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Expression and functional assembly into bacterial ribosomes of a nuclear-encoded chloroplast ribosomal protein with a long NH_2-terminal extension

Klaus Giese* and Alap R. Subramanian

Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin 33, Germany

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Chloroplast ribosomal protein L13 is encoded in the plant nucleus and is considerably larger than its eubacterial homologue by having NH_2- and COOH-terminal extensions with no homology to any known sequences (Phua et al., J. Biol. Chem. 264, 1968–1971, 1989). We made two gene constructs of L13 cDNA using the polymerase chain reaction (PCR) and expressed them in *Escherichia coli*. Analysis of the ribosomes and polysomes from these cells, using an antiserum specific to chloroplast L13, shows that the expressed proteins are incorporated, in the presence of the homologous *E. coli* L13, into functional ribosomes which participate in protein synthesis (i.e. polysomes). Evidence is obtained that the large NH_2-terminal extension probably lies on the surface of these 'mosaic ribosomes'. This first report of the assembly into *E. coli* ribosomes of a nuclear-coded chloroplast ribosomal protein with terminal extensions thus suggests an extraordinary conservation in the function of eubacterial type ribosomal proteins, despite the many changes in protein structure during their evolution inside a eukaryotic system.

Chloroplast ribosome; Endosymbiont hypothesis; L13 protein expression; Chloroplast–bacterial hybrid ribosome

1. INTRODUCTION

Chloroplast ribosomes are assumed to be of eubacterial origin, derived from one or more photosynthetic endosymbionts over 10^9 years ago [1,2]. Due to a presumed massive transfer [3] of the endosymbiont's gene into the host nucleus, todays chloroplast genomes (reviewed in [4]) contain only about 3% (~150 kb) of the total DNA found in eubacteria. The great majority of chloroplast proteins are therefore encoded in the nucleus, including over 40 proteins of the chloroplast ribosome [5]. Recently it has been shown that two ribosomal proteins (r-proteins), L21 and L35, and the elongation factor Tu (EF-Tu) are chloroplast-encoded in lower plant taxa [6–8] but nuclear-coded in flowering plants [9–11], indicating a temporal gradation in the presumed gene transfer.

Several nuclear-coded chloroplast r-proteins have now been characterized at the cDNA level [9,10,12–16] and/or by protein purification and sequencing [9,10,14–16]. This work has shown the presence of novel proteins in the chloroplast ribosome that have no counterparts in the *E. coli* ribosome [12–14]. Another character of the nuclear-coded r-proteins is that, as a rule, they contain long NH_2- and/or COOH-terminal extensions [9,16] as compared to the homologous counterparts in *E. coli* or *Bacillus stearothermophilus* [17]. Such extensions are absent, generally, in the chloroplast r-proteins that are encoded in the organelle DNA [4,5].

Since chloroplast ribosomes are of the eubacterial type by many structural and functional criteria [18], it is an open question whether these novel or lengthened proteins, encoded in the plant nucleus, will assemble into bacterial ribosomes, if expressed in *E. coli*. Here we report the efficient assembly into *E. coli* ribosomes of nuclear-coded spinach chloroplast L13, which has N- and C-terminal extensions of 52 and 9 amino acid residues, respectively. Polysome analysis shows that these 'mosaic ribosomes' are functional and participate in protein synthesis. In polysomes they are present in approximately the same proportion as in total ribosomes. The N-terminal extension is removable by mild protease digestion indicating its location on the ribosome surface.

Abbreviations: r-protein, ribosomal protein; EF-Tu, elongation factor Tu; PCR, polymerase chain reaction; CTE, COOH-terminal extension; NTE, NH_2-terminal extension; SDS, sodium dodecyl sulfate

**Present address:* Department of Microbiology and Immunology, University of California, San Francisco, CA 94143, USA

Correspondence address: A.R. Subramanian, Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin 33, Germany. Fax: (49) (30) 8307 380.

