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Authors
Yang, Austin J.
Mulligan, R. Michael

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Identification of a 4.5S-like ribonucleoprotein in maize mitochondria

Austin J. Yang* and R. Michael Mulligan*

Department of Developmental and Cell Biology, University of California, Irvine, CA 92697-2300, USA

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ABSTRACT

Escherichia coli has a ribonucleoprotein complex that is composed of a 114 nucleotide 4.5S RNA and a 48 kDa polypeptide (P48) that has been demonstrated to function in translation and in the secretion of periplasmic polypeptides. A small RNA of ~220 nucleotides has been identified in maize mitochondria that includes sequence identity with the highly conserved domain of the bacterial 4.5S RNA. The transcript is mitochondrially encoded and maps to a region upstream of the gene for ATP synthase subunit I. The mitochondrial 4.5S-like RNA has 15 nucleotides of sequence identity with the highly conserved region of the bacterial 4.5S RNA. Sucrose density gradient centrifugation of a maize mitochondrial lysate demonstrated that the 4.5S RNA is a component of a high molecular weight complex under native conditions, and could be disrupted by phenol. Anti-P48 immune serum immunoprecipitated a mitochondrial protein of ~48 kDa, and RNA gel blot analysis of the immunoprecipitation reaction indicated that the 4.5S-like RNA co-immunoprecipitated with the 48 kDa polypeptide. The mitochondrial 4.5S ribonucleoprotein complex could function in translation or protein targeting.

INTRODUCTION

The 4.5S RNA is a small bacterial RNA that has been identified from a number of procaryotes (1,2). The 4.5S gene (ffs) is essential for growth of Escherichia coli (3), and other procaryotic 4.5S genes can complement E.coli strains depleted in the 4.5S RNA (2). The 4.5S RNA of E.coli is a 114 nucleotide (nt) transcript, and ffs genes from other procaryotes express small structural transcripts of 105–271 nt when expressed in E.coli (2). The procaryotic 4.5S RNAs have a highly conserved 22 nt domain and conserved secondary structure, although the 4.5S RNAs differ in size and have relatively little (29%) sequence identity outside of the conserved domain (2,4).

The function of the 4.5S RNA has been controversial and continues to be an intriguing puzzle. Suppression analysis of a 4.5S RNA depletion strain of E.coli lead to the identification of suppression mutations that mapped to elongation factor G (EF-G) and the EF-G binding site on the 23S rRNA (5), or resulted in increased concentrations of uncharged valyl-, glutamyl- or alanyl-tRNA (6). In addition, the 4.5S RNA is known to sediment with ribosomes or polyribosomes in sucrose gradients (5), and is released by puromycin (5–7). Thus, genetic and biochemical evidence support a role for the 4.5S RNA in translation.

The 4.5S RNA associates with a 48 kDa polypeptide (P48, product of the ffh gene) to form a ribonucleoprotein (RNP) complex that has considerable similarity to the cytosolic signal recognition particle (SRP) of eucaryotic cells. The SRP binds to the hydrophobic signal sequence of nascent polypeptide chains and targets the synthesis of these polypeptides to the rough endoplasmic reticulum. The SRP is composed of a ~300 nt RNA (SRP-RNA) and six polypeptides (8) and has secondary structure and sequence identity with the bacterial 4.5S/P48 RNP (9,10).

The conserved 22 nt domain of the 4.5S RNA is also conserved in mammalian SRP-RNAs, as is the predicted secondary structure in this region of the SRP-RNA (11). P48 and SRP54, the signal sequence binding subunit of the SRP, have 31% sequence identity over the entire length of the polypeptides, as well as a highly conserved GTP-binding domain (12). In addition, SRP54 and P48 interact with the 4.5S or SRP-RNAs interchangeable: P48 binds both 4.5S RNA and human SRP-RNA (10); and SRP54 binds SRP-RNA or 4.5S RNA (10,12).

