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Sequences of four tRNA genes from *Caenorhabditis elegans* and the expression of *C. elegans* $tRNA^{Leu}$ (anticodon IAG) in *Xenopus* oocytes

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ABSTRACT

Four tRNA genes have been identified in cloned segments of Caenorhabditis elegans DNA by tRNA hybridisation and expression after injection into Xenopus laevis oocyte nuclei. From DNA sequencing these are (with DNA anticodon sequences) $tRNA^{Asp}$ (GTC), $tRNA^{Leu}$ (AAC), $tRNA^{Lys}$ (CTT) and $tRNA^{Pro}$ (TGG). Their flanking DNA sequences are compared. Two identical $tRNA^{Lys}$ (CTT) genes from different regions of the genome have quite unrelated 5' flanking sequences. The tRNA synthesised in Xenopus oocytes after injection of the $tRNA^{Leu}$ cloned DNA has the modified anticodon IAG. The $tRNA^{Leu}$ gene precursor transcript from injected oocytes has short 5' and 3' additional sequences and lacks certain of those modified bases found in the processed tRNA.

INTRODUCTION

The study of the structure and expression of eukaryotic tRNA genes has developed using recombinant DNA techniques and RNA polymerase III dependent transcription systems, in particular the Xenopus oocyte in vivo system. Recognition of two separate sequences within tRNA structural genes is essential for initiation of their transcription by RNA polymerase III (1-4). However features of the 5' flanking sequences can act as modulators in transcription (1,5,6). One approach to finding such regulatory sequences is a comparison of 5' flanking sequences from tRNA genes of the same and different eukaryotes. We describe the DNA sequences, with flanking regions, of five tRNA genes from the nematode, Caenorhabditis elegans.

Eukaryotic tRNA genes may be expressed in Xenopus oocytes after injection of DNA containing these sequences into the oocyte germinal vesicle. In some cases the transcripts are processed slowly allowing study of tRNA precursor maturation (7-9). Each of the cloned nematode tRNA genes can be expressed in Xenopus oocytes as an RNA corresponding in size to the mature tRNA. Injection of the $tRNA^{Leu}$ gene resulted in accumulation of tRNA and also of a precursor RNA differing from mature tRNA by the presence of short additional 5' and 3' sequences. We describe the properties and sequences of

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this precursor and its relationship to $tRNA^{Leu}$.

MATERIALS AND METHODS

Plasmids, phages and bacterial strains

Cet 1, 7 and 18 are ColE1 plasmids with segments of C. elegans DNA containing tRNA genes cloned into the plasmid EcoR1 site (7). Plasmid DNA was isolated according to Clewell (10). The bacteriophage vector λ1059 and the cloning into the λ1059 Bam H1 site of a partial Bam H1 restriction digest of C. elegans DNA (fragment size 15000-20000 bp) has been described by Karn et al. (11) who made these phages available to us. The bacteriophage m13mp3 vector is a derivative of m13mp2 containing a Bam H1 cloning site (12). Procedures for cloning in m13mp2 derivatives using E. coli JM101 as host were according to Messing (13).

Biochemicals and enzymes

^{32}P labelled compounds were from the Radiochemical Centre, Amersham. Restriction endonuclease Taq 1 was a gift of the late Dr. J.I. Harris and purified E. coli RNaseP (14,15) was a gift from Dr. S. Altman. Other restriction endonucleases were from Biolabs (Beverly, Massachusetts, U.S.A.); T_4 RNA ligase, T_4 DNA ligase and polynucleotide kinase were from P-L biochemicals. DNA polymerase I and DNA polymerase I Klenow fragment were from Boehringer.

^{32}P labelling and separation of tRNA

Nematode tRNA (7) was labelled at the 3' terminus with [5'-^{32}P]pCp using T_4 RNA ligase (16) or with [α-^{32}P]ATP using yeast tRNA nucleotidyl transferase (7). ^{32}P labelled tRNA complementary to cloned DNA segments was isolated by hybridisation to DNA immobilised on cellulose nitrate filters and elution as previously described (7). Transfer RNAs were separated by electrophoresis on 12.5% polyacrylamide gels in 0.089 M tris base, 0.089 M boric acid, 2.5 mM EDTA, 7 M urea, pH 8.3. Two-dimensional separations used the same buffer solution containing 4 M urea. The first dimension was on a 40 cm long 10% polyacrylamide thin denaturing gel run for 2h at 1200V (30 mA). The second separation was for 20h at 200V on a 20% acrylamide gel. (We are indebted to Dr. P. Arcari for this method.)

Identification of DNA fragments from restriction enzyme digests

DNA from clones identified as containing tRNA genes was digested in 6.6 mM tris-chloride pH 7.4, 6 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol with endonuclease Sau 3A or Alu 1 at 37° or with Taq 1 at 67°. EcoR1 digests were in 80 mM tris-chloride pH 7.5, 10 mM $MgCl_2$, 80 mM NaCl, 1 mM dithiothreitol.

DNA fragments were separated on 5% or 10% polyacrylamide non-denaturing tris-glycine gels. After transfer to cellulose nitrate sheets (17) those containing tRNA sequences were identified by hybridisation with [5'-^{32}P]pCp labelled nematode tRNA (2-4 x 10^7 dpm/µg) in 5 x SSC, 50% v/v formamide at 37° for 16h. (SSC is 0.15 M NaCl, 0.015 M sodium citrate pH 7.) The filters were washed in 5 x SSC, 50% v/v formamide at 37° and in 2 x SSC at 20° and autoradiographed (18). DNA was recovered from gels by extraction (19) or by electroelution (20).

