ISOLATION AND TRANSLATION OF HORDEIN MESSENGER RNA FROM WILD TYPE AND MUTANT ENDOSPERMS IN BARLEY

by

ANDERS BRANDT and JOHN INGVERSEN

Agricultural Research Department, Risø National Laboratory, DK-4000 Roskilde

and

Department of Physiology, Carlsberg Laboratory,
Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby

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Messenger RNA has been isolated from developing barley endosperms by sucrose gradient sedimentation, Sepharose 4B gel filtration and preparative gel electrophoresis. Hordein messenger RNA was found to be a major constituent of the total messenger RNA population of the endosperm cell. Polyadenylated hordein messenger RNA sedimented at 11S in sucrose gradients and electrophoretic analysis reveals the presence of at least three RNA species with apparent molecular weights of 0.45, 0.36 and 0.30 megadaltons. The 11S messenger RNA was translated in vitro into hordein precursor polypeptides which are 2-4 kilodaltons larger than the native hordein polypeptides. The endosperm cell of mutant No. 1508 contained twice as much RNA as the wild type endosperm cell but the same amount of polyadenylated 11S RNA. The template activity of the latter was 10% of that found for the 11S hordein messenger RNA from the wild type and was limited to translation into one hordein precursor polypeptide.

1. INTRODUCTION

The prolamine storage protein of the barley endosperm, termed hordein, is preferentially synthesized in the developing kernel 15-25 days after fertilization (6). The hordein polypeptides are synthesized on polyribosomes bound to the endoplasmic reticulum (7, 17), and transferred across the membrane into its lumen (10). From there they are transported into the vacuoles and deposited in protein bodies (26, 31). In a short

period of time the barley endosperm cells thus carry out a large scale synthesis and transport of hordein. High levels of messenger RNA's coding for hordein polypeptides can be expected to be present in the endosperm cells 15-25 days after fertilization.

In vitro synthesis of specific plant proteins with the aid of isolated RNA or DNA templates has recently been achieved for the small and large subunits of ribulose 1,5-bisphosphate

carboxylase (11, 13, 15, 20, 22, 24) light harvesting chlorophyll a/b protein (1), phenylalanine ammonia lyase (36), leghaemoglobin (41), globulin-1 storage protein (19) and zein (8,30). With such experiments it is possible to determine the cellular site of synthesis of a protein, the location of the gene coding for its primary structure or the nature of a mutant. Zein, the small subunit of ribulose 1,5-bisphosphate carboxylase, and the light harvesting chlorophyll a/b protein are apparently synthesized as larger sized precursors which are subsequently processed in connection with their transport to other cell compartments.

Single gene mutations exist in barley, which affect the synthesis of hordein in the endosperm. One such mutant, the high lysine mutant Risø No. 1508, synthesizes only 20% of the amount of hordein found in the wild type endosperm (6, 27). Isolated polysomes from this mutant produced only small amounts of hordein (7). Allelic differences for hordein polypeptide patterns in electropherograms are determined by two loci on chromosome 5 (32, 39), whereas the gene mutated in No. 1508 is located on chromosome 7 (29, 40).

In order to study the synthesis of hordein in the barley endosperm and to gain an insight into the nature of the mutation No. 1508, we have isolated messenger RNA coding for the hordein polypeptides using Poly(U)-Sephadex affinity chromatography, Sephadex 4B chromatography, preparative gel electrophoresis and sucrose gradient sedimentation. This paper describes the partial purification of the hordein messenger RNA molecules and gives evidence for their translation into larger sized precursors of hordein polypeptides. Some characteristics of the RNA from the mutant are given.

2. MATERIALS AND METHODS

2.1. Chemicals

Micrococcus nuclease (E.C. No. 3.2.4.7), RNAase A (E.C. No. 3.1.4.22), t-RNA mixture (Type I) and spermidine-phosphate were obtained from Sigma Chemicals. CsCl (Supra-

pur^R) and phenol from Merck. Phenol was redistilled before use. E.coli ribosomal RNA was purchased from Miles Laboratories, Poly(U)-Sephadex 4B from Pharmacia and Oligo(dT)-cellulose from Collaborative Research. ³H-polyuridylic acid (specific activity 20-74 Ci-mmol⁻¹, 41-147 nucleotide residues) was obtained from Radiochemical Center, Amersham.

2.2. Plant materials

Barley plants (*Hordeum vulgare* L) cv. Bomi and its mutant Risø No. 1508 were either field grown or grown in a growth chamber as described previously (10). Spikes were harvested 20 days after anthesis. If spikes were not to be used immediately after harvest, they were placed in polyethylene bags, frozen in liquid nitrogen and stored at -70°C. Stored plant material was used within 3 months after harvest.

