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SYNTHESIS OF POLY(A)$^+$ RNA AND POLY(A)$^-$ RNA IN SLICES OF SWEET POTATO ROOT

KAYOKO MATSUMOTO-FURUSE, KAZUKO ÔBA and IKUZO URITANI

Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

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Key Word Index—*Ipomoea batatas*; Convolvulaceae; cell-free translation; poly(A)$^+$ RNA; poly(A)$^-$ RNA; slicing

Abstract—Poly(A)$^+$ RNA and poly(A)$^-$ RNA from sweet potato root tissue were examined for translational activity using a wheat germ cell-free system. The activity of poly(A)$^+$ RNA was 20 times higher than that of poly(A)$^-$ RNA. Time course studies of *in vivo* radioactive labelling in tissue slices indicated the appearance of two peaks of poly(A)$^+$ RNA synthesis, the first at *ca* 6 hr after slicing and the second at about *ca* 24 hr, and one of poly(A)$^-$ RNA synthesis at *ca* 12 hr. The synthetic rates of both poly(A)$^+$ RNA and poly(A)$^-$ RNA at 24 hr were not changed by subsequent treatment with mercuric chloride. In the 6 hr incubation period, i.e. the period of the highest increase in poly(A)$^+$ RNA labeling, no significant change in the content of poly(A)$^+$ RNA occurred, although the poly(A)$^-$ RNA content increased. Larger MW populations of poly(A)$^+$ RNA were synthesized during the early stage (6 hr) of incubation after slicing, but smaller MW populations were formed during the later stage (24 hr) of incubation.

INTRODUCTION

When storage organs are cut into thin slices and incubated aerobically, metabolic processes are rapidly activated in the tissue [1, 2], including RNA and protein synthesis. In fact, the synthesis of rRNA, tRNA and heterogeneous RNA have been shown to increase during incubation after slicing of storage organs [3, 4]. In addition, it has been demonstrated that chromatin-bound DNA-dependent RNA polymerase activity is enhanced markedly [5, 6], and that polysomes are formed rapidly after slicing [7–10]. However, there is little evidence that mRNA species are actively changed during incubation after slicing. The present paper deals with the changes in the synthetic rate, the content and the size distribution of poly(A)$^+$ RNA and poly(A)$^-$ RNA in slices of sweet potato root.

RESULTS

Fractionation of RNA from fresh or cut tissue and its translational activity

The RNA preparation from either fresh tissue or cut tissue which had been incubated for 6 hr was fractionated by poly(U)-Sepharose CC into bound RNA, poly(A)$^+$ RNA, and unbound RNA, poly(A)$^-$ RNA. To characterize the two RNAs, their translational activities in a wheat germ cell-free system were examined following determination of the optimum reaction condition. Both poly(A)$^+$ RNA and poly(A)$^-$ RNA from fresh or incubated cut tissue stimulated the incorporation of [5,6-^{3}H]leucine into acid-insoluble material (Fig. 1). The stimulation of [5,6-^{3}H]leucine incorporation by the addition of poly(A)$^-$ RNA was not marked and almost the same level as that by total RNA (data not shown). In contrast, with poly(A)$^-$ RNA, [5,6-^{3}H]leucine incorporation was greatly enhanced by poly(A)$^+$ RNA. The translational activity of poly(A)$^+$ RNA was 20 times higher than that of poly(A)$^-$ RNA and also higher than that of standard TMV RNA (data not shown). These data showed that poly(A)$^+$ RNA was composed of functional mRNA. [5,6-^{3}H]leucine incorporation by the addition of

