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A tRNA gene transcription initiation site is similar to mRNA and rRNA promoters in plant mitochondria

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ABSTRACT

The gene for tRNA^{Phe} is located 292 nucleotides upstream of the tRNA^{Pro} gene in the *Oenothera* mitochondrial genome. Hybridization with *in vitro* capped primary transcripts indicates a transcription initiation site in the 5′ region of the gene for tRNA^{Phe}. Primer extension experiments show the presence of precursor transcripts covering tRNA^{Phe} and adjacent sequences up to a transcription initiation site 181 or 180 nucleotides upstream of the tRNA gene. The genomic sequence at this transcription initiation site contains the consensus motif derived for putative promoters of mitochondrial protein and rRNA coding genes in dicotyledonous plants. This sequence similarity suggests that tRNAs, rRNAs and mRNAs can be transcribed from homologous promoters in plant mitochondria.

INTRODUCTION

In all genetic systems structural RNAs are subject to extensive, specific expressional control. Particularly rRNAs and tRNAs are required in quantities exceeding the abundance of any mRNA molecule species. To achieve and ensure the necessary supply of rRNAs and tRNAs specific modes of transcription have become established in many genetic systems. In the nucleus of eukaryotes for example, tRNA and the 5S rRNA genes are transcribed by a specific RNA polymerase (RNA-Polymerase III) from gene internal promoters (1), while a high rate of transcription of the other rRNA genes is provided by amplification of these genomic sequences (2).

In the compact organellar systems of plastids and mitochondria similar strategies are used to meet the requirements for abundant RNA molecules. In plastids rRNA genes are duplicated and evidence for an additional RNA-polymerase (and/or its respective cofactors) has been described, which may provide a differential transcriptional activity (3–5). Only few of the plastid tRNA genes are located in the inverted repeat region, while most of the others appear to be cotranscribed with protein coding genes (6–8).

In mammalian mitochondria the two rRNA genes are part of a more frequently transcribed gene cluster including two tRNA genes, while all other tRNA genes are interspersed between protein coding genes functioning as processing signals in the polycistronic precursor transcripts (9, 10). Since only two promoters are present on the animal mitochondrial genomes (11), no specific transcription signals are known for the tRNA genes. The greater stability of the tRNA molecules presumably provides the sole basis for the higher steady state level of these molecules.

Cotranscription of mRNAs, rRNAs and tRNAs is also observed in fungal mitochondria, although here tRNA genes tend to be clustered in a few genomic regions (12). The promoters identified for these tRNA clusters, however, are indistinguishable from mRNA and rRNA promoter sequences, suggesting likewise no elevated transcriptional activity, but rather molecule stability for regulation of abundances (13).

In plant mitochondria tRNA genes are scattered throughout the genome and are located in genomic contexts varying between the different species, similar to the high variation in neighbouring sequences of many of the protein coding regions (8, 14, 15). Many tRNA genes are found to be rather distant from any recognizable other gene (16, 17). This dispersed arrangement of genes in plant mitochondria has prompted speculations about the number and the nature of promoters required to transcribe the essential genomic information in plant mitochondria (8, 18). Investigations by *in vitro* capping and *in vitro* transcription have indeed revealed the presence of several distinct promoter regions for rRNA and protein coding genes (18, 19).

Analysis of tRNA genes and their surrounding sequences led to the identification of small sequence elements that are conserved just upstream of the coding regions of some, but not all tRNA genes (8, 20). Besides these motifs, gene internal elements as in cytoplasmic tRNAs have been suggested as potential promoter signals. However, the lack of clear mapping data of tRNA transcription initiation sites makes an evaluation of these interpretations difficult. It is therefore necessary to obtain more information on tRNA transcription in plant mitochondria.

We have now investigated the transcription initiation site of the tRNA^{Phe} gene in *Oenothera* mitochondria, that most likely acts as promoter also for the downstream tRNA^{Pro}. The sequence surrounding the transcription initiation site is very similar to the dicot promoter consensus derived for mRNAs and rRNAs, suggesting that all three classes of RNAs are transcribed by the same RNA polymerase system in plant mitochondria.

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MATERIALS AND METHODS

Mitochondrial nucleic acids

O.berteriana mitochondrial DNA (mtDNA) was purified from tissue culture cells as described elsewhere (21). For extraction of mtRNA a crude fraction of mitochondria was isolated by differential centrifugation and was further purified on discontinuous percoll gradients. Mitochondria were lysed in a buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5 % sarkosyl and 0.1 M β-mercaptoethanol. After incubation at room temperature for 5 min, 0.1 vol of 2 M NaOAc (pH 4.0) was added, followed by an extraction with 1 vol water saturated phenol and 0.2 vol of chloroform:isoamylalcohol (24:1). The sample was kept on ice for 15 min and phases were separated by centrifugation in a Beckman SJ 13.1 rotor at 10,000 rpm for 15 min. Mitochondrial RNA was precipitated from the aqueous phase by addition of 1 vol isopropanol and incubation at −20°C for at least 2 hrs prior to centrifugation as above. The RNA was resuspended in water and the extraction/precipitation step was repeated. On average between 200−400 μg mitochondrial RNA were obtained from 1.5 kg callus culture cells.

