Volume 8 Number 4 1980 Nucleic Acids Research

The nucleotide sequence of a major species of leucine tRNA from bovine liver

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Received 20 November 1979

ABSTRACT

Through the use of a variety of post-labeling techniques, the nucleotide sequence of a major species of leucine tRNA from bovine liver was determined to be pG-G-U-A-G-C-G-U-G-m^2G-C-ac^4C-G-A-G-C-G-G-D-C-Ψ-A-A-G-G-C-m^2_2G-C-U-G-G-A-Ψm-U-I-A-G-m^1G-C-Ψ-C-C-A-G-U-C-Ψ-C-Ψ-U-C-G-G-G-G-G-m^5C-G-U-G-G-G-T-Ψ-C-G-m^1A-A-U-C-C-C-A-C-C-G-C-U-G-C-C-A-C-C-A_{OH}. A comparison of known sequences of leucine tRNAs shows a consistent set of features which clearly distinguish prokaryotic and eukaryotic leucine tRNAs from each other.

INTRODUCTION

All tRNAs, with the exception of tyrosine, leucine and serine, contain small minor loops in both prokaryotes and eukaryotes (1). Tyrosine tRNA appears to contain a large minor loop in prokaryotes and a small minor loop in eukaryotes. Leucine and serine tRNAs are unusual in that they contain large minor loops in both prokaryotes and eukaryotes. In part because of this unusual property we have decided to study the mammalian leucine tRNAs and their corresponding tRNA genes. As an initial step in this study we have isolated and determined the nucleotide sequence of a major species of leucine tRNA of bovine liver.

MATERIALS AND METHODS

Materials. Ribonuclease PhyM (U+A-specific) from Physarum polycephalum and the pyrimidine-specific ribonuclease from B. cereus were gifts of Drs. Helen Donis-Keller and John Dunn, respectively. Radioisotopes were purchased either from New England Nuclear or from Amersham. Other reagents, enzymes, and materials have been listed previously (2-8).

Partial formamide hydrolysis and ^{32}P-labeling reactions. Intact bovine liver $tRNA^{Leu}_{IAG}$ was partially hydrolyzed with formamide (4,9). The [5'-^{32}P]-labeling of lyophilized partial formamide or complete RNase T_2 digestions of bovine liver $tRNA^{Leu}_{IAG}$ was done essentially as previously described (2,3,8).

The precipitated [5'-^{32}P]-labeled formamide fragments were dissolved in 100µl of water and stored at -90°C until further use. The reaction mixture (40µl) for labeling the 3'-terminus of the tRNA (10,11) contained 40 pmoles tRNA, 350 units/ml T4 RNA ligase, 500 pmoles ATP, and 90 pmoles [5'-^{32}P]pCp, and was incubated at 4°C for 19 hr. The incubation mixture was then heated at 90°C for 2 min, quickly cooled, diluted with 0.5ml of 0.25M sodium acetate (pH 5.2) followed by 2µl of wheat germ 5S RNA (42 mg/ml), and precipitated and centrifuged. The precipitate was dissolved in 12µl of 50% urea, 10mM Tris-HCl (pH 7.5), 25mM NaCl, 4mM EDTA and 0.04% each of xylene cyanol FF (XC) and bromphenol blue (BPB), heated at 80°C for 2 min, and applied to a 20% polyacrylamide gel for purification.

Polyacrylamide gel electrophoresis. For separation of the [5'-^{32}P]-oligonucleotides generated by formamide hydrolysis, 5µl portions of the 100µl stock digest mentioned above were lyophilized, dissolved in 4µl of gel loading buffer (12), heated to 80°C for 90 sec, rapidly cooled, and applied to thin 12%, 15%, or 20% polyacrylamide gels (40x20x0.05 cm) (7,12,13). The bands from the gel ladder were excised after autoradiography.

The major portion of the formamide digest (80µl of the 100µl stock) was lyophilized, dissolved in 15µl of a solution containing 50% urea, 10mM Tris-HCl (pH 7.5), 25mM NaCl, 4mM EDTA, and 0.04% each of XC and BPB, heated at 80°C for 2 min, rapidly cooled, and applied to a 90x20x0.17 cm polyacrylamide gel (9,12). The excised gel slices from this 90 cm gel were extracted essentially as described (8), except that the bands were crushed with siliconized glass rods and vortexed rather than homogenized. Further purification of the partial formamide fragments used for "mobility-shift" analyses or analyses on RNA sequencing gels was achieved on 23.5% polyacrylamide gels (4) and elution was done(8). The thin 20% polyacrylamide sequencing gels were prepared and run as described (12,13). The purification of the [3'-^{32}P]tRNA was done similarly on a 20% polyacrylamide gel (40x20x0.17 cm).

