In vitro processing of mitochondrial and plastid derived tRNA precursors in a plant mitochondrial extract

Anita Marchfelder, Wolfgang Schuster and Axel Brennicke
Institut für Genbiologische Forschung, Ihnestraße 63, D-100 Berlin 33, FRG

Received January 2, 1990; Revised and Accepted February 22, 1990 EMBL accession nos X07922, X07923

ABSTRACT

A lysate of purified mitochondria of the higher plant *Oenothera* processes *in vitro* synthesized tRNA precursors to the mature tRNA size. *In vitro* synthesized transcripts containing genuine plant mitochondrial tRNAs and analogous RNAs from mitochondrial loci with plastid derived tRNA sequences are accurately processed by an RNAase P-like activity to yield the mature 5'-terminus. A four nucleotide deletion in the anticodon stem-loop structure, however, prevents processing. The results show that *in vitro* transcripts containing tRNAs from sequence fragments of plastid origin integrated in plant mitochondrial genomes can be processed correctly in plant mitochondria, if tRNA sequences and structures are intact.

INTRODUCTION

A number of sequence fragments of plastid and nuclear origin have been found in the mitochondrial genomes of a number of different plant species (1). Many of these transferred sequences contain plastid tRNA genes that could potentially be activated in the mitochondrial translation system. Several tRNA molecules with sequence homologous to plastid insertions have indeed been found in plant mitochondria (2–7).

Transcription of these plastid fragments would presumably depend on the upstream position of a mitochondrial promoter from which transcripts could be initiated containing the tRNA sequence of the plastid fragment. The mitochondrial tRNA processing enzymes must then be able to recognize the originally plastid tRNA structure as a substrate.

Particularly interesting in this respect is a plastid fragment identified in the mitochondrial genomes of several species (wheat, maize and *Oenothera*; 5–7) with the sequences of two tRNA genes. Sequences homologous to the plastid $tRNA^{Pro}$ and $tRNA^{Trp}$ are linked in the plastid and mitochondrial genomes of the three plant species, and an ancient transfer event from the plastid to the mitochondrial genome before the divergence of these plant species has been accordingly suggested for the origin of this sequence (5). The plastid derived $tRNA^{Pro}$ gene is not essential in plant mitochondria since another gene with the same anticodon is encoded in plant mitochondria, presumably the 'genuine' gene (8). This redundancy may explain why in *Oenothera* and wheat mitochondria the $tRNA^{Pro}$ gene has been destroyed by independent deletions of only four internal nucleotides in the *Oenothera* sequence, but two thirds of the coding sequence in wheat mitochondria (5,6). The $tRNA^{Trp}$ gene on the other hand is the only gene for a tRNA with this specificity so far identified in higher plant mitochondria. The single $tRNA^{Trp}$ species found in the mitochondrial tRNA population of bean appears to be transcribed from the bean mitochondrial homologue of this very plastid insert. This plastid sequence is present in the main mitochondrial genomes of wheat and *Oenothera* but on a 2.3 kb linear plasmid in maize mitochondria which is therefore essential in the maize mitochondrion for synthesis of the $tRNA^{Trp}$ (5,7). The $tRNA^{Trp}$ gene on the maize mitochondrial plasmid is transcribed into a tRNA-sized transcript whereas no such transcript could be found for the upstream encoded $tRNA^{Pro}$ (7). Any longer precursor transcript molecule of the $tRNA^{Trp}$ initiated from upstream mitochondrial promoters contains both 5'- and 3'-flanking sequences that are (although considerably diverged) still recognizeable derived from the plastid insertion. The mitochondrial processing activities will have to recognize correctly the plastid-like sequence of $tRNA^{Trp}$ to excize the mature length. Therefore a $tRNA^{Trp}$ transcript is one of the first candidate templates to test for the mitochondrial processing competence of transcripts from plastid sequences integrated in plant mitochondrial genomes.

The *in vitro* processing experiments with plant mitochondrial lysates described in this communication were aimed at investigating this specificity and the requirements of plant mitochondrial tRNA processing activities in processing tRNA precursors derived from plastid inserts in the mitochondrial genome. The results indicate that the enzymatic activities required for tRNA maturation of longer precursors from both 'original' mitochondrial loci and from transferred and integrated plastid sequences are present in plant mitochondrial extracts.

MATERIAL AND METHODS

Tissue cultures from *Oenothera berteriana* were grown and propagated in the dark as described (9).

