Characterization and Messenger Activity of Poly(A)-containing RNA from *Chlorella*

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SUMMARY

Poly(A)-containing RNA was isolated by cellulose column chromatography from total RNA extracted from *Chlorella fusca* var. *vacuolata* 211/8p. RNA retained by the column was identified as poly(A)-containing RNA because it contained ribonuclease-resistant tracts, 25 to 55 nucleotides in length, from which not less than 80 % of base was found to be adenine after acid hydrolysis. The base composition of poly(A)-containing RNA differed from that of RNA (largely ribosomal) which did not adsorb to cellulose, having a higher adenine content and a lower guanine content. Poly(A)-containing RNA was polydisperse including molecules with mobilities from 10S to 40S with a mean of about 20S. In an *in vitro* system derived from wheat-germ, protein synthesis was stimulated by adding poly(A)-containing RNA from *Chlorella*. Optimum conditions were established in this system with respect to the amount of poly(A)-containing RNA added and the concentration of KCl and Mg^{2+} . It is proposed that, in *Chlorella*, poly(A)-containing RNA includes cytoplasmic mRNA as has been shown for some other eucaryotic organisms.

INTRODUCTION

RNA molecules containing polynucleotide sequences rich in adenylate residues [RNA containing poly(A)-tracts] have been isolated from a wide range of eucaryotic cells (Brawerman, 1974). In both mammalian and insect cells, poly(A)-containing RNA has been found in the nucleus, cytoplasm and mitochondria (Darnell, Jelinek & Molloy, 1973; Perlman, Abelson & Penman, 1973; Hirsch, Spradling & Penman, 1974). Isolation of poly(A)-containing RNA has been reported from a wide range of organisms such as chickens (Schutz, Beato & Feigelson, 1972), fungi (Reed & Wintersberger, 1973; Silver & Horgen, 1974), slime moulds (Lodish, Firtel & Jacobson, 1973; Fouquet *et al.* 1974), higher plants (Higgins, Mercer & Goodwin, 1973; Van de Walle, 1973) and *Euglena* (Sagher, Edelman & Jakob, 1974).

Present evidence suggests that mRNA in the cytoplasm of eucaryotic cells is largely poly(A)-containing RNA (Darnell *et al.* 1973; Lodish *et al.* 1973) although histone mRNA is probably an exception (Adesnik & Darnell, 1972). In contrast, whilst mitochondrial mRNA is poly(A)-containing in mammalian and insect cells (see above), poly(A)-tracts are not found in mRNA isolated from yeast mitochondria (Groot *et al.* 1974; Eggitt & Scragg, 1975) or in chloroplast mRNA (Ellis, 1975). We report here the isolation of poly(A)-

containing RNA from the unicellular green alga, *Chlorella*, together with some properties of this RNA which suggest that it has messenger activity.

METHODS

Organism and cultural conditions. *Chlorella fusca* var. *vacuolata*, Cambridge culture collection, strain 211/8p (previously called *C. pyrenoidosa*) was grown autotrophically as described by John, Thurston & Syrett (1970). Growth was followed by measurement of cell number density with a Coulter Counter Z_{B1} (Coulter Electronics Ltd, Dunstable, Bedfordshire).

Extraction of total RNA. Samples of culture were centrifuged at 4 °C and all subsequent operations were performed at 0 to 4 °C. Organisms were washed and resuspended in 0.1 M-potassium phosphate pH 7.0 and disrupted by passage twice through a French pressure cell. The broken-organism suspension was immediately mixed with an approximately equal volume of ice-cold, redistilled, water-saturated phenol. The mixture was held in crushed ice with occasional shaking for 20 min, after which aqueous and phenol phases were separated by centrifugation. The aqueous phase was extracted twice more with phenol, after which RNA was precipitated by the addition of 2 vol. ethanol. The phenol phase from the first extraction was mixed with a fresh sample of 0.1 M-potassium phosphate pH 7.0 and the mixture was again allowed to stand and separated by centrifugation. This (second) aqueous phase was extracted twice more with phenol and RNA was precipitated with ethanol. The two RNA precipitates were recovered by centrifugation, dissolved in distilled water, combined and immediately reprecipitated with ethanol. Such total RNA precipitates were stored at -20 °C. Typically, 15 mg RNA were obtained from 10¹⁰ cells.

RNA fractionation on cellulose columns. The method of Schutz *et al.* (1972) was used. A column with a volume of 10 ml was filled with Whatman CC 41 cellulose powder suspended in TKM buffer (0.5 M-KCl, 0.002 M-MgCl₂, 0.01 M-tris-HCl, pH 7.5). Total RNA was dissolved in the same buffer for application to the column. The concentration of RNA was adjusted so that sample E_{260} was less than 20.0. Unadsorbed RNA was washed through the column with TKM buffer until the E_{260} of the eluate was less than 0.04. Adsorbed RNA (poly(A)-containing RNA) was eluted with distilled water and precipitated at -20 °C after addition of 2 vol. ethanol. Where RNA was to be used in cell-free protein-synthesis experiments, the ethanol-precipitated RNA was dissolved in distilled water and freeze-dried.