Similarities between the 4.5S/P48 RNP and the SRP have lead to speculation and experimentation on the possible role of the RNP in protein targeting in bacteria (9,10). Expression of 4.5S RNA levels through a repressible promoter have been utilized to study the phenotype of 4.5S RNA limitation (9,10). Depletion of the 4.5S RNA lead to accumulation of unprocessed precursors of β-lactamase; in addition, maltose binding protein was depleted, although accumulation of precursors was not evident (9,10). Thus, processing of the β-lactamase precursor was dependent on the 4.5S RNA. In addition, the role of P48 has been analyzed through mutation of the ffh gene and expression through a regulated promoter (13,14). Depletion of P48 polypeptide results in the accumulation of precursors for secretory polypeptides such as lambda B, maltose binding protein, alkaline phosphatase and ribose binding protein. These results strongly indicate the 4.5S/P48 RNP must play some role in the bacterial secretory pathway.

Recently a chloroplast homologue of the P48 polypeptide, 54CP, has been identified (15). The 54CP protein has 44%
sequence identity with the ffl gene product, and acts as a molecular chaperone in the chloroplast stroma (16). 54CP binds the light harvesting chlorophyll a/b binding protein after import into the chloroplast, and may be involved in targeting the protein to the thylakoid.

The importance of the 4.5S/P48 RNP in bacterial gene expression is clear, although the function would appear to include both roles in protein synthesis and secretion. The presence of a procaryotic homologue of the SRP in bacteria and chloroplasts prompted us to determine whether higher plant mitochondria may have retained a structurally related RNP.

MATERIALS AND METHODS

Plant material and isolation of maize mitochondria

Mitochondria were isolated from 7–10 day old seedlings of Zea mays (B37N) and purified by centrifugation on a discontinuous sucrose density gradient and resuspended in homogenization buffer containing 0.4 M mannitol (17).

Isolation and hybridization of maize mtRNA

Total maize mitochondrial RNA (mtRNA) was extracted at 65°C with phenol equilibrated with 0.3 M sodium acetate (pH 5.0) and subsequently with chloroform (18). Mitochondrial RNA was fractionated in an 8 M urea/6% polyacrylamide gel in 1× TBE buffer [TBE: 89 mM Tris-borate, 2 mM EDTA (pH 8.0)]. Gel temperature was maintained at 55°C during the electrophoresis. RNAs were electro-transferred to a nylon membrane (Hybond-N+) at 25–40 V (100 mA) at 4°C for 16 h. The RNAs were UV cross-linked and the RNA blot was pre-hybridized [prehybridization at 25–40 V (100 mA) at 4°C] for 4–6 h. The annealed RNA was hybridized at 45°C for 4–6 h. The sequence of the oligonucleotide probe for the 4.5S RNA (5′-GCTGCTTCCTT-GCTGCTTCCTT-GCTGCTTCCTT-GCTGCTTCCTT-3′) was end-labeled oligonucleotide probe to the conserved domain of the 4.5S RNA (5′-GCTGCTTCCTT-GCTGCTTCCTT-GCTGCTTCCTT-GCTGCTTCCTT-3′); 2). Blots were washed with 2× SSPE at room temperature for 10 min, 2× SSPE plus 0.1% SDS for 1 h at 42°C and 0.1% SSPE for 10 min at 42°C. The RNA was detected by autoradiography.

RNase protection

A 1.3 kb BamHI–PstI restriction fragment from maize mitochondrial DNA that encoded the 4.5S-like RNA sequence was cloned into pBluescript and identified as pBP1.3. Maize mtRNA (5–10 µg) and 1 × 10⁶ c.p.m. T7 or T3 promoted transcripts of pBP1.3 were precipitated and the RNA was collected by centrifugation. The RNA was resuspended in 10µl hybridization buffer (40 mM PIPES–NaOH pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide). After denaturation at 85°C for 10 min, the RNA was hybridized at 45°C for 4–6 h. The annealed RNA was treated with 300 U RNase T1 and 100 ng RNase A in 300µl RNase buffer (10 mM Tris–HCl pH 7.7 and 300 mM NaCl) at 30°C for 1 h. The RNase digestion was terminated with 0.1% SDS, 100 µg protease K. The protected RNA fragments were extracted with phenol/chloroform and precipitated. The protected fragments were fractionated in an 8 M urea/6% polyacrylamide sequencing gel and autoradiographed.

