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Accurate transcription and processing of 19 *Euglena* chloroplast tRNAs in a *Euglena* soluble extract

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Summary

The transcription and accurate processing of 19 different *Euglena gracilis* chloroplast tRNAs in a homologous chloroplast soluble extract is described. The chloroplast DNA dependent-RNA polymerase present in the extract selectively transcribes the tRNA genes (Greenberg *et al.*, 1984, J. Biol. Chem., 259: 14880–14887). Two dimensional polyacrylamide gel electrophoresis and RNA fingerprint analysis were used to show that the tRNAs are correctly processed at the 5′- and 3′-ends. The *Euglena* chloroplast soluble extract contains a 5′-processing or 'RNase P-like' activity and RNases responsible for processing tRNA termini. However, it was not determined if the 3′-CCA was added. Therefore, the soluble extract contains activities that are quite similar to an extract of spinach chloroplasts (Greenberg *et al.* (1984), Plant Mol. Biol., 3: 97–109). After transcription of total chloroplast DNA in the *Euglena* soluble extract, thirty-three tRNA sized products were resolved by two dimensional polyacrylamide gel electrophoresis. Nineteen tRNAs could be identified in this mixture.

Introduction

Chloroplasts from *Euglena gracilis* contain a genome of 145 kilobase pairs (18, 19). The chloroplast DNA most likely encodes all the tRNA genes necessary for chloroplast protein synthesis. These genes have been extensively characterized (19, 25, 29). To date, 23 *Euglena* chloroplast tRNA genes have been sequenced (22–24, 30–33; Manzara & Hallick, unpublished results). The tRNA genes are found in both monocistronic and polycistronic transcription units.

Recently, two types of chloroplast RNA polymerase activities were described (11–13). One of these, the transcriptionally active chromosome, is composed of chloroplast DNA with a tightly bound RNA polymerase activity (4, 20, 21). It is active in rRNA synthesis (3, 33). The other RNA polymerase activity is isolated in a soluble form (2, 11, 13, 15). This soluble RNA polymerase(s) is active in the transcription of tRNA and mRNA genes (13, 15, 26, 34). The soluble RNA polymerase preparations from *Euglena* and spinach chloroplasts are also active in tRNA processing (10, 11, 15), yielding tRNAs that are mature at the 5′ and 3′-termini.

Since *Euglena* chloroplast tRNA genes are well characterized, and a soluble extract active in tRNA transcription and processing is available, it is possible to begin to investigate the regulation of chloroplast tRNA gene expression *in vitro*. As a first step toward understanding this process, the transcription of several *Euglena* chloroplast tRNA genes and the subsequent processing steps were investigated.

In this paper we report the accurate transcription and processing of 19 different *Euglena* chloroplast tRNAs. The tRNAs were transcribed from either cloned chloroplast tRNA genes or intact chloroplast DNA in the *Euglena* chloroplast soluble extract. Ribonucleases in the soluble extract converted the transcripts to mature tRNAs with respect to

their 5′- and 3′-termini. The mature tRNAs were purified by two dimensional gel electrophoresis and characterized by subsequent RNase T_1 fingerprint analyses. However, from this analysis it could not be determined if the 3′-CCA was added. Thirty-three distinct RNA products were resolved by two dimensional gel electrophoresis. It was possible to identify 19 different tRNAs in the two dimensional gel pattern.

Materials and methods

Euglena *chloroplast DNA*

The recombinant plasmid DNAs used as templates for *in vitro* transcription are listed in Table 1. The organization of the chloroplast genes on the recombinant plasmids are shown in Fig. 1. All of these tRNA genes have been sequenced (22–24, 28, 30). Plasmid DNAs were isolated according to previously published methods (30). In experiments where pEZC800 was the template, only the replicative form of the DNA was used.

Preparation of the Euglena *chloroplast soluble extract*

Euglena gracilis Klebs, Pringsheim Strain Z cells were grown and chloroplasts were isolated as previously described (11). The *Euglena* chloroplast soluble extract was prepared as previously described (11, 15).

In vitro *transcription*

A reaction mixture was made containing 1 mM ATP, 1 mM CTP, 100 μM GTP, 100 μM UTP, 3.0 μCi/μl each of [α-^{32}P]-GTP and [α-^{32}P]-UTP, 10 mM Tris-HCl (pH 7.9), 7.5 mM $MgCl_2$, 4 mM dithiothreitol, 1.9% glycerol and 200 μg/ml DNA. 10 μl of the reaction mixture was combined with 10 μl of *Euglena* chloroplast soluble extract for a final reaction volume of 20 μl. The soluble extract contains 20 mM HEPES (pH 7.9), 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% (v/v) glycerol and a mixture of chloroplast proteins. The reaction was allowed to proceed for 60 min at 25 °C and was then stopped by adding 5 μl of 1 mg/ml proteinase K, 5% SDS, 1.0 μg/μl polyadenylic acid followed by a 15 min incubation at 37 °C. Proteins were extracted from the RNA products with phenol, $CHCl_3$, isoamyl alcohol (25:24:1, v:v:v). The RNA solution was brought to 0.8 M in ammonium acetate and the nucleic acids were precipitated with 3 volumes of 95% ethanol.

Two-dimensional gel electrophoresis and RNA isolation

Transfer RNAs produced *in vitro* were purified by two dimensional polyacrylamide gel electrophoresis. This method was adapted from the procedure of Fradin *et al.* (7). The electrophoresis buffer was 90 mM Tris base, 90 mM boric acid, 0.5 mM EDTA (pH 8.3) and the acrylamide/bisacrylamide ratio was 19:1 for all gels. The first dimension was

Table 1. Plasmid DNAs used for *in vitro* transcription.

