Ribonucleic Acids with Messenger Activities in the Cotyledons of Soybean Seeds

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Ribonucleic acids from the cotyledons of soybean seeds (H-RNA) stimulated the amino acid incorporation in a cell-free system prepared from soybean seedlings at a concentration of 5 mM magnesium acetate. In a reticulocyte cell-free system H-RNA stimulated the amino acid incorporation at a concentration of 10 mM Mg^{2+} , but not at 4.3 mM Mg^{2+} . Aurintricarboxylate, an inhibitor for the initiation of protein synthesis, inhibited the H-RNA and TMV-RNA-stimulated amino acid incorporation by about 90% and 70% at a concentration of 50 μM in *E. coli* and soybean seedling systems, respectively. When the H-RNA preparation was passed through a Millipore filter, about 1% of the charged RNA was retained on the filter. Chromatography on plain cellulose of H-RNA resulted in slight enrichment of an RNA fraction with the characteristics of mRNA.

We have been studying the ribonucleic acids that have messenger activities in the soluble fraction prepared from the cotyledons of soybean seeds. It has been suggested that the messenger activity was due to that some mRNA species existed in the RNA preparation, while major RNA components might be rRNAs and their derivatives. In dry wheat embryos, a subcellular fraction which stimulated *in vitro* amino acid incorporation¹⁾ and a ribosomal fraction having endogenous template activity²⁾ have been isolated. The messenger fractions were suggested to be a ribonucleoprotein¹⁾ or in complex form within the ribosomal fraction.²⁾ We have also reported that ribonucleic acids with template activities were in the particulate forms, 37 S and 59 S in the post-ribosomal supernatant of soybean seeds.³⁾ On the other hand, in the cotyledons of cottonseeds, it has been demonstrated that the mRNA for two enzymes (a

carboxypeptidase and isocitratase) was transcribed in embryogenesis and translated in the germination process.⁴⁾ These results may indicate the existence of conserved mRNA not only in the embryos but also in the cotyledons of the seeds.

In this paper we attempt to examine some characteristics of the messenger activity of H-RNA preparation to support the presence of mRNA species in the RNA preparation.

MATERIALS AND METHODS

Materials. Leucine- $U^{14}C$ (282 mCi/mmol) was purchased from Daiichi Pure Chem. Co. Sigmacell type 38 obtained from Sigma Chemical Co. was used as cellulose. Millipore filter (HA 0.45 μm) was purchased from Millipore Filter Corp. The ammonium salt of aurintricarboxylic acid (ATA) was purchased from Nakarai Chemicals Ltd.

Preparation of H-RNA. Soybean seeds were soaked overnight in water at 4°C. Cotyledons detached from germs were homogenized in a mortar with 50 mM Tris-HCl buffer (pH 7.8) containing 0.25 M sucrose, 1 mM magnesium acetate and 5 $\mu g/ml$ of PVS. The homogenate was squeezed through gauze and centrifuged at $10,000 \times g$ for 30 min. The supernatant was centrifuged at $100,000 \times g$ for 1 hr. RNA was prepared from the supernatant (S-100 RNA) as described previously⁵⁾ except that SDS was added in a final concentration of 0.5% before phenol extraction. H-RNA

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Abbreviations used: H-RNA, high molecular weight RNA of soybean seeds prepared as shown in the text; ATA, aurintricarboxylate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PVS, polyvinyl sulfate (potassium salt); SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TMV, tobacco mosaic virus.

was prepared from S-100 RNA by Sephadex G-200 column chromatography as described previously⁶⁾ except that the elution was done in the presence of PVS (5 μ g/ml).

