1975). Water was removed from the gel with DMSO (Dimethyl sulfoxide, Sigma) by shaking gently for 30 minutes in a closed container. After another treatment in fresh DMSO for 30 minutes, the gel was impregnated with 20 % (w/v) PPO (2,5 diphenyl oxazole: scintillation grade from Amersham) in DMSO with gentle shaking for 3 hours. The gel was washed with double distilled water to precipitate PPO and remove DMSO, and shaken overnight in double distilled water to remove the DMSO completely. When the wash was completed, the gel was placed on 3MM paper and dried in a gel drier. The dried gel was exposed to X-ray film at -80°C for up to 2 weeks.
RESULTS

This study has investigated whether 27K zein has a transmembrane feature that permits 27K zein to remain in the ER despite the indiscriminate bulk flow, to serve as an anchor for other classes of zeins by way of specific zein-zein interactions, and, consequently, to act as a nucleating factor for zein protein body formation in the ER. Following injection of the in vitro transcribed 27K zein mRNA into the oocytes, the potential transmembrane features of 27K zein have been analyzed by the three categories of assays: proteinase K digestion, biotinylation of protein vesicles containing 27K zein, and pulse-chase experiments. The results from the present study are described in this chapter.

A. 27K zein is not affected by proteinase K treatment in situ.

In determining whether 27K zein spans the ER membrane, proteinase K has been used to treat protein vesicles from Xenopus laevis oocytes having equal density to maize endosperm protein bodies. The rationale for this approach is that if 27K zein is a transmembrane protein, a new band should be evident on Western transfers corresponding to a fragment of this protein protected within the protein body membrane from the proteinase K digestion in the absence of detergent, which is known to affect the integrity of the membrane surrounding the protein bodies. Following injection of in vitro transcribed 27K zein mRNA and incubation for 24 hours at 14°C, the oocytes were homogenized to isolate protein vesicles as described in Materials and Methods. Aliquots of the homogenates were incubated with proteinase K (0, 0.5, 1.0 mg/ml) on ice 30 minutes either in the absence or presence of 1% Triton X-100. Following incubation, proteins were extracted in 70% ethanol containing 5% 2-mercaptoethanol. The alcohol-
soluble fraction was collected and dried completely after the insoluble portion was pelleted by centrifuging at 10,000g for 5 minutes. The dried remnant was subjected to electrophoresis in SDS-polyacrylamide gels.

Proteins were transferred to nitrocellulose electrophoretically. Following blocking and washes, the filters were incubated in a TBS buffer containing antibodies against γ-zein as described in Materials and Methods. The filters were incubated in TBS containing protein A conjugated Horse Radish Peroxidase (HRP, BioRad), washed in 0.5% Tween-20 TBS, developed in ECL (Enhanced Chemiluminescence, Amersham) reagents for 10 minutes in the dark, and exposed to X-ray film as described in Materials and Methods.

Figure 1A is a Western blot of such an experiment in which protein vesicles were treated with proteinase K at concentrations of 0, 0.5, and 1.0 mg per ml and resolved by electrophoresis. In Figure 1, each lane contains the equivalent of 10 oocytes. Lane a shows the control reaction in which the protein vesicles are incubated without adding proteinase K. Lanes b-d represent the results from proteinase K treatment of the protein vesicles. When the protein body vesicles were treated with proteinase K (even at the lowest concentration used, 0.5 mg/ml) in the presence of 1% Triton X-100, 27K zein was totally degraded (lane b). However, 27K zein remained intact (lane c) at the same proteinase K concentration when the detergent was omitted from the reaction. Even with the doubled proteinase K concentration (1.0 mg/ml), 27K zein was not affected without detergent added (Lane d). In both cases, the size of the product resistant to proteinase K appears unchanged relative to native 27K zein (Lane a). When protein bodies were incubated either at 2.0 mg/ml of proteinase K or for prolonged period (5 hours), most zeins have been degraded as evidenced by their greatly reduced mass (data not shown). This effect is probably due to a loss of the integrity of the membrane surrounding the protein bodies as membrane proteins, which stabilize the structure, are degraded.
Figure 1 Proteinase K treatment of protein vesicles from oocytes.

Forty oocytes were injected with synthetic 27K zein mRNA (25 ng/oocyte in a 50 nl injection sample), incubated for 24 hours, and homogenized in the oocyte homogenization buffer. The homogenate was divided into four aliquots, and treated with proteinase K at 0 (Lane a), 0.5 (Lane b and c), and 1.0 mg/ml (Lane d) either in the presence or absence of 1% Triton X-100. Each lane contains the equivalent of protein vesicles isolated from 10 oocytes. The result of each reaction was detected by Western transfer using antibody against 27K zein and ECL (Amersham) protein detection system following electrophoresis on a 12.5% polyacrylamide gel containing SDS (Figure 1-A) or on a higher percentage (15%) polyacrylamide gel (Figure 1-B). The +/- below the lanes denote the presence/absence of 1% Triton X-100 during incubation with proteinase K.
Figure 1. Proteinase K treatment of protein vesicles from oocytes
The result shown in Figure 1A indicates that 27K zein is not affected by proteinase K; thus, it may not protrude out of the protein bodies. The size of the product resistant to proteinase K treatment corresponds to that of the untreated counterpart. This result also indicates that 27K zein is completely contained and protected within the membrane.

