PERGAMON

Phytochemistry 52 (1999) 233–238

PHYTOCHEMISTRY

Degradation of Bowman-Birk protease inhibitor mRNA with a cell-free extract

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Received 21 July 1998; received in revised form 15 January 1999

Abstract

A cell-free mRNA degradation system consisting of the polysomal and postpolysomal fractions was obtained from cultured soybean cotyledons for studying Bowman-Birk protease inhibitor (BBPI) mRNA stability. This in vitro system reflected the shorter in vivo half-life of BBPI mRNA of cotyledons cultured in basal-medium as compared to cotyledons cultured in methionine-supplemented medium. Most of the BBPI mRNA degradative activity was found to be present in the postpolysomal supernatant fraction. The higher rate of BBPI mRNA degradation in basal medium-cultured cotyledons was due to an increased destabilizing activity specific to BBPI mRNA in the postpolysomal fraction from basal-medium cultured cotyledons. The specificity was absent when purified RNA was used as the substrate. Degradation of the mRNA was not divalent cation-dependent and was inhibited in the presence of higher concentrations of monovalent and divalent cations.

Keywords: *Glycine max*; Leguminosae; Soybean; Cell-free mRNA degradation; Bowman-Birk protease inhibitor

1. Introduction

In previous studies, we found that when soybean cotyledons are cultured in a complete medium supplemented with methionine (met) (met-cultured), the β-subunit of the 7 S storage protein and its mRNA are absent, but Bowman-Birk protease inhibitor (BBPI) and its mRNA are abundant. In contrast, when cotyledons are cultured in complete medium without met (basal-cultured), the β-subunit and its mRNA are prominent, but amounts of BBPI and its mRNA are low compared to those in met-cultured cotyledons (Holowach, Thompson & Madison, 1984; Holowach, Madison & Thompson, 1986; Biermann, de Banzie, Handelsman, Thompson & Madison, 1998). Levels of α/α′ subunits of the 7 S protein and their mRNAs are not different under these two culturing conditions (Holowach et al., 1986). The difference of BBPI mRNA levels is caused by greater stability of BBPI mRNA in met-cultured cotyledons as compared to basal-cultured cotyledons (Cheng, Madison & Thompson, 1999).

It appears that both *cis*-acting elements and *trans*-acting factors are involved in the control of mRNA degradation (Abler & Green, 1996). However, it is difficult to dissect mechanisms that determine differential rates of mRNA degradation using intact cells or tissues. Genetic approaches, which have been successfully used in prokaryotes and yeast (Sachs & Deardorff, 1992) are difficult when applied to higher eukaryotes except for those with small genomes and short generation times like Arabidopsis (Abler & Green, 1996). In light of these difficulties, a cell-free mRNA degradation system which mimics the in vivo situation becomes valuable. For studying histone mRNA degradation, Ross and Kobs (1986) developed such a cell-free system from human erythroleukemia cells.

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0031-9422/99/$ - see front matter
PII: S0031-9422(99)00108-9