2.3. Isolation of membrane bound polyribosomes

Membrane bound polyribosomes were isolated by a procedure which was modified from that previously described (7) in the following manner: The initial 500 × g centrifugation was omitted, which improved the yield of membrane bound polysomes two-fold. K- and Mg-salts were present as acetates and beta-mercaptoethanol was replaced with 2 mM-dithiothreitol in the buffers. Analysis of the polysomes was performed in linear 0.3-1.0 M-sucrose gradients made in a solution containing 0.05 M-Tris, pH 7.5, 0.1 M-KCl, 0.01 M-Mg-acetate and 0.002 M-beta-mercaptoethanol. Centrifugation was for 3 hrs at 65,000 × g_{av} in a Spinco S.W. 25 rotor.

2.4. Extraction of total cellular and membrane bound RNA

RNA extractions were performed essentially as described by AVIV and LEDER (3). Total cellular RNA was extracted from 25 g endosperms (fresh weight) by homogenization in 60 ml 0.1 M-TRIS, 0.1 M-NaCl, 0.01 M-EDTA pH 9.0 at

Abbreviations: TCA = trichloroacetic acid, SDS = Sodium dodecyl sulfate, TRIS = Tris(hydroxymethyl)-amino-methan, EGTA = Ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra acetic acid.

0°C with mortar and pestle. The suspension was further homogenized five times for 30 seconds in a Potter-Elvehjem homogenizer operated at 3,000 r.p.m. SDS was added to give a concentration of 1% and the homogenate brought to room temperature for 10 minutes. The homogenate was then centrifuged at $10,000 \times g$ for 10 minutes at 2°C. An equal part of a solution of phenol:chloroform:isoamylalcohol (50:50:1 v/v/v) was added to the supernatant, whereafter the mixture was shaken for 10 minutes at room temperature and cooled to 4°C before the phases were separated by centrifugation at $10,000 \times g$ for 5 minutes at 4°C. The organic lower phase was re-extracted with an equal volume of buffer. The aqueous phases were combined and extracted twice with a mixture of 24 parts of chloroform and 1 part of isoamylalcohol (v/v). To the final aqueous phase, which contained most of the DNA and RNA of the endosperm, was added solid CsCl to give a concentration of $1 \text{ mg} \cdot \text{ml}^{-1}$. After the CsCl had dissolved the extract was layered on a solution of 5.7 M-CsCl in 0.1 M-EDTA (18). After centrifugation at $65,000 \times g_{av}$ for 18 hrs at 20°C in a Spinco S.W. 25 rotor, a clear pellet of RNA was obtained. Alternatively the nucleic acid was precipitated from the aqueous phase by addition of two volumes of ethanol. After storage over night at -20°C, the nucleic acids were collected by centrifugation at $10,000 \times g$ for 10 minutes, redissolved in 50 mM-TRIS, 1 mM-EDTA pH 7.5 and NaCl added to a final concentration of 3 M. RNA was precipitated by storage at 4°C for 18 hrs and collected by centrifugation at $10,000 \times g$ for 10 minutes.

2.5. Cell free protein synthesis

A wheat germ extract was prepared according to ROBERTS and PATTERSON (37) omitting the pre-incubation step. Endogenous wheat germ template activity was destroyed by treating the extract with *Micrococcus* nuclease (35). The standard reaction mixture was as described previously (10), but contained 1.8 mM-Mg-acetate, 80 mM-K-acetate and 0.4 mM-spermidine-phosphate (2). TCA precipitable and isopropanol soluble polypeptides were prepared for electrophoresis as described pre-

viously (10) and were analyzed on 12.5% SDS-polyacrylamide slab gels using the buffer system of CHUA and BENNOUN (12). The gels were processed for fluorography by the procedure of BONNER and LASKEY (4).

2.6. Poly(U)-Sephacrose 4B and oligo(dT)-cellulose affinity chromatography

Small columns of pre-swollen beads were prepared in pasteur pipettes. The Poly(U)-Sephacrose 4B columns were washed with ten volumes of 0.7 M-NaCl, 10 mM-TRIS pH 7.5, five volumes of 90% formamide and ten volumes of 0.7 M-NaCl, 10 mM-TRIS pH 7.5 before the RNA sample in 0.7 M-NaCl, 10 mM-TRIS pH 7.5 was applied to the columns. The columns were washed with the 0.7 M-NaCl buffer until all material absorbing at 260 nm had eluted. The retained material was eluted with 90% formamide. The RNA was ethanol precipitated. Oligo(dT)-cellulose columns were washed with 0.7 M-NaCl buffer, two volumes of H_2O , three volumes of 0.1 M-NaOH and five volumes of the 0.7 M-NaCl buffer before the RNA was applied in the 0.7 M-NaCl buffer. The columns were washed with the 0.7 M-NaCl buffer until no more material absorbing at 260 nm had eluted. The polyadenylated fraction was eluted with water.

