The Effect of Age on the Properties of Poly(A)-containing Messenger RNA in *Physarum polycephalum*

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The effect of ageing on the properties of polysomal poly(A)-containing messenger RNA [poly(A)+ mRNA] of *Physarum polycephalum* has been investigated. Using poly(U)-Sephadex affinity chromatography it was shown that shortening of the poly(A) tract occurred as the age of the mRNA population increased. Analysis of the poly(A) segments by use of polyacrylamide gel electrophoresis, after digestion of polysomal poly(A)+ mRNA molecules with RNAase A and RNAase T1, revealed that their lengths ranged from 140 to 220 nucleotide residues. A reduction in the efficiency of utilization of mRNA for translation as the age of the mRNA population increased was demonstrated by measuring the proportion of poly(A)+ mRNA present in the polysomal fraction as compared with post-polysomal material.

INTRODUCTION

Physarum polycephalum is an attractive organism for studies of the control of gene expression for a number of reasons. For example, synchronous nuclear division occurs during growth in *Physarum* plasmodia, affording an opportunity to study expression during the cell cycle without the requirement for artificial methods of cell synchronization. Also, several stages of differentiation exist during the life-cycle of *Physarum*, a property that has led to extensive use of this organism as a model system for studies of differential gene expression (Dee, 1975). Despite these advantages, limited progress has been made so far with investigation of the properties of mRNA in *Physarum*. This is, in part, due to technical problems related to the high endogenous levels of ribonuclease present in the organism. The conflicting reports concerning the length of the 3' poly(A) tract in *Physarum* mRNA may be another manifestation of these problems; the poly(A) segments derived from poly(A)-containing mRNA [poly(A)+ mRNA] were reported to be in the range 100 to 250 nucleotides by Fouquet *et al.* (1974), whereas Adams & Jeffery (1978) estimate the average length to be 65 nucleotides. In the present study an attempt was made to determine the length of poly(A) tracts in *Physarum* mRNA under conditions which minimize artefactual degradation by ribonuclease, and with reference to appropriate RNA size standards.

A comparison of the relative lengths of poly(A) segments in different RNA populations was achieved by thermal elution of poly(A)+ mRNA from poly(U)-Sephadex. This provides an alternative to estimating the size of the poly(A) tracts by polyacrylamide gel electrophoresis and avoids the need to expose RNA to ribonucleases in order to obtain the ribonuclease-resistant poly(A) core. The effect of ageing of *Physarum* poly(A)+ mRNA on its translation was also examined.

METHODS

Materials. The Colonia Leicester (CL) strain of *Physarum polycephalum* was used in this study. Poly(U)-Sephadex was from Pharmacia, [2-³H]adenosine (24 Ci mmol⁻¹, 890 GBq mmol⁻¹) and L-[4,5-³H]-

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leucine (52 Ci mmol^{-1} , $1.92 \text{ TBq mmol}^{-1}$) from The Radiochemical Centre, Amersham, pancreatic ribonuclease A [$3.23 \times 10^3 \text{ units (mg protein)}^{-1}$] and ribonuclease T1 [$4.24 \times 10^5 \text{ units (mg protein)}^{-1}$] from Worthington Biochemical Corp., *Escherichia coli* 5S RNA and yeast tRNA^{Phe} from Boehringer, creatine phosphokinase ($110 \text{ units mg}^{-1}$) and polyvinyl sulphate from Sigma, and fungal proteinase K from BDH. Freshly-ground wheat germ was a generous gift from W. P. H. Marriage and Sons, Chelmsford, Essex.

Poly(A)+ mRNA. Polysomal and post-polysomal fractions were isolated from exponentially growing *Physarum* microplasmodia by procedures adapted from those of Brewer (1972), as described previously (Brown & Hardman, 1980). Growth medium for microplasmodia was that described previously (Dee & Poulter, 1970). Proteinase K digestion of the fractions, phenol/chloroform/isoamyl alcohol extraction, followed by poly(U)-Sephadex affinity chromatography were performed as described previously (Brown & Hardman, 1980).

