Isolation of Polysomes from *Nostoc* sp. MAC and Translation of Messenger RNA in a Heterologous Cell-free System

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A method has been established which isolates polysomes from the lysozyme/EDTA-shocked cyanobacterium, *Nostoc* sp. MAC. In a typical preparation the total recovery of RNA as polysomes was 83%, in which 77% of the polysome fraction was present at sizes > 5-mers and 23% as 2–4-mers. Messenger RNA isolated from such a preparation of polysomes produced a 10-fold stimulation in the incorporation of [³⁵S]methionine into polypeptides by a cell-free system of *Escherichia coli*. The *in vitro*-synthesized polypeptides were analysed on an SDS-polyacrylamide gradient gel together with *in vivo*-labelled proteins of *Nostoc* sp. MAC: seven polypeptides co-migrated with the *in vivo*-synthesized products. This is the first report of the expression of cyanobacterial messenger RNA in a heterologous cell-free system from *E. coli*; the efficiency of the system is discussed.

INTRODUCTION

In the first report of the *in vitro* translation of cyanobacterial mRNA, [¹⁴C]leucine was shown to be incorporated into an insoluble fraction by crude extracts of *Anacystis nidulans* (Bazin, 1970). Leach & Carr (1974) examined the *in vitro* protein synthesis in extracts of *Anabaena variabilis* and estimated the half-life of the mRNA to be 12 min. In both these reports the mRNA was translated by cell-free preparations of its own organism and the products were not characterized with respect to size. Careful separation procedures can lead to the isolation of polysomes in which a number of ribosomes are attached to the same mRNA molecule. Polysomes have been successfully used as a means of separating mRNA in a functionally intact form from a wide range of organisms, including higher plants, yeast and microalgae (Dunham & Thurston, 1980; Hall, 1979; Kraig & Haber, 1980; Turner *et al.*, 1981). Many cyanobacteria change the amount and identity of their phycobiliprotein light-harvesting pigments in response to both qualitative and quantitative change in incident light. The total amounts of such pigments are, generally, inversely proportional to light intensity and some species show chromatic adaptation, in which different phycobiliproteins are synthesized so as to maximize the harvesting of the light available. The phycobiliproteins comprise a major proportion, up to 50%, of the total soluble protein of exponentially growing cyanobacteria, and therefore alteration in their rates of synthesis involves considerable changes in gene expression (see Glazer, 1976; Doolittle, 1979; Cohen-Bazire & Bryant, 1982). The understanding of the control of phycobiliprotein synthesis will be aided by characterization of the mRNAs involved. This communication describes the isolation, and expression in a heterologous system, of mRNA from polysomes prepared from *Nostoc* sp. MAC, a cyanobacterium which chromatically adapts to green or red light.

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Abbreviations: DOC, sodium deoxycholate; DTT, dithiothreitol; LDS, lithium dodecyl sulphate.

METHODS

Culture and growth. *Nostoc* sp. MAC (obtained from Dr A. J. Smith and deposited as PCC 8009, Institut Pasteur, Paris, France) was cultured at 37 °C in medium C of Kratz & Myers (1955) supplemented with 0.1% (w/v) sodium bicarbonate and gassed with air/CO₂ (95:5, v/v) at a light intensity of 75 µE m⁻² s⁻¹; 2-litre or 10-litre culture volumes were employed for polysome isolation.

Harvesting. When the culture density had reached approximately 0.25 g wet wt l⁻¹, chloramphenicol (3 mg l⁻¹) was added 10–15 min prior to harvesting. This prevented the 'run-off' of the ribosomes from mRNA during harvesting and cell lysis, and maximized polysome yield. The culture was cooled by addition of an equal volume of crushed ice and organisms were collected on filter paper in a Buchner funnel under gentle vacuum. The whole process took 4–5 min for 2-litre cultures.

Polysome isolation. The basic procedure was modified from Godson (1967). In principle, a precise EDTA/lysozyme shock was given to the cells followed by lysis with neutral detergents in the presence of magnesium ions. Every step was carried out at 0–4 °C using cold buffers and sterile glassware to minimize RNAase activity.