Plasmid	Vector	Chloroplast DNA	Genes	Reference
pPG14	pMB9	*Eco*RI-G[a]	trnV-UAC, trnN-GUU, trnR-ACG, trnL-UAG[b]	30
pEZC300	pBR325	*Eco*RI-Q	trnT-UGU, trnG-GCC, trnM-CAU, trnS-GCU, trnQ-UUG	24
pPG76	pBR322	*Bam-Sal*9	trnY-GUA, trnH-GUG, trnM-CAU, trnW-CCA trnE-UUC, trnG-UCC	22
pEZC2	pBR322	*Hind*III 21[c]	trnF-GAA, trnC-GCA, trnX-CAT[f]	19
pEZC800	m13mp8	A *Sau*3a-*Hin*dIII fragment[d]	trnF-GAA, trnC-GCA	19
pEZC514	pBR325	*Eco*RI I	trnL-UAA, psbA, psbC[e]	19

[a] All chloroplast DNAs are from the *Euglena gracilis* chloroplast genome. They are designated as restriction endonuclease fragments.
[b] tRNA genes are designated by trn followed by the single letter code indicating the amino acid. Gene nomenclature is described in reference 17.
[c] *Hin*dIII 21 is located in *Eco*RI A.
[d] This *Sau*3a-*Hin*dIII fragment is a 600 base pair subclone from the *Hin*dIII 21 insert of pEZC2.
[e] psbA is the locus coding for the 32 kd protein from photosystem II. *Eco*RI I also contains the carboxy terminal portion of psbC.
[f] Identity of tRNA gene not yet determined.

a 40 cm×20 cm×0.4 mm 10% acrylamide, 7 M urea gel. The RNAs were electrophoresed at 1200 volts for 4 h (or until the xylene cyanol blue had moved 25 to 30 cm from the origin). The gel was covered with plastic wrap (or Saran Wrap) and the RNAs were detected by autoradiography at −70 °C (27). For the second dimension, the tRNAs were located in the gel from the autoradiogram. A 1 cm×10 cm gel slice containing the tRNAs was excised from the gel and placed at the origin of the second dimension gel between the two glass plates. A 35 cm×20 cm×0.4 mm 20% polyacrylamide, 4 M urea gel was poured below the tRNA gel slice, and a 5 cm 10% acrylamide, 4 M urea stacking gel was poured around the tRNA containing gel slice. A diagram of this gel is shown in a publication by Burkard *et al.* (5). Electrophoresis was carried out for 36 h at 900 volts. The gel was wrapped in plastic wrap and the tRNAs were detected by autoradiography at 4 °C.

For further analysis, individual tRNAs were located in the polyacrylamide gel using the autoradiogram as a template, and excised. The tRNAs were eluted from the gel matrix by incubating the gel slice in 300 μl of 0.5 M NaCl, 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 1% phenol at 4 °C overnight. The eluant was brought to 0.8 M in ammonium acetate and 10 μg of *E. coli* tRNA was added. The nucleic acids were then precipitated with 3 volumes of ethanol.

RNase T_1 fingerprinting and secondary analysis of oligonucleotides with pancreatic nuclease (RNase A)

RNase T_1 fingerprints were performed as previously described (14). The oligonucleotides were detected by autoradiography at −70 °C (27). They were eluted from the second dimension polyethylene imine (PEI) thin layer chromatography (TLC) plate with 2 M triethylamine bicarbonate (pH 8) as described by Volckaert *et al.* (36). The sequences of the oligonucleotides were determined by secondary digestion with RNase A followed by two dimensional TLC. This method is described in detail by Volckaert and Fiers (35). Individual RNase T_1 oligonucleotides were digested in 5 μl of 0.5 mg/ml RNase A, 10 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 8 mg/ml *E. coli* tRNA for 4 h at 37 °C. The RNase A digested oligonucleotide was then applied to a 6.7 cm×10 cm PEI-cellulose TLC plate (Brinkman Instruments, Polygram Cel 300 PEI) 1 cm in from each of two edges. The first dimension of chromatography was 22% (v/v) formic acid. This buffer was run to the top of the TLC plates, after which the plates were dried under a stream of air. The second dimension was in 1.1 M pyridine formate (pH 4.3), and the buffer was run to the top of the plates. The chambers for this procedure are shown diagramatically by Volckaert and Fiers (35). The 2 dimensional TLC plates were dried under a stream of air and the oligonucleotides were detected by autoradiography at −70 °C.

Results

Transcription of Euglena *chloroplast tRNA genes in the* Euglena *soluble extract*

It was previously shown that the *Euglena* chloroplast soluble extract transcribes tRNA-sized products from cloned chloroplast tRNA genes (11, 15). Additionally, $tRNA^{Leu}$ from *Eco* G was accurately processed at the 5′ and 3′-ends post-transcriptionally (ref. 11, see Fig. 1 and Table 1). A similar spinach chloroplast soluble extract also accurately transcribes and processes chloroplast tRNA genes (10, 13). For the experiments described in this report, 19 chloroplast tRNA genes of known sequence were chosen as templates for the *Euglena* chloroplast soluble extract. This project was undertaken to determine if several *Euglena* chloroplast tRNA genes organized in a variety of transcription units could be transcribed in the soluble extract. This information will be useful in subsequent characterization of transcriptional regulatory signals, and in determining the total number of tRNA genes required to yield the known *in vivo* spectrum of tRNAs. It was also of interest to determine if all of these tRNAs would be accurately processed by endogenous ribonucleases at the 5′- and 3′-ends.