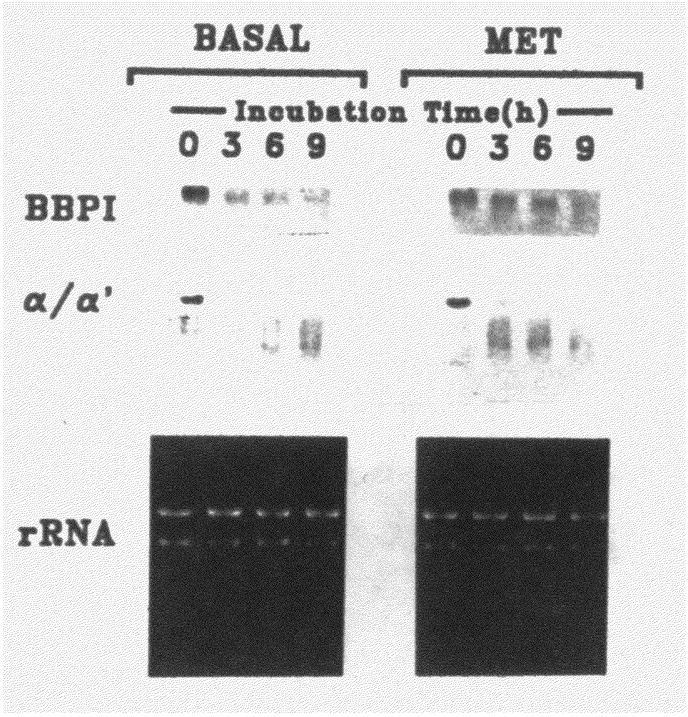

Fig. 1. mRNA degradation in the cell-free system. Standard cell-free degradation reactions were performed by incubating polysomal and postpolysomal fractions from basal-cultured cotyledons or polysomal and postpolysomal fractions from methionine-cultured cotyledons. RNA samples recovered at each time point were analyzed by gel blots (upper and middle panel). Ribosomal RNA was photographed with UV light after ethidium bromide staining (lower panel). For BBPI mRNA, the difference in band intensity between basal and methionine shown here does not reflect the actual difference of BBPI mRNA levels between basal-cultured and methionine-cultured cotyledons, because different film-exposure times were used.

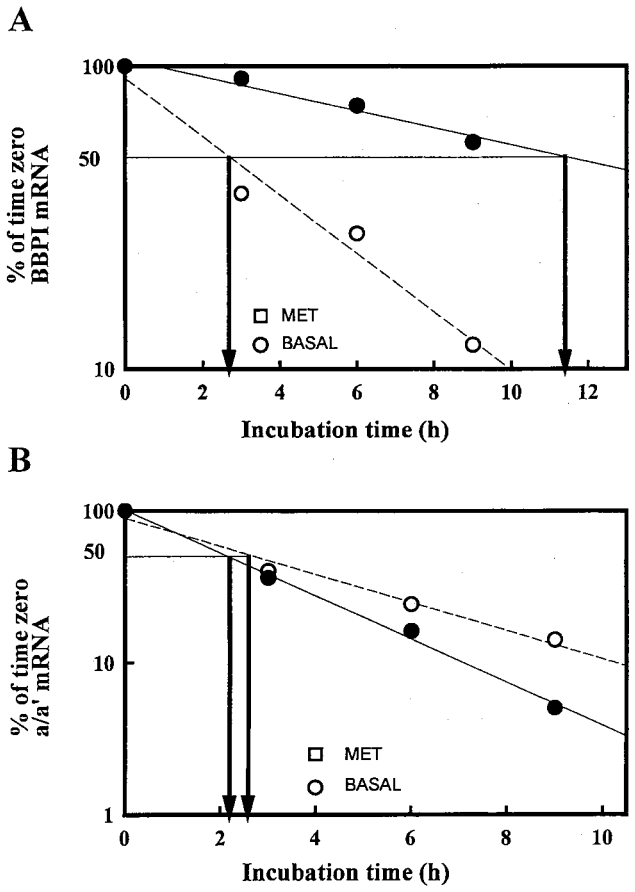

Fig. 2. Quantitation of autoradiograms shown in Fig. 1. The quantity of RNAs remaining at each time point relative to that at time zero was plotted. Lines were drawn based on the least squares method. (A) Decay kinetics of BBPI mRNA in the cell-free system. (B) Decay kinetics of α/α′ mRNAs in the cell-free system. ● Cultured on methionine medium. ○ Cultured on basal medium.

