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Novel mechanisms for maturation of chloroplast transfer RNA precursors

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Despite the prokaryotic origins of chloroplasts, a plant chloroplast tRNA precursor is processed in a homologous *in vitro* system by a pathway distinct from that observed in *Escherichia coli*, but identical to that utilized for maturation of nuclear pre-tRNAs. The mature tRNA 5′ terminus is generated by the site-specific endonucleolytic cleavage of an RNase P (or P-type) activity. The 3′ end is likewise produced by a single precise endonucleolytic cut at the 3′ terminus of the encoded tRNA domain. This is the first complete structural characterization of an organellar tRNA processing system using a homologous substrate. In contrast to eubacterial RNase P, chloroplast RNase P does not appear to contain an RNA subunit. The chloroplast activity bands with bulk protein at 1.28 g/ml in CsCl density gradients, whereas *E.coli* RNase P bands as ribonucleoprotein at 1.73 g/ml. Chloroplast RNase P activity survives treatment with micrococcal nuclease (MN) at levels 10- to 100-fold higher than those required to totally inactivate the *E.coli* enzyme. The chloroplast system is sensitive to a suppression of tRNA processing, caused by binding of inactive MN to pre-tRNA substrate, which is readily overcome by addition of carrier RNA to the assay.

Key words: chloroplast/RNA processing/RNase P/3′ endonuclease/micrococcal nuclease

Introduction

All cellular RNA molecules are synthesized as immature precursors. RNA maturation or processing is the crucial step in gene expression whereby precursor RNA transcripts are converted to functional species. We are interested in the biochemical mechanisms and evolutionary history of RNA processing in cellular organelles, in particular the chloroplast. Studies of chloroplast gene organization and DNA sequences reveal chloroplasts to be closely related to contemporary eubacteria, specifically the cyanobacteria, and only slightly less related to the Bacilli or to *Escherichia coli* (Woese, 1987; Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Gray and Doolittle, 1982). Although many aspects of bacterial gene organization are retained in chloroplast DNA (summarized in Gray, 1986), there are significant differences which might necessitate altered mechanisms of RNA metabolism. For example, bacterial tRNA genes are frequently clustered (Fournier and Ozeki, 1985; Vold, 1985; Fukada and Abelson, 1980) and transcribed into polycistronic precursors (reviewed by King *et al.*, 1986; Deutscher, 1984; Gegenheimer and Apirion, 1981). In plant chloroplasts, just as in eukaryotes, tRNA genes are generally not clustered (Shinozaki *et al.*, 1986). In addition, some chloroplast tRNA genes lack conventional bacterial 5′-flanking promoters but appear to contain eukaryotic-type internal promoter elements (Gruissem *et al.*, 1986a).

tRNA molecules are synthesized as precursors with 5′ (leader) and 3′ (trailer) extensions. The overall steps in tRNA maturation are similar in all cells, from bacteria and their viruses to fungi, plants and animal cells (Deutscher, 1984). The 5′ leader is removed by the site-specific endonuclease RNase P, which cleaves precisely at the 5′ side of the mature tRNA domain (Robertson *et al.*, 1972; see Gegenheimer and Apirion, 1981). The specific sequence of events employed for maturation of tRNA 3′ termini, however, is quite different in prokaryotes versus eukaryotes. Processing of *E.coli* pre-tRNA$^{Tyr}_2$ (suIII$^+$) *in vivo* and *in vitro* is initiated by an endonucleolytic attack ~7 nucleotides (nt) downstream from the 3′ end of the mature tRNA domain (Bikoff *et al.*, 1975; Sekiya *et al.*, 1979). The remaining 3′ extension is trimmed exonucleolytically by RNase D, exposing the mature 3′ terminus CCA_{OH} (Bikoff *et al.*, 1975; Sekiya *et al.*, 1979; Cudny and Deutscher, 1980). In contrast, all characterized eukaryotic 3′ maturation endonucleases cleave precisely at the 3′ end of the encoded tRNA domain, which does not include the CCA_{OH} sequence (Deutscher, 1984). This specificity has been conclusively demonstrated for *Xenopus* (Hagenbüchle *et al.*, 1979; Garber and Gage, 1979; Castano *et al.*, 1985), *Drosophila* (Frendewey *et al.*, 1985) and rat liver mitochondria (Manam and Van Tuyle, 1987). Only in the processing of yeast dimeric tRNA precursors (Mao *et al.*, 1980; Engelke *et al.*, 1985) was a 3′ endonuclease not required.

RNase P has been extensively characterized in *E.coli* (Robertson *et al.*, 1972; Stark *et al.*, 1978; Kole *et al.*, 1980) and in *Bacillus subtilis* (Gardiner and Pace, 1980; Gardiner *et al.*, 1985). The enzymes contain an RNA subunit 377–400 nt long and a protein subunit of ~14 kd (Stark *et al.*, 1978; Reed *et al.*, 1982; Gardiner *et al.*, 1985; Ogasawara *et al.*, 1985; Reich *et al.*, 1986). The RNA subunit contains all the sites for substrate binding, cleavage specificity and catalysis (Guerrier-Takada *et al.*, 1983). The role of the protein subunit is to shield electrostatic repulsion between substrate and enzyme (Reich *et al.*, 1988). Endonucleases with RNase P specificity have been well characterized in yeast nuclei (Mao *et al.*, 1980; Kline *et al.*, 1981; Engelke *et al.*, 1985; Krupp *et al.*, 1986), and in human and rat liver mitochondria (Doerson *et al.*, 1985; Manam and Van Tuyle, 1987). In many other systems, accurate 5′ processing was detected but not proven to be endonucleolytic. The involvement of RNA in eukaryotic RNase P activity is controversial (Castano *et al.*, 1986; Krupp *et al.*, 1986). RNase Ps from yeast, *Xenopus* and

