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Three High-Lysine Mutations Control the Level of ATP-Binding HSP70-like Proteins in the Maize Endosperm

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The synthesis and deposition of seed storage proteins in maize are affected by several dominant and recessive mutants. The effect of three independent mutations, *floury-2 (fl2), Defective endosperm-B30 (De-B30),* and *Mucronate (Mc),* that reduce zein level in the endosperm were investigated. These mutations also control the level of b-70, a polypeptide bound to protein bodies, which is separable into the two isoforms b-70I and b-70II by two-dimensional gel electrophoresis. Both isoforms are overexpressed 10-fold in *fl2*; however, only b-70I is present in *De-B30* and *Mc*, which contain an amount of total b-70 isoforms fivefold higher than in the wild type. Both b-70I and b-70II resemble heat shock protein (HSP70) in that they bind ATP, cross-react with anti-HSP antibodies, and have N-terminal sequence homology to HSP70. All maize protein body-located b-70 characteristics are typical of those of chaperone-like HSPs. A third protein, b-70III, similar in size to but slightly more acidic than b-70I and b-70II, also binds ATP and reacts with the same antibody, providing evidence for the presence in endosperm extracts of a cytosolic chaperone-like protein. The level of b-70III was not altered by the mutations studied. The results suggested that the repression effect of the three mutations on zein accumulation may be mediated by the alteration of a zein transport or zein assembly process involving b-70I and b-70II.

INTRODUCTION

An increasing amount of evidence indicates that the correct assembly of oligomeric proteins requires structural information resident in other protein molecules called "molecular chaperones," which prevent the formation of incorrect structures while not being part of the final oligomer (Hemmingsen et al., 1988). Molecular chaperones enable the precursor protein to be maintained in a soluble state and promote its binding to the membrane. This interaction triggers a change in protein conformation that is followed by insertion into the membrane. The 57-kD GroEL and 10-kD GroES (heat shock) proteins from *Escherichia coli* are regarded as chaperonins because they assist in the assembly of protein oligomers in the phage T4 capsid. GroEL shares amino acid homologies with the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)-binding protein that assists in the assembly of Rubisco (Hemmingsen et al., 1988). Yeast mutants with defective 70-kD heat-shock-like proteins (HSP70) are unable to translocate pro-α-factor to the endoplasmic reticulum (ER) (Deshaies et al., 1988). Because HSP70 has an ATP binding site, it was suggested that HSP70-like proteins require ATP hydrolysis to maintain newly synthesized proteins in an unfolded state (Chirico et al., 1988; Deshaies et al., 1989; Flaherty et al., 1990). The rat liver HSP70-related protein is identical with GRP78—a protein identified in fibroblasts when cells are starved of glucose—and with the immunoglobulin binding protein (BiP), which binds to the immunoglobulin heavy chains of B lymphocyte lineage (Munro and Pelham, 1986; Kassenbrock et al., 1988; Kozutsumi et al., 1988). Despite their different cellular locations, these proteins are all thought to be involved in the assembly of newly synthesized multimeric proteins in an ATP-dependent manner (Beckmann et al., 1990).

Zein synthesis and deposition are major functions of developing maize endosperm. Zein storage proteins account for 55% of the total endosperm proteins at seed maturity (Murphy and Dalby, 1971). Zein secondary structure is dependent on the presence of many hydrophobic residues and prolines that prevent regular α-helix formation and confer significant hydrophobic properties on the molecule (Argos et al., 1982). The amino terminus of the nascent polypeptide contains signal sequences that are

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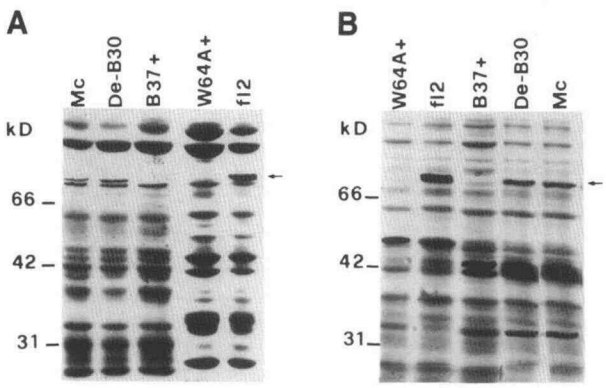

Figure 1. Genetic Control of the b-70 Protein Expression.

(A) and **(B)** 8% SDS-PAGE of albumins [**(A)**] and protein bodies [**(B)**] extracted from maize endosperms carrying either the wild-type (+) or the mutant *fl2*, *Mc*, or *De-B30* alleles. *fl2* was in the background of the W64A maize inbred line; *Mc* and *De-B30* were in that of B37. Arrows indicate the position of the b-70 proteins.

cleaved cotranslationally (Larkins and Hurkman, 1978). Zeins are synthesized by membrane-bound polysomes and transported into the lumen of the ER, where they assemble into protein bodies (Burr and Burr, 1976).

