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Processing of precursor tRNAs in a chloroplast lysate

Processing of the 5′-end involves endonucleolytic cleavage by an RNase P-like enzyme and precedes 3′-end maturation

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An in vitro tRNA processing system using either a spinach or tobacco chloroplast lysate has been constructed. Monomeric $tRNA^{Phe}$ precursors were prepared using an SP6 transcription vector system. tRNA precursors were processed to mature tRNA molecules by incubation in an S30 fraction of a chloroplast lysate. Both 5′ leader and 3′ extension sequences were processed endonucleolytically. Processing of the 5′ leader was demonstrated to precede 3′-end maturation. RNase P-like endoribonuclease is likely to function in the 5′-end processing.

tRNA processing; Chloroplast lysate; RNase P-like enzyme; Transcription vector SP6; 3′-Endonucleolytic cleavage

1. INTRODUCTION

Chloroplasts contain a complete apparatus for protein synthesis. All the chloroplast tRNAs are believed to be coded for by chloroplast DNA. In tobacco, 37 tRNA genes have been identified in the complete nucleotide sequence of the chloroplast genome [1,2], seven of which are located in the inverted repeat region. In higher plants, chloroplast tRNA genes are not usually cotranscribed except for $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in the spacer region of 16 S–23 S rRNA genes [3,4] and $tRNA^{Glu}$, $tRNA^{Tyr}$ and $tRNA^{Asp}$ genes [5]. In contrast, *Euglena* chloroplast tRNA genes tend to be clustered like prokaryotic tRNA genes [6].

A number of processing reactions are required for maturation of initial tRNA precursors. The nascent tRNA transcripts have extra sequences at their 5′- and 3′-ends. These flanking sequences are removed by processing nucleases. After trimming of flanking sequences the CCA sequence is added to the 3′-end and several bases are modified in chloroplasts in a similar way to other prokaryotic and eukaryotic tRNA maturation events [7].

In vitro transcription and processing of chloroplast tRNA genes have been studied in extracts of HeLa cells, spinach chloroplasts and *Euglena* chloroplasts [7,8]. However, the order of tRNA maturation events and characteristics of the processing enzymes in chloroplasts are not precisely known. Here, we report processing of monomeric $tRNA^{Phe}$ precursors to mature tRNAs in chloroplast lysates of spinach and tobacco. Our results indicate that both 5′- and 3′-processing is accomplished by endonucleolytic cleavage and that the 5′ leader sequence is removed before the 3′ extension trimming. The 5′ processing endoribonuclease of chloroplasts appears to possess both protein and nucleic acid subunits like RNase Ps of prokaryotes, eukaryotes and mitochondria [9–16].

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2. MATERIALS AND METHODS

2.1. *Recombinant plasmids*

Plasmid pSF19 contains a 507 bp *Sau*3A fragment isolated from pTBa3 (former pTCB3) DNA [17,20] cloned in the *Bam*HI site of the plasmid pSP65 [18]. This *Sau*3A fragment contains the tobacco chloroplast $tRNA^{Phe}$(GAA) gene (*trn*F, fig.1).

2.2. *In vitro transcription*

Recombinant plasmid pSF19 DNA was linearized by digestion with *Sna*BI or *Xba*I. The DNA was extracted with phenol, precipitated with ethanol, and redissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The in vitro transcription mixture for SP6 RNA polymerase was supplied from Amersham. Transcription was initiated by addition of SP6 RNA polymerase, and continued for 2 h at 37°C. The reaction mixture was incubated at 37°C for 10 min with 2 U RNase-free DNase and 20 U human placenta RNase inhibitor (HPRI), extracted with phenol, and passed through a Bio-Rad P-30 column. The precursor RNAs were precipitated with ethanol and dissolved in distilled water.

2.3. *In vitro processing of precursor RNAs*

Preparation of spinach and tobacco chloroplast extracts was carried out as described by Bard et al. [19]. The labeled precursor RNAs were incubated with 2 μl spinach chloroplast extract, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NH_4Cl and 60 U HPRI for various times at 37°C (final volume 20 μl). They were extracted with phenol followed by addition of 160 μl of 0.3 M NaCl, 1 mM EDTA and 10 mM Tris-HCl (pH 7.5), and ethanol precipitated. The processed RNAs were redissolved in 1 μl of 0.2% XC and analyzed by electrophoresis on a 3% polyacrylamide-8 M urea gel.

2.4. *Micrococcal nuclease inactivation*

Chloroplast extracts containing RNase P activity were incubated in the presence of 25 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 5, 15 or 25 U micrococcal nuclease (Cooper) per μl with or without 100 mM EGTA for 15 min at 37°C. Aliquots from each reaction were assayed for RNase P activity as described above.

3. RESULTS AND DISCUSSION

A gene for $tRNA^{Phe}$(GAA) is located in the middle of the large single copy region of tobacco chloroplast DNA and is likely to be transcribed monocistronically [17]. We believe that it is necessary to use monomeric tRNA precursors as substrates to study the order of processing events in vitro.