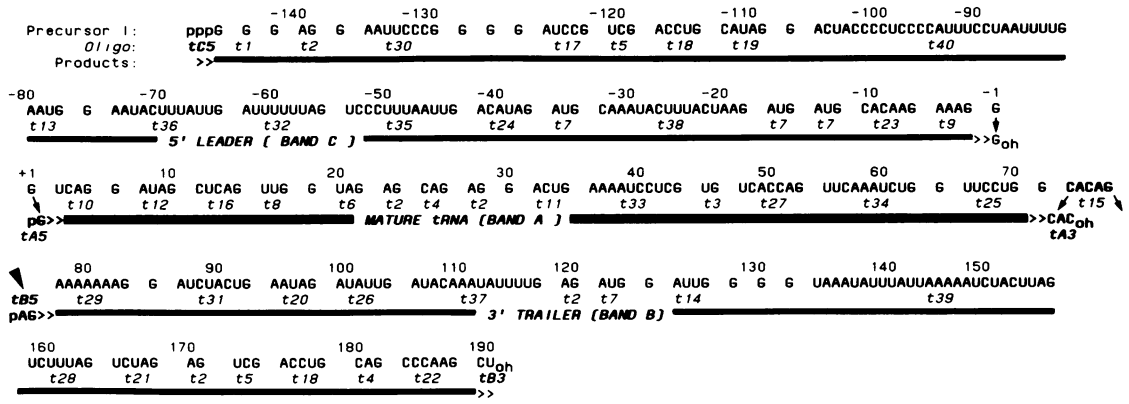

Fig. 1. Pre-tRNA substrates. Plasmid pTUC8-NXPhe was linearized with *Hin*dIII and transcribed with phage T7 RNA polymerase. Below the predicted RNA sequence of the runoff pre-tRNA transcript are shown the RNase T1 oligonucleotide numbers and the extent of sequences in the processed RNA species (bands A, B, C and D of Figure 2) as determined by RNA fingerprinting and secondary analysis (Figure 3; see Materials and methods). In precursor II linearized with *Bam*HI, nucleotides −138 to −117 are replaced with ACCCAAGCUUGG and the *in vitro* runoff transcript terminates with C_{OH} at position +88.

HeLa cells have been reported either to lack RNA (Castão *et al.*, 1986) or to be nuclease-sensitive and denser than bulk protein (Akaboshi *et al.*, 1980; G.Carrara *et al.*, cited in Gandini Attardi *et al.*, 1985), and associated with specific small RNA molecules (Krupp *et al.*, 1986; Gold and Altman, 1986; Hollingsworth and Martin, 1986).

Chloroplast tRNA genes have previously been transcribed and processed to mature tRNAs in *in vitro* systems from spinach (Gruissem *et al.*, 1983; Greenberg *et al.*, 1984) and *Euglena gracilis* (Greenberg and Hallick, 1986). Leader and trailer segments were not detected, however, so the processing activities involved could not be characterized. It was suggested that pre-tRNA maturation in chloroplasts was analogous to that in *E.coli*, with the participation of an RNase P-like 5′ endonuclease and both 3′ endo- and exonucleases (Greenberg *et al.*, 1984). Based on the evolutionary relationship between chloroplasts and eubacteria, it was generally assumed that chloroplast RNase P would have an RNA subunit (e.g. Yamaguchi-Shinozaki *et al.*, 1987).

Results

Properties of a chloroplast tRNA maturation system

To fully delineate the pathway of chloroplast tRNA processing *in vitro*, we prepared homogenous radiolabeled monomeric precursor tRNAs by *in vitro* transcription, with phage T7 RNA polymerase, of two different clones of the maize chloroplast $tRNA^{Phe}$ gene *trnF-GAA*. The two constructs, called precursors I and II, are shown in Figure 1. Precursor I has 143 nt of 5′ leader, 74 nt of mature tRNA and 116 nt of 3′ trailer. Precursor II has 133 nt of leader but only 14 nt of trailer. The *trnF-GAA* locus contains a single 3′-terminal C residue which could serve as the first nucleotide of the CCA sequence. Preparation of Fraction II spinach chloroplast tRNA processing activities followed the protocol of Gruissem *et al.* (1986b) as described in Materials and methods.

Uniform labeled pre-$tRNA^{Phe}$ (I) was incubated with excess Fraction II protein and the products were fractionated by denaturing gel electrophoresis. The identity of each product was confirmed by RNA fingerprinting (see below). Figure 2 demonstrates that the precursor is rapidly and efficiently cleaved to yield 5′ mature $tRNA^{Phe}$ (Band A)

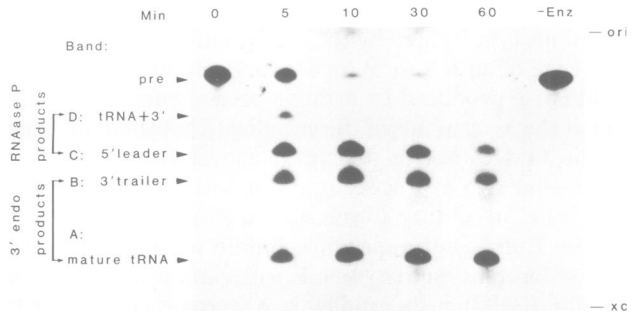

Fig. 2. Accurate *in vitro* processing of maize chloroplast pre-$tRNA^{Phe}$. [^{32}P]pre-$tRNA^{Phe}$ I (0.07 pmol, 0.033 μCi/pmol) was incubated with 45 μg Fraction II chloroplast protein at 37°C. Separate 10 μl reactions were incubated for the times indicated. Products were analyzed by denaturing gel electrophoresis and detected by autoradiography.

plus leader (Band C) and trailer (Band B) segments. In addition, we detect another species (Band D) consisting of tRNA plus trailer sequences. This is the expected product of cleavage by RNase P only. Mature tRNA is quantitatively released from the precursor and is stable in the extract. By contrast, the precursor-specific fragments are unstable, with leader being degraded more rapidly than trailer. We found that production of mature tRNA is optimal between 20 and 42°C. Product yield increases with increasing pH up to at least 8.7. The complete processing reaction requires 5−20 mM $MgCl_2$ and is stimulated by 50−100 mM KCl (data not shown).

Specificity of processing endonucleases

The 5′ and 3′ cleavage sites were pinpointed by RNA fingerprinting and secondary redigestion analysis of gel-purified products. Figure 3 presents RNase T1 fingerprints of each processed RNA; the expected sequence and location of each oligonucleotide is given in Figure 1. Band A is 5′ mature tRNA. Band B is the complete 3′ trailer and Band C is the intact 5′ leader sequence. Band D consists of 5′ mature tRNA covalently linked to trailer RNA. Oligonucleotides tA5 (pGp) (panel A) and tB5 (pAGp) (panel B) are of particular interest, as they define the 5′ termini of mature tRNA and of trailer RNA, respectively. The presence of a 5′ phosphate is a hallmark of authentic processing. Every nucleotide of the