After the discovery of the high-lysine mutants *opaque-2* (*o2*) and *floury-2* (*fl2*) (Mertz et al., 1964; Nelson et al., 1965), other mutations have been found to affect zein deposition (reviewed in Motto et al., 1989). Mutation of any of these regulatory genes results in an opaque phenotype, reduced level of zein, and increased lysine content in the endosperm.

In this paper, we provide evidence that the three semidominant or dominant mutations *fl2*, *Defective endosperm-B30* (*De-B30*), and *Mucronate* (*Mc*), which have reduced kernel zein contents, alter the level of HSP70-like proteins, suggesting a role for these proteins in the transport and/or accumulation of zein into protein bodies.

RESULTS

Relationship between b-70 Content and Zein Deposition

Galante et al. (1983) reported the presence of a major protein band absent in the wild type in total protein extracts from *fl2* maize endosperm. This protein was named b-70 and its quantity was correlated strictly to the dosage of the *fl2* allele. (The maize endosperm is triploid, and allelic dosages from 0 to 3 can be obtained by appropriate crosses.) The b-70 protein binds tightly to protein bodies from which it can be eluted by detergent treatment. This topographical location was considered circumstantial evidence for a functional relationship between the zein secretory accumulation system and the b-70 protein itself.

Albumins and protein body extracts from *fl2*, *Mc*, *De-B30* mutants, and their normal counterparts were analyzed by gel electrophoresis, as shown in Figure 1A. In albumin extracts of the three mutants examined, an increase of the b-70 protein was observed compared with the wild type. This protein accumulated specifically in the protein body fractions of the three mutants (Figure 1B).

In Table 1, the relationships between the protein b-70 and zein deposition were reconsidered for the mutant *fl2* and extended to the dominant zein regulatory mutants *De-B30* and *Mc*. These results established a quantitative correlation between the level of the b-70 protein and the mutant effect on zein synthesis in the three mutations. The relative amount of b-70 protein was reported for several maize genotypes as the ratio between the percentage of b-70 in the mutant and in the corresponding wild type. These values were derived from spectrophotometric scanning of stained 8% SDS gels of albumins extracted from developing endosperms. Routinely, peak areas corresponding to the b-70 and remaining proteins were cut out, weighed, and compared, as described in Methods. The amount of b-70 in the mutant *fl-2* was increased markedly (10.0 to 14.3 times) over the wild-type level; *Mc* and *De-B30* caused an increase of 4.7-fold to 5.8-fold. The

Table 1. Quantitative Evaluation of the b-70 Proteins (Isoforms I and II) and Zein Contents in Several Maize Genotypes

Line	Genotype	b-70 Ratio (M/WT[a])	Total Zein (%)[b]
B37	+	1.0	49.8
	fl2	11.1	30.2
	Mc	4.7	43.3
	De-B30	5.2	38.9
	o2De-B30	1.6	11.8
	o2	0.7	30.6
W64A	+	1.0	52.5
	fl2	14.3	40.5
	o2fl2	1.8	26.0
	o2	0.8	28.5
A69Y	+	1.0	61.8
	fl2	10.0	30.6
	Mc	4.9	43.6
	De-B30	5.8	34.2
	o2Mc	0.7	12.0
	o2	0.7	33.5

[a] M/WT indicates the ratio between the percentage of b-70 in the mutant (M) and in the corresponding wild type (WT).
[b] Soave et al. (1979); DiFonzo et al. (1980, 1988).

three mutations also did not enhance the amount of b-70 when in combination with the recessive allele of the *O2* locus. The *o2* mutant alone slightly reduced the level of b-70 compared with the wild type. The higher the content of b-70, the lower the accumulation of zeins: reduction of zein content was higher for *fl2* and lower for *De-B30* and *Mc*. In the double mutant strains, the recessive allele *o2* was completely epistatic over *fl2*, *Mc*, and *De-B30* in controlling zein deposition.

The Protein Body-Bound b-70 Consists of the Isoforms I and II; a Cytosolic b-70III Is not Affected by Mutations

Further experiments demonstrated that the protein body-bound b-70 protein, as characterized by one-dimensional electrophoresis, can be resolved into two species when analyzed by two-dimensional electrophoresis, as shown in Figure 2A. This result was observed clearly in extracts of *fl2* protein bodies (Figure 2B), which were enriched in these proteins compared with those of the wild type. Figure 2 shows that the two forms, with molecular weights of approximately 70,000, have isoelectric points of about 5.2 (b-70I) and 5.4 (b-70II). Both forms accumulate on the protein body membrane. The two-dimensional electrophoresis also provided evidence for the presence of a third component, b-70III, not associated with protein bodies, found at similar levels in the albumin fraction of all genotypes examined, and not affected by the three dominant and semidominant mutations (Figure 2A).

