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Increased Expression of the Maize Immunoglobulin Binding Protein Homolog b-70 in Three Zein Regulatory Mutants

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Plants carrying *floury-2*, *Defective endosperm-B30*, or *Mucronate* mutations overproduce b-70, a maize homolog of the mammalian immunoglobulin binding protein. During endosperm development in these mutants, levels of both b-70 protein and RNA increase dramatically between 14 days and 20 days after pollination. At later stages, b-70 RNA levels decline while protein levels remain high. The increase in b-70 RNA levels is endosperm specific and dependent on gene dosage in the *floury-2* mutant. In all three mutants, the increases in b-70 RNA and protein levels are inversely proportional to changes in zein synthesis. Although b-70 polypeptides can be extracted from purified protein bodies, they carry a carboxy-terminal endoplasmic reticulum retention signal, HDEL. We propose that induction of b-70 in these mutants is a cellular response to abnormally folded or improperly assembled storage proteins and probably reflects its role as a polypeptide chain binding protein.

INTRODUCTION

Developing endosperm of maize is characterized by rapid accumulation of zeins, the major alcohol-soluble storage proteins within protein bodies. Synthesis and accumulation of zeins are decreased in several endosperm regulatory mutants of maize (reviewed in Salamini et al., 1985). In at least three dominant or semidominant mutants, the decreases in zein levels are accompanied by increases in levels of b-70, a water-soluble protein associated with both the endoplasmic reticulum (ER) and protein bodies (Galante et al., 1983; Salamini et al., 1985).

In the accompanying paper, we used both molecular cloning and biochemical characterization to identify b-70 as a homolog of the mammalian immunoglobulin binding protein (BiP) (Fontes et al., 1991). Both b-70 and BiP are members of the heat shock protein (hsp) 70-related protein family and share such properties as (1) a capacity to bind ATP, (2) post-translational modification by phosphorylation and ribosylation, (3) induction by treatment with the glycosylation inhibitor tunicamycin, (4) localization within the lumen of the ER, and (5) conservation of primary amino acid sequence (Lee, 1987; Kozutsumi et al., 1989; Fontes et al., 1991; Haas, 1991).

BiP has been implicated in sorting and transport of both normal and abnormal proteins from the ER (Hendershot, 1990). It has been hypothesized to act as a molecular chaperone that binds to other polypeptide chains and prevents improper folding, assembly, or oligomerization without becoming part of the final product (Ellis et al., 1989; Pelham, 1989). Although direct binding of b-70 to zeins has not been reported, several previous observations are consistent with a possible molecular chaperone activity. Lending and Larkins (1989) have demonstrated that the assembly of zeins in protein bodies is not random, but follows definite spatial and developmental patterns. In the *floury-2* (*fl2*) mutant, protein bodies have altered morphology, a new 24-kD zein band can be observed in SDS-polyacrylamide gels, and the normal pattern of zein aggregation within protein bodies is disturbed (Christianson et al., 1974; Di Fonzo et al., 1977; Jones, 1978; Soave et al., 1978; C. Lending and B. Larkins, personal communication). Concomitant with these changes in zein synthesis and deposition in protein bodies are increases in b-70 (Galante et al., 1983).

The endosperm mutants *Defective endosperm-B30* (*De*-B30*) and *Mucronate* (*Mc*) are similar to *fl2* in their effects on zeins and b-70. All three mutants are phenotypically normal except for an opaque endosperm. The *fl2* and *Mc* mutations negatively affect all zein size classes and exhibit dosage dependence and incomplete dominance, respectively. *De*-B30* is dominant and primarily affects the 22-kD zeins. The *fl2* mutation has the largest effect on zein levels, followed by *De*-B30* and *Mc* (Salamini et al., 1979, 1983).

We investigated increases in the accumulation of b-70 RNA and protein during endosperm development in the maize mutants *fl2*, *De*-B30*, and *Mc*. We present evidence

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that the increased accumulation of b-70 in the ER and protein bodies can be detected at the level of mRNA abundance and reflects the severity of the mutation with respect to zein synthesis. For *fl2*, b-70 RNA levels are correlated positively with gene dosage. We show that b-70 has the post-translational modifications and COOH-terminal ER retention signal characteristic of mammalian BiP. We discuss possible stimuli for specific induction of b-70 in *fl2*, *De*-B30*, and *Mc* endosperm in relation to a molecular chaperone function for facilitating zein deposition in protein bodies.

