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The existence of pre-mature 16S rRNA species in plastid ribosomes

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Plastid 70S ribosomes were prepared from heterotrophic cultured cells of tobacco (*Nicotiana tabacum*, BY2), and the 5′ termini of the 16S rRNA molecules present in the ribosomes were analyzed. RNase protection and primer extension experiments showed that a minor fraction of the 16S rRNA species carries a leader sequence of 30 nucleotides, coinciding with a putative RNase III cleavage site. The results suggest that an RNase III-like activity is present in plastids and that ultimate 5′ maturation of 16S rRNA takes place within the ribosome.

Plastid; 16S rRNA; rRNA processing; Tobacco

1. INTRODUCTION

According to the endosymbiotic theory [1] chloroplasts of higher plants are intracellular organelles of the plastid type of prokaryotic origin. Plastid ribosomes are 70S in size similar to those from bacteria, and their protein components are encoded both in the nuclear and the organellar genomes, whereas the rRNA genes are clustered in plastid DNA in the order 16S–23S–4.5S–5S, with tRNA genes interspersed, resembling the prokaryotic arrangement (see [2–4] for recent reviews). In either plastids or bacteria, large precursor rRNA molecules spanning the whole operon are produced which must be processed into the individual mature products [4,5]. In bacteria, several enzymes, including endonucleases, exonucleases and modifying enzymes, are thought to be involved in rRNA processing [6]. However, none of these activities has been reported in plastids with the exception of the tRNA specific enzymes [4].

In *E. coli*, RNase III cleaves duplex structures formed between regions of the rRNA transcript flanking the 5′ and 3′ ends of 16S or 23S sequences, yielding intermediate products slightly larger than the mature rRNAs [5,7,8]. The 5′ terminus of the precursor 16S rRNA (p16) still contains some 60 extra nucleotides the removal of which requires the participation of RNase M16 [5,8,9]. This activity fails to process naked RNA, but needs at least the formation of pre-30S ribosome particles [5,8]. In a mutant strain lacking this activity, together with the mature 16S rRNA, a 16.3S species accumulates even in 30S subunits on polysomes [5,8]. This strain is a slower-growing but viable one, hence it remains obscure whether this extra sequence affects ribosome function or whether it must be eliminated before the 30S subunit becomes functional [8]. In plastids, the mechanisms and pathways of rRNA processing remain speculative based on the bacterial model. The flanking regions of the 16S rRNA sequence are predicted to form a structure susceptible to be cleaved by an RNase III-like activity [10,11] albeit no experimental demonstration is available. Likewise, nothing is known about the final maturation of the 5′ end in plastid rRNA.

Here we provide evidence on the presence of pre-mature 16S rRNA in isolated plastid ribosomes. The 5′ end of this extra fragment has been mapped at position −30 with respect to mature 16S rRNA, situated in a single stranded bulge embedded in a putative RNase III site [4,11], which suggests that such an activity is involved in plastid rRNA processing.

2. MATERIALS AND METHODS

2.1. *Material*

Culture of heterotrophic tobacco cells (*Nicotiana tabacum*, BY2) and enzymatic digestion of cell walls were carried out essentially as described [12]. Plastid 70S ribosomes were prepared from 500 ml of digested material according to [13]. Total cell RNA and 70S ribosome RNA (70 RNA) were extracted as in [14] and treated with RNase-free DNase to remove trace amounts of contaminating DNA.

2.2 *Ribonuclease protection assay*

A tobacco chloroplast DNA fragment corresponding to the first 203 nucleotides (nt) of 16S rRNA together with the upstream 77 nt was subcloned from the plasmid pTB9 [15] into the *Bam*HI and *Hin*dIII sites of Bluescript SK+ (Stratagene, USA) precut with *Bam*HI, *Pst*I and *Hin*dIII. The resultant plasmid, PR16, was linearized with *Bam*HI and used as template for the synthesis of antisense RNA with T7 RNA polymerase as reported [16] except that [α-^{32}P]rUTP (60 μCi, 800 Ci/mmol) was included. Ribonuclease protection assay was performed as described [16] using a commercial kit (RPAII Ambion, USA).