2.7. Sucrose gradient sedimentation

RNA samples heated for 10 minutes at 60°C were layered on a 5-20% linear sucrose gradient prepared with a solution containing 10 mM-TRIS, 1 mM-EDTA, 1% SDS pH 7.5. Sedimentation was for 18 hrs at 20°C at $151,000 \times g_{av}$ in a Spinco S.W. 40 rotor. RNA in the collected fractions was precipitated with ethanol. When the fractions contained only small amounts of RNA, t-RNA was added as carrier.

2.8. RNA gel electrophoresis

RNA gel electrophoresis was carried out in composite 0.7% agarose-2.5% polyacrylamide slab gels according to PEACOCK and DINGMAN (33) using a 10 mM-Na-phosphate buffer, pH 7.0. The low ionic strength buffer was re-circu-

lated during the electrophoresis. RNA samples were denatured in 50% dimethyl sulfoxide by heating at 60°C for 10 minutes before the electrophoresis. The latter was carried out at 30 mA for 4.5 hrs at 4°C. Gels were stained with 0.2% methylene-blue in 0.4 M-Na-acetate pH 4.7 and destained in H₂O. Ethidium bromide staining was performed with a solution containing 2 µg·ml⁻¹ in 0.5 M-NH₄-acetate.

2.9. Extraction of RNA from agarose-polyacrylamide gels

Gel slices were extracted with 0.1 M-NaCl, 0.05 M-TRIS, 0.005 M-EDTA pH 7.4 by grinding with a teflon coated pestle (25). SDS was added to give a final concentration of 1%. One volume of buffer saturated phenol was added, the mixture shaken and kept over night at 4°C. The phases were separated by centrifugation at 10,000 × g for 10 minutes and the lower phenol phase re-extracted with the buffer. The combined aqueous phases were extracted two times with chloroform:isoamylalcohol 24:1 (v/v), NaCl added to a final concentration of 0.7 M, and the solution applied to a 1 ml oligo(dT)-cellulose column. Bound RNA was eluted as described under 2.6.

2.10 Hybridization of RNA to ³H-polyuridylic acid

RNA was hybridized to 80 nCi ³H-polyuridylic acid (specific activity 1.63 mCi·mg⁻¹) in 0.5 M-NaCl, 10 mM-TRIS, 1mM-MgCl₂ pH 7.0 at 28°C for 15 minutes (38). The hybridization mixture was diluted ten-fold with the hybridization buffer and single stranded RNA was digested with 20 µg pancreatic RNAase A at 28°C for 15 minutes. 0.5 mg bovine serum albumin was added and the RNAase resistant RNA-poly(U) hybrids were precipitated with 10% TCA at 0°C for 30 minutes. The hybrids were collected on glass fiber filters and counted in Omnifluor-toluene (New England Nuclear).

2.11. Sepharose 4B chromatography of RNA

A 1.5 × 30 cm column was packed with Sepharose 4B equilibrated with 0.1 M-Na-ace-

tate, 0.001 M-EDTA pH 5.0. RNA samples in the column buffer heated for 2 minutes at 70°C and quickly cooled to 4°C were applied to the Sepharose 4B column. The column was eluted at 6 ml·h⁻¹ and the absorbance at 254 nm was measured continuously. Three ml fractions were collected and the RNA precipitated with ethanol.

3. RESULTS

3.1. Amounts and distribution of cellular RNA in wild type and mutant endosperms

Sucrose gradient centrifugation of initially membrane bound polyribosomes from the two genotypes resulted in profiles as exemplified in Figure 1. A large UV absorbing peak representing monosomes is observed together with six minor peaks corresponding to polyribosomes. Mutant No. 1508 endosperms yielded fewer polysomes ranging from dimers to heptamers than Bomi endosperms. Correspondingly the

Figure 1. Sucrose density gradient profile of membrane bound polysomes isolated from Bomi and mutant No. 1508 endosperms and separated on 0.3-1.0 M-sucrose gradients.

Table I

Yield of RNA (μg in 100 endosperms) obtained in polysomal and total cellular extracts from endosperms of wild type and mutant barley. RNA was extracted from polysomes and from the endosperm as described in Materials and Methods (2.3; 2.4). The amount of RNA was calculated using an extinction coefficient $E_{260}^{0.1\%} = 25$.

Genotype	Polysomes		Total cells
	membrane-bound	free	
Bomi	400	240	1060
Mutant No. 1508	300	320	2240

mutant preparations were richer in monosomes. This implies that there are either fewer initially membrane bound polysomes in the mutant No. 1508 than in wild type or the polysomes are less stable.

As can be seen from Table I about the same amount of RNA is found in the initially membrane bound polysomes of wild type and mutant endosperm. A similar amount is recovered from the free polysomes in the two genotypes. Two times more total cellular RNA is obtained from the mutant endosperm than from the wild type, a difference which is not expressed in the yields of the RNA from the polysomes.

Analysis of the RNA from the initially membrane bound polysomes and monosomes in sucrose gradients resulted in two broad peaks with maxima at 18S and 26S indicative of the RNAs of the small and large subunits from cytoplasmic ribosomes (Figures 2A,B). In most preparations from Bomi endosperms, the 26S peak was larger than the 18S peak, whereas in the mutant the 26S peak was equal to or lower than the 18S peak.