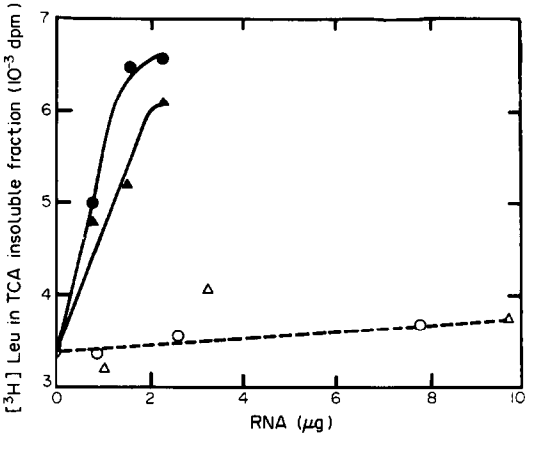

Fig. 1. Response of the wheat germ cell-free system to the addition of RNAs from fresh or 6 hr incubated cut tissues. RNA extracted from fresh or 6 hr incubated cut tissue according to method 2 (see text) was applied to a poly(U)-Sepharose column and separated into poly(A)$^+$ RNA and poly(A)$^-$ RNA. The composition of the cell-free reaction mixture and the incubation condition are described in the text. The incorporation of radioactivity from [5,6-^{3}H]leucine into the hot acid-insoluble fraction was determined after a 60-min incubation, using poly(A)$^+$ RNA (●, ▲) and poly(A)$^-$ RNA (○, △) from fresh (●, ○) or 6 hr incubated cut (▲, △) tissue.

Abbreviations: poly(A)$^+$ RNA, polyadenylated RNA; poly(A)$^-$ RNA, non-polyadenylated RNA; poly(U), polyuridylic acid; SDS, sodium dodecyl sulfate; SSC, standard saline citrate (150 mM sodium chloride + 15 mM sodium citrate).

poly(A)$^+$ RNA from incubated cut tissue was almost the same as that of poly(A)$^+$ RNA from fresh tissue when the same amount of RNA was added to the wheat germ cell-free system. The results indicated that fresh tissue also contained functional mRNA with comparable activity to cut tissue.

Changes in the rate of synthesis of poly(A)$^+$ RNA and poly(A)$^-$ RNA following slicing or mercuric chloride treatment

The synthetic rate of poly(A)$^+$ RNA and poly(A)$^-$ RNA in fresh, cut or mercuric chloride-treated tissue was examined by measuring the rate of incorporation of the label from [5-^{3}H]uridine into the two RNAs. The incorporation rate of [5-^{3}H]uridine into poly(A)$^-$ RNA increased after slicing, reached a maximum at *ca* 12 hr and then decreased (Fig. 2). This pattern was similar to that of the changes in the rate of total RNA synthesis [4]. The rate of incorporation of [5-^{3}H]uridine into poly(A)$^+$ RNA increased dramatically soon after slicing, reached a maximum at *ca* 6 hr and then decreased sharply. The incorporation rate then increased slowly from 12 to 24 hr, reached a second maximum at 24 hr, and then fell to a level that was maintained constant from 30 hr to the end of the experiment. Mercuric chloride treatment after 24 hr of incubation scarcely changed the rate of incorporation of the label from [5-^{3}H]uridine into poly(A)$^+$ RNA and poly(A)$^-$ RNA (Fig. 2).

Both poly(A)$^+$ RNA and poly(A)$^-$ RNA were most heavily labelled in cut tissue which had been incubated for 6 hr. (see Figs. 2 and 3). On a radioactivity basis, the poly(A)$^+$ RNA synthesized was 2.6% of the total RNA