Analysis of base composition of RNA. The method of Monjardino (1972) was used. RNA (120 to 200 µg) was dissolved in 0.1 ml distilled water and hydrolysed with an equal volume of 2 M-HCl for 1 h at 100 °C in sealed glass tubes. Hydrolysates (20 to 50 µl) were chromatographed on cellulose thin layers in t-butanol-2.67 M-HCl, 7:3 (v/v). Adenine, guanine, cytidine 2'(3')-monophosphate and uridine 2'(3')-monophosphate standards were run on the same plates. Spots were detected by examination of the dried plates under ultraviolet (253 nm) light. For each spot the cellulose was scraped from the backing material of the plate and extracted with 1.5 ml of 0.01 M-HCl for 2 h, with occasional shaking. Amounts of base and nucleotide extracted were determined spectrophotometrically (Burton, 1969). Recovery of base and nucleotide standards was 91 to 105 %.

Analysis of poly(A)-tracts. Poly(A)-tracts were isolated from poly(A)-containing RNA by digestion with T₁ and pancreatic ribonucleases as described by Perlman *et al.* (1973). For analysis of electrophoretic mobility ribonuclease digests were applied directly to 10 % (w/v) acrylamide gels cross-linked with ethylene diacrylate, prepared as described by Weinberg *et al.* (1967). [³H]tRNA from *Escherichia coli* and [³H]ATP markers were run on a separate

gel stick. Gels were divided into 1 mm slices which were digested for 2 h at room temperature with 0.2 ml ammonium hydroxide sp.gr. 0.88. Radioactivity from each digested gel slice was determined in a liquid scintillation spectrometer after the addition of 10 ml of dioxane-based scintillation fluid.

Density-gradient centrifugation. Sucrose gradients (5 to 20 %, w/v) containing formamide were prepared as described by Anderson *et al.* (1974). RNA samples were layered on to 5 ml gradients and centrifuged at 84000 g for 15 h at 4 °C. Fractions (100 µl) were collected as 8 drops from the bottom of each tube. Bovine plasma albumin (50 µg) and 50 µl of 20 % (w/v) trichloroacetic acid (TCA) were added to each fraction. Precipitates which had formed after 1 h at 4 °C were collected on glass-fibre filters, washed twice with 5 ml of 5 % (w/v) TCA and once with 5 ml of ethanol. When dry, the discs were counted in a toluene-based scintillation fluid. Fractions from a separate tube to which unlabelled marker RNA had been applied were diluted with 0.4 ml of 0.5 M-NaCl-0.05 M-sodium acetate, pH 5.5, for measurement of E_{260} .

Cell-free protein synthesis assay. A wheat-germ cell-free system was prepared and used as described by Roberts & Paterson (1973). The S-30 extract had a protein concentration of 17.0 mg/ml and was stored in 1.0 ml amounts at -70 °C. Except for the experiment described in Fig. 4, which was scaled up five times, the cell-free assay system had a final volume of 50 µl and contained the following: ATP, 0.07 µmol; GTP, 0.02 µmol; creatine phosphate, 0.42 µmol; creatine phosphokinase, 4.0 µg; dithiothreitol, 0.04 µmol; *N*-2-hydroxymethyl-piperazine-*N'*-2-ethane sulphonic acid-KOH pH 7.6, 0.9 µmol; cysteine, 0.055 µmol; 19 L-amino acids, each 0.015 µmol; 21 µCi [³⁵S]-methionine (185 Ci/µmol); S-30 extract, 20 µl. Magnesium acetate (5 mM) and 74 mM-KCl were included except in the experiments described in Fig. 6. Amounts of *Chlorella* RNA added are specified in the legends to the Figures. After incubation at 30 °C, for the times given in the legends to the Figures, hot trichloroacetic acid-insoluble radioactivity was determined as described by Bollum (1959).

Materials. Whatman CC 41 cellulose powder and Whatman GF/C glass-fibre filters were from W. & R. Balston Ltd, Maidstone, Kent. Deoxyribonuclease 1 (ribonuclease-free) and T₁ ribonuclease were from Worthington Biochemical Co., Freehold, New Jersey, U.S.A. Ethylene diacrylate was from Monomer-polymer Laboratories, Philadelphia, Pennsylvania 19124, U.S.A. Radiochemicals were from the Radiochemical Centre, Amersham, Buckinghamshire. Other chemicals used were analytical grade.

