A chloroplast system capable of translating heterologous mRNAs

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Received 19 October 1982

A system capable of incorporating amino acids into protein has been prepared from chloroplasts isolated from spinach leaves. The activity of the system is inhibited by chloramphenicol or RNase but not by rifampycin or cycloheximide. After reducing the endogenous activity by treatment with micrococcal nuclease, the system responds to homologous or heterologous mRNAs. The RNA from MS-2 phage is translated faithfully as demonstrated by the isolation from the translation products of a protein with the same mobility of the phage coat protein. Partial proteolytic digestion confirmed that the protein synthesized in vitro is indeed the phage protein.

Chloroplast protein synthesis *Phage MS-2 RNA translation* *Translation in vitro*

1. INTRODUCTION

Chloroplasts contain a complete protein synthetic apparatus different from those present in the cell cytoplasm and in mitochondria [1,2]. So far chloroplast mRNAs extracted from higher plants or unicellular algae have been translated only in heterologous systems [3,4]. Translational fidelity in such systems appears to vary according to the type of mRNA and/or the organism from which the chloroplasts have been prepared. Thus it has been reported that some chloroplast mRNAs are translated faithfully, preferentially or exclusively, in cell-free systems derived from prokaryotes but not in those prepared from eukaryotes. Other chloroplast mRNAs are translated in both types of cell-free systems. Finally, certain chloroplast mRNAs seem to be translated with good efficiency and fidelity only in systems prepared from the cytoplasm of eukaryotes.

An homologous, sub-organellar system prepared from chloroplasts, would be of considerable interest as it would allow us to probe how translational fidelity is controlled at least in vitro. To this end we have developed from spinach chloroplasts a system capable of translating homologous and heterologous mRNAs. The system appears to translate faithfully spinach chloroplast mRNAs and the RNA from coliphage MS-2. The RNA from tobacco mosaic virus stimulates the activity of the system and gives rise to protein products one of which has the mobility of the viral coat protein.

2. MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves as in [5] but using a modified extraction buffer containing: 50 mM Tris-acetate (pH 8.2), 2 mM EDTA, 1 mM magnesium acetate, 4 mM β-mercaptoethanol and 330 mM mannitol. The chloroplast pellet was resuspended by vortexing in 0.5 vol. (v/w) 10 mM Tris-acetate (pH 8.2), 14 mM magnesium acetate, 60 mM potassium acetate, 1 mM dithiotreitol and 20 μg/ml phenylmethylsulphonyl fluoride (PMSF), homogenized with 30–50 strokes in a Teflon–glass Potter homogenizer and centrifuged at 27000 $\times$ g for 30 min. After addition of 10% glycerol (v/v), the supernatant was stored frozen in small aliquots at −80°C.

Translation was carried out at 26°C for 30 min using the salt mixture described for the *Escherichia coli* cell-free system [6] with the addition of 0.2 mg

Published by Elsevier Biomedical Press
00145793/82/0000—0000/$2.75

PMSF/ml and 0.5–40 μCi [^{35}S]methionine/assay. Treatment with micrococcal nuclease (Boehringer) (250 units/ml) was performed as in [7] at 26°C for 2 min.

Preparation of *E. coli* cell-free system, RNA extraction, translation conditions, SDS–polyacrylamide gel electrophoresis, fluorography and peptide mapping were performed as in [6]. Poly(U)-directed polyphenylalanine synthesis was assayed as in [8].

3. RESULTS AND DISCUSSION

3.1. *Characterization of the amino acid incorporating system*

The general features of the incorporation of [^{35}S]methionine directed by the endogenous mRNAs present in a non-preincubated extract are reported in fig. 1. Incorporation appears to be linear within [extract] of 100–300 μg protein/assay (fig. 1a). The synthetic activity proceeds linearly for a short period of time. After 5 min incubation, the incorporation of radioactive methionine slows down and levels off after ~30 min incubation (fig. 1b). An indication that the incorporation of methionine may be due, at least in part, to initiation of translation and not only to elongation of peptide chains is reported in fig. 1c. Aurintricarboxylic acid (ATA), a specific inhibitor of initiation of protein synthesis, reduces the incorporation of methionine directed by the endogenous mRNAs at concentrations that do not affect peptide chain elongation, as measured by assaying poly(U)-directed polyphenylalanine synthesis. The activity of the system is drastically affected by chloramphenicol (85% of inhibition at 400 μg/ml) and RNase (97% inhibition at 200 μg/ml) whereas it is inhibited very little by rifampicyn (25% of inhibition at 2 μg/ml) and cycloheximide (15% inhibition at 200 μg/ml). Concentration dependence curves (not reported) indicated that optimal [Mg^{2+}] was 8.8 mM. The optimal pH for activity was found to be 8.2 (Tris-acetate buffer). The extracts may be stored frozen at −80°C for several months without significant loss of activity. However, once frozen they cannot be re-frozen.

