Phytochemistry, Vol 23, No 7, pp 1361–1366, 1984
Printed in Great Britain

0031–9422/84 $3 00 + 0 00

NON-POLYADENYLATED 10S RNA IN PAUL'S SCARLET ROSE CELL SUSPENSION CULTURES

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(*Received* 12 *August* 1983)

Key Word Index—*Rosa* sp, Rosaceae, Paul's Scarlet rose, cell culture, growth control, RNA synthesis, mRNA

Abstract—Paul's Scarlet rose cells in suspension culture synthesized a non-ribosomal, non-polyadenylated RNA which had a sedimentation value of *ca* 10S in sucrose gradients At all stages of growth, the 10S RNA was preferentially associated with the non-polysomal particles in the cytoplasm Of the newly synthesized polyA(−)RNA in the cell, 20 to 30% was 10S RNA and its synthesis was regulated during growth It was transcribed throughout the growth cycle except for a short period in lag phase Late logarithmic and stationary phase cells synthesized more than the exponentially growing cultures Several lines of evidence suggest that the 10S RNA is messenger RNA

INTRODUCTION

The cytoplasmic RNA in eukaryotic cells can be resolved into two main classes, a poly A(+)RNA and a poly A(−)RNA, depending on their ability or inability to bind to an affinity column such as oligo(dT) cellulose or poly(U)-Sepharose respectively The poly A(+)RNA obtained in this way has been shown to contain long stretches of A residues (60–200) at its 3′ end and represents the cellular mRNA The poly A(−)RNA class, on the other hand, may lack a poly A segment entirely or may contain a short stretch of 10–15 residues This fraction which is predominantly rRNA has also been shown to contain mRNA species [1, 2] At the moment, the exact role of poly A at the 3′ end of mRNA molecules largely remains unclear Both RNA forms apparently coexist in most higher plant and animal cells and their kinetics of synthesis and ability to function as a template in polypeptide synthesis appear to be similar [3–6]

While studying the RNA metabolism in Paul's Scarlet rose cells, it was found that both the polyA(+) and A(−)RNAs are synthesized in this cell line [7–9] This cell line, which can be grown in suspension culture, has now been used to investigate the *de novo* synthesis and regulation of poly A(−)RNA in higher plants The present study provides evidence for the existence of a distinct class of poly A(−)RNA of *ca* 10S and shows its synthesis is regulated during the growth cycle of the culture Further analysis of this RNA fraction demonstrates that it is an mRNA

RESULTS

Identification of 10S RNA in the newly-made ribosomes

A culture was labeled with [^{3}H]uridine and the RNA from total ribosomes was extracted It was freed of contaminating tRNA and DNA by washing with 2 M lithium chloride [7] and the polyadenylated mRNA fraction [poly A(+)RNA] was removed by binding to an oligo(dT) cellulose column The sucrose gradient analysis of the final non-polyadenylated RNA [poly A(−)RNA], as shown in Fig 1, displayed three distinct peaks Two of them correspond to ribosomal 18S and 26S peaks and a new, third peak sedimented at *ca* 10S in sucrose gradients and will be referred to as '10S RNA' hereafter for the purpose of discussion The 10S RNA was present in cells labeled for 3 or 6 hr