To further analyze for smaller polypeptide fragments as a result of proteinase K digestion, similar extracts as in Figure 1A were electrophoresed on 15% gels, transferred to nitrocellulose and challenged with antibodies directed against γ-zein (Figure 1B). Lanes in the blots did not show any additional bands other than that corresponding to the original 27K zein (Figure 1B, lanes c and d).

**B. 27K zein is not Biotinylated in Isolated, Intact Protein Bodies**

A second approach to assay the potential transmembrane feature of 27K zein involved the chemical modification (biotinylation) of exposed proteins on the outside of intact protein bodies. As for the proteinase K experiments, the rationale for this experiment is that fractions of protein, exposed on the outer surface of protein bodies, will be affected, to the exclusion of other proteins contained completely in the ER membrane, by the biotinylating reagent, biotin amidocaproate N-hydroxysuccinimide ester. Isolated protein bodies were reacted with this reagent as described in Materials and Methods. The incubation was maintained at room temperature for four hours, and quenched for 20 minutes by adding a 100 fold molar excess of lysine. Proteins were extracted either in 70% ethanol or in 30% urea, containing 5% 2-mercaptoethanol and 1% Triton X-100. The extracted proteins were dried in a vacuum desiccator, electrophoresed on SDS-polyacrylamide gels, and transferred to nitrocellulose filters as described in Methods for proteinase K digestion.
The blocked filters were incubated with avidin labeled HRP (BioRad; 1:1,500 dilution) in buffer A (see Materials and Methods). The biotin-avidin complexes were detected by impregnating the filters in ECL (Amersham) protein detection reagent while 27K zein was detected by antisera against gamma-zein as described in the proteinase K assay.

In Figure 2, lanes a and b represent the ethanol soluble fraction of the biotinylated protein from oocytes, non-injected and injected with 27K zein mRNA, respectively. Little or no portion of the ethanol soluble protein was biotinylated from the oocytes injected or non-injected with 27K zein mRNA although numerous bands were detected from the urea extracted fraction from the injected oocytes (lane c). The existence of the 27K zein in the biotinylation reaction was confirmed by ECL detection system (Enhanced Chemiluminescent, Amersham) using 27K zein antisera (Lane d). These results are in good agreement with those obtained from the proteinase K experiment. In situ biotinylation and proteinase K digestion suggest that 27K zein polypeptide does not protrude on the outside of the ER membrane.

C. Pulse-Chase experiment to monitor movements of 27K and total zeins in ER

In order to analyze 27K zein’s ability to move away from the site of synthesis, 27K zein was monitored by pulse-chase experiments. If 27K zein is a transmembrane protein or anchored to the ER membrane, it should be localized at the site of synthesis. The movement of 27K zein was analyzed by monitoring the distribution of labeled 27K zein using a pulse-chase protocol as described in Materials and Methods.

The validity of this pulse-chase experiment was checked by measuring the velocity of $^3$H-leucine quenching during the chase period and mRNA diffusion during the labeling
Figure 2. Biotinylation of protein vesicles from oocytes.

After injection of 25 ng of the synthetic 27K zein mRNA in a 50 nl injection volume, protein vesicles were isolated as described in Materials and Methods. Protein vesicles were also isolated from 10 non-injected oocytes. Those vesicles were reacted with biotin amidocaproate N-hydroxysuccinimide ester. Proteins were extracted from the protein vesicles either with 70% ethanol (lanes a, c, and d) or 30% urea (lane b), separated on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose. Biotinylated proteins were detected by avidin labeled Horse Radish Peroxidase and Enhanced Chemiluminescent (Amersham) reagent (Lanes a, b, and c). 27K zein was detected by reaction with antisera against 27K zein and ECL detection (Lane d). Each lane accommodates the equivalent of 10 oocytes. Lane a: non-injected oocytes; Lanes b, c, and d: oocytes injected with synthetic 27K zein mRNAs.
Figure 2. Biotinylation of protein vesicles from oocytes.
period. If the labeling $^3$H-leucine is not rapidly diluted in the oocyte during the chase period or if the injected mRNAs diffuse rapidly across hemispheres during labeling, the distribution of radioactive proteins may not be a valid measurement of protein movement within the lumen of the ER. The speed of $^3$H-leucine quenching and mRNA diffusion was measured indirectly by the change in $^3$H-leucine incorporation rate into zeins and distribution of the labeled proteins between oocyte hemispheres after injection of the mRNAs.