Gene specific clones

Mitochondrial DNA fragments containing the genes for the different tRNAs and/or the respective upstream and downstream sequences were isolated from *Hin*dIII libraries of total mitochondrial DNA from *O.berteriana*. Clone H21/1 was isolated from a library in the vector pKUN and clones H8/47, H4/69 and H3/31 from a library in the vector pBR 322. Subclones were constructed from the *Hin*dIII clone H21/1 by inserting an *Eco*RI/*Sfu*I (subclone 21/1-5) and a *Sfu*I/*Hin*dIII (subclone 21/1-4) fragment respectively into pBluescript vectors following standard protocols (22).

Blotting and hybridization conditions

For Southern blot and dot blot analysis Genescreen plus (DuPont) membranes were used according to manufacturer's instructions. Hybridizations and prehybridizations were done in 50 % formamide, 1 % SDS, 1 M NaCl and 10 % dextranesulphate at 42°C with 100 μg of *in vitro* capped mitochondrial RNA. Filters were washed for 5 min each once in 2×SSC at RT and twice in 2×SSC, 1 % SDS at 60°C for 30 min.

RNase digestion

To remove unannealed RNA, hybridized filters were digested with 100 μg RNase A and 20 units RNase T_1 in a buffer containing 10 mM Tris−HCl (pH 7.8), 5 mM EDTA and 300 mM NaCl for 2 hrs at 37°C.

In vitro capping

For *in vitro* labelling by capping 50−100 μg of mitochondrial RNA were incubated in a total volume of 20 μl containing 50 mM Tris−HCl (pH7.9), 1.25 mM $MgCl_2$, 2.5 mM DTT, 80 U RNase inhibitor, 1 μM GTP, 250 μCi alpha−^{32}P GTP (3,000 Ci/mMol) and 15 U of guanylyltransferase for 30 min at 37°C. The capping reaction was stopped by digestion with 20 μg proteinase K for 15 min at 37°C. After one extraction with 0.5 vol watersaturated phenol and 0.5 vol of chloroform:isoamylalcohol (24:1) and two extractions with 1 vol of chloroform:isoamylalcohol (24:1), RNA was precipitated from the aqueous phase by addition of ammonium acetate to a final concentration of 2.5 M and 70 % ethanol. Most of the unincorporated label was removed by three washes with 70 % ethanol.

Miscellaneous methods

Primer extension experiments were done following standard protocols (22). Primers used in these experiments were : P1: 5′-CTTGAATTTCCAAATCCGGC-3′ and P3: 5′-CTCTTGTCTTCCGTCTTTTG-3′. DNA sequencing was performed with a T7 sequencing kit (Pharmacia).

RESULTS

Identification of primary transcripts of tRNA genes

The first step in this investigation was to determine primary transcripts levels of tRNA genes in *Oenothera* mitochondria and to identify candidate transcripts abundant enough for detailed

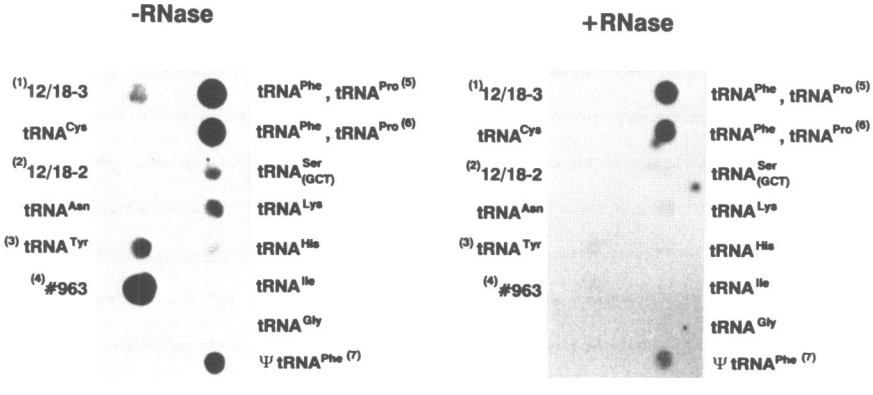

Figure 1. Dot blot analysis of various tRNA coding regions with *in vitro* cap-labelled mitochondrial transcripts. Digestion of the hybridized and washed blot (−RNase) with RNase A (+RNase) removes most of the signals suggesting the presence of larger primary transcripts extending beyond the cloned regions probed here or similarities with other transcribed regions due to sequence duplications common to plant mitochondria. The identities of the tRNAs encoded on the different fragments are indicated in the margins. Cloned fragments correspond in most instances to those reported in ref. (23). Several upstream regions and control fragments were included in this analysis: (1) Clone 12/18-3 contains the region 5′ of $tRNA^{Cys}$; (2) clone 12/18-2 covers the region 3′ of $tRNA^{Cys}$ and 5′ of $tRNA^{Asn}$ (19); (3) this clone contains the $tRNA^{Tyr}$ gene and the downstream located *nad2* exons c−e (24); (4) clone #963 contains the *nad2* exons a and b (24); (5) the complete genes for $tRNA^{Phe}$ and $tRNA^{Pro}$ are covered in clone H21/1 (see also Fig. 4) and (6) in clone H8/47; (7) is clone H4/69 with the incomplete $tRNA^{Phe}$ gene. Data for $tRNA^{Ile}$ are as yet unpublished.