Preparation and purification of mitochondria

Cells were harvested after four weeks of subculture and suspended in ice-cold isolation buffer (300mM mannitol; 10 mM KH_2PO_4; 50 mM Tris (pH 7.4); 5 mM EGTA; 4mM mercaptoethanol). Cells were broken in a waring blendor by three

strokes of six seconds each. Unbroken cells and large debris was removed by centrifugation at 1,000 g for 15 min and subsequent filtration through four layers of muslin cloth and four layers of miracloth. Mitochondria were pelleted by centrifugation at 10,000 g for 20 min. Mitochondria were resuspended in isolation buffer and layered onto preformed percoll step gradients. Gradients were spun at 40,000 g for 45 min, mitochondria were taken from the 45% to 28% interface, transferred to a 30 ml corex tube and diluted to 25 ml with isolation buffer. Mitochondria were pelleted by centrifugation at 17,000 g for 10 min, the supernatant was carefully removed, the pellet was resuspended in the residual buffer and transferred to a sterile Eppendorf tube. Mitochondria were repelleted at maximum speed in an Eppendorf centrifuge for 5 min. The mitochondrial pellet was taken up in processing buffer (40 mM Tris (pH 7.6); 40 mM KCl; 10 mM $MgCl_2$; 20 mM spermidine; 2 mM DTT) and repelleted. The supernatant was discarded and the pellets stored in aliquots at −20°C.

Lysis of mitochondria and incubation conditions

For preparation of the mitochondrial lysate mitochondria were disrupted by three rounds of freeze-thawing and resuspended in processing buffer. In each assay about 100,000 cpm of *in vitro* synthesized, uniformly with ^{32}P-UTP labelled precursor tRNA was added and incubated at 25°C. Samples were taken at different incubation times and extracted immediately with hot phenol at 60°C. Residual phenol was removed by chloroform extraction and nucleic acids were ethanol precipitated.

Reaction products were analysed on 8% polyacrylamide gels and identified by autoradiography.

Preparation of mitoplasts and matrix fractions

The outer membrane was stripped from purified mitochondria by digitonin treatment (10) and pelleting of the resulting mitoplasts at 100,000 g for 10 min. Mitoplasts were washed in extraction buffer, repelleted and disrupted by osmotic shock in a twofold diluted extraction buffer and a freeze-thawing cycle. Membranes were pelleted by centrifugation at 100,000g for 120 min and the supernatant was taken as the matrix fraction (11). In parallel experiments mitoplasts were obtained by disruption of the outer membrane through osmotic shock in low sucrose concentration. Mitoplasts were alternatively sonified to obtain inside-out vesicles with the matrix released. Membrane vesicles were then spun down from the matrix supernatant at 184,000 g for 60 min.

Template preparation

RNAs were synthesized as run-off products from T7 or T3 promoters (Fig. 1) in the presence of 50 μCi α-^{32}P UTP and 2 μg DNA, 40 mM Tris (pH 7.4), 6 mM $MgCl_2$, 20 μg/ml BSA, 2 mM spermidine, 0.4 mM each of ATP, GTP, CTP, 0.1 mM UTP, 20 U RNasin, 20 U T7 or T3 polymerase. The entire reaction mix was loaded onto 4% polyacrylamide gels to purify the RNA product. The RNA was identified by autoradiography, excised and eluted from the gel and further purified by a second round of gel electrophoresis and elution.

Primer extension

The RNA to be analysed was produced in an unlabelled synthesis reaction, processed and cut out from the product analysis by analogy with a parallel assay of labelled substrate. For the primer extension reaction approximately 150 ng eluted RNA (intermediate reaction product) was incubated with 70 ng oligonucleotideprimer for 10 min at 60°C and annealed for 30 min at 37°C. A tRNA internal primer was used for both cDNA strand synthesis and the sequencing reactions of the plasmid template for length determination. The primer extension was done in 50 mM Tris (pH 8.2), 6 mM $MgCl_2$, 10 mM DTT, 100 mM NaCl, 50 U RNasin, 0.4 mM each of dCTP, dGTP, dTTP, 40 μCi α-^{35}S dATP and 25 U AMV reverse transcriptase for 30 min at 37°C and chased with 1 μl of 2 mM dNTPs and 25 U reverse transcriptase for 30 min at 37°C. The ethanol precipitate from this reaction was redissolved in denaturing loading buffer and electrophoresed in 8% polyacrylamide gels.

Proteinase K and micrococcal nuclease treatment

The mitochondrial lysate was incubated with 100 μg proteinase K for 20 min at 4°C before addition of the RNA. The lysate was digested with 50 μg of micrococcal nuclease with 10 mM $CaCl_2$ for 20 min at 4°C. The nuclease was inhibited with 50 mM EGTA before addition of the RNA processing substrate. In controls lysate was preincubated for 20 min at 4°C before addition of the RNA.