1. The injected $^3$H-leucine is rapidly diluted during the chase period

To assess the rate at which the labeling $^3$H-leucine is quenched by unlabeled leucine in the incubation medium during the chase period, the rate was measured by way of $^3$H-leucine incorporation into zein. Incorporation of $^3$H-leucine into zein was analyzed when oocytes were incubated in OR2 medium containing 100 fold excess of non-radioactive leucine either immediately or after three hours of incubation in OR2 following coinjection of native zein mRNA (1 µg/µl) and $^3$H-leucine (10 µCi/µl) in a 50 nl injection per oocyte. At 2, 3, 4, 6, and 15 hours after the coinjection, zein polypeptides were extracted from two groups of five oocytes after dissection and pooling by half (see Materials and Methods). The ethanol-soluble fraction, prepared from 70% ethanol containing 5% 2-mercaptoethanol, was precipitated on 3MM paper in 10% TCA and counted in a scintillation spectrometer. Total counts in zeins per oocyte are presented against the time in the main graph (Figure 3). The incorporation of $^3$H-leucine is sharply reduced within 1/2 to 2 hours during the incubation.
Incorporation of $^3$H-leucine was analyzed when oocytes were incubated in OR2 medium containing 100 fold excess leucine either immediately (---) or after three hour incubation in OR2 (--------) following coinjection of native zein mRNA (1 $\mu$g/$\mu$l) and $^3$H-leucine (10 $\mu$Ci/$\mu$l, 115 Ci/mmol) in a 50 nl injection per oocyte. At 2, 3, 4, 6, and 15 hours after the coinjection, samples were taken from two groups of five oocytes by dissecting and pooling by half. The ethanol-soluble fraction, prepared from 70% ethanol containing 5% 2-mercaptoethanol, was precipitated on 3MM paper in 10% TCA and counted in a scintillation spectrometer. Total zein counts per oocyte are presented against time. Insert: Incorporation of $^3$H-Leu into zeins was measured at 0.5, 1, 2, 3, and 4 hours to analyze the kinetics for a narrower time range after coinjecting the identical amounts of mRNA and $^3$H-Leu. The proteins were extracted with ethanol from two sample groups of three oocytes without dissecting the oocytes by half.
Figure 3. Time-dependent $^3$H-leucine Incorporation into zein.
In separate experiments, the incorporation of $^{3}$H-Leu into zeins was measured at 0.5, 1, 2, 3, and 4 hour to analyze the kinetics for a narrower time range after coinjecting the identical amount of mRNA and $^{3}$H-Leu used in the aforementioned experiment. At each time point, the proteins were extracted with ethanol from two sample groups of three oocytes without dissecting the oocytes by half. The kinetics of zein synthesis are presented in the insert (Figure 3). When the oocytes were initially incubated in the OR2 free from leucine, the $^{3}$H-leucine was also incorporated into zein in linear fashion. In all cases, the rate of $^{3}$H-leucine incorporation into zeins became minimized within one half to two hours after the oocytes were placed in OR2 medium containing 100 fold excess of unlabeled leucine. These parallel experiments indicate that the labeling leucine pool can be diluted in the oocytes no later than two hours within the chase period, and that zeins are no longer significantly labeled during synthesis after two hours into the chase period.

2. Zein mRNA does not diffuse during the labeling period

Since diffusion of the mRNA is limiting the effective labeling period, the extent of zein mRNA diffusion was analyzed indirectly by the distribution of labeled zein by hemisphere. Oocytes were injected with 50 ng of native zein mRNAs into the vegetal pole and incubated in OR2 medium for the times indicated (0.5, 2, 4, 6, and 8 hours). After those time periods, two groups of three oocytes were injected with 0.5 $\mu$Ci of $^{3}$H-leucine at the equator and allowed to incubate three hours in OR2. After the three hour labeling, oocytes from each incubation were dissected into halves and the halves were collected. Proteins were extracted in 70% ethanol containing 5% 2-mercaptoethanol and counted in a scintillation spectrometer after precipitation on 3MM paper by 10% TCA.

Figure 4 shows the distribution of radioactive zein measured after 3 hour labeling following mRNA injection at the indicated times. Based upon the distribution of the
labeled zeins, zein mRNAs do not significantly diffuse up to seven hours following the zein mRNA injection, since the majority (93%) of labeled zeins is still localized in the vegetal half, where the zein mRNAs have been initially injected (4 hr bar). Taken together with results from the $^3$H-leucine quenching experiments (Figure 3), these results strongly indicate that zein mRNA diffusion is insignificant during the labeling period and the time it takes to dilute the pool of $^3$H-leucine and that newly synthesized zeins are not significantly labeled during the chase period. Thus, the velocity of the $^3$H-leucine dilution and the insignificant diffusion of zein mRNA do not jeopardize the interpretation of zein movement within the lumen of ER based on the data from the pulse-chase experiments.

3. 27K zein and total zein are free to move in the lumen of the ER.

The potential transmembrane feature of the 27K zein was further investigated by a pulse-chase assay according to Ceriotti and Colman (1988). 27K mRNA (25 ng; see Materials and Methods) was coinjected with $^3$H-leucine (0.5 µCi) in a 50 nl injection volume either into the vegetal or animal hemisphere. Oocytes were incubated in OR2 for three hours; then half of the oocytes were manually dissected and pooled by hemisphere. The remaining half of the oocytes were chased in OR2 containing 100 fold excess of non-radioactive leucine for 12 hours.