3.2. Isolation of messenger RNA

Polyadenylated RNA was obtained by Poly(U)-Sephadex 4B affinity chromatography and tested in the wheat germ cell free protein synthesizing system. Both polyadenylated and non-polyadenylated RNA had optimum template activity in the presence of 1.8

mM-Mg^{2+} and 80 mM-K^{+} . A 10-30 fold stimulation of protein synthesis was obtained at saturating levels of RNA. For non-polyadenylated RNA from initially membrane bound polysomes, 40 μg of RNA were necessary for maximum activity, whereas only 4 μg of adenylated RNA resulted in maximum activity (Figure 3).

In Bomi barley (Table II) the polyadenylated fraction of the total RNA reveals a 137-fold higher specific template activity than the non-polyadenylated fraction. An equivalent increase in the specific template activity is observed for the polyadenylated fraction of the RNA derived from the membrane bound polysomes. As will be shown below (3.3.) the two types of polyadenylated RNAs gave the same

Figure 2. Sedimentation profile of RNA isolated from the pellet of membrane bound polysomes prepared as described under Materials and Methods 2.7.

A: Endosperm RNA from Bomi

B: Endosperm RNA from mutant No. 1508

Figure 3. Incorporation of ^3H -leucine into material insoluble in 10% hot TCA. The cell free protein synthesizing system was directed by polyadenylated and non-polyadenylated RNA isolated from membrane bound polyribosomes of Bomi endosperms.

translation products. The isolation of polyadenylated RNA via polysomes is less efficient than isolation of this RNA directly from the total cellular RNA. The 4 μg of polyadenylated RNA obtained from the polysomes is probably only a fraction of that present in vivo. Therefore it could well be that most of the 10 μg of polyadenylated messenger RNA found in the total cellular RNA is derived from membrane bound polysomes.

In mutant No. 1508 (Table II) the specific template activity of both the total RNA and the polysome derived RNA is only 15-20% of that of the wild type. The specific template activity of the polyadenylated fraction of the total RNA is low compared to wild type. The 340 fold increase in specific template activity over the non-polyadenylated RNA fraction reveals that the large amount of RNA in the mutant is mostly without terminal poly(A) sequences. On the other hand, the polyadenylated fraction of the polysome derived RNA is of the same magnitude as in the wild type. The specific template activity of this RNA in the mutant, however, is poor. This provides evidence that mutant No. 1508 contains considerable amounts of untranslatable polyadenylated messenger RNA.

RNA extracted from the initially membrane bound polysomes from Bomi endosperm was fractionated by sepharose 4B gel filtration and the fractions analyzed for the amount of polyadenylated RNA as well as for template activity. As seen in Figure 4 the major part of the RNA was separated from the polyadenylated RNA which also was most active as template for isopropanol soluble polypeptides.

A separation of the polyadenylated RNA into different molecular species was attempted by sucrose gradient sedimentation. The RNA retained by poly(U)-sepharose 4B chromatography amounted to about 1% of the total cellular RNA (cf. Table III), most of which is ribosomal RNA. Figure 5 illustrates that the 18S and 26S ribosomal RNA could be separated from an 11S polyadenylated RNA fraction of Bomi endosperms by sucrose gradient sedimentation. This RNA fraction contained more template activity than the other fractions and its specific activity was 8 fold higher than that of polyadenylated RNA (Table III). Although a

Table II

Messenger RNA activity in the polyadenylated and non-polyadenylated RNA fractions of membrane bound polysomal and total cellular extract. The RNA fractions were obtained by poly(U) Sepharose 4B chromatography. Messenger RNA activities were measured in the wheat germ cell free protein synthesizing system using ^{35}S -methionine as label.

	RNA in μg per 100 endosperms	specific activity $\text{cpm} \times 10^{-3} \mu\text{g RNA}^{-1}$
Bomi cells	1200	5
non-poly(A)	1165	2
poly(A)	10	275
polysomes	415	5
non-poly(A)	405	2
poly(A)	4	300
Mutant 1508 cells	2380	0.8
non-poly(A)	2350	0.4
poly(A)	5	135
polysomes	325	1
non-poly(A)	315	0.3
poly(A)	3	65

Figure 4. Sepharose 4B chromatography of RNA isolated from initially membrane bound polyribosomes of Bombyx mori endosperms. Messenger RNA was identified as polyadenylated RNA and by its template activity for synthesis of 55% isopropanol soluble polypeptides. The major absorbance peak signifies column fractions containing large amounts of ribosomal RNA.

polyadenylated fraction could also be separated from ribosomal RNA in a comparable experiment with mutant No. 1508 it had low messenger RNA activity (figure 5).