Organisms (0.50 g) were resuspended in 2.0 ml 25% (w/v) sucrose in 10 mM-Tris/HCl, 12 mM-dithiothreitol (DTT), pH 8.1, and 0.5 ml EDTA/lysozyme (lysozyme at 10 mg ml⁻¹ in 250 mM-Tris/HCl, pH 8.1 plus an equal volume of 8 mM-EDTA) was added and the suspension left on ice with occasional gentle swirling. After 10 min, 2.5 ml lytic mixture [0.5 ml each of 300 mM-MgCl₂, 10% (w/v) sodium deoxycholate (DOC), 10% (w/v) Brij 35 and 10% (v/v) Triton X-100 each dissolved in 100 mM-Tris/HCl, pH 8.1 and 200 mM-EGTA] was added. The final concentrations of reagents at the time of lysis were: 46 mM-Tris/HCl, pH 8.1, 10% sucrose, 0.5 mg lysozyme ml⁻¹, 0.4 mM-EDTA, 1% DOC, 1% Triton X-100, 1% Brij 35, 20 mM-EGTA and 30 mM-MgCl₂. The suspension was moved on and off the ice alternately for 5 min, thus raising the temperature of the suspension and increasing lysis. Lysis was detected microscopically 10 min after the addition of lytic mixture, but 20 min more were required for its completion, when 90–95% of the cells were broken. At this stage, 0.55 ml 4 M-KCl in 200 mM-Tris/HCl, pH 8.1 was added and cells and cell-debris were removed by centrifugation at 27000 g for 25 min. The supernatant was layered on to 3 ml 60% sucrose made up in 400 mM-KCl, 15 mM-MgCl₂, 5 mM-EGTA, 5 mM-DTT and 40 mM-Tris/HCl, pH 8.0 (polysome buffer) and centrifuged at 200000 g for 3 h. The supernatant was carefully removed with a Pasteur pipette and the sides of the tubes were washed with distilled water and carefully wiped. The opalescent polysome pellet was resuspended as required.

Test for polysome integrity. The polysome pellet was resuspended in 300 µl polysome buffer containing 0.2 M-sucrose. About 5.0 A₂₆₀ units of polysomes were put on each 16 ml sucrose gradient formed by layering 2, 4, 4 and 6 ml of 15, 25, 35 and 45% solutions, respectively, of sucrose in buffer (40 mM-Tris/HCl, 10 mM-MgCl₂, 60 mM-KCl, 5 mM-DTT, pH 8.0) and centrifuged at 95000 g for 105 min. The profile of the polysome distribution, measured at A₂₅₄, was analysed by piercing the bottom of the tube with an ISCO density gradient fractionator (model 183) and displacing the contents upwards into a 2 mm light path of a ISCO model UA-4 absorbance monitor. Optical density was later multiplied by 5 to give unit absorbance in a 1 cm light path.

Isolation of polysomal RNA. Polysome pellets were resuspended in buffer (10 A₂₆₀ units ml⁻¹) containing 10 mM-Tris/HCl, 100 mM-LiCl, 1 mM-EDTA and 0.5% (w/v) lithium dodecyl sulphate (LDS), pH 7.4 and deproteinized two or three times with an equal volume of redistilled phenol/chloroform (1:1, v/v). RNA was precipitated from the aqueous phase with 2 vol. redistilled ethanol in the presence of 0.1 vol. 2.4 M-ammonium acetate at -20 °C. After 2 h, the RNA precipitate was collected by centrifugation at 50000 g for 15 min and dissolved in 1 ml 10 mM-Tris/HCl containing 150 mM-lithium acetate, 2 mM-EDTA and 0.5% LDS at pH 6.0. RNA was reprecipitated by the addition of 2 vol. ethanol, held at -20 °C for 2 h and centrifuged. The precipitate was washed twice with 70% (v/v) ethanol containing 50 mM-KCl and put under vacuum for 5 min to evaporate the remaining ethanol. When required for use in *in vitro* translation systems, polysomal RNA was dissolved in buffer (10 mM-Tris/HCl, 30 mM-NH₄Cl, 10 mM-MgCl₂, 6 mM-β-mercaptoethanol, pH 8.5) and used immediately; storage in liquid nitrogen for several days did not reduce messenger activity. All operations were performed at 4 °C to avoid degradation of polysomal RNA, which was shown to occur at room temperature.