Zeins were extracted directly from the separately homogenized hemispheres in 70% ethanol containing 5% 2-mercaptoethanol. Ten microliter of the zein extraction was spotted on 3MM, precipitated in 10% TCA, and radioactivity was quantitated in a scintillation spectrometer. The remaining zein were completely dried in a vacuum desiccator, electrophoresed on a 15% polyacrylamide gel, and subjected to fluorography.
Figure 4. Zein mRNA diffusion

Zein mRNA diffusion was analyzed indirectly by the distribution of the radioactive zein by oocyte hemisphere. Oocytes were injected with 50 ng of native zein mRNAs into the vegetal pole and incubated in OR2 medium for the times indicated (0.5, 2, 4, 6, and 8 hours). After each of those time periods, two groups of three oocytes were injected with 0.5 μCi (115 Ci/mmole) of 3H-leucine to allow the labeling of zeins by incubation for three hours in OR2. Oocytes from each incubation were dissected into hemispheres, which were pooled separately. Proteins were extracted in 70% ethanol containing 5% 2-mercaptoethanol and counted in a scintillation spectrometer after precipitation on 3MM paper by 10% TCA. Dark bars refer to the portion of radioactive protein found in the animal half, whereas, light bars refer to that in vegetal half.
Figure 4. Zein mRNA diffusion
Figure 5 shows the fluorograph in which the distribution of 27K zein is visualized after 3 hour labeling (A) and at 12 hour chase (B) by PAGE and fluorography following polar injection at the vegetal pole. Most of the labeled 27K zein is restricted to the injected vegetal half at three hours following coinjection of 27K zein mRNA and $^3$H-leucine. After 12 hours of chase with 100 fold excess non-radioactive leucine, however, the 27K zein appears to have reached an equilibrium between the two hemispheres (B). This movement pattern is very similar to that of ovalbumin.

In control experiments to measure the rate of a secretory protein's movement in the ER, twenty oocytes were injected with ovalbumin mRNA (10 ng) and $^{35}$S-methionine (0.4 μCi, 1170 Ci/mmmole) and incubated in OR2 for three hours. Half of the incubated oocytes were removed after 3 hours of labeling and the remaining half were chased in OR2 containing 100 fold excess non-radioactive methionine. After the oocytes were dissected and pooled as described in the pulse-chase for 27K zein, the pooled halves were homogenized by half in a homogenization buffer (see Materials and Methods). Following homogenization, ovalbumin was immunoprecipitated as described in Materials and Methods and the labeled ovalbumin visualized by fluorography following SDS-gel electrophoresis. In parallel control experiments with the ER transmembrane protein HA<sub>env</sub>, twenty oocytes were injected with 5 ng of <i>in vitro</i> transcribed HA<sub>env</sub> mRNA and 0.4 μCi $^{35}$S-methionine per oocyte. The movement of HA<sub>env</sub> was determined by immunoprecipitation and fluorography as for ovalbumin (see Materials and Methods).

The movement of ovalbumin in the lumen of the ER is visualized in Figure 6 after PAGE and fluorography. As is expected for a secretory protein, the ovalbumin, restricted to the vegetal pole during the three hour labeling period (Figure 6A), is shown to move readily in the lumen of the ER during the 12 hour chase (Figure 6B). The mutant
Figure 5. 27K zein movement after localized translation.

Following coinjection of mRNAs (25 ng) and labeling $^3$H-leucine (0.5 μCi, 115 Ci/m mole) in a 50 nl injection, proteins made in oocytes were analyzed both immediately after 3 hour labeling (A) and after 12 hour chase (B). Oocytes from each incubation were dissected into halves and the halves were pooled by hemisphere. Proteins, extracted in 70% ethanol containing 5% 2-mercaptoethanol, were electrophoresed on a 15% SDS-polyacrylamide gel and subjected to fluorography as described in Materials and Methods. Each lane contains the equivalent of 10 oocyte halves. VH: Vegetal Half; AH: Animal Half.
Figure 5. 27K zein movement after localized translation.
Oocytes were injected with 10 ng of ovalbumin mRNAs and 0.4 μCi $^{35}$S-methionine in a 50 nl injection sample and incubated in OR2 medium for three hours. After this incubation, half of the oocytes were dissected into hemispheres which were pooled separately. The rest were incubated in OR2 containing 100 fold excess non-radioactive methionine for 12 hours and dissected by hemisphere. The collected hemispheres were homogenized in a homogenization buffer (see Materials and Methods). Ovalbumin was immunoprecipitated using specific antisera, electrophoresed, and subjected to fluorography as described in the Materials and Methods. Each lane holds the equivalent of 10 oocyte halves. VH: Vegetal Half; AH: Animal Half.
Figure 6. Ovalbumin movement after localized translation.
Figure 7. Distribution of the mutant hemagglutinin (HA\textsubscript{env}) after localized translation.

Following coinjection of \textit{in vitro} transcribed HA\textsubscript{env} mRNA (5 ng) and \textsuperscript{35}S-methionine (0.4 \(\mu\)Ci, 1170 Ci/m mole) in a 50 nl volume, twenty oocytes were incubated in OR2 medium for three hours. After this time period, ten oocytes were dissected into halves and pooled by half. The remainder of oocytes were incubated in OR2 containing 100 fold excess non-radioactive methionine. After the pooled halves were homogenized in a homogenization buffer, HA\textsubscript{env} was immunoprecipitated, electrophoresed, and subjected to fluorography as described in Materials and Methods. VH: Vegetal Half; AH: Animal Half.
Figure 7. Distribution of the mutant haemagglutinin (HAenv) after localized translation
hemagglutinin ($\text{HA}_{\text{env}}$), the ER transmembrane control, does not show any movement even after the 12 hour chase periods (Figure 7). Its labeled protein distribution appears identical after the labeling and chasing periods (Figure 7A and 7B).

Symmetrical distributions of protein were observed in all cases when the mRNAs were injected either at the vegetal pole or the animal pole (data not shown). In later experiments, therefore, samples were injected into the vegetal pole in order to monitor the protein movements.