RESULTS

Accumulation of b-70 during Endosperm Development

The accumulation of b-70 during kernel development was assayed by SDS-PAGE and immunostaining with b-70 antibodies. Figure 1A shows the Coomassie-stained protein pattern of soluble fractions of *fl2* endosperm between 10 days after pollination (DAP) and 44 DAP. The corresponding immunoblot in Figure 1B shows an increase in b-70 between 14 DAP and 24 DAP. Beyond 24 DAP, levels remained high. The apparent decrease in b-70 levels between 35 DAP and 44 DAP resulted most likely from incomplete extraction of the protein from kernels undergoing desiccation because proteins extracted by buffer containing SDS and 2-mercaptoethanol did not show such a decline in b-70 at late stages of development (D.N. Martin and R.S. Boston, unpublished results). The increased accumulation of b-70 paralleled the developmental pattern of zein accumulation in the *fl2* mutant (Jones, 1978). Although the immunoblot did not allow precise quantitation of b-70 levels, a comparison of b-70 in 24 DAP extracts from *fl2* endosperm with b-70 extracted from an equal fresh weight of normal endosperm showed a significant difference in b-70 levels (Figure 1B, lanes fl2 and A^+, 1×). Increasing the normal extract concentration by 10-fold still resulted in less immunostaining than in the lower amount of extract from the *fl2* mutant (Figure 1B, lane A^+, 10×).

Increases in b-70 Reflect b-70 RNA Levels

RNA gel blots of total RNA extracted from kernels at 10 DAP to 36 DAP were probed with a ^{32}P-labeled partial b-70 cDNA clone (Fontes et al., 1991). Figure 2 shows a comparison of b-70 RNA levels in normal and *fl2* kernels. In normal kernels (Figure 2A), levels of b-70 RNA were fairly constant throughout endosperm development. In *fl2* kernels (Figure 2B), b-70 RNA levels increased during mid-maturation but declined at later stages of kernel development. The temporal pattern of b-70 RNA levels reflected the increases in b-70 protein shown in Figure 1B.

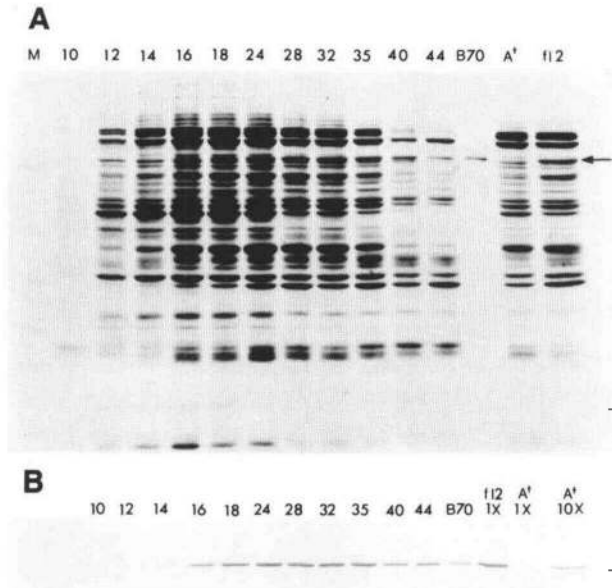

Figure 1. Accumulation of b-70 during Endosperm Development.

Soluble protein extracts from normal and *fl2* endosperm were fractionated by SDS-PAGE and immunoblotted using b-70 antisera. Developmental stages of *fl2* samples are indicated at top in DAP.

(A) SDS-polyacrylamide gel stained with Coomassie Brilliant Blue. B70 is partially purified b-70. A^+ and fl2 are extracts from equal fresh weights of normal and *fl2* endosperm, respectively. M denotes molecular size markers of 66 kD, 45 kD, 34.7 kD, 24 kD, 18.4 kD, and 14.3 kD. Arrowhead shows the position of b-70.

(B) Immunoblot of b-70. An SDS-polyacrylamide gel was prepared similarly to that shown in **(A)** except that one-third the amount of protein was loaded in each lane. Lanes labeled fl2 1×, A^+ 1×, and A^+ 10× refer to extracts of equal amounts of endosperm from 24 DAP *fl2* and normal maize and a 10-fold greater amount from normal maize, respectively.