3.2. *Translation of homologous and heterologous mRNAs*

Addition of heterologous mRNAs to the system does not result in an appreciable increase in the incorporation of [^{35}S]methionine, probably because

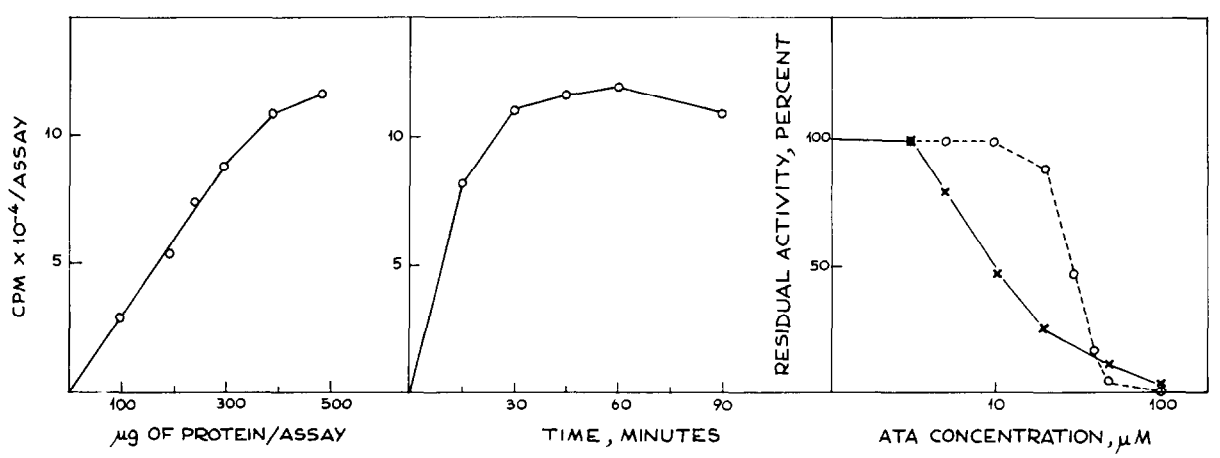

Fig. 1. Characteristics of the chloroplast system: (A) Effect of different concentrations of chloroplast extract. Assay system was as in section 2. Each assay contained 1 μCi of [^{35}S]methionine; (B) Time course of [^{35}S]methionine incorporation. Each assay contained chloroplast extract corresponding ~480 μg protein; (C) Effect of ATA (aurintricarboxylic acid) on the incorporation of [^{35}S]methionine directed by the endogenous mRNAs (×—×) and of [^{14}C]phenylalanine directed by poly(U) (○—○). Each assay system contained chloroplast extract corresponding ~480 μg protein.

of the saturation by endogenous mRNAs. Thus, several attempts were made to lower the endogenous activity. Preincubation of the extracts leads to a dramatic loss of activity as do dialysis or chromatography on Sephadex G-25. However, treatment with micrococcal nuclease decreases the endogenous activity without a total loss of the capacity to incorporate radioactive methionine. Under these conditions, addition of different mRNAs stimulates the incorporation of methionine (2–3-fold over the residual endogenous activity). As depicted in fig. 2, the profile of the radioactive polypeptides produced when spinach chloroplast mRNAs (slot 3) were added to nuclease-treated extracts (slot 2) was practically identical to that produced in untreated extracts (slot 1). Addition of phage MS-2 RNA gave rise to a rather prominent radioactive band (app. M_r 14000) with the same mobility of authentic phage coat protein (slot 4). When the RNA from TMV was added, a number of new protein bands were evident one of which