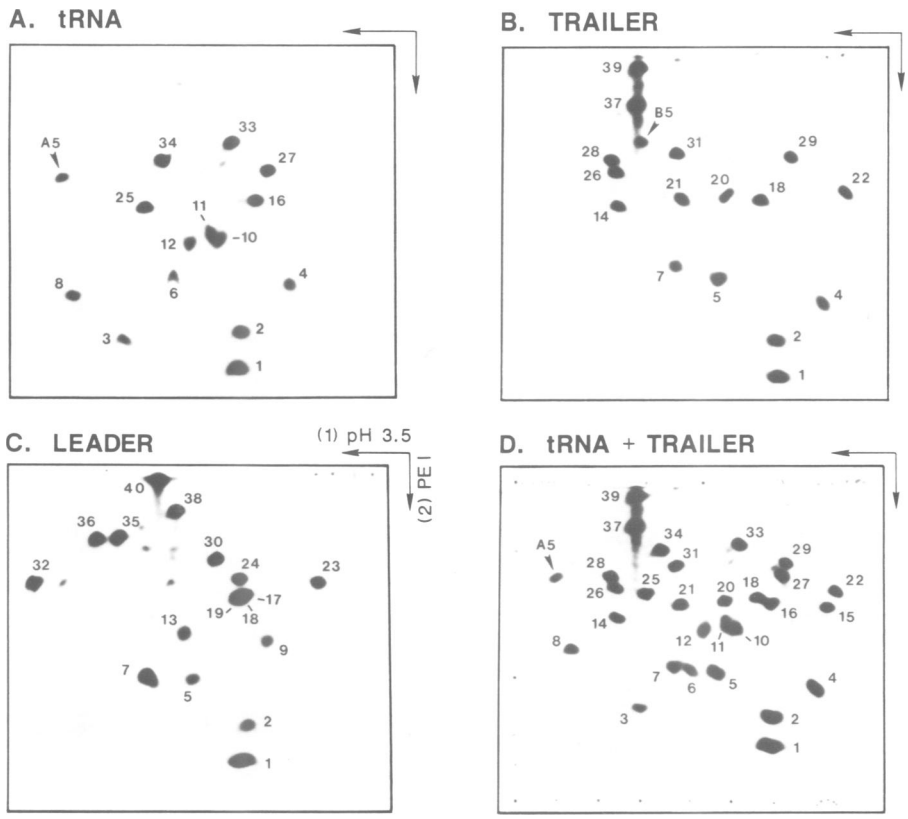

Fig. 3. Fingerprint identification of *in vitro* processed RNAs. Pre-tRNA^{Phe} I (2.0 pmol, 0.411 μCi/pmol) was cleaved in a scale-up reaction similar to that shown in Figure 4A, lane 7. Products were electroeluted and fingerprinted as detailed in Materials and methods. Oligonucleotides are numbered according to Figure 1. Fingerprints of RNA Bands A–D are displayed in **panels A–D**. For simplicity, the prefix 't' for RNase T1 oligonucleotide numbers is omitted.

precursor is contained within the sum of bands A, B and C, or of bands C plus D. [The 3′ end of the leader, G_{OH}, is not detectable in a T1 fingerprint. We did not analyze the 5′ and 3′ termini of the precursor, nor did we recover the 3′ CAC_{OH} fragment of Band A tRNA. Because Band A RNA is a competent substrate for CMP and AMP addition (see below), we are confident that it does possess the correct 3′ terminus]. Band D RNA (Figure 3D) contains every oligonucleotide of the tRNA and trailer RNAs except that oligonucleotide tB5 (pAGp), the 5′ end of the trailer, is replaced with oligonucleotide t15, CACAGp, representing the junction between tRNA and trailer sequences. When Band D RNA was isolated and re-incubated with Fraction II protein, it was cleaved further to yield two species precisely comigrating with mature tRNA and trailer RNA (data not shown).

The tRNA-sized product should be a substrate for subsequent addition, by nucleotidyl transferase, of a 3′ CA_{OH} sequence. Band A tRNA was therefore purified and incubated with Fraction II protein in the presence of ATP and/or CTP. The products were separated on a sequencing gel. Incubation with only CTP results in the addition of a single nucleotide, whereas incubation with both CTP and ATP allows addition of two nucleotides. No significant addition occurs during incubation with ATP only (data not shown).

Do processing activities have RNA components?

Chloroplast RNase P was anticipated to contain a functional RNA subunit like that of the bacterial enzymes. Bacterial RNase Ps possess three properties diagnostic of a ribonucleoprotein composition. These are (i) tight binding to DEAE chromatographic resins (Robertson *et al.*, 1972); (ii) inactivation by pre-treatment with nuclease as well as with protease (Stark *et al.*, 1978); and (iii) either exhibiting, like the *E.coli* enzyme, a buoyant density in CsCl of ~1.7 g/ml, or dissociating, like the *B.subtilis* enzyme, into inactive protein and RNA components (Gardiner and Pace, 1980).

We examined chloroplast 5′ and 3′ processing activities by these criteria. In preliminary trials, the 5′ and 3′ activities eluted from DEAE–Sephacel at low ionic strength (80–180 mM KCl) (P.A.Gegenheimer, M.J.Wang and L.Hedrick, unpublished observations). Processing activity was tested for sensitivity to protease and nuclease. Reactions were performed in two steps consisting of a pre-incubation with protease or nuclease followed, after inactivation of nuclease, by an assay for RNA processing. Both 5′ and 3′ endonucleases are sensitive to proteinase K and are resistant to DNase I pre-treatment (data not shown). The effect of micrococcal nuclease (MN) is seen in Figure 4A. In the presence of $CaCl_2$-activated MN, tRNA processing is increasingly inhibited with increasing time of pre-incubation (Figure 4A, lanes 2–4). Both 5′ and 3′ processing are inhibited, with 3′ processing being more sensitive. When the extract is pre-incubated with EGTA-inactivated MN, RNase P activity is not significantly reduced, but 3′ maturation is selectively inhibited (Figure 4A, lanes 5–7).

To determine whether the block in tRNA maturation resulted from degradation of an RNA component(s) integral to one or more processing enzymes, or from hydrolysis of