The expression of b-70I and b-70II proteins was influenced differentially by the three mutations. In Figure 3, two-dimensional analysis of protein bodies is presented for the wild type and the mutants *Mc* and *De-B30*. The results showed that whereas *fl2* was characterized by a derepressed state of both b-70I and b-70II, *Mc* and *De-B30* accumulated abnormal levels of only b-70I. This result correlates with the lower quantity of b-70 noted for the mutants *Mc* and *De-B30* in monodimensional electrophoresis and with their less drastic effect on the accumulation of zeins (see Figure 1 and Table 1). Similar analyses were obtained for double mutant combinations involving mutations *o2*, *fl2*, *Mc*, and *De-B30* and confirmed the suppressive action of the recessive *o2* allele on the abnormal levels of b-70I and b-70II induced by the three semidominant and dominant mutations (data not shown; see Table 1 for the level of b-70 in double mutants as measured from one-dimensional gels).

Purified b-70 Proteins Bind ATP

More direct proof of the nature of the b-70 proteins was provided by the following experiments. A mixture of b-70I and b-70II proteins purified by one-dimensional electrophoretic separation of *fl2* protein bodies extract was tested for its capacity to bind ATP. In an experiment similar to that reported for the 70-kD protein of yeast by Chirico et al. (1988), it is shown in Figure 4 that the *fl2*-purified b-70s were capable of binding ATP. Labeling was dependent on the presence of b-70 and was absent when marker proteins were subjected to the same photoaffinity cross-linking reaction.

We were not able to separate sufficient quantities of b-70I and b-70II from *fl2* endosperm to test whether both isoforms are capable of binding ATP. Nevertheless, b-70I and b-70III were purified respectively by ATP-agarose chromatography of *Mc* and *De-B30* protein bodies and wild-type albumins, and it was shown that both components, the protein body b-70I and the cytosolic b-70III, are able to bind ATP. To avoid the possibility that a contaminating protein of the same size class competing with b-70 proteins in one-dimensional electrophoretic gels also binds ATP, protein body extracts of the wild-type lines W64A and B37 were subjected to the same photoaffinity reaction. Only a very faint band was detected, indicating that the level of binding reported in Figure 4 was dependent specifically on the amount of b-70. In this experiment, the labeled band was localized in the gel in the usual position occupied by the overexpressed b-70s.

b-70s Are Related Immunologically to Chaperone-like HSP70 Proteins

Because of the apparent size of maize b-70s in SDS gels, their relative isoelectric points, the protein body location of b-70I and b-70II, and their ability to bind ATP, we suspected that maize b-70s belong to the family of HSP70-related proteins that include molecular chaperones.

To obtain additional evidence, wild-type and *fl2* protein body extracts were separated on one-dimensional and two-dimensional gels and electroblotted onto nitrocellulose filters. The filters were then reacted with monoclonal antibodies to *Drosophila* chaperone-like HSP70. Bound antibody was detected with ^{125}I-labeled protein A. The results shown in Figure 5 established that both b-70I and b-70II cross-react with an antibody specifically directed against a conserved domain of a chaperone-like HSP70 protein from *Drosophila* (Kurtz et al., 1986), and which recognizes the 70-kD protein product of the yeast genes *SSA1* and *SSA2* (Ingolia et al., 1982).

Protein gel blot analysis using the above-mentioned antibody was carried out on albumins isolated from W64A+ and W64A*fl2* and separated by one-dimensional electrophoresis. The b-70III protein also reacted with the HSP70 antibody, providing further evidence for the presence of an additional HSP-related endosperm albumin not associated with protein bodies.