The following procedures were from Maxam and Gilbert (19) with minor modifications: 3' terminal labelling of DNA with [α-^{32}P] deoxyribonucleoside triphosphates using the E. coli DNA polymerase I Klenow fragment. 5' terminal labelling of DNA with [α-^{32}P]ATP and T_4 polynucleotide kinase after removal of the 5' terminal phosphates with E. coli phosphomonoesterase; strand separation of denatured DNA fragments by gel electrophoresis; chemical sequencing of 5' terminally labelled DNA strands.

Sub-cloning into the ml3mp3 vector and sequence determination

DNA from clones positively identified as containing tRNA genes was digested with endonuclease Sau 3A and inserted by ligation into the Bam Hl site of ml3mp3 replicative form DNA. After transfection of E. coli JM101, recombinant DNA phages were identified by the inability of infected cells to synthesise β-galactosidase (13). Those with inserts containing tRNA genes were identified by the plaque hybridisation technique (21) using as probe [5'-^{32}P]pCp labelled nematode tRNA.

Growth of ml3 clones, phage DNA isolation and sequence determination by the di-deoxyribonucleotide chain termination were by the methods of Sanger et al. (22), in some instances using a synthetic 17 bp primer (23).

All cloning was with the approval of GMAG.

Transcription of DNA by E. coli RNA polymerase

The reaction mixture (100 µl) contained 2.5 µg DNA, 40 mM tris-chloride pH 7.9, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM KCl, 2.5 µM each of GTP, CTP and UTP, 5 µc [α-^{32}P]ATP (400 Ci/mmole) and E. coli RNA polymerase. After 30 min at 30° DNase (RNase free) was added and incubation continued for 10 min. Labelled RNA was recovered by phenol extraction and ethanol precipitation from 0.2 M sodium acetate.

Analysis of tRNA synthesised after microinjection of DNA into Xenopus laevis oocyte nuclei

Germinal vesicles of Xenopus oocytes were co-injected with plasmid DNA and [α-^{32}P] ribonucleoside triphosphates, the oocytes incubated, the labelled

RNAs isolated and separated by gel electrophoresis (7). After elution from the gels and digestion with ribonuclease T_1 the labelled digest was separated either by two dimensional electrophoresis on cellulose acetate in pyridine-acetate buffer pH 3.5 containing 7 M urea and on Whatman DE81 paper in 7% v/v formic acid, or by the pH 3.5 electrophoresis followed by homochromatography on DEAE cellulose thin layer plates (23). The labelled oligonucleotides were analysed by digestion with ribonuclease A and separation of the products by electrophoresis at pH 3.5 on DEAE paper (Whatman DE81), or by alkaline hydrolysis followed by electrophoresis on Whatman 3 MM paper at pH 3.5 (24). Radioactive spots were detected by autoradiography (18).

RESULTS AND DISCUSSION

Cet 1, 7 and 18 are ColE1 plasmids with *C. elegans* DNA fragments cloned into the plasmid EcoR1 site and characterised as containing *C. elegans* tRNA genes (7). The three plasmids contain unrelated nematode DNA sequences. This was shown by preparing ^{32}P labelled *in vitro* transcripts with *E. coli* RNA polymerase and hybridising these against the EcoR1 digestion products of each plasmid DNA separated by electrophoresis on 1% agarose gels and transferred to cellulose nitrate sheets (17). Each RNA transcript only hybridised to the insert of the clone from which it was derived.

The tRNAs specified by each plasmid DNA were examined by two-dimensional gel electrophoresis of the [5'-^{32}P]pCp labelled tRNA which hybridised to the plasmid DNA immobilised on cellulose nitrate filters. Cet 1 and Cet 7 DNA each hybridised to a unique single tRNA species resolved on the gel, but Cet 18 DNA hybridised to two closely migrating tRNAs implying that the 2500 bp insert encodes two different tRNAs. Cet 18 DNA was injected into *Xenopus* oocyte nuclei together with [α-^{32}P]GTP and after 24h the labelled RNAs separated by two-dimensional electrophoresis. Two RNAs with mobilities similar to those from the hybridisation experiment were labelled.

Sequences of the tRNA genes

$tRNA^{Pro}$ (Cet 1). A single 280 bp Taq 1-EcoR1 digestion fragment of Cet 1 DNA hybridises to labelled nematode tRNA suggesting that the Cet 1 5500 bp nematode DNA insert codes for one tRNA gene (7). Using synthetic linker oligonucleotides this fragment was inserted into the EcoR1 site of the vector m13mp2 in both orientations and the DNA sequence determined by the di-deoxyribonucleotide chain termination method (21). The sequence of the purified Taq 1-EcoR1 DNA fragment was also determined by direct chemical

sequencing (19) using an internal restriction endonuclease Sma 1 site. This gene codes for a $tRNA^{Pro}$ (Figs. 1-3).

