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A Maize Ribosome-Inactivating Protein Is Controlled by the Transcriptional Activator *Opaque-2*

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Although synthesis of the cytosolic maize albumin b-32 had been shown to be controlled by the *Opaque-2* regulatory locus, its function was unknown. We show here that b-32 is a member of the large and widely distributed class of toxic plant proteins with ribosome-inactivating activity. These ribosome-inactivating proteins (RIPs) are RNA *N*-glycosidases that remove a single base from a conserved 28S rRNA loop required for elongation factor 1α binding. Cell-free in vitro translation extracts were used to show that both maize and wheat ribosomes were resistant to molar excesses of b-32 but not to the dicotyledonous RIP gelonin. We extracted RIP activity from kernels during seed maturation and germination. The amount of RIP activity increased during germination, although the amount of b-32 protein remained fairly constant. Expression of a maize RIP gene under the control of an endosperm-specific transcriptional regulator may be an important clue prompting investigation of the biological basis for RIP expression in seeds of other plants.

INTRODUCTION

Ribosome-inactivating proteins (RIPs) are a widely distributed group of toxic plant proteins that catalytically inactivate eukaryotic ribosomes (for review, see Stirpe and Barbieri, 1986). RIPs function as *N*-glycosidases to remove a specific adenine in a conserved loop of the large rRNA (Endo and Tsurugi, 1987; Endo et al., 1987). This irreversible modification renders the ribosome unable to bind elongation factor 1α, thereby blocking translation. Because this translational inhibitory activity is toxic, RIPs have been tested extensively for use as immunotoxins and antiviral agents and more recently for their effects on protozoa, insects, and fungi (Barbieri and Stirpe, 1982; Cenini et al., 1988; Gatehouse et al., 1990; Leah et al., 1991).

RIP activities have been found in the seed, root, leaf, or sap of more than 50 different plant species (Gasperi-Campani et al., 1985). Two forms of RIPs have been described (Stirpe and Barbieri, 1986). Type 1 RIPs such as pokeweed antiviral protein, trichosanthin, the barley translation inhibitor, and gelonin are monomeric enzymes, each with an approximate M_r of 30,000 (Irvin, 1975; Stirpe et al., 1980; Asano et al., 1984; Maraganore et al., 1987; Yeung et al., 1988). Type 2 RIPs such as ricin, abrin, and modeccin are highly toxic heterodimeric proteins, each with an approximate M_r of 60,000 in which one polypeptide with RIP activity (A-chain) is linked by a disulfide bridge to a galactose-binding lectin (B-chain; Olsnes and Pihl, 1973, 1982; Stirpe et al., 1978).

Type 1 RIPs and the A-chain of type 2 RIPs have basic isoelectric points, and many have signal peptides that are not present in the mature protein (Stirpe and Barbieri, 1986). Although RIPs share biological activity, they typically exhibit similarities of <50%, and antibodies raised against RIPs seldom cross-react with RIPs from distantly related species (Ready et al., 1988). The maize b-32 protein has homology with several previously characterized RIPs, yet it is a single-chain acidic protein that lacks a signal peptide and fractionates with soluble cytoplasmic components during density gradient centrifugation (Di Fonzo et al., 1986, 1988; Lohmer et al., 1991).

Control of b-32 gene expression is mediated by the endosperm regulatory locus *Opaque-2* (Mertz et al., 1964). Opaque-2 has been shown to be a DNA binding protein that affects expression of the major seed storage protein genes, particularly those encoding the 22-kD α zeins (Jones et al., 1977; Hartings et al., 1989; Schmidt et al., 1990). Levels of b-32 and 22-kD zeins are greatly decreased in *opaque-2* mutants, and transcription from a b-32 promoter can be specifically activated by the Opaque-2 protein in transient expression assays (Soave et al., 1981; Lohmer et al., 1991). Regulation of the b-32 gene by *Opaque-2* thus appears to be a mechanism for producing high levels of protein in an endosperm-specific manner.

The exact nature of the b-32 protein has been unknown because of differences in the amino acid sequences deduced from DNA sequences of b-32 clones. Hartings et al. (1990) compared sequences of b-32 genomic clones and showed that although deduced amino- and carboxy-terminal sequences were identical, the central domains were very different. These differences within the internal region of b-32 were

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attributed to three single base deletions that would result in three reading frame shifts. In the absence of an activity for the b-32 protein, the importance of either sequence could not be determined.

We have isolated a b-32 cDNA clone and shown that it encodes a protein with striking sequence similarity to RIPs. We have assayed b-32 from developing maize kernels and germinating seeds and demonstrated that it has ribosome-inactivating activity. Ribosomes from rabbit were very sensitive to inactivation by b-32, but maize and wheat ribosomes were resistant. Although b-32 gene expression is controlled by the *Opaque-2* locus, low levels of b-32 and RIP activity were detectable in an *opaque-2* mutant.

RESULTS

B-32 Has Homology to RIPs

A comparison of the deduced amino acid sequence of the b-32 clone ZmcRIP-9 (see Methods) with that of barley RIP 30, a type 1 RIP, and the A-chain of ricin, a type 2 RIP, is shown in Figure 1 (Lamb et al., 1985; Leah et al., 1991). Several positions with identity among the three proteins have been previously implicated as being important for RIP activity (see Figure 1 legend for numbering scheme). Conserved amino acids E-230, R-233, and W-268 fell within the proposed active site cleft for the A-chain of ricin, as determined by x-ray crystallography (Montfort et al., 1987; Ready et al., 1988). At a fourth conserved residue in the cleft (266), the b-32 sequence has undergone a chemically conservative substitution of Q for N. Recent refinement in the crystallographic structure confirms that in addition to E-230, R-233, and W-268, invariant residues Y-106 and Y-149 are also part of the active site cleft (Katzin et al., 1991). All of these are present in b-32, as indicated in Figure 1.

