Phytochemistry, 1978, Vol. 17, pp. 1859–1861 © Pergamon Press Ltd Printed in England 0031-9422/78/1101-1859 $02.00/0

FRACTIONATION OF PLANT POLYADENYLATED RNA ON THE BASIS OF POLY(A) SIZE

G. TRAPY and R. ESNAULT
Physiologie Cellulaire Végétale, Universite Paris VII, 2 Place Jussieu, 75005 Paris, France

(*Received* 4 *April* 1978)

Key Word Index—*Vicia faba*; Leguminosae, broad bean roots; polyadenylated RNA; poly(U) Sepharose chromatography.

Abstract—Polyadenylated RNA from *Vicia faba* meristematic root cells was fractionated on the basis of mean poly(A) size by a thermal stepwise elution from poly(U) Sepharose. Such a procedure allowed the elimination of contaminating RNA at 30° and the collection of two populations of purified polyadenylated RNA at 40° and 50°, respectively. RNA eluting at the higher temperature carried a poly(A) segment (mean size of 100 nucleotides), twice as large as the RNA eluting at the lower temperature.

INTRODUCTION

One of the most fascinating problems in eukaryotic molecular biology is the role of the polyadenylic segment present in many, but not all, animal and plant *m*RNAs. Studies with animal cells have shown that, as the age of *m*RNA increases, the poly(A) segment undergoes a progressive shortening [1, 5–7] which suggests that the poly(A) segment might determine the life of the molecule [1, 6, 8]. In higher plants, this research is very much less advanced.

Systems able to isolate single *m*RNA species [9, 10], and techniques capable of fractionating polyadenylated RNA on the basis of poly(A) length hove now been developed. In this communication, we demonstrate that *Vicia faba* meristematic root cell polyadenylated RNA can be separated into populations differing in their mean poly(A) size using poly(U) Sepharose affinity chromatography.

RESULTS

Conditions of stepwise thermal elution from poly(U) Sepharose

Table 1. Influence of temperature in elution of poly(U)Sepharose bound RNA*

Fractions	Counts/min	RNA eluted as % of RNA recovered
Applied sample	56×10^4	
Filtrate $\frac{+\text{NaCl}}{T: 25°}$	43×10^4	72.7
1st eluate $\frac{-\text{NaCl}}{T: 25°}$	2.1×10^4	3.6
2nd eluate $\frac{-\text{NaCl}}{T: 50°}$	14×10^4	23.7
RNA recovered	59.1×10^4 (105%)	

*Sample was applied to the column in 2 ml saline–SDS buffer at 25°. After washing with 5 ml, elution was performed in 2 ml of the same buffer without NaCl first at 25° and then at 50°.

Previous results [4, 11] showed that total elution of poly(U) Sepharose bound RNA could be achieved by removing salt from the wash buffer and by raising the temperature to 50°. Two experiments were done in order to evaluate the temperature influence in this procedure.

The first experiment (Table 1) showed that removal of NaCl was not sufficient to recover all the bound RNA, which was completely eluted only when the temperature was raised to 50°. In the second experiment removing the NaCl and raising the temperature to 30°, 40° and 50° successively permitted the fractionation of all the fixed RNA (Fig. 1). Repetitive experiments showed that, considering total collected RNA, the percentage of 40° and 50° fractions was remarkably constant, in contrast to the filtrate and 30° eluate which were highly variable.

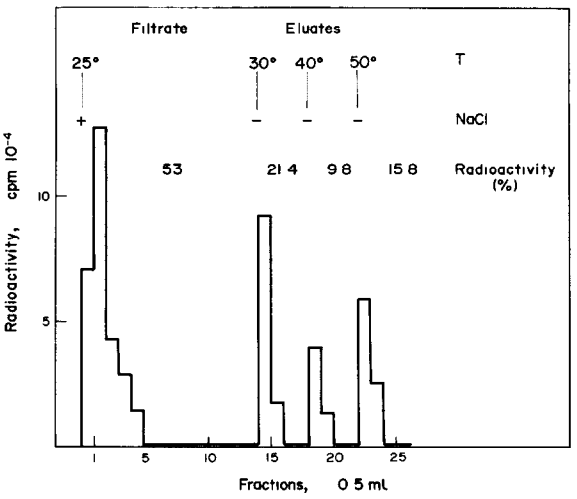

Fig. 1. Stepwise thermal elution of poly(U) Sepharose bound RNA. Application of the sample in 2 ml of saline–SDS buffer at 25° was followed by washing the column with 5 ml of the same buffer. After removal of NaCl from the buffer, elution was accomplished by raising the temperature to 30°, 40° and 50° successively. Fraction volume: 0.5 ml.