Preparation of TMV-RNA. The ordinary strain of TMV (strain OM) was used. Virus-infected leaves were made available by the courtesy of Dr. F. Sakai, Institute for Plant Virus Research, Chiba, and the virus was prepared by differential centrifugation according to the method of Boedtker and Simmons⁷⁾ with slight modification as follows. The juice from the frozen leaves was clarified by centrifugation at $10,000\times g$ for 15 min, and the supernatant fluid was centrifuged at $100,000\times g$ for 1 hr. The pellet was dispersed in 0.1 M potassium phosphate (pH 7) by allowing it to soak overnight. It was subjected to the same cycle of low speed then high speed centrifugations. The pellet was dispersed in water by allowing it to soak overnight, followed by the addition of an equal volume of 20 mM EDTA (pH 7.5). The suspension was centrifuged at $10,000\times g$ for 15 min, and the supernatant fluid was centrifuged at $100,000\times g$ for 1 hr. The pellet (TMV) thus obtained was dispersed in water. TMV-RNA was prepared by bentonite-phenol extraction from the TMV.⁸⁾

Preparation of ribosomal and supernatant fractions from soybean seedlings. Ribosomal fraction was prepared from hypocotyls of 2-day-old soybean seedlings which were germinated in vermiculite at 25°C in the dark. Hypocotyls were homogenized in a mortar with a solution containing 1 mM magnesium acetate, 2 mM CaCl_2 and 50 mM KCl and the homogenate was centrifuged at $24,000\times g$ then $100,000\times g$, as described by Marcus *et al.*⁹⁾ The ribosomal pellet (crude ribosomes) was washed once by repelleting from the suspending medium containing 50 mM Tris-HCl buffer (pH 7.6), 0.5 M KCl, 5 mM magnesium acetate, 1 mM 2-mercaptoethanol and 0.25 M sucrose. Ribosomal pellet (washed ribosomes) was dissolved in a suspending medium containing 40% glycerol. Supernatant was prepared from cotyledons of 2- or 3-day-old soybean seedlings by centrifugation at $100,000\times g$ and treated with Sephadex G-25 as described previously.¹⁰⁾

Preparation of ribosomal and pH 5 fractions from reticulocytes. Male white rabbits were used in the experiments. Induction of reticulocytosis by phenylhydrazine injection and the preparation of lysate, ribosomes and $100,000\times g$ supernatant fraction was performed according to the procedures reported by Schreier and Staehelin.¹¹⁾ The pH 5 fraction was prepared from the post-ribosomal supernatant by the method described below.

Preparation of rat liver pH 5 fraction. A pH 5 fraction was prepared according to the method de-

scribed by Hoagland *et al.*¹²⁾ with slight modifications.¹³⁾ Rat livers were homogenized in 10 mM Tris-HCl buffer (pH 7.5) containing 0.3 M sucrose, 5 mM magnesium acetate and 1 mM DTT, and the post-mitochondrial supernatant was prepared by centrifugation at $17,500\times g$ for 15 min. The supernatant was then centrifuged at $100,000\times g$ for 3 hr. Approximately two-thirds of the supernatant fluid was then diluted with 2 volumes of cold distilled water containing 1 mM DTT and the pH was adjusted to 5.1 to 5.2 by dropwise addition of 1 M acetic acid with constant stirring. The suspension was centrifuged at $10,000\times g$ for 10 min and the precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NH_4Cl , 5 mM magnesium acetate and 1 mM DTT. The pH was adjusted to 7.5 by addition of small amounts of 1 N KOH.

Preparation of *E. coli* S-30 fraction. *E. coli* strain K-12 cells harvested at 0.6 of absorbance at 520 nm with 1 cm light path were ground with alumina. The S-30 fraction ($30,000\times g$ supernatant) was prepared by differential centrifugation and preincubated at 37°C for 80 min as described by Nirenberg.¹⁴⁾

All operations were carried out at 0~5°C. The subcellular preparations obtained above were stored in liquid nitrogen and RNA preparations were stored at -20°C.

Cell-free amino acid incorporation systems

Reticulocyte system. The reaction mixture of 0.2 ml contained 0.146 mg protein of ribosomes, 5.834 O.D.₂₆₀ units of H-RNA preparation, 0.639 mg protein of pH 5 fraction and the following ingredients per ml: 30 μ moles Tris-HCl (pH 7.6), magnesium acetate as indicated, 70 μ moles KCl, 1 μ mole DTT, 30 nmoles each of 19 amino acids except for leucine, 1 μ mole ATP, 0.4 μ mole GTP, 20 μ moles creatine phosphate, 50 μ g creatine kinase and 2.5 μ Ci leucine-U-¹⁴C. Incubation was carried out at 37°C for 30 min.