Analyses of the 5'-termini of the formamide fragments. The excised gel slices containing the [5'-^{32}P] labeled fragments were eluted essentially as previously described (9). Following ethanol precipitation the fragments were dissolved in 10µl of a solution containing 2 units/ml of RNase T_2 and 0.05 mg/ml each of RNases T_1 and A in 0.1M ammonium acetate (pH 4.5) (6) and digested for 4 hrs or overnight at 37°C. Aliquots of 1-2µl were spotted and developed on prewashed PEI cellulose sheets (7). The fragments extracted from the gels were also digested with nuclease P_1, liberating radioactive nucleoside-5'-monophosphates from the 5'-end of each fragment. Aliquots of the

nuclease P_1 digest were spotted on 10x10 cm cellulose plates, together with 5µg each of pA, pG, pC and pU standards (Schwarz-Mann). The plates were developed (without wicks), using the solvent A and solvent C system of Silberklang et al. (2).

"Mobility-shift" analysis. For "mobility-shift" analysis, partial alkaline hydrolysates of ^{32}P-labeled RNA were electrophoresed on cellulose acetate in 5% acetic acid, 5mM EDTA and 7M urea (pH 3.5) at 16°C for 45-50 min at 4800 V (14). For the second dimension, homochromatography was carried out on DEAE-cellulose plates at pH 4.7 and 65°C using the 3% RNA hydrolysates ("homomixes") described by Silberklang et al. (2), usually with 20-50mM KOH-strength homomixture (pH 4.7). The limited alkaline hydrolyses were carried out as described (12).

Sequence gels. The tRNA used for sequence analysis on 20% polyacrylamide gels was partially digested at pH 5.0 with ribonucleases T_1, PhyM and U_2, essentially as described by Donis-Keller et al. (12). Partial digestions at pH 3.5 with ribonuclease U_2 were done as described by Krupp and Gross (15). Partial digestions using a pyrimidine-specific ribonuclease from B. cereus were performed as described by Silberklang et al. (2).

RESULTS

Purification of tRNA. Crude tRNA was isolated from bovine liver by the procedure of Roe (16). Pure $tRNA^{Leu}_{IAG}$ was isolated by three successive chromatographic procedures as shown in Figs. 1A-C. Fig. 1D is a double-label chromatographic run which shows that the $tRNA^{Leu}_{IAG}$ isolated above corresponds to a predominant species, perhaps the major species of leucine tRNA in bovine liver, and it elutes considerably later than the other leucine tRNAs in this chromatographic procedure.

Modified base composition of the tRNA. The nucleoside trialcohol procedure of Randerath and Randerath (5) showed that $tRNA^{Leu}_{IAG}$ contains the following modified nucleosides: Ψ, I, m^5C, m^1A, m^2G, m^2_2G, ac^4C, D, m^1G, and T. In addition, the [5'-^{32}P]nucleoside-monophosphate procedure (2) showed that pΨm, which comigrates with pT in the solvent system of Silberklang et al. (2), is also present, as determined by the 5'-end analysis of band 33 from the gel in Fig. 2A.

Sequence analysis. The fractionation of the [5'-^{32}P]oligonucleotides generated by a 10 min formamide hydrolysis is shown in Fig. 2A. The bands are numbered in accordance with the sequence shown in Fig. 5. The shorter [5'-^{32}P]oligonucleotides from the formamide digest were fractionated on a