The level of b-70 RNA at 10 DAP in the *fl2* mutant was significantly greater than that with the 12 DAP sample. Likewise, 14 DAP and 20 DAP samples from the normal genotype fluctuated with respect to samples at other stages. These results were not due to unequal amounts of RNA in these lanes because reprobing the blot with an rDNA probe showed very little sample-to-sample variation. [Densitometric quantitation of the autoradiograph resulted in all values falling within a 10% difference of the mean. The highest and lowest values differed by 16% of the highest value for normal kernel RNA and 11% for *fl2* RNA (data not shown).] RNA gel blot analysis of normal plants revealed that levels of b-70 RNAs in shoot and root tissues were comparable with levels in kernels (Figure 2A). However, the level of b-70 RNA in preemerged tassel tissue from the normal genotype was comparable with the higher

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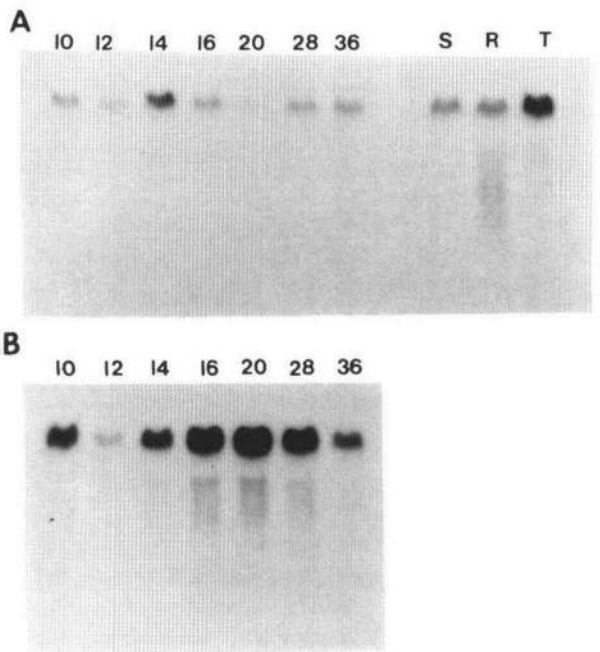

Figure 2. Developmental RNA Gel Blot of b-70.

RNA (15 μg/lane) from kernels at the stages noted above lanes was separated by agarose gel electrophoresis, transferred to a nylon membrane, and probed for b-70 sequences. Both normal and *fl2* samples were probed on a single RNA gel blot. The filter was washed at high stringency to remove any signal contributed by related members of the hsp70 gene family.
(A) Developmental profile of b-70 RNA in normal kernels. Developmental stage of endosperm (above lanes) is noted in DAP. S, R, and T refer to RNA from shoot, root, and preemerged tassel, respectively.
(B) Developmental profile of b-70 RNA in *fl2* kernels.

levels of b-70 found in *fl2* kernels (compare Figures 2A and 2B).

The Effect of *fl2* on b-70 RNA Levels Is Endosperm Specific

The developmental increase in *fl2* b-70 parallels phenotypic changes in kernels of plants that are otherwise normal. We examined b-70 RNA levels in normal and *fl2* seedlings for changes that might be attributable to the *fl2* allele. Figure 3 shows an RNA gel blot of total RNA from 5-day-old etiolated seedlings compared with the same amount of RNA from normal and *fl2* kernels at 20 DAP. No differences were detected between normal and *fl2* seedling samples, suggesting that the increase in abundance of b-70 RNA in *fl2* is organ specific.

Increases in b-70 RNA Are Dependent on *fl2* Gene Dosage

The *fl2* mutation has been shown to be semidominant and negatively to affect zein gene expression in a dosage-dependent manner (Jones, 1978). The triploid nature of endosperm allowed us to generate plants with endosperm genotypes +/+/+, +/+/*fl2*, *fl2*/*fl2*/+, and *fl2*/*fl2*/*fl2*. Results of densitometric quantitation of b-70 RNAs in kernels from this dosage series are shown in Figure 4. In the absence of an *fl2* allele, very low levels of hybridizable RNAs were detected in normal endosperm. Introduction of a single copy of the *fl2* allele, however, resulted in a significant induction of b-70 mRNA. A similar increase was observed between one and two doses of *fl2*, in agreement with the semidominance of the mutation. Comparison of b-70 levels in a dosage series from different developmental stages indicated that at 16 DAP to 20 DAP, b-70 levels were strictly correlated with *fl2* gene dosage. At 24 DAP, however, levels increased with one and two doses of *fl2* but declined slightly between two doses of *fl2* and fully mutant kernels. This effect resembles the plateau of b-70 protein accumulation shown in Figure 1.

Accumulation of b-70 in Other Endosperm Mutants

The coordinate increase in b-70 RNA and protein in the *fl2* mutant prompted us to investigate the molecular basis for increases in b-70 previously reported for *De*-B30* and *Mc*

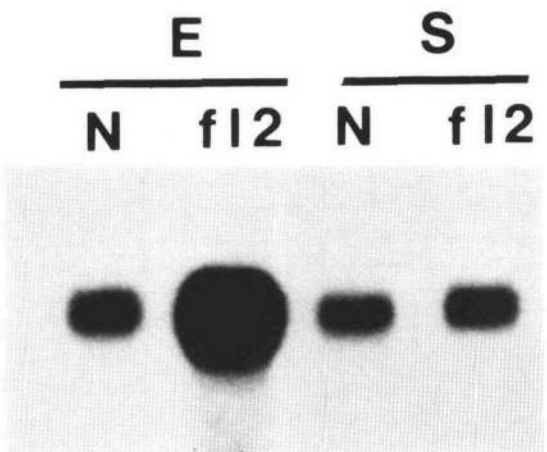

Figure 3. RNA Gel Blot Showing Effect of *fl2* on b-70 Levels in Seedlings.