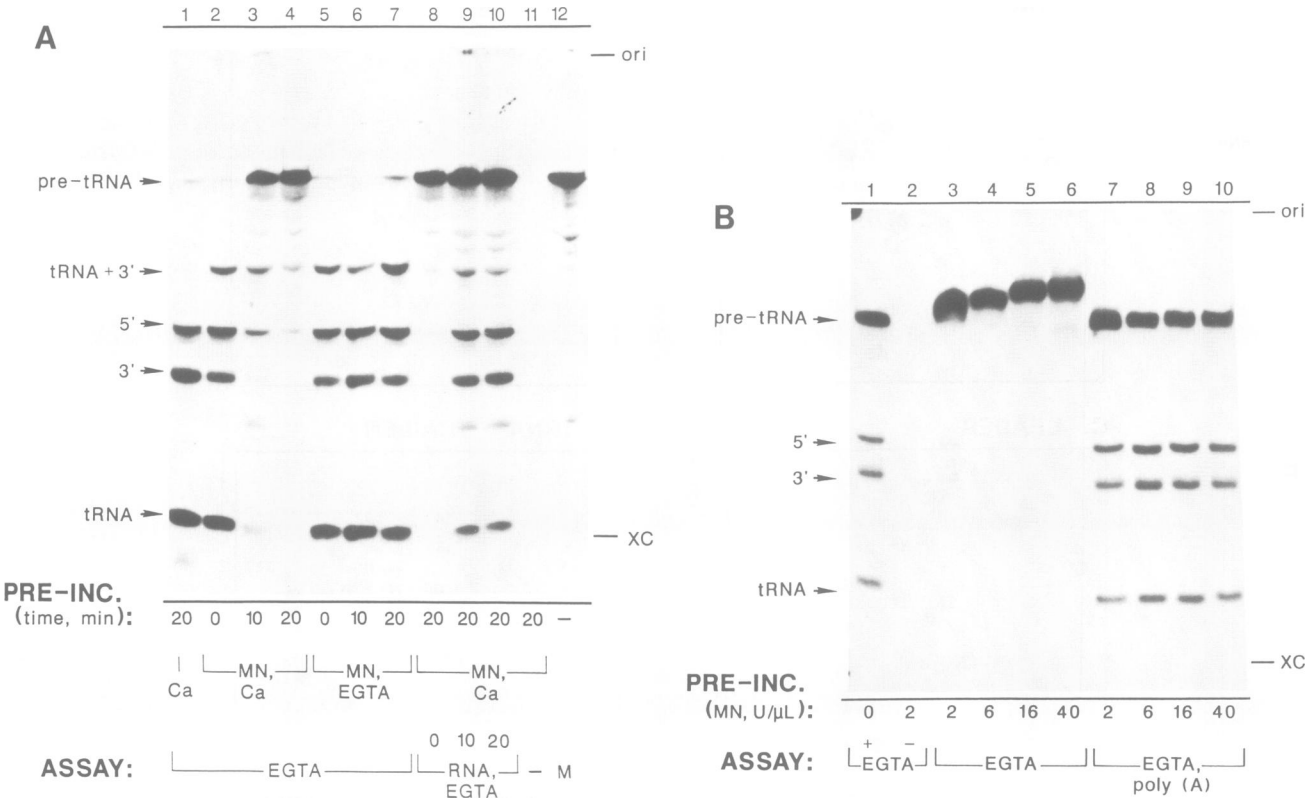

Fig. 4. Micrococcal nuclease suppression and restoration of tRNA processing activities. Processing reactions were performed and analyzed according to Materials and methods. (**A**) Fraction II protein (7.8 μg) was treated as indicated in a 3 μl volume with either 5 nmol $CaCl_2$ or 50 nmol EGTA plus either 10 U/μl MN (in water) or water. After incubation for the indicated times, 50 nmol EGTA (1 μl) was added (if necessary), and 2 μl of this mix was added to 8 μl of substrate mix containing precursor I. Lanes 8–10 contain 0, 10 or 20 μg yeast RNA in water. In **lane 11**, MN was not inactivated prior to substrate addition. **Lane 12** is untreated substrate. (**B**) Fraction II protein (9 μg) was pre-incubated in 5 μl as described in Materials and methods. EGTA was then added in **lanes 1** and **3–10**, as well as poly(A) in **lanes 7–10**, followed by substrate mix containing precursor II.

endogenous non-specific RNA species, we attempted to restore processing by addition of heterologous RNA to the MN-treated extract (Ryner and Manley, 1987). The surprising result was that tRNA maturation was restored to ~50% of control levels by addition of bulk yeast RNA (Figure 4A, lanes 8–10). To demonstrate that MN was indeed active during the pre-incubation, EGTA was omitted from a control reaction, resulting in total hydrolysis of the added [^{32}P]pre-tRNA (Figure 4A, lane 11).

Restoration of processing activity by RNA is independent of the source of RNA. Figure 4B demonstrates that when assayed in the presence of 1 mg/ml synthetic poly(A), an MN-treated enzyme preparation exhibits levels of tRNA processing activity identical to the control (Figure 4B, lanes 7–10). Different lots of poly(A) from three manufacturers gave identical results. At 2.5 mg/ml, poly(A) substantially inhibited tRNA maturation. The possibility that RNA was acting simply as a polysaccharide (for example, as a phase exclusion polymer) was eliminated by experiments showing that neither glycogen, Ficoll nor polyethylene glycol (each at 1 mg/ml) could restore RNase P activity to MN-treated extracts (data not shown). Fingerprint analysis of mature tRNA produced by an MN-treated (20 U/μl), poly(A)-restored extract showed the presence of all the expected oligonucleotides, including the authentic RNase P-generated 5′ terminus pGp (data not shown).

The restorative effect of poly(A) is most simply described by the phenomenon which we call 'substrate masking'. We have found that EGTA-inactivated micrococcal nuclease can bind tightly to RNA (M.J.Wang, in preparation). Inactivated MN bound to radiolabeled pre-tRNA physically blocks or 'masks' the sites of 5′ and 3′ endonuclease cleavage, thus suppressing tRNA processing. Addition of excess carrier RNA competes with pre-tRNA for MN binding, and thus permits normal processing. This concept is developed further in the Discussion.

A more direct test for the presence of an integral RNA component in a complex is its buoyant density in CsCl. We therefore subjected chloroplast Function II enzyme to equilibrium density centrifugation in CsCl. The results are displayed in Figure 5A; quantitative data are presented in Figure 5B. The peak of RNase P activity is coincident with the center of the protein peak at 1.28 g CsCl/ml (Figure 5B). An RNA content of 5% would be detectable as a density shift of one fraction (0.25 ml), to 1.31 g/ml. The enzyme preparation from CsCl gradients is designated Fraction III. To test the homogeneity of Fraction III, another preparation was subjected to a second round of CsCl ultracentrifugation, and again RNase P activity banded at the top (data not shown). When the gradient was assayed with a 5′ mature, 3′-extended substrate specific for tRNA 3′ endonuclease (described in Materials and methods), activity was found only in the same fractions as RNase P, indicating that 3′ endonuclease also consists of protein.