The relative distribution of these proteins is quantitated in Figure 8. Figure 8A summarizes the distribution of proteins, after the three hour labeling period. At this time, all of the four sets of proteins used in this study show a similar pattern; the majority of the labeled proteins are restricted to the vegetal half, where mRNAs and labeling isotope were injected. After the 12 hour chase (Figure 8B), the distribution of $27K$ zein exhibits a very similar pattern to ovalbumin, which is indicative of free movement in the lumen of the ER. Whereas, the mutant hemagglutinin shows lack of movement, different from the other proteins used in these experiments.

Figure 8 also displays that total zeins are capable of freely moving in the lumen of the ER like ovalbumin and $27K$ zein. After the 12 hour chase, the radioactive total zeins are located evenly across the hemispheres, different from the transmembrane control $\text{HA}_{\text{env}}$ (Figure 8B). In addition, that all zeins were present in the total zein complexes were verified after gel electrophoresis and visualization of zeins by fluorography and immunodetection on Western blots (data not shown).
Figure 8. Time-dependent distribution of proteins in ER.

The time-dependent distribution of 27K, total zeins, ovalbumin, and a mutant hemagglutinin in the ER. (For injections see Figures 5-7.) Immediately after 3 hour incubation in OR2, the distribution of radioactive proteins was measured by half (Panel A). The oocytes were then incubated in OR2 containing 100 fold excess of non-radioactive leucine/methionine for 12 hours and the distribution of the radioactive proteins was analyzed by half (Panel B). Dark bars represent the portion of radioactive protein found in animal half while the light bars represent that in vegetal half. (HA_{env}: an ER-transmembrane hemagglutinin envelope protein; Oval: Ovalbumin)
Figure 8. Time-dependent distribution of proteins in ER.
DISCUSSION

The 27K zein plays a major role in protein body formation. The 27K zein accumulates in the earliest (smallest) endosperm protein bodies (Lending and Larkins, 1989). Heterologous studies using Xenopus oocytes also support 27K zein's significance in the protein body formation (Wallace et al., 1988, Lending and Larkins, 1989). Oocytes injected with mRNAs of 27K zein and α–zein assembled zein polypeptides into denser vesicles which were of density equivalent to native zein protein bodies whereas those injected singularly with α–zein mRNA assembled zein polypeptides into less dense vesicles (Wallace et al., 1988; Lending and Larkins, 1989).

Recent studies suggest that the 27K zein may have a potential transmembrane feature which facilitates zeins' retention and protein body formation in the lumen of the ER. The 27K zein has a primary structure which is typical for a transmembrane protein (Johnson, 1989). The 27K zein is detected primarily at the proximity to the protein body membrane by immunogold labeling (Ludevid et al., 1984; Lending et al., 1988; Lending and Larkins, 1989). Larkins et al. (1991) and Geetha et al. (1991) have suggested that in quality protein maize (QPM) an increased amount of the 27K zein is responsible for converting the opaque phenotype to the vitreous phenotype. They believe that the 27K zein functions in this phenotype conversion through its transmembrane domain which facilitates the close packing of starch granules.

Despite these circumstantial evidences, this present study suggests that the 27K zein may not span the membrane of protein vesicles. Throughout the three categories of assays, the potential transmembrane feature of zein has not been supported. The 27K zein associated with the protein vesicles was not affected in situ by proteinase K treatment (lanes c-d in Figure 1, A and B) unless a non-ionic detergent was added in the reaction (lane b in Figure 1, A and B). Likewise, 27K zein did not react with biotinylating agents
(lane b in Figure 2) although its association with the protein vesicles was confirmed by ECL detection system using 27K antisera (lane d in Figure 2). These two assays indicate that 27K zein may not protrude out of the protein vesicles. These observations are in agreement with an in vitro assay by Torrent et al (1986) showing that in vitro translated 27K zein is protected from proteinase K by microsomal membranes, suggesting that it is inside the membranes. Therefore, 27K zein appears to be contained within the protein vesicles and may not serve as a protein body nucleating factor by virtue of its ER transmembrane feature.

An argument can be made that 27K zein's resistance to proteinase K digestion and biotinylation may be the outcome of a blocking effect by oocyte yolk that is non-specifically bound to the 27K zein or of an insufficient size of the 27K zein exposed. If a small segment of the N-terminus or the C-terminus is exposed, it may not show a visible size reduction. In addition, only the N-terminal amine can be biotinylated so the N-terminus must protrude in order to be labeled by this procedure. The pulse-chase experiment, therefore, was used to assay whether 27K zein is stationary in the ER based on its redistribution pattern after the protein synthesis.

Observations from the pulse-chase experiment strongly indicate that the 27K zein does not span the ER membrane; rather, it distributes itself across both oocyte hemispheres during the chase period (Figures 5 and 8) and obtains an equilibrium of nearly equal mass in the two hemispheres (Figures 5 and 8), indicating that it freely moves through the lumen of the ER like a secretory protein such as ovalbumin (Figures 6 and 8), totally different from a mutant hemagglutinin (HAenv), a mutagenized ER transmembrane protein (Figures 7 and 8). Since the incorporation of \(^{3}\text{H}-\text{leucine}\) into zein was quenched within one half to two hours after placing the oocytes in excess unlabeled leucine (Figure 3) and zein mRNAs did not significantly diffuse for seven hours (Figure 4), the radioactive
zeins' distribution during the pulse-chase correctly represented the extent of the time-dependent movement. This free movement even excludes the possibility that 27K zein interacts with ER membrane or membrane-bound factors to be retained in the ER. The free movement of 27K zein also implies that, for a significant period of time after zein polypeptides is completed, interactions between membrane and 27K zein may not be responsible for zein's ER retention.