Isolation of mRNA by stripping from polysomes. Polysomes isolated from 10-litre cultures were resuspended in the buffer containing 20 mM-Tris/HCl, 20 mM-EDTA, 100 mM-LiCl and 10 mM-DTT, pH 8.0 (25 A₂₆₀ units ml⁻¹), left on ice for 1 h with occasional swirling to allow complete separation of mRNA from ribosomes, and then centrifuged at 200000 g for 2 h to pellet ribosomes. The supernatant containing mRNA was deproteinized and first made ready for *in vitro* translation as described above for polysomal RNA except that RNA precipitation was carried out overnight at -20 °C, followed by centrifugation at 100000 g for 30 min.

Preparation of cell-free extracts for *in vitro* synthesis. *Escherichia coli* strain PR7, which is a mutant lacking both RNAase I and polynucleotide phosphorylase, was used for the preparation of ribosomal and supernatant fractions for *in vitro* protein synthesis. It was grown aerobically in yeast/tryptone medium (16 g Bactotryptone, 10 g yeast extract and 5 g NaCl l⁻¹) at 37 °C to an A₅₅₀ of 2, cooled with equal volume of ice, harvested and used immediately to purify the different fractions of the protein-synthesizing apparatus. Ribosomes were isolated from

a pre-incubated S30 extract followed by high-speed centrifugation according to Crawford & Gesteland (1973). Crude initiation factors were extracted from ribosomes after treatment with buffered 1 M-NH₄Cl; the ribosomes were centrifuged and the supernatant was precipitated with ammonium sulphate. Fraction 'S100 DE' was the high-speed supernatant of cell-free extract after passage through a DEAE-cellulose column to free it of low molecular weight proteins and RNA. Both these fractions were purified as described by Traub *et al.* (1971). All three fractions were stored as small samples in liquid nitrogen. Transfer RNA was purified from total RNA of *E. coli* as described by Leach & Carr (1974).

***In vitro* protein synthesis.** The reaction mixture (final volume 25 µl) contained, in 5.4 µl: Tris/acetate, 50 mM; NH₄Cl, 75 mM; magnesium acetate, 11.2 mM; ATP, 3 mM; GTP, 0.5 mM; phosphoenolpyruvate, 5 mM; pyruvate kinase, 50 µg ml⁻¹; DTT, 2 mM; 19 L-amino acids, 0.25 mM; [³⁵S]methionine (840–1445 Ci mmol⁻¹), 10 µCi (370 kBq). The cell-free fractions comprised: ribosomes (18 mg ml⁻¹), 7.5 µl; crude initiation factors (13.44 A₂₈₀ units), 2 µl; S100 DE (26 A₂₆₀ units), 2 µl; tRNA (4 mg ml⁻¹), 0.625 µl; mRNA from *Nostoc* sp. MAC and distilled water made up to the final volume. Each component of the reaction mixture and cell-free fractions was tested individually in different concentrations to maximize protein synthesis. The reaction was carried out at 37 °C for 30 min and stopped by addition of 12 mM-methionine (5 µl) and 6 M-NaOH (15 µl) with further incubation for 2 min at 37 °C to hydrolyse RNA. Samples were chilled on ice and the proteins precipitated by the addition of 150 µl 10% (w/v) TCA, washed once with ethanol/ether (1:1, v/v) and once with ether and then dried. The precipitate was dissolved in 50 µl sample buffer [60 mM-Tris/HCl, 10% (v/v) glycerol, 5 mM-DTT, 1% (w/v) SDS and 0.001% bromophenol blue] and heated for 10 min at 100 °C, and triplicate samples (3 µl) were placed in Aquasol for radioactive counting. The remainder was saved for gel electrophoresis.