analysis. Total mitochondrial RNA was labelled by *in vitro* capping and hybridized to dot blots of various tRNA coding regions of *Oenothera* mitochondrial DNA (Fig. 1; 19, 23, unpublished results). The presence of primary transcripts accessible to *in vitro* capping was observed for cloned DNA fragments containing coding sequences for $tRNA^{Phe}$ and $tRNA^{Pro}$ and a truncated, partial $tRNA^{Phe}$ gene. Weak signals were obtained with clones covering genes for $tRNA^{Ser}$(GCT), $tRNA^{Lys}$, and $tRNA^{Tyr}$. No primary transcripts were detected for clones carrying genes for $tRNA^{Cys}$, $tRNA^{Asn}$, the region between these two genes (i.e. clone 12/18-2), and clones with the $tRNA^{His}$, $tRNA^{Ile}$ and $tRNA^{Gly}$ genes.

The genomic region encoding the first two exons of the *nad2* gene (24) was included in this experiment (clone #963 in Fig. 1). RNase digestion of the hybridized dot blot removed the adhering primary transcripts of this probe and most other clones, suggesting that the sites of the added cap structures and thus the sites of transcription initation lie outside of the cloned genomic regions. Since the *nad2* clone contains little sequence upstream of exon a, the observed cap-site may nevertheless be the primary transcript end for these exons. An alternative explanation for RNase sensitive hybridizations may lie in duplicated sequence elements often found in plant mitochondrial genomes (18, 25) which may result in retention of primary transcripts by such similarities of several hundred nucleotides, but no obvious functional connection.

Only the hybridization observed for the $tRNA^{Phe}$ and pseudo-$tRNA^{Phe}$ loci are largely resistant to RNase attack. The region with the complete $tRNA^{Phe}$ and $tRNA^{Pro}$ gives the strongest signal, corresponding to the most abundant primary transcript of the tRNA coding regions tested, and was therefore selected for detailed analysis.

Genomic recombinations at the $tRNA^{Phe}$-$tRNA^{Pro}$ locus

Sequence analysis of the genomic environment of $tRNA^{Phe}$ beyond the previously investigated region (23) revealed the presence of a $tRNA^{Pro}$ gene 292 nucleotides downstream (Fig. 2). The $tRNA^{Pro}$ gene shows very high sequence similarity with the respective genes from other plant mitochondria (26) rather than from plastids, suggesting this gene to be of genuine mitochondrial ancestry.

Complete or partial sequences of the $tRNA^{Phe}$-$tRNA^{Pro}$ locus are present in the mitochondrial DNA of *Oenothera* in several different arrangements (Fig. 3). Southern hybridizations to digested total mtDNA reveal at least four genomic loci with divergent vicinities for the complete two tRNAs. All of these have been cloned as *Hin*dIII fragments and were mapped in detail (Fig. 3). All four clones contain the complete $tRNA^{Phe}$ and