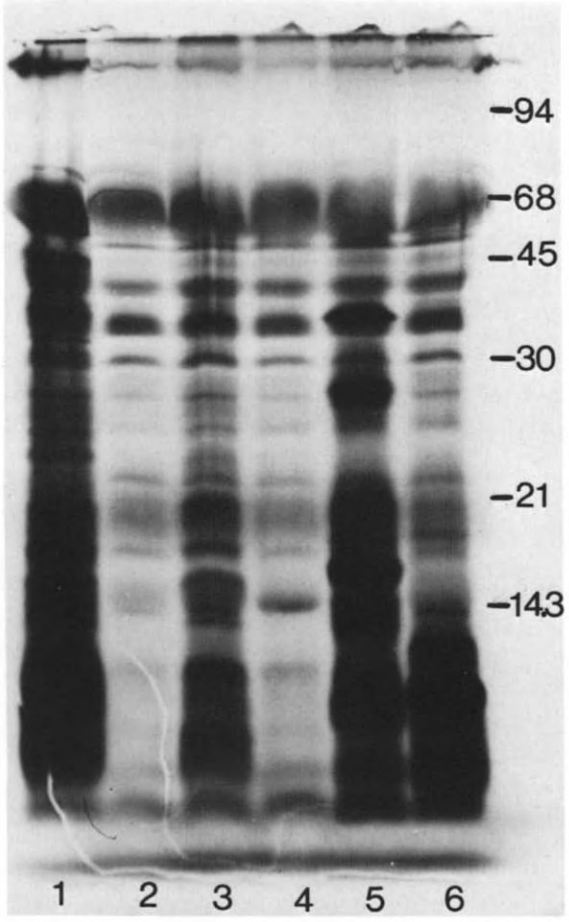

Fig. 2. Products of the translation of different mRNAs. Fluorography of monodimensional SDS–polyacrylamide gel electrophoresis of the products of: (1) endogenous activity (no nuclease treatment); (2–6) nuclease-treated extracts; (2) residual endogenous activity; (3) spinach chloroplast mRNAs (14 μg); (4) MS-2 phage RNA (25 μg); (5) TMV RNA (8.8 μg); (6) *E. gracilis* chloroplast mRNAs (13 μg); numbers represent $M_r \times 10^{-3}$.

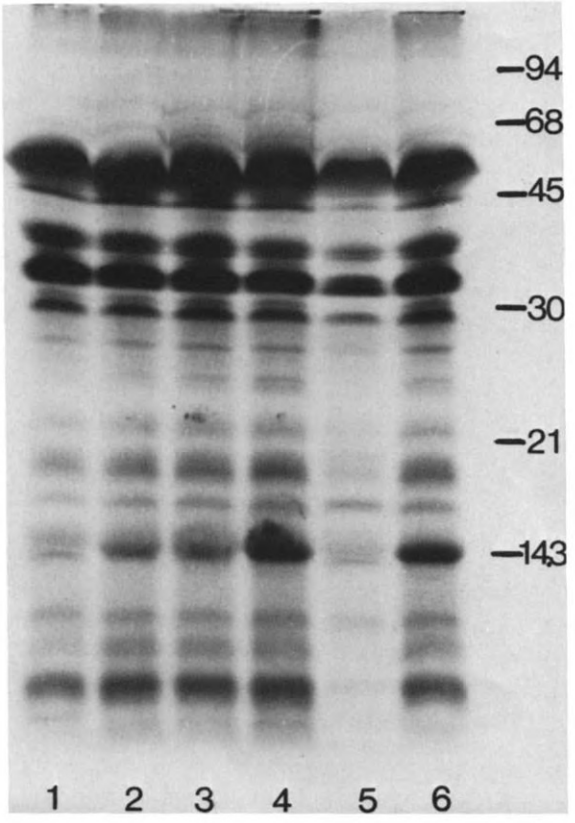

Fig. 3. Translation of phage MS-2 RNA. Fluorography of monodimensional SDS–polyacrylamide gel electrophoresis of the products synthesized by the nuclease-treated system in the presence of: (1) no addition; (2) 10 μg MS-2 phage RNA; (3) 12.5 μg MS-2 phage RNA; (4) 25 μg MS-2 phage RNA; (5) 25 μg MS-2 phage RNA and 100 μg chloramphenicol; (6) 25 μg of MS-2 phage RNA and 50 μg cycloheximide; numbers represent $M_r \times 10^{-3}$.

has an app. M_r (~17000) similar to that of the virus coat protein [9]. Finally, mRNAs from *Euglena gracilis* chloroplast gave rise to a polypeptide pattern different in the low-M_r range from that observed when spinach chloroplast mRNAs were used. An increase in the radioactivity in the band corresponding to the large subunit of ribulose-biphosphate carboxylase (LS) was also evident but no effort was made to establish if the band corresponds to *E. gracilis* LS.