Poly(A) tracts. Polysomal poly(A)+ mRNA ($50 \mu\text{g}$) was digested at 37°C in 1.2 ml of solution containing pancreatic RNAase A ($2.5 \text{ units ml}^{-1}$), RNAase T1 (25 units ml^{-1}), 10 mM-EDTA, 0.2 M-NaCl, 10 mM-Tris/HCl, pH 7.5. Portions of the reaction mixture ($10 \mu\text{l}$) were withdrawn periodically to determine the amount of radioactivity rendered soluble in 5% (w/v) trichloroacetic acid. After 30 min, 1.0 ml of the reaction mixture was applied directly to a 0.3 g oligo(dT)-cellulose column, previously washed with 5 ml 0.1% (v/v) diethyl pyrocarbonate, and equilibrated prior to use by washing with 5 ml 0.5 M-NaCl, 10 mM-Tris/HCl, pH 7.5 (buffer A). Unbound material was washed from the column using $5 \times 1.0 \text{ ml}$ portions of buffer A, and the bound fraction was eluted using $5 \times 1 \text{ ml}$ washes of 10 mM-Tris/HCl, pH 7.5 (buffer B). Fractions of the eluate ($10 \mu\text{l}$) were used to determine the amount of radioactivity precipitated in the presence of 5% (w/v) trichloroacetic acid. Those fractions containing [^3H]RNA bound to the column in the presence of buffer A were pooled and precipitated using 2 vol. 95% (v/v) ethanol in the presence of $100 \mu\text{g}$ calf thymus DNA carrier.

Gel electrophoresis. Poly(A)-containing RNAase-resistant material obtained from polysomal poly(A)+ mRNA was dissolved in 30 mM-sodium phosphate, 1 mM-EDTA, 36 mM-Tris/HCl, pH 7.5, and electrophoresed for 9 h at 80 V (60 mA) on a 10% (w/v) polyacrylamide slab gel (Laemmli & Favre, 1973; Loening, 1969). Oligo(dT)₁₀, *E. coli* 5S RNA and yeast tRNA^{Phe} were used as size standards. RNA samples were denatured before use by heating to 60°C for 2 min immediately before application to the gel. After electrophoresis the position of markers was determined by staining with acridine orange (McMaster & Carmichael, 1977). [^3H]RNA was located by measuring the radioactive content of gel slices (Adamson & Woodland, 1977). Gel slices (1.5 mm) were solubilized using 0.5 ml 2% (v/v) 0.880 sp.gr. ammonia in 30% (v/v) H_2O_2 for 48 h at 37°C .

Thermal elution from poly(U)-Sephadex. The methods used were adapted from those described by Palatnik *et al.* (1979). RNA samples were dissolved in 1.0 ml 1% (w/v) sodium dodecyl sulphate, 30 mM-EDTA, 50 mM-Tris/HCl, pH 8.0, heated to 60°C for 3 min, cooled in solid CO_2 /ethanol and re-equilibrated to 20°C . Samples were then diluted fivefold with 20% (v/v) formamide, 0.7 M-NaCl, 10 mM-EDTA, $100 \mu\text{g}$ polyvinyl sulphate (PVS) ml^{-1} , 30 mM-Tris/HCl, pH 7.5 (buffer C) and loaded on to 2 ml poly(U)-Sephadex columns, previously equilibrated at 20°C with buffer C. Poly(A)- RNA was eluted from the columns at 20°C with $10 \times 1.0 \text{ ml}$ fractions of buffer C and $10 \times 1.0 \text{ ml}$ fractions of 20% (v/v) formamide, 0.1 M-NaCl, 10 mM-EDTA, $100 \mu\text{g}$ PVS ml^{-1} , 50 mM-Tris/HCl, pH 7.5 (buffer D). The temperature of the columns was raised from 20 to 84°C in 2°C increments. At each temperature $3 \times 1.0 \text{ ml}$ volumes of buffer D were passed through the columns and the ^3H radioactivity was monitored.

Protein synthesis *in vitro*. The method used was that described by Marcu & Dudock (1974) as modified by M. Cannon (personal communication). Viable embryos (20 g) were selected from the crude wheat germ by flotation on CCl_4 /cyclohexane (2.5:1, v/v). These were ground whilst dry for 60 s at 4°C with an equal weight of washed sand. After mixing with 40 ml 100 mM-potassium acetate, 1 mM-magnesium acetate, 2 mM- CaCl_2 , 1 mM-dithiothreitol, 20 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), pH 7.4, the slurry was centrifuged at $30000 g$ for 15 min at 4°C . The supernatant was applied to a Sephadex G-25 column ($15 \times 1.6 \text{ cm}$) previously equilibrated with 120 mM-potassium acetate, 5 mM-magnesium acetate, 1 mM-dithiothreitol, 20 mM-HEPES, pH 7.4 (buffer E). The samples were eluted using buffer E. The first 8 ml eluate after the void volume of the column was collected and centrifuged at $30000 g$ for 10 min. The supernatant was stored at -70°C under nitrogen.