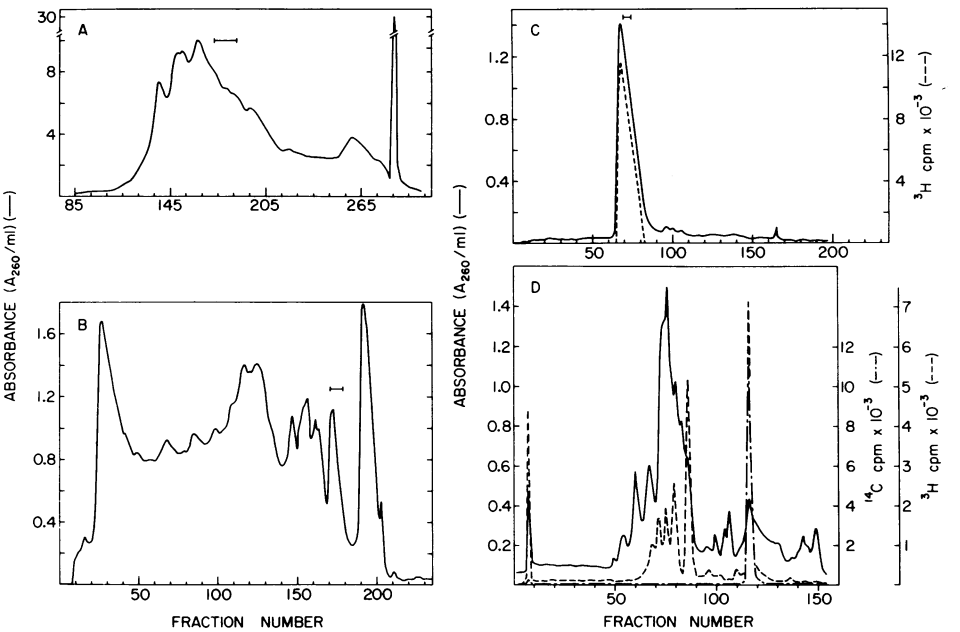

Fig. 1. Chromatographic purification and characterization of leucine tRNA from bovine liver. A) Crude bovine liver tRNA (18,700 A_{260} units) was dissolved in 80 ml of buffer A (0.01M Tris pH 7.5, 5.0mM $MgCl_2$) containing 0.38M NaCl and applied to a 600 ml BD cellulose column. The column was eluted with three consecutive linear gradients, all in buffer A, as follows: gradient 1, 1200 ml each of 0.38M-0.60M NaCl; gradient 2 (started at tube 118), 1500 ml each of 0.6M-0.9M NaCl; gradient 3 (started at tube 221), 500 ml each of 0.8M-1.5M NaCl. At tube 245 the column was eluted with a high salt wash of 2M NaCl in buffer A containing 15% ethanol. B) 704 A_{260} units of partially purified leucine tRNA (tubes 173-187 of Fig. 1A) were dissolved in 10 ml of buffer B (0.01M NaOAc pH 4.5, 5.0mM $MgCl_2$) containing 0.37M NaCl and applied to a 70 ml RPC-5 column. The column was eluted with three consecutive concave gradients, all in buffer B, as follows: gradient 1, 0.37M NaCl (400 ml)-0.7M NaCl (200 ml); gradient 2 (started at tube 159), 0.7M NaCl (100 ml)-1.0M NaCl (50 ml); gradient 3 (started at tube 199), 1.0M NaCl (100 ml)-1.5M NaCl (50 ml). C) 24 A_{260} units of purified leucine tRNA (tubes 171-179 of Fig. 1B) were dissolved in 0.5 ml of buffer A containing 0.37M NaCl and applied to a 10 ml RPC-5 column. The column was eluted with a concave gradient of 0.45M NaCl (150 ml)-1.0M NaCl (75 ml) both in buffer A. The dashed line indicates the leucine acceptor activity. The leucine peak ($tRNA^{Leu}_{IAG}$) gave a single sharp band upon electrophoresis in a denaturing 20% gel (12) (data not shown), and tubes 70-75 were used for sequence analysis. D) Total bovine liver tRNA and the $tRNA^{Leu}_{IAG}$ were aminoacylated by wheat germ synthetase with [^{3}H]-leucine (sp.act. 130 Ci/mmole) and with [^{14}C]-leucine (sp.act. 325 mCi/mmole), respectively. The reactions were done as described previously, except that the total volume was 20µl (17). [^{14}C]-leucine tRNA (70,000 cpm), [^{3}H]-leucine tRNA (40,000 cpm) and carrier *E. coli* tRNA (1 mg) were applied to a 10 ml RPC-5 column. The column was eluted with a concave gradient of 0.4M NaCl (100 ml)-1.2M NaCl (50 ml) both containing 0.01M NaOAc pH 4.5, 0.01M $MgCl_2$ and 1.0mM $Na_2S_2O_3$.