2. EXPERIMENTAL

2.1. *PCR-mediated construction of L13 expression clones*

L13 cDNA was isolated from the recombinant λgt11 phage CL13-1 [16] by restriction with *SacI-KpnI*, ligate [20] into the vector

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pT7T318U, and used to transform *E. coli* strain WK6 [21]. Positive plasmid clones were identified by restriction analysis and confirmed by DNA sequencing by the dideoxy method [22]. PCR amplification [23] of two defined parts of the chloroplast L13 coding region was done with 100 ng of the plasmid DNA and 1 μM each of either primer (L13', 5'dGAATGGCGTCAACTCAAAGAT or L13", 5'dCCTAAATCTGCTGACCATGT) and λgt11 reverse primer (New England Biolabs), 200 μM dNTP (dATP, dTTP, dGTP, dCTP) and 7.5 U of *Taq* DNA polymerase in a final volume of 100 μl on an Intelligent Heating Block (Biometra) in 25 cycles of 1 min at 95°C followed by 1 min at 45°C and 2 min at 74°C. The final extension step was done at 72°C for 10 min. The amplified products were purified on Qiagen columns (Diagen, Düsseldorf), phosphorylated at the 5' ends by treatment with T4 polynucleotide kinase and 1 mM ATP [20], and ligated into the expression vector pJLA502 [24]. After transforming *E. coli* WK6 [21] the recombinant clones were identified by restriction analysis and verified by DNA sequencing.

2.2. *Cell culture, ribosome isolation and protein analysis*

E. coli WK6 cultures containing the individual plasmids were grown to A_{560} of 0.1 (Lange-Photometer) at 30°C and shifted to 42°C for several hours or overnight for L13 expression. Aliquots of the cells were boiled in 4% SDS and analyzed on 15% polyacrylamide gel [25]. Ribosomes were isolated by pelleting a lysate (French Press) for 3 h at 100 000 × *g* [26]. 100 μg TP70 were used for 2D gel electrophoresis [26]. The proteins were blotted onto Immobilon membrane (Millipore) using Pegasus (Phase, Mölln) semi-dry blotter [14], and immuno-stained with chloroplast L13 antiserum [16] at 1:1500 dilution. A monoclonal antiserum to *E. coli* L13 (1:100 dilution: gift from G. Stöffler and M. Stöffler-Meilicke) was used to detect *E. coli* L13. Color development after the second antibody (peroxidase-conjugated IgG) binding was with 0.5 mg/ml 4-chloro-1-naphthol/0.03% H_2O_2 [14].

2.3. *Polysome isolation and analysis*

Cells carrying chloro L13" or L13' construct were induced by temperature shift and further grown for 3 doublings. Cells were isolated, lysed by the freeze–thaw lysozyme method, and polysomes isolated [27]. Aliquots were loaded onto 10–40% sucrose gradients and centrifuged in a SW40 rotor (Beckman), 5 h, 26 000 rpm, 4°C. The gradients were fractionated and ribosomes in pooled fractions pelleted (16 h, 110 000 × *g*). Electrophoresis and immuno staining were done as described. For histogram, equivalent amounts of the different fractions were electrophoresed, immuno-stained and quantitated by densitometry.

2.4. *Protease digestion of chloroplast L13-containing ribosomes*

100 μg of the hybrid ribosomes in 100 μl TKM buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM Mg-acetate) were incubated with or without 2 μg Glu-C proteinase (Boehringer-Mannheim) at 37°C for different times. The ribosomes were then boiled in 2% SDS, electrophoresed and, after blotting, the membrane was immuno-stained for chloroplast L13.

2.5. *Materials*

Restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were purchased from Boehringer-Mannheim, *Taq* polymerase and [α-^{35}S]dATP from Amersham, T7 sequencing kit and pT7T318 vector from Pharmacia, the expression vector pJLA502 from Medac and peroxidase-conjugated, goat anti-rabbit IgG from Dianova. Oligonucleotide primers were synthesized on an Applied Biosystems DNA Synthesizer.