Subsequently, several similar systems have been developed for other mammalian cell lines and organs (Altus & Nagamine 1991; Bandyopadhyay, Coutts, Krowczynska & Brawerman, 1990; Narayanan, Fujimoto, Geras-Raaka & Gershengorn, 1992; Pastori, Moskaitis & Schoenberg, 1991; Pei & Calame, 1988; Sunitha & Slobin, 1987; Wager & Assoian, 1990), yeast (Vreken, Buddelmeijer & Raue, 1992) and, recently, plants (Byrne, Seeley & Colbert, 1993; Tanzer & Meagher, 1994). Here, we report that a cell-free system for studying mRNA stability can be obtained from cultured soybean cotyledons. This system reflected the shorter half-life of BBPI mRNA in vivo of basal-cultured cotyledons as compared to met-cultured cotyledons.

2. Results

2.1. Degradation of BBPI mRNA in a cell-free system

Standard reactions for mRNA degradation in a cell-free system obtained from cultured soybean cotyledons were carried out as described in the Experimental section. As shown in Figs. 1 and 2A, BBPI mRNA, in reactions containing polysomal and postpolysomal fractions from met-cultured cotyledons, was degraded at a much slower rate than in reactions containing the same fractions from basal-cultured cotyledons (the ratio of the BBPI mRNA half-life of met to basal was 4.6 ± 0.3, mean $\pm$ std error, $n = 3$). This result is in good agreement with the ratio of about 5 between the in vivo half-life of BBPI mRNA in met-cultured cotyledons and that in basal-cultured cotyledons (Cheng et al., 1999). To show that the differential stabilities of BBPI mRNA observed above did not result from higher nonspecific degradative activities of reaction mixtures prepared from basal-cultured cotyledons, the same filters hybridized with the BBPI probe were stripped and rehybridized with the probe of α/α′ subunits. As shown in Figs. 1 and 2B, the decay rate of α/α′ mRNAs in reactions containing fractions from basal-cultured cotyledons was similar to that from

Table 1
Localization of mRNA degradative activities[a]

Source of		Percentage of zero time mRNA	
Polysomal fraction	Postpolysomal fraction	BBPI[b]	α/α′[c]
Basal	None added	104 ± 3 (n = 3)[d]	82 ± 6 (n = 6)[d]
Methionine	None added	116 ± 8 (n = 3)	85 ± 3 (n = 3)
None added	Basal	58 ± 5 (n = 3)	Not detected
None added	Methionine	102 ± 8 (n = 3)	Not detected
Methionine	Basal	49 ± 4 (n = 3)	24 ± 4 (n = 3)
Basal	Basal	46 ± 8 (n = 2)	31 ± 1 (n = 2)
Methionine	Methionine	95 ± 5 (n = 2)	27 ± 7 (n = 2)

[a] Cell-free mRNA degradation reactions were set up with different combinations of polysomal fractions and postpolysomal fractions from basal or methionine-cultured coyledons, incubated for 4 h at 25° and analyzed as described in the Experimental section.
[b] BBPI stands for Bowman-Birk Protease Inhibitor.
[c] α/α′ refers to two subunits of the 7 S soybean storage protein.
[d] Mean ± standard error; n = number of independent measurements.

met-cultured cotyledons (the ratio of met to basal is 1.0 ± 0.1, mean ± std error, n = 3). Also ribosomal RNA (in both reaction mixtures) was intact throughout the whole incubation as judged from agarose gel electrophoresis and ethidium bromide staining (Fig.1). Thus, from these results, we concluded that this cell-free system reflected the in vivo situation and could be used for studying BBPI mRNA degradation.