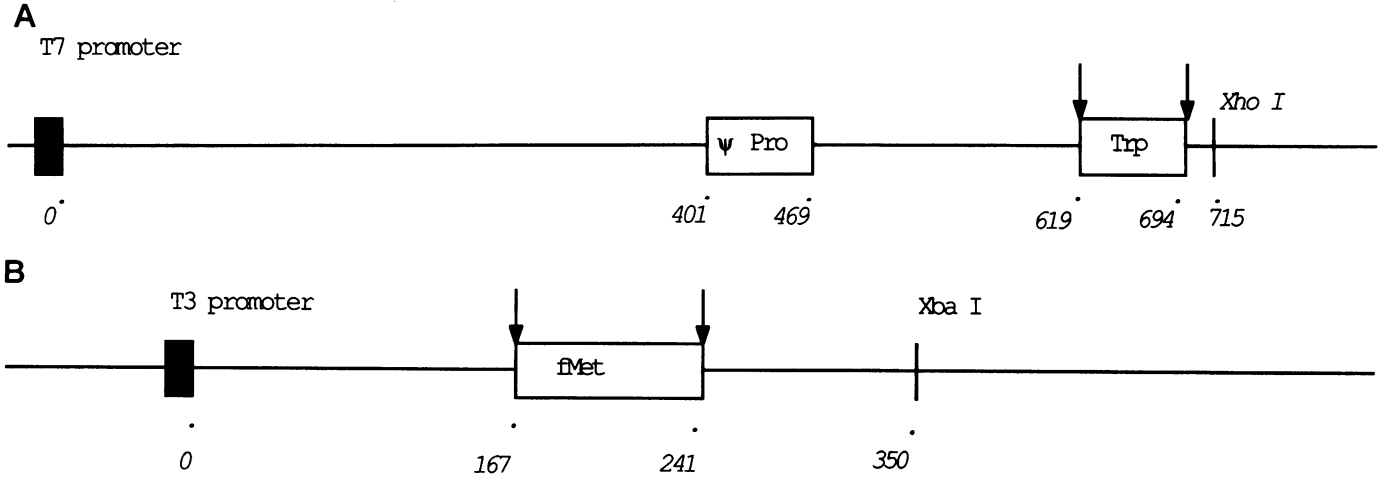

Fig.1 Templates for the production of the artificial tRNA containing substrates. Part A shows the construct for the *Oenothera* mitochondrial locus with the integrated plastid fragment containing the $tRNA^{Pro}$ pseudogene and the $tRNA^{Trp}$ gene and flanking sequences cloned behind a T7 phage promoter of the Bluescript vector. Part B depicts the 'native' *Oenothera* mitochondrial $tRNA^{fMet}$ locus cloned behind a T3 phage promoter for *in vitro* transcription. Both drawings show the templates for the run-off transcripts used in the experiments detailed in figures 2,3 and 5.

RESULTS

Preparation of templates

The mitochondrial loci encoding the plastid derived $tRNA^{Trp}$ - $tRNA^{Pro}$ region and the mitochondrial $tRNA^{fMet}$ in *Oenothera* have been described previously (6,12,13). The $tRNA^{fMet}$ gene is distinct from the plastid encoded counterparts and appears to be a 'genuine' mitochondrial gene. The $tRNA^{Trp}$ is encoded on a sequence fragment of plastid origin and shows more than 90% similarity with the plastid homologues. The only $tRNA^{Trp}$ gene so far identified in plant mitochondria from several species is transcribed in plant mitochondria (5).

Both genomic clones were further subcloned in Bluescript vectors to allow the *in vitro* synthesis from T7 or T3 phage promoters of RNA molecules containing the tRNAs (Fig. 1). The *in vitro* synthesized RNA templates were purified by polyacrylamide gel electrophoresis to reduce the background of non-specific breakdown products. The RNA eluted from the gel was still highly contaminated with smaller RNAs as evidenced by the comparison of the control lanes in figure 2 and 3. A second gel purification step removed most of the background of the $tRNA^{Trp}$ precursor (fig.2, lane E and 1) in comparison with the $tRNA^{fMet}$ precursor purified only through one gel run which is nevertheless sufficient for the reaction (Fig. 3).

Optimization and purity of the mitochondrial lysate

Several procedures for the isolation and purification were tested for the optimal processing activity. The finally adopted scheme is described in the preceding section yielding the highest activity towards the substrates assayed in the crude mitochondrial lysates.

Purity of the mitochondrial extracts was tested in parallel experiments with matrix and membrane fractions from mitochondria. The bulk of processing activity cofractionated with the mitochondrial matrix prepared by different methodes with specificities identical with the crude lysate towards the membrane fractions indicating low contamination by adherent cytoplasmic or plastid processing activities. The unlysed mitochondrial preparation had no activity in the assays. Increasing percentages of lysed mitochondria also increased the processing activity. Intact mitoplasts likewise had no processing activity, which was only released from the matrix after disruption of the inner mitochondrial membrane. These results suggest that the activity observed is indeed contained within the mitochondrion and not due to contamination from other cellular compartments.