RESULTS

RNA fractionation on a cellulose column

When total *Chlorella* RNA was applied to a cellulose column, the bulk of the RNA (hereafter called unadsorbed RNA) washed through, but a small proportion was adsorbed (Fig. 1). The adsorbed fraction was eluted with distilled water. Its base composition showed a higher proportion of adenine than unadsorbed RNA (Table 1) and its identification as poly(A)-containing RNA was confirmed as described below. From 30 measurements, poly(A)-containing RNA was 6.9 ± 2.6 % of total RNA. The DNA content of fractions was estimated by measurement of the E_{260} of the cold TCA-soluble material, after 170 µg of RNA had been incubated for 20 min at 37 °C with 100 units of ribonuclease-free deoxyribonuclease 1. DNA was less than 5 % of both unadsorbed and poly(A)-containing RNA fractions.

Fig. 1

Fig. 2

Fig. 1. Chromatography on a cellulose column of total RNA from *Chlorella*. RNA (11.7 mg) was applied in 20 ml of TKM buffer (see Methods) and washed through with the same buffer. Elution of adsorbed RNA with distilled water is indicated by an arrow. Fraction volume was 5 ml.

Fig. 2. Estimation of the size of poly(A)-tracts by polyacrylamide gel electrophoresis. Poly(A)-containing RNA was isolated from a *Chlorella* culture which had been incubated for 3 h in the presence of 0.5 μ Ci [3 H]adenine (17 Ci/mmol)/ml. Poly(A)-containing RNA (350 μ g; 5000 d.p.m./ μ g) was digested with ribonucleases and deoxyribonuclease and subjected to electrophoretic analysis as described in Methods. A second gel was loaded with 7.5 μ g [3 H]tRNA from *E. coli* (15000 c.p.m.) and 2.5 μ Ci [3 H]ATP (20 Ci/mmol). Both gels were run under the same conditions. ○, Poly(A)-tracts; ●, *E. coli* tRNA and ATP.

Table 1. *Base composition of RNA fractions separated by cellulose column chromatography*

Fractions of *Chlorella* RNA which had been separated by cellulose-column chromatography were subjected to acid hydrolysis and thin-layer chromatography as described in Methods.

	Composition (mol %)			
	Adenine	Guanine	Cytosine	Uracil
Poly(A)-containing RNA				
Expt. 1	26.8	31.7	19.6	21.9
Expt. 2	26.6	32.7	17.8	22.4
Mean	26.7	32.2	18.7	22.4
Unadsorbed RNA	21.5	41.0	17.5	20.0

Isolation of poly(A)-tracts from poly(A)-containing RNA

By means of procedures described in Methods, a sample of putative poly(A)-containing RNA was digested with T_1 and pancreatic ribonucleases and applied to a cellulose column after dilution in TKM buffer. By measurement of E_{260} , it was shown that 4.3 to 4.8 % of the sample was ribonuclease-resistant as it was adsorbed and eluted with water from the column. This material did not contain mononucleotides as it was excluded by Sephadex G-25. After acid hydrolysis and thin-layer chromatography of this fraction, only adenine was detected when areas of the thin-layer plate parallel with the base or nucleotide markers were eluted and measured for extinction between 240 and 280 nm. Although absorbance of the eluates was small, it was estimated that not less than 80 % of base in the acid hydrolysate was adenine.

The electrophoretic mobility of ribonuclease-resistant tracts from poly(A)-containing

Fig. 3

Fig. 4

Fig. 3. Formamide-sucrose gradient centrifugation of poly(A)-containing RNA. Poly(A)-containing RNA was isolated from a culture which had been incubated for 1 h with $0.5 \mu\text{Ci } [^3\text{H}]\text{adenine}$ (17 Ci/mmol)/ml. RNA ($270 \mu\text{g}$; $3400 \text{ d.p.m.}/\mu\text{g}$) was centrifuged in a 5 ml, 5 to 20% (w/v) sucrose gradient containing 50% (v/v) formamide for 12 h at 84000 g . Analysis of the gradient contents is described in Methods. rRNA and tRNA from *E. coli* and from *Chlorella* were centrifuged in separate tubes to determine the S values (arrows). Fraction volume was 0.1 ml .

Fig. 4. Effect of *Chlorella* RNA on the time course of *in vitro* peptide synthesis in the wheat-germ system. Samples ($50 \mu\text{l}$) were taken from $250 \mu\text{l}$ wheat-germ systems (see Methods). ●, No RNA added; ○, $96 \mu\text{g}$ poly(A)-containing RNA added; □, $250 \mu\text{g}$ unadsorbed RNA added.

RNA is shown in Fig. 2. By comparison with tRNA from *E. coli* and ATP markers, the tracts were 25 to 55 nucleotides in length.