Because Fraction III RNase P has been freed from endogenous RNA, we re-tested its sensitivity to micrococcal nuclease. Figure 5C demonstrates that Fraction III activity is resistant to high levels of MN. When Fraction III is pre-

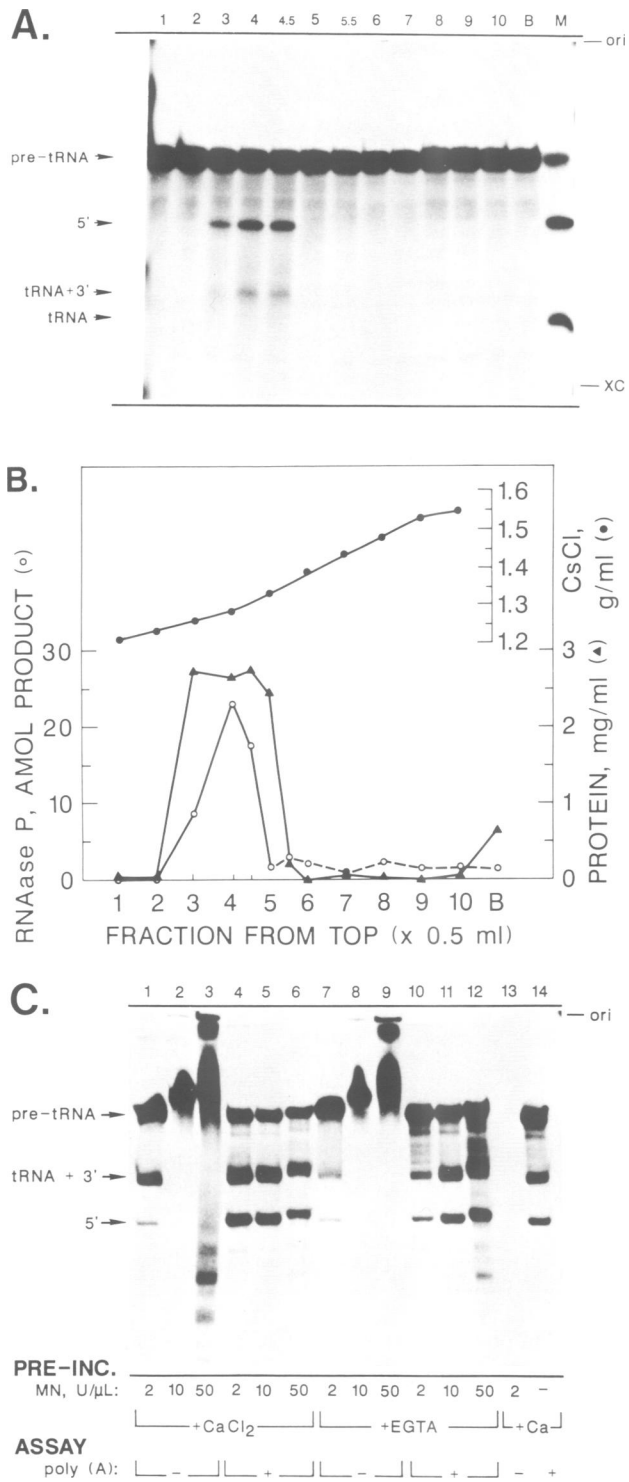

Fig. 5. Buoyant density of chloroplast RNase P activity. Gradients were prepared, centrifuged to equilibrium and analyzed according to Materials and methods. The lowest-density fraction is number 1. (**A**) Gradient from 1.2 to 1.5 g CsCl/ml. Fractions 4, 4.5, 5, and 5.5 were 250 μl; all others were 500 μl. The substrate was precursor II. (**B**) Quantitation of RNase P activity and protein and CsCl concentrations. Solid lines (○——○) indicate radioactivity in authentic RNase P products, and dashed lines (○---○) denote non-specific material which is not an RNase P product (see **A**). (**C**) 4 μl samples of the peak fraction from a different gradient were pre-incubated in 6 μl, according to Materials and methods, with the concentration of micrococcal nuclease shown. Where noted, 20 μg poly(A) was added prior to substrate mix (containing precursor I). Retardation of substrate in **lanes 2, 3, 8** and **9**, and of products in **lanes 6** and **12**, is attributable to residual bound MN. In **lane 13**, MN was not inactivated.

incubated either with $CaCl_2$-activated or with EGTA-inactivated MN, tRNA processing is completely suppressed by substrate masking if the assay is performed in the absence of poly(A) (Figure 5C, lanes 1−3, 7−9). Addition of poly(A) restores processing to control levels (lanes 4−6, 10−12). In other experiments, MN at 32 U/μl gave results comparable to 10 U/μl in Figure 5C (data not shown). Some reduction in the yield of RNase P products is evident at the highest level of MN tested (50 U/μl; Figure 5C, lanes 6, 12). A concomitant increase in unprocessed substrate is not seen, however (compare Figure 5C, lanes 6, 7), suggesting that the reduced yield is attributable to incomplete inactivation of MN.

Comparison of chloroplast and E.coli RNase P

To demonstrate that our protocols for micrococcal nuclease treatment of CsCl gradient centrifugation would correctly identify an RNase P which did contain an RNA subunit, we prepared RNase P from *E.coli* and subjected it to the same analyses. The *E.coli* enzyme was analyzed by CsCl isopycnic ultracentrifugation (final density 1.45−1.75 g/ml). As seen in Figure 6A, *E.coli* RNase P bands near the bottom of the gradient at a density of 1.72−1.73 g/ml. When the chloroplast enzyme was analyzed on a similar gradient, activity was detected only in the top fraction (data not shown). The peak fraction was re-assayed after digestion with micrococcal nuclease. Figure 6B demonstrates that *E.coli* RNase P is irreversibly inactivated by MN digestion regardless of whether the assay is performed in the absence or presence of poly(A). Activity is virtually abolished at 0.5 U MN/μl and is eliminated at 2 U MN/μl. By comparison, the chloroplast 5′ processing activity is resistant to 20−50 U MN/μl (Figures 4B, 5C and data not shown). These results validate our experimental techniques and demonstrate a striking physical difference between chloroplast and bacterial RNase P.

Discussion

Processing pathway for chloroplast tRNA

We summarize the proposed pathway of chloroplast transfer RNA maturation as illustrated in Figure 7. The initial processing event is an RNase P (or P-type) endonucleolytic cut which separates leader sequences from a tRNA plus trailer molecule, thereby generating the mature 5′ terminus of the tRNA. The second endonucleolytic cleavage occurs precisely at the 3′ end of the encoded mature RNA sequence domain. The (C)CA_{OH} terminus is then added stepwise by CTP,ATP:tRNA nucleotidyl transferase activity. In $tRNA^{Phe}_{GAA}$, as in one-third of tobacco chloroplast tRNA genes (Shinozaki *et al.*, 1986), the encoded tRNA domain includes the first C of the 3′ CCA_{OH} sequence, and tRNA 3′-terminal maturation is completed by the addition of single cytidylate and adenylate residues. This specificity of chloroplast tRNA 3′ maturation is distinctly different from the mechanism best described in bacteria (see Introduction), but is identical with that reported for nuclear and mitochondrial pre-tRNAs (Castāno *et al.*, 1985; Frendewey *et al.*, 1985; Manam and Van Tuyle, 1987).