The results of the transcription of various chloroplast tRNA genes and gene clusters are shown in Fig. 2. If no DNA is added to the extract, only a very low level of tRNA sized transcripts are observed (Fig. 2, lane 1). This background transcription is due to residual chloroplast DNA in the soluble extract that serves as a template for both the soluble and DNA bound RNA polymerases (11).

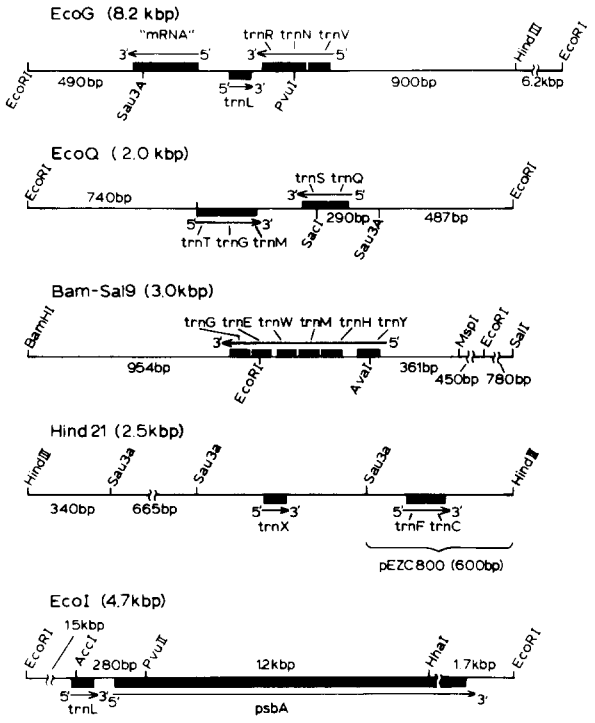

Fig. 1. Physical maps of plasmid DNAs used for *in vitro* transcription. The *Euglena* chloroplast DNA inserts from the plasmid DNAs are illustrated. Details of the plasmids are in Table 1. Boxes on the maps indicate the sites of genes. The name of the gene is given above the locus (see reference 17 for gene nomenclature). The direction of transcription is indicated by the arrows. Sizes of the inserts are in kilobase pairs (kbp). The sizes of the restriction fragments are given in kbp or in base pairs (bp) as indicated. All the maps are drawn to the same scale.

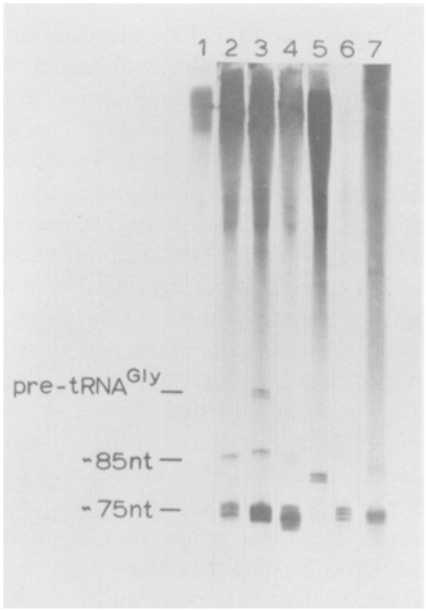

Fig. 2. Transcription of cloned *Euglena* chloroplast tRNA genes in the soluble extract. Polyacrylamide gel electrophoresis of *in vitro* transcription products. All reactions were performed as described in the text. The gel was 10% polyacrylamide-7 M urea. The direction of electrophoresis was top to bottom. RNA was detected by autoradiography. DNAs used as templates are as follows: Lane 1, No DNA; lane 2, pPG14; lane 3, pPG76; lane 4, pEZC300; lane 5, pEZC514; lane 6, pEZC800; lane 7, pEZC2. Transcript sizes (in nucleotides) were determined from known standards (not shown).

When a plasmid DNA containing a tRNA gene or a cluster of tRNA genes is added as template, tRNA sized product(s) are generated (Fig. 2, lanes 2–7). Because this transcription is inhibited by heparin, it must be due to the soluble RNA polymerase since the DNA bound RNA polymerase is heparin insensitive (11, 15). If the added plasmid DNAs contain tRNA genes with both large and small variable loops, i.e. pPG14, pPG76, pEZC300, and pEZC2, tRNAs of different size are produced reflecting the sizes of these genes (Fig. 2, lanes 2, 3, 4 and 7). If pEZC800 DNA, which contains two tRNA genes with small variable loops is transcribed, then only the smaller size tRNAs appear (Fig. 2, lane 6). Conversely, when pEZC514, which contains only a large variable loop tRNA gene is the template, only a large tRNA is produced (Fig. 2 lane 5). Note, that in a few cases multiple bands are observed for one tRNA product (Fig. 2, lanes 5 and 6). However, by two dimensional gel electrophoresis and RNA fingerprint analysis, it was shown that the heterogeneous RNA products are the result of transcription from the same gene (see below). It can be concluded, therefore, that the RNA polymerase in the *Euglena* chloroplast soluble extract transcribes the tRNA genes contained in the six plasmid DNAs tested. The only exceptions are the trnI and trnA loci of the chloroplast rDNA spacer, which are not transcribed in the soluble extract (11). The primary transcripts are then processed by enzymes in the extract into molecules that correspond in size to tRNAs.

An interesting result was obtained from the transcription of pPG76 DNA (Fig. 2, lane 3). In addition to the mature tRNA-sized products a tran-

script of approximately 120 nucleotides was also produced. This molecule was analyzed by RNase T_1 fingerprinting (data not shown), and was found to be $tRNA^{Gly}$ containing a mature 5′-end and an unprocessed 3′-extension of 46 to 48 nucleotides (pre-$tRNA^{Gly}$). This would make this RNA molecule 118 to 120 nucleotides, in concert with the one dimensional-gel analysis. It could not be determined if this tRNA precursor was the result of transcription termination or processing of an RNA with a longer 3′-extension.