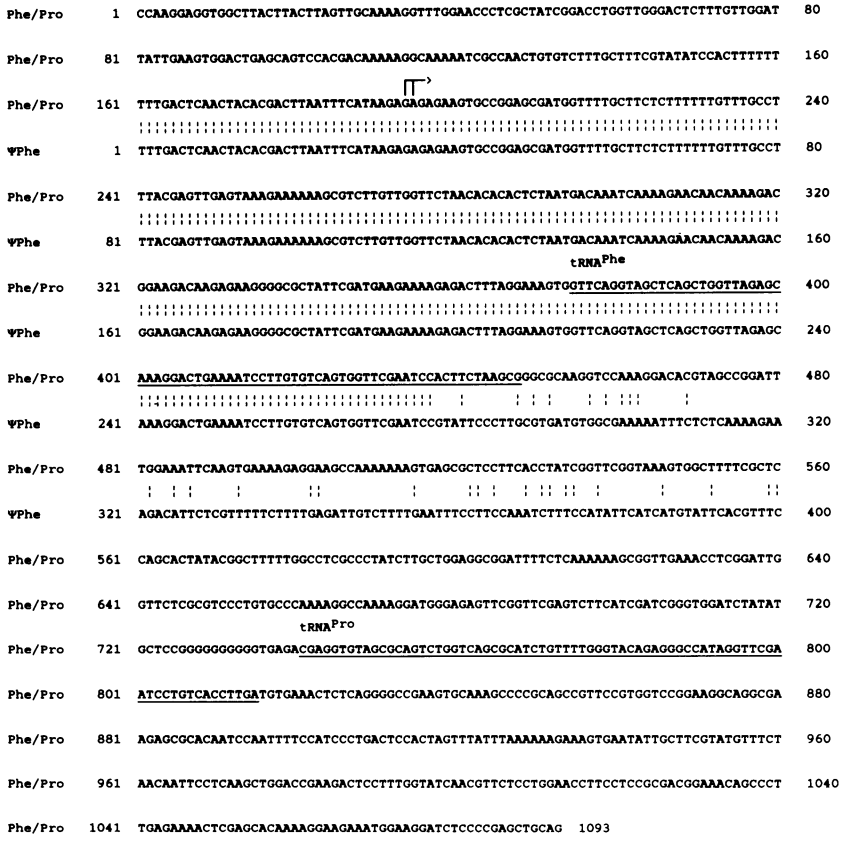

Figure 2. Nucleotide sequence of the region containing the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$. tRNA coding regions are underlined and the position of the transcription initiation site upstream of the gene for $tRNA^{Phe}$ is indicated by the arrow above the two nucleotides between which the primary transcript terminus has been mapped. Nucleotides identical between the loci encoding the intact two tRNA genes and the transcription initiation site (upper line marked Phe/Pro as represented by clones H3/31, H21/1 and H17/4) and the locus recombined in the $tRNA^{Phe}$ gene (lower sequence marked Phe as derived from clone H4/69) are indicated by vertical lines. These sequences are available under EMBL accession numbers X74449 and X74450.

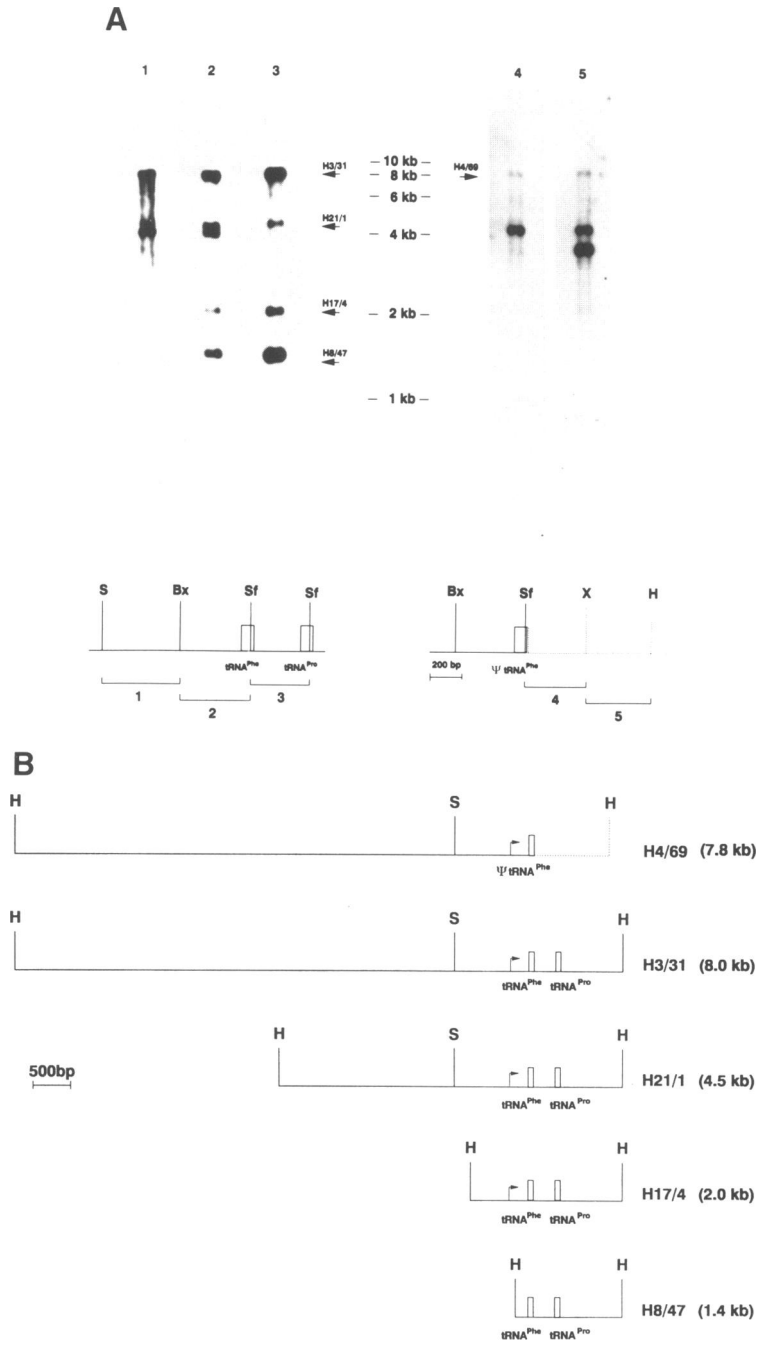

Figure 3. Several loci in *Oenothera* mitochondria contain sequences of $tRNA^{Phe}$ and $tRNA^{Pro}$. **(A)** Southern blot analysis of the *Oenothera* mitochondrial loci with sequences of the $tRNA^{Phe}$ and $tRNA^{Pro}$ genes. Total mitochondrial DNA was digested with *Hin*dIII, size-fractionated and the blots were hybridized with the probes indicated underneath in the schematic restriction maps. The mitochondrial fragments isolated as clones are indicated by arrows with the respective clone designations. The four genomic regions containing the entire genes for $tRNA^{Phe}$ and $tRNA^{Pro}$ (clones H3/31, H21/1, H17/4 and H8/47) and one of the two regions with the truncated pseudo-$tRNA^{Phe}$ (H4/69) have been cloned. **(B)** Schematic comparison of the cloned loci. The mapped transcription initiation site is present in four loci as indicated by the flag. DNA size markers are indicated in kb and restriction sites are given for *Bst*XI (Bx), *Hin*dIII (H), *Sal*I (S) and *Sfu*I (Sf).