The fluidity of biological membranes may provoke an argument that the distribution pattern of 27K zein may result from the membrane fluidity even though 27K zein is anchored to the membrane. This argument has been negated by contrasting the distribution pattern of 27K zein to the H1Env (an ER transmembrane protein) which did not show any change in terms of the protein distribution after the chase period (Figures 7 and 8). In addition, the estimated diffusion time for a membrane-bound factor also supports this interpretation. A membrane-bound factor, for example vertebrate rhodopsin, normally has a diffusion coefficient of 5 X 10^-9 cm^2/sec. It would take at least 250 hours for a membrane-bound factor to show the pattern of 27K zein observed in these experiments (Ceriotti and Colman, 1988). Along with the proteinase K and biotinylation experiments, therefore, results from the pulse-chase experiments strongly suggest that 27K zein does not span the ER membrane.

The 27K zein has a strong tendency for oligomerization through cross-linking by disulfide bonds (Lopes and Larkins, 1991). Most likely, 27K zein polypeptides form high-molecular-weight oligomers immediately after their synthesis and translocation into the lumen of the ER. With the amount of mRNAs injected in this study (more than 20 ng per oocyte), the ER membranes in the entire oocyte would be saturated with 27K zein mRNA when the mRNA distribution is complete based on competitive translations of membrane bound zein and ovalbumin mRNA (Richter and Smith, 1981; Richter et al., 1983; Ceriotti
et al., 1991). The 27K zein is, thus, abundant in the ER lumen. Based on calculations (Shih et al., 1978; Larkins et al., 1979) assuming that the size of the internal leucine pool is 160 pmoles (Larkins et al., 1979), 27K zein should be accumulating up to 39.5 ng (1.7 pmoles or 1.02 X 10^{12} molecules) at the end of the 15 hour incubation. This amount may be sufficient for the ER lumen, whose volume is less than 100 nl or 15% of the oocyte volume, to be densely loaded with 27K zein. Following protein synthesis, therefore, the majority of 27K zein molecules are believed to form oligomers through the disulfide bonds in the ER. The observed free movements of 27K zein strongly suggest that 27K zein is capable of moving in the lumen of the ER in aggregated forms.

Total zeins were also shown free to move in the lumen of the ER (Figure 8). On the basis of the similar calculation for the 27K zein, total zeins were synthesized approximately in 69 ng (3 pmoles or 1.8 X 10^{12} molecules) which filled the ER lumen at an estimated concentration of 4.3 X 10^{-3} M. Zeins have a dissociation constant (Kd) of 4.6 X 10^{-6} M (Johnson, 1989). With this estimated zein concentration, zeins are expected to aggregate following their synthesis. In addition, the presence of all classes of zeins in both oocyte hemispheres was verified both by immunodetection and fluorography. The free movement of total zeins, therefore, also suggests that the total zeins are capable of moving as aggregates containing all classes of zeins. According to the observed free movements of 27K zein and total zeins, it is tempting to suggest that the interactions among the various zein classes, leading to insolubility and exclusion from ER luminal fluid, may not be an important factor for zein's ER retention even though the zein interactions are likely to be the driving force in zein protein assembly and protein body formation.

The free movement of zein in this study may be interpreted in several ways. The free movement may be indicative of heterogeneous conditions (spatial discrimination) for
zein deposition within the lumen of the ER. In other words, zein deposition preferentially occurs at certain locations along the ER cisternae. Zein may freely move until it finds an appropriate location for accumulation. The same mechanisms appear to govern protein body formation in both maize endosperm and *Xenopus* oocytes since zein vesicles are formed with identical densities. Because 27K zein and total zeins are abundantly present in the lumen of the ER, their free movements may be an indication that zeins require a certain period of time to grow into a threshold mass before precipitating out from the luminal fluid. Before the threshold point, zeins may be free to move in the lumen of the ER at concentrations where it is expected that zeins form aggregates. Therefore, zeins may have to be accompanied by a retention mechanism to assist zeins' retention in the lumen of the ER for sufficient period of time, until zeins find a suitable location or reach the threshold aggregate in the presence of the indiscriminate bulk flow. The free movements of 27K zein and total zeins in this study imply that additional retention mechanisms are required for zeins to remain in the lumen of the ER where their protein bodies are assembled.

Zein polypeptides may be retained in the lumen of the ER because they spontaneously form oligomers which are unsuitable for transport out of the ER compartment due to misfolding. Except for some soluble proteins, acquisition of proper conformation is absolutely required for proteins to exit the ER compartment (Hurtly and Helenius, 1989). Zeins may not acquire proper conformation following oligomerization or aggregation in the ER. In most case, however, the misfolded (or unsuitably assembled) proteins are assisted in the ER compartment by molecular chaperons (e.g., BiP) to reach the proper conformation; then, the corrected proteins are included in the transport pathway. If the suitable conformation is not achieved, the proteins are eventually degraded in the ER. Therefore, the noticeable presence and accumulation of zeins in the ER cannot be explained by their exclusion from the default transport because of the improper
conformation for exiting from the ER.