3. RESULTS AND DISCUSSION

Two different constructs (L13' and L13") of chloroplast L13 were made using the L13 cDNA [16] and PCR amplification as illustrated in Fig. 1. The L13' encodes 194 amino acid residues and contains N- and C-terminal extensions of 43 and 9 residues, respectively. The shorter L13" encodes 150 residues and includes only the C-terminal extension. The specific DNA fragments were then cloned into the expression vector pJLA502 and introduced into *E. coli*. The expressed proteins were analyzed in total cell extracts and in isolated ribosomes. SDS-gel electrophoresis showed two new protein bands migrating at positions corresponding to the calculated molecular weights (22408 for L13' and 17068 for L13"), and reacting with an antiserum to chloroplast L13 (Fig. 2). The incorporation into ribosomes was analyzed by two-dimensional gel electrophoresis. The new protein spot in Fig. 2B corresponded to L13" in size and pI. The final identification was made by immuno staining as shown in Fig. 2C.

It has been previously shown in our laboratory that several antisera against *E. coli* L13 cross-reacted with spinach chloroplast L13 [28]. Interestingly, the antiserum against chloroplast L13, which we prepared and used in the present and an earlier experiment [16], showed little or no cross-reaction to *E. coli* L13. Hence the immunostaining of *E. coli* L13 in the Western blots was carried out using a monoclonal antiserum to *E. coli* L13 (see section 2). In Fig. 2C the arrowhead shows *E. coli* L13 so identified whereas the two arrows show chloroplast L13' and L13" immunostained using the chloroplast L13 antiserum.

To test if the ribosomes containing chloroplast L13 constructs were functional, polysomes were isolated from induced early log-phase cultures expressing the chloroplast constructs. After SDS-gel electrophoresis and immunoblotting both protein constructs were identified in the polysomes (Fig. 3). Quantitation of the amounts of expressed L13' and L13'' in ribosomes, supernatant and cell debris (Fig. 3B) showed that a major part occurs in ribosomal particles; the proportion in polysomes was approximately the same as that in total ribosomes. Thus chloroplast L13 is incorporated into functional *E. coli* ribosomes in the presence of the homologous bacterial L13, despite a $\simeq$50% change in the primary structure and the presence of NTE and CTE.

Because the L13 r-protein functions as an early assembly protein by binding to the 23S rRNA [29] it was of interest to determine whether the chloroplast-specific NTE and CTE are in the interior or on the surface of these hybrid ribosomes. The isolated ribosomes were incubated with endoproteinase Glu-C (specific to glutamic acid residues) and shifts in the mobilities of the chloroplast constructs were determined after electrophoresis and immuno staining. As indicated in Fig. 1B, there are 5 Glu-X peptide bonds in the N-terminal extension; only one Glu-X bond is present in the C-terminal extension, but it is at the last two residues [16]. There was a shift (Fig. 4) in M_r for L13', but no change for L13'' which lacks the NTE but con-