Amino acids E-230, R-233, and W-268 have also been implicated in RIP function based on mutational analysis of the ricin A-chain in yeast. Amino acid substitution at any of these positions resulted in production of ricin A-chains that retained immunoreactivity but had reduced levels of enzymatic activity (Frankel et al., 1989). These three residues were identical in b-32 and the A-chain of ricin. In addition to the common RIP regions, the b-32 sequence had a putative RNA binding domain of the ribonucleoprotein consensus sequence (RNP-CS) type (Bandziulis et al., 1989). This domain is characterized by two conserved motifs (RNP 1 and RNP 2) flanking a 20- to 40-residue region. The putative RNA binding region of b-32 contained both RNP 1 (158 to 166) and RNP 2 (105 to 110) motifs that were separated by 38 amino acids.

Three other regions in b-32 that repeatedly had identity with other RIPs were PVLPP (65 to 69), TLAIR (97 to 101), and GFGG (144 to 147). Although the significance of these regions is unknown, two of them were found near invariant tyrosine residues at positions 106 and 149, respectively, and

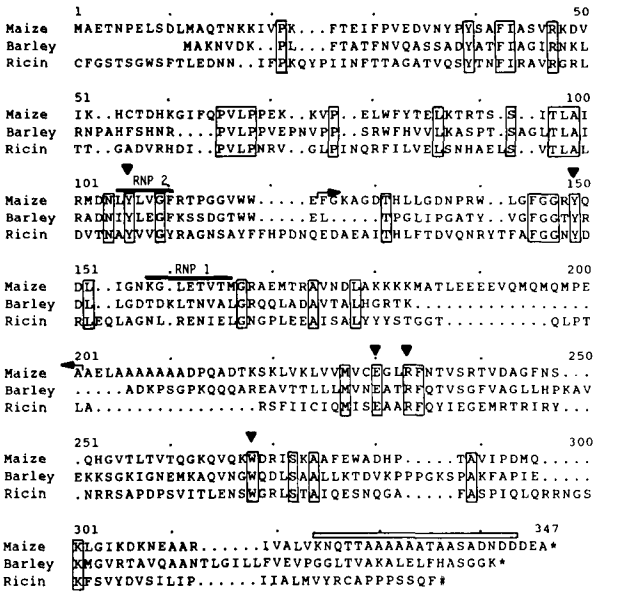

Figure 1. Comparison of Amino Acid Sequences of b-32 and Two Other RIPs.

Maize, barley, and ricin denote amino acid sequences derived from b-32 clone ZmcRIP-9, RIP 30 from barley, and ricin A-chain from castor bean, respectively (Lamb et al., 1985; Leah et al., 1991). Sequences have been aligned by introducing gaps (. . .) to maximize similarity. Numbers indicate positions within the alignment. Boxes enclose amino acids that are identical in the three proteins. Two motifs in b-32, RNP 1 and RNP 2, within a putative RNA binding domain (see text) are overlined with a solid bar. Five active site cleft residues (Y-106, Y-149, E-230, R-233, and W-268), invariant among RIPs published to date, are indicated by arrowheads. The peptide sequence determined from a purified tryptic peptide of b-32 is overlined with an open bar. Asterisks mark termination codons, and the pound sign follows the C-terminal amino acid in the mature ricin A-chain. Bent arrows delineate the location of the variant central region predicted by two previously reported b-32 sequences (Hartings et al., 1990).

may be involved in formation of the active site cleft (Katzin et al., 1991).

B-32 Has RIP Activity

To determine whether b-32 could enzymatically inactivate ribosomes, we assayed purified b-32 both for its capacity to modify rRNA specifically and to inhibit protein synthesis in vitro. The b-32 used in these assays was isolated from kernels harvested 28 days after pollination (DAP), as described in Methods. Purification relied on three anion exchange chromatography steps and thus differed significantly from other RIP purification procedures that routinely include chromatography over cation exchange columns. As judged by SDS-PAGE (Laemmli, 1970) and immunoblot analysis with

b-32 antiserum, the protein with an M_r of 32,000 was quantitatively retained on DEAE-Sepharose CL-6B (data not shown).

The RNA modification assay relied on aniline-induced cleavage of the sugar phosphate chains at modified nucleotides in the RNA (Peattie, 1979). At pH 4.5, aniline will induce strain scission at sites of modification, whereas unmodified nucleotides are unaffected. Thus, after RIP-catalyzed cleavage of the *N*-glycosidic bond at A^{4324} in the 28S rRNA, treatment of purified RNA with aniline should produce two fragments of ~4300 and ~425 nucleotides. (The numbering of A^{4324} is based on rat cytosolic 28S rRNA; Endo et al., 1987.)

RNA modification assays were performed using purified b-32 and target ribosomes from a rabbit reticulocyte cell-free translation lysate. Subsequent isolation of RNA, treatment with aniline, and fractionation through a denaturing polyacrylamide gel yielded a 425 nucleotide product visible in

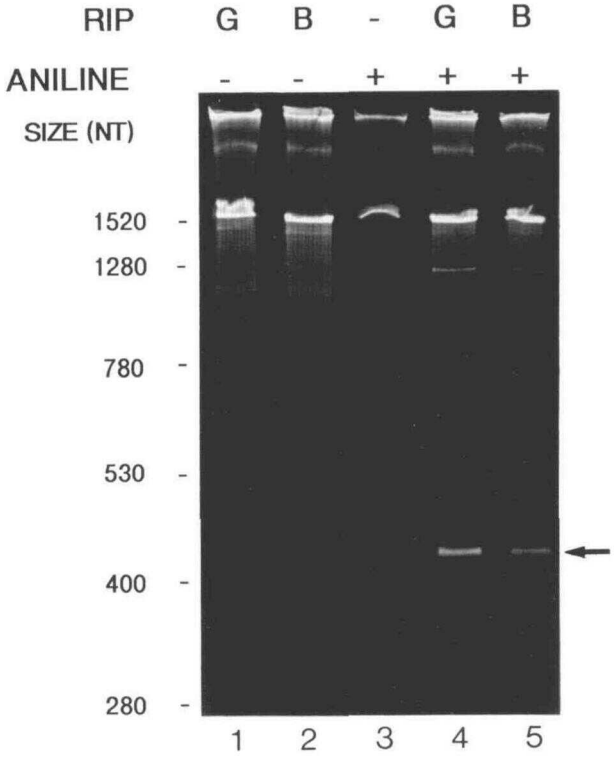

Figure 2. Aniline Cleavage of RIP-Modified rRNA.