Monomeric $tRNA^{Phe}$ precursors were prepared by using the SP6 transcription vector system. The plasmid pSF19 was constructed as described, cleaved by *Sna*BI or *Xba*I, and transcribed by SP6 RNA polymerase. Two $tRNA^{Phe}$ precursors of 439 and 292 bases (b) were obtained (fig.1A and B, respectively) and used as substrates for in vitro processing reactions (fig.2, lanes 1,6). These two tRNA precursors have the same 5′ leader sequence of 135 b but different 3′ extension sequences of 231 and 84 b, respectively. With these two substrates we can distinguish processed RNA molecules on the basis of their sizes.

Intact chloroplasts were prepared from spinach or tobacco using Percoll gradient sedimentation and lysed by sonication. Then S30 fractions were prepared from the chloroplast lysate according to Bard et al. [19]. This S30 fraction could be used for in vitro processing as well as transcription and translation. The tRNA precursors were incubated at 37°C in the S30 fraction and the processed

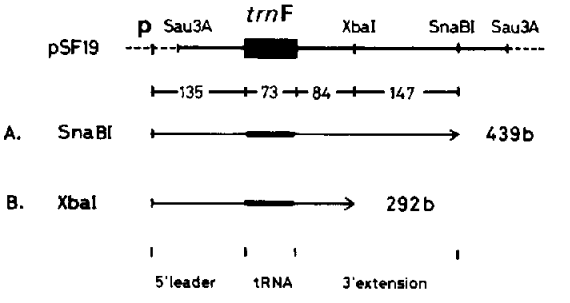

Fig.1. Structure of the DNA template used in synthesis of RNA substrate containing a $tRNA^{Phe}$ sequence. The top line represents a restriction map of a part of recombinant plasmid pSF19 DNA. Solid line, *Sau*3A insert; dotted line, vector; numeral, bp and P, a SP6 promoter site. Arrows indicate RNA substrates synthesized on a pSF19 DNA linearized by *Sna*BI (A) or *Xba*I (B).

products were analyzed by polyacrylamide gel electrophoresis.

To determine the order of processing events we incubated two different $tRNA^{Phe}$ precursors (fig.1) in the S30 fraction of spinach chloroplast lysate and analyzed RNA products at various times up to 40 min. Processing of two tRNA precursors resulted in the formation of four smaller RNAs (fig.2). Common RNA species of 76 and 135 b corresponding to the mature tRNA and the 5′ leader sequences, respectively, were found with the two different substrates. The 76 b RNA appears to be a mature tRNA containing the CCA sequence at the 3′-end as it is 3 b longer than the $tRNA^{Phe}$ coding region. Two RNA products of 231 and 84 b correspond to the 3′ extension sequences. Judging from RNA sizes, products of 304 and 157 b are likely to be processing intermediates which contain tRNA and the 3′ extension sequences. Similar results were obtained with a tobacco chloroplast S30 fraction (not shown). As discrete RNA bands corresponding to both the 5′ leader and 3′ extension sequences are detected during the in vitro processing reaction, the 3′-end as well as the 5′-end is

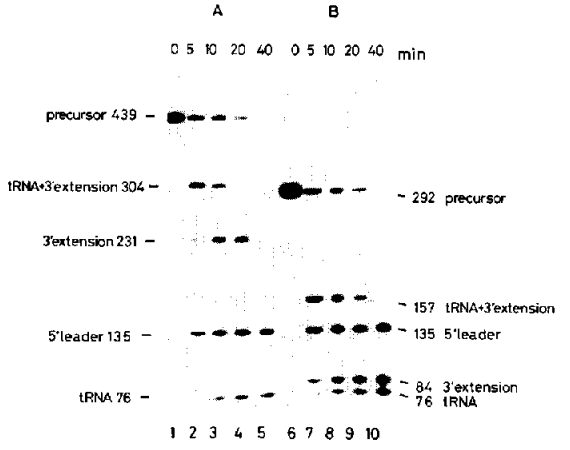

Fig.2. Time course of the appearance of processing products in a chloroplast extract using $tRNA^{Phe}$ precursors. Precursor RNAs of 439 b (A) or 292 b (B) labeled with [α-^{32}P]UTP were incubated in a chloroplast extract for the times indicated above the lanes. The RNA products were analyzed on a 3% polyacrylamide-8 M urea gel. Numbers show RNA sizes (b). RNA sizes were calculated from [^{32}P]DNA markers (*Hin*cII digest of ϕX174 DNA, *Hin*dIII digest of λDNA) and in vitro transcribed RNA markers (634, 440, 109 b).

likely to be processed endonucleolytically. The 3′ extension sequences of eukaryotic tRNAs are also processed endonucleolytically in extracts from *Xenopus laevis* nuclei and *Drosophila* cells [24,25], while the 3′-end maturation proceeds exonucleolytically in *E. coli* [26].

We could not detect processing intermediates of the 5′ leader plus tRNA molecules (fig.2). Furthermore, the 5′ leader sequence of 135 b appeared before the 3′ extension sequence of 231 b during a 5 min incubation (fig.2A). Thus we concluded that the 5′-end processing precedes the 3′-end maturation in chloroplasts like many other organisms [25,27]. The processed 5′ leader sequence was more stable than the processed 3′ extension sequence during incubation with the S30 fraction (fig.2A).