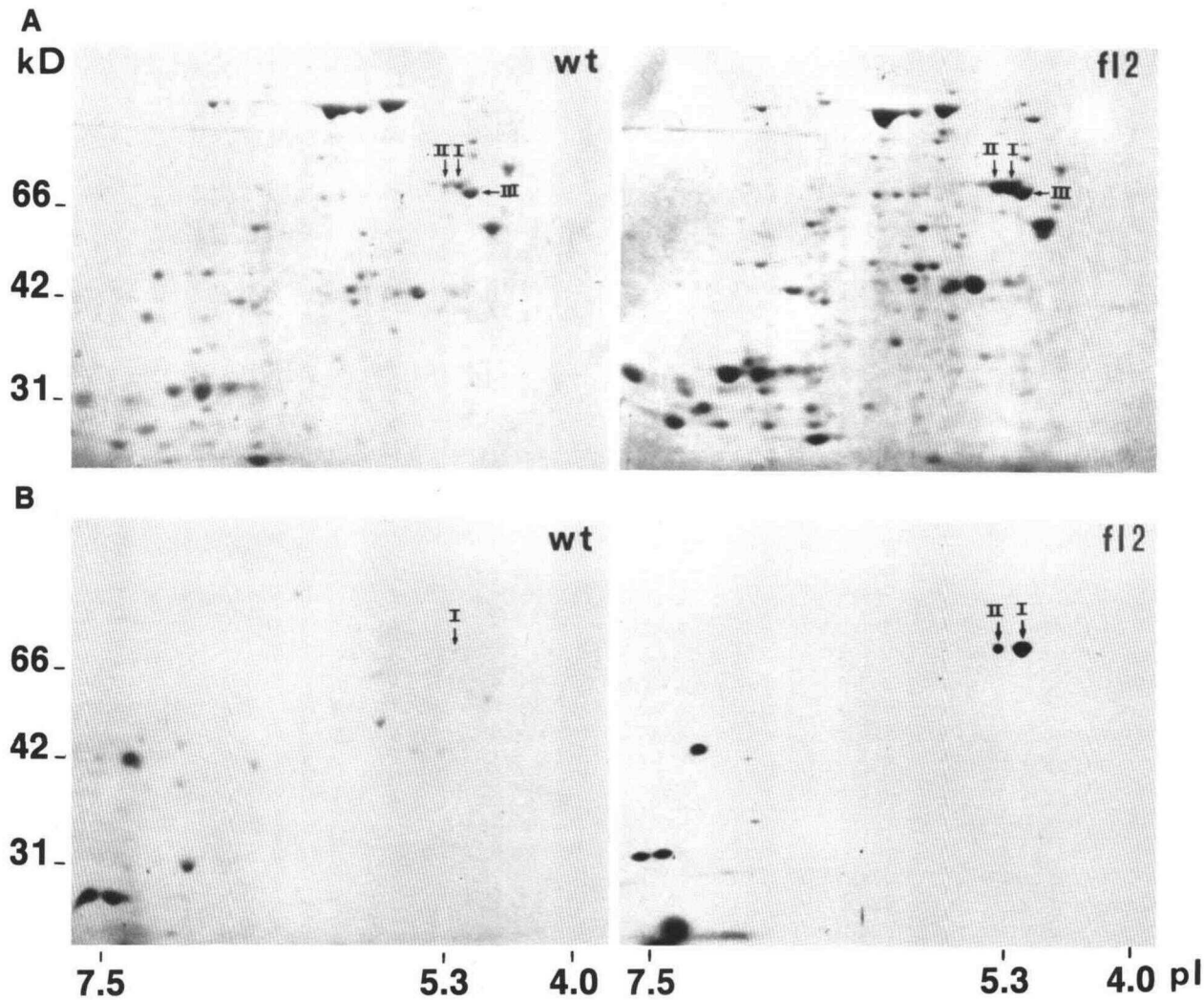

Figure 2. Two-Dimensional Isoelectric Focusing (IEF)/SDS Gel Fractionation of *fl2* and Wild-Type (wt) Albumins and Protein Body Extracts.

(A) and **(B)** Albumins [**(A)**] and protein bodies [**(B)**] were extracted from endosperms of the maize inbred line W64A *fl2* and wild type. In IEF (first dimension, left to right), the pI range is from 7.5 to 4.0. In the second dimension (run from top to bottom), molecular mass markers were BSA (66 kD), ovalbumin (42 kD), and carbonic anydrase (31 kD) (Bio-Rad). Arrows indicate the b-70I, b-70II, and b-70III proteins.

b-70s and Molecular Chaperones Have Homologous NH_2-Domains

The homology of b-70I and b-70II with HSPs was further supported by the data in Figure 6. Microsequencing of protein regions highly conserved in HSP-like chaperones revealed that b-70I and b-70II have identical primary structure. They are also highly homologous to several induced or constitutive HSP70s, as reported in other species (Craig, 1985; Lindquist, 1986) and with other transport-related proteins (Munro and Pelham, 1986). The b-70 proteins were purified from two-dimensional electrophoresis of *fl2* protein bodies, electroblotted onto polyvinylidene difluoride membranes, and visualized by brief Coomassie Blue staining. Areas of the membrane containing well-resolved proteins were excised and sequenced (Matsudaira, 1987). Based on two separate analyses, it was found that b-70I and b-70II proteins have the same NH_2-terminal sequence. Computer-assisted alignment (Devereux et al., 1984) of this partial sequence with those of HSP70 from maize, *Drosophila*, and yeast (Craig, 1985) produced a match of 15 to 22 identical amino acids over