Size distribution of poly(A)-containing RNA molecules

Density-gradient centrifugation of poly(A)-containing RNA in formamide-sucrose showed a spread of sizes from about 40S to 10S (Fig. 3). That little low-molecular-weight material was present suggests that the extraction procedure for RNA did not allow significant degradation.

Stimulation of in vitro incorporation of amino acids into peptides by Chlorella poly(A)-containing RNA

Poly(A)-containing RNA increased the rate of incorporation of labelled amino acid into the hot TCA-insoluble fraction in a wheat-germ cell-free system (Fig. 4). Incorporation was approximately linear for 80 min. Unadsorbed RNA from a cellulose column did not show comparable stimulation. Figure 5 shows that stimulation of incorporation was proportional to the amount of poly(A)-containing RNA added, up to about $4 \mu\text{g}/\text{assay}$. With the addition of larger amounts of RNA the system became saturated, or stimulation progressively diminished with increasing RNA. Reduction of stimulation when more than $6 \mu\text{g}$ RNA was added was most pronounced with RNA from rapidly growing cells. In the culture system used, growth became light-limited at about 10^7 cells/ml so that growth rate at the time of sampling decreased with increase in cell-number density.

Fig. 5

Fig. 5. Effect of concentration of *Chlorella* poly(A)-containing RNA on *in vitro* protein synthesis in the wheat-germ system. The assays were incubated for 1 h. Cell-number densities of *Chlorella* cultures at the time of harvesting for poly(A)-containing RNA isolation were (cells/ml): ○, 5.7×10^6 ; ●, 15.7×10^6 ; □, 27.7×10^6 .

Fig. 6

Fig. 6. Effect of magnesium acetate and potassium chloride on peptide synthesis, stimulated by $3.5 \mu\text{g}$ *Chlorella* poly(A)-containing RNA in a wheat-germ cell-free assay. The assays were incubated at 37°C for 30 min. Magnesium acetate concentration (○) was varied in the presence of 64 mM-potassium chloride. Potassium chloride concentration (●) was varied in the presence of 3.5 mM-magnesium acetate.

Concentration of magnesium acetate and KCl affected the cell-free system. When the system was stimulated with *Chlorella* poly(A)-containing RNA, 5 mM-magnesium acetate and 74 mM-KCl gave maximum incorporation rates (Fig. 6).

DISCUSSION

Our results suggest that some 7 % of the RNA in *Chlorella* is mRNA or its nuclear precursor. It is not known whether mRNA in the chloroplast or mitochondrion is poly(A)-containing in *Chlorella* and so included in this estimate. Poly(A)-tracts 25 to 55 nucleotides in length are amongst the smallest so far reported, being comparable with those isolated from yeast polysomal RNA (Reed & Wintersberger, 1973) and from mitochondrial poly(A)-containing RNA of mammalian and insect cells (Hirsch *et al.* 1974). Clearly there is great variability amongst eucaryotic micro-organisms, as poly(A)-tracts from *Euglena* are 150 to 250 nucleotides long (Sagher *et al.* 1974) as found in higher animals (Darnell *et al.* 1973) and higher plants (Sagher *et al.* 1974).

The relatively large average size of *Chlorella* poly(A)-containing RNA (Fig. 3) contrasts with *Dictyostelium* where the average size is about 14S (Lodish *et al.* 1973). It may be that *Chlorella* mRNA contains much larger untranslated sequences than *Dictyostelium* mRNA, but it is also possible that the nuclear mRNA precursors in *Chlorella* are much larger than cytoplasmic mRNA and contribute significantly to the size distribution of total poly(A)-containing RNA. From the size of poly(A)-tracts and the observation that the tracts represent about 4.5 % of poly(A)-containing RNA it appears that there is approximately one poly(A)-tract per 1000 nucleotides in *Chlorella* poly(A)-containing RNA. As the whole molecules are mostly very much larger than this, there must be several poly(A)-tracts per

molecule. The most probable arrangement is a few tracts of 25 to 55 adenylate residues, separated by at least one guanosine riboside, as digestion with pancreatic ribonuclease alone yields some resistant tracts about 200 nucleotides in length (C. A. Lambe and P. C. L. John, personal communication).

Stimulation of peptide synthesis in the wheat-germ cell-free system is evidence for the messenger function of *Chlorella* poly(A)-containing RNA. Similar results were obtained when this RNA was injected into *Xenopus* oocytes, but an *E. coli* cell-free system was not stimulated (A. H. Scragg, unpublished results). It is hoped that work in progress will give positive identification of a *Chlorella* protein amongst the products of poly(A)-containing-RNA-stimulated protein synthesis in the wheat-germ cell-free system.

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