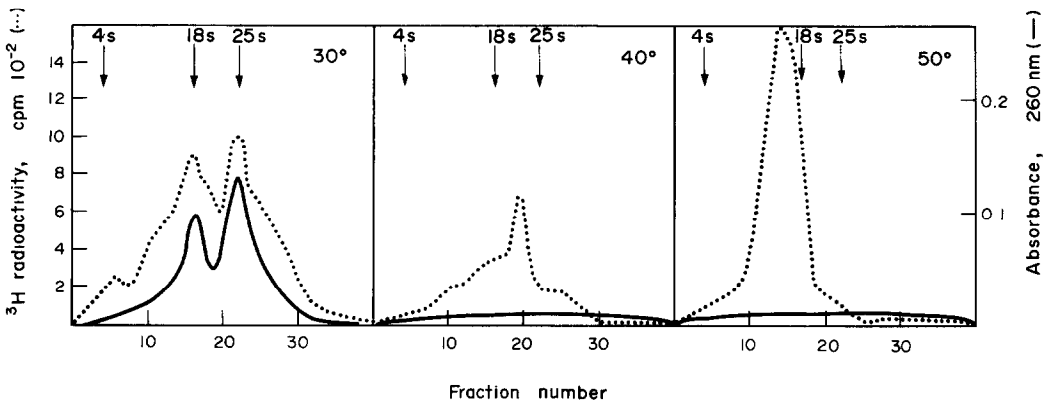

Fig. 2. Sucrose gradient centrifugation profiles of RNA eluted at 30°, 40° and 50° from poly(U) Sepharose. Each eluate was directly layered over 20 ml of a linear sucrose gradient (5–20% w/v) and centrifuged at 105000 *g* for 15 hr. After centrifugation, the solution was pumped from the bottom of the tube and the effluent monitored at 260 nm. Radiactivity was determined on 0.5 ml fractions.

Furthermore, no degradation leading to acid-soluble radioactivity occurred during this chromatographic procedure (data not shown). Further analysis of stepwise eluted fractions with regard to RNA species and poly(A) characteristics was carried out in order to explain this behaviour.

Characteristics of eluted fractions

Fractionation by sucrose gradient centrifugation of adenine-labelled RNA eluted from poly(U) Sepharose by the stepwise thermal procedure showed that the molecular species recovered at 30° were mainly *r*RNA (Fig. 2a). No such species seemed to be present in the 40° (Fig. 2b) and the 50° (Fig. 2c) fractions, which was confirmed by densitometry readings of acrylamide gels, after electrophoresis of 50 µg of unlabelled RNA from each population (unpublished results).

The sedimentation profiles for the radioactivity distribution of the polyadenylated RNA were relatively polydisperse (Figs. 2b and c), but the species eluted at 40° showed an overall size higher than that of RNA collected at 50° (9–30*S* and 9–20*S*, respectively).

Determination of the amount of poly(A) in fractions collected by either the one step or three step procedure was done using a fluorimetric technique as described in the Experimental section.

The results reported in Table 2, show that, among the poly(A)-plus populations, the poly(A) content of the 40° and 50° fractions was strongly enriched with respect to the one step 50° eluate. The elimination of contaminating poly(A)-minus RNA in the 30° eluate is a likely explanation for this result as suggested by the absence of poly(A) in this fraction and the fact that poly(A) content in the total three step eluate (0.6% calculated from the percentage of each contributing fraction in absorbance) was very similar to that measured in the one step 50° eluate. Further experiments were carried out to determine the possible contribution the length of the poly(A) segment made to the different elution positions of the poly(A) plus RNA contained in the 40° and 50° fractions. The mean size of poly(A) was estimated by measuring the radioactivity ratio AMP/adenosine in the alkaline hydrolysate of poly(A) isolated from the 40° and 50° eluate (see Experimental section).