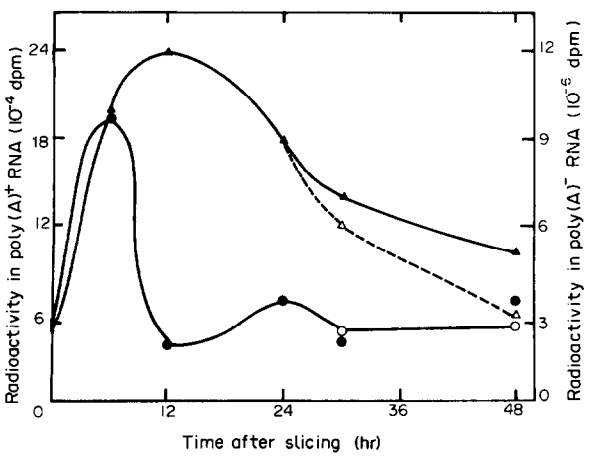

Fig. 2. Changes in the rate of incorporation of radioactivity from [5-^{3}H]uridine into poly(A)$^+$ RNA or poly(A)$^-$ RNA in tissue during incubation after slicing or mercuric chloride treatment. Fresh tissue, cut tissues incubated for appropriate periods, and mercuric chloride treated tissues were incubated with [5-^{3}H]uridine for 1 hr, after which the RNA was extracted by method 1. The RNA preparations were separated into poly(A)$^+$ RNA and poly(A)$^-$ RNA by poly(U)-sepharose CC. Mercuric chloride treated tissue: the discs were administered 0.1% mercuric chloride 24 hr after slicing, then incubated for another 6 or 24 hr. Poly(A)$^+$ RNA, (●, ○); poly(A)$^-$ RNA, (▲, △); cut tissue, (●, ▲); mercuric chloride treated tissue, (○, △).

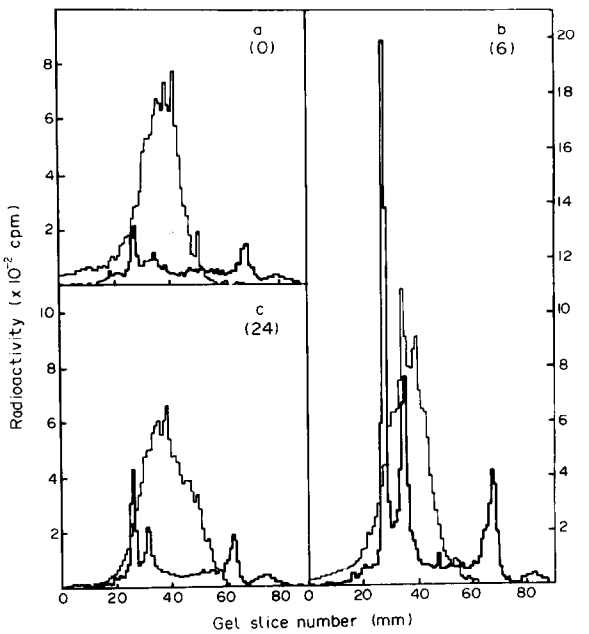

Fig. 3. PAGE profiles of poly(A)$^+$ RNA and poly(A)$^-$ RNA synthesized in the 0–1 hr, 6–7 hr and 24–25 hr periods after slicing. [8-^{3}H]Adenine (1.01 mCi) was administered to 160 discs of each type of tissue after which the treated discs were incubated for 1 hr at 30°. RNA was extracted from the [8-^{3}H]adenine labelled tissue according to method 2, then separated into poly(A)$^+$ RNA and poly(A)$^-$ RNA by poly(U)-Sepharose CC. The fractions of poly(A)$^+$ RNA from fresh tissue (9.94 μg) and 6 hr incubated (13.2 μg) and 24 hr incubated (27 μg) cut tissues and of the respective poly(A)$^-$ RNAs (25 μg) were subjected to PAGE. The radioactivity data have been corrected for background (cpm). a(0), b(6) and c(24): zymograms (cpm in RNA) of poly(A)$^+$ RNA (——) and poly(A)$^-$ RNA (——) synthesized during 0–1 hr, 6–7 hr and 24–25 hr after slicing, respectively.

synthesized during the 0–1 hr labelling period, and fell to 1.3% and 1.4% over the 6–7 hr and 24–25 hr labelling periods, respectively, when calculated from the data in Fig. 3.

Contents of poly(A)$^+$ RNA and poly(A)$^-$ RNA in fresh and cut tissue

The poly(A)$^-$ RNA content increased 30% during a 6 hr incubation period after slicing, but the poly(A)$^+$ RNA content was unchanged (Table 1). Since the incorporation rate of the label from [5-^{3}H]uridine into poly(A)$^+$ RNA was markedly enhanced after 6 hr of incubation after slicing (Fig. 2), poly(A)$^+$ RNA may have a higher turnover rate than poly(A)$^-$ RNA.