2.2. Localization of the mRNA degradative activity in the postpolysomal fraction

Since there is evidence that some mRNA degradative activities are associated with polysomes (Ross, 1993), we first attempted to study BBPI mRNA degradation in vitro by incubating the polysomal fraction alone. However, as shown in Table 1, no significant RNA degradative activity was found in our polysomal fractions prepared either from basal- or met-cultured cotyledons. Similar results were obtained when extraction buffers containing different K^+ and Mg^{++} concentrations (60 mM K^+ and 30 mM Mg^{++}, or 1.5 mM K^+ and 1 mM Mg^{++} were used, data not shown). Thus, the RNA degradative activity, observed in Fig. 1, was present in the postpolysomal fraction. To verify this point, we incubated the postpolysomal fraction alone. As shown in Table 1, BBPI mRNA in the postpolysomal fraction from basal-cultured cotyledons was degraded about 40% after a 4 h incubation, but that in the postpolysomal fraction from met-cultured cotyledons was stable. Degradation of α/α′ mRNA (from 7 S storage protein subunits) was measured to determine specificity. There was no detectable α/α′ mRNA remaining in either postpolysomal fraction (in Table 1 indicated by 'not detected') indicating the possibility of two degradative activities.

To determine whether there is any special modification (e.g. methylation) or conformational change of BBPI mRNA that renders it resistant to nucleases in met-cultured cotyledons, the polysomal fraction from met-cultured cotyledons and the postpolysomal fraction from basal-cultured cotyledons were incubated together. BBPI mRNA in the polysomal fraction from met-cultured cotyledons was degraded about 50% after a 4 h incubation with the postpolysomal fraction from basal-cultured cotyledons, which is in contrast to the absence of degradation when the polysomal and postpolysomal fractions from met-cultured cotyledons were incubated together (Table 1). On the other hand, the α/α′ mRNAs in the polysomal fraction from met-cultured cotyledons were degraded by the degradative activity in the postpolysomal fraction from basal-cultured cotyledons to the same extent as that in the postpolysomal fraction from met-cultured cotyledons (Table 1). These results suggested that the difference in BBPI mRNA stabilities obtained from the cell-free system was the result of the difference in a degradative activity specific to BBPI mRNA present in the postpolysomal fraction.

2.3. Evidence for the presence of a BBPI mRNA destabilizing activity in the postpolysomal fraction from basal-cultured cotyledons

Two possible reasons for the higher BBPI mRNA degradative activity in the postpolysomal fraction from basal-cultured cotyledons than in the fraction from met-cultured cotyledons are: (1) a higher level of a destabilizer in the postpolysomal fraction from basal-cultured cotyledons, or (2) a stabilizer present in the postpolysomal fraction from met-cultured cotyledons. To address this question, the polysomal fraction from met-cultured cotyledons was incubated with postpolysomal fractions from basal-cultured or from met-cul-

Table 2
Effect of mixing the postpolysomal fraction from methionine-cultured cotyledons and the postpolysomal fraction from basal-cultured cotyledons on Bowman-Birk protease inhibitor mRNA[a]

Postpolysomal fraction	Percentage of zero time mRNA
Basal	50 ± 5 ($n = 2$)[b]
Basal + methionine	42 ± 1 ($n = 2$)
Heated basal + methionine[c]	81 ± 4 ($n = 2$)
Basal + heated methionine[c]	52 ± 11 ($n = 2$)

[a] Cell-free mRNA degradation reactions were set up by mixing the polysomal fraction from methionine-cultured cotyledons with different combinations of postpolysomal fractions as indicated, incubated for 6 h at 25° and analyzed as described in Table 1.
[b] Mean ± standard error; n = number of independent measurements.
[c] Heated at 65° for 10 min.

tured cotyledons. As shown in Table 2, the presence of the postpolysomal fraction from met-cultured cotyledons in the mixture did not stabilize the BBPI mRNA. This result indicated that the degradation of BBPI mRNA was caused by higher levels of a degradative activity in the postpolysomal fraction from basal-cultured cotyledons.

To further confirm this point, the polysomal fraction from met-cultured cotyledons was incubated with the heated postpolysomal fraction from basal-cultured cotyledons and the unheated postpolysomal fraction from met-cultured cotyledons, and vice versa. As shown in Table 2, BBPI mRNA was more stable in the reaction mixture containing the heated postpolysomal fraction from basal-cultured cotyledons, but the presence of heated or unheated postpolysomal fractions from met-cultured cotyledons did not have an effect on BBPI mRNA degradation. This result again suggested that the presence of a destabilizer (heat labile) in the postpolysomal fraction of basal-cultured cotyledons was the cause of the degradation of BBPI mRNA.