Two dimensional gel electrophoresis of the soluble extract transcription products

To determine the number of individual tRNAs transcribed from each plasmid DNA template, two dimensional polyacrylamide gel electrophoresis of the *in vitro* reaction products was performed (Fig. 3C–3F and Table 2). In a parallel experiment, the number of different tRNAs transcribed from total chloroplast DNA was determined (Fig. 3B). The same pattern of tRNAs can be obtained with either exogenous or endogenous total chloroplast DNA as template (data not shown). As shown in Fig. 3A

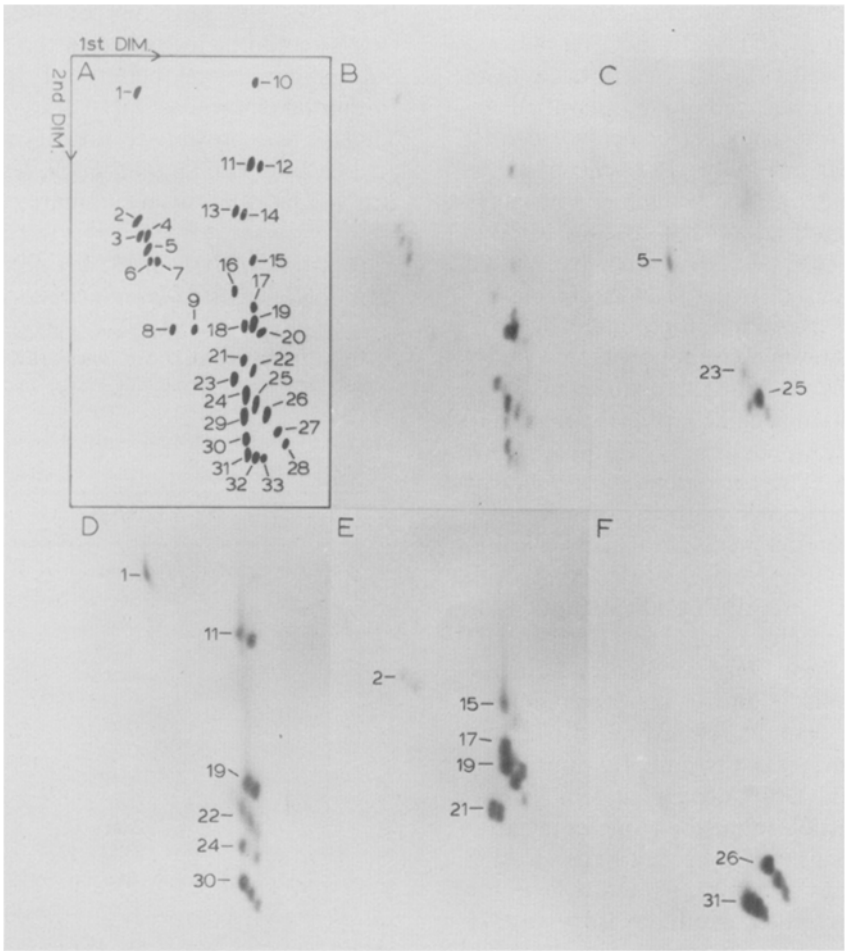

Fig. 3. Two-dimensional polyacrylamide gel electrophoresis of tRNAs transcribed *in vitro*. RNA transcribed in the *Euglena* chloroplast soluble extract was separated by two-dimensional gel electrophoresis. The autoradiograms of those gels are shown. The first dimension was 10% acrylamide-7 M urea, the second dimension was 20% acrylamide-4 M urea. The electrophoresis system is described in detail in reference 5. DNAs used for transcription are as follows: (panel A) Diagramatic representation of panel B. (panel B) endogenous chloroplast DNA. (panel C) pPG14. (panel D) pPG76. (panel E) pEZC300. (panel F) pEZC800. The numbering in panel A is used throughout the figure. The identified tRNAs are listed in Table 2.

and 3B, at least 33 different tRNAs can be resolved by two dimensional gel electrophoresis of RNA transcribed in the extract with the endogenous DNA. This is in good agreement with the report that 31 to 37 different tRNAs are resolved by two dimensional gel electrophoresis of *in vivo Euglena* chloroplast RNAs (25). The *in vitro* transcribed tRNAs must therefore represent all or nearly all *Euglena* chloroplast tRNAs.

When plasmid DNAs containing tRNA genes are used as templates in the *Euglena* chloroplast soluble extract, and the products are characterized by two dimensional gel electrophoresis, distinct tRNAs are produced. Two dimensional gels are shown for the products of transcription from pPG14, pPG76, pEZC300, and pEZC800 plasmid DNAs (Fig. 3C–3F). The tRNAs isolated from these two dimensional gels were identified by RNase T_1 fingerprint analysis (see below). *Eco* G, which is contained in pPG14 DNA encodes four tRNA genes (30). trnL (See Hallick & Bottomley (17) for gene nomenclature) has a large variable loop, and trnV, trnN, and trnR each have small variable loops. Four different tRNAs are present among the pPG14 transcription products. $tRNA^{Val}$ and $tRNA^{Asn}$, however, were not separated under these conditions and only three different RNA products are apparent (Fig. 3C). pPG76, pEZC800, and pEZC300 DNAs contain 6, 5, and 2 tRNA genes, respectively. The corresponding number of tRNAs are resolved in each of the two dimensional gels of the transcription products of these plasmid DNAs (Fig. 3D–3F). Note that $tRNA^{Met}$ from pPG76 and $tRNA^{Thr}$ from pEZC300 migrate to the same spot in the two dimensional gels (Fig. 3A, 3D and 3E, spot 19). Spot 19 is therefore a mixture of $tRNA^{Met}$ and $tRNA^{Thr}$ and is more pronounced than other spots. Spot 25, a mixture of $tRNA^{Val}$ and $tRNA^{Asn}$ from pPG14, is similarly prominent.

