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HETEROGENEITY AND EXPRESSION OF THE ZEIN STORAGE PROTEIN GENE FAMILY OF MAIZE

(zein, maize, multiple gene families, in vitro processing, protein synthesis, cloning, low molecular weight PAGE)

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SUMMARY

The storage protein zein is synthesized by polysomes on the endoplasmic reticulum in certain cells of the maize seed. As zein accumulates within the membrane lumen the cisternae are transformed into vesiculate protein bodies. Zein is comprised of a large family of structurally related polypeptides that fall into two classes of 19,000 and 22,500 daltons. On two-dimensional IEF:SDS polyacrylamide gels, the number and positions of the electromorphs within each class differ greatly from one strain to another. Typically, however, there may be 8-12 spots for each class. Within any strain that has been examined the polypeptides constituting each molecular weight class have a similar amino-terminal sequence, but there are a number of position differences when the sequences are compared between strains.

Zeins are made as preproteins that are 1,000 and 2,000 daltons larger than the respective mature light and heavy zein polypeptide classes. As the growing preprotein chain is moved through the ER membrane, the leader sequence is removed by an exopeptidase. One glucose residue is added to the mature portion of the molecule at this time. An in vitro system using maize endosperm membranes has been developed to study the synthesis and processing of maize preproteins. Assessment of the products made indicates that the method faithfully reproduces the in vivo processes.

Cloned cDNA zein sequences were employed to examine the extent of genetic heterogeneity. Based on restriction analyses and dot hybridizations, eighteen zein clones could be divided into three classes for the light zein class and two for the heavy zein class. The Southern transfer hybridizations showed a very complex picture that suggests that zein is encoded by many genomic sequences which share partial homologies. Many more sequences are recognized than there are polypeptides. Possibly some of these represent relic sequences and pseudogenes but until more is known about the structures of the different zein genes, an estimate of the number of copies in the maize genome by hybridization with cloned sequences cannot be made.

INTRODUCTION

Proteins, along with carbohydrates, constitute the main reserve materials of the corn seed. They are both stored principally in the major tissue of the seed, the endosperm. The endosperm is a triploid tissue derived from the second event in double-fertilization, which is the fusion of the second male gamete of the pollen grain with the two polar nuclei of the central egg cell. The reserves stored in the endosperm are not used until seed germination, at which time they are degraded and used to synthesize new compounds required by the emerging plant before photosynthetic competence has been attained.

Four categories of seed storage proteins have been traditionally recognized and these are distinguished primarily by their solubility characteristics: The albumins are soluble in water; the globulins in salt solutions; the prolamines in aqueous alcohols; and the glutelins in dilute alkali. Zein, the storage protein that we have been working on, is a prolamine. The prolamines in each cereal genus have been assigned different names - e.g. gliadin in wheat, hordein in barley (MOSSÉ 1966). They are extremely hydrophobic, as their solubility requirements might indicate. In addition, all contain elevated amounts of proline and share some other similarities in their overall amino acid compositions. The extent of structural similarities between the prolamines of the different cereal genera is not known in detail; however, examining each as a total pool, they are found to have different solubility characteristics and to display different electrophoretic patterns.

Zein is the major protein of the seed and it can represent more than 50% of the storage protein at maturity. It is localized in protein bodies which are concentrated in the outer cells of the endosperm subadjacent to the aleurone. The aleurone, which is the outermost layer of the

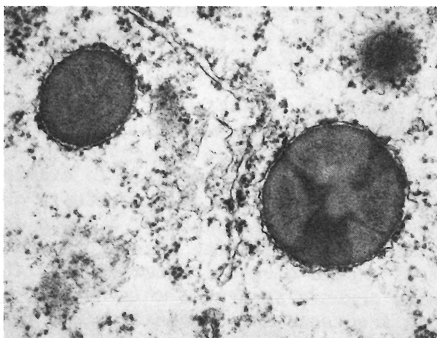

FIGURE 1. Electron micrograph of zein protein bodies and associated polysomes in endosperm cells 18 days after pollination.

endosperm, does not contain zein (Burr, F., unpublished). The zein protein bodies are vesicular, single-membrane bound organelles, 1-2 μm in diameter with an osmiophilic homogeneous matrix (Fig. 1). Attached to the outer surface of the limiting membrane are numerous polyribosomes containing an average of 7-8 monomers (BURR, B. and F. BURR 1976; LARKINS and TSAI 1977; BURR, B. et al. 1978). The protein bodies were first postulated as being the site of zein accumulation by Duvick (1961) on the basis of light microscopic observations. Wolf and Khoo (WOLF et al. 1967; CHRISTIANSON et al. 1968; KHOO and WOLF 1970) substantiated this hypothesis by ultrastructural studies and also documented the apparent origin of the protein bodies from the rough endoplasmic reticulum. The protein bodies were subsequently isolated by sucrose gradient centrifugation and the polysomes eluted from the membrane surface were shown to direct the synthesis of zein (BURR, B. and F. Burr 1976). Similar results were reported using polysomes prepared from total seed membranes (LARKINS et al.) (1976).