Protein gel blots

Maize mitochondrial protein (10 µg) was separated on a 10% SDS–PAGE and electrotransferred to a nitrocellulose membrane. The membrane was blocked with 10% (w/v) dried milk in the TBS solution (0.5% Tween 20, 20 mM Tris–HCl pH 7.5, 500 mM NaCl) at 4°C overnight. The blot was washed three times with 50–100 µl TBS at room temperature for 5 min. The blot was incubated with either rabbit anti-E.coli P48 immune or preimmune serum at a dilution of 1:1000 for 1 h at room temperature. The immunoblot was washed three times with TBS for 10 min. The primary antibodies were detected by the alkaline phosphatase conjugated secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate; Sigma) at a dilution of 1:3000. Immunoblot was then washed three times with TBS and incubated with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium for 5–30 min.

Preparation of mitochondrial extracts

Maize mitochondria were washed twice with 1 ml native buffer [50 mM triethanolamine–HCl (pH 7.5), 100 mM potassium acetate, 16 mM magnesium acetate, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% Nikkol (octaethylene glycol dodecyl ether)] at 4°C and collected by centrifugation at 10 000 g for 10 min (9). The mitochondrial pellet was resuspended in 1 ml native buffer containing 0.5 mM phenylmethylsulfonyl fluoride, and 1 mg/ml of each of leupeptin, pepstatin, chymostatin and antipain. The mitochondrial suspension was sonicated four times with a Biosonicator at maximum setting for 10 s. The mitochondrial lysate was clarified by centrifugation at 10 000 g for 10 min; and the supernatant was saved and stored at −80°C.

Immunoprecipitation of the RNPs

The experiment was performed essentially as described (9). Anti-E.coli P48 antibody was bound to protein G–agarose beads for 1 h in phosphate buffered saline (PBS) containing 2% Triton X-100 at room temperature. The antibody–protein G beads were washed once in PBS containing 1 M NaCl, and twice with native buffer at room temperature. The antibody–protein G beads were then resuspended with 3 vol native buffer.

The mitochondrial lysate (~100 µg protein) was mixed with 40 µl of a 25% slurry of antibody–protein G beads containing in a microfuge tube. The reaction was incubated at room temperature for 1 h with constant rotation. The beads were washed twice with 1 ml native buffer, transferred to a new microfuge tube, and washed twice with 1 ml PBS. Immunoprecipitated RNA was eluted with 1% SDS, 50 mM Tris–HCl (pH 7.5), 25 mM EDTA and 20 µg glycogen. RNA was extracted with phenol/chloroform, precipitated with ethanol, and subjected to RNA gel blot hybridization with the oligonucleotide probe for the 4.5S RNA.

Sucrose gradient analysis of mitochondrial lysates

KCl was added to the mitochondrial extract to a final concentration of 100 mM, and the extract was clarified by centrifugation at 10 000 r.p.m. in the microfuge as described (10). The supernatant was then layered on a 5–20% linear sucrose density gradient containing 10 mM Tris–HCl pH 7.0, 5 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol, 0.01% (w/v) Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride. Gradients were centrifuged in a Beckman SW41TI rotor at 30 000 r.p.m. (154 000 g) for 15 h. Fractions
RESULTS

Maize mtDNA encodes and expresses a 4.5S-like RNA

In order to determine whether a small mitochondrial transcript with sequence similarity to the procaryotic 4.5S RNA was present in maize mitochondria, RNA gel blots were hybridized with an oligonucleotide probe for the 22 nt conserved domain of 4.5S RNA. The probe detected a transcript of ~220 nt (Fig. 1), and indicated that a small transcript with sequence similarity to the procaryotic 4.5S RNA was present in maize mitochondria.