Soybean seedling system. The reaction mixture of 0.1 ml contained ribosomes and supernatant from soybean seedlings (or rat liver pH 5 fraction), H-RNA or TMV-RNA preparations and the following ingredients per ml: 50 μ moles Tris-HCl (pH 7.8), 5 μ moles magnesium acetate, 40 μ moles KCl, 1 μ mole DTT, 20 nmoles each of 19 amino acids, 1 μ mole ATP, 0.25 μ mole GTP, 10 μ moles creatine phosphate, 50 μ g creatine kinase and 2 μ Ci leucine-U-¹⁴C. Incubation was carried out at 37°C for 20 min.

***E. coli* system.** The amino acid incorporation system used was basically the same as that of Nirenberg.¹⁴⁾ The reaction mixture of 0.1 ml contained 0.147 mg protein of S-30, H-RNA or TMV-RNA preparations and the following ingredients per ml: 50 μ moles Tris-HCl (pH 7.8), 10 μ moles magnesium

acetate, 75 μ moles KCl, 1 μ mole DTT, 20 nmoles each of 19 amino acids, 3 μ moles ATP, 0.25 μ mole GTP, 10 μ moles creatine phosphate, 50 μ g creatine kinase and 2 μ Ci leucine- 14 C. Incubation was carried out at 37°C for 15 min.

Measurement of radioactivity. Reaction was stopped by applying an aliquot of the incubation mixture to a filter paper disk and the disk was plunged into a beaker of ice-cold 10% TCA.¹⁵ The disk was washed with cold 5% TCA, heated at 90°C for 15 min in 5% TCA, washed again with cold 5% TCA, washed with ethanol followed by acetone and dried in air. The disk was placed into a counting vial containing 10 ml of toluene-PPO-POPOP scintillation liquid and counted in a Packard Tricarb Scintillation Spectrometer at 48.8% efficiency.

Adsorption on Millipore filter. The RNA preparations were diluted with 10 mM Tris-HCl buffer (pH 7.6) containing 0.5 M KCl and 1 mM magnesium acetate (around 0.25 mg/ml), and passed through a Millipore filter presoaked in the same solution at a rate of approximately 1 ml/min. The filter was kept in 1 ml of an ice-cold solution of 0.5% SDS in 0.1 M Tris-HCl buffer (pH 9.0) for 30 min, with occasional shaking. The adsorbed material was thus eluted with this solution. A potassium dodecyl sulfate crystals formed were removed by centrifugation and absorbance at 260 nm was measured.

Cellulose chromatography. RNA preparation was applied to a 5 ml cellulose column which had been equilibrated with 10 mM Tris-HCl buffer (pH 7.6) containing 0.5 M KCl and 0.2 mM MgCl₂. The column was washed with application buffer until the absorbance at 260 nm had dropped below 0.05. The adsorbed RNA was eluted with neutralized water. The non-

adsorbed RNA was precipitated with ethanol, dissolved in water and dialyzed against water. The adsorbed material was concentrated by ultrafiltration using collodion bag and used for the *in vitro* amino acid incorporation system. The chromatography was performed at 20°C.

RESULTS AND DISCUSSION

Effects of H-RNA on the amino acid incorporation in the soybean and reticulocyte systems

When rabbit reticulocyte cell-free system was used as an *in vitro* amino acid incorporation system, the increase of the incorporation by the addition of H-RNA was observed at 10 mM Mg²⁺ (Table I). The stimulative effect of H-

TABLE I. EFFECTS OF H-RNA ON THE AMINO ACID INCORPORATION IN RABBIT RETICULOCYTE SYSTEM

Concentration of Mg ²⁺	H-RNA	Leucine- ¹⁴ C incorporated (cpm per ml)
4.3 mM	minus	102,860
	plus	97,097
10.0 mM	minus	41,989
	plus	67,383

RNA was not observed at lower Mg²⁺ concentration (4.3 mM) where specific chain initiation will occur. On the other hand, H-RNA stimulated the amino acid incorporation at 5 mM Mg²⁺ in the cell-free system prepared

TABLE II. EFFECTS OF H-RNA AND TMV-RNA ON THE AMINO ACID INCORPORATION IN SOYBEAN SEEDLING SYSTEM

The amounts of components used in experiments were 0.148 mg of crude ribosomes or 0.131 mg of washed ribosomes and 0.4 mg of supernatant fractions from soybean seedlings or 0.362 mg of rat liver pH 5 fraction, as protein amounts and 2.288 OD₂₆₀ units of H-RNA or 1.282 OD₂₆₀ units of TMV-RNA.