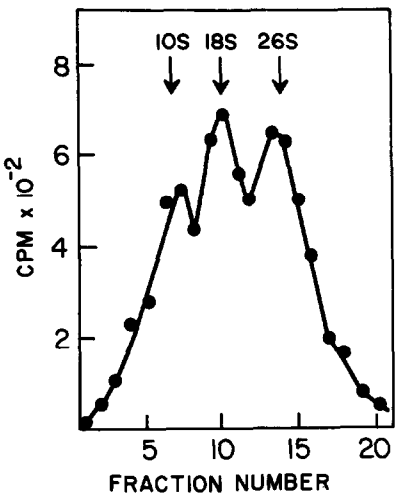

Fig 1 Sedimentation of newly made, poly A(−)RNA from total ribosomes on a sucrose gradient A stationary phase culture was diluted in fresh medium and labeled with 5 μCi/ml of [5,6^{3}H]uridine (10^{-5} M) for 3 hr Total ribosomes prepared, RNA extracted and a poly A(−)RNA fraction purified by oligo(dT) cellulose chromatography as described in Experimental It was sedimented through a 5–20% linear sucrose gradient in 10 mM Tris–HCl, pH 7 6, 50 mM NaCl and 10 mM Na_2EDTA, pH 7 2 (7), fractions collected and precipitated with cold 5% TCA

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To further localize the 10S RNA in the cytoplasm, pulse-labeled ribosomes were fractionated into two groups by differential ultracentrifugation One group contained primarily polysomes and the second, the remainder of the particles (non-polysomal fraction) Figure 2 shows an example of a preparation from 6-day-old cultures labeled with [^{3}H]uridine The non-polysomal fraction contained a trace amount of smaller polysomes but mainly particles that sediment less than 80S (Fig 2) The two types of particles were treated with 10 mM Na_2EDTA and separated on sucrose gradients as described before [7] Gradients of polysomes showed two peaks corresponding to 40S and 60S subunits On the other hand, the EDTA-treated non-polysomal fraction displayed three distinct peaks two representing the 40S and 60S subunits and a third at *ca* 20S (data not shown) Furthermore, the poly A(−)RNA was prepared from the two kinds of ribosome preparations and analysed on sucrose gradients As seen in Fig 3, only RNA from the non-polysomal fraction showed three peaks, one of which corresponds to 10S RNA Similar results were obtained in the non-dividing (lag phase) or rapidly dividing (5 day) cells (Fig 3)

In an experiment to find whether the 10S RNA originated from the membrane-bound or free ribosomal particles, lysates were prepared with and without treating with the non-ionic detergent, Triton X-100 The data showed that 10S RNA was associated only with the non-polysomal fractions and its relative amount was similar in both the detergent treated and untreated lysates (not shown)

The poly A content of the poly A(−)RNA fractions obtained after oligo(dT) cellulose chromatography was further analysed by hybridization with [^{3}H]poly U The poly A(−)RNA fractions from the non-polysomal and polysomal particles indicated negligible amounts of poly A compared to the poly A(+)RNA fractions of the corresponding particles (Table 1) The sedimentation behaviour and the proportion of 10S RNA in the poly A(−)RNA were unchanged even after recycling through oligo(dT) column 2–3 times (not shown)

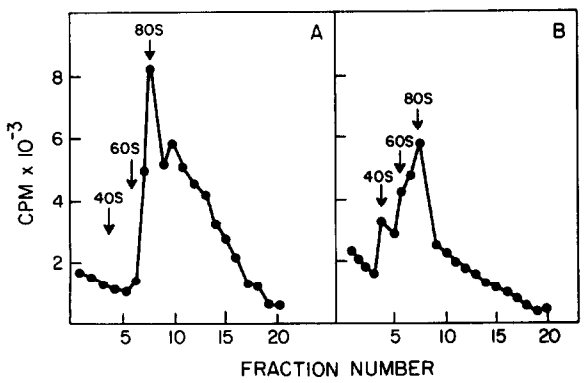

Fig 2 Sucrose gradient analysis of polysomal and non-polysomal particles A 6-day-old, logarithmically growing culture was labeled with [^{3}H]uridine for 4 hr and polysomal and non-polysomal components prepared as described in Experimental They were separated on 5–45% linear sucrose gradients in 50 mM Tris–HCl, pH 7 6, 50 mM KCl and 5 mM $MgCl_2$, individual fractions precipitated with cold 5% TCA and counted