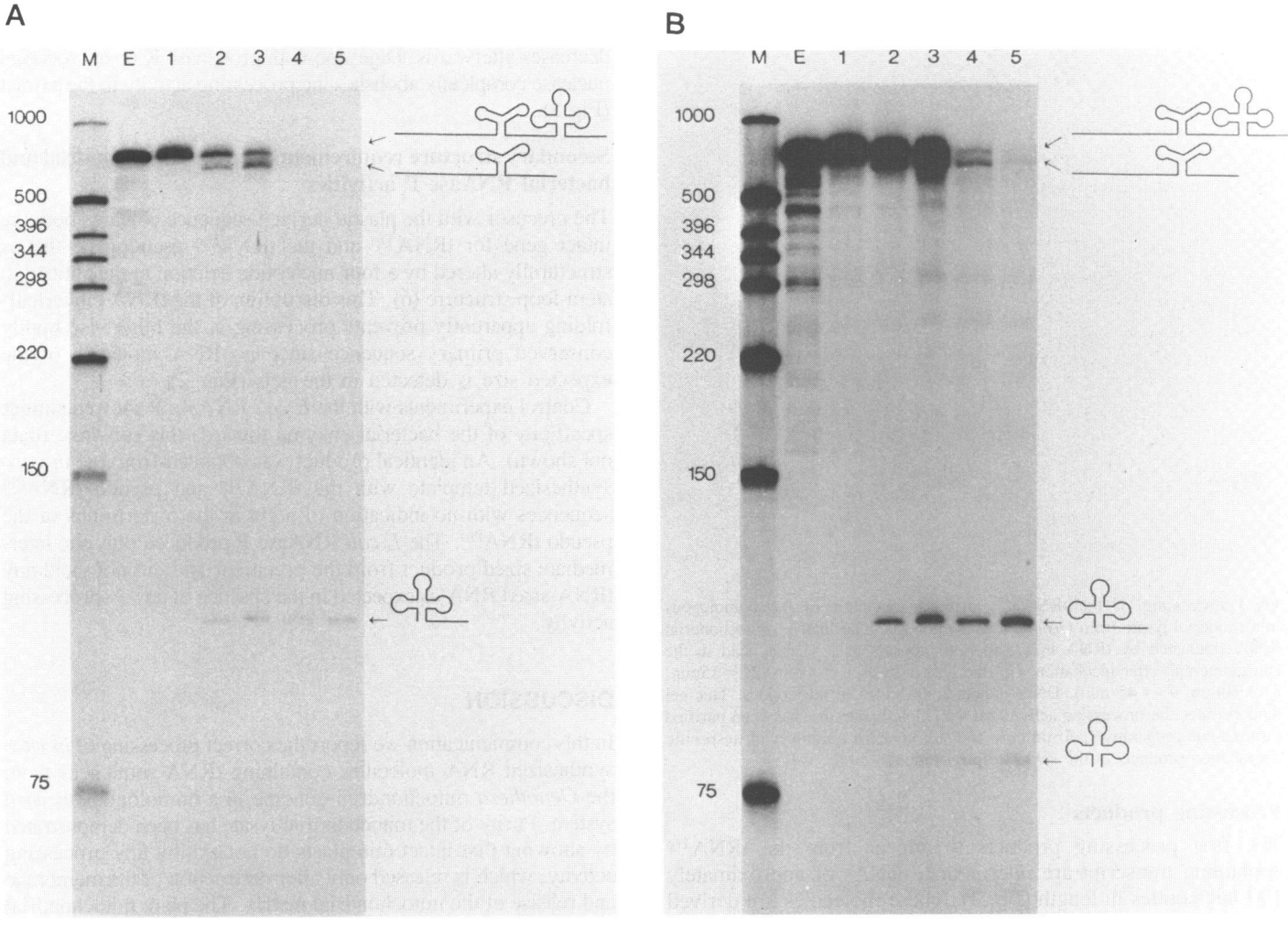

Fig.2 Analysis of the reaction products obtained by incubation of the artificial transcript containing the mitochondrial locus of the plastid insertion ($tRNA^{Trp}$ and pseudo $tRNA^{Pro}$) with the *Oenothera* mitochondrial lysate. Aliquots were taken at different times (lanes 1 = 0min, 2 = 5min, 3 = 15min, 4 = 35min, 5 = 60min; M = DNA marker in nucleotides; E = *in vitro* synthesized RNA substrate loaded in excess amount to demonstrate the still inherent degree of unspecific degradation products) and electrophoresed to show the progressive processing. The RNA species observed in the reaction are schematically depicted at their corresponding positions. The schematic secondary structure of the $tRNA^{Pro}$ pseudogene is given as predicted by optimal computer folding (20). Short (**A**) and long (**B**) exposures of a gel are shown to allow the identification of both the 5'-processing product and the mature tRNA molecules.