Table 3
Effect of monovalent and divalent cations of the degradation of mRNA in the cell-free system[a]

K^+ (mM)	Mg^{++} (mM)	Percentage of zero time mRNA	
		BBPI[b]	α/α′[b]
10[c]	5[c]	50 ± 7 ($n = 4$)[d]	21 ± 2 ($n = 4$)[d]
5	5	54 ± 2 ($n = 2$)	21 ± 1 ($n = 2$)
100	5	85 ± 9 ($n = 2$)	55 ± 15 ($n = 2$)
10	2.5	51 ± 10 ($n = 2$)	13 ± 1 ($n = 2$)
10	20	81 ± 10 ($n = 2$)	48 ± 7 ($n = 2$)
10	5 + 20 mM EDTA	50 ± 4 ($n = 2$)	22 ± 5 ($n = 2$)

[a] Cell-free mRNA degradation reactions using the polysomal fraction from methionine-cultured cotyledons and the postpolysomal fraction from basal-cultured cotyledons were incubated for 4 h at 25° and analyzed as described in Table 1, except that K^+ and Mg^{++} concentrations were varied as indicated.
[b] See footnote to Table 1.
[c] The concentration used in standard reactions.
[d] Mean ± standard error; n = number of independent measurements.

Table 4
Degradation of purified RNA in the cell-free system[a]

Postpolysomal fraction	Purified RNA	Percentage of zero time RNA		
		rRNA[b]	BBPI[c]	α/α′[c]
Basal	Basal	41	52	25
Methionine	Methionine	44	51	31

[a] Polysomal RNA was purified by phenol/chloroform extraction from the polysomal fraction. Ten μg of purified RNA was mixed with the postpolysomal fraction (containing 100 μg of protein), incubated for 4 h at 25° and analyzed as described in Table 1.
[b] Ribosomal RNA was quantified by scanning the ribosomal bands on a Polaroid film of the agarose gel stained with ethidium bromide and exposed to ultraviolet light.
[c] See footnote to Table 1.

2.4. Some properties of the cell-free mRNA degradation system

Table 3 shows that the degradation of mRNA (both BBPI mRNA and α/α′ mRNAs) did not require Mg^{++} because the addition of excess EDTA did not affect the degradation rate; however, the presence of 20 mM Mg^{++} inhibited the degradation of both mRNAs. Also the degradation of both BBPI mRNA and α/α′ mRNAs was reduced in the presence of a higher concentration of K^+ (100 mM).

We also tested purified total RNA as the substrate for studying mRNA degradation in vitro. However, as shown in Table 4, ribosomal RNA was rapidly degraded, and the differential stability of BBPI mRNA observed between basal-cultured and met-cultured cotyledons disappeared (even when only half of the amount of the postpolysomal fraction (100 μg protein) was incubated with purified RNA (data not shown).

3. Discussion

In this study, we have obtained a cell-free mRNA degradation system which reflected the fact that regulation of BBPI mRNA stability is the major factor in the accumulation of higher amounts of BBPI mRNA and its protein in met-cultured cotyledons as compared to basal-cultured cotyledons (Cheng et al., 1999). Most of the mRNA degradative activity in this system was located in the postpolysomal fraction. Although nuclease activities in several cell-free mRNA degradation systems derived from mammalian cells (Ross & Kobs,