Chloroplast RNase P can process pre-tRNA prior to cleavage by the 3′ maturation endonuclease, as illustrated in Figures 2, 4 and 5. No 3′ mature, 5′-extended intermediates are detected. Furthermore, substrates which are

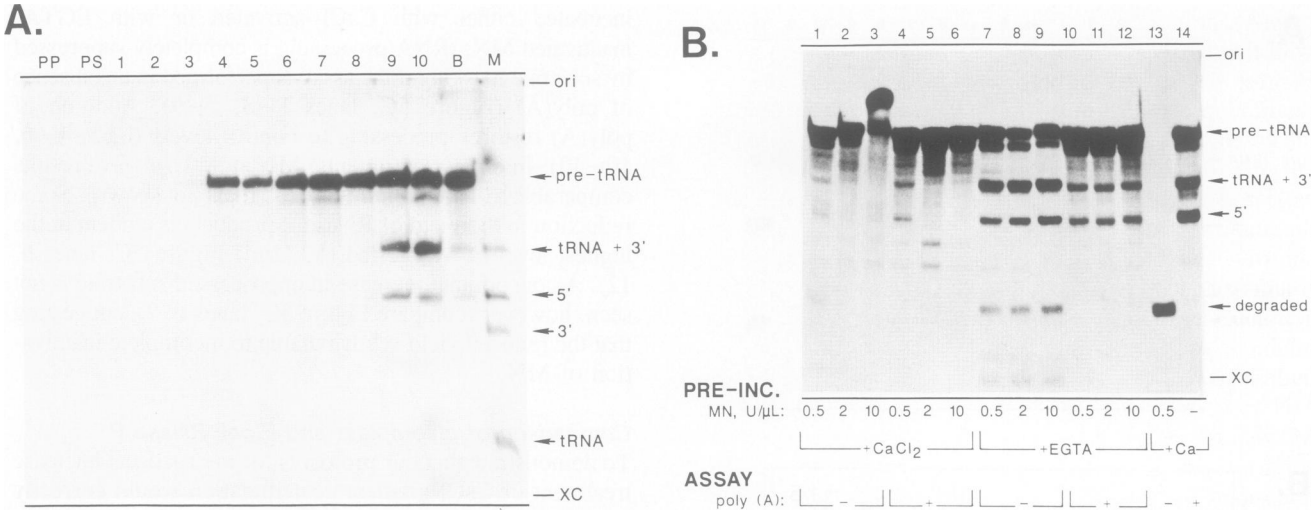

Fig. 6. Buoyant density of *E.coli* RNase P. (**A**) *E.coli* S100 fraction was mixed with CsCl solution, centrifuged and assayed as detailed in Materials and methods, using precursor I. Fractions PP and PS are the detergent-soluble and the buffer-soluble fractions of the pellicle. No substrate is detected in fractions PP, PS and 1–3, which contain essentially all the protein recovered from the gradient, because they have concentrated the non-specific nucleases present in the S100. (**B**) 4 μl aliquots of gradient fraction 10 were treated with micrococcal nuclease just as in Figure 5C. In **lane 13**, MN was not inactivated. The band labeled 'degraded' represents an MN partial degradation product of [^{32}P]pre-tRNA^{Phe} which accumulates at low MN concentration. Its presence in **lanes 7–9** indicates that MN was not completely inactivated.

5′ mature can be processed into tRNA and trailer RNA by the Fraction II chloroplast processing preparation. We have chromatographically resolved 3′ endonuclease from RNase P. The 3′ cleavage activity is highly specific for 5′ mature substrate (X.Q.Li, unpublished data). These observations suggest that an ordered processing pathway exists in which RNase P cleavage is followed by 3′ maturation.

While this work was in progress, Yamaguchi-Shinozaki *et al.* (1987) presented preliminary findings on the processing of tobacco *trnF-GAA* transcripts in S30 lysates of spinach or tobacco chloroplasts. Their results are similar to ours in that both presumptive 5′ and 3′ endonuclease activities were detected. Products were identified on the basis of size only; the positions of processing cleavages were not determined. Consistent with our results, these investigators observed only a putative 5′ mature, 3′-extended processing intermediate.

Novel aspects of chloroplast tRNA 5′ maturation

Plant chloroplasts contain a tRNA 5′ maturation activity which is the functional analogue of eubacterial RNase P. We designate the chloroplast activity as RNase P based solely on the reaction catalyzed. Both activities cleave tRNA precursors precisely at the 5′ side of the mature tRNA domain, releasing a single leader RNA fragment and exposing the mature tRNA 5′ terminus. Nonetheless, the plastid activity differs fundamentally from the eubacterial enzyme. Chloroplast RNase P activity is refractory to digestion with micrococcal nuclease and co-fractionates with protein at 1.28 g/ml on a CsCl buoyant density gradient. By comparison, *E.coli* RNase P, which contains a well-characterized catalytic RNA subunit and is ~80% (w/w) RNA, is sensitive to nuclease treatment and bands at a hybrid density (~1.7 g/ml) in CsCl (this work; Stark *et al.*, 1978).

We cannot exclude that chloroplast RNase P could contain a small amount of RNA which is inaccessible to micrococcal nuclease. A definitive answer will come only after final purification of the enzyme, which is in progress. At this stage, the most straight-forward explanation is that plant chloroplast RNase P does not have an RNA subunit comparable to that of *E.coli* or *B.subtilis*.

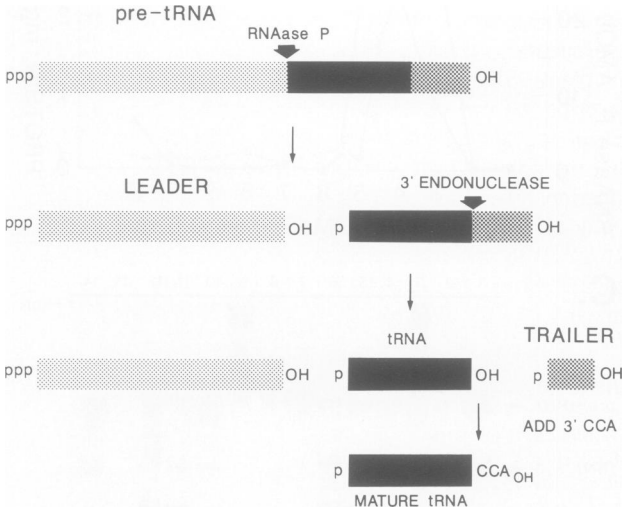

Fig. 7. Pathway for chloroplast tRNA maturation. Details are discussed in the text.

In addition to the experimental results described here, we searched the tobacco chloroplast genome for seven regions (9–35 nt) common to all eubacterial RNase P RNAs (James *et al.*, 1988; Lawrence *et al.*, 1987). Even when examined at relaxed stringency, the chloroplast DNA sequence did not contain homologues of all conserved sequence blocks in the appropriate order. We conclude that the chloroplast genome does not encode a eubacterial type of RNase P RNA. Similar results have also been obtained by G.Olsen (N.R.Pace, personal communication).