(A) Polysomal fraction, (B) non-polysomal fraction

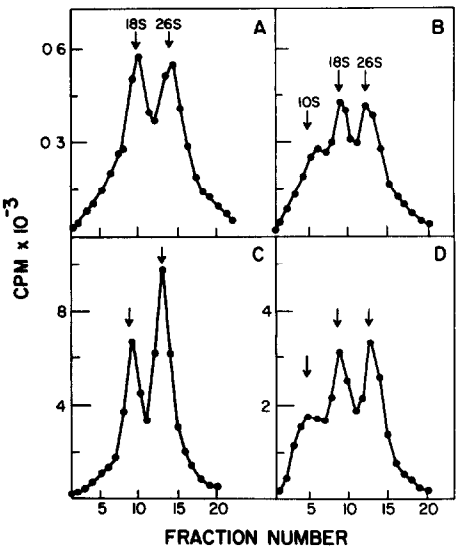

Fig 3 Localization of 10S RNA in subcellular fractions Cells from lag (0–4 hr) and exponential phase (5-day) of growth cycle were labeled for 4 hr with [^{3}H]uridine Polysomal (P) and non-polysomal (nP) components were prepared as described in Experimental The poly A(−)RNA fractions were analysed as in Fig. 1 (A) lag phase, P, (B) lag phase, nP, (C) growing, P, (D) growing, nP

Table 1 Determination of the poly A content of the various RNAs by hybridization with [^{3}H]poly U

RNA	cpm in hybrid/μg RNA
Non-polysomal, poly A(−)RNA	21
Non-polysomal, poly A(+)RNA	17182
Polysomal, poly A(−)RNA	26
Polysomal, poly A(+)RNA	14961

RNA samples were prepared from the non-polysomal and polysomal particles of a 5-day culture Fractions of poly A(−)RNA and poly A(+)RNA obtained after 2 cycles of oligo(dT) cellulose chromatography were hybridized with [^{3}H]poly U as described in Experimental

Synthesis of 10S RNA during the growth cycle

In these studies, the cells were labeled with [^{3}H]uridine and the poly A(−)RNA was prepared from the non-polysomal particles The kinetics of 10S RNA synthesis in a growing culture is compared to that of rRNA in Table 2 In the initial 45 min, over 50% of the RNA made is 10S RNA Although the synthesis continues, its proportion is reduced later on as the transcription of rRNA increases (Table 2) The relative synthesis of 10S RNA at various periods of culture growth is given in Table 3 Two features are noteworthy First is the complete absence of 10S RNA synthesis on days 1 and 2 (Fig 4), it was not associated with the polysomal fraction either (not shown) Secondly, late logarithmic and stationary phase cells synthesize

Table 2 Time course of synthesis of 10S RNA and rRNA

Period of labeling	Incorporation (cpm) 10S RNA	rRNA (18S + 26S)	10S RNA (% of total)
45 min	2045	1888	52
2 hr	10265	21813	32
4 hr	17584	45216	28

A 5-day-old, growing culture was labeled with [^{3}H]uridine and the poly A(−)RNA was isolated from non-polysomal fractions It was further analysed on a sucrose gradient and radioactivity under 10S and rRNA (18S + 26S) peaks are shown

Table 3 Relative synthesis of 10S RNA during the growth cycle

Duration of culture (days)	10S RNA (% of total)
0	31
1	0
2	0
3	22
4	22
5	21
6	22
12	34
16	26

A stationary phase culture (21-day) was inoculated into fresh medium and a 20 ml aliquot was labeled immediately (0 day) Other cultures were labeled after incubation for different times during the growth cycle In all cases, cells were labeled for 4 hr with [^{3}H]uridine, poly A(−)RNA from non-polysomal fractions prepared and analysed on sucrose gradients as described in Experimental The synthesis of 10S RNA relative to total poly A(−)RNA is presented