The rate of *in vitro* protein synthesis was determined in $50 \mu\text{l}$ reaction mixtures containing 20 mM-HEPES, pH 7.4, 2 mM-magnesium acetate, 130 mM-potassium acetate, 2 mM-dithiothreitol, 1 mM-ATP, 0.25 mM-GTP, 8 mM of each of the 20 L-amino acids other than leucine, $2.5 \mu\text{Ci L-}[^3\text{H}]\text{leucine}$, $1 \mu\text{g}$ RNA template and $20 \mu\text{l}$ wheat germ extract.

RESULTS AND DISCUSSION

Properties of poly(A) segments derived from poly(A)+ RNA

An exponentially growing culture of microplasmodia was labelled for 2.5 h in the presence of $5 \mu\text{Ci } [^3\text{H}]\text{adenosine ml}^{-1}$. The organism was harvested and polysomal poly(A)+ mRNA

Fig. 1. Isolation of poly(A) segments from *Physarum* poly(A)+ RNA. (a) Time course of digestion of polysomal poly(A)+ mRNA (50 μ g) using 2.5 units RNAase A ml⁻¹ and 25 units RNAase T1 ml⁻¹. The vertical bar indicates the point at which samples were taken for the analysis of poly(A) segment length. (b) Oligo(dT)-cellulose chromatography of RNAase-resistant material derived from polysomal poly(A)+ mRNA after 30 min digestion with RNAase A and RNAase T1, as shown in (a). O, Acid-soluble ³H radioactivity; $\blacktriangle$, acid-precipitable radioactivity.

was isolated. Samples (50 μ g) of the RNA were subjected to digestion using RNAase A and RNAase T1. Preliminary experiments were performed to determine the optimum conditions for digestion (results not shown) since it was known that RNAase A degrades poly(A) tracts slowly (Beers, 1960). The conditions finally employed were such that the minimum amounts of ribonuclease were added to digest the RNA completely in 30 min (Fig. 1a), thus minimizing the 'nibbling' effect of the enzymes on the 20% of the RNA which was resistant to the action of the enzymes, and presumed to represent the poly(A) core. Analysis of the digestion products using oligo(dT)-cellulose affinity chromatography revealed that 85% of the ³H radioactivity that was acid-precipitable remained bound to the column, whereas 99% of the soluble radioactivity was eluted in the presence of 0.5 M-NaCl (Fig. 1b), thus confirming the nature of the RNAase-resistant material. The size distribution of the poly(A)-containing core material was determined by polyacrylamide gel electrophoresis with reference to *E. coli* 5S RNA (120 nucleotides; Brownlee *et al.*, 1967), yeast tRNA^{Phe} (76 nucleotides; Rich & Bhandary, 1976) and oligo(dT)₁₀. Difficulties that were encountered when 10% polyacrylamide gels were sliced leave the possibility that the minor peaks and troughs of radioactivity on the gels (Fig. 2) were artefactual. However, it was clear that the distribution of lengths for the poly(A) segments in poly(A)+ mRNA was in the range 140 to 220 nucleotides.

These results are consistent with those of Fouquet *et al.* (1974), but disagree with the average length of 65 nucleotides reported by Adams & Jeffery (1978). The reason for these discrepancies is not known, though it is likely to reside in differences in the conditions of digestion with ribonucleases. The average length of the poly(A) tract in *Physarum* poly(A)+ RNA found here is similar to those reported for mammalian-cell poly(A)+ RNA (Edmonds & Caramela, 1969; Edmonds *et al.*, 1971; Greenberg & Perry, 1972; Mendecki *et al.*, 1972), that is, approximately 200 nucleotides. In contrast, lengths of poly(A) segments ranging between 50 and 100 nucleotide residues have been reported in a variety of lower eukaryotic organisms, including *Dictyostelium discoideum* (Firtel *et al.*, 1972), *Saccharomyces* (Groner *et al.*, 1974), *Blastocladiella emersonii* (Jaworski, 1976) and *Naegleria gruberi* (Hickey *et al.*, 1979).