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2.3. *Primer extension*

The oligonucleotide PER16, 5′-CTCTCCATGAGATTCAT-AGTTGC-3′, complementary to the upstream 11 nt and the first 12 nt of 16S rRNA, was prepared with a DNA synthesizer (Applied Biosystems, USA, Type 380 A), labeled at its 5′ end with ^{32}P and used as a primer. Reverse transcription reactions were carried out with 30 μg of total cell RNA or 90 μg 70S RNA as in [16] except that 400 units of M-MLV reverse transcriptase (BR, USA) were used.

3. RESULTS AND DISCUSSION

Tobacco heterotrophic cultured cells (BY2) containing proplastids were the source of plastid 70S ribosomes because large amounts of cells are generated in short periods and rRNA genes are actively transcribed ([17], unpublished data).

To investigate the presence of 16S rRNA precursor sequences, RNA was extracted from 70S ribosome fractions (designated 70 RNA), annealed to a specific riboprobe, digested with RNases A/T1 and the protected fragments were separated in denaturing polyacrylamide gels. For comparison, total cell RNA was subjected to the same treatment. As shown in Fig. 1, the most intense band (band A) from 70 RNA and total cell RNA (TC RNA) represents mature 16S rRNA. In total cell RNA but not in 70 RNA, two larger fragments of about 280 and 265 nt appear (Fig. 1A, lane 6; Fig. 1B, bands D and C), the first of which coincides with the maximum protecting capacity of the probe (280 nt), very likely corresponding to primary transcripts ([4,11], see below). Some 50 nt from the Bluescript vector sequence account for the difference in length between the probe and this band (Fig. 1A, lanes 3, 6 and 7). The 265 nt band might be a processing intermediate although an additional initiation of transcription can not be ruled out. In vitro capping experiments [18] are necessary to distinguish between these two possibilities.

The most interesting in this assay is a band comigrating with the 234 nt marker and protected by either total cell RNA and 70 RNA (Fig. 1A, lanes 4 and 6; Fig. 1B, band B). This band indicates the existence of a pre-mature 16S rRNA species with about 30 nt extra sequence at its 5′ end. Our trials to cleave this extra sequence using distinct tobacco plastid lysates were unsuccessful (not shown), suggesting that processing of this 16S rRNA species occurs within ribosomal particles. As judged by the situation in *E. coli* (see Introduction) this scenario is not unlikely. The bands corresponding to pre-mature and 16S RNA are less intense in 70 RNA than in total cell RNA in spite of using equal amounts of RNA. A higher rate of degradation in 70 RNA due to a longer extraction procedure could explain this as deduced from the many shorter bands in lanes 4 and 6 of Fig. 1A.

In order to ascertain the presence of pre-mature 16S RNA sequences in plastid ribosomes and to assign the precise positions of the 5′ ends, high resolution primer extension was carried out with 70 RNA and the oligonucleotide PER16 which can only prime transcripts longer than mature 16S RNA (see materials and methods). Again, total cell RNA was used in parallel and two close extension products were found at positions −116 and −114 with respect to the 5′ end of mature 16S rRNA sequence (Fig. 2A, lane TC). At least the first position most probably represents a starting site of transcription since likely prokaryotic-type-35 (TTGACG) and -10 (TATATT) promoter motifs are found 35 and 11 nt, respectively, upstream from this site (see [19] for sequence data). This is in good agreement with the −117 position for the initiation site identified in the maize chloroplast rRNA operon [11].

Reverse transcriptase also pauses at position −64 which fits the 265 nt band in Fig. 1. This extension product was visible after prolonged exposure (not shown). Finally, a strong pausing site is found at position −30 in both total cell RNA and 70 RNA (Fig. 2A,