$tRNA^{Leu}$ (Cet 7). A Sau 3A digest of Cet 7 DNA was cloned into the Bam H1 site of ml3mp3. Phages with DNA inserts containing the tRNA gene were identified by plaque hybridisation (21) using as probes either [5'-^{32}P]pCp labelled nematode tRNA or a short [5'-^{32}P] labelled Cet 7 Taq 1-Alu 1 restriction enzyme digest DNA fragment known to contain part of the tRNA gene sequence (see below). Phages with the tRNA gene inserted in both directions were isolated and the inserts sequenced by the chain termination method. A sequence coding for a $tRNA^{Leu}$ (DNA anticodon AAC) was recognised (Figs. 1-3).

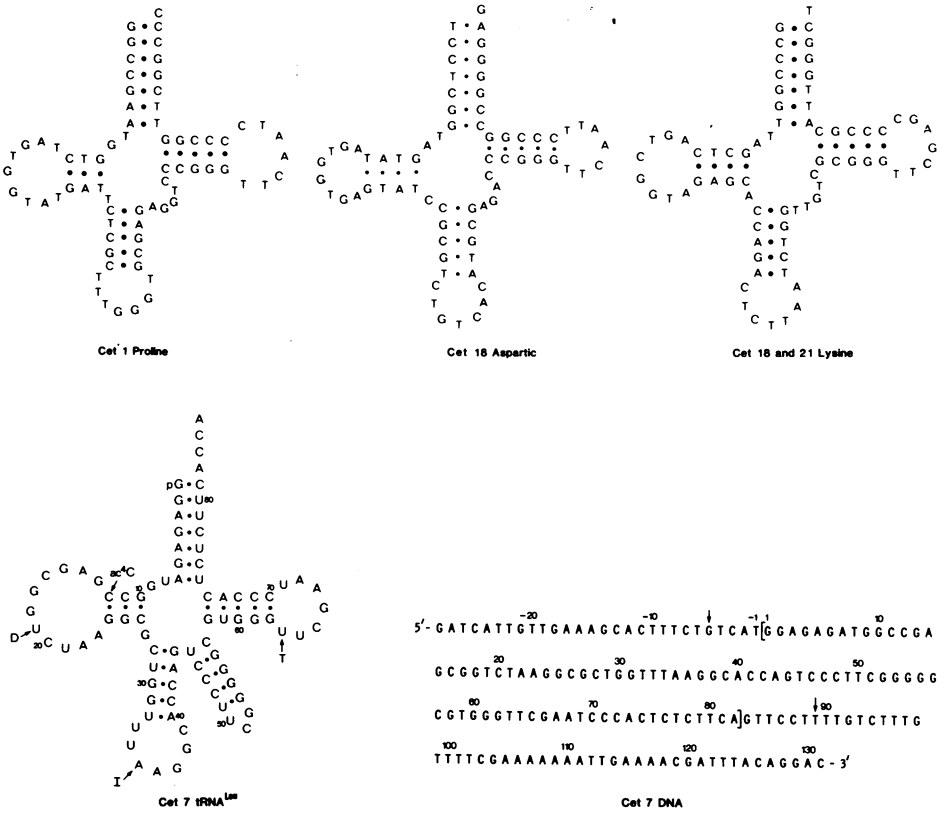

Figure 1. Cloverleaf arrangements of the DNA sequences of the nematode tRNA genes. The RNA sequence of cet 7 $tRNA^{Leu}$ shows those modified bases identified in the oocyte processed transcript. Pseudouridine residues would not have been distinguished by the analysis. The arrows in the Cet 7 DNA sequence show the start and termination points deduced from analysis of the precursor RNA.

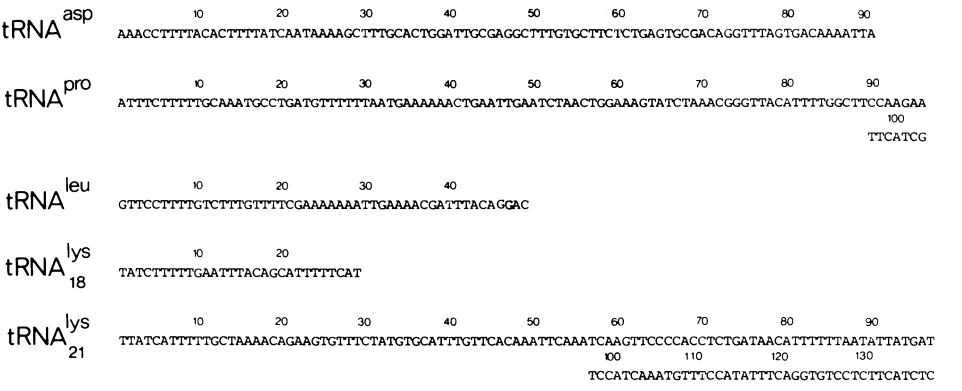

Figure 2. 3' trailer sequences immediately following the nematode structural tRNA genes.