RNA isolated from RIP-treated rabbit reticulocyte lysates was separated on a 4.5% denaturing polyacrylamide gel and visualized by ethidium bromide staining. Treatments consisted of incubation with H_2O (lane 3), 0.5 μg/mL gelonin (G; lanes 1 and 4), or 15 μg/mL b-32 (B; lanes 2 and 5) for 30 min at 30°C. Aliquots of purified RNA from each treatment were prepared for electrophoresis (lanes 1 and 2) or treated with aniline (as described in Methods) before electrophoresis (lanes 3 to 5). The positions of RNA size markers are indicated in nucleotides (NT) at left. The arrow indicates the small aniline cleavage product diagnostic for RIP-and aniline-treated samples.

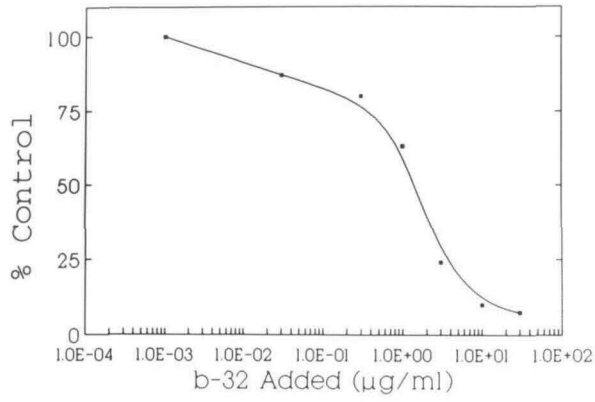

Figure 3. Translational Inhibition by b-32.

Purified b-32 from kernels 28 DAP was preincubated with rabbit reticulocyte lysates for 20 min at 25°C prior to initiation of a 20-min translation, as described in Methods. Trichloroacetic acid–precipitable radioactivity in each sample was normalized to controls with no b-32 added. The control value was 4.4×10^7 cpm/mL reaction. Data points represent the average value from duplicate samples. A 50% inhibition of translation occurs at 1.3 μg/mL.

Figure 2. RNA from a complete reaction (Figure 2, lane 5) contained the 425 nucleotide band indicative of RIP activity. In untreated rabbit reticulocyte lysates (Figure 2, lane 3) and a control lacking aniline (lane 2), the band diagnostic of RIP activity was not detectable. Thus, b-32 itself was not acting as an endo-ribonuclease, and neither was the protein preparation contaminated by a general RNase activity that might have interfered with in vitro translation assays. As a positive control, rabbit reticulocyte lysates were treated with gelonin (Figure 2, lanes 1 and 4), a well-characterized type 1 RIP from the seeds of *Gelonium multiflorum* (Stirpe et al., 1980). Treatment with gelonin and aniline produced a band that comigrated with the 425 nucleotide band produced by b-32 and aniline treatment (compare lanes 4 and 5).

The aniline reaction clearly demonstrated the specificity of the b-32 RIP activity. To quantify this activity, a translation inhibition assay was also used. Figure 3 shows a representative dose response curve for in vitro protein synthesis in rabbit reticulocyte cell-free translation reactions preincubated with purified b-32. A 50% reduction in the amount of radioactivity incorporated into TCA insoluble material (ID_{50}) was obtained at 1.3 μg/mL (41 nM) b-32 in a reaction containing 3 μM ribosomes, indicating that b-32 was acting catalytically. Under the same assay conditions, an ID_{50} of 6 ng/mL was obtained for gelonin.

Maize and Wheat Ribosomes Are Resistant to Maize b-32

Finding a ribosome inactivating activity in b-32 prompted us to further investigate this unusual RIP for the capacity to

Table 1. Effect of RIPs on Cell-Free Translation

Source of Ribosomes + Supernatants[a]	b-32[b]		Gelonin[b]	
	(% Control ± SD)			
Rabbit + Rabbit[c]	2.4 ± 1.6	(3)[d]	0.9 ± 0.1	(2)
Wheat[e] + Wheat[f]	100.5 ± 12.4	(9)	3.0 ± 1.1	(9)
Maize[g] + Wheat	92.3	(1)	3.6	(1)
Maize polyribosomes + Wheat or Maize	108.4 ± 4.0	(4)	12.0 ± 4.2	(4)

[a] Components were prepared as described in Methods.
[b] Values obtained with RIP to ribosome ratios between 0.3 and 15 were combined because there was no difference. See Methods for ranges for each ribosome type.
[c] Intact lysate.
[d] Numbers in parentheses refer to number of experiments.
[e] Ribosomes and salt-washed ribosomes showed no difference.
[f] Intact lysate or reconstruction.
[g] Puromycin-treated ribosomes.

distinguish between homologous and heterologous ribosomes. In general, nonplant ribosomes seem to be susceptible to RIP inactivation. However, susceptibility of plant ribosomes depends on the source of both the ribosome and the RIP, with homologous ribosomes having at least some resistance to inactivation (Owens et al., 1973; Harley and Beevers, 1982; Reisbig and Bruland, 1983; Battelli et al., 1984). We tested the capacity of purified b-32 to inactivate both maize and wheat germ cell-free translation systems. The experiments were carried out under the conditions described in Methods, and the results are presented in Table 1.