3.3. Polypeptides coded for by the messenger RNA fractions

Endogenous template activity of the wheat germ extract could be destroyed by treat-

ment with Micrococcus nuclease and subsequent removal of the nuclease activity by complexing Ca^{++} with EGTA. No labelled polypeptides were detectable when such a wheat germ extract was used in the reaction mixture for cell free protein synthesis without added messenger RNA (cf. Figure 7, track 1).

Total cellular RNA from wild type endosperm coded for a considerable number of ³⁵S-methionine labelled polypeptides (Figure 6,

Table III

Purification of the barley endosperm 11S messenger RNA fraction. Messenger RNA activity was determined in the wheat germ cell free protein synthesizing system. Poly(A) RNA = poly(U) Sepharose retained RNA. 11S RNA = poly(A) containing RNA sedimenting at 11S. Yield was calculated as $\mu\text{g RNA} \times \text{specific mRNA activity}$.

	μg	Specific template activity cpm $\times 10^{-3}$ $\mu\text{g RNA}^{-1}$	purification	yield %
Total cell RNA	6720	5	1	100
poly(A) RNA	60	275	55	49
11S RNA	7	2175	435	43

track 2). These include polypeptides with apparent molecular weights larger than, equal to and smaller than those of *in vivo* labelled hordein (Figure 6, track 1). The fluorogram

Figure 6. Fluorograms of translation products of mRNA isolated from Bomi endosperms. The molecular weights refer to some characteristic hordein polypeptides synthesized *in vivo*. Pairs originating from the same electrophoretic run are: Tracks 1 and 2; tracks 3 and 4; tracks 5 and 6.

Track 1: *In vivo* ^{14}C -labelled hordein marker polypeptides.

Track 2: *In vitro* ^{35}S -methionine labelled polypeptides synthesized with total cellular endosperm RNA.

Track 3: *In vivo* ^{14}C -labelled hordein marker polypeptides.

Tracks 4 and 5: *In vitro* ^{35}S -methionine labelled polypeptides made with 11S polyadenylated RNA as template and extracted with 55% isopropanol.

Track 6: *In vitro* ^{35}S -methionine labelled polypeptides made with initially membrane bound polysomes as template and extracted with 55% isopropanol.

Figure 7. Fluorograms of translation products of mRNA isolated from Bomi and mutant No. 1508 endosperms.

Track 1: *In vitro* ^{35}S -methionine labelling in the wheat germ cell free protein synthesizing system without added RNA (control).

Track 2: *In vitro* ^{35}S -methionine labelled polypeptides using non-poly-adenylated total cellular RNA from Bomi endosperms as template.

Track 3: *In vitro* ^{35}S -methionine labelled polypeptides using polyadenylated total cellular RNA from Bomi endosperms as template.

Track 4: *In vitro* ^{35}S -methionine labelled polypeptides using RNA isolated from initially membrane bound Bomi polysomes as template.

Track 5: *In vitro* ^{35}S -methionine labelled polypeptides using RNA isolated from initially membrane bound mutant No. 1508 polysomes as template.

Track 6: *In vivo* ^{14}C -labelled hordein marker polypeptides.

Figure 5. Isolation of 11S mRNA from barley endosperms of Bomi and its mutant No. 1508 by sucrose gradient sedimentation. RNA retained on Poly(U)-Sepharose 4B columns was eluted and then separated on 5-20% sucrose gradients. After the centrifugation fractions were collected, the RNA was precipitated with ethanol and then dissolved in H_2O . An aliquot of this RNA was hybridized to ^3H -polyuridylic acid and RNAase resistant radioactivity determined. Another portion of the RNA was used for analysis of template activity in the wheat germ cell free protein synthesizing system using ^{35}S -methionine as label.

indicates a large portion of the incorporated ^{35}S -methionine to be present in low molecular weight peptides which is interpreted to indicate premature chain termination in the translational system.

In tracks 3 and 4 of Figure 6 a comparison is made between the native labelled hordein polypeptides and the 55% isopropanol soluble translation products of 11S polyadenylated RNA from the sucrose gradient of Figure 5A. The translation products of the 11S RNA (track 4) contain 3 groups of bands with apparent molecular weights slightly higher than those of the three groups of native hordein bands. The latter are characterized by the presence of polypeptides with molecular weights at 27, 38 and 48 kilodalton. Faint bands with a molecular weight greater than 67 kilodalton were occasionally seen among the translation products of messenger RNA.

In tracks 5 and 6 of Figure 6 a comparison is made between 55% isopropanol soluble translation products of 11S RNA and of initially membrane bound polysomes. The latter preparation yielded bands very similar if not identical with those of native hordein, although the 67 kilodalton band was faint. A comparison of tracks 4 and 5 with those of 3 and 6 indicates that the translation of isolated messenger RNA gives hordein polypeptides with a larger molecular weight than those of native hordein. The initially membrane bound polysome preparations apparently contain the components necessary for the processing of these hordein precursor molecules.