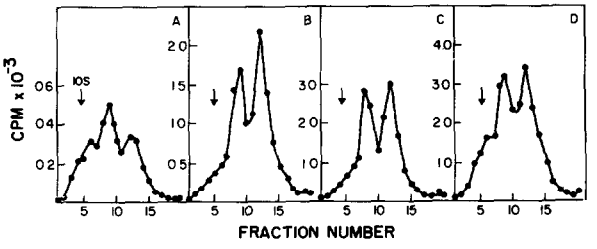

Fig 4 Synthesis of 10S RNA during the resumption of growth cycle Cultures were sampled at 0 (A), 1 (B), 2 (C) and 3 (D) days of growth cycle and each labeled for 4 hr as in Fig 1 Only poly A(−)RNA from the non-polysomal fractions were analysed on sucrose gradients

more than the logarithmic phase cultures (Table 3) The extensive synthesis of 10S RNA during the early hours of lag phase (i e 0 day) probably reflect a 'carry over' of the process in the stationary state cells Lag phase culture did not transcribe the 10S RNA if labeled after 12 hr of starting the growth cycle (data not shown)

Characterization of 10S RNA

Since the poly A(−)RNA may contain both rRNA and mRNA, it was of interest to determine to which category the 10S RNA belonged As the mRNA and rRNA have characteristic methylated oligonucleotides [10], they can be identified by labeling the RNA with [Me^{3}H]-methionine first and separating the nucleotides on DEAE-Sephadex columns in the presence of urea [8] Fractions from the 10S region and rRNA (18S + 26S) peaks were pooled and digested exhaustively with RNase T2 For comparison, poly A(+)RNA from polysomes was similarly treated The elution of RNase T2 resistant nucleotides on a DEAE-Sephadex column is shown in Fig 5 and the distribution of radioactivity in different peaks calculated in Table 4 It is clear from the data that 10S RNA resembles poly A(+)RNA than rRNA (Fig 5, Table 4) The peak eluting at a charge of −4 5 is seen only in 10S RNA and poly A(+)RNA It would represent a cap O structure, m^{7}GpppX [10], found only in mRNA The peak eluting at −2 has been shown to include m^{6}A found internally in mRNA [11] The prominent radioactivity in 10S RNA and poly A(+)RNA at this peak compared to rRNA (Table 4) suggests the presence of internal methylation Further analysis of these peaks could not be carried out because of low radioactivity Only a trace of the −4 5 peak is seen in rRNA, presumably due to contamination of 10S RNA Obviously 10S RNA and poly A(+)RNA were also contaminated with some rRNA as evidenced by radioactivity in the −3 peak (Table 4)

Since the cap structure in 10S RNA suggested that it is an mRNA, its function in protein synthesis was examined in a wheat germ cell-free system (Table 5) Its activity was compared with a poly A(+)RNA preparation from polysomes Both RNA fractions stimulated incorporation of labeled amino acid into TCA-insoluble proteins However, the incorporation was non-linear with the 10S RNA fraction, probably due to the unavoidable contamination with rRNA (Table 4) An analysis of translational products by SDS gel electrophoresis revealed that distinct polypeptides were coded for by both the RNA fractions (Fig 6) The translation of more than one protein from the 10S RNA preparation suggests that the 10S RNA fraction may, in fact, represent more than one mRNA species Only proteins below 40 000 Mr were observed in the 10S RNA sample, most of which also appeared in the poly A(+)RNA products One protein of 10S RNA (arrow, Fig 6) apparently was not made by the poly A(+)RNA

DISCUSSION

The results of this study show that a non-polyadenylated RNA of about 10S in size is synthesized in rose cells grown in suspension culture It was important to characterize this RNA further because no rRNA species of this size class is known in the cytoplasm of plant cells [12] The following properties of 10S RNA demonstrate that it is mRNA (a) in short term pulses mRNA is known to