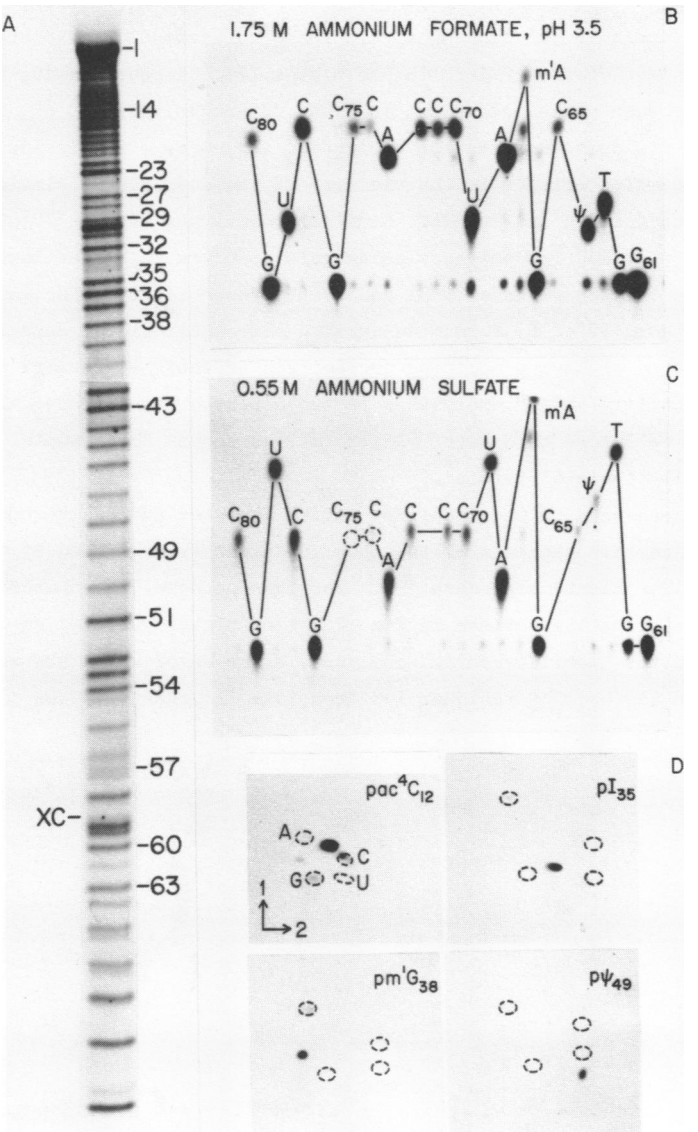

Fig. 2. A) Fractionation of [5'-^{32}P]oligonucleotides derived by partial formamide hydrolysis of $tRNA_{TAG}^{Leu}$ on a 90 cm, 20% polyacrylamide gel. B and C) One dimensional separation of [5'-^{32}P] 3',5' nucleoside diphosphates on PEI cellulose plates with ammonium formate (Fig. 2B) or ammonium sulfate (Fig. 2C) solvent systems. D) Two-dimensional chromatography on cellulose plates of [5'-^{32}P]nucleoside monophosphates generated by nuclease P_1 digestion of oligonucleotides fractionated in Fig. 2A. Dashed circles indicate non-radioactive markers. Numbers refer to locations in the sequence.

40 cm, 20% polyacrylamide gel (not shown), extracted, and digested to obtain [5'-^{32}P]nucleoside-3',5'-diphosphates. These nucleoside diphosphates were spotted and developed on PEI-cellulose plates (7). Representative autoradiograms of two such plates are shown in Figs. 2B and 2C. The 40 cm, 20% gel encompassed residues 48-80 in the nucleotide sequence, with missing bands and/or compression effects in the vicinity of residues 55-60, similar to the corresponding portion in Fig. 2A. Similarly, analyses of [5'-^{32}P]oligonucleotides from 12 and 15% polyacrylamide gels aided in the determination of the sequence between residues 1-31 and 29-69, respectively. Nuclease P_1 digestions of some [5'-^{32}P] formamide-derived oligonucleotides, especially those suspected of having modified nucleotides at their 5'-termini and those used for "mobility shift" analysis were subjected to two-dimensional chromatography on cellulose thin-layer plates (2), as shown for example, in Fig. 2D, to identify their 5'-termini.

It was necessary to use "mobility shift" analyses and RNA sequence gels to sequence certain segments of the tRNA inadequately sequenced by the Stanley and Vassilenko procedure (9). For this purpose, the numbered bands from the fractionation pattern in Fig. 2A were further purified on 23.5% gels (4) and used for "mobility-shift" analyses, examples of which are shown in Fig. 3. The sequence of residues 1-8 from [5'-^{32}P] $tRNA^{Leu}_{IAG}$ (band 1 of Fig.