On SDS-polyacrylamide gels zein has two bands corresponding to M_r = 22,500 and 19,000 (MISRA 1975; BURR, B. and F. BURR 1976; LARKINS et al. 1976; GIANAZZA et al. 1976). In some maize strains and with other extraction procedures minor components have also been reported (SOAVE et al. 1981). The messenger RNAs coding for zein have been purified from protein body RNA and have been shown to average 1-1.2 kilobases in length (BURR, B. et al. 1978). They have a 110 poly(A) tract at the 3' terminus and a G+C content of 50% (BURR, B. et al. 1981). The zein mRNA can, in fact, be electrophoretically fractionated into two populations which code for the large and small zein polypeptide classes (WIENAND and FEIX 1978). When zein mRNA is translated in vitro, the products are respectively about 2,000 and 1,000 daltons larger than the mature, authentic heavy and light polypeptide classes (BURR, B. and F. BURR 1978; LARKINS and HURCKMAN 1978; VIOTTI et al. 1979). The additions have been shown to be at the amino terminal ends (BURR, F. and B. BURR 1979) and, therefore, constitute presequences typical of those that have been reported for many secretory and membrane proteins (ZIMMERMAN et al. 1980). Xenopus oocytes have been used to examine in vivo synthesis and processing of zein (LARKINS et al. 1979).

Righetti et al. (1977) reported that zein was negative in the periodic acid-Schiffs (PAS) staining test for carbohydrates. However, Burr and Burr (BURR, F. 1979; BURR, F. and B. BURR 1979) using another PAS procedure and ovalbumin and cytochrome c as internal positive and negative controls obtained positive results. Moreover, the latter authors demonstrated by four different quantitative tests that there was one mole of neutral sugar/mole 20,000 dalton polypeptide. Based on tests with glucose oxidase (negative with galactose oxidase), in vivo labeling with ^{3}H-glucose (negative with ^{3}H-galactose), and thin-layer chromatography with reference sugars, the sugar moeity was identified as glucose. While monosaccharide adducts are not common, they

have now been found on lysine side chains of hemoglobin, lens crystallin, collagen, and red cell membrane proteins (see BUNN and HIGGINS 1981 for references). For these proteins the attachment is covalent and appears to be a non-enzymatic process. The covalent binding of glucose to zein remains to be demonstrated although it is unlikely to involve lysine as zein seems to be lacking this particular amino acid.

SYNTHESIS AND PROCESSING OF PREZEINS IN VITRO

We have recently developed a cell-free mRNA-dependent system from maize which synthesizes, processes, and sequesters zein and other preproteins (BURR, F. and B. BURR 1981). Rough endoplasmic reticulum (RER) cisternae vesicles that were isolated from developing maize endosperms were treated with Streptococcus aureus (micrococcal) nuclease to destroy endogenous messages. The treated membranes were added to the wheat germ translation system along with zein mRNA. The >20 zein polypeptides produced in the presence of RER vesicles co-migrated precisely with authentic zeins in two-dimensional isoelectric focussing (IEF):SDS polyacrylamide gels indicating that the synthesis and processing was identical to that occurring in the endosperm cells (Fig. 2a). The removal of the amino terminal zein presequences is a co-translational process for, if completed zein preproteins were incubated with the membrane vesicles, no processing took place. Only five spots which could not be associated with particular authentic polypeptides were seen on the fluorograms (Fig. 2b). A SDS:urea:polyacrylamide gel system which we devised for the analysis of polypeptides below M_r = 10,000 was used to search for possible presequence fragments. As no polypeptides were observed in the expected classes of 1,000 and 2,000 daltons, the presequences must be digested by an exopeptidase rather than cleaved off by an endopeptidase (BURR, F. and B. BURR 1981). The digestion is probably occurring as soon as the amino terminus protrudes into the RER lumen as polypeptides seen in some in vitro translations, which we believe to be premature termination chains, have already lost their presequences.