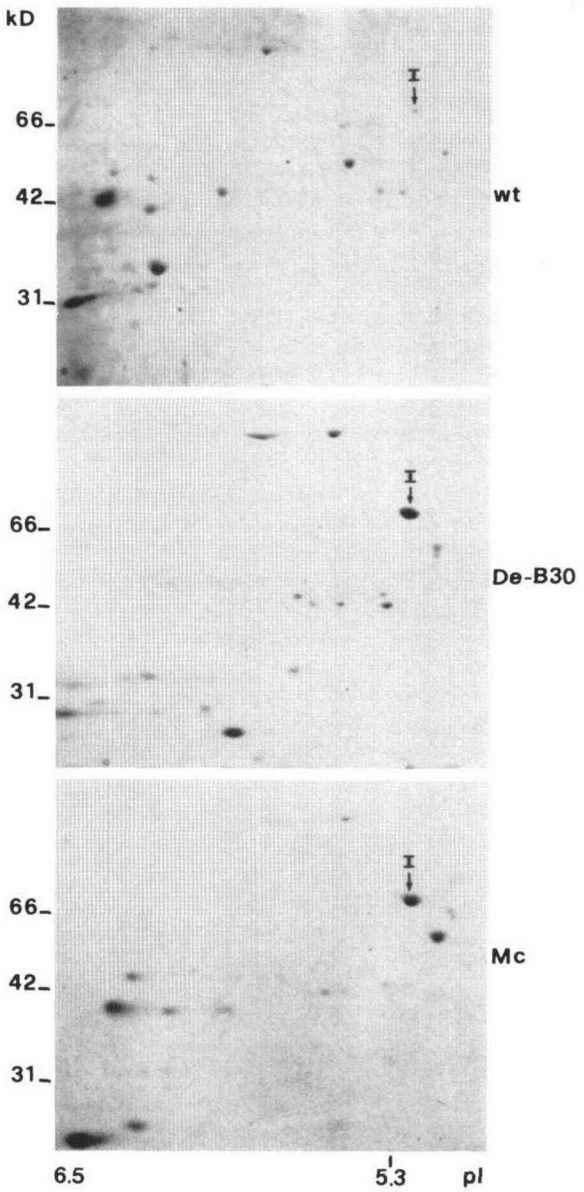

Figure 3. Two-Dimensional Gel Separations of Protein Body Extracts of the Wild Type (wt) and the Mutants *De-B30* and *Mc*.

Wild-type and mutant alleles were in the background of the B37 maize inbred line. Arrows indicate the b-70I proteins. Molecular mass markers were as described in Figure 2.

a 33-amino acid stretch. In addition, comparison of the b-70 sequences with those of GRP78 and BiP revealed a striking homology, with a match of 18 to 23 identical amino acids. GRP78, a fibroblast protein whose synthesis is increased when cells are starved of glucose, is very similar to BiP, which binds to immunoglobulin heavy chains in pre-B cells (Munro and Pelham, 1986). No NH_2-terminal sequence was obtained for the b-70III protein, most likely because of the presence of a blocked NH_2 terminus, resulting either from biosynthesis or extraction.

DISCUSSION

We have shown that in maize endosperm two protein body-bound proteins, or two alternative states of a protein, exist with several attributes typical of HSP-related chaperones. The protein has intrinsic specific biochemical properties such as the ability to bind ATP tightly. Among the ATP-dependent chaperones are representatives of the HSP70 family, which are found in bacteria and in eukaryotic cells (Horwich et al., 1990). It has been demonstrated that the specific ATP-dependent functions of members of the HSP70 family include disassembly of clathrin cages by 70-kD heat shock cognates (Schlossman et al., 1984) and assembly of oligomeric proteins in the ER by BiP (Hurtley et al., 1989). The folding reactions require hydrolysis of

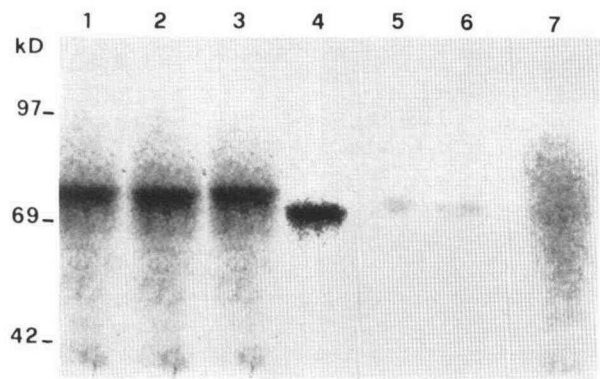

Figure 4. Purified b-70 Proteins Bind ATP.

Lanes 1 to 4 represent the products of photoaffinity cross-linking reactions containing α-^{32}P-ATP and 5.0 μg, respectively, of b-70 proteins purified from protein bodies of genotypes *fl2* (lane 1), *Mc* (lane 2), *De-B30* (lane 3), and from albumins (lane 4). Protein body-purified b-70 corresponds to isoforms I and II. The albumin extract contains only isoform III. Lanes 5 and 6 contain 80 μg of protein body extracts from W64A and B37 wild-type lines, respectively; lane 7 contains 5 μg of marker proteins subjected to the same treatment. Molecular mass markers are shown in kilodaltons (kD).