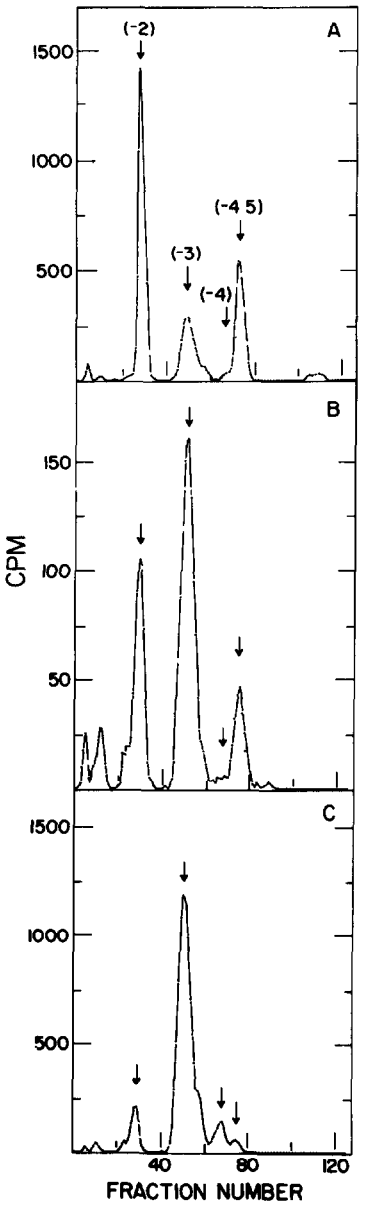

Fig 5 DEAE-Sephadex chromatography of RNase T2 resistant nucleotides in poly A(+)RNA, 10S RNA and rRNA A stationary phase culture was diluted and labeled with 6 μCi/ml of [Me^{3}H]methionine (80 Ci/mmol, New England Nuclear Corporation, Boston) for 3 hr as before [7] and polysomal and non-polysomal fractions prepared Poly A(+)RNA was isolated from polysomal fraction and the non-polysomal, poly A(−)RNA was separated into 10S and rRNA (18S + 26S) peaks on sucrose gradients They were reprecipitated with ethanol and washed once with 70% ethanol and 0 1 M sodium acetate Hydrolysis and separation were done as described in Experimental (A) Poly A(+)RNA, (B) 10S RNA, (C) rRNA (18S + 26S)

label rapidly and in fact the labeling kinetics of 10S RNA followed such a pattern (Table 2) (b) EDTA treatment of non-polysomal particles released a 20S component in addition to ribosomal subunits Such a treatment has been demonstrated to release mRNA from ribosomes [13]

Table 4 Distribution of RNase T2 resistant nucleotides in 10S RNA, rRNA and poly A(+)RNA

	% Distribution			
Source of RNA	(−2)	(−3)	(−4)	(−4 5)
10S	28 6	55 8	2 2	13 3
18S + 26S	9 5	80 7	7 4	2 4
poly A(+)	50 1	23 0	1 3	25 6

The data from Fig 5 were used to calculate the distribution of radioactivity in different oligonucleotide peaks

Table 5 Stimulation of amino acid incorporation in a wheat germ cell-free system

RNA	μg	Incorporation (cpm)
Poly A(+)	0 09	64 080
	0 17	122 209
	0 34	196 809
Poly A(−)	0 08	2953
	0 17	4238
	0 34	3729

RNA samples were prepared from a 5-day growing culture Fractions of poly A(−)RNA (from nonpolysomal particles) and poly A(+)RNA (from polysomes) were passed twice through oligo(dT) cellulose, washed in 0 1 M KOAc, 70% ethanol and resuspended in water The poly A(−) 10S RNA was obtained from a sucrose gradient fraction (Fig 5) The incorporation of [^{35}S]methionine into TCA insoluble products was determined as described in Experimental

(c) The methylation pattern of 10S RNA was similar to poly A(+)RNA (Fig 5, Table 4) (d) 10S RNA directed the synthesis of proteins in a wheat germ cell-free translational system (Fig 6)