Analytical polyacrylamide gel electrophoresis. The *in vitro* translation product was fractionated on SDS-polyacrylamide gels using a discontinuous buffer system (Laemmli, 1970). A 12–22% gradient of polyacrylamide slab gel (15.5 × 15 × 0.1 cm) containing 0.18 (w/v) bisacrylamide and 0.1% (w/v) SDS was run initially at 10 mA for 1 h, and then at 20 mA for 5 h, along with marker proteins. The lanes corresponding to the protein markers were separated from the rest of the gel, stained overnight at room temperature with 0.1% (w/v) kenacid blue R in 45.2% (v/v) methanol and 9.6% (v/v) glacial acetic acid and destained with 7% acetic acid and 20% methanol; the remainder of the gel was processed for fluorography (Bonner & Laskey, 1974). After impregnation with PPO, the gel was immersed for 1 h in 1% glycerol and dried overnight between two sheets of cellophane paper. The gel was fluorographed in contact with Ilford X-ray film at –70 °C.

***In vivo* labelling of proteins.** ³⁵SO₄²⁻ (2 mCi per 150 ml) was added to a suspension (1.2 mg wet wt ml⁻¹) of exponentially growing *Nostoc* sp. MAC and the suspension was incubated for 4 h under growth conditions. The culture was then cooled on ice, harvested and washed three times with 100 mM-Tris/HCl, pH 7.2, sonicated in the same buffer and centrifuged at 10000 g for 10 min at 4 °C. An equal volume of 10% TCA was added to supernatant, which was then left on ice for 1 h. The precipitate was centrifuged, washed three times with 5% TCA, twice with acetone and once with ether, and then dried. The residue was dissolved in the sample buffer as used for the *in vitro* product and heated for 10 min in a boiling water bath, and samples were electrophoresed.

Chemicals. Sources were as follows: ATP, GTP, EGTA, Brij 35, lysozyme and phosphoenolpyruvate from Sigma; sucrose (density gradient grade) from Schwarz/Mann; DNAase (RNAase-free) from Millipore; DTT and pyruvate kinase from Boehringer-Mannheim; [³⁵S]methionine and Na₂³⁵SO₄ from Amersham; Aquasol and PPO from New England Nuclear; DEAE-cellulose from Whatman; electrophoresis reagents from Bio-Rad; Bactotryptone and yeast extract from Difco. All other chemicals used were of high purity grade and purchased from BDH.

RESULTS

Although the cell wall of *Nostoc* sp. MAC was more resistant to lysozyme treatment than those of many bacteria, a good yield of polysomes was obtained after a brief lysozyme shock followed by cell lysis. Under standard conditions of lysis, about 30–35 A₂₆₀ units of polysomes were routinely obtained per g broken cells. The ratio of A₂₆₀ to A₂₈₀ of the suspended isolated polysomes varied from 1.72 to 1.8 (mean 1.76), characteristic of undegraded polysomes. The total time required to lyse 90–95% of a *Nostoc* sp. MAC suspension was about 45 min, which was considered reasonable and avoided lengthy steps of centrifugation.

A typical sedimentation profile of intact polysomes on a sucrose density gradient is shown in Fig. 1(a). The concentrations of subunits and monosomes and polysomes were calculated by counting the areas below their respective peaks. Polysomes containing two or more ribosomes are subsequently referred to as 'mers' (Davies & Larkins, 1972). The total recovery of the polysomes was 83%, and only 10% was present as monosomes. A peak of polysomes present in the >7-mers region contributed 40% of the total polysomes, which was greater than the

Fig. 1. Sucrose density gradient profiles of polysomes isolated from *Nostoc* sp. MAC under the conditions described in Methods. (a) Profile obtained after the addition of 20 mM-EGTA into the lytic mixture. (b) Without EGTA addition, the lytic mixture being the same as in (a) except that MgCl_2 was decreased to 10 mM and DNAase (RNAase-free; $5 \mu\text{g ml}^{-1}$) was added. The dashed line represents the control in which buffer was put on top of the gradient. s, subunits; m, monosomes; peaks 2–9 represent polysomes.

percentage of polysomes present as 5–7-mers (37%) or 2–4-mers (23%). When EGTA was omitted from the lytic mixture and buffers, a slightly better yield of polysomes was obtained (35–40 A_{260} units per g broken cells) after addition of DNAase at the time of lysis. However, under these conditions the polysomes were comparatively more degraded, as indicated by the sucrose density gradient profile (Fig. 1b). Monosomes and subunits contributed 30% of the total yield, and the ratio of > 5-mers to < 5-mers was also low (2.8) when compared with the value from the lytic mixture containing EGTA.