Substrate masking by inactive micrococcal nuclease

We found, unexpectedly, that inactive MN inhibits tRNA processing if the final assay is performed in the absence of carrier RNA. Our molecular explanation for this phenomenon is as follows. We have recently demonstrated both by native gel electrophoresis and by nitrocellulose filter retention that EGTA-inactivated MN forms a complex with free RNA which can be dissociated by addition of yeast RNA,

poly(A), or heparin (M.J.Wang, in preparation). Consistent with this finding, many of our processing assays, such as Figures 4B and 5C, exhibit progressive electrophoretic retardation of substrate with increasing amounts of MN in the absence but not in the presence of poly(A). Our starting enzyme fractions contain endogenous RNA which acts as carrier to prevent substrate masking by inactive MN. After digestion with active MN, endogenous RNA is degraded and substrate masking is observed (Figure 4A). In enzyme fractions lacking RNA, substrate masking is observed with active or inactive MN (Figure 5C). This result proves that inhibition of RNA processing by MN does not involve RNA hydrolysis. Subsequent addition of carrier poly(A) binds the MN and permits normal processing. In these experiments, MN is present in saturating molar excess over pre-tRNA substrate, and poly(A) is further in excess over MN. Reversible suppression of RNA processing by MN was first noted by Ryner and Manley (1987; cited in Hashimoto and Steitz, 1986) during investigation of a possible requirement for RNA in nuclear mRNA 3′ cleavage and polyadenylylation reactions.

Conclusion

Despite the comparatively close evolutionary relationship between chloroplasts and eubacteria, they differ in their approaches to transfer RNA maturation. RNase P activity in plant chloroplasts differs fundamentally from that described at present in eubacteria. From a comparison of *E.coli* and spinach chloroplast RNase P activities, we infer that the chloroplast enzyme does not contain a substantial functionally required RNA component. We are currently comparing the properties of plant nuclear and chloroplast RNase P to test the hypothesis that chloroplast RNase P might be a nuclear-encoded protein related to the nuclear enzyme.

Materials and methods

Construction of clones

Maize chloroplast pre-tRNA^{Phe} subclones were constructed from maize chloroplast DNA *Bam*HI fragment 5, provided by E.Orozco,Jr and N.-H.Chua. Plasmid pTUC8-NXPhe contains an *NsiI*−*XbaI* fragment spanning positions −116 to +165 from the 5′ end of *trnF-GAA* (Steinmetz *et al.*, 1983) inserted into pTUC8. [Plasmid pTUC8 (G.Morin and T.R.Cech, personal communication) consists of pUC8 with a synthetic phage T7 promoter at the *Eco*RI site.] Plasmid pT72-NBPhe consists of pT7-2 (US Biochemicals) plus the *trnF*-containing *NsiI*−*BglII* fragment from −116 to +84 flanked by the *Hin*dIII−*NsiI* and *Bam*HI−*Eco*RI polylinker regions of pTUC8. Both strands of the 5′ leader, and the non-coding strand of the 3′ trailer, were sequenced via the supercoil method (Chen and Seeburg, 1985) using T7 DNA polymerase (Sequenase, US Biochemicals).

Preparation of pre-tRNA substrates

Radiolabeled tRNA^{Phe} precursors were produced by transcription with phage T7 RNA polymerase (US Biochemicals or Pharmacia). Precursor I was synthesized from plasmid pTUC8-NXPhe linearized with *Hin*dIII, and precursor II was transcribed from pT72-NBPhe cut with *Bam*HI. A precursor to *E.coli* tRNA^{Phe}, mature at the tRNA 5′ terminus but extended 124 nt at the 3′ end, was transcribed from plasmid p67CF0 (obtained from O.Uhlenbeck) cut with *Pvu*II. Typical reactions (100 μl) contained 40 mM Tris−HCl (pH 8.0), 15 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.5 mg/ml nuclease-free bovine serum albumin (BSA), 40−50 μg/ml template DNA, 3500−4500 U/ml T7 RNA polymerase, 1 mM each rNTP, and 50 μCi each [α-^{32}P]rNTP (New England Nuclear). RNAs destined for preparative-scale processing were synthesized with 0.4 mM each rNTP. The specific activity of pre-tRNA^{Phe} transcripts was thus 0.165 or 0.411 μCi/pmol pre-tRNA. After 2 h at 37°C, 50 μl of 3 × loading mix (7.5% Ficoll, 0.075% bromophenol blue, 0.075% xylene cyanol, 30 mM Na·EDTA (pH 8), 10 M urea) was added. The mixture was heated 2 min at 65°C and fractionated by electrophoresis on 8% or 10% polyacrylamide gels (acrylamide:bisacrylamide, 19:1) containing 0.5 × TBE (1 × TBE is 89 mM Tris, 89 mM boric acid, 2.5 mM Na_2EDTA) and 7 M urea. Transcripts were located by autoradiography, excised and eluted either by diffusion in 10 mM Tris−HCl, pH 7.4, 1 mM Na·EDTA, 0.5 M NH_4OAc, 1% phenol or by electrophoresis for 1.5−2 h at 200 V in an autoclaved Elutrap apparatus (Schleicher and Schuell) filled with sterile 0.2 × TBE. The products were ethanol precipitated twice from 2 M NH_4OAc, dried, and resuspended in sterile water.

Processing assay

Routine assays contained ~0.1 pmol [^{32}P]pre-tRNA and 3−30 μg protein in 10 or 20 μl of 20 mM Hepes-KOH (pH 8.0), 50 mM KCl, 15 mM $MgCl_2$, 0.2 mM Na·EDTA (pH 8), 2 mM DTT, and ~5% total glycerol. Reactions were incubated 10−30 min at 37°C and stopped by digestion with 0.1 vol of stop mix [2 mg/ml proteinase K (Boehringer Mannheim), 0.15 M Na·EDTA (pH 8), 5% SDS]. Loading mix (0.5 vol of 3 × stock) was added; the samples were heated 2 min at 65°C and loaded onto 0.5 or 1.5 mm thick 10% polyacrylamide gels containing 0.5−1 × TBE and 7 M urea. Electrophoresis was at 40°C (15−25 W) until xylene cyanol dye was 1−2 cm above the bottom of the gel. Following autoradiography, Cerenkov radiation in gel slices corresponding to each RNA species was determined, corrected for a background from each lane, and converted to fmols of RNA.