This contains a Taq 1 site in a position corresponding to loop IV of the tRNA structure. A Taq 1 digest of Cet 7 DNA was separated on a 5% polyacrylamide tris-glycine gel and after transfer to a cellulose nitrate sheet (17) a single DNA fragment hybridised to [5'-^{32}P]pCp labelled nematode tRNA. The 5' terminal phosphates of this DNA fragment were labelled with ^{32}P using T_4 polynucleotide kinase and the DNA split with endonuclease Alu 1 to give two 5' labelled Taq 1-Alu 1 fragments. After strand separation the sequences of these were determined (19). One included a sequence complementary to residues 1-66 of the tRNA^{Leu} structural gene from the Taq 1 site. This

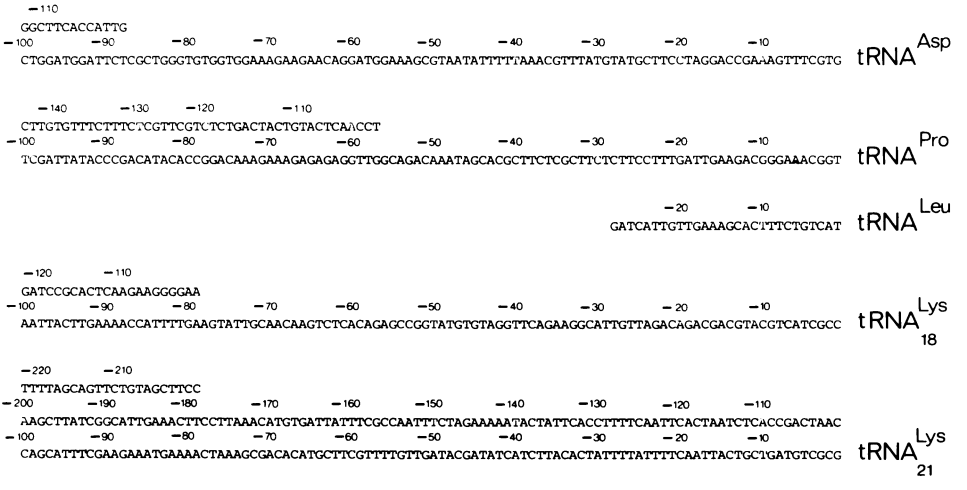

Figure 3. 5' leader sequences immediately preceeding the nematode structural tRNA genes.

confirmed the identity of the tRNA gene and suggested that the 5000 bp Cet 7 nematode DNA insert contained only one tRNA gene.

tRNA^{Lys} and tRNA^{Asp} (Cet 18). A Sau 3A digest of Cet 18 was subcloned into the Bam H1 site of ml3mp3 and phages from plaques hybridising to [5'-^{32}P]pCp labelled nematode tRNA isolated. One contained a 600 bp insert which was recloned in both orientations in ml3mp3. This contained a sequence encoding tRNA^{Asp} (DNA anticodon GTC). A second phage with a 300 bp insertion encoded a tRNA^{Lys} (DNA anticodon CTT). These sequences are in Figs. 1-3.

tRNA^{Lys} Cet 21. An identical tRNA^{Lys} structural gene from a different region of the C. elegans genome was isolated by screening λ1059 phages with insertions from a partial Bam H1 digest of C. elegans DNA, size range 15000-20000 bp (11). λ1059 Cet 21 hybridised to three separable species of nematode tRNA labelled with [5'-^{32}P]pCp or with [α-^{32}P]ATP using tRNA nucleotidyl transferase. One of these tRNA genes was cloned into mpl3mp3 as a Sau 3A fragment using the same probes and then recloned to give both orientations. Sequence determination (22) identified a tRNA^{Lys} gene identical to that of Cet 18 but with different flanking regions (Figs. 1-3).

DNA from Cet 21 was injected into oocyte nuclei (10-50 ng DNA/oocyte) together with [α-^{32}P]GTP. No tRNA transcript was detected although co-injection of Cet 21 DNA together with 5 ng Cet 7 DNA gave transcription of tRNA^{Leu} alone. However the gene was transcribed on injection of ml3mp3 viral DNA with the Cet 21 tRNA^{Lys} sense strand (in oocytes the viral DNA is rapidly converted by synthesis to double-stranded DNA). Failure of the tRNA gene in the λ1059 cloned DNA to be transcribed may be a property of the vector or of the additional nematode DNA sequences.

Comparison of the sequences

In Fig. 1 the DNA sequences of the four tRNA genes are arranged in the tRNA cloverleaf secondary structure. They show no exception to the generalised structure reviewed by Clark (25). Comparison with a compilation of tRNA sequences (26) shows they share the comparatively high degree of sequence homology found in comparable higher eukaryotic tRNAs.

The 3' flanking sequences immediately following the tRNA structural genes (Fig. 2) all have at least one run of four or more T residues in common with other eukaryotic tRNA genes (26,27) where the T residues act as termination signals. Oocyte transcription of the nematode tRNA^{Leu} gene terminates at the first of these sequences.

Transfer RNA genes have promoter sequences within the structural gene which suffice for transcription (1-4). However in Drosophila tRNA$_2^{Lys}$ genes

(1,5) and in Bombyx mori $tRNA_2^{Ala}$ genes (5) the 5' flanking sequences can modulate tRNA transcription. Regions of homology are found in the 5' flanking sequences of some related sets of Drosophila tRNA genes (1,29-31). Comparison of the 5' flanking sequences of the nematode tRNA genes (Fig. 3) shows no direct sequence homology common to all five, and those of the two copies of the $tRNA^{Lys}$ gene are quite dissimilar. Regions of homologous sequence are found between residues -22 and -11 of the $tRNA^{Pro}$ and $tRNA^{Leu}$ genes, TTC(A)TTCAA$^{GA}_{AC}$C, and between residues -7 and -1 of the $tRNA^{Asp}$ and Cet $tRNA^{Lys}$ genes; T$^{T}_{G}$TCG$^{T}_{C}$G. In the Cet 18 $tRNA^{Lys}$ gene only the sequence TCGC (residues -5 to -2) resembles this.