RNA (10 μg/lane) was isolated from 5-day-old etiolated normal (N) or *fl2* (fl2) seedlings or 20 DAP kernels of the same genotypes. E designates endosperm samples, S designates seedling samples. Blots were washed at moderate stringency.

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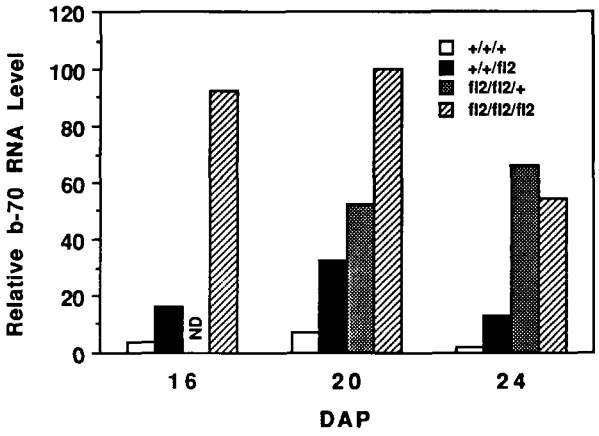

Figure 4. Histogram of Relative b-70 RNA Levels in Response to *fl2* Gene Dosage.

RNA (10 μg/lane) was separated by agarose gel electrophoresis, transferred to nylon membranes, and probed for b-70 sequences at moderate stringency. RNA was isolated from developing endosperm at 16 DAP, 20 DAP, and 24 DAP. Samples were prepared from plants with endosperm genotypes of +/+/+, +/+/*fl2*, *fl2*/*fl2*/+, and *fl2*/*fl2*/*fl2*. Relative RNA levels determined by densitometric scanning of the autoradiograph were normalized to the highest value.

(Salamini et al., 1985). In the companion paper (Fontes et al., 1991), we demonstrated by two-dimensional gel electrophoresis that in W64A*fl2* the band of approximately 70 kD identified originally as b-70 actually contains several different polypeptides, but only two of them are overproduced in *fl2* mutants. These two proteins have apparent molecular sizes of 75 kD and are referred to collectively as b-70.

Figure 5 shows results of two-dimensional electrophoretic separation of aqueous-soluble proteins from *De*-B30* and *Mc* in a B37 background. Coomassie-stained gels in Figures 5A, 5F, and 5K (first column) show clearly the resolution of distinct acidic polypeptides of 73 kD and 75 kD. In the mutants, only the 75-kD polypeptide was overproduced. The induction of b-70 in the mutants can also be seen in immunoblots probed with b-70 polyclonal antisera, as shown in Figures 5B, 5G, and 5L (second column).

Induction of a single b-70 species in *De*-B30* and *Mc* was unexpected because two induced polypeptides of pI 5.3 and pI 5.4 were resolved in W64A*fl2*. Only the pI 5.3 form, however, is post-translationally modified by phosphorylation and ADP-ribosylation (Fontes et al., 1991). To determine whether or not the proteins induced in *De*-B30* and *Mc* can be post-translationally modified, we incubated crude protein extracts with adenylate-^{32}P-NAD^{+} and separated the products by two-dimensional gel electrophoresis. Autoradiograms are shown in Figures 5C, 5H, and 5M (third column). The in vitro labeling resulted in incorporation of radioactivity into the 75-kD species but not the 73-kD polypeptide, as judged by comparison of the Coomassie-stained gel with the x-ray film after autoradiography (data not shown). The differential labeling intensities correlated with the relative amounts of b-70 in those genotypes (compare first column with third column).

It is unlikely that the two b-70 polypeptides in W64A*fl2* differ only by the presence or absence of post-translational modifications because removal of ADP-ribose and phosphate did not significantly change the polypeptide pattern on two-dimensional gels (Fontes et al., 1991). Thus, the detection of a single 75-kD species in the B37 background was unexpected. We reasoned that if only one of the two 75-kD polypeptides in W64A*fl2* were a BiP homolog, it would be unlikely that a single polypeptide in B37 mutants would be able to contribute both BiP function and the unknown function of the other 75-kD polypeptide.