A similar observation was made when chloramphenicol was omitted before harvesting, or when cells were incubated on ice after harvesting for 1 h or less prior to lysis without chloramphenicol addition. Under both conditions, degradation of polysomes was reflected in a high monosome peak. An attempt to lyse the cells in a higher concentration of Tris (100 mM) and salt (KCl, 200 mM), prolonged the lysis to 1 h, though no significant effect on recovery of polysomes was observed. Therefore, cells were always lysed under optimal Tris concentration (46 mM) at pH 8.1. No inhibitor of ribonuclease activity was tested except DTT, and the temperature was kept between 0 and 4 °C unless stated.

The kinetics of [^{35}S]methionine incorporation into proteins by a cell-free system of *E. coli* directed by polysomal RNA from *Nostoc* sp. MAC as mRNA source is shown in Fig. 2. Amino acid incorporation was linear with increasing RNA concentration up to 25 μg RNA per assay and then declined at concentrations above 35 μg RNA per assay. This system gave threefold stimulation over the endogenous level. Different buffers, having various concentrations of Tris, salt and EDTA, were tested for their effects on the stripping of mRNA from ribosomal complexes (Table 1). Buffer 3, with 20 mM-Tris/HCl pH 8.0, 20 mM-EDTA, 100 mM-LiCl and 10 mM-DTT, was most effective with respect to both mRNA release and amino acid incorporation into polypeptides per unit polysome. This procedure gave about 85% reduction in rRNA content from total polysomal RNA. Such RNA, when expressed in the *in vitro* system, resulted in the incorporation of [^{35}S]methionine into protein as a function of RNA concentration (Fig. 2). The incorporation was linear up to 100 μg RNA per assay, giving a sixfold stimulation. Above this concentration a non-linear response was observed, yielding a 10-fold stimulation of incorporation with 300 μg RNA. The time course of amino acid incorporation *in vitro* reached a plateau after 30 min. The effect of omitting different constituents from the standard reaction mixture was examined (Table 2). When fraction S100 DE was isolated from *Nostoc* sp. MAC using the same procedure as for *E. coli* and employed in the reaction mixture alone or in different combinations with *E. coli* S100 DE, no enhancement of polypeptide synthesis was observed; furthermore, omission of S100 DE reduced incorporation by only 50%. Mercaptoethanol (4 mM) and reduced glutathione (10 mM)

Fig. 2. Relationship between added *Nostoc* sp. MAC mRNA and incorporation of [^{35}S]methionine into polypeptides in a cell-free system of *E. coli*. ●, mRNA stripped from the ribosomal complex was used as RNA source as described in Methods; ○, total polysomal RNA was used as RNA source.

Table 1. *Effect of buffer composition on the release of mRNA from ribosomes and its subsequent cell-free translation in an E. coli system*

Buffer*	Constituents (mm)			Polysomes (A_{260} units)	RNA released (μg per unit polysomes)	[^{35}S]Methionine incorporation (c.p.m. per μg RNA)
	Tris/HCl	EDTA	LiCl			
1	10	20	50	6.57	14.8	3.6×10^3
2	20	10	100	7.25	12.1	2.1×10^3
3	20	20	100	5.63	13.9	6.1×10^3
4	40	20	200	7.71	8.5	3.9×10^3

* Each buffer was at pH 8.0 and contained 10 mM-DTT.

Table 2. *Incorporation of [^{35}S]methionine into proteins directed by Nostoc sp. MAC mRNA in an E. coli cell-free system*

	Relative incorporation (%)
Complete system	100*
– Ribosomes	7
– Initiation factors	40
– S100 DE	50
– mRNA	11
+ RNAase (50 $\mu\text{g ml}^{-1}$)	10
– DTT	60
– tRNA	49

* 100% = 0.5×10^6 c.p.m.

in place of DTT inhibited the incorporation by 53% and 100%, respectively. Crude S30 extract of *E. coli*, when used in place of purified ribosomes, initiation factors and S100 DE fractions, gave no incorporation of amino acids in polypeptides on addition of mRNA from *Nostoc* sp. MAC. The efficiency of the system was tested periodically using total polysomal RNA from *E. coli*.