Two Drosophila $tRNA^{Leu}$ (anticodon CAA) genes share a sequence GANTTTGG beginning at residues -12 and -21 and a similar sequence GCNTTTTC is shared by several $tRNA^{Ile}$ genes beginning at residues -23 or -24 (31). Drosophila $tRNA_2^{Lys}$ genes (DNA anticodon CTT) have a consensus sequence GGCACTTTTTA approximately from residue -25 (1,29,30). In C. elegans $tRNA^{Leu}$ the closest approximation to the Drosophila sequence is GACTTTA (residues -48 to -41); in C. elegans $tRNA^{Lys}$ (Cet 18) that resembling the Drosophila sequence is GGCATGTTA (residues -32 to -24). The C. elegans tRNA 5' leader sequences all have direct repeats of 3 or more bases (7 in the case of $tRNA^{Pro}$) within the first 30 residues. Comparison of different regions of the nematode gene 5' flanking sequences by matching 7 out of 11 consecutive bases did not give any significant extended homologies.

Expression of Cet 7 $tRNA^{Leu}$ in Xenopus oocytes

We have previously shown (7) that microinjection of Cet 7 DNA together with [α-^{32}P]GTP into Xenopus oocyte germinal vesicles results in the synthesis of two labelled RNAs distinguished by their migration on polyacrylamide gels (Fig. 4). One corresponds in size to the nematode tRNA species hybridisable to Cet 7 DNA while the second RNA product, approximately 10 nucleotides longer, has the properties of a precursor to the tRNA. Injection of the purified larger RNA species into oocyte germinal vesicles results in its conversion to the tRNA-like species (8). The biosynthesis of these RNAs is sensitive only to high concentrations of α-amanitin as expected for RNA polymerase III transcription. Both labelled RNAs were also detected after the in vitro transcription of Cet 7 DNA using an unfractionated extract from manually isolated germinal vesicles and labelled with [α-^{32}P]GTP.

A partial sequence analysis of the tRNA and precursor RNA was obtained by analysis of [^{32}P] labelled ribonuclease T_1 digestion products. Cet 7 DNA was injected into the germinal vesicles of oocytes together with either [α

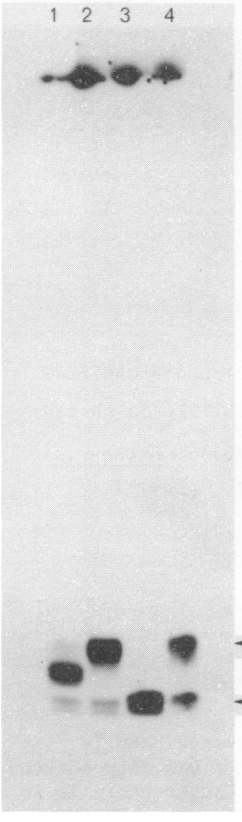

Figure 4. Cleavage of Cet 7 precursor RNA by E. coli RNase P. [^{32}P] precursor RNA from in vivo labelled oocytes (germinal vesicles injected with Cet 7 DNA and [α-^{32}P]GTP) was incubated with RNase P in 0.01 M tris-HCl pH 8.0, 0.1 M NH_4Cl, 0.005 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol at 37° for 40 min. The products were separated by electrophoresis on a 12.5% acrylamide gel containing 7 M urea (Methods). Lanes: 1, incubated with enzyme; 2, incubated without enzyme; 3, mature Cet 7 tRNA from oocytes; 4, a mixture of precursor and mature tRNA.

-^{32}P] labelled GTP, ATP, CTP or UTP. The oocytes were incubated for 5 h and the labelled RNAs were extracted, separated by polyacrylamide gel electrophoresis, eluted and digested with ribonuclease T_1. The products were separated by two-dimensional electrophoresis or by electrophoresis at pH 3.5 followed by homochromatography on DEAE cellulose thin layer plates. After elution they were hydrolysed either with ribonuclease A or with alkali and the products analysed as described in methods.

Fig. 5 shows the separation of ribonuclease T_1 products from the tRNA labelled with [α-^{32}P]GTP or [α-^{32}P]UTP and Table 1 summarises the analytical results on these products. Essentially this gives a type of nearest neighbour sequence analysis. The sequences of some of the larger oligonucleotides, for example nucleotides 15 and 16, cannot be determined in this way but their analysis and positions on the two dimensional separation enables them to be assigned to the DNA sequence. The sequences of the other nucleotides are those expected from the DNA sequence. The tRNA appears to be