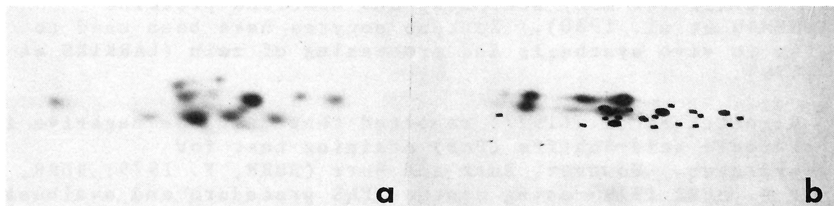

FIGURE 2. Fluorograms of two-dimensional IEF:SDS polyacrylamide gel electrophoresis of: a) processed zein made by zein mRNA + treated endosperm RER - radiogram spots coincided with the Coomassie blue-stained positions of authentic zein electrophoresed in the same gel; b) unprocessed zein synthesized by zein mRNA alone - inked spots indicate the positions of authentic zein.

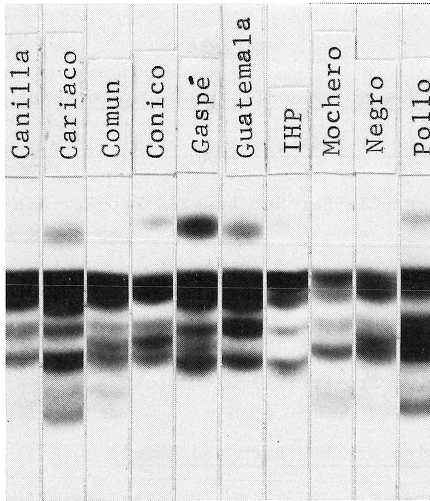

FIGURE 3. 8 M urea, pH 4.5:polyacrylamide gel electrophoresis of zeins from mature seed of selected maize races and Illinois High Protein (IHP), the inbred that we have used for most of our studies.

ELECTROPHORETIC AND AMINO ACID SEQUENCE DATA INDICATING LIMITED HETEROGENEITY OF ZEIN

Righetti et al. (1977) have examined zein from many maize strains by one-dimensional isoelectric focusing and we have analyzed zein from a large number of Central and South American races on acidic urea-polyacrylamide gels (examples of which are shown in Fig. 3). By either method zein separates into as many as twelve or more bands depending on the strain being tested. The resolution is increased by two-dimensional IEF:SDS polyacrylamide gel electrophoresis, and for most strains we have worked with, the two molecular weight classes seen on one-dimensional SDS polyacrylamide gels can be resolved into 15-20+ (Fig. 2). Each inbred strain (HAGEN and RUBENSTEIN 1980) or race (F. BURR, unpublished) appears to have its own characteristic two-dimensional pattern. While some spots are held in common by many strains, others seem to be unique. No changes in the one-dimensional IEF pattern of zein during development were reported for the strain W64A+ or its opaque-2 counterpart (RIGHETTI et al. 1977) although examination of the figure presented in their paper shows that there are at least some quantitative changes between days 21 and 50 postpollination.

Bietz and co-workers (1979) have published amino-terminal sequences for the first 36 residues of a reduced and alkylated total zein mixture prepared from two normal hybrids, (B37TMS x H84) x (Oh43RF x A619) and P-A-G SX52, and P-A-G 50001 opaque-2, the near isogenic counterpart of P-A-G SX52. Alternative amino acids were detected at only a few positions. The

TABLE I

Comparison of Amino-Terminal Sequences of Zeins

I. Heavy class zeins of W64A (Larkins et al. 1979):

Ser/Phe-Ile-Ile-Pro-Gln-Ser-Ser-Leu-Ala-Pro-Ser-Ala-Ile/Ser-Ile-
5 10

II. Heavy class zeins of IHP (Burr and Burr):

Phe-Ile-Phe-Pro-Gln-Asp-(Ser)-Leu-Ala-Pro-(Pro)-Ala-Ile-Leu-Pro-
5 10 15
Gln-Phe-Leu-Pro-Pro-Val-Gln-Asp-(Ala)-
20

Light class zeins of IHP (Burr and Burr):

Thr-Ilu-Phe-Pro-Gln-(Ser)-(Ser)-Gln-Ala-Pro-Ilu-Ala-(Ser)-Leu-Leu-
5 10 15
Pro-Pro-Tyr-Leu-Gln-Pro-Ala-Val-Arg-(Ser)-Phe-Arg-
20 25