The addition of gelonin inhibited translation in both wheat germ and rabbit reticulocyte cell-free systems at RIP to ribosome molar ratios of less than one. Furthermore, gelonin also inhibited translation in a wheat germ postribosomal supernatant complemented with maize ribosomes. In contrast, at b-32 concentrations capable of inhibiting rabbit reticulocyte lysates, b-32 had no effect on either the wheat germ system or a wheat germ postribosomal supernatant complemented with maize ribosomes. Increasing the molar ratio of b-32 to ribosomes above one had no effect on translational capacity. The immunity of the wheat system to b-32 inactivation was not altered by a high salt wash of the ribosomes (Table 1), suggesting that either the ribosome itself or a tightly associated factor contributed to the resistance.

Accumulation of b-32 in Normal and *opaque-2* Maize

The recent identification of *Opaque-2* as a transcriptional activator prompted us to assay for b-32 expression in the absence of the Opaque-2 protein. A comparison of b-32 RNA and protein from normal and *opaque-2* kernels is shown in Figure 4. In normal kernels (Figure 4A), b-32 RNA levels reflected the increases reported for other *Opaque-2*–regulated RNAs during kernel development (Marks et al., 1985). In the *opaque-2* mutant, b-32 RNA was detectable during mid-maturation stages, but amounts were greatly decreased relative to those in normal kernels (Figure 4B).

To determine whether or not the b-32 RNA in *opaque-2* mutants was functional, we used an immunoblot assay to detect b-32 protein. As shown in Figure 4C, accumulation of b-32 protein is coordinated with that of b-32 RNA in normal corn. In *opaque-2* kernels harvested 30 DAP, protein was detectable but amounts were much lower than in normal kernels. Our data demonstrated that Opaque-2 is not absolutely required for seed-specific expression of b-32 but appears to be important for quantitative accumulation of b-32 protein.

RIP Activity from Immature Seed

A rabbit reticulocyte translation inhibition assay was used to compare RIP activity of b-32 extracted from *opaque-2* kernels and RIP activity from normal kernels at various stages of development. The small amounts of b-32 in *opaque-2* kernels complicated detection of the protein in the early stages of purification. To insure uniformity among purification steps, the procedure was modified by substituting step elutions for gradient elutions during chromatography. Dose response curves of RIP activity from normal kernels 14, 24, and 35 DAP

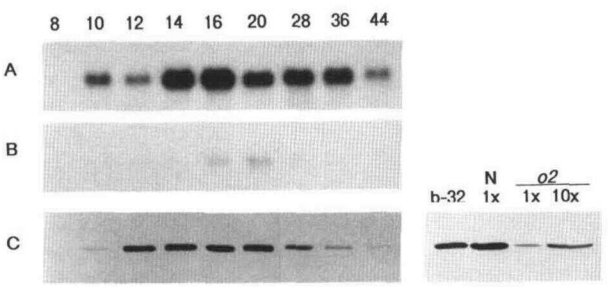

Figure 4. Accumulation of b-32 Throughout Kernel Development.

(A) and **(B)** RNA gel blots of equal amounts of total RNA from maize kernels were incubated with a radiolabeled b-32 cDNA clone. The age of the ear at the time of harvest is indicated at top as DAP.
(A) RNA gel blot of b-32 RNA in normal kernels. Total RNA was glyoxalated, fractionated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with b-32 cDNA at 68°C in 1 × SSC-containing buffers.
(B) RNA gel blot of b-32 RNA in *opaque-2* kernels.
(C) Immunoblot of b-32 protein in normal kernels. Soluble protein extracts of endosperm protein were fractionated by SDS-PAGE and immunoblotted using anti-b-32 antisera. Protein from equal fresh weights of endosperm was loaded in each lane for the developmental series. A direct comparison of b-32 levels in 28 DAP normal (N) and 30 DAP *opaque-2* (*o2*) endosperm can be made by comparing lanes with equal (1x) or 10-fold (10x) excess fresh weight equivalents of protein. The lane marked b-32 contains purified b-32.

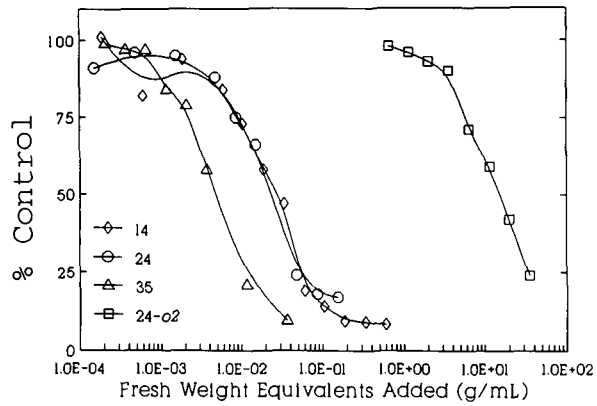

Figure 5. Dose-Response Curves for Translation Inhibition by b-32–Containing Extracts.

Translation inhibition reactions were performed with preincubation for 15 min at 20°C and translation for 30 min at 30°C. All protein additions were normalized to fresh weight of starting material (fresh weight equivalents). The samples used were partially purified as described in Methods from 200 g of normal kernels harvested at 14, 24, and 35 DAP or from *opaque-2* kernels harvested at 24 DAP. The ID_{50} values based on kernel fresh weight were 30 mg/mL (14 DAP sample), 22 mg/mL (24 DAP sample), 4.2 mg/mL (35 DAP sample), and 12,000 mg/mL (24 DAP *opaque-2* sample).

and *opaque-2* kernels 24 DAP are presented in Figure 5. The ID_{50} values for samples from normal kernels were clustered between 4 and 30 mg/mL lysate based on the fresh weight of starting material. In contrast, the ID_{50} for the *opaque-2* sample was much higher (12,000 mg/mL lysate).