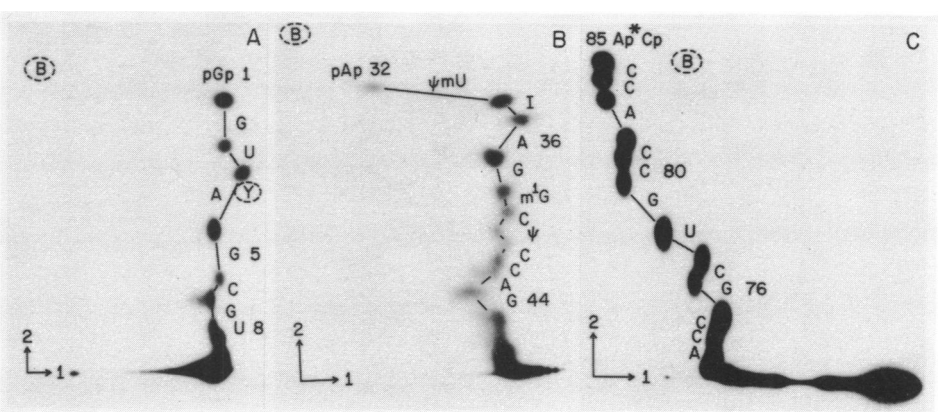

Fig. 3. "Mobility-shift" analyses by two-dimensional electrophoresis-homochromatography. The oligonucleotides for the analyses of (A) and (B) were [5'-^{32}P]oligonucleotides from the gel shown in Fig. 2A. Intact [3'-^{32}P] $tRNA^{Leu}_{IAG}$ was digested for the analysis shown in (C). The "homomixes" used for the second dimensions were 50mM KOH for (A) and (C) and 20mM KOH for (B). The blue (xylene cyanol FF) and yellow (methyl orange) dye markers are indicated by B and Y, respectively. The location of the [^{32}P]-label in (C) is indicated by an asterisk (*).

2A) and of residues 73-85 (from the [3'-^{32}P]tRNA$^{Leu}_{IAG}$) were derived from the profiles depicted in Fig. 3A and 3C, respectively. Additional confirmation of the sequence was obtained from RNA sequence gels (12,13,19) of [5'-^{32}P] and [3'-^{32}P]tRNA$^{Leu}_{IAG}$. The absence of the 5'-phosphate in a portion of tRNA$^{Leu}_{IAG}$ eliminated the necessity for a separate 5'-end labeling experiment. The gel of the long electrophoretic run of the 5'-labeled digests (Fig. 4A) confirms the sequences from residues 17-37 and 42-54. As expected, there is a gap at position 33 due to the inability to cleave the ΨmU bond. At residue 19 (D) band-doubling starts to occur in the alkaline hydrolysis lane. It is possible that these double bands arise as a result of partial breakdown of dihydrouridine during the alkaline hydrolysis (20). A short electrophoretic run of the [5'-^{32}P] tRNA from band 1 in Fig. 2A confirmed residues 4-37 of the nucleotide sequence (data not shown). A sequence gel of [3'-^{32}P] tRNA$^{Leu}_{IAG}$ is seen in Fig. 4B. From this analysis, the sequence of residues 42-56 and 62-81 in the nucleotide sequence are confirmed. There is considerable compression effect in the region of residues 57-61 (corresponding to the 5'-portion of the TΨC arm). Residue 67 (m^1A) is only cleaved by alkali and certain missing bands occur in the enzymatic lanes at residues 70-71 and several other locations in the profile, resulting from relatively stable regions of secondary structure. The relative location of all G's and A's except the modified nucleosides (e.g., m^2G_{10}, $m^2_2G_{27}$, m^1G_{38} and m^1A_{67}) can be discerned from these gels. Cleavage with the ribonuclease from B. cereus can apparently occur at T_{63}, Ψ_{64}, Ψ_{49}, Ψ_{47} (at least in the [3'-^{32}P] gel) and at Ψ_{21} in the D arm. Cleavage with RNase PhyM can occur at I_{35} and can occur only to a small extent at Ψ residues and at D_{19} and not at all at m^1A_{67}.