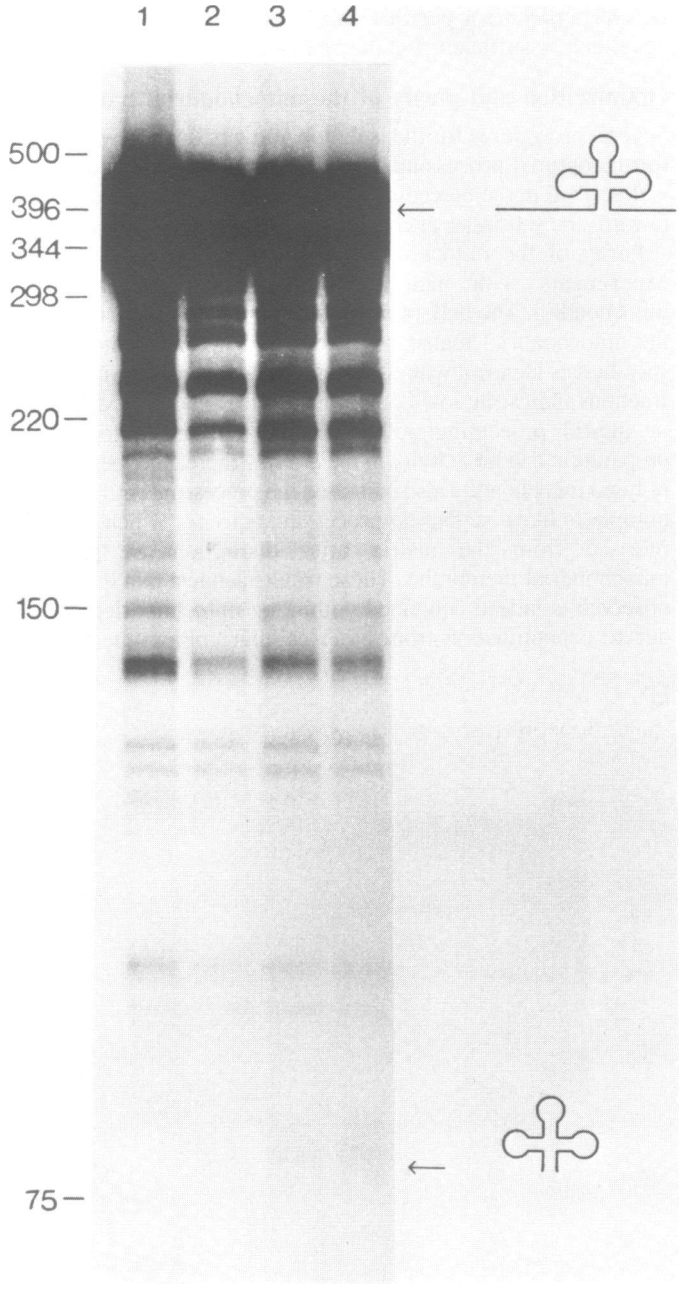

Fig.3 Processing of the $tRNA^{fMet}$ artificial precursor in the homologous mitochondrial lysate from *Oenothera* mitochondria. The 'native' mitochondrial tRNA (indicated by tRNA in the margin) appears as a visible band in the autoradiograph after incubation with the lysate (lanes 1 = 0min, 2 = 15min, 3 = 30min, 4 = 45 min). DNA standards are given in nucleotides. This gel demonstrates the processing activity on a RNA template that has been purified through one preparative gel run only and still contains a number of unspecific degradation products in the substrate preparation.

Processing products

The first processing products to appear from the $tRNA^{Trp}$ containing transcript are intermediate RNAs of approximately 100 nucleotides in length (Fig.2). These molecules are derived from the precursor by endonucleolytic processing at the 5′-termini of the tRNAs and contain the tRNA sequence together with the 3′-trailer sequence. RNA molecules with the size of the mature tRNA of about 80 nucleotides in length are detectable after about 15 min incubation with the lysate. The slow migration of the mature tRNA reaction product with 75 nucleotides as a band at about 80 nucleotides may be due to incomplete resolution of secondary structures of the tRNA in this gel system or addition of the CCA terminus in the lysate. No intermediate product was detectable in the processing assay of the $tRNA^{fMet}$ containing precursor at the 5′- and 3′-tRNA termini. The precursor molecules of both tRNAs are substrates for the 5′-mitochondrial processing activity with approximately equal efficiency (Figs. 2 and 3).

Identification of the 5′-processing site

Primer extension experiments were performed on the intermediate processing product of 100 nucleotides from the $tRNA^{Trp}$ transcript to determine the sequence of events and to avoid contamination of the mature, endogenous mitochondrial $tRNA^{Trp}$ present in the mitochondrial lysate. The cDNA strands synthesized on these templates from a $tRNA^{Trp}$ internal oligonucleotide yielded molecules with homogenous 5′-termini at the very first nucleotide of the mature tRNA (Fig. 4). No unspecific side products were observed indicating a very precise cut of the 5′-tRNA terminus.

Stability of the activity

The processing activity in the crude mitochondrial lysate is stable for about 30 min under the incubation conditions and rapidly decreases afterwards. Digestion with proteinase K or micrococcal nuclease completely abolishes the processing activity in the extract (Fig.5).

Secondary structure requirements of plant mitochondrial and bacterial RNAase P activities

The precusor with the plastid derived sequence contains both the intact gene for $tRNA^{Trp}$ and the $tRNA^{Pro}$ pseudogene that is structurally altered by a four nucleotide deletion in the anticodon stem-loop structure (6). This disruption of the tRNA cloverleaf-folding apparently prevents processing at the otherwise highly conserved primary sequence since no RNA molecule of the expected size is detected in the gels (Fig. 2).