In these plasmid DNA dependent tRNA transcription experiments, more than one spot is resolved by two dimensional electrophoresis for each tRNA product. This is evident, for example, in the analysis of transcription products from pPG76 (Fig. 3D). The extra products for a given tRNA always appear on a diagonal. The species on a given diagonal were shown by fingerprint analysis to be derived from the same tRNA gene (data not shown). It is assumed these tRNAs differ by either a nucleotide modification or an incomplete tRNA processing step. There is precedent for electrophoretic variants of trnM transcripts produced in a spinach chloroplast transcription extract due to partial pseudouridylation (10). These diagonal arrays of spots do not appear in the two dimensional gel of tRNAs transcribed from the endogenous chloroplast DNA (Fig. 3A and 3B). However, much less RNA is produced in this reaction and the enzymes responsible for processing and/or nucleotide modification may be able to keep pace with the RNA polymerase.

The migration of tRNAs transcribed from plasmid DNAs (Fig. 3C–3F) was compared with tRNAs transcribed from total chloroplast DNA (Fig. 3B). The positions of nineteen individual tRNAs could be identified in the two dimensional polyacrylamide gel pattern of RNA transcribed from total chloroplast DNA (Fig. 3A and Table 2). Thus, it was possible to identify the tRNAs transcribed from *Eco* G, *Bam-Sal* 9, *Eco* Q, *Eco* A, and *Eco* I. This analysis is summarized in Table 2).

Fingerprint analysis of tRNAs produced in the Euglena *chloroplast soluble extract*

To confirm that the RNAs transcribed from the

Table 2. Transfer RNAs identified by two-dimensional gel electrophoresis.

Spot number	tRNA	Locus
1[a]	Tyr	*Bam-Sal*9
2	Ser	Eco Q
5	Leu	Eco G
6	X	Eco A
8	Leu	Eco I
11	Glu	*Bam-Sal*9
15	Gln	Eco Q
17	Gly	Eco Q
19	Met[b]	*Bam-Sal*9
19	Thr[b]	Eco Q
21	Met	Eco Q
22	Gly	*Bam-Sal*9
23	Arg	Eco G
24	His	*Bam-Sal*9
25	Val + Asn[c]	Eco G
26	Cys	Eco A
30	Trp	*Bam-Sal*9
31	Phe	Eco A

[a] The spot numbers correspond to those in Fig. 3.
[b] $tRNA^{Met}$ and $tRNA^{Thr}$ do not separate.
[c] $tRNA^{Val}$ and $tRNA^{Asn}$ do not separate.

plasmid DNA templates in fact represent the correct mature tRNAs, RNase T_1 fingerprint analysis was performed. Transfer RNAs were transcribed from pPG14, pPG76, pEZC300, pEZC514, pEZC2, and pEZC800 DNAs (see Fig. 1, and Table 1) and purified by two dimensional gel electrophoresis. The individual tRNAs were recovered from the gel and analyzed by RNase T_1 fingerprinting (Fig. 4). The RNase T_1 oligonucleotides generated from the fingerprints are listed in Table 1. These assignments were made based on secondary analysis using digestion with pancreatic Ribonuclease A (data not shown). Because [α-^{32}P]-UTP and -GTP were the only source of radiolabel in the transcription reactions, the secondary digestions resulted in a nearest neighbor analysis. All the RNase T_1 products