To investigate the possibility that both modified and unmodified b-70 polypeptides from *De*-B30* and *Mc* comigrated in our gel system, we treated crude protein extracts with alkaline phosphatase and snake venom phosphodiesterase. These enzymes cleave covalently linked phosphate and ADP-ribose groups, respectively. An immunoblot probed with b-70 polyclonal antibody is shown in Figures 5D, 5I, and 5N (fourth column). Treatment with the enzymes under saturating conditions resulted in the appearance of a single, new 75-kD polypeptide with a slightly different pI in addition to the 73-kD and 75-kD polypeptides detectable before enzyme treatments (compare with Figures 5B, 5G, and 5L, second column). These data reconcile the previous data from W64A*fl2* with results from B37*De*-B30* and B37*Mc* by suggesting that there are actually two b-70 polypeptides in B37*Mc* and B37*De*-B30*, only one of which is post-translationally modified by ADP-ribosylation and phosphorylation.

Previous work indicated that b-70 was associated with protein bodies but was not enclosed within a membrane (Galante et al., 1983). In ER fractions, however, b-70 is localized within the membrane, as judged by its resistance to proteinase K digestion (Fontes et al., 1991). These findings raised the possibility that b-70 is a resident ER protein that is released from the lumen during formation of protein bodies. Proteins that are localized in ER have been shown to have COOH-terminal amino acids HDEL (yeast) or KDEL (animals; reviewed in Pelham, 1989). The deduced amino acid sequence of b-70 terminates in HDEL (Fontes et al., 1991). However, a maize auxin-binding protein has been shown to terminate in KDEL, suggesting that both signals may be functional in maize (Inohara et al., 1989).

To determine whether or not the b-70 polypeptides carry such an ER-retention signal and to investigate the relationship between the 73-kD and 75-kD proteins, we immunostained a second set of protein gel blots with antibody specific for the carboxy-terminal HDEL (Hardwick et al.,

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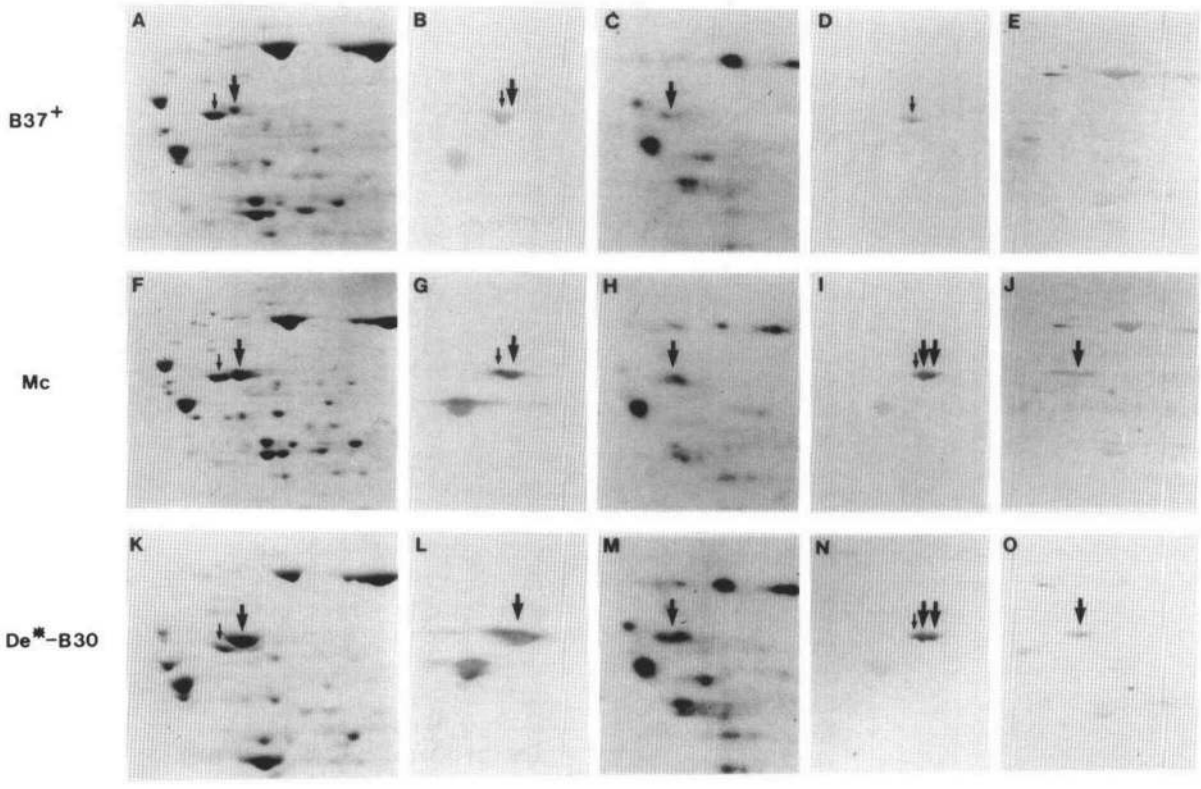

Figure 5. Two-Dimensional Gel Analysis of Endosperm Proteins in Normal, *De*-B30*, and *Mc* Endosperm.