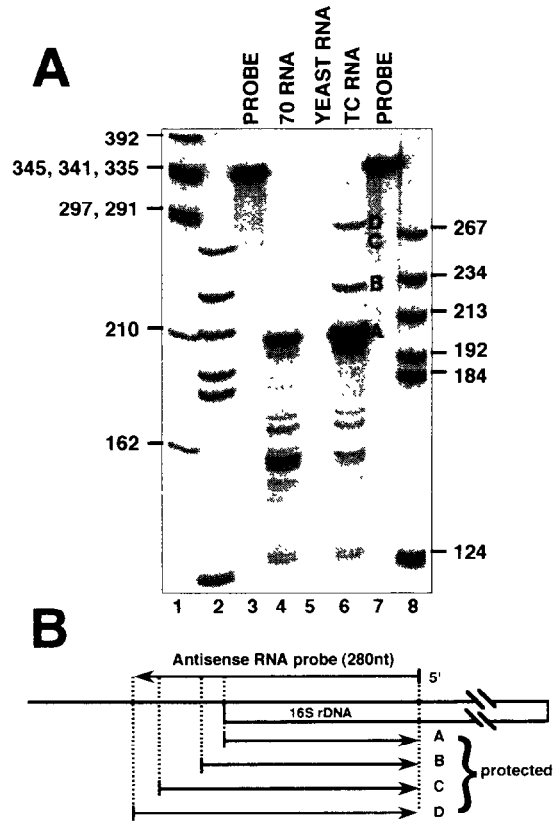

Fig. 1. Ribonuclease protection assay to detect the 5′ end of 16S rRNA and its precursors. (A) Denaturing electrophoresis (6% polyacrylamide, 1 × TBE, 7.5 M urea) of the protected fragments. Ten μg of 70 RNA (lane 4), total yeast RNA (lane 5) and total BY2 cell RNA (lane 6) were annealed to a saturating amount of riboprobe and digested with 40 units RNase T1/0.2 units RNase (A) Undigested probe (lanes 3 and 7) was run in parallel. Radiolabeled molecular weight markers are HincII digest of ϕ x174 RF-DNA (lane 1) and HaeIII digest of pBR322 DNA (lanes 2 and 8). Electrophoresis was carried out at 2500 V for 1 5 h. (B) Diagrammatic representation of the experiment. A, B, C and D refer to the protected fragments identified in (A) and discussed in the text.

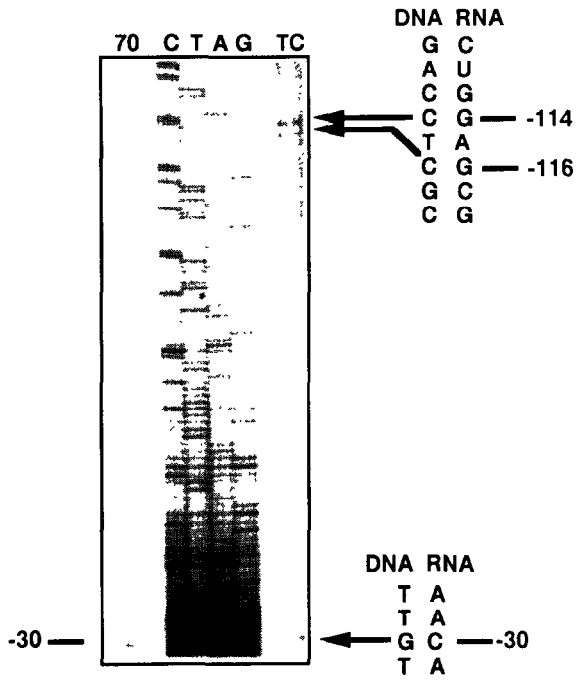

Fig. 2. High resolution primer extension mapping of the 5′ ends of 16S rRNA and its precursors. Reverse transcription products with primer PER16 and total BY2 cell RNA (TC lane) or 70 RNA (70 lane). G, A, T and C lanes indicate dideoxy sequencing ladders prepared from the same primer on a PCR fragment template (600 bp, the first 203 bp of the 16S rRNA-encoding gene together with the upstream 397 bp) obtained from PTB9 [15] using a double stranded cycle sequencing kit (BRL). Electrophoresis conditions were as in Fig. 1 during 3 h.

lanes 70 and TC), confirming our protection assay results.

Position −30 is particularly noteworthy in that an RNase III cutting site was predicted in between nucleotides −30 and −31, in the light of the potential secondary structures formed by the 16S rRNA flanking sequences in both monocot and dicot plastid genomes ([4] and references therein). Position −30 was also identified as a major stop point for reverse transcriptase with maize chloroplast RNA [11]. This might indicate a common maturation pathway of 16S rRNA in diverse organisms, that is, a RNase III-like activity creating the substrate for enzyme(s), like prokaryotic RNase M16 [8], acting in the ribosome and/or the 30S ribosomal subunit. In vitro studies on these processes are essential for further progress.

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