III. Unseparated light and heavy classes of (B37TMS x H84) x (Oh43F x A619) (Bietz et al. 1979):

Thr/Phe-Ile-Phe/Ile-Pro-Gln-Cys-Ser-Gln/Leu-Ala-Pro-Ile-Ala-Ile-Leu-Leu-
5 10 15
Gln-Phe/Pro-Tyr-Leu/Phe-Pro-Val-Ala-Val-Met-Gly-Val/Ile-Ala-Phe-Tyr-Gln-
20 25

authors, therefore, concluded that the two molecular weight classes seen on SDS-polyacrylamide gels had common sequences to a great extent. Moreover, when the hybrids were compared, the zeins for each were found to differ at only two positions. Normal and opaque zeins had few minor differences. Larkins et al. (1979) have reported data on the first twelve amino acids of the heavy class zeins from W64A. Two alternative residues were noted for the first and thirteenth positions. We obtained partial sequences for the amino-terminal ends of the separated heavy and light zein classes of the strain Illinois High Protein. The analyses were performed as follows: Heavy and light chains of zein prepared from 15-18 day postpollination endosperms were separated on 0.1% SDS:15% polyacrylamide preparative gels. Five mg, or approximately 250 mmoles of protein, at a concentration of 10 mg/ml in 1% SDS (Bailey et al., 1977) were subjected to automated Edman degradation on a Beckman 890C sequencer with the N,N-dimethyl-N-allylamine program No. 102974. The yields were higher using the dimethylallylamine buffer rather than Quadrol. Phenyl-thiohydantoin amino acids were identified by thin layer chromatography on Merck Silica Gel 60 F-254 sheets in chloroform-ethanol (98:2) and chloroform-methanol (90:10). After some cleavages,

derivatives were hydrolysed in 6 N HCl with 0.01% $SnCl_2$ and subjected to automated amino acid analysis. Although some carry-over of residues into the subsequent cleavage was noted due to the solubility difficulties of zein, there was no indication of heterogeneity at any position as might be expected if zein were heterogeneous. Minor variants (<5%), however, would have escaped detection. Comparison of the heavy and light class sequences (Table 1) shows that at least seven of the first fourteen residues are identical but the subsequent positions are quite dissimilar. The sequences from Bietz et al. (1979) and Larkins et al. (1979) are included for comparison. While the data shows some similarities, there are also many differences. It is not known whether these differences can be ascribed to technical problems caused especially by the extreme hydrophobicity of zein or whether they are due to strain differences as each group has used different strains. Assuming, however, that the zein polypeptides are derived from the same ancestor sequence, the homology found in the first fourteen residues of both classes may reflect the necessity of a common tertiary structure that is required for proper presequence recognition and processing.

The picture that emerges from the electrophoretic analyses and amino acid sequencing suggests that zein is a very large family of polypeptides with limited heterogeneity. It would be important to know whether each polypeptide spot seen on a two-dimensional IEF:SDS polyacrylamide gel corresponds to a different structural gene or whether co-translational or posttranslational modifications such as amidation (or deamidation), phosphorylation, or glycosylation are responsible for generating some of the observed diversity. It is estimated that about 85% of the glutamic and aspartic acid residues are amidated. Deamidation had been suggested earlier as a possible contributor to zein heterogeneity (BURR, B. 1979; RIGHETTI et al. 1977). If partial deamidation were occurring in vivo it would be expected to be a random, uncontrolled event that would probably increase with age. No qualitative changes in development, however, have been observed (SOAVE et al. 1978). Our in vitro processing results have shown an exact correspondence of position of the synthetic and authentic spots on two-dimensional gels and the particular pattern produced is dependent on the strain used to prepare the mRNA. If polypeptide modifications are occurring, the changes must be specific, genetically controlled steps.

The extent of genetic diversity has also been studied with the aid of cloned zein sequences. Double-stranded cDNA was constructed from purified zein mRNA and inserted into the bacterial plasmid pMB9 by dA:dT ligation (BURR, B. 1979; BURR, F. and B. BURR, 1980; BURR, B. et al. 1981). From the recombinant clones constructed, a random selection of twenty clones was selected for characterization. The inserts, including the poly dA:dT tails varied from 90 to 1450 base pairs. Eighteen of the twenty clones were found to have sequences homologous to zein mRNA by hybridization of total

poly(A)+ endosperm RNA to the recombinant plasmid and translation of the specifically bound mRNA in the wheat germ cell-free system. The remaining two did not hybridize any translatable mRNA. On this basis the clones could be subdivided into light or heavy class zein polypeptide clones (PARK et al. 1980; BURR, B. et al. 1981). This had also been shown for other zein cDNA clones by Wienand et al. (1979).