Ribosomes	RNAs	Leucine- ¹⁴ C incorporated (cpm per ml)		
		2-Coty ^{a1}	Supernatant fractions 3-Coty ^{b1}	Rat liver pH 5 fraction
Crude ribosomes	None	1240	544	8002
	H-RNA	1594	1008	7846
	TMV-RNA	1866	1200	8186
Washed ribosomes	None	378	208	2502
	H-RNA	540	402	2432
	TMV-RNA	600	398	2592

^{a1} Cotyledons of 2-day-old soybean seedlings.

^{b1} Cotyledons of 3-day-old soybean seedlings.

from soybean seedlings (Table II). TMV-RNA which was used as a natural mRNA showed similar stimulative effect in a crude ribosome system. H-RNA and TMV-RNA stimulated the amino acid incorporation in 0.5 M KCl-washed ribosome system at 5 mM Mg^{2+} in the presence of the supernatant fraction. The pH 5 fraction from rat liver enhanced the amino acid incorporation level both in crude and washed ribosome systems but was not effective for the stimulation of the incorporation by the addition of exogenous mRNA. The initiation factors have recently been found in the soluble fraction in wheat embryos^{16,17)} and in either the ribosomal or the soluble fractions in developing pea cotyledons.¹⁸⁾ In pea cotyledons, it was shown that the initiation factors associated with ribosomes could be extracted with 0.5 M KCl from the ribosomes. The results obtained with washed ribosomes from soybean seedlings (Table II) may be explained by the existence of the initiation factors in the supernatant of soybean seedlings. It is not clear at present why H-RNA did not show the stimulative effect in the reticulocyte system at lower Mg^{2+} concentration.

Effect of ATA on exogenous RNA-stimulated amino acid incorporation in E. coli S-30 and soybean seedling systems

The effects of ATA which is known as an inhibitor for the initiation of protein synthesis on the amino acid incorporation stimulated by H-RNA and TMV-RNA were shown in Table III. The amino acid incorporation corresponding to the addition of H-RNA was inhibited by 84% and 70% at a concentration of 50 μ M ATA in *E. coli* and soybean seedling systems, respectively. ATA inhibited similarly the TMV-RNA-stimulated amino acid incorporation by 92% and 63% in *E. coli* and soybean seedling systems, respectively. In *E. coli* cell-free system, Grollman and Stewart¹⁹⁾ described that f2-RNA-stimulated peptide synthesis was inhibited completely by 50 μ M ATA, and 30 μ M ATA allows endogenous protein synthesis to continue at 50% of

TABLE III. EFFECTS OF ATA ON THE AMINO ACID INCORPORATION STIMULATED BY H-RNA AND TMV-RNA

In soybean seedling system 0.224 mg protein of ribosomes and 0.609 mg protein of supernatant fraction were used. The amounts of RNAs used were 0.532 OD₂₆₀ unit of H-RNA and 0.125 OD₂₆₀ unit of TMV-RNA. ATA was added in a concentration of 50 μ M to the reaction mixture containing ribosomes prior to the addition of RNA preparations.