DISCUSSION

The nucleotide sequence of bovine liver tRNA$^{Leu}_{IAG}$ is shown in Fig. 5. This tRNA contains 85 nucleotides including 15 modified residues. The tRNA structure exhibits all the invariant and semi-invariant nucleotides in their respective loci in comparison to other tRNAs (21). This sequence was derived using strictly in vitro labeling methods utilizing the recently developed and complementary methods of Silberklang et al. (2,3), Stanley and Vassilenko (9), Gupta and Randerath (7) and Donis-Keller et al. (12).

Comparison of leucine tRNAs shows a set of characteristics which unambiguously distinguish eukaryotic from prokaryotic leucine tRNAs. These characteristics, based upon the sequences of three prokaryotic and three eukaryotic

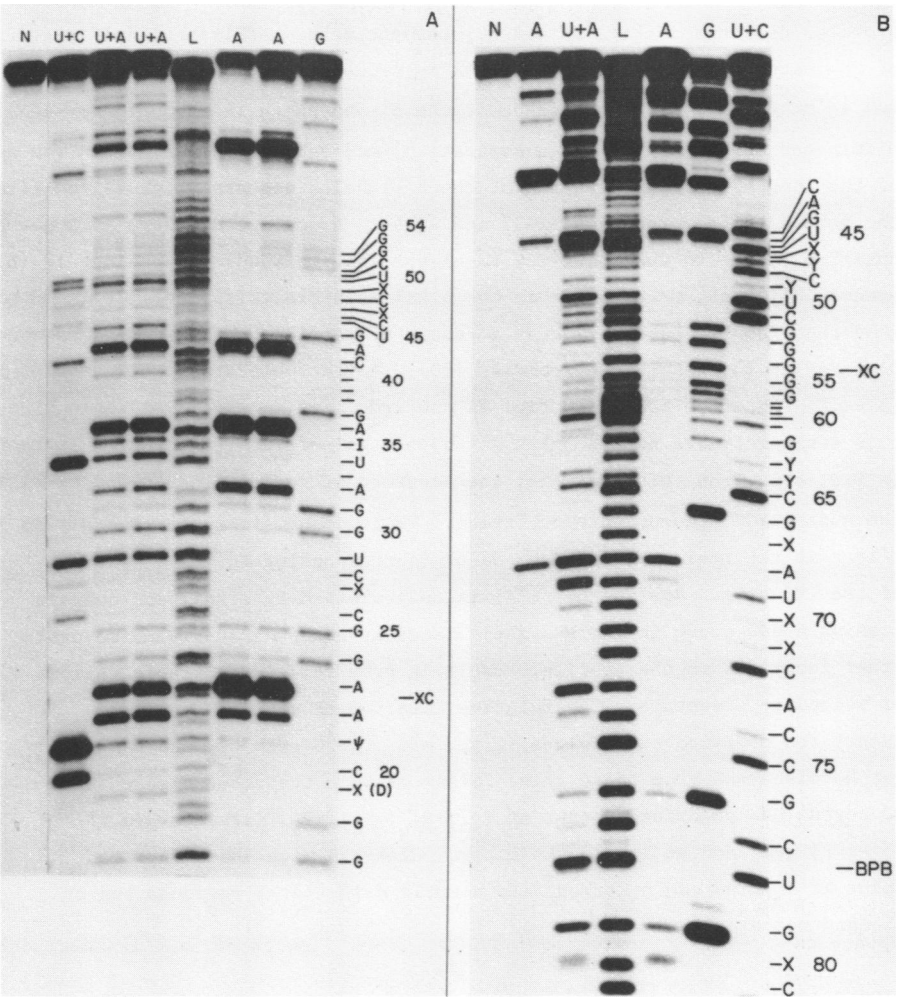

Fig. 4. Autoradiograms of RNA sequencing gels of oligonucleotides from partial digestions of (A) [5'-^{32}P]-labeled and (B) [3'-^{32}P]-labeled $tRNA^{Leu}_{IAG}$. The base-specific cleavages were obtained using ribonuclease T_1 (G), U_2 (A) and PhyM (U+A) and the pyrimidine-specific ribonuclease from B. cereus (U+C). The ribonuclease U_2 digestions were done at pH 3.5 in (A) and at pH 5.0 in (B). Limited alkaline hydrolyses were used to obtain the "ladders" (L). The [5'-^{32}P] $tRNA^{Leu}_{IAG}$ control was untreated (N). The products of these digestions were fractionated by electrophoresis on 20% polyacrylamide gels (40x33x0.05cm).