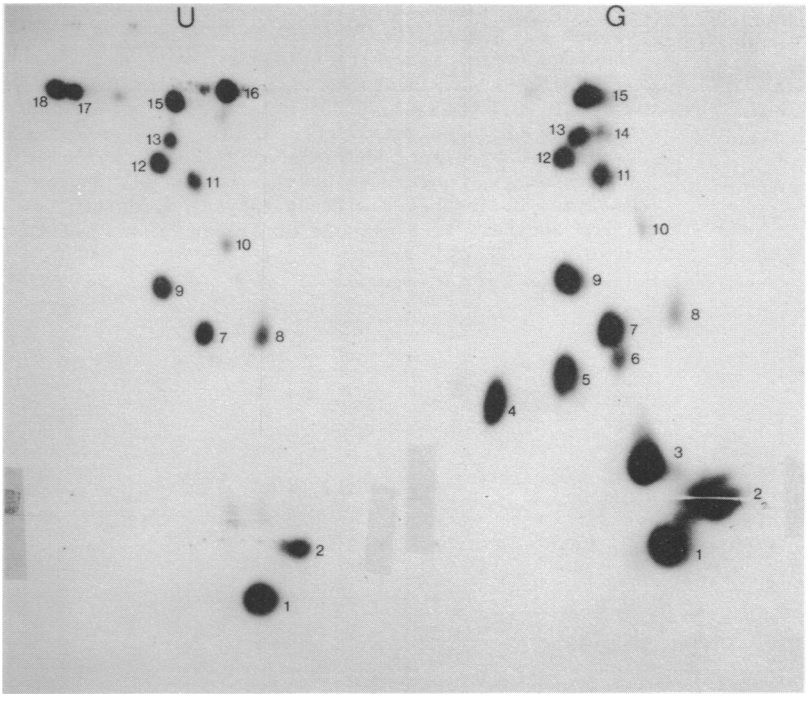

Figure 5. Two-dimensional electrophoretic separation of ribonuclease T_1 digests of Cet 7 $tRNA^{Leu}$ synthesised after injection of Cet 7 DNA into oocyte nuclei and labelled with [α-^{32}P]UTP(U) or [α-^{32}P]GTP (G). Electrophoresis is in pyridine-acetate buffer, 7 M urea, pH 3.5 (right to left) and on Whatman DE81 paper in 7% v/v aqueous formic acid (top to bottom). The numbered spots on the autoradiograph are described in Table 1.

transcribed and processed correctly at the 5'-terminus but we cannot conclude whether the CCA terminus is present.

Modified nucleotides of $tRNA^{Leu}$

2-dimensional thin layer chromatographic separation (32) of the labelled oocyte tRNA hydrolysed with venom phosphodiesterase showed several modified nucleoside residues. Four of these (inosine, N^4acetylcytidine, dihydrouridine and thymine riboside) were recognised and placed in the tRNA sequence on the basis of the electrophoretic and hydrolytic properties of their nucleotides as isolated from ribonuclease T_1 digestion products. Other modified nucleotides which could not be distinguished on the electrophoretic separations may have been overlooked.

Inosine was recognised at position 35 in the tRNA sequence by analysis of nucleotides 17 and 18 (Fig. 5, Table 1), both of which are only labelled

Table 1. Identification of the T_1 ribonuclease products from $tRNA^{Leu}$ and precursor shown in Figs. 5 and 6.

Spot number (Figs. 5 and 6)	Identification (3' adjacent nucleotides in brackets)	tRNA	Precursor
1	G (G,A,C,U)	+	+
2	CG (G,C,U)	+	+
2a	CCG		+
3	AG(A,C)	+	+
4	pGp(G)	+	-
5	UG(G)	+	+
6	Cac^4CG(A)	+	-
7	CUG(G)	+	+
8	CACCAG(U)	+	+
9	AUG(G)	+	+
11	DCU^*AAG(G)	+	-
12	TUCG(A)	+	+
13	UCUAAG(G)	+	+
14	See Legend		
15	UCCCUUCG(G)	+	+
16	3' terminus from A67	+	-
17	UUUI(A)	+	faint
18	UUUI(A)	+	faint
A	UCAUG(G)	-	+
B	UUUAAG(G)	-	+
C	UUCCUU	-	+
D	A_{67}-G_{83}	-	+

The RNAs synthesised in oocytes after injection of Cet 7 DNA were labelled separately with each [α-^{32}P] ribonucleoside triphosphate and their ribonuclease T_1 products separated. Sequences of these were deduced from ribonuclease A and alkaline digestion products, the position of the RNase T_1 product on the electrophoretic separation and, with nucleotides 15, 16, A, C and D comparison with the DNA sequence. Modified nucleotides are described in the text (ψ and U could not be distinguished in this analysis), * is an unidentified derivative. Nucleotide 14, Fig. 5, is an unidentified minor component. Nucleotide 14, Fig. 6, is only labelled with C and U (nucleotides C and 14 are related alternative 3' terminal fragments).

with ATP and UTP. After labelling with [α-^{32}P]ATP, alkaline or ribonuclease A hydrolysis of 17 and 18 gave labelled Up and a labelled nucleotide corresponding in electrophoretic mobility to Ip at pH 3.5 on 3MM paper or on DEAE ion exchange paper. 17 and 18 labelled with [α-^{32}P]UTP gave only Up on hydrolysis. This shows that two or more U residues precede the I residue. The position of 17 on the two-dimensional separation corresponds to UUUI while that of 18 is compatible with UUUI terminated by a cyclic 2',3'-phosphate resulting from the slow rate of hydrolysis of inosine 2',3'-cyclic phosphate by ribonuclease T_1. From the DNA sequence this places the fragment at residues 32-35 of the tRNA where A_{35} has been modified to I and so is hydrolysed by RNase T_1. We conclude that Cet 7 codes for a $tRNA^{Leu}$ which when expressed in the frog oocyte has the anticodon IAG (residues 35-37).