1986; Altus & Nagamine, 1991; Bandyopadhyay et al., 1990; Pastori et al., 1991; Sunitha & Slobin, 1987; Wager & Assoian, 1990) yeast (Vreken et al., 1992) and from oat and soybean leaves (Byrne et al., 1993; Tanzer & Meagher, 1994) are polysome-associated (Bandyopadhyay et al., 1990; Pastori et al., 1991; Sunitha & Slobin, 1987; Wager & Assoian, 1990; Byrne et al., 1993; Tanzer & Meagher, 1994; Ross, 1993), the result here is similar to what has been found in a cell-free system derived from rat pituitary GH_3 cells, where the nuclease activity that degrades the thyrotropin-releasing hormone receptor mRNA has been found in the postpolysomal fraction (Narayanan et al., 1992). However, it is possible that in cultured soybean cotyledons the association between polysomes and nucleolytic activities is of lower affinity than in some of the other systems and that dissociation occurs during the isolation process. Nevertheless, the presence of the mRNA degradative activity in the postpolysomal fraction should allow its characterization and isolation to proceed more readily.

Specific destabilizing activities in the postpolysomal fraction are involved in the control of c-*myc* mRNA degradation (Brewer & Ross, 1989), in the autoregulation of histone mRNA stability (Peltz & Ross, 1987), and also in the degradation of urokinase-type plasminogen activator (uPA) mRNA (Altus & Nagamine, 1991). A virion host shutoff protein, which is not a nuclease itself, from herpes simplex virus type 1, has been shown to be a nonspecific destabilizer for host mRNAs in vitro (Sorenson, Hart & Ross, 1991). Our results also indicated that the increase of BBPI mRNA degradation in basal-cultured cotyledons compared to met-cultured cotyledons was due to an increased BBPI-specific destabilizing activity in the postpolysomal fraction from basal-cultured cotyledons. We do not know whether degradation is due to a BBPI mRNA specific nuclease, due to an activator that activates a nuclease specific to BBPI mRNA, or due to a RNA-binding protein that recognizes BBPI mRNA and, upon binding, changes the conformation of BBPI mRNA and increases its susceptibility to nucleases. An examination of the secondary structure in the 3′-half of the BBPI mRNA encoded by by the cDNA clone 6-7 (Biermann et al., 1998) revealed a stem-loop structure (residues 245–276), similar to those that have been shown to be important in mRNA turnover (Stern & Gruissem, 1987). The relationship between BBPI mRNA stability and this structure has not been studied.

We also found that nuclease activities that degrade BBPI and α/α′ mRNAs were not dependent upon Mg^{++}. Higher concentrations of both monovalent and divalent cations inhibited the degradation process. It is possible that, instead of affecting nucleases per se, higher monovalent and divalent cation concentrations result in modifying the structure of RNA molecules and subsequently affect their accessibility to nucleases (Barnard, 1969). The specific BBPI mRNA degradative activity in the postpolysomal fraction from basal-cultured cotyledons was not observed when purified RNA was used as the substrate. Under such conditions, ribosomal RNA, BBPI mRNA and α/α′ mRNAs (Table 4) were all degraded by postpolysomal fractions either from basal-cultured or met-cultured cotyledons. In addition, results in Table 4 also suggest that total nuclease activities are similar between postpolysomal fractions from basal-cultured cotyledons and those from met-cultured cotyledons.

Other reports have shown that free RNA is nonspecifically degraded. For example, polysome-associated c-*myc* mRNA is destabilized by a cytosolic factor, but in vitro synthesized c-*myc* mRNA is not destabilized under identical conditions (Ross, 1993). Also, in vitro synthesized c-*myc* mRNA is at most two- to three-fold less stable than in vitro synthesized γ-globin mRNA. In contrast, polysome-associated c-*myc* mRNA is at least 40-fold less stable than γ-globin mRNA, which reflects their relative intracellular stabilities (Ross, 1993). Similar results have been reported concerning the degradation of isolated urokinase-type plasminogen activator mRNA (Altus & Nagamine, 1991) and eEF-Tu mRNA in vitro (Sunitha & Slobin, 1987). These findings suggest that the structure of native ribonucleoprotein particles in vivo is important in determining stability of mRNA.