Previous studies have shown the presence of poly A(−) mRNA in both plant and animal cells [1, 6] The present data provide further evidence for the existence of poly A(−)RNA in another higher plant and extend information on the regulation of synthesis of a 10S RNA *in vivo* Unfortunately, we cannot conclude on the number of component mRNA molecules in the 10S RNA preparation because of the other contaminants It appears from its translational ability that it must contain many A major difficulty in obtaining a pure 10S RNA fraction has been that it was present in cells in extremely small amounts While it was not difficult to localize the 10S RNA peak from a labeled RNA preparation, no such peak could be discerned from the unlabeled RNA preparations (data not shown) These technical difficulties hinder any additional detailed studies of the individual species in the 10S RNA fraction

The data suggest that the synthesis of 10S RNA in the

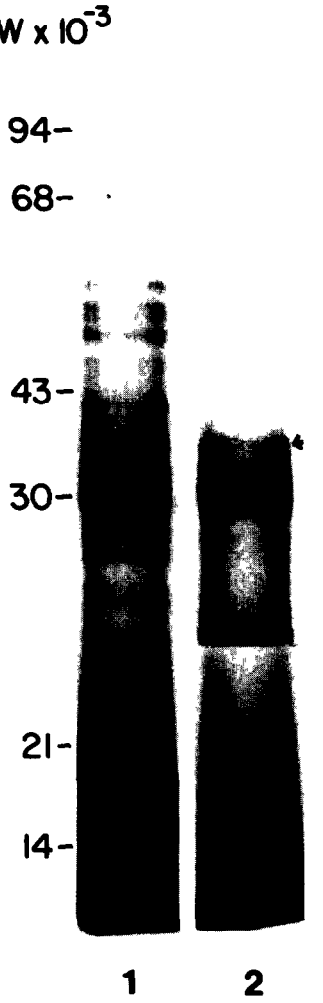

Fig 6 Translation products of the RNA fractions in a wheat germ system RNA was translated as described in Table 5 and the products separated by SDS gel electrophoresis The gel was treated with PPO, dried and exposed to a prefogged X-ray film (1) Poly A(+)RNA (2) Poly A(−)10S RNA (3) No RNA blank

rose culture was controlled by the state of cell growth The proportion of 10S RNA that was transcribed was greater in late logarithmic and stationary phase cells compared to exponential phase cells Except for a short period during the lag phase, its synthesis could be detected throughout the growth cycle of the culture The significance of the absence of its synthesis during this time is not yet clear We have earlier shown that the synthesis of other RNA species, DNA and proteins proceeded normally at this period [7–9] Synthesis of 10S RNA was observed initially on day 3, coincident with the first cell division in the culture This suggests that this RNA may preferentially code for histones Both the size of the RNA fraction and the MWs of products translated from it suggest the presence of histone-like products

It is also interesting to note that this 10S RNA fraction was found only in the nonpolysomal particles throughout the growth cycle of the culture Experiments in rat liver have shown the preferential association of ferritin mRNA with the post-ribosomal particles whose transport into polysomes was regulated by iron [14] That nonpolysomal particles in rose culture might serve as intermediate storage units for the 10S RNA before being mobilized into polysomes remains an attractive hypothesis

EXPERIMENTAL

Cell line and labeling Cells of *Rosa* sp (Paul's Scarlet Rose) were grown in suspension culture in a synthetic medium [15] Details of isotope incorporation and processing of cells have already been described elsewhere [7–9] Other particulars are explained in figures and tables

Isolation of polysomes and non-polysomal particles Frozen cells were ground in dry ice and resuspended in 6 ml of extraction buffer (40 mM Tris–HCl, pH 7 6, 2 mM $MgCl_2$, 20 mM KCl, 5 mM 2-mercaptoethanol and 0 25 M sucrose) The lysate was treated with 1% Triton X-100 and a 14000 *g* supernatant was prepared [9, 15] For the preparation of total ribosomes, the supernatant was layered over 1 5 ml of 0 75 M sucrose in extraction buffer and centrifuged for 2 5 hr at 160000 *g* in a Spinco 65 rotor to obtain the pellet For polysomes, the 14000 *g* supernatant was layered over 1 5 ml of 1 8 M sucrose in extraction buffer, sedimented for 90 min at 160000 *g* and the pellet saved, the post-polysomal supernatant was recentrifuged at 160000 *g* for 2 hr to obtain the non-polysomal fraction The pellet in each case was resuspended in a suitable buffer and clarified for 5 min at 12000 *g*