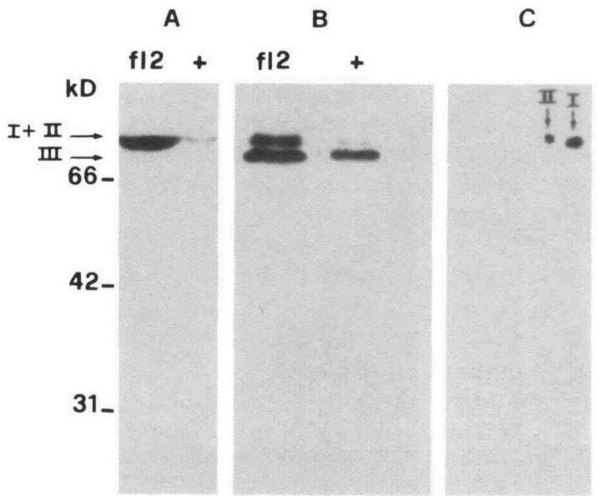

Figure 5. Antigenic Cross-Reaction between b-70 Maize Proteins and 70-kD Heat Shock-Related Proteins.

(A) and **(B)** Protein bodies [**(A)**] and albumins [**(B)**] extracted from 25 DAP endosperms of the maize inbred W64A wild type (+) and *fl2* mutant were electrophoresed in one dimension.
(C) Protein extracted from *fl2* protein bodies was fractionated by two-dimensional IEF/SDS-PAGE.
Arrows indicate the position of b-70I, b-70II, and b-70III proteins that are recognized by monoclonal antibody raised to a conserved epitope present in *Drosophila* HSP70 protein.

ATP (Flaherty et al., 1990). The ability of a monoclonal antiserum raised against chaperone-like HSP70 proteins from *Drosophila* (Kurtz et al., 1986) to recognize both b-70I and b-70II, combined with their observed affinity for ATP, indicates that b-70 represents a new member of the HSP70 family. This finding is confirmed by the comparison of the amino-terminal sequences of b-70I and b-70II with other HSP70 proteins: they are highly homologous to different members of the HSP70 family. Biochemical, immunological, and amino acid sequence data indicate that the protein overexpressed in *fl2*, *Mc*, and *De-B30* mutants and associated on protein bodies is the equivalent of maize HSP70 proteins.

The finding that b-70III, a major albumin protein not located on the protein body, is also an ATP-binding protein and reacts with an HSP70 specific antibody supports the notion either that HSP70 proteins may perform functions in a number of cellular compartments or that a number of major compartments of the cell may contain HSP70-related proteins (Craig et al., 1989).

The homology of b-70I and b-70II to other molecular chaperones is strongly suggestive of their potential role in facilitating the proper assembly of the multimeric zein proteins in normal and mutant maize endosperms. The role of these two proteins in assisting zein synthesis, processing, and deposition is supported further by three observations: (1) the protein(s) reside on the membrane of the protein body, a structure generated by the transport and assembly into the ER lumen of zein molecules synthesized by membrane-bound polyribosomes (Burr and Burr, 1976); (2) in yeast, ER translocation-promoting polypeptides correspond to the constitutively expressed *SSA1* and *SSA2* gene products which, like b-70I and b-70II, belong to the HSP70 family (Chirico et al., 1988; Deshaies et al., 1988). SSA proteins participate in the import of precursor proteins into both the ER lumen and the mitochondrial matrix (Deshaies et al., 1989); (3) mutations like *fl2*, *Mc*, and *De-B30* that lead to the overexpression of b-70I and b-70II are defective in zein accumulation. It is not clear whether the decreased zein accumulation results directly from the overproduction of b-70 or whether the two phenomena are linked by a third defect. The overproduction of b-70I in three nonallelic mutants eliminates the possibility that all of them encode the protein.

It is concluded that at least two of the three genes are not coding for b-70(s) but, most likely, affect the level of expression of this chaperone-like protein. A hypothesis for the putative role for the wild-type alleles of genes *fl2*, *Mc*, and *De-B30* could be that they code membrane receptors of the molecular chaperone: mutation in the binding capacity of the receptor(s) may lead to the accumulation of the b-70 polypeptides on the surface of the protein body and, consequently, to a reduction of zein processing and deposition. The dominant nature of all three mutants supports this suggestion.

The accumulation of b-70 also depends on a further regulating locus, *O2*. The function of the gene is apparently needed because in *o2fl2*, *02Mc*, and *o2De-B30* double mutants the level of b-70I and b-70II decreases. *O2* codes for a leucine zipper-based transcriptional activator affecting zein gene expression (Hartings et al., 1989; Schmidt

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b-70 I and II    SEE-TKK--LGTVIGIDLGTTYSCVGLWQHDXVEII  33
Yeast                    MSKAV..........AHFAM.A.D..    27
Drosophila                MPA.............VY.MGK...N   26
Maize                  MA.-SE.PA.................DR....  31
GRP78            E..DD..EDV...V-........P..VFKNGR.---  32
BiP              E..-D..EDV...V............VFKNGR....  53
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Figure 6. Comparison of the Amino-Terminal Sequences of Maize b-70s and HSP70-like Proteins.