3.3. *Translation of coliphage MS-2 RNA*

Further evidence that the chloroplast system translates faithfully the RNA from this phage was obtained in fig. 3. An increase in the radioactivity of the band with the mobility of the coliphage coat protein was evident on increasing the amount of phage RNA added to the reaction mixtures (slots 2–4). Addition of chloramphenicol drastically reduced the radioactivity incorporated in this band (slot 5) while cycloheximide had no effect (slot 6). The production in vitro of MS-2 coat protein was confirmed by comparing the partial proteolytic digestion with chymotrypsin (fig. 4) and papain (not shown) of the bands with the mobility of authentic phage MS-2 coat protein produced in the *E. coli* and chloroplast systems.

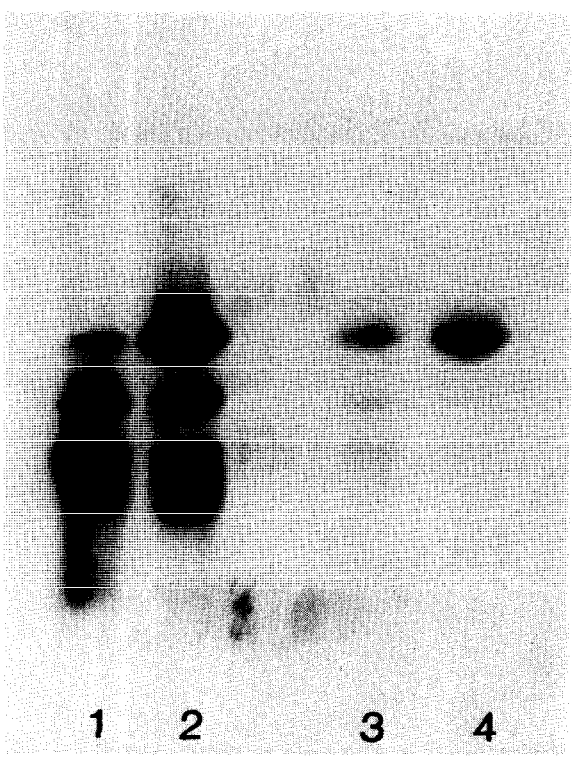

Fig. 4. Partial proteolytic digestion of the MS-2 phage coat protein synthesized in the chloroplast and *E. coli* systems. Fluorography of the radioactive bands with the same mobility of authentic MS-2 coat protein synthesized in the *E. coli* (1,2) or the chloroplast system (3,4) digested with 5 μg/ml (2,4) or 50 μg/ml (1,3) of chymotrypsin.

4. CONCLUSION

The system that we have developed from isolated spinach chloroplasts appears to be of the prokaryotic type since its activity is inhibited by chloramphenicol and not by cycloheximide. Heterologous mRNA are translated by the system with fidelity in the case of the RNA from phage MS-2 and, possibly, TMV.

This system may be utilized to study the conditions that control the fidelity of translation of chloroplast mRNAs. It would be of interest to determine whether it translates faithfully the mRNAs for the M_r 32000 thylakoid polypeptide from *Spirodela oligorrhiza* or the LS from *E. gracilis*, reported to be translated only, or predominantly, by the wheat-germ system [10,11]. Finally, such a system may be of considerable importance for determining the pattern of proteins that are expressed at different stages of development of plastids or to assess the protein synthetic activity in vitro of plastids at different stages of development. Its usefulness could be further enhanced if it were possible to obtain a transcriptional activity coupled to translational.

ACKNOWLEDGEMENTS

G.C. is a postdoctoral fellow of Consiglio Nazionale delle Ricerche. This investigation was supported by grants from Consiglio Nazionale delle Ricerche.

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