Difference in the size distribution of poly(A)$^+$ RNA synthesized at 0–1, 6–7 and 24–25 hr after slicing

The rate of synthesis of poly(A)$^+$ RNA was enhanced after slicing and exhibited two maxima, the first at *ca* 6 hr and the second at *ca* 24 hr after slicing (Fig. 2). Therefore, [^{3}H]adenine was applied for 1 hr to fresh (0–1 hr labelled) tissue and 6 hr incubated (6–7 hr labelled) and 24 hr

Table 1. Poly(A)$^+$ RNA and poly(A)$^-$ RNA contents of fresh tissue and 6 hr incubated cut tissue

	RNA contents (μg/g tissue)*	
	poly(A)$^+$ RNA	poly(A)$^-$ RNA
Fresh tissue	0.148(1)	166(1)
Cut tissue	0.155(1.05)	218(1.34)

*RNA extracted by method 2 was applied to a poly(U)-Sepharose column and the fractions of poly(A)$^+$ RNA and poly(A)$^-$ RNA were separated as described in the text. Each fraction was precipitated with ethanol and its RNA content was analysed. The values in parentheses show the ratios of RNA content in cut tissue to those in fresh tissue.

incubated (24–25 hr labelled) cut tissue, and then the labelled RNA was separated into poly(A)$^+$ RNA and poly(A)$^-$ RNA by poly(U)-Sepharose chromatography. Each poly(A)$^+$ RNA and poly(A)$^-$ RNA was then subjected to gel electrophoresis. Typical electrophoretic profiles of poly(A)$^+$ RNA and poly(A)$^-$ RNA synthesized during the three experimental periods are shown in Fig. 3. Poly(A)$^-$ RNA was separated into heavy- and light-rRNA, 5S RNA plus tRNA; poly(A)$^+$ RNA, however, was separated into heterogeneous RNAs. To compare the differences in the species among the fractions of poly(A)$^+$ RNA synthesized in the different time periods after slicing, the data of Fig. 3 were recalculated and arranged as shown in Fig. 4. The figure shows that the synthesis of the larger MW populations of poly(A)$^+$ RNA was enhanced in the early stage of incubation (6 hr incubation), and that synthesis of the smaller MW populations of poly(A)$^+$ RNA was activated but that of the larger RNA species was suppressed in the later stage of incubation (24 hr incubation), compared with the early stage (6 hr incubation).

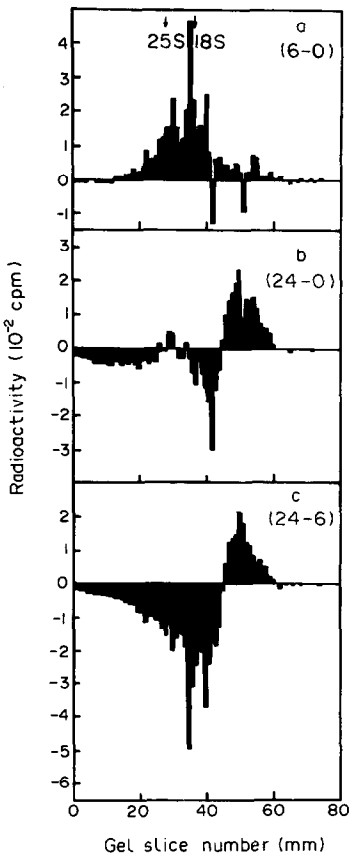

Fig. 4. Comparison of sizes of poly(A)$^+$ RNA synthesized during 0–1 hr, 6–7 hr and 24–25 hr after slicing. (a) The differences between the counts in each gel slice of poly(A)$^+$ RNA (equivalent to 10 μg) labelled during 6–7 hr and during 0–1 hr. (b) The differences between the RNA labelled during 24–25 hr and during 0–1 hr. (c) The differences between the RNA labelled during 24–25 hr and during 6–7 hr.