Fig. 2

Fig. 3

Fig. 2. Polyacrylamide gel electrophoresis of poly(A) tracts derived from *Physarum* polysomal poly(A)+ mRNA. Poly(A) segments were isolated and electrophoresed on a 10% polyacrylamide slab gel (see Methods). The radioactive content of the gel slices is shown with reference to co-electrophoresed *E. coli* 5S RNA, yeast tRNA^{Phe} (4S) and oligo(dT)₁₀ as size standards. The position of migration of standard RNA species was determined by staining the gel.

Fig. 3. Time course of incorporation of [³H]adenosine into *Physarum* poly(A)+ RNA. The incorporation of [³H]adenosine (20 μ Ci ml⁻¹) into poly(A)+ RNA during exponential growth of microplasmodia was followed. Experimental details are given in the text.

Length of the poly(A) tract and mRNA age

Adams & Jeffery (1978) reported that the length of the poly(A) tract of *Physarum* mRNA decreases in parallel with the age of the message. Since the results obtained here, concerning the average length of poly(A) segments, were in conflict with this previous study, experiments were performed to investigate the shortening of poly(A) segments in more detail.

Comparison of mRNA populations that differ with respect to age is dependent on the kinetics of incorporation of radioactive substrate into mRNA. [³H]Adenosine was added to an exponentially growing culture of microplasmodia to a final concentration of 20 μ Ci ml⁻¹ and portions of the culture were analysed for acid-insoluble radioactivity. Microplasmodia were harvested and resuspended in 2% (w/v) sodium citrate, 0.5% (w/v) Triton X-100 (Ouellette *et al.*, 1976) and homogenized at 4 °C using a Dounce ball-type homogenizer. Post-mitochondrial supernatants were obtained by centrifugation of homogenates at 10000 *g* for 10 min at 4 °C. The supernatants were then made 75 mM with respect to MgCl₂, incubated at 4 °C for 45 min, and precipitated ribonucleoprotein (Iwabuchi *et al.*, 1970; Akalehiyot *et al.*, 1977) was harvested by centrifugation at 10000 *g* for 10 min at 4 °C. Control experiments showed that equal proportions of polysomal and monosomal ribonucleoproteins were precipitated using these procedures (results not shown). The incorporation of [³H]adenosine into poly(A)+ RNA was measured by determining the radioactivity present in the poly(A)+ RNA fraction after purification using poly(U)-Sepharose chromatography. The results (Fig. 3) indicated that [³H]adenosine was depleted after about 5 h, after which the level of incorporated label decreased, probably due to turnover of the poly(A)+ mRNA. Hence, the use of 2.5 and 24 h labelling periods allowed analysis to be made of newly synthesized and relatively long-lived poly(A)+ mRNA, respectively.

The strength of the interaction between poly(U)-Sepharose and the poly(A) tract of mRNA is dependent on the length of the poly(A) segment (Palatnik *et al.*, 1979). Hence, thermal elution chromatography using poly(U)-Sepharose can be used to reveal differences

Fig. 4. Thermal elution chromatography of *Physarum* polysomal RNA using poly(U)-Sephacrose. Polysomal RNA was isolated from exponentially growing microplasmodia after labelling for 2.5 h (—) or 24 h (---) with $20 \mu\text{Ci } [^3\text{H}]\text{adenosine ml}^{-1}$. RNA was subjected to thermal elution chromatography from poly(U)-Sephacrose: (a, b) elution profiles using buffer C and buffer D at 20 °C; (c) elution profiles of RNA using buffer D at increasing temperatures.

in the size of poly(A) tracts in different mRNA preparations. This technique was used here since it permits comparisons to be made between poly(A) populations without recourse to the use of ribonuclease digestion, thus excluding the disadvantages in the application of the ribonuclease method.

Two polysomal RNA populations, isolated from exponentially growing cultures of microplasmodia labelled for 2.5 and 24 h, respectively, with $20 \mu\text{Ci } [^3\text{H}]\text{adenosine ml}^{-1}$, were subjected to thermal elution from poly(U)-Sephacrose. The profiles of elution of the labelled RNA with increasing temperature (Fig. 4) showed that the major proportion of the long-labelled poly(A)+ mRNA was released from the poly(U)-Sephacrose column at a lower temperature than that labelled for 2.5 h, indicating that the lengths of the poly(A) segments were shorter in most cases in longer-labelled mRNA. This confirms the observations of Adams & Jeffery (1978) and is also consistent with the shortening of the poly(A) segment with mRNA age that has been observed previously in animal-cell poly(A)+ mRNA (Sheiness *et al.*, 1975; Wilson *et al.*, 1978).