Purification of RNA and analysis The procedures described earlier [7] were followed RNA from the various subcellular fractions was extracted with 0 5% sodium dodecyl sulfate (SDS) and phenol–$CHCl_3$–isoamyl OH soln and the EtOH ppts were washed extensively with 2 M LiCl [7] The final RNA was dissolved in 0 5 M NaCl, 10 mM Tris–HCl, pH 7 6 and 0 1% SDS and applied to an oligo(dT) cellulose (Type T-2, Collaborative Research, Mass) column The unbound fraction was reprecipitated with EtOH and washed once with 70% EtOH, 0 1 M NaOAc The resultant RNA, termed the 'poly A(−)RNA' was resuspended in 10 mM Tris–HCl, pH 8 0, 50 mM NaCl and 10 mM Na_2EDTA, pH 7 2 and clarified The poly A(+)RNA was the fraction bound to the column, and was eluted with 10 mM Tris–HCl, pH 7 6 and 0 1% SDS Before precipitation with EtOH, 50 μg of wheat germ tRNA and NaCl to 0 1 M were added to this fraction RNA was analysed on linear sucrose gradients as before [7]

Hybridization with ^{3}H-*poly U* The various RNA fractions from the polysomal and non-polysomal particles were hybridized with ^{3}H-poly U (Miles, 360 μCi/μmol) and analysed as before [15]

DEAE-Sephadex chromatography of nucleotides RNA was resuspended in 50 mM NaOAc, pH 4 5 and incubated with 10 units of RNase T2 (Sigma) at 37° for 19–20 hr The hydrolysate was diluted in starting buffer (20 mM Tris–HCl, pH 7 6, 50 mM NaCl and 7 M urea) and applied to a 0 4 × 55 cm column of DEAE-Sephadex A-25 (Pharmacia Fine Chemicals) The column was developed with 200 ml of a linear gradient of NaCl (0 05 to 0 35 M in above buffer), fractions (1 8 ml each) collected, and counted in Aqueous Counting Scintillant (Amersham-Searle) The charge of the nucleotide peaks was determined by co-chromatography of ^{14}C-labeled (Ap)n markers [7, 8]

Cell free protein synthesis and product analysis The various RNA fractions were translated in a wheat germ *in vitro* system modified from [16] A 50 μl reaction mixture contained 20 mM HEPES–KOH (pH 7 4), 2 5 mM ATP, 0 4 mM GTP, 2.5 mM dithiothreitol, 3 mM $Mg(OAc)_2$, 90 mM KOAc, 10 mM creatine

phosphate, 50 μg/ml creatine phosphokinase, 0 5 mM spermidine (neutralized with KOH to pH 7 4), 0 06 mM unlabeled amino acids minus methionine, 14 μCi of ^{35}S-methionine (1300 Ci/mmol, Amersham), 20 μl of wheat germ S30 treated with micrococcal nuclease, S1 [17] and RNA The incubation was carried out at 24° for 90 min Radioactivity in a 5 μl aliquot was determined after treating with alkali (0 5 ml of 0 5 N NaOH, 10 min at 37°) and precipitation with cold 5% TCA [9] The translation products were separated on a polyacrylamide gel in SDS buffer [18], impregnated with PPO and visualized by fluorography [19]

Acknowledgements—This study was financed by USPHS grants GM20664, CA06927 and RR-00539 from NIH to A Marcus and by an appropriation from the Commonwealth of Pennsylvania I want to thank Abe Marcus for the facilities

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