$tRNA^{Pro}$ genes and are in 3′ direction identical at least up to the common *Hin*dIII site, but differ in their 5′ regions. Sequences between the two clones H21/1 and H8/47 diverge upstream of nucleotide −42 relative to the first nucleotide of the $tRNA^{Phe}$ gene (nucleotide 333 in figure 2) and show no similarity further upstream. The third locus represented by clone H17/4 is in this upstream region colinear with clone H21/1, but diverges about 400 nucleotides upstream of the $tRNA^{Phe}$. The fourth locus (clone H3/31) is identical with H21/1 at least as far upstream as the *Sal*I site.

Another recombination has occurred within the $tRNA^{Phe}$ coding sequence and has disrupted this gene by replacing the last 11 nucleotides of the tRNA with completely unrelated sequences (represented by clone H4/69; Figs. 2 and 3). This sequence

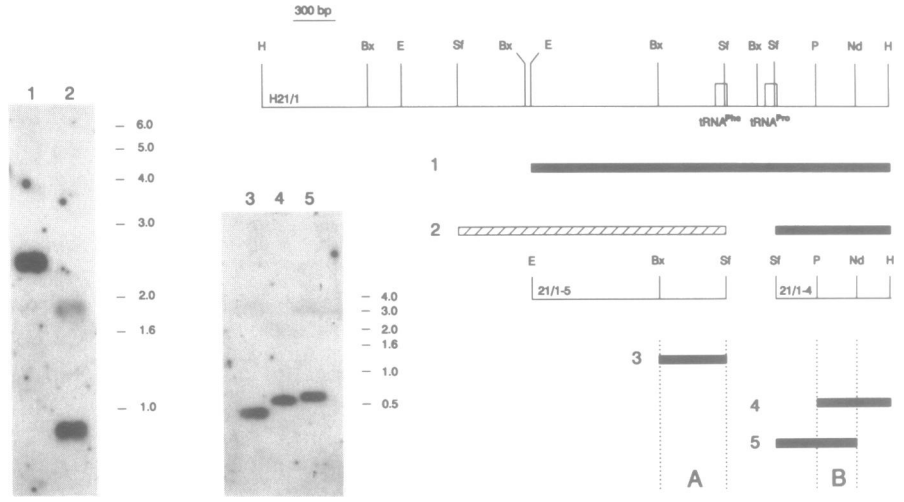

Figure 4. Localization of primary transcript termini on restriction fragments of the region of clone H21/1 encoding the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$. Blots of different restriction digests were hybridized with *in vitro* cap-labelled total mitochondrial RNA. Clone H21/1 was digested with various combinations of restriction enzymes, *Hin*dIII/*Eco*RI in lane 1 and *Hin*dIII/*Sfu*I in lane 2 of the left panel. In the right panel subclone 21/1-5 was digested with *Bst*XI/*Sfu*I in lane 3 and subclone 21/1-4 was cut with *Pst*I/*Hin*dIII in lane 4 and with *Sfu*I/*Nde*I in lane 5 respectively. The schematic restriction maps show the positions of the strongly (black bars) and less intensively (hatched bar) hybridizing restriction fragments in each of the digests and are numbered accordingly. The regions marked A and B denote the locations of the two independent transcription initiation sites in the regions delineated by the dotted vertical lines. DNA size markers are indicated in kb and restriction sites are given for *Bst*XI (Bx), *Eco*RI (E), *Hin*dIII (H), *Nde*I (N), *Pst*I (P) and *Sfu*I (Sf).

replacement results in complete disruption of the acceptor stem of the tRNA. While in the intact $tRNA^{Phe}$ the acceptor stem is stabilized by 5 Watson–Crick pairs, 1 G·U pair and a C/A mismatch, only 3 G·U pairs and 4 mismatches are present in the analogous folding of the truncated tRNA gene.

These recombinations at the $tRNA^{Phe}$-$tRNA^{Pro}$ locus have to be taken into consideration when identifying transcription initiation sites.

Two transcription initiation sites are located near the $tRNA^{Phe}$ and $tRNA^{Pro}$ genes

To define the transcription initiation site(s) in this genomic region *in vitro* cap-labelled mitochondrial RNA was hybridized to restriction fragments of the cloned mtDNA fragments (Fig. 4). Hybridization was observed with several fragments containing two separate genomic regions, one upstream of the tRNA genes, the other downstream as represented by the *Sfu*I/*Sfu*I and *Sfu*I/*Hin*dIII fragments respectively (Fig. 4, upper left panel, lane and fragments labelled 2). Significantly no hybridization was observed to the 0.38 kb *Sfu*I fragment, running at the very bottom of lane 2 in the upper left panel of figure 4. This fragment is located between the two hybridizing regions and covers the entire spacer sequence from the 3′ end of the $tRNA^{Phe}$ to the 3′ end of the $tRNA^{Pro}$ genes. Rapid processing of the potential tRNA containing precursors may be responsible for the lack of hybridization to this fragment by lowering the abundance of primary transcripts containing these downstream sequences below the threshold of detection in this experiment.