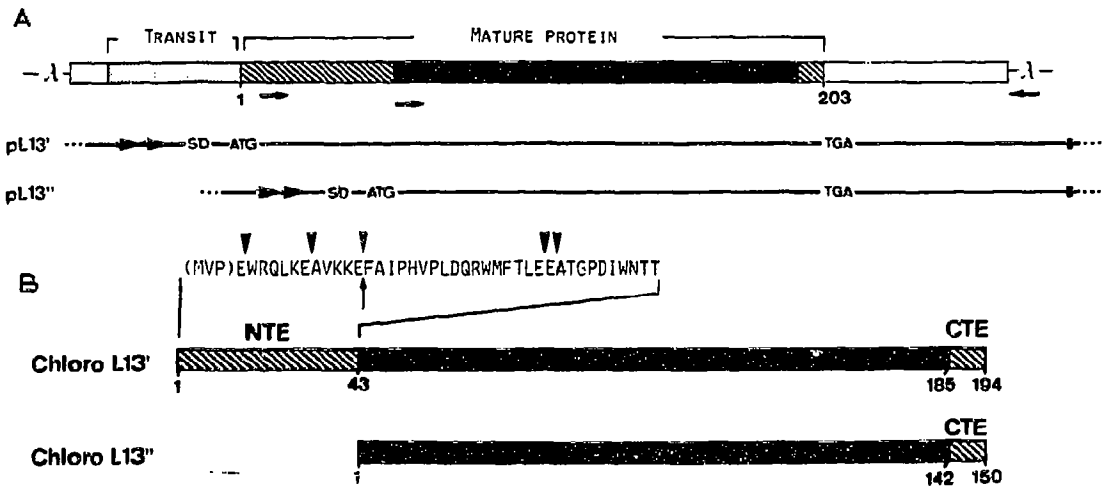

Fig. 1. Schematic diagram of the L13 cDNA and the two constructs used in this study. The shaded and lined areas show, respectively, the *E. coli* L13 homologous region and the N- and C-terminal overhangs (NTE, CTE). A. The three arrows indicate the positions and directions of the three oligonucleotide primers used for PCR amplification. Arrowheads represent the two promoters ($P_R + P_L$) in the expression vector. SD, Shine–Dalgarno site. B. Chloro L13' (194 amino acid residues) represents the almost complete mature chloroplast L13 (203 residues). Chloro L13'' (150 residues) lacks NTE but includes CTE (9 residues). It corresponds in length to *E. coli* L13, which has 142 residues [17]. The complete amino acid sequence of the NTE is given, with arrowheads showing the cleavage sites for endoproteinase Glu-C. The arrow below indicates the putative cleavage site from the protease experiment (Fig. 4). The three amino acids in brackets are derived from the expression vector.