Fig. 3. Fluorogram showing analysis of (a) *in vivo*-labelled and (b) *in vitro*-labelled polypeptide products on 12–22% SDS-polyacrylamide gels. Marked bands correspond to the seven polypeptides, labelled *in vitro*, which co-migrated with *in vivo*-labelled products. The diagram on the right indicates the positions of these bands.

The pattern of polypeptides obtained after *in vivo* labelling of proteins of *Nostoc* sp. MAC with $^{35}\text{SO}_4^{2-}$ was examined by analytical SDS-polyacrylamide gel electrophoresis. A number of polypeptide bands became distinct after a labelling period of 4 h and all of them showed molecular weights less than 35000 (Fig. 3a). The corresponding pattern of *in vitro*-labelled polypeptides on polyacrylamide gel is shown in Fig. 3(b). Seven polypeptides co-migrated with the *in vivo*-labelled proteins and fell in the molecular weight range 17500–30000; however, most of the synthesis resulted in small molecular weight polypeptides.

DISCUSSION

It has been emphasized that the ratio of higher (> 5-mers) to lower (< 5-mers) polymerized forms of polysomes is a more reliable criterion to judge polysome degradation resulting from RNAase activity than the ratio of polysomes to monosomes from a sucrose density gradient profile (Jackson & Larkins, 1972). In our present findings, when EGTA was omitted from the lytic mixture, a lower value was obtained for both ratios, indicating polysome degradation to be a result of RNAase activity. Divalent cations are known to affect polysomes in two ways. Firstly, they cause aggregation of polysomes in cell-free extracts thereby reducing recovery of the polysomes (Jackson & Larkins, 1972) and secondly, Ca^{2+} especially contributes to the

degradation of polysomes by activating RNAases (Larkins & Davies, 1973). EGTA is known to chelate divalent cations (Marhol & Cheng, 1970); although we did not observe any improvement in the final yield of polysomes on EGTA addition, it is possible that it improved polysome recovery into higher polymers by inhibiting RNAase activity. Godson & Sinsheimer (1967) reported a 10% lower yield of polysomes from *E. coli* when DNAase was omitted from the lytic mixture, the reason being that the entrapment of large polysomes by DNA molecules during low-speed centrifugation was increased. A 10–15% lower yield of polysomes in our experiments with EGTA (in which DNAase had no effect) may be due to the same phenomena.

Translation of total polysomal RNA in the cell-free system of *E. coli* produced only threefold stimulation of incorporation of labelled amino acid, and adding more RNA to the system produced inhibition in polypeptide synthesis. This may be due to the large amount of rRNA contaminating mRNA (Doolittle *et al.*, 1979). There is no published report regarding the occurrence of poly(A)-containing mRNA in cyanobacteria, although recent experiments indicate that it may be present in *Nostoc* sp. MAC (Ownby *et al.*, 1982). Therefore, in the experiments reported here deproteinization of polysomes was postponed until separation of mRNA from the ribosomal complex by chelation of Mg^{2+} ions, a method used for separation of mRNA in higher plants before the development of oligo-(dT) cellulose columns (Hall, 1979). This step reduced the level of rRNA present and produced enrichment of mRNA, which yielded a 10-fold stimulation in polypeptide synthesis by the cell-free system.

The results reported here demonstrate for the first time the expression of cyanobacterial mRNA in a heterologous cell-free system of *E. coli*. The similar electrophoretic mobility of *in vivo*- and *in vitro*-produced polypeptides coded for by *Nostoc* sp. MAC mRNA indicates that similar proteins are also synthesized in *E. coli*. The synthesis of small polypeptides in the *E. coli* cell-free system may be due either to the degradation of *Nostoc* sp. MAC mRNA in the system or to the premature switch-off in the polypeptide synthesis (Crawford & Gesteland, 1973). Experiments are under way to identify the proteins synthesized in the cell-free system directed by mRNA isolated from polysomes prepared from *Nostoc* sp. MAC.

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