The sequence for b-70I and b-70II is identical. The yeast, *Drosophila*, and maize heat-induced HSP70 protein sequences are shown. Sequences for GRP78 and BiP are also shown. GRP78 is an induced mammalian fibroblast protein and BiP is the mammalian immunoglobulin heavy chain binding protein. Amino acids identical for all proteins appear as dots. Gaps introduced to optimize alignment are represented by dashes.

et al., 1990), and it is conceivable that the *O2* gene is also involved in the control of genes responsible for transport and processing of the same molecules.

METHODS

Plant Material

The mutant *fl2* was obtained from Maize Genetics Cooperation Stock Center, Urbana, IL. *De-B30* was isolated as a spontaneous mutation from an open-pollinated variety (Salamini et al., 1979). *Mc* was isolated from a selfed ear of an *o2* synthetic (Salamini et al., 1983). Maize inbreds A69Y, B37, and W64A and their homozygous mutants *o2*, *fl2*, *De-B30*, and *Mc* as well as double mutant gene combinations *o2/o2,fl2/fl2*, *o2/o2,De-B30/De-B30*, and *o2/o2,Mc/Mc* were produced at the Istituto Sperimentale per la Cerealicoltura, Section of Bergamo, Italy.

Albumin Extraction, SDS-PAGE, and b-70 Quantification

Seeds were collected at 25 days after pollination (DAP), and the endosperm was dissected out and homogenized (with a mortar and pestle) in 2 mM NaH_2PO_4, 10 mM Na_2HPO_4, 0.15 M NaCl (200 μL of buffer per endosperm). The extraction was continued for 15 min on ice, and samples were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatants were denatured for 1.5 min at 90°C and about 80 μg of protein was loaded on an 8% SDS-polyacrylamide slab gel (Laemmli, 1970). Staining was with 0.1% Coomassie Brilliant Blue R-250. Molecular weight markers were phosphorylase *B*(94.7 kD), BSA (66.2 kD), ovalbumin (45 kD), and carbonic anydrase (31 kD) (Bio-Rad). Stained gels were quantitatively evaluated by scanning: a Beckman Gel Transport system connected with a DU-65 spectrophotometer and an Epson FX-800 printer was used. The areas under the individual b-70 peak and remaining peaks were calculated by tracing the recorder plots and cutting out appropriate areas and weighing them. The percentages of b-70 proteins of the total proteins were calculated by comparing peak areas.

Preparation of Protein Bodies

Dissected endosperms (25 DAP) were homogenized with a mortar and pestle in 50 mM Tris-HCl, pH 8, 100 mM KCl, 5 mM $MgCl_2$, and 1 mM EDTA (1 mL of buffer per gram of seed). The homogenate was passed through cheesecloth and the filtrate was centrifuged at 2000 rpm for 5 min and at 6000 rpm for 8 min at 4°C. The pellet was resuspended in 0.3 mL of 0.6 M sucrose per gram of seed in extraction buffer; 3 mL were applied to a 14-mL ultracentrifuge tube (Kontron polyclear) containing a discontinuous sucrose gradient consisting of 3 mL of 2 M sucrose, 3 mL of 1.5 M sucrose, 3 mL of 1 M sucrose in the same buffer. After centrifugation at 35,000 rpm for 2 hr at 4°C in a Kontron TST41.14 rotor, the material between 1.5 M and 2 M sucrose gradient interphase (zein protein bodies) was recovered. The zein protein body fraction was diluted with 3 vol of extraction buffer and pelleted at 35,000 *g* for 1 hr at 4°C; the pellet was resuspended in 10 mM potassium phosphate, pH 7.6, 2% Nonidet P-40, 1% DTT, 1 mM PMSF.

Two-Dimensional Electrophoresis

Approximately 100 μg of proteins were separated by IEF in the first dimension and by SDS-PAGE on an 8% acrylamide concentration gel in the second dimension, followed by staining with 0.1% Coomassie Brilliant Blue R-250. The procedure used for IEF was essentially that described by Bravo (1984) with the following modifications: gel tubes were 18 cm long, acrylamide solution contained 30% acrylamide and 1.6% bisacrylamide, lysis buffer contained 9.5 M urea, 5 mM K_2CO_3, 0.125% SDS, 0.5% DTT, 2% ampholyte, pH 3.5 to 10 (LKB), 0.6% nonionic detergent P40; overlay buffer was 8 M urea. A 10-mL gel mix was prepared by adding 4.4 g of urea, 1.62 mL of acrylamide solution, 1.6 mL of 10% Nonidet P-40, 1.58 mL of distilled water, 80 μL of ampholyte, pH 3.5 to 10, 80 μL of ampholyte, pH 5 to 8, 160 μL of ampholyte, pH 4 to 6, 80 μL of ampholyte, pH 7 to 9. Gels were prerun as follows: 20 min at 200 V, 30 min each at 300 V, 400 V, 500 V. Runs were at 800 V for 15 hr, followed by 1 hr at 1000 V. Gels were equilibrated in 0.1 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.5% DTT. Second dimensions were made according to the SDS gel system described by Laemmli (1970) by using 4% and 8% acrylamide concentrations in stacking and resolution gels, respectively. A melted solution of 1% agarose in equilibration buffer, with 0.5% DTT, was used to keep the first-dimension gels in place.

b-70 Purification

The b-70 proteins were purified by either electroelution or ATP-agarose chromatography. Protein bodies extracted from endosperms of the W64A *fl2* maize inbred line were electrophoresed on 8% SDS gels, and the b-70 protein was electroeluted and resuspended in 50 mM Tris-HCl, pH 9, 1% Triton X-100, 2% SDS.