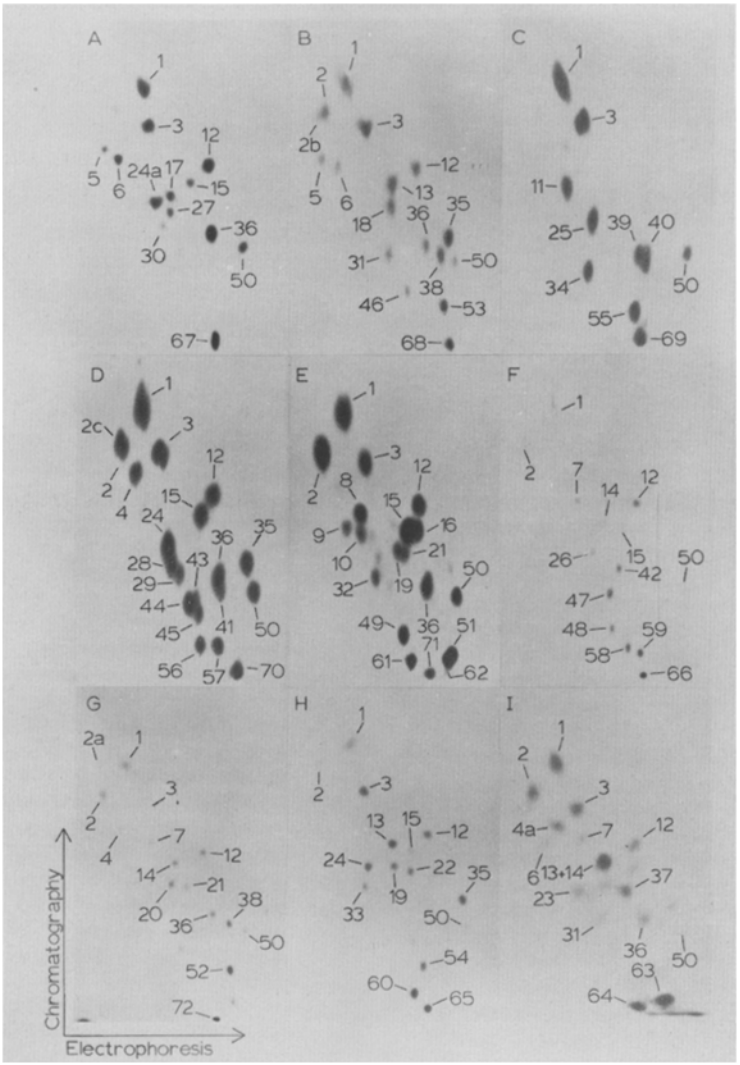

Fig. 4. RNase T_1 fingerprints of *Euglena* chloroplast tRNAs transcribed *in vitro*. RNA was transcribed from cloned chloroplast tRNA genes in the soluble extract. The individual tRNAs were then purified by two dimensional gel electrophoresis and subjected to RNase T_1 digestion. The resulting oligonucleotides were separated by a two dimensional technique using paper electrophoresis in the first dimension and thin layer chromatography in the second dimension (14). The fingerprints of the tRNAs are as follows. (panel A) $tRNA^{Arg}$/pPG14, (panel B) $tRNA^{Tyr}$/pPG76, (panel C) $tRNA^{Glu}$/pPG76, (panel D) $tRNA^{Gly}$ and $tRNA^{Met}$/pPG76, (panel E) $tRNA^{Trp}$ and $tRNA^{His}$/pPG76, (panel F) $tRNA^{Leu}$/pEZC514, (panel G) $tRNA^{Cys}$/pEZC2, (panel H) $tRNA^{Phe}$/pEZC2, (panel I) $tRNA^{X}$/pEZC2. Oligonucleotide compositions are given in Table 3 and are according to the numbering used here.

could be unambiguously assigned to oligonucleotides predicted from the tRNA gene sequences (Table 3).

Nine representative fingerprints are shown in Fig. 4; these include $tRNA^{Arg}$ from *Eco* G, all six tRNAs from *Bam-Sal* 9 $tRNA^{Leu}$ from *Eco* I, and $tRNA^{Phe}$, $tRNA^{Cys}$, and $tRNA^{X}$ from *Eco* A. Eight other tRNAs were also fingerprinted (data not shown). A fingerprint of $tRNA^{Leu}$ transcribed from *Eco* G in the *Euglena* chloroplast soluble extract was published (11). $tRNA^{Asn}$ and $tRNA^{Val}$, also from *Eco* G, which were not separated by two dimensional gel electrophoresis, were fingerprinted after transcription in the spinach chloroplast soluble extract (10). The analyses of these two tRNAs transcribed in the *Euglena* extract were identical to

Table 3. RNase T_1 oligonucleotides identified by fingerprint analysis.

Spot[a] number	RNase T_1[b] oligonucleotide	Spot number	RNase T_1 oligonucleotide
1	**Gp**[c]	35	**UpUpGp**
2	**CpGp**	36	UpC**p**UpGp + U**p**UpC**p**GpC**p**U**p**U**p**Gp
2a	UpC**p**U_{OH}	37	ApA**p**UpGp + A**p**UpA**p**Gp
2b	ApC**p**UpCpA$_{OH}$	38	**ApUpUpGp**
2c	C**p**U_{OH}	39	UpC**p**UpA**p**Gp
3	**ApGp**	40	**ApUpUpCpGp**
4	CpA**p**Gp	41	UpA**p**UpA**p**Gp
4a	ApC**p**Gp + CpA**p**Gp	42	ApAp**ApUpGp**
5	CpCpC**p**Gp	43	**UpUpApCpCpCpGp**
6	CpApC**p**Gp	44	**CpCpCpUpUpGp**
7	ApA**p**Gp	45	UpCpCpAp**ApUpGp**
8	ApApC**p**Gp	46	UpApAp**ApUpCpCpGp**
9	CpCpAp**ApGp**	47	**CpCpUpUpCpGp**
10	CpApAp**ApGp**	48	ApCpApC**p**UpCpA**p**UpGp
11	CpApApC**p**Gp	49	ApA**p**UpCpC**p**UpApCpA**p**Gp
12	**UpGp**	50	**pGp**
13	**CpUpGp**	51	**CpUpUpUpApGp**
14	UpC**p**Gp	52	**ApUpUpCpUpGp**
15	UpA**p**Gp	53	**UpUpApApUpGp**
16	**ApUpGp**	54	**UpUpCpCpUpApGp**
17	UpCpA**p**Gp	55	ApA**p**UpUpCpCpCpC**p**UpGp
18	C**p**UpA**p**Gp	56	UpCpC**p**UpUpCpCpApA**p**Gp
19	ApC**p**UpGp	57	A**p**UpUpCpA**p**UpApA**p**Gp
20	CpA**p**UpGp	58	**UpUpCpApApCpUpCpCpGp**
21	UpApA**p**Gp	59	**CpUpUpApUpApApApGp**
22	A**p**UpA**p**Gp	60	ApApApA**p**UpCpC**p**UpUpGp
23	C**p**UpCpC**p**Gp	61	UpC**p**UpCpCpApApApApCpC**p**UpGp
24	C**p**UpCpA**p**Gp	62	**UpUpCpApApUpUpGp**
24i	C**p**UpCpA**p**Gp + C**p**UpApC**p**Gp	63	**CpApUpUpUpApUpPGp**
25	CpC**p**UpA**p**Gp	64	ApC**p**UpCpA**p**UpApA**p**UpC**p**UpC**p**Gp
26	UpApCpC**pGp**	65	**UpUpCpApApApUpCpUpGp**
27	ApC**p**UpA**p**Gp	66	ApC**pUpUpApApApApUpCpApUpGp**
28	UpCpApCpA**pGp**	67	ApA**p**UpCpC**pUpUpC**p**UpUpGp**
29	ApA**p**UpCpA**pC**p**Gp**	68	**UpUpCpApUpC**p**UpUpUpC**p**Gp**
30	ApApC**p**UpApC**p**Gp	69	ApCpA**p**UpC**p**UpCpCpC**p**UpUpUpCpApC**pGp**
31	ApA**p**UpCpCpA**p**Gp	70	**UpUpCpApApApUpCpUpUpGp**
32	**ApUpCpCpCpC**p**Gp**[d]	71	ApC**p**UpCpC**pUpUpCpApUpUpC**pGp**
33	UpCpApCpCpA**pGp**	72	CpApApA**p**UpC**p**UpU**p**U**p**UpA**p**UpUpCpCpCpCpA**pGp**
34	CpCpCpCpCpA**p**UpC**pGp**		