Restriction enzyme recognition sites of the DNA inserts were examined with Alu I, Bam HI, Bst EII, Eco RI, and Pst I. Clones having inserts that were cleaved by common enzymes were placed in the same subgroup. On this basis the heavy class zein clones were divided into three subgroups and the light class clones into four subgroups. The classification is provisional for a more detailed restriction analysis which included other restriction enzymes could further subdivide the groups. Examination of other clones could also increase the number of groups. It should be noted that these subgroups only serve to indicate very crude similarities and should not be construed to imply identity. The data can only be used to give an idea of the heterogeneity existing among the cloned representatives.

The eighteen clones identified as having zein sequences were also analyzed by dot hybridizations (BURR, B. et al. 1981). Equivalent amounts of DNA from each clone was spotted onto a nitrocellulose filter. Each filter was then hybridized with a radioactive probe prepared from the insert of one of four selected zein clones for the light and heavy classes. The size of each spot on the autoradiograph of the filter was used to measure the relative degree of homology between the labeled probe and the particular clone being tested. The results obtained essentially confirmed the classification constructed by the restriction enzyme analyses - that is, members of the same subgroup were recognized by the same probe. The number of subgroups, however, was reduced from three to two for the heavy class and from four to three for the light class. The conditions used for these hybridizations were very stringent; if they were relaxed all the clones hybridized with the probes.

The same four clones employed in the dot hybridizations were also used to prepare probes to assess the extent of zein sequence heterogeneity in maize genomic DNA. Maize DNA was prepared from shoot axes of etiolated seedlings and digested with the restriction enzymes Bam HI, Eco RI, and Hind III. The products were electrophoresed on neutral agarose gels, transferred to nitrocellulose filters, and hybridized with a radioactively labeled probe. Each probe recognized between three to 12 bands depending on the enzyme used. The fragments varied from 1-33 kilobase pairs in length. Although each probe recognized a distinctive set of fragments, certain fragments were apparently cross-recognized by different probes indicating shared homologies.

The genes for chorion, which is made in large amounts at a specific time in development, are known to undergo

amplification (SIM et al. 1979; SPRADLING AND MAHOWALD 1980). The genomic sequences of zein were probed for amplification using DNA prepared from endosperm and shoot material. A cDNA clone for the enzyme sucrose synthetase (BURR, B. and F. BURR 1981) was used for comparison. Transmission genetics had previously shown that this enzyme is encoded by a single gene. While only one band was detected for sucrose synthetase by Southern transfer hybridizations, many bands were found for zein - one clone recognized about 24 bands (BURR, B. et al. 1981). Some of the bands were much more intense than the sucrose synthetase band indicating that they contained multiple fragments. The multiplicity is not due to specific amplification of one sequence but to the coincidence of different sequences of similar sizes as no differences in intensity were noted in comparing shoot with endosperm DNA. The identical patterns of the restriction fragment blots seen for both types of tissues showed that there were no rearrangements or amplifications occurring during development.

While it would be desirous to have an estimate of the number of zein genes, this cannot be done with the present clones because of the extensive cross-reactivity of the cloned sequences. More information is also required on the structure of the different zein genes. Wienand et al. (1981) have recently isolated a genomic zein clone and found that it had no intervening sequences by heteroduplex analysis. But other zein genes may well turn out to contain introns as has been reported for the soybean storage proteins, some of which have, and others lack, intervening sequences (GOLDBERG, personal communication). If introns were present and a particular probe being used spanned several introns that had been cleaved by restriction enzymes, then multiple fragments of that gene would be observed by blotting experiments. Finally, some of the genomic sequences may turn out to be relic sequences (or pseudogenes) that are no longer being actively transcribed. This has been shown to be the case for one of the actin genes of Dictyostelium (McKEOWN and FIRTEL 1981).

Knowledge of the chromosomal location and copy number is important to maize geneticists and plant breeders. The search for, and use of, electrophoretic variants has been complicated by the size, of the zein family and the lack of much overall charge for the polypeptides. Soave et al. (1981) have been able to associate a cluster of seven zein polypeptide genes with chromosome 7. A few additional genes have been found to be on different chromosomes.