RIP Activity Increases during Seed Germination

Detection of b-32 and RIP activity at late stages of kernel development suggested that RIP activity might also be present during seed germination. Figure 6 shows an analysis of RIP activity in germinating seeds. At 3 days after germination, RIP activity increased above that of mature seeds, and this increase continued until 11 days after germination. The increase in activity was correlated with an increase in proteolytic activity in germinating seeds (Moureaux, 1979; de Barros and Larkins, 1990). Even though RIP activity was increasing, there was marked reduction of the b-32 protein band detectable by immunoblotting but no decrease in b-32 cross-reacting material quantitated by ELISA analysis (data not shown). Such results were consistent with enhanced RIP activity due to post-translational activation of b-32.

DISCUSSION

We have shown that an RIP from developing maize kernels is the same protein that was previously identified as b-32. A cDNA clone (ZmcRIP-9) corresponding to this RIP was identical to the corresponding region of the maize genomic clone b32-129 reported previously and thus demonstrated that b32-129 is an active gene (Hartings et al., 1990). A different b-32 cDNA clone (b32.66) and its corresponding genomic clone (b32-120) have more than 99% identity with ZmcRIP-9 and b32-129, yet predict proteins with different central domains (Di Fonzo et al., 1988; Hartings et al., 1990). To better understand the relationship between the two types of b-32 genes, a codon usage analysis was performed. If a common ancestral origin is assumed, either three single insertions or three single deletions gave rise to the duplicate sequence. Monocot nuclear genes have a strong codon bias for a C or G in the third position with a mean percentage of 73.5, and maize exhibits even greater third position C/G bias (Campbell and Gowri, 1990). The alignment for ZmcRIP-9 and b32.66 in Figure 7 shows the third position C/G trend for the two b-32 gene types. ZmcRIP-9 maintained the strong third position C/G bias but b32.66 diverged significantly across the variant central domain. Analysis of these two sequences scored against a codon usage table from 39 maize nuclear genes gave similar results (J. Krawetz, H.W. Bass, and R.S. Boston, unpublished results). We inferred from these data that ZmcRIP-9 represented the ancestral sequence from which b32.66 most likely arose as a recent duplication. This central region in ZmcRIP-9 includes the RNP-CS region of the amino acid sequence (see Figure 1) as well as several amino acids conserved among other RIPs. Thus, it seems unlikely that ZmcRIP-9 and b32.66 both encode proteins with RIP catalytic activity.

Ribosome inactivation by an RIP requires a specific RNA binding event as well as hydrolysis of a single *N*-glycosidic

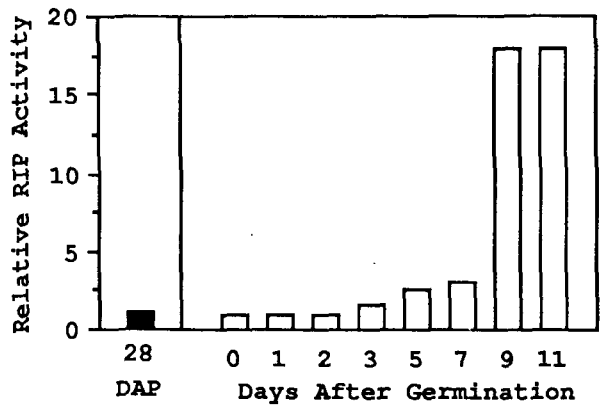

Figure 6. Maize Seed RIP Activity Increases during Germination.

Protein from a 55 to 85% ammonium sulfate fraction of a crude extract was used to generate translation inhibition curves under conditions described in Figure 5. ELISAs were used to determine the relative amount of b-32 cross-reacting material in each sample. RIP activities were normalized to that of a sample harvested at 28 DAP as shown in the left panel (b-32 ELISA-normalized ID_{50}^{-1} = 1.0).

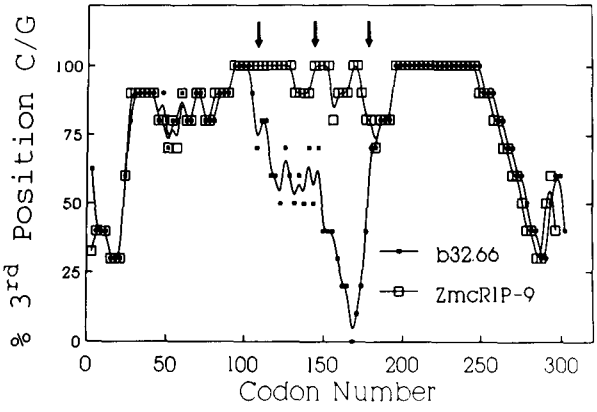

Figure 7. Comparison of Codon Bias for Two Cloned b-32 cDNAs.

Coding regions of ZmcRIP-9 and b32.66 (Di Fonzo et al., 1988) were scored for presence of C or G in the third position of each codon. Overlapping segments of 10 codons were evaluated consecutively, and the average score (%) of each segment was plotted at its midpoint. Arrows indicate positions of frameshifts caused by single base changes that give rise to the different central domain (Hartings et al., 1990).