Effect of mRNA ageing on translation

The efficiency of utilization of *Physarum* mRNA for translation was estimated by determining the proportion of the RNA that was present in actively translating polysome structures, as compared with monosomes (Harvey, 1973). The validity of this approach was

Fig. 5. Utilization of *Physarum* poly(A)+ RNA in a wheat germ protein-synthesizing system. Polysomal and post-polysomal poly(A)+ RNA were isolated from exponentially growing cultures of microplasmodia. Samples of each RNA preparation (1 μ g) were added to an *in vitro* protein-synthesizing system prepared using wheat germ extract, and the rate of incorporation of L-[³H]leucine into acid-insoluble material was monitored. ●, Polysomal poly(A)+ RNA template; ■, post-polysomal poly(A)+ RNA template.

Table 1. *Effect of the age of poly(A)+ RNA on its utilization for translation*

Identical exponentially growing microplasmodial cultures were labelled for 2, 12 and 24 h, respectively, using 10 μ Ci [³H]adenosine ml⁻¹. Poly(A)+ RNA was prepared, and the efficiency of utilization of the mRNA for translation was estimated by determining the ratio of [³H]poly(A)+ RNA in polysomes to that in post-polysomal material.

Time of labelling (h)	Polysomal radioactivity
	Post-polysomal radioactivity
2	7.6
12	5.5
24	2.9

assessed by comparing the relative template ability of *Physarum* polysomal and post-polysomal poly(A)+ mRNA using an *in vitro* wheat germ protein-synthesizing system. Polysomal or post-polysomal poly(A)+ RNA (1 μ g) was added to each assay mixture and portions (6 μ l) were taken over a period of 60 min to determine the extent of incorporation of L-[³H]leucine into acid-precipitable material. The results (Fig. 5) showed that polysomal poly(A)+ RNA is a more efficient template than monosomal poly(A)+ RNA under the conditions used and hence probably has a greater integrity and inherent functional capacity *in vivo*. It is conceivable, though unlikely, that selective degradation of post-polysomal poly(A)+ mRNA during isolation might also account for these observations. However, similar functional differences between polysomal and post-polysomal mRNA populations have been observed in other systems (Dworkin *et al.*, 1977; Lee & Engelhardt, 1979).

Three exponentially growing cultures of microplasmodia of identical culture density were labelled with 10 μ Ci [³H]adenosine ml⁻¹ for 2, 12 and 24 h prior to harvesting. Poly(A)+ mRNA was isolated from polysomal and post-polysomal fractions and the ratio of ³H radioactivity in polysomes to that in post-polysomal material was determined for each labelling period (Table 1). A reduction in this ratio was observed as the length of the labelling period increased. Electrophoretic analysis of the poly(A)+ RNA from polysomes and monosomes showed that the observation was not due to changes in the level of contamination

of the poly(A)+ fractions with ribosomal RNA. Hence, the results suggest that the efficiency of utilization of mRNA for translation decreased as the age of the messenger RNA increased.

Such an observation is consistent with a previous report which described a reduction in the capacity of reticulocyte mRNA to support protein synthesis as the mRNA ages (Lodish & Small, 1976). In contrast, the translational capacity of poly(A)+ mRNA for α - and β -globin, when injected into *Xenopus laevis* oocytes, does not decrease with time (Gurdon *et al.*, 1973; Huez *et al.*, 1974). The high stability of mRNA in the *Xenopus laevis* system may be responsible for some of the inconsistencies in the relationship between the age of an mRNA and its capacity for translation.

Conclusions

Three conclusions can be drawn from the data presented here. First, the length of the poly(A) tracts in *Physarum* poly(A)+ RNA is shown to range from 140 to 220 nucleotide residues, using experimental conditions which minimize the artefactual degradation of the poly(A) segment by ribonucleases. The conditions used here differ from those used previously (Fouquet *et al.*, 1974; Adams & Jeffery, 1978) in that we have employed milder treatments with RNAase and have determined the length of the poly(A) segments with reference to appropriate RNA standards of known chain size. Second, thermal elution chromatography from poly(U)-Sephadex has shown that the poly(A) segments become shorter as the population of mRNA molecules ages. Third, it has been possible to determine the efficiency with which mRNA populations in *Physarum* are utilized for translation by comparing the proportion of poly(A)+ RNA present in polysomes with that in monosomes. As the age of the poly(A)+ mRNA increases, its ability to be utilized for translation appears to decrease. This is probably related to the turnover of poly(A)+ RNA or some other *in vivo* phenomenon.

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