Munro and Pelham (1987) suggested that a membrane-bound receptor exists which shuttles between ER and a post ER compartment, most likely cis-Golgi. This receptor might be responsible for retrieval of an ER resident protein from the cis-Golgi. This postulated retrieval mechanism is not contradictory to the observations made in this study since this mechanism does not require a transmembrane feature and the protein would be retrieved from the cis-Golgi to random locations along the ER. However, the postulated retrieval mechanism cannot be tested in vivo for zein proteins since they are not posttranslationally modified (e.g., glycosylation, phosphorylation). Without any possible assay focusing on the posttranslational modification, the postulated trip of zein to the cis-Golgi is very difficult to verify in vivo. In most cases, the retrieval is considered to be mediated by an ER retention amino acid signal.

Most of ER resident proteins have the typical signal sequence: KDEL/HDEL (Lys/His-Asp-Glu-Leu). This message is so powerful that a post ER protein was retrieved from the cis-Golgi and retained in the ER compartment. Munro and Pelham (1987) showed that a KDEL attached secretory protein was retrieved from the Golgi complex in an animal system. Also in a plant, KDEL attached to vicilin, a vacuolar storage protein, inhibited vicilins to exit from the ER compartment (Wandelt et al., 1992). In this case, probably by avoiding degradation of the protein both in vacuole and during the protein targeting process, the ER retention increased the level of protein 100 times in comparison to the wild type counterpart.

Zeins do not have retention signals similar to KDEL/HDEL. Zeins may have unknown ER retention signals; the possibility may be minimal since the sorting signal for the ER retention is highly conserved across animal and plant systems during evolution (Denecke et al., 1992). In most cases, single amino acid substitution within KDEL/HDEL
can cause a complete loss of its function as an ER retention signal (Denecke et al., 1992). This fact strongly suggests that zein's ER retention and its protein body formation may not depend on their own ER retention signal.

Recent studies may provide a solution to the above contradiction: zeins behave in the lumen of the ER as a protein retained by an ER sorting signal; however, they do not have an appropriate ER retention signal. Zein can be retained in the ER as a result of interaction with other proteins which do have the ER retention signal (HDEL) (Boston et al., 1991). Maize b-70 has been suggested as a candidate to assist zein to remain in the lumen of the ER (Boston et al., 1991; Chrispeels and Raikohl, 1992). B-70 carries a HDEL sequence at the proximity to the carboxyl terminal and has sequence homology to polypeptide chain binding proteins (Boston et al., 1991). The maize b-70 is associated both with zein protein bodies and with the rough endoplasmic reticulum (Galante et al., 1983; Salamini, 1985). The level of b-70 expression appears to correlate to the extent of deformation in protein body morphology. The flo2 mutation, carrying the most severely altered protein bodies, shows the highest level of b-70 expression while the o2 mutation, whose zein level appears most reduced, shows the level of b-70 equivalent to wild type (Marocco et al., 1991; Boston et al., 1991). Except for the o2 mutation, the expression of b-70 is inversely proportional to the level of zein deposition: the more severe the reduction of zein content, the higher the level of b-70 (Marocco et al., 1991; Boston et al., 1991). In wild type endosperm, the level of b-70 is very low compared to other mutations affecting zein accumulation although the wild type would need more b-70s to retain zeins. Even if b-70 functions as a zein-specific retainer in maize endosperm, zeins' ER retention in the oocytes cannot be explained by the postulated mediation of b-70 since the oocytes do not contain an equivalent of b-70 or BiP. Therefore, b-70 does not seem to be a likely candidate for the ER retention of zeins.
The b-70 has been suggested as a plant homolog of immunoglobulin heavy chain binding protein (BiP), known as a molecular chaperone (Marocco et al., 1991; Fontes et al., 1991; Boston et al., 1991; Miernyk et al., 1992). Sequence homology and cross-reactivity reveal that b-70 is a plant version of the heat shock protein-70 superfamily (hsp-70) and a homolog of Binding Protein (BiP). BiP, an ER-resident protein, plays a very important role in catalyzing the correct folding and assembly of polypeptides into oligomers by binding to the nascent protein bearing a misfolded domain. On the basis of the biochemical studies (Marocco et al., 1991; Fontes et al., 1991; Boston et al., 1991; Miernyk et al., 1992), b-70 has been postulated to function as a BiP in maize endosperm. However, this suggestion cannot be further supported. If b-70 functions as a BiP, misfolded zeins should be predominantly retained in the ER excluding normal zeins.

The results presented in this study rule out the possibility that 27K zein serves as an anchor for protein body formation. The observed free movements of 27K zein and total zeins suggest that the interactions among the various zein classes, leading to insolubility and exclusion from the ER lumenal fluid, may not play an important role in zeins' ER retention even though the zein interactions are likely the driving force in zein protein assembly and protein body formation. Zein's ER retention may be a separate occurrence from zeins' precipitation into a hydrophobic aggregate finalizing the protein body formation. Zein may be initially retained in the lumen of the ER largely based upon binding by an ER resident protein; subsequently, the hydrophobic interaction among zeins will increase as the concentration of zeins rises in the lumen of the ER and as the endosperms become mature.