bond (A^{4324}) in an RNA loop essential for translation elongation. An RNA binding consensus sequence, RNP-CS, initially identified in mRNA poly(A) binding proteins has now been noted for a number of RNA binding proteins including human and yeast poly(A) binding proteins, mammalian heterogeneous nuclear ribonucleoprotein particle proteins A1, C, and E, and a maize abscisic acid–induced protein (Adam et al., 1986; Bandziulis et al., 1989). The deduced b-32 amino acid sequence also included an RNP-CS that may facilitate the b-32-rRNA interactions required for RIP activity. RNP 1 and RNP 2 were identified in b-32 (Figure 1), and the significance of the homology was confirmed by repeated computer alignments of b-32 with RNP 1 or RNP 2 motifs from individual and consensus sequences compiled by Bandziulis et al. (1989). Recently, Brennan and Platt (1991) used site-directed mutagenesis to demonstrate the functional importance of an RNP 1-like sequence in RNA binding by the *Escherichia coli* transcriptional terminator protein rho. Alteration of a G-F-G-F in the rho RNP 1 site to G-L/A-G-L/A resulted in mutant proteins that were defective for RNA binding. The sequence G-F-G-G (144-147) in b-32 lies adjacent to the putative b-32 RNP 1 site within a block of homology that is even greater than that found within the active site cleft. Pairwise searches were also performed on the barley RIP 30 and ricin A-chain sequences. Although several sequence blocks had some homology with either RNP 1 or RNP 2, none of them was organized as an RNP-CS RNA binding domain (H.W. Bass and R.S. Boston, unpublished results).

We have shown that preparations of b-32 exhibited ribosome-inactivating properties characteristic of RIPs. The unusual acidic properties of b-32 compared to the high isoelectric points reported for other RIPs can be attributed in part to a 35-amino acid region from M-184 to K-218 and an aspartate-rich region at the carboxy terminus (Figure 1). Proteolytic processing of the heterodimeric RIP, ricin, occurs by both terminal and internal removal of amino acids before formation of the active form (Butterworth and Lord, 1983). Walsh et al. (1991) have observed an increase in RIP activity in preparations of b-32 treated with papain and subtilisin and have demonstrated that the activation is due to proteolytic processing of the central acidic region. Furthermore, in mature maize kernels, RIP activity is found in a protein with tightly associated polypeptide subunits of M_r 16,500 and 8500. These two polypeptides are homologous to regions flanking the amino and carboxy termini of the acidic domain of b-32, respectively. This protein may represent the 25-kD maize RIP previously reported by Coleman and Roberts (1982). Such findings are consistent with our repeated observations of increases in RIP activity correlating with the appearance of smaller molecular weight products with basic isoelectric points after storage of b-32 at either 4°C or −20°C (H.W. Bass and R.S. Boston, unpublished results). These lower molecular weight forms are not detected in freshly ground endosperm. However, they have been detected by others in b-32 preparations from both normal kernels and *proline-1* mutants and may reflect cleavage by endogenous proteases during purification (Manzocchi et al., 1986; Di Fonzo et al., 1988). Further support for a proteolytic activation of b-32 was found in the demonstration of increases in RIP activity (Figure 6) coincident with the onset of protease synthesis and storage protein degradation during germination (Moureaux, 1979; Torrent et al., 1989; de Barros and Larkins, 1990; Hay et al., 1991).

Synthesis of b-32 as an inactive preprotein could provide an additional self-resistance mechanism against ribosome inactivation. Many RIPs, like ricin, abrin, and trichosanthin, are targeted to endomembrane systems whose spatial separation from ribosomes could provide a mechanism for resistance. Unlike these RIPs, b-32, RIP 30 in barley, and tritin in wheat are cytoplasmic proteins. We have shown that maize ribosomes in the presence of maize or wheat supernatants were resistant to b-32 inactivation at levels 50-fold greater than those necessary for inactivation of rabbit ribosomes (Table 1). We are currently investigating the possibility that soluble factors can contribute to the resistance of cereal ribosomes to b-32 inactivation. Certainly, the cereal ribosomes were not intrinsically resistant to all RIPs, however, because gelonin, a dicotyledonous seed RIP, was capable of catalytic inactivation of ribosomes from both maize and wheat. In the case of ricin, ribosomes from a wide variety of plant families have greater resistance to ricin than do rat ribosomes (Harley and Beevers, 1982). Interestingly, none of the plant ribosomes assayed in that study was completely resistant to ricin and castor bean ribosomes were no more resistant to ricin than were wheat ribosomes. Such data suggest that sufficient self-immunity can be obtained by the combination of partial resistance of ribosomes and compartmentalization of ricin. For barley, RIP 30 is not synthesized in

endosperm at early stages of development but does accumulate at late stages (Leah et al., 1991). In contrast, synthesis of b-32 is initiated in kernels as early as 10 DAP and levels at maturity account for 1 to 3% of the soluble seed protein (Soave et al., 1981). Perhaps the early onset of b-32 synthesis results in high cytosolic quantities of b-32 that necessitate an additional level of self-protection.

The biological role of RIPs is not known. They have features of some constitutive defense agents in that they are preferentially toxic to nonplant cells, accumulate to high levels in seeds, and have no apparent effect on endogenous processes. A role for b-32 in defense against pathogens is suggested by an increase in susceptibility to fungal attack (Loesch et al., 1976) and insect feeding (Gupta et al., 1970) in *opaque-2* mutant kernels, although contributions resulting from pleiotropic effects of *opaque-2* cannot be ruled out. Of the numerous RIPs that have been identified, only a few have been characterized at the genetic level. We suggest that the coordinate *Opaque-2*-controlled synthesis of this maize RIP and the major seed storage proteins provides the germinating seedling with both nutritional benefits and protection against pathogen invasion of the surrounding endosperm.

METHODS

Plant Material

Maize (*Zea mays*) inbred W64A and its near isogenic mutant *opaque-2* were grown and self-pollinated at the Purdue University Agronomy Farm (West Lafayette, IN) from 1985 to 1987 and at the NC State University Research Unit I (Raleigh, NC) from 1988 to 1991. Ears were harvested at the developmental stages indicated and rapidly frozen in liquid nitrogen. Kernels were shelled onto dry ice and stored at −80°C.