Values reported in Table 3 led to the conclusion that polyadenylated RNAs, fractionated in two parts from poly(U) Sepharose were mainly differentiated by the mean size of their poly(A) segment.

DISCUSSION

Our results indicate the importance the degree of purification of polyadenylated RNA makes in the determination of poly(A) length using the method of alkaline hydrolysis of adenosine-[^{3}H] labelled poly(A). The

Table 2. Content in poly(A) of fraction collected from poly(U) Sepharose

Fractions		Concentration of sample (molarity in nucleotides) RNA*	poly(A)†	% of poly(A) $\frac{C^{tion} \text{ of poly(A)}}{C^{tion} \text{ of RNA}} \times 100$
One step elution	Filtrate	4.2×10^{-5}	0	0
	Eluate 50°	3×10^{-5}	2.3×10^{-7}	0.8
Three step elution	Filtrate	2.1×10^{-5}	0	0
	Eluate 30°	3.7×10^{-6}	0	0
	Eluate 40°	5.7×10^{-6}	30×10^{-8}	5.3
	Eluate 50°	1.77×10^{-5}	1.2×10^{-7}	6.8

Determination of the content in poly(A) is performed by a fluorimetric method (See Experimental for details).

*$\varepsilon_M \times A$ 260 where ε_M is the molar extinction coefficient for a nucleotide included in RNA (8000).

†$\varepsilon_A \times A$ 260 where: ε_A = molar extinction coefficient for A included in RNA (10000); *A* 260 = absorbance corresponding to poly(A) in RNA; it is equal to *A* 260 poly(U) added to reach the plateau of fluorescence.

Table 3. Mean size of poly (A) segment carried by polyadenylated RNA eluted at 40° and 50° from poly (U) sepharose

Fractions eluted	Length of poly(A) in nucleotides 1st Expt	2nd Expt	Mean value
40°	45	63	54
50°	90	109	100

Analysis of nucleotides and adenosine of adenosine-[3H] labelled poly (A) segment is carried out by paper chromatography of the alkaline hydrolysate. Length of poly (A) in nucleotides is given by the ratio of $\frac{\text{AMP + adenosine}}{\text{adenosine}}$ radioactivity.

values of poly(A) size measured from 40° and 50° eluates (50–100 nucleotides) are different from our previous results [4] obtained using a one step elution (150–200 nucleotides). Such a difference can be explained by the presence of AMP in alkaline hydrolysate of 30° eluate (data not shown), probably arising from the internal oligo(A) sequences of contaminating RNAs [12]. Under these conditions, the presence of these latter species in the one step 50° eluate led to the overestimation of the AMP/adenosine ratio and hence the poly(A) segment size.

Analysis of the 40° and 50° eluted RNA leads to the conclusion that the main criteria for distinct elution of polyadenylated RNA is the poly(A) size (Table 3). These results are in good agreement with those obtained by Rhoads [13] concerning the fractionation of ovalbumin *m*RNA using a stepwise thermal elution from oligo(dT) cellulose. However, under our condition, it must be noted that the resolving power of the column, with respect to polyadenylated RNA separation and increased temperature, is somewhat less. This difference is probably due to the greater chain length of poly(U) which permits a more extensive interaction with the relatively long poly(A) segments. The major role that poly(A) size plays in the stepwise thermal elution of *m*RNA from poly(U) Sepharose does not exclude the possibility of other factors. For example, the difference between the mean overall sizes of polyadenylated RNA fractionated at 40° and 50° (Figs. 2b and c) might be a modulating factor in the hybridization between the poly(A) segment of RNA and the poly(U) chain attached to the matrix. We are presently investigating such a possibility and also the eventual influence of the degree of secondary structure of the non poly(A) part.

The retention of purified polyadenylated RNA, mainly differentiated by the mean length of its poly(A) segment, may afford a basis for investigations into the role poly(A) size plays in the processing of plant *m*RNA.