Control experiments with the *E.coli* RNAase P showed similar specificity of the bacterial enzyme towards this substrate (data not shown). An identical product was obtained from the *in vitro* synthesized template with the $tRNA^{Trp}$ and pseudo $tRNA^{Pro}$ sequences with no indication of a cut at the 5′-terminus of the pseudo $tRNA^{Pro}$. The *E.coli* RNAase P produced only the intermediate sized product from the precursor and did not yield any tRNA-sized RNA as expected in the absence of any 3′-processing activity.

DISCUSSION

In this communication we report the correct processing of *in vitro* synthesized RNA molecules containing tRNA sequences from the *Oenothera* mitochondrial genome in a homologous *in vitro* system. Purity of the mitochondrial lysate has been demonstrated by showing that intact mitoplasts do not exhibit any processing activity, which is released only after destruction of the membrane and release of the mitochondrial matrix. The plant mitochondrial lysate processes both 'native' tRNA precursors and RNAs derived from a mitochondrial sequence of plastid origin. A similar activity has been mentioned but not yet described in detail for a wheat mitochondrial extract processing both plastid-like and 'native' mitochondrial containing RNAs (4).

The plastid derived fragment in the mitochondrial genome of

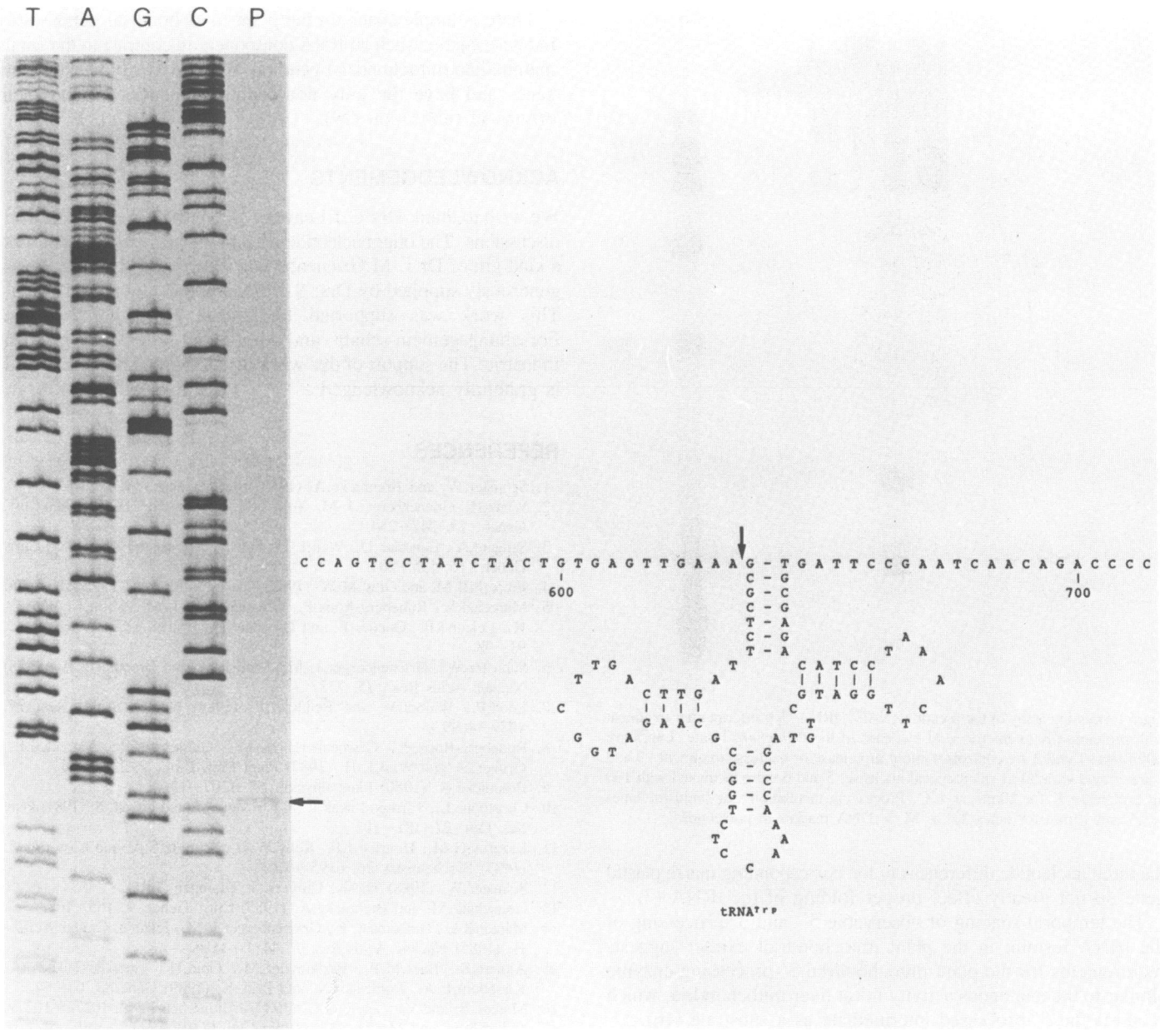

Fig.4 Primer extension with a $tRNA^{Trp}$ terminal oligonucleotide (5'-GGCACGCTCTGTAGGATTTGAACCTAG-3') indicates a homogeneous terminal nucleotide at the precise 5'-terminus of the mature $tRNA^{Trp}$ as predicted from the sequence after the first cleavage step investigated in this experiment with the intermediate processing product as substrate. The four nucleotide specific sequencing reactions (T,A,G,C) were derived from the cloned DNA fragment with the same primer. The DNA sequence is shown with its complementary sequence to allow the direct comparison with the terminal nucleotide of the primer extension (P). The arrow denotes the processing site in the primer extension gel and the schematic folding of the sequence.