The E. coli 4.5S RNA probe also hybridized to a genomic maize mitochondrial DNA gel blot. The oligonucleotide probe hybridized intensely to a 4.3 kb BamHI restriction fragment, and weakly to an additional restriction fragment (data not shown). The 4.3 kb BamHI restriction fragment was mapped to a region upstream of the atp1 promoter by hybridization of the 4.5S RNA probe to restricted cosmid clones that represent the entire maize mitochondrial genome (19, 20). DNA gel blot analysis of restricted plasmid DNA demonstrated that 4.5S RNA probe hybridized to DNA sequences between the Apal and Kpnl sites. DNA sequence analysis identified a region with sequence identity to the 4.5S RNA probe (Fig. 2A).

The maize mtDNA sequence represented in subclone pBP1.3 was shown to encode a 220 nt 4.5S-like RNA by an RNase protection assay. RNA probes were prepared by in vitro transcription with either T3 or T7 RNA polymerase and utilized in RNase protection assays (Fig. 2B). A 220 nt fragment was protected from the T3 promoted probe, demonstrating that the 4.5S-like RNA was encoded by the pBP1.3 subclone.

The sequence of the 4.5S-like RNA is shown in Figure 3A. The transcribed sequences are underlined (approximately nucleotides 101–321) and the region of sequence identity to the 4.5S RNA (nucleotides 192–210) is double underlined. The region of sequence identity with the 4.5S RNA includes an 18 nt sequence (GTCTAGAAGGGAAAGCAGC) with 15 nt of sequence identity within the 22 nt conserved domain (GTCCGAAGGAAGCAGC) of the E. coli 4.5S RNA. The nucleotide sequences of bacterial 4.5S RNAs that are outside of the conserved region have a very low degree of sequence identity (2, 4), and the remainder of the maize mitochondrial 4.5S-like RNA and the procaryotic 4.5S RNAs have very little sequence identity. Although the procaryotic 4.5S RNAs have little sequence identity outside the conserved domain, the genes for these small transcripts complement the phenotype of the 4.5S RNA-depleted bacterial strain (1), and the sequence of the conserved domain and overall secondary structure of the 4.5S RNAs are apparently sufficient to confer function of these small transcripts.

Bacterial 4.5S RNAs have a highly conserved domain that can be folded into a stem–loop structure with characteristic bulges (4). Figure 3B shows the predicted secondary structure of the E. coli 4.5S RNA. The corresponding region of the mitochondrial 4.5S-like RNA sequence can be folded into a similar structure.

Interaction of the 4.5S-like RNA with a P48-like polypeptide

The E. coli 4.5S RNA has been shown to interact with a 48 kDa polypeptide (P48) to form a ribonucleoprotein complex. The presence of a maize mitochondrial polypeptide that was immunologically related to the P48 antigen of E. coli was tested by reaction with anti-P48 immune sera (Fig. 4A). Immune sera specifically recognized a polypeptide from maize mitochondria (lane 1), that was comparable in size with purified P48 from E. coli (lane 2). Preimmune sera reacted non-specifically with a small polypeptide present in the maize mitochondrial fractions (lanes 1 and 3).

The P48 polypeptide of E. coli has substantial amino acid sequence identity with the mammalian SRP54 polypeptide of the cytosol (12). The polypeptide present in the maize mitochondrial fraction was specifically associated with the mitochondrial fraction (Fig. 4B). The P48-like antigen was detected only in the mitochondrial fraction (lane 2), and not in the post-mitochondrial fraction (lane 3).

Figure 1. RNA gel blot analysis for 4.5S-like RNA from maize mitochondria. Maize mtRNA was fractionated on a 6% urea/polyacrylamide gel and electroblotted to a nylon membrane. The RNA gel blot was probed with a 22 nt oligonucleotide that is complementary to the conserved domain of the 4.5S RNA of E. coli. The migration of DNA size standards are indicated.