DISCUSSION

The present investigation on *in vivo* labelling of poly(A)$^+$ RNA and poly(A)$^-$ RNA of sweet potato roots has been undertaken in order to examine the induction of synthesis of mRNA and rRNA in response to wounding or chemical injury in relation to the metabolic activation of wounded, infected and chemically injured tissues. Polysome formation is observed over a period of 1 hr after slicing, although the RNA content of ribosomes remains constant for 6 hr [7]. The experiment shown above demonstrated that the synthesis of both poly(A)$^+$ RNA and poly(A)$^-$ RNA is markedly enhanced during a 6 hr incubation after slicing of sweet potato roots (Figs. 2 and 3), and the highest synthetic rates of poly(A)$^+$ RNA and poly(A)$^-$ RNA are seen *ca* 6 hr and 12 hr after slicing, respectively. These results indicate that polysome formation in sweet potato root tissue following slicing [7] is paralleled by the synthesis of new mRNA. However, fresh tissue contains functional poly(A)$^+$ RNA whose activity is comparable to that of cut tissue (Fig. 1) and the content of poly(A)$^+$ RNA remains constant for 6 hr after slicing (Table 1). These results may indicate that the increased synthetic rate of mRNA in cut tissue of sweet potato roots is not the cause of the initiation of polysome formation and increase of protein synthesis in cut tissue, and that there is another mechanism which controls polysome formation, different from the case of potato tuber [3].

The proportion of radioactivity incorporated into poly(A)$^+$ RNA to that in total RNA is higher (2.6%) in fresh tissue (0–1 hr labelling) than in cut tissue (1.3%, in the case of 6–7 hr of labelling, and 1.4% in the case of 24–25 hr of labelling). This does not reflect a drop in the absolute rate of poly(A)$^+$ RNA synthesis in cut tissue (Figs. 2 and 3), but seems to be a result of the relatively high synthesis of rRNA during either the 6–7 hr or 24–25 hr labelling period after slicing, that is the result of a late acceleration of rRNA synthesis. Delayed synthesis of rRNA has been reported for cell cultures and germinating embryos which undergo transition from a resting state to an actively growing one [11–13].

The size distribution of poly(A)$^+$ RNA synthesized in different incubation periods after slicing changes (Fig. 4). Thus, the size distribution of poly(A)$^+$ RNA synthesized during a 6–7 hr labelling period after slicing is heterogeneously dispersed with a mean of *ca* 18S, i.e. close to that of poly(A)$^+$ RNA from soybean hypocotyl [14], whilst the size of poly(A)$^+$ RNA synthesized during the 24–25 hr labelling period is smaller. This is consistent with the recent observation by Ishizuka *et al.* [15] that the

proportion of mRNA coding for high MW polypeptides decreases in potato tuber slices with time of incubation while that for low MW polypeptide increases.

When cut tissue is treated with toxic chemicals, such as mercuric chloride, the tissue produces many kinds of furano-sesquiterpene phytoalexins, i.e. compounds which are not present in fresh or cut tissue [16]. The activities of some of the enzymes involved in furano-sesquiterpene production have been found to increase markedly after such treatment [16–18]. In the present paper, we have examined the change in the rate of synthesis of poly(A)$^+$ RNA and poly(A)$^-$ RNA in response to slicing and mercuric chloride treatment in connection with the above observations on furano-sesquiterpene production and the pertaining enzyme synthesis. The results indicate that mercuric chloride treatment enhances the synthesis of neither poly(A)$^+$ RNA nor poly(A)$^-$ RNA (Fig. 2). This may suggest that the completion of the enzymic pathway on furano-sesquiterpene production in response to mercuric chloride treatment does not directly depend on the enhancement of synthetic rates of mRNAs for various enzymes in the pathway. Thus, the above observations may be helpful in the elucidation of the mechanism of furano-sesquiterpene production in relation to the transcriptional and translational activities in the tissue.

EXPERIMENTAL

Plant material. Sweet potato (*Ipomoea batatas* Lam. cv. Norin 1) roots were harvested in the autumn, and stored at 10–13° until use. Roots were dipped in 0.1% NaClO soln for 20 min for sterilization, washed with H_2O for 20 min, then cut transversely into blocks *ca* 40 mm thick. After being peeled, the blocks were immersed in the NaClO for another 10 min then washed with a large vol. of sterile H_2O. Tissue cylinders, 15 mm diameter, were obtained from parenchymatous tissue with a cork borer. Discs 1 or 2 mm thick were prepared from the cylinders by cutting with a razor blade. They were then washed with a large vol. of sterile H_2O and lightly blotted. All manipulations were carried out under aseptic conditions. The discs were placed on a wire net in a plastic box under high humidity and incubated at 30° in the dark. Freshly cut discs and incubation discs were called 'fresh tissue' and 'cut tissue', respectively. Some of the discs which had been incubated for 24 hr were administered 20 μl/disc of 0.1% $HgCl_2$ soln at one side, then incubated for a further 6 or 12 hr under the same condition. These were called '$HgCl_2$ treated tissue'.