Oenothera investigated here encodes the only $tRNA^{Trp}$ gene found in plant mitochondrial genomes of several species (5,6) and a $tRNA^{Trp}$ derived from this sequence has been identified in wheat (5) and bean (14) mitochondria. This tRNA gene is linked to a $tRNA^{Pro}$ gene in plastid genomes, that has been either completely or partially cotransferred to the mitochondrial genomes. The *Oenothera* plastid insert in the mitochondrial genome, however, has a four nucleotide deletion in the $tRNA^{Pro}$ gene sequence that precludes a proper folding on the anticodon stem-loop structure (6). The processing activity in the *Oenothera* mitochondrial lysate does not recognize this pseudo-tRNA structure as substrate and cannot process the precursor transcript at its termini.

This observation implies that not only the linear sequences are determinants of the RNAase P-like specificity in plant mitochondria, but also the complete, intact structural folding is required for recognition. The intact structure of the tRNA moieties has been shown to be the determinant of the *E.coli* RNAase P (15) orientating the enzyme on the precursor molecule to cleave at the right nucleotide. The bacterial enzyme indeed shows the same specificity on the plant mitochondrial substrate. Both the plant mitochondrial and the bacterial tRNA-processing activities thus appear to have similar broad specificities as to the tRNA identities they will recognize and the determinants of the precise cleavage site.

It will be interesting to test the accessibility of the maize mitochondrial $tRNA^{Pro}$ sequence to processing since no mature tRNA-sized transcript can be found from this sequence although

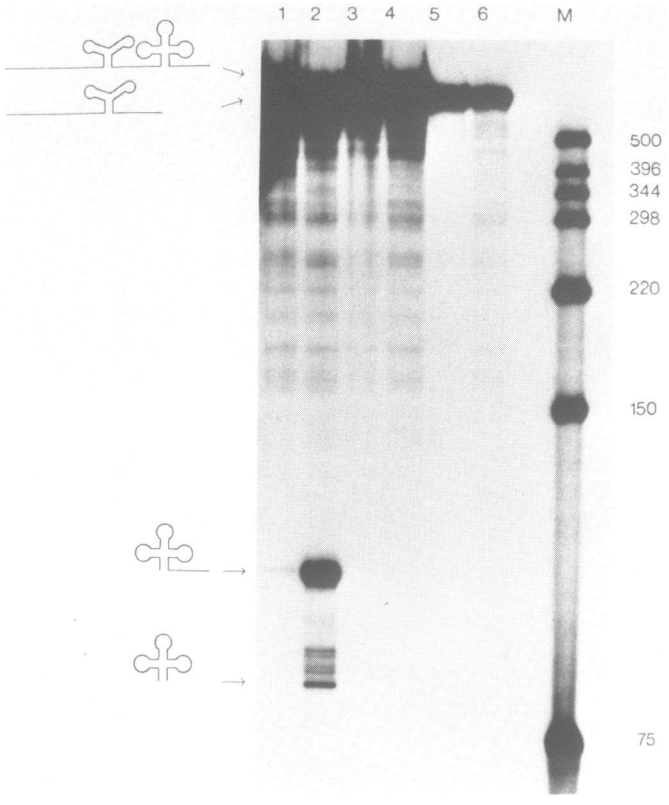

Fig.5 Processing assay of the pseudo $tRNA^{Pro}$- $tRNA^{Trp}$ transcript after treatment with proteinase K or micrococcal nuclease of the *Oenothera* lysate. Lanes are as follows: 1 and 2 are controls without proteinase or nuclease treatment ; 3 and 4 are treated with 50 μg micrococcal nuclease; 5 and 6 were incubated with 100 μg proteinase K for 20min at 4°C. Processing incubation was 0min for lanes 1,3,5 and 15min for lanes 2,4,6. M = DNA marker in nucleotides.

the three nucleotide differences to the corresponding maize plastid gene do not greatly effect proper folding of the tRNA (7).