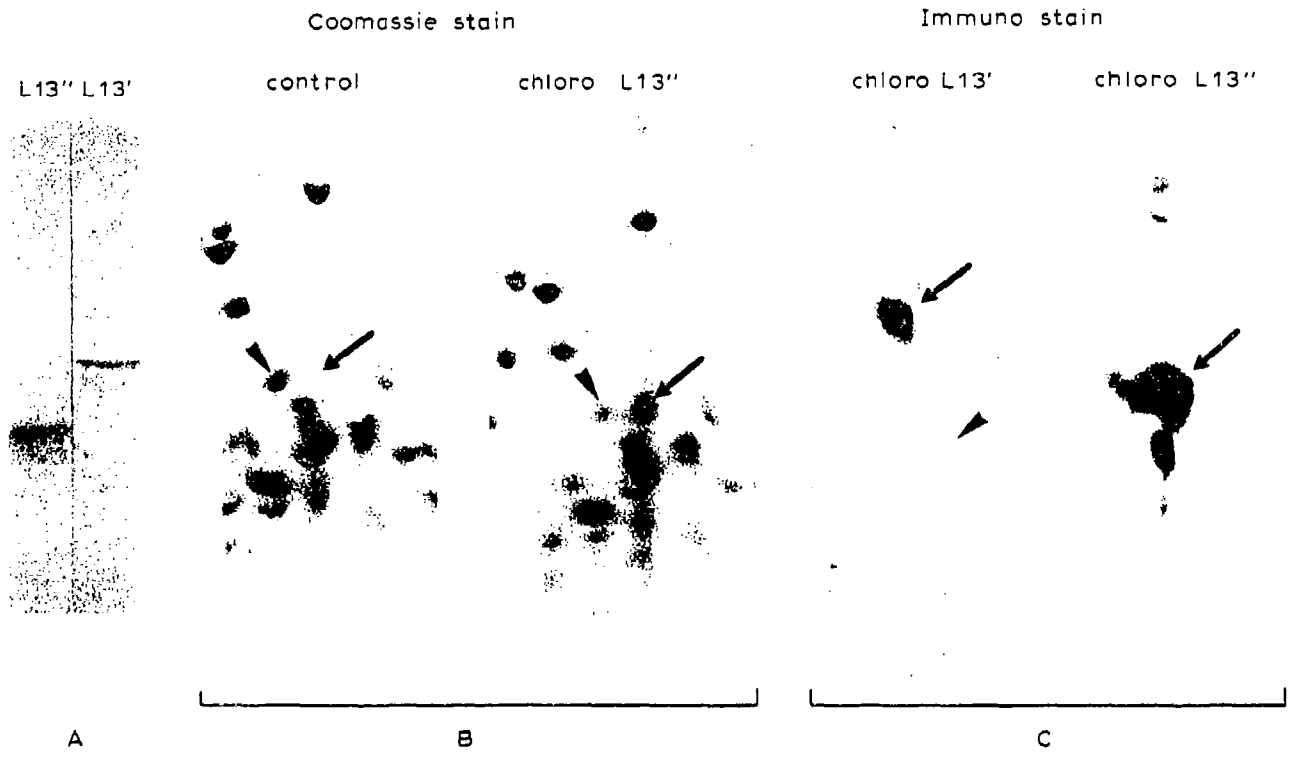

Fig. 2. Detection of expressed chloroplast protein constructs in *E. coli* cell lysates and isolated 70S ribosomes (TP70). A. Cell lysate after one-dimensional gel electrophoresis and immuno staining with chloroplast L13 antiserum. B. Isolated ribosomes after two-dimensional gel electrophoresis and Coomassie brilliant blue staining. Part of the gel where *E. coli* L13 (arrowhead) and chloro L13'' (arrow) migrate are shown. C. Immuno-stained 2D gel blots (Western) of TP70 from cells expressing chloro L13' and chloro L13''. The arrowhead shows *E. coli* L13. Since the chloroplast L13 antiserum used in this study did not cross-react with *E. coli* L13, a monoclonal antiserum specific to *E. coli* L13 was used to identify the latter on the same blot.