Crude protein extracts were subjected to two-dimensional isoelectric focusing (IEF)/SDS-PAGE and immunoblotting. Migration was from basic (right) to acidic (left) for IEF gels and top (cathode) to bottom (anode) for SDS gels. Large arrows denote the 75-kD polypeptide and small arrows denote the 73-kD polypeptide. Panels within each vertical row represent samples that received the same treatment.

(A) Coomassie-stained gel of soluble proteins from B37$^+$.

(B) Immunoblot of soluble proteins from B37$^+$ probed with b-70 polyclonal antibody.

(C) In vitro ADP-ribosylation of soluble proteins from B37$^+$.

(D) Immunoblot of soluble proteins from B37$^+$ probed with b-70 polyclonal antibody after treatment with phosphatase and phosphodiesterase.

(E) Immunoblot of soluble proteins from B37$^+$ probed with anti-HDEL polyclonal antibody.

(F) Coomassie-stained gel of soluble proteins from B37*Mc*.

(G) Immunoblot of soluble proteins from B37*Mc* probed with b-70 polyclonal antibody.

(H) In vitro ADP-ribosylation of soluble proteins from B37*Mc*.

(I) Immunoblot of soluble proteins from B37*Mc* probed with b-70 polyclonal antibody after treatment with phosphatase and phosphodiesterase.

(J) Immunoblot of soluble proteins from B37*Mc* probed with anti-HDEL polyclonal antibody.

(K) Coomassie-stained gel of soluble proteins from B37*De*-B30*.

(L) Immunoblot of soluble proteins from B37*De*-B30* probed with b-70 polyclonal antibody.

(M) In vitro ADP-ribosylation of soluble proteins from B37*De*-B30*.

(N) Immunoblot of soluble proteins from B37*De*-B30* probed with b-70 polyclonal antibody after treatment with phosphatase and phosphodiesterase.

(O) Immunoblot of soluble proteins from B37*De*-B30* probed with anti-HDEL polyclonal antibody.

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1990). The results shown in Figures 5E, 5J, and 5O (last column) demonstrate that in the size range of 70-kD species, only the 75-kD polypeptides were stained. Thus, they alone terminate with the HDEL consensus ER-retention sequence. In addition, the absence of signal at the position of the 73-kD protein in immunoblots of normal and mutant B37 endosperm probed with anti-HDEL antiserum eliminates the possibility that this protein is a differentially modified form of the 75-kD b-70 polypeptides.

Levels of b-70 RNA Are Increased in Some but not All Endosperm Regulatory Mutants

Figure 6A shows RNA gel blots of total RNA from the B37$^+$ inbred and mutants *Mc* and *De*-B30* in the B37 background at 10 DAP, 14 DAP, and 18 DAP. Both mutants had higher b-70 RNA levels than the normal genotype at 14 DAP and 18 DAP. However, the increase in *De*-B30* was significantly larger and could be detected at an earlier developmental stage (10 DAP) than that for *Mc*. Levels of b-70 RNA in the *Mc* and *De*-B30* mutants reflected the increase in the corresponding proteins (Figure 5).

The relative effects of *De*-B30* and *Mc* on b-70 RNA and protein levels corresponded inversely to their effects on zein accumulation. Repression of zein accumulation is much greater in *De*-B30* than in *Mc* (Salamini et al., 1979, 1983). Even more dramatic effects on zein accumulation, however, are found in the *opaque-2* (*o2*) regulatory mutant (Soave et al., 1976). Figure 6B shows the results of an RNA gel blot of the *o2* mutant and a mutant in starch biosynthesis, *sugary-2*, that also has decreased levels of zein. Both mutants were in a W64A background. Levels of b-70 RNA in both mutants were very similar to levels in the normal W64A genotype (H.W. Bass and R.S. Boston, unpublished results). Thus, the increase in b-70 gene expression cannot be explained simply as a result of altered zein levels in the mutants. Furthermore, the induction of b-70 by mutations in any of three unlinked loci rules out direct effects of the mutations on the b-70 gene itself and suggests a common *trans*-acting control.