Application of ^{3}H-labelled compounds to discs. 25 μl/disc [5-^{3}H]uridine (100 μCi/ml, 26 Ci/mmol, Radiochemical Centre, Amersham) or[8-^{3}H]adenine (200 μCi/ml, 22 Ci/mmol, Radiochemical Centre, Amersham) soln containing chloramphenicol (50 μg/ml) was applied to one side of each of the discs, which then were incubated for 60 min at 30° unless otherwise stated. The labelled discs (20 discs) were washed once with 500 ml and twice with 250 ml 0.02% uridine or adenine in cold H_2O then lightly blotted, and the RNA was extracted by the PhOH–$CHCl_3$ method as described below.

Extraction of RNA. Method 1. 20 discs (equivalent to 5 or 10 g fr. wt) were chopped into small pieces with a razor blade, then ground with a mortar and pestle in 20 ml Tris–KCl–$MgCl_2$ buffer (0.02 M Tris–HCl buffer, pH 7.6, containing 0.05 M KCl, 2 mM $MgCl_2$), 0.5% SDS, 0.1 g bentonite, 0.7 M 2-mercaptoethanol, 8 g sea sand and 20 ml PhOH–$CHCl_3$ (1:1) containing 0.1% hydroxyquinoline which had been satd with 0.01 M acetate buffer, pH 6.0, containing 0.1 M NaCl and 1 mM EDTA [4]. When [5-^{3}H]uridine had been applied to the discs, 1 mg uridine was added to the above grinding medium. The homogenate was centrifuged at 3000 rpm for 20 min and the upper aq. phase removed with a pipette. The PhOH–$CHCl_3$ phase with cell debris was stirred vigorously for a few min using a glass rod, with 10 ml Tris–KCl–$MgCl_2$ buffer. The suspension was centrifuged as above to obtain the aq. phase. The procedure was repeated once. The aq. phases were combined and shaken vigorously with an equal vol. of PhOH–$CHCl_3$ (1:1) for a few min. After low-speed centrifugation, to the upper aq. phase was added solid NaCl in 3% concn, and then 2.5 vols. 95% EtOH was mixed with the above soln. The mixture was allowed to stand overnight at 4°. The ppted RNA preparation was dissolved in 10 ml standard saline citrate (SSC), and the soln shaken vigorously with an equal vol. of PhOH–$CHCl_3$ (1:1). After low-speed centrifugation, the RNA in the upper phase was ppted with EtOH as described above and this procedure was repeated again. The RNA preparation thus obtained was stored at −20° until use.

Method 2. 40 discs (equivalent to *ca* 20 g of fresh tissue or cut tissue) was frozen in liquid N_2 and ground to a fine powder using a chilled mortar and pestle. Just before the powder started to thaw, it was homogenized with 40 ml grinding buffer and the other ingredients (see method 1). In this case, the buffer was adjusted to pH 8.4 instead of pH 7.6, and bentonite and uridine were omitted. RNA was extracted as described in method 1 and the final EtOH–ppted RNA preparation was dissolved in autoclaved distilled H_2O and dialysed against the same H_2O. This procedure was repeated twice and the RNA preparation was stored at −80° until use.

Estimation of RNA. The final ppt of RNA was dissolved in 5 ml SSC or autoclaved distilled H_2O, and the amounts of RNA estimated spectrophotometrically by using the following equation according to the method reported previously [4]:

$$\text{RNA } (\mu g/ml) = (A_{260} - A_{320}) \times 50.$$

Since the amount of DNA extracted by this method was negligible, it was disregarded in estimating the amount of RNA.