Additions	Leucine- ¹⁴ C incorporated (cpm per ml)	
	Soybean seedling system	<i>E. coli</i> S-30 system
None	636	888
ATA	562	—
H-RNA	1086 (450) ^{a)}	7136 (6248)
H-RNA+ATA	696 (134)	1896 (1008)
TMV-RNA	936 (300)	19,944 (19056)
TMV-RNA+ATA	668 (106)	1444 (556)

^{a)} Net incorporations corresponding to the addition of RNAs were shown in parenthesis.

the control rate. They concluded that ATA prevented the attachment of phage RNAs to ribosomes, thereby inhibiting viral peptide synthesis. Analogous results have been shown in wheat embryo system,²⁰⁾ in which the effects of ATA on TMV-RNA-dependent amino acid incorporation and on amino acid incorporation catalyzed by endogenous polyribosomes were investigated. At a concentration of 25 μ M ATA, the TMV-RNA dependent system was completely inhibited. In contrast, polyribosome-catalyzed incorporation was unaffected at the same concentration of ATA and slightly inhibited at a concentration of 50 μ M ATA. From the experimental results, they demonstrated that the initial phase of amino acid incorporation dependent on exogenous mRNA involved an initiation reaction between ribosome and mRNA. Considering from the results obtained here and by other investigators, it appears likely that H-RNA preparation contains mRNA.

Binding of H-RNA to Millipore filter

The presence of polynucleotide sequences rich in adenylic acid in mRNA has been

recognized as a characteristic of eukaryotic mRNAs with only the exception of mRNAs for histones. RNA containing adenine-rich sequences have been shown to be retained on nitrocellulose membrane-filters (Millipore filters) at high ionic strength.²¹⁾ As shown in Table IV a small portion of H-RNA was re-

TABLE IV. BINDING OF H-RNA TO MILLIPORE FILTERS

H-RNA used (OD ₂₆₀ units)	RNA recovered (OD ₂₆₀ units)	% Yield
13.3	0.185	1.39
26.6	0.244	0.92
53.2	0.513	0.96

tained on Millipore filters when the filtration was performed in the presence of 0.5 M KCl. The adsorbed materials were about 1% of the total amount of charged RNAs by three experiments. As pointed out by Brawerman *et al.*,²²⁾ however, the possibility of the contamination of rRNAs in the adsorbed RNA could not be excluded.

Chromatography of H-RNA on cellulose

RNA containing adenine-rich sequences has been separated selectively by binding to oligo(dT) or poly(U) covalently linked to cellulose. Recently, Kitos *et al.*²³⁾ have demonstrated that at high ionic strength untreated cellulose can adsorb poly(A) and poly(A)-containing RNA molecules. Schutz *et al.*²⁴⁾ also reported that poly(A) and poly(A)-rich RNA (mRNA for globin and ovalbumin) was selectively bound to the Sigma microgranular cellulose, but rRNA and tRNA was not bound. As can be seen in Fig. 1, 1.3% of H-RNA was retained by plain cellulose under conditions of high ionic strength. The retained material was eluted with water as a sharp peak. The elution profile was similar to that obtained with the RNA from rabbit reticulocyte polyribosomes. The unfractionated RNA and the cellulose-fractionated RNAs were checked for their ability to stimulate the amino acid incorporation in *E. coli* S-30 system. Although

FIG. 1. Chromatography on Cellulose Column of H-RNA.

H-RNA preparation in 10 mM Tris-HCl (pH 7.6), 0.5 M KCl and 0.2 mM MgCl₂ containing 228.8 OD₂₆₀ units in 20 ml was applied and washed extensively with the same buffer solution. Elution with water was indicated by the arrow.

FIG. 2. Effects of Unfractionated and Cellulose-Fractionated RNAs on the Amino Acid Incorporation in *E. coli* S-30 System.

Incorporation of leucine-¹⁴C into protein was measured in 0.1 ml reaction mixture in the presence of the indicated amounts of RNA preparations as described in MATERIALS AND METHODS.

○—○, unfractionated RNA (H-RNA); □—□, cellulose-adsorbed RNA; △—△, cellulose-through RNA.

there was no large difference in the messenger activity of the RNA fractions obtained from the column chromatography, the material retained by the column was more effective than the unfractionated RNA in stimulating the amino acid incorporation (Fig. 2). Thus, the enrichment of RNA species containing poly(A) with messenger activity seems to be obtained by the cellulose chromatography. On the other hand, it has recently been reported that globin mRNA lacking the poly(A) sequence retained considerable messenger activity.²⁵⁻²⁷⁾ The result obtained here (Fig. 2) together with the findings of others suggests that the RNA fraction which could not be adsorbed on the cellulose column may still contain some mRNAs deficient in poly(A).

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