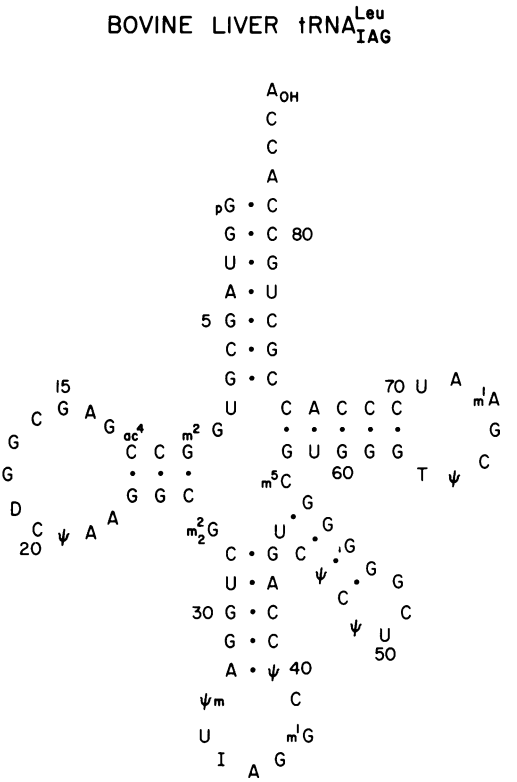

Fig. 5. The nucleotide sequence of tRNA$^{Leu}_{IAG}$ from bovine liver.

leucine tRNAs (listed in ref. 1), as well as the recently determined S. pombe Sup 8-e tRNA^{Leu} (18) and the bovine liver tRNA$^{Leu}_{IAG}$ reported in this paper are: A) Eukaryotic leucine tRNAs contain 85 nucleotides as compared to 87 nucleotides found in prokaryotic leucine tRNAs. Apparently, the observation that eukaryotic tRNAs are smaller in size compared to their prokaryotic counterparts is generally valid for tRNAs in which there is a size difference between eukaryotes and prokaryotes. This generalization applies not only to leucine tRNAs, but also to arginine, aspartic acid, glutamic acid, methionine elongator, methionine initiator, serine, tryptophan, and tyrosine tRNAs (see tRNA compilation-ref. 1). B) The position of the universal GG sequence (or GmG in some leucine tRNAs) in the D loop is different in prokaryotic and eukaryotic leucine tRNAs. In prokaryotic leucine tRNAs the GmG is separated from both A_{14} (numbered according to ref. 1) and A_{22} by three nucleotides,

while in eukaryotic leucine tRNAs the GmG (or GG) is separated from A_{14} by only two nucleotides and from A_{22} by four nucleotides. C) Prokaryotic leucine tRNAs have a G at position 6 while eukaryotic leucine tRNAs have a pyrimidine at that site. D) Prokaryotic leucine tRNAs have an A at position 20-1 (numbered according to ref. 1) while eukaryotic leucine tRNAs do not have an A at that site. E) Prokaryotic leucine tRNAs have the following sites in common: G_{12}, A_{15}, C_{23}, U_{48}, G_{59}, and C_{67} while these sites in eukaryotic leucine tRNAs are ac^4C_{12}, G_{15}, G_{23}, m^5C_{48}, A_{59} and G_{67} respectively. F) Finally, eukaryotic but not prokaryotic leucine tRNAs have m_2^2G at position 26. In total, there are 12 different features which clearly distinguish prokaryotic and eukaryotic leucine tRNAs from one another.

A comparison of the sequences of the double-stranded stem regions of all nine leucine tRNAs of known structure (1,18, and bovine liver $tRNA^{Leu}_{IAG}$) shows that, other than invariant nucleotides, only one base pair has been conserved. This base pair is G_{60}-C_{72}, located in the TΨC stem, and it is found in all leucine tRNAs sequenced to date. Since this specific base pair has remained unaltered in nine different leucine tRNAs from a wide variety of sources, it appears likely that this base pair plays a specific role in the functioning of leucine tRNA.

ACKNOWLEDGEMENTS

We wish to thank H. Donis-Keller and J. Dunn for generous gifts of enzymes used in this study, A. Diamond for the isolation of bovine liver tRNA and H. Mae Sprouse for excellent technical assistance. This investigation was supported in part by grants from the National Institute of Health (GM-25254) and the American Cancer Society (NP 146).

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