[a] The spot numbers correspond to the numbers in Fig. 4.

[b] RNase T_1 cuts after G, leaving a 3′-phosphate on the G. The RNase T_1 oligonucleotides were assayed by digestion with RNAse A, which cuts after U and C, leaving a 3′-phosphate on the U or C.

[c] Bold face p indicates a ^{32}P.

[d] This oligonucleotide may also contain the 3′-end of $tRNA^{His}$, which is UpCpApUpUpCpApCpCpU$_{OH}$.

those which were previously published. The tRNAs transcribed from *Eco* Q were fingerprinted and the results were consistent with the gene sequences (data not shown).

From the fingerprint data, it can be concluded that the tRNAs are transcribed from the respective genes and they are correctly processed at the 5′-end. Additionally, the data is consistent with the argument that the tRNAs are correctly processed at the 3′-end by ribonuclease action. The constellations of oligonucleotides can be uniquely assigned to the transcripts of the tRNA genes used in the experiments. This is evident from the RNase T_1 oligonucleotides that are unique to each tRNA. For example, oligonucleotide 67, 5′-AAUCCUUCUUGp, is a unique internal sequence in $tRNA^{Arg}$ from *Eco* G (Fig. 4A), and oligonucleotide 68, 5′-UUCAUCUUUCGp, can only be derivied from $tRNA^{Tyr}$ transcribed from *Bam-Sal* 9 (Fig. 4B). Each of the tRNAs produced in these experiments is predicted to have at least one unique internal oligonucleotide and all of them were unambiguously identified by fingerprint analysis followed by secondary RNase A analysis (Fig. 4 and Table 3).

Two oligonucleotides predicted from the sequence of trnY and trnM in *Bam-Sal* 9 (22) were not found: 5′-CAGp at positions 43–45 in RNA^{Tyr}, and 5′-AUAG from positions 20–22 in $tRNA^{Met}$ (see Hallick *et al.* (19) for numbering of nucleotide positions). However, if 5′-CAGp is actually 5′-CUAGp, then it would be represented by oligonucleotide 18 in the fingerprint of $tRNA^{Tyr}$ (Fig. 4B). Also, if 5′-AUAG is actually 5′-UAG, then it may be assigned to oligonucleotide 15 in the fingerprint of $tRNA^{Met}$ (Fig. 4D). To test this possibility, the $tRNA^{Tyr}$ molecule was isolated from *Euglena* and sequenced directly. By this analysis it was found to contain 5′-CUAGp as predicted by the fingerprint (data not shown). Therefore, we assume both of these discrepancies are due to errors in the tDNA sequence.

Accurate 5′-termini processing was shown for each tRNA. The correct RNase T_1 5′-oligonucleotide for the eleven tRNAs that were fingerprinted is 5′-pGp. This is oligonucleotide 50, and it appears in all nine fingerprints (Fig. 4). Accurate 3′-processing is supported by three lines of evidence. First, all the tRNAs are the correct length as indicated by polyacrylamide gel electrophoresis (Fig. 2) and all have the correct 5′-end. Therefore, with respect to length, the 3′-end must be processed very close to the predicted nucleotide. Secondly, any 3′-terminal RNase T_1 oligonucleotide that should have been labeled under the reaction conditions used, was identified by fingerprint analysis. These are oligonucleotide 2a, 5′-UCU_{OH}, from $tRNA^{Cys}$ (Fig. 4G), oligonucleotide 2b, 5′-$ACUCA_{OH}$, from $tRNA^{Tyr}$ (Fig. 4B), and oligonucleotide 2c, 5′-CU_{OH}, from $tRNA^{Gly}$ (Fig. 4D). The 3′-end of $tRNA^{His}$ 5′-$UCAUUCACCU_{OH}$, is probably oligonucleotide 32 (Fig. 4E). Thirdly, in all the fingerprints the penultimate RNase T_1 oligonucleotide was found. For example, oligonucleotide 6, 43, and 54 (5′-CACGp, 5′-UUACCCGp, and 5′-UUCCUAGp) are unique oligonucleotides in $tRNA^{Tyr}$, $tRNA^{Gly}$, and $tRNA^{Phe}$, respectively (Fig. 4B, D, and H; and ref. 19). Therefore it can be concluded that the RNAs are transcribed from the tRNA genes and are then correctly processed at the 5′ and 3′-ends in the *Euglena* chloroplast soluble extract. We could not determine from this analysis if the 3′-CCA_{OH} had been added to the tRNAs or if any base modifications were made.