RNase digestion of the hybridized blot did not alter the obtained hybridization signals, suggesting both to be derived from bona fide transcription intiation sites within the respective restriction fragments.

This observation can only be explained by the presence of two independent and separate promoter regions upstream and downstream of the tRNA genes respectively. The different hybridization intensities of the equimolar restriction fragments covering these two regions (fragments and lane 2 at the top of figure 4) suggest transcripts with primary 5′-termini to be less abundant for the promoter region upstream of the tRNA genes than for the transcription initiation site downstream of these tRNA genes.

This interpretation is supported by the dot-blot signal strength obtained with clone H4/69, which is clearly less intensive than the signals observed with clones H21/1 and H8/47 as estimated by visual inspection of the signals in figure 1. Clone H4/69 covers only the region upstream of the tRNAs, while the second potential promoter region downstream of the two genes is substituted by a different sequence due to the recombination within the $tRNA^{Phe}$ pseudogene. The tRNA genes encoded on clone H8/47 are probably not transcribed, since only the second promoter region downstream of these genes is present. This clone H8/47 although containing only the downstream promoter region shows a signal strength in the cap-labelled RNA hybridization comparable with clone H21/1 as judged by visual inspection of figure 1. These data are consistent with primary transcripts being more abundant from the second transcription initiation site than from the tRNA promoter.

Mapping of the transcription initation site for the $tRNA^{Phe}$-$tRNA^{Pro}$ locus

The smallest fragment of cloned mitochondrial DNA hybridizing with *in vitro* capped RNA upstream of the two tRNA genes is the 430 bp *Bst*XI/*Sfu*I fragment which contains the 5′ part of the $tRNA^{Phe}$ and 387 upstream nucleotides (Fig. 4). This hybridization narrows the location of the transcription initiation site for the tRNAs to the 387 upstream nucleotides. The low efficiency of the capping reaction at these primary transcript molecules precluded further direct analysis of the capped end by RNA sequencing or RNase protection.

The location of the RNA terminus was therefore analysed with primer extension walks from the tRNA coding regions to identify all detectable RNA 5′ termini in this region (Fig. 5). Starting

from a primer (P1) located downstream of the tRNA^{Phe} gene in the transcribed spacer region, the expected strong signals at the mature 3′ (Fig. 5A, product a) and 5′ (Fig. 5A and B, termini b) ends of this tRNA were observed. However, longer precursor molecules can also be identified that contain the unprocessed tRNA and additional upstream sequences. The 5′ most extended product detected from this primer (Fig. 5B, terminus c) is identical with the RNA terminus detected from a primer (P3) upstream of the tRNA gene (Fig. 5C, product c). This primer is located upstream of the recombination point of clone H8/47 and thus only detects transcripts of the genomic arrangement represented by the other three clones H3/31, H17/4 and H21/1. The longest RNA precursor molecules detected in these primer extensions start with the G or A 181 and 180 nucleotides upstream of the 5′ end of the tRNA^{Phe}. The absence of any larger primer extension products in these experiments is consistent with the *in vitro* capped hybridization data, suggesting this terminus to be derived from *de novo* initiation of transcription.

Precursor transcripts are present in steady state RNA

The above described *in vitro* capping analysis suggests the presence of primary transcripts covering the genomic region upstream of the tRNA genes in the steady state mitochondrial RNA population. To identify these precursor RNA species present in the steady state transcripts total mitochondrial RNA was blotted and probed with several genomic regions for the presence of homologous transcripts.

Strong hybridization signals were obtained in the size range of the mature tRNAs in addition to larger RNA species (Data not shown). An abundant transcript of 500 nucleotides in length is detected only with sequences downstream of the two tRNAs and is probably derived from the second transcription initiation site downstream of tRNA^{Pro}.

The most likely candidate for the primary precursor transcript of the two tRNAs is a much less abundant 3,000 nucleotides long RNA molecule. This transcript is detected in Northern blots with the probe upstream of the two tRNAs covering the region which hybridizes with the cap-labelled RNA, with a probe containing the spacer between the tRNAs and with the sequence downstream of the tRNA^{Pro} gene (Data not shown). In addition several smaller transcripts are detected, which may be processing intermediates.

Northern blot probing with the region downstream of the incomplete tRNA^{Phe} locus revealed an abundant 1.5 kb mRNA which is also detected with sequences upstream of the tRNA gene. This suggests that the incomplete tRNA is excised from the precursor molecules very inefficiently.

Besides several cDNA clones containing the region downstream of the two tRNA genes and probably corresponding to the abundant 500 nucleotides long transcript with as yet unidentified information content, one cDNA clone identified is derived from an unprocessed RNA containing the pseudo-tRNA^{Phe} sequence further supporting the evidence for transcription and slow processing of this unprocessed incomplete tRNA locus (data not shown).