Purification of b-32

All purification steps were performed at 4°C. The b-32 protein was purified from kernels of W64A 28 days after pollination (DAP) according to the following protocol modified from that of Di Fonzo et al. (1986). Briefly, coarse meal was stirred for 2 hr with 4 volumes (w/v) of 50 mM Tris-HCl (pH 6.8 at 25°C), 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation. Proteins recovered from a 50 to 75% saturated ammonium sulfate precipitation of the supernatant were chromatographed over DEAE-Sepharose CL-6B (two times) developed with a linear NaCl gradient and hydroxylapatite washed with 1.0 M NaCl and developed with a linear phosphate gradient. Fractions containing b-32 as judged by migration through SDS-polyacrylamide gels were then dialyzed against 50 mM Tris-HCl (pH 6.8 at 25°C), applied to a 2 × 1 cm DEAE-Sepharose CL-6B column, and eluted with 50 mM Tris-HCl, 200 mM NaCl. Fractions containing b-32 were dialyzed against 10 mM sodium phosphate, pH 7.0, and stored frozen at −20°C.

For production of antiserum, b-32 was prepared as described above from kernels harvested 35 DAP except that the 1.0 M NaCl wash was omitted from the hydroxylapatite chromatography step. Approximately 200 μg of b-32 mixed 1:1 with Freund's complete adjuvant was injected subcutaneously into a New Zealand white rabbit. The rabbit was injected again after 2 weeks and subsequently was bled from the ear at 1-week intervals.

To insure uniformity in preparation procedures from plant materials with very different amounts of b-32, gradient elutions from the DEAE-Sepharose CL-6B and hydroxylapatite chromatography steps were replaced with step elutions for the comparative experiments presented in Figure 5. Frozen kernels from normal corn 14, 24, or 35 DAP or *opaque-2* corn 24 DAP were used as starting material. The DEAE-Sepharose CL-6B column was developed with 50 mM Tris-HCl (pH 6.8 at 25°C), 50 mM NaCl, and the hydroxylapatite column was developed with 150 mM potassium phosphate.

For the experiments in Figure 6, crude preparations of b-32 were obtained from kernels that had been surface-sterilized, soaked overnight in distilled H_2O, and germinated in the dark in vermiculite for the times indicated in the figure. Kernels were separated from seedlings, frozen in liquid nitrogen, ground to meal, and extracted for 2 hr with 50 mM Tris-HCl (pH 6.8 at 25°C). Cell debris was removed by centrifugation, and proteins recovered from a 50 to 85% ammonium sulfate precipitation of the supernatant were dialyzed for 2 hr against 10 mM potassium phosphate, pH 7.0.

Immunoblot Analysis

Flour was prepared from acetone-extracted, dissected endosperms at developmental stages noted in the figure legends, homogenized with 50 mM Tris-HCl (pH 6.8 at 25°C) and 1 mM phenylmethylsulfonyl fluoride (50 mL/g flour) for 3 hr at 4°C, and clarified by brief centrifugation. Equal volumes (4 μL per lane) were loaded onto 12% SDS polyacrylamide gels and electrophoresed, and the proteins were electroblotted using a semidry apparatus according to manufacturer's instructions (Polyblot, American Bionetics, Hayward, CA). Immunoblot analysis, in which anti-b-32 polyclonal antisera was used at a 1:3000 dilution, was performed using a horseradish peroxidase color reaction as described previously (Lending et al., 1988).

RIP-Specific RNA Modification Analysis

Rabbit reticulocyte lysate (translation grade, Green Hectares, Oregon, WI) was preincubated for 15 min on ice in 25 mM Hepes-KOH, pH 7.6, 10 mM MgOAc, 10 mM ATP, and 0.1 mg/mL yeast tRNA at a final dilution of 45% (v/v). RIPs (b-32 or gelonin [Calbiochem Corp., San Diego, CA]) were added and the reactions were incubated at 30°C for 30 min in a final volume of 20 μL and terminated by the addition of TLES (0.2 M Tris-HCl [pH 8.0 at 25°C], 0.1 M LiCl, 20 mM EDTA-NaOH, pH 8.0, 0.2% [w/v] SDS, and 15 mM DTT) to a final volume of 200 μL. RNA was purified by sequential phase extractions with TLES-buffered phenol, phenol/$CHCl_3$ (v/v, 1:1), and $CHCl_3$ and concentrated by ethanol precipitation. The aniline used (Aldrich Chemical Co., Milwaukee, WI) was redistilled and stored in small aliquots at −80°C. For aniline treatment, an aliquot of RNA was vacuum dried and redissolved in 1 M aniline/acetate, pH 4.5, for 45 min on ice in darkness (based on Peattie, 1979). The reaction was stopped by freezing at −80°C, and the buffer was removed by vacuum drying the frozen pellet. The RNA was redissolved in 20 μL of H_2O, frozen, and vacuum dried before final dissolution in running buffer and electrophoretic fractionation through a 4.5% denaturing polyacrylamide

gel. RNA molecular weight markers were purchased from Promega (Madison, WI).

Translation Inhibition Assays

For dose-response curves presented in Figures 3 and 5, rabbit reticulocyte lysate (Green Hectares or Promega) was incubated with b-32 or gelonin in the presence of 13 units per milliliter of Inhibit-Ace (5 Prime-3 Prime, West Chester, PA). Aliquots were added from a single master mix (Promega) supplemented with 20 μM hemin, 0.1 mM GTP-KOH, pH 7.6, 5 units per milliliter Inhibit-Ace, 15 μg/mL brome mosaic virus (BMV) RNA (Promega), and 1 mCi/mL ^{35}S-L-methionine (ICN Biomedicals, Inc., Costa Mesa, CA) for a final reaction volume of 20 μL containing 55% (v/v; Green Hectares) or 40% (v/v; Promega) lysate. Incubation times are noted in the figure legends. Translation reactions were stopped by the addition of 4 volumes of 10 mM EDTA-NaOH, pH 8.0, 1 mM DTT. Incorporation of ^{35}S-methionine into protein translated in vitro was measured by hot TCA precipitation. For individual dose response curves, all values including control (no RIP) were derived from the same experiment. Samples with no RIPs (typical values of 0.4 to 1 × 10^8 cpm/mL reaction) were assigned a value of 100%.