Figure 2. Identification of the coding sequence for the 4.5S-like RNA from maize mitochondria. (A) Restriction map of the 4.5S-like mtDNA sequence. (B) RNase protection analysis for transcription products from the pBP1.3 clone. Radioactive BPI.3 RNA probes were hybridized with mtRNA and digested with RNase T1 and RNase A. Protected RNA fragments were separated by electrophoresis and visualized by autoradiography. Lanes 1–3 labeled the T7-promoted RNA probe with: 1, no mtRNA; 2, no RNase; 3, complete reaction. Lanes 4–6 used the T3-promoted RNA probe with: 1, no mtRNA; 2, no RNase; 3, complete reaction. End-labeled DNA fragments were utilized as size standards.
Figure 3. (A) Sequence of the 4.5S-like RNA from maize mitochondria. Sequence of the 4.5S-like RNA gene from maize mitochondria. The approximate transcribed sequence is single underlined, and the region of sequence identity with the 22 nt conserved domain of the E. coli 4.5S RNA is double underlined. (B) Secondary structure of the E. coli 4.5S RNA and possible secondary structure of the maize mitochondrial 4.5S-like RNA.

Figure 4. Maize mitochondria have a P48-like polypeptide. (A) Ten micrograms of maize mitochondrial protein (lanes 1 and 3) or 100 ng purified E. coli P48 protein (lane 2) was separated by SDS–PAGE and electroblotted to nitrocellulose. The protein gel blots were probed with anti-P48 sera (lanes 1 and 2) or preimmune sera (lane 3). (B) P48-like polypeptide is associated with the mitochondrial fraction. Total cell lysate was prepared from 7 day old maize seedlings by centrifugation at 10 000 g for 15 min to separate the post-mitochondrial supernatant from mitochondrial pellet. Protein gel blots (30 µg) of the post-mitochondrial supernatant (lane 1) and crude mitochondrial pellet (lane 2) were analyzed by hybridization with anti-P48 antibody. Protein size standards are shown along the margins.

If the sample was extracted with phenol to disrupt protein–nucleic acid interactions prior to centrifugation, the 4.5S RNA failed to enter the gradient (Fig. 5B). These results indicated that the 4.5S RNA associates in a large complex under native conditions.

The interaction of the 4.5S-like RNA with the P48-like antigen to form an RNP was demonstrated by co-immunoprecipitation of the transcript by immune sera to the polypeptide. The anti-P48 immune serum was incubated with mitochondrial or E. coli extracts; and the antibody–antigen complex was then collected by the adsorption onto protein G–agarose beads. The immunoprecipitated complexes were extracted by phenol/chloroform and the 4.5S RNA was then detected by RNA gel blot analysis (Fig. 6). Control reactions with the E. coli lysate demonstrated that the E. coli 4.5S RNA was immunoprecipitated with immune serum (lane 2), but not with preimmune serum (lane 1). A 220 nt transcript was detected in the mitochondrial lysate that has been treated with anti-P48 antisera (lane 4), but was not detectable in the lysate that was treated with pre-immune sera (lane 3). Thus, this result suggests that the 4.5S-like RNA associated with a P48-like polypeptide in maize mitochondria.

**DISCUSSION**

An RNP in maize mitochondria has been characterized that bears similarity to the bacterial 4.5S RNA/P48 RNP. A gene for a 4.5S-like RNA is mitochondrial encoded and expresses a small transcript of ~220 nt. The mitochondrial 4.5S-like RNA has 15 nt of sequence identity within the 22 nt conserved domain of the prokaryotic 4.5S RNAs. The 4.5S-like RNA from maize mitochondria has sedimentation behavior indicated that it is associated in a high molecular weight complex. In addition, maize mitochondria have a ~50 kDa polypeptide recognized by P48 antibody that is capable of immunoprecipitation of the 4.5S-like RNA. These results strongly support the presence of an
stroma before integration into or translocation across the polypeptides of the thylakoid must pass through the aqueous membranes are localized in the thylakoids, an internal membrane. Light harvesting complex and other photosynthetic polypeptides of the chloroplast system. 54CP apparently acts as a molecular chaperone.