DISCUSSION

Plant mitochondrial tRNA genes are transcribed as larger primary transcripts

Primary transcript termini are detected in the total steady state RNA for several tRNA coding regions in the *Oenothera* mitochondrial genome. The primary transcript termini of three

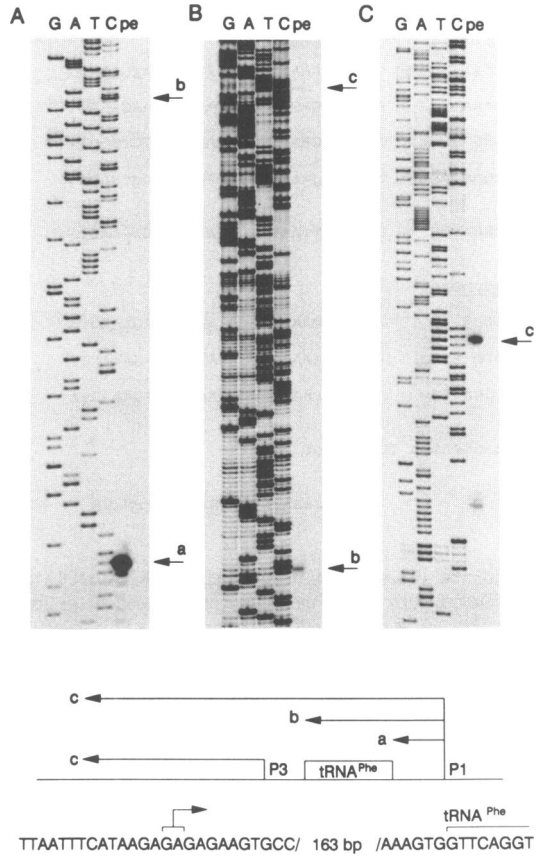

Figure 5. Primer extension experiments identify the borders of tRNA^{Phe} and the 5′ end of the longest detectable precursor molecule. Panels **A** and **B** show extensions from primer P1 located downstream of tRNA^{Phe}. Panel **C** shows the fine mapping of the upstream RNA terminus from primer P3. Schematic representation of the obtained products shows that terminus a corresponds to the 5′ end of the spacer between the two tRNAs at the junction with the tRNA^{Phe}. Terminus b identifies the 5′ end of the tRNA^{Phe} and terminus c corresponds to the location of the transcription initiation region identified by hybridization upstream of the tRNAs (Fig. 4). The sequence surrounding extension product c is given at the bottom of the figure and the site of transcription initiation is indicated by an arrow.

of the cloned mitochondrial regions tested are either outside of the clones or due to spurious similarities with transcripts derived from other loci, since the hybridizing primary transcript ends are removed by RNase digestion. Such similarities are frequently found in plant mitochondrial genomes as duplications of transcribed coding and intron regions for example (e.g. 25), which in hybridization analyses of genome-transcript relationships have to be taken into account. The correlation between primary transcript end and tRNA transcription thus needs to be verified rigourously in each instance, particularly since most of the assayed clones are large enough to potentially contain additional genes.

The extensive analysis of the genomic region encoding tRNA^{Phe} and tRNA^{Pro} for example shows the presence of two transcription initiation sites, one for the tRNA(s) and a second for as yet unknown sequence information. The primary end of this second site is much more abundantly present in the mitochondrial RNA than the tRNA precursor. Comparison of hybridization efficiencies to restriction fragments containing only one of the transcription initiation sites each (e.g. lane 2 in figure 4) shows far stronger signals for the second site than for the tRNA

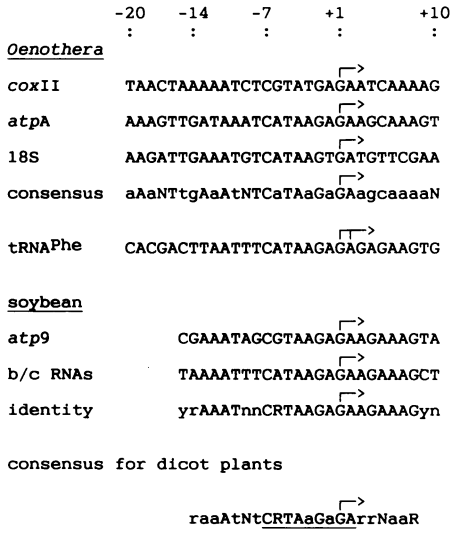

Figure 6. Conserved sequence motifs at promoters for tRNA, rRNA and mRNA genes. The sequence surrounding the transcription initiation site for $tRNA^{Phe}$ shows high similarity with the consensus sequence derived for several promoters analysed in *Oenothera* mitochondria for mRNAs and the 18S rRNA (19). Sequences at identified primary transcript termini from soybean mitochondria (34) are also aligned to show the high conservation of this motif in dicotyledonous plants. Inclusion of the putative tRNA promoter identified here allows further refinement of the deduced consensus sequence (19).

precursor. This comparatively close spacing of unrelated transcription initation sites in a small region of the *Oenothera* mitochondrial genome shows that numerous promoters are present in these plant organelles, confirming the previous minimal estimates (19). Such coinciding unrelated transcription initation sites must be included in any consideration and identification of plant mitochondrial promoters.