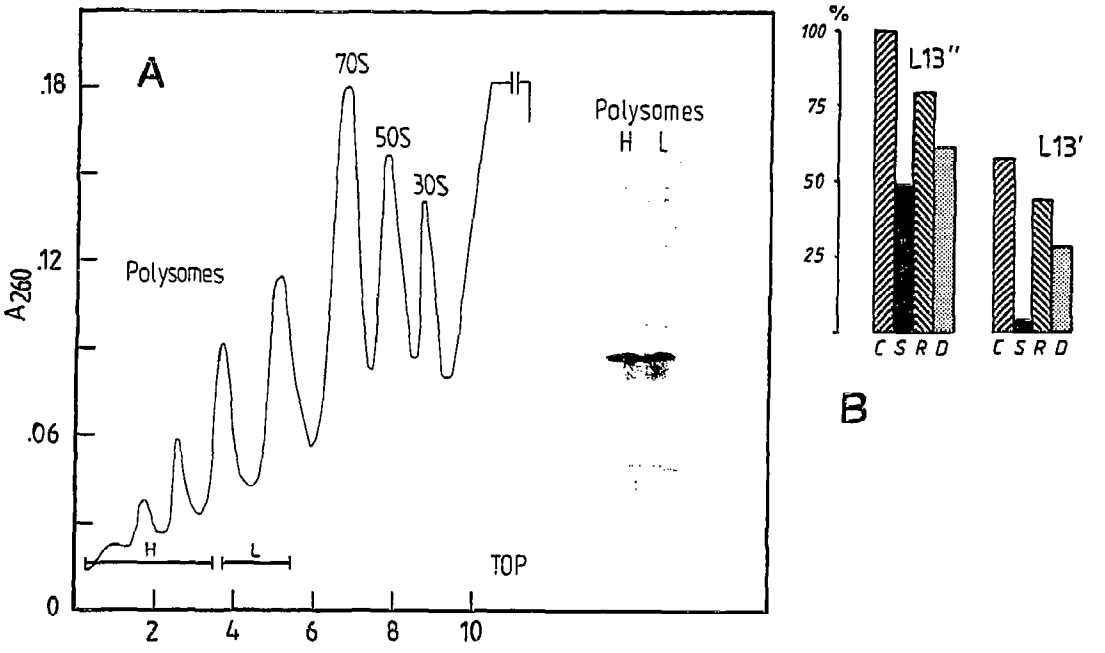

Fig. 3. Demonstration of chloro L13'' in functional ribosomes by separating monosomes and polysomes and immuno staining. A. Sucrose gradient separation of ribosomal particles and on the right the detection of chloro L13'' in both heavy and light polysomal fractions (similar results were obtained with the chloro L13' construct). L, disomes and trisomes; H, heavier polysomes. B. Distribution of the two chloroplast construct proteins in various cell fractions. C, whole cells; S, high-speed supernatant (S-100); R, ribosomes, D, cell debris.

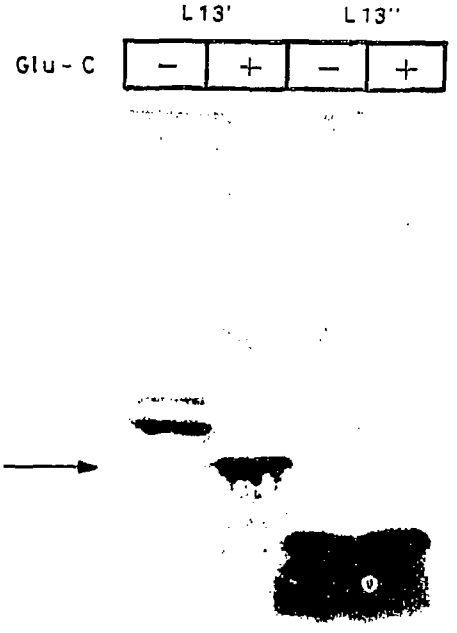

Fig. 4. Glu-C protease experiment on ribosomes showing the removal of NTE from L13' and the protection of CTE in both L13' and L13''. After protease digestion (see section 2) L13' migrated with M_r ≈ 20.800 (M_r of L13' = 22.408) corresponding to a loss of ≈ 15 amino acid residues (see Fig. 1 for the putative cleavage site). The L13'' on the other hand was not detectably affected by the protease.

tains CTE. The shift in the M_r of L13' corresponded to the removal of ≈ 15 residues from the N-terminus (see Fig. 1). It thus appears that the NTE probably lies exposed on the ribosomal surface. The complete protection of L13'' indicates that this molecule is inaccessible when incorporated in ribosomes.

Bacteria expressing the chloroplast L13 did not show any striking phenotypic defects other than a slower growth rate (factor of ≈ 1.5), which could be due to the difference in codon usage between chloroplast L13 [16] and the highly expressed genes in *E. coli* [30]. Comparison shows a number of striking differences: codons GGA (Gly), AGG/AGA (Arg) and ATA (Ile) are not used in the highly expressed *E. coli* genes [30] but are frequently (20–35%) used in the chloroplast *rpl*13 [16].

In conclusion this study shows the expression and efficient assembly into *E. coli* ribosomes of two r-protein constructs derived from a plant nuclear gene. The assembly occurs in the presence of the competing homologous protein of *E. coli*, which is an early assembly protein of the 50S subunit. One of the chloroplast constructs contains a long NH_2-terminal extension and both contain shorter C-terminal extensions, but the hybrid 'mosaic ribosomes' accommodating the constructs were functionally active, as deduced from their presence and proportion in polysomes. These results thus show a surprising degree of conservation in the functionality of eubacterial type ribosomal proteins during a long period of evolution ($>10^9$ years) inside the eukaryotic system.

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