Investigations aimed at determining the rate of interaction between zeins and an ER-resident protein, having an ER retention signal, may be very fruitful in understanding the zein's ER retention. Kinetics of association and dissociation of the ER protein against
increments of zein concentration may provide a meaningful lead in determining the postulated role as the zein binding factor. In the future, similar investigation described in this present study must be performed in plant cells, hopefully in a transgenic maize plant.

This study rules out a possible mechanism for zein's ER retention and protein body formation in the ER through anchorage of 27K \( \gamma \)-zein to the ER membrane. This study provides data that 27K zein does not span the membrane and suggests that 27K zein freely moves within the lumen of the ER based upon comparisons of the \textit{in vivo} migration rate against a secretory protein (ovalbumin) and an ER transmembrane protein (HA\textsubscript{env}). The free movement of 27K zein and total zeins under conditions where zein aggregates should form necessitates a reevaluation of the significance of zein aggregation toward zein polypeptides' ER retention and protein body formation. This study concludes that 27K zein is not a protein body nucleating factor by virtue of an ER transmembrane feature or association with the ER membrane and that the significance of zein solubility should be reconsidered to explain the zeins' ER retention leading to protein body formation in the ER.
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APPENDIX 1

Compositions of Poly (A)+ RNA Isolation Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>HEPES (M, pH 7.5)</th>
<th>NaCl (M)</th>
<th>KCl (M)</th>
<th>EDTA (M)</th>
<th>SDS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Binding</td>
<td>0.02</td>
<td>1</td>
<td></td>
<td>0.004</td>
<td>1</td>
</tr>
<tr>
<td>1X Binding</td>
<td>0.01</td>
<td>0.5</td>
<td></td>
<td>0.002</td>
<td>0.5</td>
</tr>
<tr>
<td>Wash</td>
<td>0.01</td>
<td>0.5</td>
<td></td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0.01</td>
<td></td>
<td>0.5</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Low KCl</td>
<td>0.01</td>
<td></td>
<td>0.3</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Elution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX 2

Composition of *in vitro* Transcription by SP6 Polymerase

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc. Rxn$^1$ (mM)</th>
<th>Stock solution (mM)</th>
<th>V. Added$^2$ (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H-UTP</td>
<td>0.125</td>
<td>40 Ci/m mole</td>
<td>50</td>
</tr>
</tbody>
</table>

(Dried in an Eppendorf microtube and followings are added into the tube by mixing)

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc. Rxn$^1$ (mM)</th>
<th>Stock solution (mM)</th>
<th>V. Added$^2$ (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer</td>
<td>10</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>ATP</td>
<td>0.5</td>
<td>20</td>
<td>2.5</td>
</tr>
<tr>
<td>CTP</td>
<td>0.5</td>
<td>20</td>
<td>2.5</td>
</tr>
<tr>
<td>GTP</td>
<td>0.05</td>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>UTP</td>
<td>0.4875</td>
<td>20</td>
<td>2.4</td>
</tr>
<tr>
<td>CAP (m$^7$ GpppG)</td>
<td>0.5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Spermidine</td>
<td>4</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>SP6 polymerase</td>
<td>2 units/µg DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$O</td>
<td>variable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSP6 DNA (linearized)</td>
<td>0.5 -1.0 µg/µl</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>(Incubation for 45 minutes at 37°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>0.5$^3$</td>
<td>20</td>
<td>2.25</td>
</tr>
<tr>
<td>(Incubation for 1 hour at 37°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$Final concentration in the reaction mixture

$^2$Volume of stock solution added to the reaction mixture

$^3$Including the previous addition before 45 minute incubation
APPENDIX 3

LIST OF ABBREVIATION

SDS-PAGE: SDS-Polyacrylamide gel electrophoresis

10K zein: zein having apparent molecular weight of 10,000 on polyacrylamide gel
15K zein: zein having apparent molecular weight of 15,000 on polyacrylamide gel
16K zein: zein having apparent molecular weight of 16,000 on polyacrylamide gel
19K zein: zein having apparent molecular weight of 19,000 on polyacrylamide gel
22K zein: zein having apparent molecular weight of 22,000 on polyacrylamide gel
27K zein: zein having apparent molecular weight of 27,000 on polyacrylamide gel

Kd: Kilodalton

DAF: Days after flowering

DAP: Days after pollination

DTT: Dithioreitol

EM: Electron Microscope
Dong-Hee Lee was born in Seoul, Korea July 1, 1957. His parents always emphasized the importance of knowledge and faith. They encouraged him to read and study hard to become a good scientist. He was very interested in the phenomenon of life when he had witnessed his grandfathers' death. At Seoul National University, he majored in life sciences. He served the compulsory military service from 1977 to 1980. During the military service, he stationed near DMZ where he learned how to endure and cope with adversity. Fortunately, he had a opportunity to learn advanced chemistry and physics while he was a student at Korean Armed Forces Medical School. After returning to Seoul National University to continue his undergraduate work, he prepared to study abroad to further his interest in life science. He was married to Hyunsook who was also eager to advance her interest in Family Studies at the graduate level. In 1986, he came to the States to study plant physiology at Bowling Green State University where he spent considerable time to study plant regeneration and photosynthesis. After graduating from Bowling Green State University, his interest was slightly modified to study plant molecular biology at Virginia Polytechnic Institute and State University. He graduated from the VPI and SU with a Ph D in Biology October 1992. His dissertation title is "Study of zein protein body formation in a heterologous system (Xenopus laevis oocyte).