Primary transcripts for $tRNA^{Phe}$ include leader and trailer sequences

The presence of larger precursor molecules for tRNA genes is here verified and analyzed in detail for the region encoding $tRNA^{Phe}$ and $tRNA^{Pro}$. The investigation shows that at least in this instance transcription is not initiated at the first nucleotide of the tRNA guided by external or gene internal signals, but rather from distant upstream promoter elements. Correct processing of such larger precursors to mature tRNA molecules in plant mitochondria is ensured by the presence of 5′ and 3′ tRNA processing activities, which have been identified in wheat, *Oenothera* and potato mitochondria (27, 28).

The observation of a clean 5′ terminus of the spacer between the two tRNAs at the junction with the 3′ end of $tRNA^{Phe}$ suggests that *in vivo* 3′ processing at least of this tRNA is achieved by an endonucleolytic digestion rather than a 3′ to 5′ exonuclease activity. Only a specific endonuclease can release this spacer molecule with a precise 5′ terminus during processing (27).

The genes for $tRNA^{Phe}$ and $tRNA^{Pro}$ are probably cotranscribed

Cotranscription of the two genes for $tRNA^{Phe}$ and $tRNA^{Pro}$ as deduced from the presence of common larger precursor molecules is further confirmed by the primer extension experiments showing that transcripts of the $tRNA^{Pro}$ extend at least into the spacer between the two tRNAs. These observations suggest that the transcription initiation site identified upstream of the gene for $tRNA^{Phe}$ represents the functional promoter for the primary bicistronic transcript of both genes. This promoter region is most likely active in three of the four complete copies of the $tRNA^{Phe}$ and $tRNA^{Pro}$ coding region and in both copies of the 3′ truncated pseudo $tRNA^{Phe}$. It is not present in the 5′ recombined locus represented by clone H8/47. The 3′ extension of the primary transcript is as yet unclear, but must continue through the region of the second transcription initiation site located downstream of the two tRNA genes.

The presence of several promoters in tandem has been observed for example for the *atp9* gene in maize mitochondria (29, 30), where the individual transcripts overlap into the region downstream of the actual gene. The second promoter region observed downstream of the genes for $tRNA^{Phe}$ and $tRNA^{Pro}$ here in *Oenothera*, however, probably belongs to a different as yet unknown gene located further distal to this region.

The identified transcription initiation site for the $tRNA^{Phe}$ and $tRNA^{Pro}$ genes is present in three genomic arrangements of these two tRNAs in the *Oenothera* mitochondrial genome, suggesting that a comparatively high rate of transcription is at least in part ensured by this amplification of genomic sequences.

The tRNA, mRNA and rRNA promoter regions contain a common motif

The here reported identification of a transcription initiation site for a tRNA locus allows a comparison of the sequence surrounding this initiation site with the putative promoter motifs identified for rRNA and protein coding genes in this dicotyledonous plant (Fig. 6). Intriguingly the sequence at the transcription initiation site is highly similar to the consensus motif derived from the promoters of protein coding mRNAs and the 18S rRNA in *Oenothera* mitochondria (19). Transcription in all investigated instances starts with a conserved GA dinucleotide, which is part of the common sequence motif. The tRNA primary transcript identified here likewise starts with a GA dinucleotide, if the primer extension signal indeed corresponds to nucleotide −181 upstream of the tRNA (Fig. 5).

The primary sequence similarities between the previously identified mRNA and rRNA promoters and the tRNA transcription initiation site further confirm the identification of the location of this primary RNA terminus as a tRNA promoter. This high sequence similarity of transcription initiation sites suggests that all three types of RNA molecules—mRNAs, rRNAs and 'native' tRNAs—can be transcribed by the same RNA polymerase-transcriptional cofactor system in plant mitochondria.

Transcription of yeast mitochondrial genes similarly uses a single promoter motif for the different types of genes. The $tRNA^{Phe}$ in yeast mitochondria is also transcribed from its own promoter in the fungus (13), while other tRNA genes are transcribed in clusters or together with protein coding or rRNA genes (31, 32). The close location of e.g. the $tRNA^{fMet}$ gene just one nucleotide upstream of the 18S rRNA gene in wheat mitochondria (33) suggests that these two genes are cotranscribed and confirms different modes of cotranscription for other tRNA genes also in plant mitochondria. It is unclear at present, whether the presence of specific promoters for the $tRNA^{Phe}$ genes in both yeast and plant mitochondria is related to any specific function of this tRNA in the organelle.

How the appropriate relative stoichiometries between mRNAs, rRNAs and tRNAs are met needs to be further analysed. One possibility may be found in the unique differences in each of the

analysed promoter sequences, which may confer different promoter efficiencies. Another factor may be found in differential amplification of some coding regions, for example the $tRNA^{Phe}$ and $tRNA^{Pro}$ region investigated here, which is present in at least three genomic environments together with the identified promoter and thus presumably also transcribed in these copies. Detailed investigations of relative promoter strengths, processing velocities and RNA molecule half-lifes are required to determine the contributions of these different potentially interfering processes.

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