The temporal spacing of observable 5′- and 3′-processing of the tRNA termini in the plant mitochondrial extract suggests requirements for the plant mitochondrial 3′-processing enzyme similar to the analogous activity in rat liver mitochondria, which requires the 5′-processed intermediate as a substrate (16).

The observation of only 5′-processed intermediates in the plant mitochondrial enzymatic activity could be due to a similar hierarchy of events were the plant mitochondrial 3′-processing activity only acts on the observed 100 nucleotide intermediate. Alternatively could the 3′-processing activity in the lysate be much lower and the reaction slower and lagging behind the 5′-cleavage process. The 3′-processing activity in plant mitochondria appears to move in an exonucleolytic mode of progression since intermediates can be detected (not shown).

The identification of tRNA processing activities in mitochondrial lysates of higher plants will allow further characterization of the involved activities. Especially interesting will be the nature and coding locus of the RNAase P-like activity components in view of the observation that in yeast a mitochondrially encoded RNA is required *in vitro* and fragments of the entire RNA molecule also *in vitro* for tRNA processing (17,18). Both RNA and protein components appear to be likewise required in the plant mitochondrial RNAase P-like activity since either nuclease or proteinase K treatment abolishes the processing (Fig.5).

There is ample space on the plant mitochondrial genomes to likewise encode such an RNA component in contrast to the small mammalian mitochondrial genomes that are tightly packed with genes and have the only non-coding sequences around their origins of replication (19).

ACKNOWLEDGEMENTS

We wish to thank Drs.C.J.Leaver F.R.S. and J.Ross for fruitful discussions. The oligonucleotide used in the primer extension was a kind gift of Dr.J.-M.Grienenberger. The *E.coli* RNAase P was generously supplied by Drs. S.Altman and C.Guerrier-Takada. This work was supported by grants from the Deutsche Forschungsgemein-schaft and the Fonds der Chemischen Industrie. The support of this work by a Ciba-Geigy-ACE award is gratefully acknowledged.

REFERENCES

1. Schuster,W. and Brennicke,A. (1988) Plant Science, *54*, 1–10.
2. Wintz,H., Grienenberger,J.-M., Weil,J.-H. and Lonsdale, D.M. (1988) Curr. Genet., *13*, 247–254.
3. Sangare,A., Lonsdale,D., Weil,J.-H. and Grienenberger,J.-M. (1989) Curr. Genet., *16*, 195–201.
4. Joyce,P.B.M. and Gray,M.W. (1989) Nucleic Acids Res., *17*, 5461–5476.
5. Marechal,L., Runeberg-Roos,P., Grienenberger,J.-M., Colin,J., Weil,J.-H., Lejeune,B., Quetier,F. and Lonsdale D.M. (1987) Curr. Genet., *12*, 91–98.
6. Schuster,W., Grienenberger,J.-M., Weil,J.-H. and Brennicke, A. (1988) Nucleic Acids Res., *15*, 7737
7. Leon,P., Walbot,V. and Bedinger,P. (1989) Nucleic Acids Res. *17*, 4089–4099.
8. Runeberg-Roos,P., Grienenberger,J.-M., Guillemaut,P., Marechal,L., Gruber,V. and Weil,J.-H. (1987) Plant Mol. Biol., *9*, 237–246.
9. Brennicke,A. (1980) Plant Physiol. *65*, 1207–1210.
10. Christophe,L., Tarrago-Litvak,L., Castroviejo,M. and Litvak, S. (1981) Plant Sci. Lett. *21*, 181–192.
11. Lazarus,G.M., Henrich,J.P., Kelly,W.G., Schmitz,S.A. and Castora,F.J. (1987) Biochemistry *26*, 6195–6203.
12. Schuster,W. (1988) Thesis, Universität Tübingen, FRG.
13. Gottschalk,M. and Brennicke,A. (1985) Curr. Genet. *9*, 165–168.
14. Marechal,L., Guillemaut, P., Grienenberger,J.-M., Jeannin, G. and Weil,J.-H. (1985) Nucleic Acids Res. *13*,4411–4416.
15. Altman,S., Baer,M.F., Bartkiewicz,M., Gold,H., Guerrier-Takada,C., Kirsebom,L.A., Lumelsky,N. and Peck,K. (1989) Gene *82*, 63–64.
16. Manam,S. and Van Tuyle,G.C. (1987) J.Biol.Chem. *262*, 10272–10279.
17. Miller,D.L. and Martin,N.C. (1983) Cell *34*,911–917.
18. Morales,M.J., Wise,C.A., Holligsworth,M.J. and Martin, N.C. (1989) Nucleic Acids Res. *17*, 6865–7044.
19. Bibb,M.J., Van Etten,R.A., Wright, C.T., Walberg,M.W. and Clayton,D.A. (1981) Cell *26*, 167–180.
20. Zuker,M. and Stiegler,P. (1981) Nucleic Acids Res. *9*, 133–148.