The evolution of Zea mays is thought to span a period of roughly 5,000 years. Presumably zein was originally coded for by a single progene. The great diversity of electromorphs found in present day representatives of maize inbreds as well as in the diverse races would suggest that the heterogeneity could not have been generated simply by the accumulation of point mutations. It is highly probably instead that the principal causes of the heterogeneity are due to genetic mechanisms such as recombination, inversion, and translocation

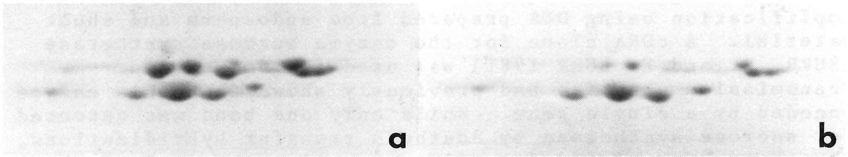

FIGURE 4. Two-dimensional IEF:SDS polyacrylamide gel electrophoresis of zeins prepared from mature seed of a) R802+ and b) R802 opaque-2. In the mutant, there is a selective suppression of some of the polypeptides in the heavier class.

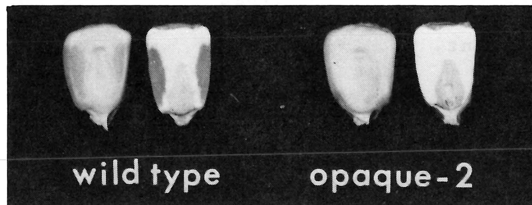

FIGURE 5. Pairs of entire (left) and longitudinally-cut (right) kernels of the inbred W22 and W22 opaque-2. Conspicuous is the loss of the vitreous lateral tissue in the mutant.

combined with gene duplication or to other more disruptive forms of reorganization (McCLINTOCK 1978). Dispersal of the genes by translocation to different chromosomes allowed for independent segregation and subsequently even greater diversity in cases of hybridization. Mutations and chromosomal rearrangements would be accumulated because zein has no (known) essential role and, therefore, would not be selected against.

There are several mutations that are known to suppress the synthesis (or accumulation) of certain zein polypeptides. As far as is known, these do not involve structural changes of zein. Opaque-2 is one such mutation - there is generally a partial to complete suppression of the heavy class polypeptides depending on the particular inbred background (Fig. 4). Phenotypically this is expressed as an opaque rather than a vitreous endosperm (Fig. 5). Soave et al. (1978) have suggested that the opaque-2 mutation may be regulatory. Two other mutations, opaque-7 and floury-2, are known to have selective suppression involving different polypeptides of both classes. Quite recently we have found another opaque among ethyl methanesulfonate-induced mutants generated by M. G. Neuffer which, in contrast to opaque-2, is associated with a decrease in many of the light class

polypeptides.

In summary it appears that zein constitutes a very large multigene family encoded by many sequences with partial homology. Although a reliable estimate of the number of zein genes cannot be provided at present, a minimum number for the strain Illinois High Protein based on the processing experiments would be perhaps 24 active genes. This number will probably have to be revised as more data is accumulated on the genetics and structure of zein genes and the nature of posttranslational processing.

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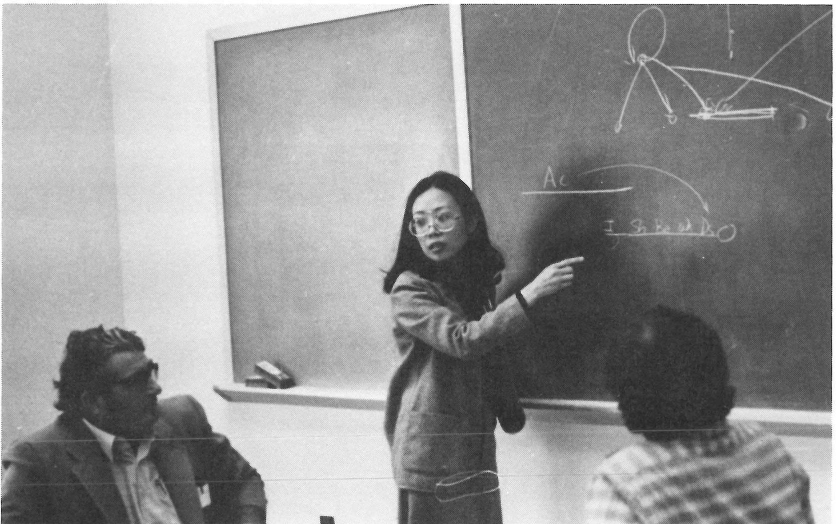

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