Greenberg et al. [7] reported that the 5′ processing proceeds endonucleolytically and that a 3′ exonuclease and an endonuclease are likely to participate in the 3′ processing. Our results indicate that the 3′ processing as well as the 5′ processing proceeds endonucleolytically. These differences might arise from the differences between the substrate tRNA precursors used. Greenberg et al. used trimeric tRNA precursors of *Euglena* chloroplasts while we used monomeric precursors of tobacco chloroplasts. We are now preparing trimeric precursors of $tRNA^{Glu}$, $tRNA^{Tyr}$ and $tRNA^{Asp}$ of tobacco to determine the order of processing events with trimeric precursors.

It is known that RNase P is responsible for processing of 5′-ends of tRNA precursors in prokaryotes, eukaryotes and yeast mitochondria [9–16]. An unusual characteristic of RNase P enzymes is the presence of an RNA component. Extensive studies on the *E. coli* RNase P enzyme have shown that the RNA subunit alone catalyzes the cleavage of tRNA precursors to produce mature 5′-termini under specific conditions in vitro [21,22]. To investigate whether the chloroplast RNase P-like 5′ endoribonuclease depends on a nucleic acid component, the activity was assayed in the presence of micrococcal nuclease with or without EGTA. Micrococcal nuclease is a nonspecific nuclease which has an absolute dependence upon Ca^{2+} for its activity. As shown in fig.3, the 5′ endoribonuclease activity could be abolished by micrococcal nuclease in the presence

of Ca^{2+}. Increasing the concentration of micrococcal nuclease resulted in a further decrease in activity (fig.3 lanes 4–6). Only a slight decrease in activity was observed when the activity was assayed with micrococcal nuclease in the absence of Ca^{2+} (fig.3 lane 3). The activity was not abolished by Ca^{2+} alone (not shown). The chloroplast RNase P-like endonuclease was assayed with or without prior treatment by protease. The activity was also abolished by protease treatment (not shown). From these experiments, it is possible to conclude that the chloroplast RNase P-like enzyme has both nucleic acid and protein components. Thus, we named the chloroplast 5′ endoribonuclease cpRNase P. The 3′ endoribonuclease activity was abolished by prior micrococcal nuclease treatment with or without Ca^{2+} (fig.3). This may be due to instability of the enzyme. We have partially purified the cpRNase P enzyme. Like the *E. coli* RNase P, cpRNase P was concentrated in the ribosome-wash fraction and by DEAE-cellulose column chromatography (unpublished). This also supports the conclusion that cpRNase P has similar characteristics to *E. coli* RNase P. We are currently studying the purified cpRNase P enzyme to determine the nature of its RNA component and whether it is encoded by the chloroplast DNA in a similar way to yeast mitochondrial 9 S RNA, which is an RNA component of mitochondrial RNase P (mtRNase P) and encoded in the organellar genome [16,23].

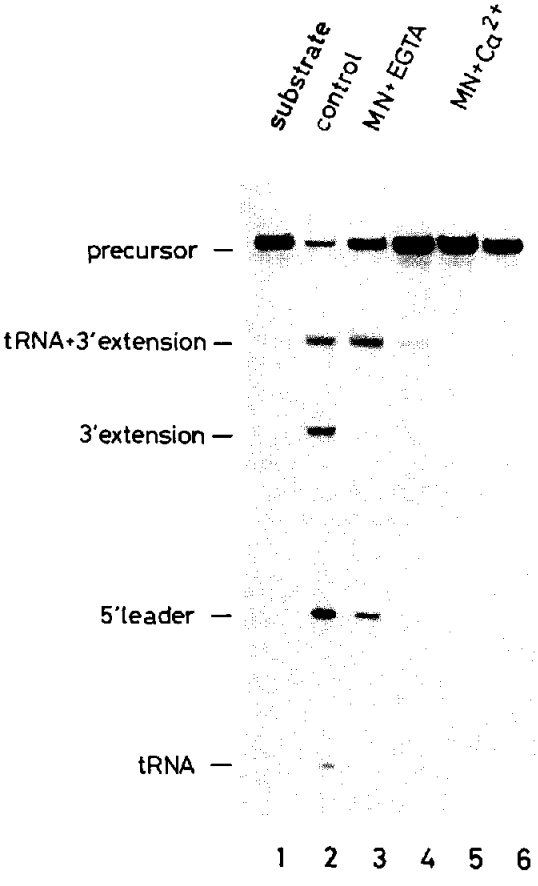

Fig.3. Nuclease-sensitive cpRNase P activity. cpRNase P was assayed in the presence of micrococcal nuclease with or without EGTA in the reaction mixture described in section 2. Lanes: 1, $tRNA^{Phe}$ precursor of 439 b; 2, control (no prior treatment); 3, incubation with 25 U micrococcal nuclease in the presence of EGTA prior to assay; 4,5,6, incubation with 5, 15, 25 U, respectively, micrococcal nuclease without EGTA prior to assay.

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