A modified residue in nucleotide 6 (Fig. 5) at position 12 in the tRNA sequence has the properties of N^4acetyl cytidine. Nearest neighbour analysis of 6 (labelled with A, C, U or G) using ribonuclease A gives the sequence $C_{(n)}$NG(A) where N is derived from C and has an electrophoretic mobility corresponding to ac^4Cp. After alkaline hydrolysis of nucleotide 6 labelled with [α-^{32}P]GTP N is converted to C as would be expected of ac^4C (32). The position of nucleotide 6 on the two-dimensional separation corresponds to that of Cac^4CG. Comparison with the DNA sequence places the ac^4C residue at position 12 in the tRNA sequence.

The U residue at position 19 in the tRNA sequence is partly modified to dihydrouridine. In nucleotide 13 (Fig. 5, Table 1), identified as UCUAAG by sequence analysis and reference to the DNA sequence, U residues 19 and 21 are unmodified. Nucleotide 11 contains the same sequence but both U residues are modified. Residue 19 identified by nearest neighbour labelling with [^{32}P]CTP has the properties expected of dihydrouridylic acid. Isolated after ribonuclease A digestion of 11 the nucleotide migrates with, or just behind, Up at pH 3.5 (Whatman 3MM paper), but alkaline hydrolysis of 11 converts it to a product with a faster electrophoretic mobility corresponding to β-ureidoipropionic acid ribotide, the alkaline breakdown product of Dp (33). The modified base corresponding to U_{21} in nucleotide 11 has not been identified.

U_{64} appears to be modified to ribothymidine judged by the mobility of the nucleotide on both pH 3.5 electrophoretic systems.

The precursor RNA

Comparison of the ribonuclease T_1 digests of the precursor RNA and the

tRNA together with the DNA sequence shows that the precursor includes the tRNA sequence, with some nucleotide modifications absent, and short additional transcribed sequences at the 5' and 3' ends.

The 5' terminal nucleotide of the tRNA was identified in ribonuclease T_1 digests as pGp (Fig. 5, Table 1). This is absent from digests of the precursor RNA (Fig. 6) being joined to a short 5' segment which is removed by E. coli RNaseP. RNaseP splits tRNA precursors adjacent to the tRNA 5' terminal nucleotide (14). Cet 7 precursor RNA, isolated from [α-^{32}P]GTP labelled oocytes and purified by gel electrophoresis, was incubated with

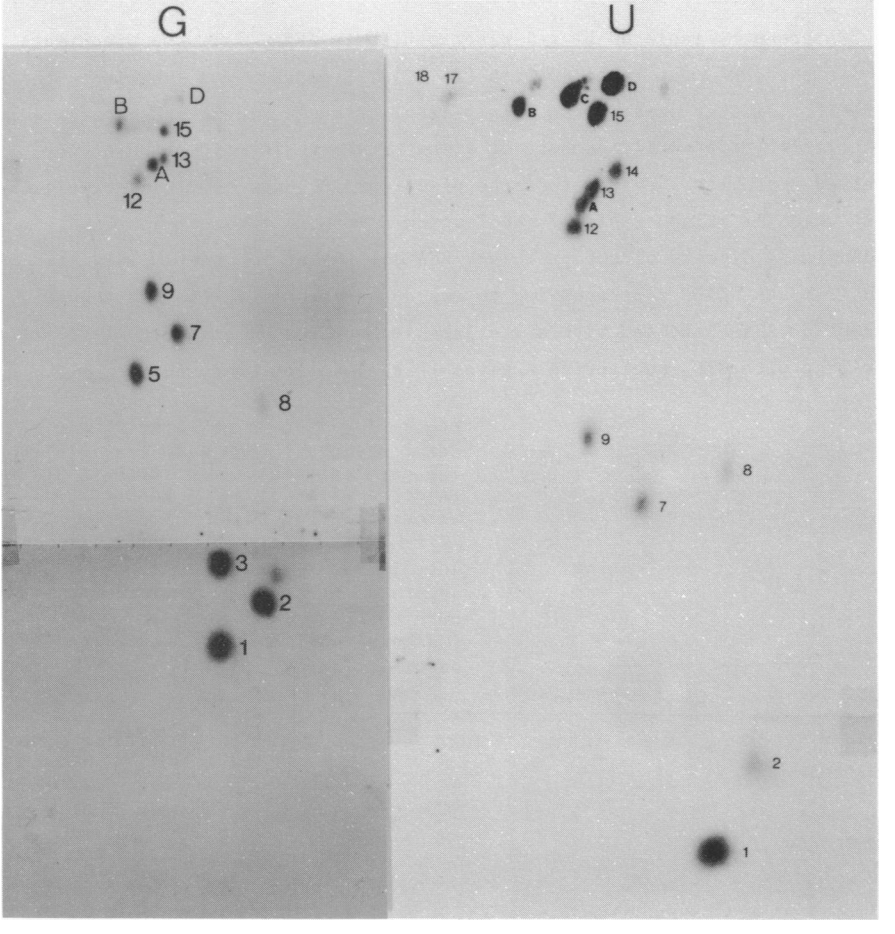

Figure 6. Two-dimensional electrophoretic separation of ribonuclease T_1 digests of Cet 7 precursor tRNA synthesised after injection of Cet 7 DNA and [α-^{32}P]UTP(U) or [α-^{32}P]GTP(G) into oocyte nuclei. Separation as in Fig. 5 and spots described in Table 1.