4.5S-like RNA has yet been identified in the higher plant sequence identity with bacterial P48 (15, 16); however no chloroplasts. 54CP is a chloroplast protein with substantial utilization of the Sec proteins (25). Thus, there is compelling evidence because these proteins are targeted by an independent system of other presecretory proteins remain unaffected, presumably to a receptor on the endoplasmic reticulum, thereby directing translation of the polypeptide with translocation into the lumen of the endoplasmic reticulum. A comparable process has recently been elucidated for bacterial secretion. The 4.5S/P48 RNP has structural and functional similarity to the cytosolic SRP (21), and the 4.5S RNP has been shown to specifically interact with the signal sequence of secretory proteins (22). A receptor for the 4.5S RNP, the ftsY gene product, has been identified and shows sequence identity with the α-subunit of the SRP receptor of the ER membrane, and both receptor subunits exhibit GTPase activity (23). Disruption of the secretory pathway through depletion of the 4.5S RNA, the P48 polypeptide, or the receptor protein results in accumulation of precursor of several periplasmic polypeptides including β-lactamase, OmpF and ribose binding protein (13, 14, 24). However, processing and translocation of other presecretory proteins remain unaffected, presumably because these proteins are targeted by an independent system utilizing the Sec proteins (25). Thus, there is compelling evidence that bacterial protein secretion and cytosolic targeting to the ER utilize a homologous pathway.

The SRP-like targeting pathway has also been identified in chloroplasts. 54CP is a chloroplast protein with substantial sequence identity with bacterial P48 (15, 16); however no 4.5S-like RNA has yet been identified in the higher plant chloroplast system. 54CP apparently acts as a molecular chaperone by interacting with the chlorophyll binding protein after import, and facilitating assembly of light harvesting chlorophyll complexes in the thylakoids. Thus, 54CP may be involved in targeting proteins to the thylakoid membrane through an ancestral pathway. Light harvesting complex and other photosynthetic polypeptides in prokaryotic algae would need to be targeted to the membrane by a secretory process. In the chloroplast, the photosynthetic membranes are localized in the thylakoids, an internal membrane system with no direct continuity to the inner envelope. Thus, polypeptides of the thylakoid must pass through the aqueous stroma before integration into or translocation across the membrane. The SRP-like targeting system of the ancestral endosymbiont may have been recruited to target nuclear encoded and imported polypeptides to the thylakoid membrane.

The role of an SRP-like RNP in maize mitochondria is unknown. It is tempting to speculate a role in protein targeting. Early work on protein targeting to the inner membrane and intermembrane space in mitochondria suggested that imported polypeptides were first translocated to the matrix, and subsequently directed through an ancestral pathway to the inner membrane or translocated across the inner membrane to the intermembrane space (26). Recent studies support a stop–transfer model for protein targeting to the inner membrane or intermembrane space (27) such that proteins would never completely enter the matrix, but would stop when associated with the inner membrane. In this case, matrix targeting proteins would not be required for protein targeting.

Regardless of how the import and targeting controversy of nuclear-encoded mitochondrial polypeptides is resolved, many mitochondrial-encoded proteins are synthesized in the matrix and targeted to the inner membrane. In several cases nuclear-encoded translation and assembly factors are known to be involved in the production of membrane complexes (28). The topological relationship between the site of synthesis and assembly is the same in mitochondria and prokaryotic progenitors, and the SRP-like system could be involved in this process. Both ATP6 and COX2 polypeptides have hydrophobic presequences which are cleaved during the biogenesis of the mature complexes (29–31). The maize mitochondrial 4.5S RNP may play a role in targeting these polypeptides to the inner mitochondrial membrane for subsequent assembly or translocation.

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REFERENCES


Figure 6. Co-immunoprecipitation of the 4.5S-like RNA with anti-P48 sera. Escherichia coli (lanes 1 and 2) or mitochondrial (lanes 3 and 4) extracts were immunoprecipitated with anti-P48–protein G complexes (lanes 2 and 4) or preimmune–protein G complexes (lanes 1 and 3). RNA was extracted from the immunoprecipitations and separated on a 6% denaturing polyacrylamide gel. RNA gel blots were probed with 32P-labeled 4.5S RNA oligonucleotide and autoradiographed. Lane 5 contains total maize mitochondrial RNA. DNA size standards are indicated.