To determine the effect of b-32 and gelonin on ribosomes from various species (Table 1), RIPs were added to translation reactions containing intact cell lysates or lysates reconstructed from various supernatant fractions and purified ribosomes or polyribosomes. The effect of RIPs on translation of endogenous (maize polyribosomal) mRNAs or BMV mRNA (added at 5 μg/mL) was then assayed by measuring incorporation of ^{3}H-leucine into protein at 30 min, as previously described (Webster et al., 1991). Micrococcal nuclease-treated wheat germ and rabbit reticulocyte lysates and BMV RNA were from Promega. Lysates were centrifuged 30 min at 205,000*g* to yield supernatant and ribosomal fractions. In addition, salt-washed wheat germ ribosomes were prepared by the method of Lax et al. (1986). A 0 to 70% ammonium sulfate fraction of maize postribosomal supernatant (RT0-70) and maize polyribosomes were prepared as described previously (Webster et al., 1991). Maize ribosomes were purified from polyribosomes by dissociation with puromycin and high salt (Blobel and Sabatini, 1971), and pelleting through 1 M sucrose. Translation reactions (20 μL) contained approximately 40 pmol rabbit ribosomes, 15 to 40 pmol wheat ribosomes, 15 pmol maize ribosomes, or 2 to 5 pmol maize polyribosomes. The molar ratios of b-32 and gelonin to ribosomes in the translation reactions were 0.3 to 2 for rabbit and wheat ribosomes, 5 for maize ribosomes, and 3 to 15 for maize polyribosomes.

Plasmid Construction and Preparation of Probes

Antisera raised against b-32 (1:1000) were used to screen a λgtII expression library from kernel RNA of a predominately TX441 maize line provided by E. Wurtzel, Lehman College, City University of NY. The genotype of the TX441 maize line was normal for *Opaque-2*. Library construction and screening have been described previously (Fontes et al., 1991). The DNA sequence of the longest positive clone isolated (ZmcRIP-3) was obtained by the dideoxy sequencing method of Sanger (1977) using a Sequenase kit (US Biochemicals). This sequence encoded a protein that corresponded to published genomic clones b32-129 and b32-152 (Hartings et al., 1990). To obtain the corresponding b-32 cDNA clone from the inbred line W64A, synthetic oligonucleotides corresponding to regions near the 5′ and 3′ ends of the sequence (5′-CATTTTAGCTGTTGATAC-3′ and 5′-GCAGCAGATCATGATGTGTC-3′, respectively) were synthesized and used as primers for amplification by the polymerase chain reaction. Oligo(dT)-primed first-strand synthesis from 1 μg of poly(A)$^+$ RNA from kernels 14 DAP was carried out according to manufacturer's specifications (Superscript RT, Gibco Bethesda Research Laboratory). The mRNA was removed by RNase A digestion and the cDNA was amplified using Vent DNA polymerase (New England Biolabs, Beverly, MA). Polymerase chain reaction products were ligated directly into SmaI-linearized pBluescript KS+ (Stratagene). The DNA sequence of ZmcRIP-9 derived by polymerase chain reaction was identical to that of the W64A genomic clone b32-129 except for substitutions of C for T^{325} and T^{439} and A for C^{829} (Hartings et al., 1990). Only the third change resulted in an amino acid substitution (I for L). The deduced amino acid sequence of ZmcRIP-9 was also identical to the amino acid sequence of a b-32 tryptic peptide purified from W64A kernels 28 DAP (Figure 1). The DNA sequences have been assigned accession numbers M83926 for the ZmcRIP-3 clone and M83927 for the ZmcRIP-9 clone in the GenBank database.

Computer Analysis

The Gap and Bestfit programs from the Genetics Computer Group software package (Madison, WI) were used to align the amino acid sequence of each RIP to that deduced from the b-32 cDNA clone ZmcRIP-9 (Devereux et al., 1984). The three sequences were then aligned using the Lineup program. Amino acid sequences of barley (*Hordeum vulgare*) RIP 30 and the A-chain of castor bean (*Ricinus communis*) ricin were derived from cloned cDNA sequences in EMBL and GenBank data bases (Lamb et al., 1985; Leah et al., 1991). In all cases, the default values for sequence analysis programs were employed.

For comparing C/G bias of the two b-32 sequences with codon bias based on maize nuclear genes, codon usage values were determined by D.A. Hamilton and J.P. Mascarenhas (as noted in *Maize Genetics Cooperative Newsletter* [1991], Vol. 65, 2–3).

RNA Gel Blotting

RNA for gel blot analysis was isolated from kernels essentially as described by Langridge et al. (1982). Equal amounts of total RNA were glyoxalated and fractionated as described by McMaster and Carmichael (1977). The RNA was transferred to a nylon membrane (Genescreen, DuPont-New England Nuclear, Boston, MA) by capillary transfer with 20 mM sodium phosphate, pH 6.9, for 12 hr at room temperature and immobilized by UV crosslinking (Stratalinker, Stratagene, La Jolla, CA). The hybridization probe was radiolabeled with α-^{32}P-dCTP by random primed labeling (Boehringer-Mannheim, Indianapolis, IN) to a specific activity of 10^9 cpm/μg. Aqueous hybridizations were performed as described for nylon membranes by Galau et al. (1986) for high (Tm −25°C) stringencies. Autoradiography was performed at −80°C using a Lightning-Plus intensifying screen (Du Pont–New England Nuclear).

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