RNA structure analysis

RNA fingerprinting and secondary analysis methods were essentially as previously described (Gegenheimer *et al.*, 1983; Gegenheimer and Apirion, 1980). The first dimension electrophoresis tank buffer did not contain urea. The second dimension was developed with an aged preparation of homomix C5 (comparable to fresh homomix C10). Each oligonucleotide from every RNA species (A−D) was quantitated by liquid scintillation spectrometry. The molar yield of nearly every oligonucleotide was within 20% of predicted values. The sequence of oligonucleotides >4 nt was confirmed by RNase A secondary digestion (data available on request). The predicted RNase T1 and RNase A products and yields, and experimental molar yields, were determined with previously-described software (Gegenheimer, 1988). Candidates for 5′ termini (pGp and pAGp) and a control (ApGp) were eluted from the RNase A TLC plates, mixed with 5 μg carrier RNA, and digested with RNase T2 (0.1 U in 5 μl of 20 mM NH_4OAc) for 1 h at 50°C. Products were analyzed on PEI TLC plates developed with 1.6 M LiCl. Unlabeled rNTPs were used as internal markers. pGp co-migrates with ATP, and pAp migrates slightly faster than CTP.

Preparation of chloroplast processing activities

Fraction II processing activity is a 13−60% saturation (at 4°C) $(NH_4)_2SO_4$ fraction of stromal protein from gradient-purified, hypotonically lysed spinach chloroplasts (Gruissem *et al.*, 1983; Gruissem *et al.*, 1986b). Fresh, young spinach leaves were obtained from local supermarkets. Chloroplasts were prepared according to Gruissem *et al.* (1986b) with the following exceptions. Homogenization mix ('GR buffer') was prepared according to Orozco *et al.* (1986) and additionally contained 2 mM DTT. Intact chloroplasts were purified on 40%/80% Percoll step gradients (Orozco *et al.*, 1986) also containing 1 mM DTT, 0.3 mM glutathione and 5 mM Na·ascorbate. The chloroplasts were lysed in hypotonic buffer (Gruissem *et al.*, 1986b), giving 'Fraction I'. Ammonium sulfate fractionation was as described, except that DEAE cellulose chromatography was omitted, and the final pellet was collected by centrifugation for 20 min at 40 000 r.p.m. in the SW 41 rotor. The final preparation, Fraction II (15−30 mg protein/ml in dialysis buffer; Gruissem *et al.*, 1986b), was quick-frozen in aliquots and stored at −70°C. tRNA maturation activity at this stage is stable for >1 year and can be thawed and quick-frozen repeatedly.

Preparation of E.coli processing activities

E.coli K12 strain MRE 600 (RNase I) was grown at 37°C in 1 l LB medium to OD_{560} = 0.6. The cells were harvested at 4°C (10 min, 6000 r.p.m., GSA rotor), washed twice with sterile TM2 buffer [10 mM Tris−HCl (pH 7.4), 10 mM $MgCl_2$] and resuspended in 5 ml sonication buffer [20 mM Tris−HCl (pH 7.4), 0.1 mM Na·EDTA, 10 mM $MgCl_2$, 60 mM NH_4Cl, 5% glycerol, 1 mM DTT] plus 'protease inhibitor mix' (1 mM PMSF, 1 mM benzamidine·HCl, 5 mM ε-amino-*n*-caproic acid). Cells were lysed on ice by three cycles of 20 s pulse (60% power) plus 40 s rest with the standard probe of a Bronson Biosonik 250 sonicator. The extract was clarified by centifugation for 40 min at 20 000 r.p.m. in the SW 50.1 rotor. The S30 supernatant was brought to 0.2 M NH_4Cl and centrifuged 3 h at 35 000 r.p.m. in the same rotor. The resulting S100 supernatant (4.3 mg protein/ml) was frozen in aliquots and stored at −70°C.

CsCl density gradients

Fraction II chloroplast enzymes (~5 mg) were treated with 1 U MN/μl and 5 mM $CaCl_2$ for 20 min at 25°C. Reaction was terminated with a 5-fold molar excess of Na·EGTA (pH 8), and the samples were chilled on ice and diluted 2-fold with gradient buffer [20 mM Hepes-KOH (pH 8.0), 60 mM KCl, 1 mM Na·EDTA (pH 8), 2 mM DTT] plus ultra-pure BRIJ-35 (Pierce) to a final 0.1%. The mixture was diluted with gradient buffer to 1.0 ml and 0.05% BRIJ-35 and layered (Pace and Spiegelman, 1966) over 4.0 ml of 1.40 g CsCl/ml gradient buffer. The gradients were then formed by centrifugation in the SW 50.1 rotor for 58 h at 40 000 r.p.m. and 8°C. Fractions of 250 μl or 500 μl were collected manually from the top. The bottom of the gradient tube was rinsed with 300 μl dialysis buffer, giving fraction 'B'. Each fraction was dialyzed for 24 h versus 4 × 1 l of dialysis buffer (Gruissem *et al.*, 1986b).

For analysis of the *E.coli* S100 fraction, 1 ml was mixed with 3.3 ml of room-temperature CsCl solution and adjusted with sonication buffer to 1.60 g/ml. 5-ml samples were subjected to centrifugation (69 h, 35 000 r.p.m., 16°C) in the SW 50.1 rotor. The pellicle fraction was removed and extracted first with gradient buffer and then with gradient buffer plus 0.5% BRIJ-35. Ten fractions of 500 μl were then collected manually from the top of the gradient. A bottom ('B') fraction was prepared as for chloroplast gradients. CsCl concentrations were determined by refractometry. Protein (10 μl samples) was assayed with 200 μl BCA reagent (Pierce); standard curves were constructed with BSA.

Micrococcal nuclease treatment

Micrococcal nuclease (MN) (15 000 U/mg) was obtained from Boehringer, dissolved in gradient buffer (lacking DTT) at 300 U/μl, and stored at −20°C. Serial dilutions were made in the same buffer. Poly(A) from Pharmacia, Boehringer, or Calbiochem was dissolved in sterile water to 20−50 μg/ml. Chloroplast Fraction II enzyme preparation was diluted with an equal volume of gradient buffer plus protease inhibitor mix. Typically, 2 μl of this dilution, or 4 μl of CsCl gradient fractions, was incubated in 4−7 μl with 4−400 U MN and 20 nmol $CaCl_2$ for 30 min at 37°C. 100 nmol of Na·EGTA [ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, pH 8 in water] was added to stop the MN reaction. For analysis of CsCl gradient fractions, 200 nmol EGTA was used. Next, 20 μg of poly(A) was added. Finally, the reaction was brought to 20 μl by addition of a substrate mix containing [^{32}P]pre-tRNA, 5% glycerol and sufficient 10 × processing buffer (as above) for a final 1 × concentration. The processing reaction was performed for 30 min at 37°C. Further treatment and analysis was as described above.

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