The improved sharpness of the polypeptide bands in track 5 (Figure 6) compared to those in track 4 is due to the different extraction procedures of the translation products. In the former case the products were first precipitated with TCA and then extracted with 55% isopropanol, whereas the latter extraction was done directly with 55% isopropanol. It is evident that lower molecular weight peptides are extracted after translation of purified messenger RNA and polysomes in the protein synthesizing system as was the case with the total RNA preparation (track 2). The hordein precursors found after translation of purified messenger RNA are predominant among the TCA

precipitable translation products from total RNA (compare tracks 4 and 5 with 2).

Identical translation products were obtained when total cellular RNA and total cellular polyadenylated RNA were used as templates in the wheat germ cell free protein synthesizing system (compare Figure 6, track 2 with Figure 7, track 3). Comparing tracks 2, 3, and 4 of Figure 7, it is evident that the total cellular non-polyadenylated RNA (track 2), total cellular polyadenylated RNA (track 3) and unfractionated RNA from initially membrane bound polysomes (track 4) from Bomi endosperms contain messenger RNA templates coding for polypeptides with identical apparent molecular weights.

The prominent polypeptides in these patterns are 55% isopropanol soluble (cf. Figure 6, tracks 4 and 5) and it is therefore concluded that all messenger RNA preparations used contain hordein template activity.

When, however, RNA from initially membrane bound polysomes of mutant No. 1508 was used to direct *in vitro* protein synthesis (track 5) only very faint polypeptide bands were present in the same apparent molecular weight range as the hordein precursors made on wild type templates.

The 11S messenger RNA from Bomi endosperms isolated by sucrose gradient sedimentation (Figure 5A) coded for polypeptides identified as hordein precursors. Figure 8 shows an experiment to determine whether a separation of different messenger RNA molecules for hordein polypeptides is possible. The translation products of fractions of a sucrose gradient were analyzed by electrophoresis and the products from 6 fractions are shown in tracks 2-7. The 11S peak of this gradient corresponds to fractions 7 and 8 (tracks 5, 6) and significant amounts of hordein precursor bands were only present in fractions 7 to 9. Figure 8 illustrates that no separation of the hordein messenger RNA was achieved.

In a comparable experiment with 11S-RNA from mutant No. 1508 endosperms only a small amount of one polypeptide with an apparent molecular weight of 30 kilodalton could be recognized (Figure 8, track 9). It corresponds to the precursor of the 27 kilodalton hordein

band. It is therefore concluded that the 11S mRNA preparations from mutant No. 1508 endosperms either lack messenger RNA for the hordein precursors with molecular weights higher than 30 kilodalton or contain untranslatable messenger RNA.

3.4. Gel electrophoresis of the hordein messenger RNA containing fractions

Agarose-polyacrylamide gel electrophoresis of 20 μ g RNA from initially membrane bound polysomes from Bomi endosperms revealed the

Figure 8: Fluorograms of translation products of polyadenylated mRNA isolated from endosperms of Bomi and its mutant No. 1508. The RNA fractions were obtained by sucrose gradient sedimentation as shown in Figure 6. Tracks 1 & 8: In vivo ^{14}C -labelled hordein marker polypeptides. Tracks 2-7: In vitro ^{35}S -methionine labelled polypeptides using Bomi mRNA fractionated on sucrose gradients as template. (Exposure of fluorogram 24 hrs). Track 9: In vitro ^{35}S -methionine labelled polypeptides using mutant No. 1508 mRNA as template. (Exposure of fluorogram 7 days).

26S and 18S ribosomal RNA molecules to be the major constituents (Figure 9, track 2), whereas the 26S RNA only comprises a minor

Figure 9. Electrophoretic separation of RNA isolated from initially membrane bound polysomes on a composite agarose-polyacrylamide gel.

Track 1: RNA from mutant No. 1508 endosperms.

Track 2: RNA from Bomi endosperms.

The bands were visualized by methylene-blue staining. The apparent molecular weights were obtained by using as markers ribosomal RNA from *E. coli* and plant cytoplasmic ribosomes. The 26S and 18S bands are ribosomal RNA from barley. The faster migrating bands are of the size seen in the fractions containing template activity. The 5S and 4S bands are considered ribosomal RNA and transfer RNA, respectively.

part of the 20 μ g RNA from mutant No. 1508 endosperms (Figure 9, track 1). On the other hand, the RNA from mutant No. 1508 contained a larger portion of RNA migrating faster than the 18S ribosomal RNA.