EXPERIMENTAL

Material and preparation of RNA. Preparation of labelled RNA was carried out from excised apices (5 mm) of broad bean roots (*Vicia faba* var. Aguadulce) incubated for 2 hr in 20 μCi adenosine-[^{3}H]/ml (5 apices/ml). Homegenization of apices, extraction of RNA by a Tris–HCl pH 9 cold buffer without salts and measurement of radioactivity were as previously described [4].

Chromatography on poly(U) Sepharose. The filtration was carried out at 25° in a saline buffer (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris–HCl pH 7.4 and 0.2% SDS) and elution was accomplished in the same buffer without NaCl and with the temp. being raised to 50° either directly (1 step procedure) or by steps (see Fig. 1 for details).

Sucrose density gradient. Centrifugation at 105000 *g* was carried out in a swinging bucket rotor of a MSE S50 centrifuge. The *A* and radioactive profiles were determined by pumping the eluted fractions through a UV analyser and a liquid scintillation spectrometer, respectively. For other details, see the legends of Fig. 2 and [4].

Determination of poly(A) content. The titration of polyadenylated RNA with poly(U) in the presence of ethidium bromide for the detection and estimation of poly(A) was conducted according to the method described in refs. [2, 14]. The RNA was prepared in 2×10^{-6} M ethidium bromide, 0.05 M NaCl, 0.05 M cacodylate buffer pH 7. The titration of each sample was performed by stepwise addition of 2 ml aliquots of $0{\cdot}5 \times 10^{-4}$ M poly(U) soln and measurement of the fluorescence signal (exc. 540 nm; em. 600 nm) read 2 min after each addition. The ref. titration of a 0.5×10^{-4} M synthetic poly(A) soln was conducted under the same conditions. The cuvette containing 500 μl of soln was inserted in a cuvette holder thermostated at 25°. The percentage of poly(A) in RNA is expressed according to the legend of Table 2.

Size evaluation of the poly(A) segment. Isolation of the poly(A) segment was performed by treatment of polyadenylated RNA with a mixture of DNase, RNases A and T_1 enzymes. Analysis of the nucleotide and adenosine content of the isolated poly(A) was carried out by PC of the alkaline hydrolysate. This procedure has been described in ref. [4].

Acknowledgements—The authors are indebted to Dr. A. Favre for helpful discussions and use of the fluorometer and to Catherine Thenevin for her expert technical assistance.

REFERENCES

1. Lim, L. and Canellakis, E. S. (1970) *Nature* **227**, 710.
2. Adesnik, M. and Darnell, J. E. (1972) *J. Mol. Biol.* **67**, 397.
3. Houdebine, L. M., Gaye, P. and Favre, A. (1974) *Nucl. Acid Res.* **1**, 413.
4. Esnault, R., Trapy, G. and Van Huystee, R. B. (1975) *Eur. J. Biochem.* **60**, 83.
5. Mendecki, J., Lee, S. Y. and Brawerman, G. (1972) *Biochemistry* **11**, 792.
6. Sheiness, D. and Darnell, J. E. (1973) *Nature New Biol.* **241**, 265.
7. Gorski, T., Morrison, M. R., Merkel, C. G. and Lingrel, J. B. (1974) *J. Mol. Biol.* **86**, 363.
8. Sussman, M. (1970) *Nature* **225**, 1245.
9. Verma, D. P. S., Nash, D. T. and Schukman, A. M. (1974) *Nature* **251**, 74.
10. Verma, D. P. S., Maclachlan, G. A., Byrne, H. and Ewings, D. (1975) *J. Biol. Chem.* **250**, 1019.
11. Trapy, G., Van Huystee, R. B. and Esnault, R. (1975) *Biochimie* **57**, 123.
12. Spohr, G., Dettori, G. and Manzari, V. (1976) *Cell* **8**, 505.
13. Rhoads, R. E. (1975) *J. Biol. Chem.* **250**, 8088.
14. Favre, A., Bertazzoni, U., Berns, A. J. M. and Bloemendal, H. (1974) *Biochem. Biophys. Res. Commun.* **56**, 273.