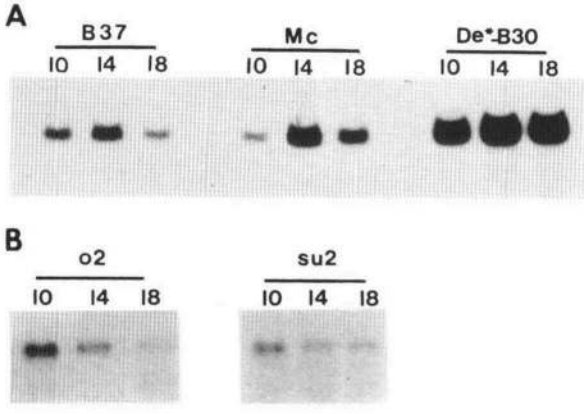

Figure 6. RNA Gel Blots of b-70 in Endosperm Mutants.

RNA (10 μg/lane) was separated by agarose gel electrophoresis, transferred to nylon membranes, and probed for b-70 sequences at moderate stringency. Blots probed at high stringency gave the same results (H.W. Bass and R.S. Boston, unpublished results).
(A) Increase in *De*-B30* and *Mc*. Total RNA was isolated from kernels at 10 DAP, 14 DAP, and 18 DAP from the B37 inbred and *Mc* and *De*-B30* mutants as noted above lanes.
(B) Levels of b-70 RNA in *o2* and *sugary-2*. Total RNA was isolated from W64A *o2* (o2) and *sugary-2* (su2) kernels at 10 DAP, 14 DAP, and 18 DAP.

DISCUSSION

In this paper, we have shown that b-70 gene expression is regulated at the RNA level. In normal maize endosperm, the b-70 RNA level showed little fluctuation during kernel development. However, in the dominant and semidominant mutants *fl2*, *De*-B30*, and *Mc*, dramatic increases in b-70 RNA accumulation were found.

The large increases in b-70 RNA and protein in the three mutants were consistent with induction in response to reduced zein synthesis. Altered zein synthesis was not strictly correlated with elevated b-70 levels, however, because b-70 RNA and protein levels were not affected in *o2* or *sugary-2* mutants that also had reduced zein levels (Figure 6; Soave et al., 1976; Galante et al., 1983). Furthermore, the increase did not appear to be due to an effect on a particular size class of zeins because *fl2* and *Mc* affect zeins nonspecifically and *De*-B30* primarily affects the 22-kD α-zeins (Lee et al., 1976; Salamini et al., 1985).

Unexpected high levels of b-70 RNA were observed at 10 DAP in *fl2* kernels and in preemerged tassels of normal plants. The increase in b-70 RNA at the 10 DAP stage of *fl2* endosperm development was similar to the difference between b-70 signals in normal and *De*-B30* kernels in a B37 background and may reflect the increased secretory activity associated with cell wall formation during the transition from liquid endosperm to individual cells. It is unlikely that the hybridization signals were due to cross-reaction of the b-70 probe with other members of the hsp70 gene family because genomic DNA gel blot analysis performed at the stringency used for this blot resulted in a simple banding pattern (B.B. Shank, unpublished results).

We previously used restriction fragment length polymorphism mapping of recombinant inbreds to show that a partial b-70 cDNA clone maps near the centromere of chromosome 5 (Shank et al., 1990). Genetic mapping of *fl2* and *De*-B30* loci placed them on chromosomes 4 and 7, respectively (Salamini et al., 1985). Thus, the effect of

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these mutations on b-70 RNA levels must occur in *trans*. The effect of the *fl2* mutation on zein gene expression was demonstrated to affect the level of transcription (Kodrzycki, 1989). The mechanism by which a mutation at a single genetic locus can up-regulate b-70 mRNA and down-regulate zein mRNAs poses an interesting problem for study.

One possible explanation for such opposing responses is a direct effect of the mutations on zein gene expression and a secondary effect on b-70 gene expression. The expression of the *fl2* allele specifically in the endosperm may cause a disturbance of normal endosperm metabolism and thus stimulate an overproduction of b-70. In *fl2* endosperm, reduction of zein accumulation is correlated with the appearance of a novel 24-kD zein component (Jones, 1978). This 24-kD zein may represent an abnormal protein, a condition that is favorable for induction of ER polypeptide chain binding proteins (Kozutsumi et al., 1988). Such a model would be consistent with a molecular chaperone role for b-70. In addition, it accounts for induction of b-70 without necessitating a change in the normal signal transduction pathway. Although the mechanism by which a signal from the ER might be transduced to the nucleus has yet to be defined, sequences 5′ to the coding region of mammalian BiP genes have been shown to confer tunicamycin induction to a chloramphenicol acetyltransferase reporter gene (Chang et al., 1987).