RNaseP and the products separated by polyacrylamide gel electrophoresis (Fig. 4). The precursor RNA was converted to a single product migrating between the precursor and mature tRNA. The ribonuclease T_1 digest of this gave pGp as expected from the tRNA 5' terminus generated by enzymic removal of a 5' leader sequence (data not shown). From the mobility of the RNaseP digestion product we conclude there is also a short additional sequence at the 3' terminus of the precursor.

At the 5' terminus of the unprocessed transcript a nucleoside 5'-triphosphate residue would be expected. To identify this, oocytes were injected with Cet 7 DNA together with either [β-^{32}P]ATP or [β-^{32}P]GTP and the two RNA products isolated by gel electrophoresis (Fig. 7). The precursor, but not the tRNA, was labelled with [β-^{32}P]GTP; neither was labelled with [β-^{32}P]ATP. Similar experiments with λ-labelled nucleoside triphosphates were inconclusive, apparently because of transfer and re-incorporation of the labelled phosphate. We conclude the precursor includes the transcriptional start which is at a G residue. Nucleotide A (Fig. 6) present in T_1 ribonuclease digests of the precursor RNA but absent in the tRNA digests was identified as UCAUG corresponding to residues -4 to +1 in the DNA sequence. Nucleotide A is labelled with all 4 labelled nucleoside triphosphates. After labelling with GTP, nucleotide A gives [^{32}P] labelled G and AU on

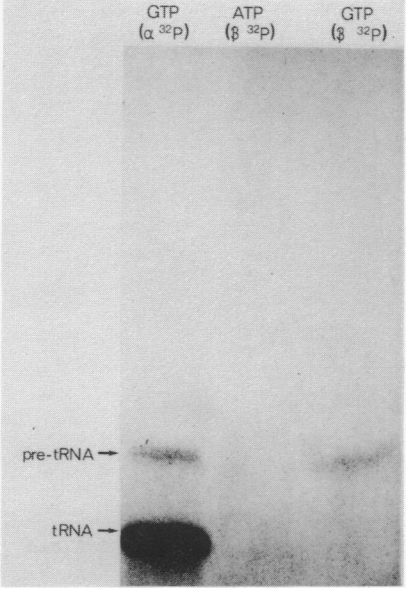

Figure 7. 5'-terminal labelling of the Cet 7 precursor tRNA with [β-^{32}P]GTP. The germinal vesicles of oocytes were injected with Cet 7 DNA together with either [α-^{32}P]GTP, [β-^{32}P]ATP or [β-^{32}P]GTP. After 5h. incubation the labelled RNAs were isolated and separated on a 12% polyacrylamide tris-borate-EDTA gel containing 7 M urea (Methods) and the bands visualised by autoradiography.

ribonuclease A hydrolysis and so has a sequence terminating in ..AUG(G) where G is the 3' neighbouring residue. The position of nucleoside A on the two-dimensional separation corresponds to that of UCAUG. The expected T_1 ribonuclease product pppGp was tentatively but not conclusively identified. From the [α-^{32}P]GTP labelling experiment and the absence of T_1 ribonuclease products corresponding to other leader sequences in the DNA we conclude the transcription start for the precursor RNA is at residue -5 in the DNA sequence (Fig. 1).

The 3' trailer sequences of the precursor tRNA were identified from nucleotides C and D (Fig. 6). Nucleotide D terminates in G and from RNaseA analysis contains the U, C, and A labelled nucleotides expected from the sequence A_{67} - G_{83}. It replaces the 3' terminus, nucleotide 16, in the tRNA digest. Nucleotide C is labelled only with U and C (and not with G) and so corresponds to the 3' terminus of the precursor RNA. Nearest neighbour analyses and positions on the fingerprint are consistant with the sequence UUCCUU expected from the fragment transcribed from residues T_{84} - T_{89} in the DNA sequence. Nucleotide 14 in Fig. 6 is also only labelled with U and C. From its position this appears to correspond to a shorter 3' terminal fragment differing from D by one U residue.

In the precursor RNA the modified nucleoside residues N^4acetylcytidine and dihydrouridine were absent. In ribonuclease T_1 digests nucleotides 6 (Cac^4CG) and 11 containing D residue 19 are absent but the corresponding products without modified bases CCG and UCUAAG (nucleotide 13 in Fig. 6) are found. Residue A_{35} is almost entirely unmodified. While there are traces of nucleotides 17 and 18 in the ribonuclease T_1 digests this residue is mainly present in nucleotide B (Fig. 6) identified by analysis as UUUAAG as expected from the tRNA sequence with an unmodified anticodon.

Transcription of the $tRNA^{Leu}$ precursor resembles that of other eukaryotic tRNA precursors (1) in being initiated a few nucleotides upstream of the gene sequence and apparently terminated at a sequence of T residues closely following the 3' end of the gene. (We cannot eliminate the possibility that the precursor seen in the oocyte is derived from a longer transcript partially processed at the 3' end). Although the precursor is a substrate for E.coli RNaseP, the relatively slow processing in Xenopus oocytes compared with other tRNA precursors suggests that a sequence preference may affect the rate of processing. The near absence of certain modified bases in the $tRNA^{Leu}$ precursor reinforces the view that processing and modification are ordered processes (8,9).

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