Gel electrophoresis was also used to analyze the RNA fractions obtained by Sepharose 4B gel filtration of RNA from initially membrane bound polysomes from Bomi endosperms (cf. Figure 4). As seen in Figure 10 an enrichment of four RNA species migrating faster than 18S ribosomal RNA was observed in the column fractions 15 and 16 as compared to fractions 12, 13 or 18, 20. The fractions 15 and 16 contained the major template activity as well as the peak for polyadenylated RNA. This provides evidence that the hordein messenger RNA's are represented among these RNA species and most likely among those with apparent molecular weights of 0.45, 0.36 and 0.30 megadaltons. Further evidence for the size of the hordein messenger RNA was obtained through electrophoretic analysis of RNA from the sucrose gradient fractions (Figure 11). Fraction 7 in track 2 of Figure 11 contains the polyadenylated 11S RNA practically free from 18S and 26S ribosomal RNA and with three major bands of apparent molecular weights of 0.45, 0.36 and 0.30 megadaltons. 18S ribosomal RNA is a major constituent of fraction 11 (track 3), whereas the 26S ribosomal RNA dominates in fractions 14 and 17 (tracks 4, 5).

For determination of the molecular weight of the hordein messenger RNA preparative gel electrophoresis was performed with RNA from initially membrane bound polysomes of Bomi endosperms (Materials and Methods 2.9.) The 55% isopropanol soluble translation products of the RNA extracted from the different gel regions are presented in the densitometer tracings of Figure 12. Only the RNA contained in fraction 5 had significant template activity for hordein polypeptides. This fraction contained $\frac{2}{3}$ of the total template activity recovered from the gel sections, emphasizing the abundance of translatable hordein messenger RNA in the developing barley endosperm. This region of the gel covered the apparent molecular weight range from 0.5-0.3 megadaltons. If hordein messenger RNA is indicated by the stained

bands of Figure 12, Figure 10 tracks 3, 4 and Figure 11 track 2, the molecular weight of hordein messenger RNA molecules can be estimated to be 0.45, 0.36 and 0.30 megadaltons.

Most eucaryotic messenger RNA molecules contain at their 5' end a 7-methylguanosine-5'-triphosphate structure (CAP) of importance for the initiation of translation (21). The 11S hordein messenger RNA directed protein syn-

Figure 10. Electrophoretic analysis of RNA isolated from initially membrane bound polysomes of Bomi endosperms and fractionated by Sepharose 4B chromatography (cf. Figure 5). Fractions 13 to 16 which contained the high template activity for hordein were enriched in the bands migrating somewhat faster than the 18S ribosomal RNA.

Figure 11. Electrophoretic analysis of polyadenylated RNA from Bomi endosperms on a composite agarose-polyacrylamide gel. The RNA was fractionated by sucrose gradient sedimentation and made visible on the gels by staining with ethidium bromide.

Tracks 1-6: RNA fractions from a sucrose gradient (cf. Figure 6). The template activity for hordein was highest in fraction 7 as evident from the polypeptide analysis of this fraction in Figure 9. This fraction is also relatively free from ribosomal RNA.

Track 7: *E.coli* ribosomal RNA marker.

Figure 12. Densitometric tracings of fluorograms from in vitro ^{35}S -methionine labelled polypeptides made with RNA fractionated by preparative gel electrophoresis.

RNA isolated from initially membrane bound polysomes from Bomi endosperms was separated on a composite agarose-polyacrylamide gel (left). The RNA was extracted from gel sections 1 to 6 and purified by oligo(dT)-cellulose affinity chromatography. The polyadenylated RNA was used as template in the wheat germ cell free protein synthesizing system. In vivo ^{14}C -labelled hordein marker polypeptides are presented at the bottom. Significant template activity for hordein is only observed with RNA fraction 5.

thesis was 95% inhibited by 0.4 mM-7-methylguanosine-5' monophosphate, an analogue to the (CAP) structure (Figure 13). Hordein messenger thus appears to have this characteristic property. The polyadenylated sequences are found at the 3' end (16). The 11S hordein messenger was titrated against a fixed amount of ^3H -polyuridylic acid which was calibrated with increasing amounts of polyadenylic acid (Figure 14). Five percent of the messenger residues consisted of a continuous segment of adenine nucleotides. For the three hordein messenger size estimates this amounts to about 70, 60 and 50 residues, respectively.

Figure 13. Increasing concentrations of 7-methylguanosine-5'-phosphate inhibit in vitro protein synthesis directed by 11S polyadenylated RNA. The RNA was isolated from initially membrane bound polysomes of Bomi endosperms and incorporation of ^{35}S -methionine into 10% hot TCA insoluble material was measured.

Figure 14. Estimation of the amount of polyadenyl sequences in a Bomi 11S mRNA isolated by sucrose gradient sedimentation. Increasing amounts of 11S mRNA and polyadenylic acid were hybridized to a given amount of ^3H -polyuridylic acid (cf. 2.10.) and the radioactivity of the hybrids was determined. The 11S RNA contains 5% polyadenyl sequences.

4. DISCUSSION

The similar polypeptide pattern obtained when using wild type total cellular and polyosomal 11S RNA as templates for in vitro protein synthesis reveals that a major part of the endosperm messenger RNA population is involved in the synthesis of a group of polypeptides which have the characteristic properties of hordein. We have previously shown that the isopropanol soluble polypeptides made on a membrane bound polysomal template comigrate with native hordein polypeptides (7). The major ^{35}S -methionine labelled polypeptides made on the isolated RNA template from the wild type barley endosperm were soluble in 55% isopropanol, whereas only little label was found in these polypeptides when RNA templates from mutant 1508 was used.