Discussion

In this paper we have shown that a soluble extract of *Euglena* chloroplasts is active in the specific and faithful transcription of a wide variety of chloroplast tRNA genes. It was previously demonstrated that the RNA polymerase activity in the soluble extract is distinguishable from the DNA bound chloroplast RNA polymerase activity of the transcriptionally active chromosome (11). The *Euglena* soluble extract is very similar to an extract from spinach chloroplasts (10, 13). As in the spinach chloroplast soluble extract, the *Euglena* extract contains ribonucleases that are active in processing the primary transcripts to mature tRNAs with respect to their 5′ and 3′-termini. Thus, the soluble extract is a good system for the study of chloroplast transcription and processing.

Twenty three *Euglena* chloroplast tRNA genes have been sequenced (19, 28; Manzara & Hallick, unpublished observations). Twenty one of these tRNA genes are transcribed in the *Euglena* chloroplast soluble extract. The genes can be tran-

scribed correctly from either intact chloroplast DNA or plasmid DNA clones. Only trnI and trnA resident in the rRNA operons are not recognized by the soluble RNA polymerase activity (11). These two loci are transcribed by the DNA bound RNA polymerase of the transcriptionally active chromosome. An interesting related finding was that the spinach chloroplast trnM2 gene is not transcribed in the *Euglena* chloroplast soluble extract (data not shown). The RNA polymerase, therefore, appears to be specific for *Euglena* chloroplast tRNA genes. This implies transcription initiation is regulated by elements contained in the chloroplast DNA sequence. These elements are probably not internal to the tRNA gene sequences, as is seen for eukaryotic nuclei RNA polymerase III initiation *in vitro* (6, 8) nor non-specific vector DNA sequences, or else one would expect trnI and trnA from the rRNA operon, and the spinach chloroplast trnM2 to be recognized. It is likely that the elements necessary for transcription are 5′-distal to the tRNA genes. This has already been shown to be the case in the spinach chloroplast soluble extract (13, 16).

The primary transcripts of tRNA genes in general are not identical to the mature, functional tRNA. Processing of tRNAs involves several enzymatic reactions (1, 9). These reactions can be divided into four classes: (1) 'RNase P-like' reactions, which produce mature 5′-termini, (2) exo- and/or endonuclease reactions which produce mature 3′-termini, (3) 3′-tRNA nucleotidyltransferase, which adds the 3′-CCA_{OH}, and (4) nucleotide modifying reactions. Although these reactions are well understood in eukaryotes and procaryotes, they have received little attention in chloroplasts.

Ribonucleases for producing mature 5′- and 3′-termini are present in the *Euglena* chloroplast soluble extract as is clearly shown by the experiments presented in this paper. All 19 *in vitro*-transcribed tRNAs were shown to have mature 5′ and 3′-termini. Most of the tRNA genes used in these experiments are clustered into multicistronic transcription units. Many of these clustered genes have very short intercistronic spacers; i.e. 1 base pair for trnF-trnC, 3 base pairs for trnN-trnR, and 5 base pairs for trnT-trnG (19, 24, 28, 30). These tRNAs are most likely transcribed as multimeric tRNA molecules. It is possible that the multimeric tRNAs are cleaved into monomeric tRNAs by the 5′-processing or 'RNase P-like' activity in the soluble extract. Thus, this activity could serve two functions, (1) to produce mature 5′-termini, and (2) to cleave multimeric transcripts into monomeric tRNAs. It is also possible that there are other endoncleolytic activities in the soluble extract which are responsible for cleaving multimeric transcripts.

As does the spinach chloroplast soluble extract (10), the *Euglena* extract contains a very active 3′-tRNA nucleotidyltransferase (B. M. Greenberg & R. B. Hallick, unpublished observations). This activity is required for tRNA maturation in chloroplasts, because the terminal CCA_{OH} is not encoded in any of the chloroplast tRNA genes (19). From the fingerprint analysis presented here, it could not be directly determined if the 3′-CCA_{OH} is added to the *in vitro*-transcribed tRNAs. Nevertheless, we believe that the *in vitro* transcribed tRNAs are largely 3′-CCA and matured *in vitro*. This is based on our observation that [α-^{32}P]-ATP and/or -CTP were preferentially incorporated into *in vivo* tRNAs that were added to the soluble extract (data not shown). It is possible the diagonals of spots following two dimensional gel analysis (Fig. 3) are due in part to incomplete 3′-CCA_{OH} addition (see Fig. 3).

The accumulation of the 120 nucleotide pre-$tRNA^{Gly}$ is noteworthy. The pre-$tRNA^{Gly}$ was found by fingerprint analysis to have a mature 5′-end (data not shown). Therefore, a mature 3′-end is not required for the 'RNase P-like' or 5′-processing activity to function. This indicates a possible order of tRNA processing steps in *Euglena* chloroplasts. That is, the 5′-end may be processed first, followed by the 3′-end. Finally, the 3′-CCA_{OH} is added and base modifications are made. Spinach chloroplasts also mature tRNAs in this order (10).

Our working hypothesis is that the pre-$tRNA^{Gly}$ is produced by transcription termination, not by RNA processing. This is because very close to the 3′-end of pre-$tRNA^{Gly}$ a weak stem-loop structure is found followed by a poly-T sequence in the noncoding strand (22). This is similar to prokaryotic RNA polymerase termination signals (32, 37). The actual identification of a chloroplast transcription terminator must however await a more direct line of experimental evidence.

The *Euglena* chloroplast soluble extract has proven to be a convenient system for the study of tRNA biosynthesis *in vitro*. The *Euglena* and spin-

ach chloroplast systems are remarkably similar. Therefore, conclusions drawn from experiments with the *Euglena* extract may be applicable to higher plants.

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