Polyacrylamide gel electrophoresis. Electrophoresis of RNA preparations was carried out on a 2.46% polyacrylamide gel (9 cm long) containing 0.5% agarose using Knowland's buffer [19] for 90 min at 5.5 mA/gel in a refrigerator. After electrophoresis, the gels were stained with toluidine blue (0.1%) for 1 hr, destained with 5% aq. HOAc then scanned at 565 nm with a densitometer (Toyo Kagaku, DMU-33C, Japan). For determination of radioactivity, each gel was frozen on a slab with dry ice and divided into 1-mm thick sections with a gel slicer (Mickle Lab. Eng., U.K.). The slices were placed separately in counting vials and digested overnight with 0.25 ml of a mixture of 30% H_2O_2 and conc. NH_4OH (99:1). To each vial was added 0.75 ml H_2O and 10 ml scintillation cocktail (toluene–Triton X-100 (2:1) containing 40 mg PPO and 1 mg POPOP). The radioactivity was measured in a Packard Tricarb scintillation spectrometer, model 3320.

Chromatography on poly(U)-Sepharose. Poly(U)-Sepharose was used for preparative affinity chromatography of mRNA according to the method of ref. [20] with some modifications. Poly(U)-Sepharose 4B (0.2 g) was washed with 0.1 M NaCl, packed in a jacketed micro-column (0.5 cm in diameter) and equilibrated with Tris–HCl buffer, pH 7.6, containing 0.1 M NaCl and 0.2% SDS. The RNA preparation was dissolved in Tris–NaCl–SDS buffer, and the soln applied to the column. Unabsorbed materials were washed out with Tris–NaCl–SDS buffer, and the RNA retained by the column was then eluted with 0.01 M Tris–HCl buffer, pH 7.6, containing 0.2% SDS and raising the temp. of the column to 50°. RNA in the effluent or in the eluate was ppted with EtOH.

Wheat germ cell-free system. This was prepared according to the procedure of Roberts and Paterson [21] with some modifi-

cations. 12 g of wheat germ (Nisshin Seifun, Tsurumi, Japan) were ground with a mortar and pestle at 4° in 28 ml homogenizing buffer (pH 7.6) composed of 0.02 M Tris, 0.1 M KCl, 1 mM $Mg(OAc)_2$, 2 mM $CaCl_2$, 6 mM 2-mercaptoethanol and 12 g sea sand. Then the homogenate was centrifuged at 30000 *g* for 10 min. The supernatant was again centrifuged at 30000 *g* for 10 min and the clear supernatant (10–12 ml) was removed by pipette, and then passed through a Sephadex G-25 column (2.6 × 25 cm) which was pre-equilibrated with 0.02 M Tris–HCl buffer, pH 7.6, containing 0.12 M KCl, 5 mM $Mg(OAc)_2$ and 6 mM 2-mercaptoethanol. Fractions with a high A_{260} value (9 ml, 46 A_{260}/0.3 ml) were pooled, frozen in aliquots in EtOH (−80°), and then stored at −80°.

Protein synthesis was measured in an incubation mixture of 50 μl which contained 30 μl wheat germ extract (4.6 A_{260}), 0.02 M Tris–HCl buffer, pH 7.6, 1 mM $Mg(OAc)_2$, 2 mM D-dithiothreitol, 0.1 mM spermine, 0.05 mM unlabelled 19 L-amino acids, 3 μCi L-[5,6-^{3}H]leucine (100 Ci/mmol Radiochemical Centre, Amersham), 1 mM ATP, 25 μM GTP, 8 mM creatine phosphate, 2.5 μg creatine kinase and RNA preparation. After incubation at 30° for 60 min, the incubation mixture was spotted on Whatmann 3MM chromatography paper (2.4 cm diameter) which had been pretreated with 50 μl 10% TCA. Then the paper was dipped into 10% TCA soln for several hr, washed successively with cold 10% TCA, hot 5% TCA, cold 5% TCA, EtOH, and Ac_2O, dried in air and assayed for radioactivity in 10 ml toluene–Triton scintillation liquid, with a Packard Tricarb scintillation spectrometer, model 3320.

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