The small subunit of ribulose-1,5-bisphosphate carboxylase synthesized in vitro with RNA templates isolated from the cytoplasm of pea seedling leaves (11,22) or *Chlamydomonas reinhardtii* cells (15) has a molecular weight of 20 kilodalton, whereas the native subunit from the chloroplast with 14 kilodalton is smaller. This protein is synthesized on cytoplasmic polysomes which are not attached to the mem-

branes of the endoplasmic reticulum. The precursor molecules have to move across the two membranes of the chloroplast envelope, where the processing of the precursor to the final size of the small subunit occurs (22). It may be inferred that the mechanism for synthesis, transport and processing of the small subunit of ribulose 1,5-bisphosphate carboxylase is quite different from the membrane bound synthesis and subsequent processing of secretory proteins including the barley endosperm hordein.

The hordein polypeptides produced with purified template were 2 kilodalton larger than native hordein polypeptides, whereas translation products of the initially membrane bound polysomes were similar in size to native hordein. In the wheat germ cell free protein synthesizing system, the only presently known post-translational modification concerns the removal of the N-terminal methionine (9, 28). In animal cells, secretory proteins have been shown to be synthesized on the endoplasmic reticulum as larger sized precursors with an extra sequence of approximately 20 amino acids at their N-terminal end (14). The extra amino acid sequence is cleaved off as the polypeptide moves into the lumen of the endoplasmic reticulum (5). It is likely that the mechanism of synthesis, processing and transport of the hordein polypeptides resembles the scheme outlined for animal secretory proteins, since the hordein polypeptides are synthesized on the endoplasmic reticulum, are transported across the membrane into the lumen of the endoplasmic reticulum (10) and are deposited in protein bodies located in vacuoles of the developing barley endosperm (26, 31). The larger sized *in vitro* synthesized hordein polypeptides are likely to contain an additional N-terminal sequence essential for their transport across the endoplasmic reticulum membrane. BURR, BURR, RUBENSTEIN and SIMON (8) have presented evidence that the maize endosperm storage protein polypeptides, collectively called zein, are synthesized *in vitro* in the form of larger sized precursors.

Three distinct RNA bands differing in molecular weights were recognizable in the purified 11S messenger RNA fraction isolated from the wild type barley endosperm. The apparent

molecular weights of these RNA bands correspond to 1450, 1150 and 950 nucleotides, respectively. The coding capacity of RNA molecules of these sizes could generate polypeptides with molecular weights of 53,000, 42,000 and 35,000 daltons. The recognizable hordein precursor polypeptides synthesized *in vitro* on the 11S template have approximate molecular weights of 50, 40 and 30 kilodaltons. Such polypeptides would require a code of approximately 1400, 1100 and 800 nucleotides. The recognizable RNA bands are thus in the right size range. The three groups of hordein polypeptides can be resolved into two or more protein bands. Two similar sized messenger RNA's could be present in each RNA band or alternatively, one messenger RNA could be present, containing the coding sequence for both polypeptides. Presence of an amber codon (UAG) near the 3' end of the coding sequence would by partial read through (34), generate polypeptides only differing little in molecular weights. Partial read through of an amber codon has been shown to occur when tobacco mosaic virus RNA is translated in cell free systems generating two polypeptides from the same initiation point on the RNA (34).

It was possible to isolate significant amounts of polyadenylated 11S RNA from mutant No. 1508. The RNA could be translated with low efficiency into only one of the hordein precursor polypeptides. At this point it is not possible to determine whether large amounts of untranslatable hordein messenger RNA are made in the mutant endosperm.

We have previously shown that the total polysomal populations were quite similar in the two genotypes when analyzed by sucrose gradient sedimentation and electron microscopy (6). Sucrose gradient analyses presented in this paper of the membrane bound polysomes, demonstrated a two fold increase in the monosome region as well as a decrease in the amount of material in the polysome region of the gradient in the mutant compared to the wild type. This indicates an abnormality in the organization of the membrane bound polysomes of the mutant. The 18S and 26S ribosomal RNA's constitute a major portion of the total cellular RNA in both genotypes as well

as of the membrane bound RNA in the wild type endosperm. In contrast, only small amounts of 26S ribosomal RNA were present in the membrane bound RNA from the mutant endosperm. A membrane factor from reticulocyte membranes has been demonstrated to make specific cuts in the 28S ribosomal RNA thus inhibiting protein synthesis at the level of elongation (42). In analogy, it is tempting to suggest, a factor of similar nature present in the mutant endosperm membranes at later stages of development. However, preliminary experiments have not demonstrated the presence of a specific nuclease activity for the 26S ribosomal RNA in Triton X-100 extracts from the membranes of the mutant endosperm.

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