Isolation of ATP-binding proteins from *Mc* and *De-B30* protein bodies and W64A wild-type albumins was carried out essentially as described by Welch and Feramisco (1985). Extracts were loaded onto a 3.0-mL ATP-agarose column (A-2767; Sigma). The column was then washed sequentially with buffer A containing 0.5 M NaCl, 20 mM Tris-acetate, pH 7.5, 20 mM NaCl, 0.1 M EDTA, 15 mM β-mercaptoethanol and with buffer A without NaCl. The proteins were eluted with buffer A containing 3 mM ATP; the fractions were tested on SDS gels and the appropriate fractions were precipitated with acetone and resuspended in the buffer described above.

Assay of ATP Binding

Each photoaffinity reaction contained 5 μg of purified b-70 proteins, α-^{32}P-ATP (5 μCi, 1500 Ci mmol^{-1}), 20 mM sodium phosphate, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 0.02% Triton X-100. Samples were incubated at 0°C for 15 min and then photolysed for 30 min using a 254-nm UV lamp, according to Chirico et al. (1988). Eighty micrograms of protein body extracts from W64A and B37 wild-type endosperms as well as 5 μg of

marker proteins phosphorylase *B*, BSA, ovalbumin, carbonic anydrase, soybean trypsin inhibitor, and lysozyme (Bio-Rad) were subjected to the same reaction. Products of cross-linking reactions were separated by 8% SDS-PAGE (Laemmli, 1970); the gel was dried at 60°C for 30 min and then exposed to Kodak XAR-5 film at −70°C.

Protein Gel Blot Analysis

Albumins and protein body extracts from 25 DAP endosperms from wild type and *fl2* mutants were electrophoresed in one dimension and two dimensions and then blotted electrophoretically at 4°C onto nitrocellulose filters (Schleicher & Schuell, 0.45 μm) in 25 mM Tris, 192 mM glycine, 0.01% SDS, and 20% methanol at 60 V, 300 mA, at 4°C for 4 hr. The blots were stained with 0.2% Ponceau-S in 3% TCA for 10 min, destained in water to locate the b-70 spots, destained with 1 M NaCl for 15 min, and finally washed with water. Subsequent steps were as described by Chirico et al. (1988) with minor modifications. The blots were incubated in 20 mM sodium phosphate, pH 7.5, 140 mM NaCl, 3% BSA for 2 hr at room temperature, washed with water, and then incubated with monoclonal (rat) anti-HSP70 gene family antibody (Kurtz et al., 1986) at a 1:200 dilution in buffer W (20 mM sodium phosphate, pH 7.5, 140 mM NaCl, 0.1% Triton X-100, 0.02% SDS, 1% BSA) for 2 hr. After several washings with buffer W, the blots were incubated with rabbit anti-rat IgG (Nordic Immunological Laboratory, Tilburg, The Netherlands) at a 1:500 dilution in buffer W for 2 hr. Further extensive washing with buffer W was followed by incubation with ^{125}I-labeled protein A (100 μCi ml^{-1}; Amersham) at a 1:2500 dilution in buffer W for 2 hr. After washing the blots with buffer W, the blots were dried and exposed to Kodak XAR-5 film with an intensifying screen.

Protein Sequencing

To purify the b-70 proteins separately, two dimensional PAGE was repeated about 40 times: 80 μg of proteins from protein bodies of the maize lines W64A *fl2* and albumins of the wild-type line was loaded each time. The gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes at 60 V, 300 mA at 4°C for 4 hr in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 5% methanol. The PVDF membranes were washed extensively in deionized H_2O to reduce the amount of Tris and glycine, stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol for 5 min, and then destained in 50% methanol, 10% acetic acid for 10 min. The membranes were finally rinsed in deionized H_2O for 10 min, and the spots corresponding to the three b-70 proteins were cut out with a clean razor, air dried overnight, and stored at −20°C. Sequence analysis was performed for each purified protein according to Matsudaira (1987). PVDF membranes were centered on the Teflon seal and placed in the cartridge block of an Applied Biosystems model 740 sequenator equipped with online phenylthiohydantoin (PTH) analysis. PTH-derivatives were separated by reverse-phase HPLC over a Brownlee C-18 column (220 × 2.1 mm).

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