Finally, questions remain as to how the destabilizer is induced in basal-cultured cotyledons and what the mechanism is for the destabilizer to cause the degradation of BBPI mRNA. Elucidating these questions by using this cell-free system may provide a better understanding of regulation of mRNA turnover in soybean seeds in response to different growing conditions.

4. Experimental

4.1. In vitro culture of soybean cotyledons

Soybean cotyledons were cultured in vitro as described (Cheng et al., 1999).

4.2. Preparation of polysomal and postpolysomal fractions from soybean cotyledons

Preparation of polysomal and postpolysomal fractions from cultured soybean cotyledons was modified from the method described by Ross and Kobs (1986). After being cultured 6 days in vitro, soybean cotyledons were harvested and ground in liquid N_2. Ten volumes of extraction buffer (200 mM sucrose, 50 mM Tris-Cl, pH 8, 10 mM KCl and 5 mM $MgCl_2$) were

added and the mixture was allowed to thaw at 4° (all subsequent preincubation procedures were done at this temperature). The mixture was centrifuged at 7840 *g* for 10 min. The resultant supernatant was layered over a sucrose pad (30% sucrose, 50 mM Tris-Cl, pH 8, 10 mM KCl and 5 mM $MgCl_2$) and centrifuged at 150,000 *g* for 3 h in a SW-40 rotor of a Beckman model L8-70 ultracentrifuge.[1] The supernatant above the sucrose pad was collected as the postpolysomal fraction and concentrated using an Amicon stirred cell with a PM 10 membrane. The protein concentration of the postpolysomal fraction was determined as described by Loffler and Kunze (1989). The polysomal pellet was resuspended in a buffer (50 mM tris-Cl, pH 7.5, 10 mM KCl and 5 mM $MgCl_2$), and A_{260} was determined. The polysomal and postpolysomal fractions were frozen in liquid N_2 and stored in small aliquots at −70°.

4.3. Assays of cell-free mRNA degradation

Standard 50 µl reactions contained the polysomal fraction (0.25 A_{260} units) and the postpolysomal fraction (200 µg protein), 50 mM Tris-Cl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$ and 20 units of RNasin (Promega). All components were added to a microfuge tube in ice and then incubated at 25°. No ATP-generating system was required in our system in contrast to the system of Ross and Kobs (1986). In some experiments, the polysomal fraction or the postpolysomal fraction was omitted in order to localize nuclease activities. For heat treatment of the postpolysomal fraction, the fraction was heated at 65° for 10 min. At indicated times, reactions were stopped by adding 400 µl of urea lysis buffer (7 M urea, 10 mM Tris-Cl, pH 7.5, 2% SDS, 0.35 M NaCl and 10 mM EDTA), and the reaction mixtures were extracted twice with phenol-chloroform (1:1) and once with chloroform. RNA was precipitated with 2.5 volumes of ethanol. The RNA recovered from each reaction was resolved by electrophoresis through an agarose-formaldehyde gel and transferred to a GeneScreen membrane (DuPont) (Maniatis, Fritsch & Sambrook, 1982). Hybridization was performed as described (Cheng et al., 1999) except that the final wash condition was 0.2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7) at 65°. Radioactivities were visualized by autoradiography on X-ray films and the RNA bands were digitized by a ScanJet Plus with the Scanning Gallery Plus 5.0 program (Hewlett Packard) and quantified using the NIH Image 1.41 program. The membrane was hybridized with the cDNA clone 6-7 which contains the BBPI mRNA sequence (Biermann et al., 1998). In some experiments, the membrane was stripped by boiling for 20 min in 0.1 × SSC, 1% SDS before probing with the α/α′ probe, genomic clone 48.2, which contains the full-length of the α′ subunit gene of the 7 S storage protein (Biermann & Madison, unpublished).

[1] Mention of company names or commercial products does not imply recommendation or endorsement by the United States Department of Agriculture over others not mentioned.

Acknowledgements

Support for Y.C. from the Wilfred S. and Elizabeth M. Martin Scholarship Fund is gratefully acknowledged.

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