The role of b-70 in zein folding and assembly is unknown. Simon et al. (1990) showed that tunicamycin treatment of *Xenopus* oocytes injected with wheat prolamin RNAs negatively affects secretion but not synthesis of the wheat proteins. Although BiP levels were not analyzed in these experiments, the data are consistent with involvement of an oocyte BiP in retaining the proteins in the ER. Similarly, cross-reaction of b-70 with anti-HDEL antisera is suggestive of a role in the maize zein secretory pathway. In other systems, proteins whose assembly and secretion are facilitated by BiP lack H/KDEL sequences. Likewise, zeins lack HDEL or KDEL sequences even though they remain within the ER until formation of protein bodies within ER membranes. Such behavior is consistent with b-70-facilitated assembly followed by precipitation of large hydrophobic protein aggregates as protein bodies.

Identification of b-70 as a plant homolog of BiP provides a novel system for study. Unlike single-celled organisms, cells in culture, or cells exposed to physiological stresses, maize endosperm represents an intact tissue within a normal physiological environment. In addition, the *fl2*, *De*-B30*, and *Mc* mutations provide an endogenous induction of b-70 gene expression in endosperm of plants that are otherwise phenotypically normal. Inbred maize lines harboring these mutations will provide an excellent system for elucidating the role of b-70 in cell physiology and the effect of the mutations on b-70 gene expression.

METHODS

Plant Material

Maize (*Zea mays*) inbreds W64A and B37 and their near isogenic mutants *fl2*, *De*-B30*, and *Mc* were grown and self-pollinated at the Purdue University Agronomy Farm, West Lafayette, IN, from 1985 to 1987 and the North Carolina State University Research Unit I, Raleigh, NC, from 1988 to 1990. Ears were harvested at stages noted in the figure legends and rapidly frozen in liquid nitrogen. The frozen kernels were shelled onto dry ice and stored at −80°C.

SDS-PAGE, Two-Dimensional Gel Electrophoresis, and Immunoblotting

Electrophoretic and immunoblotting techniques were carried out as described by Fontes et al. (1991) except that immunoblots probed with HDEL antibodies were developed using an alkaline phosphatase color reaction (Harlow and Lane, 1988). Antibodies to b-70 were used at a 1:1000 dilution and are described in the companion paper (Fontes et al., 1991). HDEL-specific antibodies were kindly provided by M. Lewis and H. Pelham and have been described elsewhere (Hardwick et al., 1990). These antibodies were used at 1:500 dilution.

In Vitro Post-translational Modification of b-70

In vitro ADP-ribosylation assays were performed with crude protein extracted from normal and mutant endosperm. Equal fresh weights of normal and mutant endosperm were homogenized intermittently in 0.2 M KCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM DTT for 15 min. Cell debris was removed by centrifugation at 16,000 *g* for 10 min. The supernatant (30 μg) was incubated with 5 μCi of adenylate-^{32}P-NAD$^+$ for 15 min at 37°C. ADP-ribosylation reactions were stopped by equilibrating the samples with isoelectric focusing buffer in the ratio of 1:1 (v/v), as described by O'Farrell (1975). After the incubation period, aliquots were also treated with 10 units/μL calf intestinal alkaline phosphatase (Promega Biotec) for 1 hr, after which 0.6 units/μL snake venom phosphodiesterase I from *Crotalus adamanteus* (Worthington, Freehold, NJ) was added. An additional incubation for 1 hr at 37°C was followed by two-dimensional gel electrophoresis and Coomassie staining of the gel. Efficiency of phosphate and ADP-ribose removal was greater than 99%, as determined in control experiments with radioactive compounds (data not shown).

RNA Preparation and Gel Blot Analysis

RNA was isolated from kernels, dissected endosperm, and 5-day-old etiolated seedlings essentially as described by Langridge et al. (1982) (see Figure 2) or Wadsworth et al. (1988) (see Figures 3, 4, and 6). RNA gel blot analysis was performed as described by Fontes et al. (1991). Moderate (T_m −32°C) or high (T_m −25°C) stringencies were determined according to Galau et al. (1986) for

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nylon membranes, as noted in the figure legends. Relative quantitation of bands was determined by scanning multiple linear exposures of x-ray films with a Zeinh Soft Laser scanning densitometer and integrating areas under peaks.

DNA Probes

The 1.2-kb b-70 cDNA clone pcb70.2 was described previously and has been assigned accession no. M59449 in the GenBank database (Bilofsky and Burks, 1988; Fontes et al., 1991). Hybridization probes were generated by isolating EcoRI inserts from vector and labeling, as described by Fontes et al. (1991).

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