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Isolation and Sequence Analysis of Two Major Leucine Transfer Ribonucleic Acids (Anticodon Mm-A-A) from a Rat Tumor, Morris Hepatoma 5123D†

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ABSTRACT: The nucleotide sequences of two major $tRNA^{Leu}$ species (anticodon Mm-A-A) isolated from Morris hepatoma 5123D were determined by a combination of a newly developed thin-layer readout sequencing method [Gupta, R. C., & Randerath, K. (1979) *Nucleic Acids Res. 6*, 3443–3458] and additional ^3H- and ^{32}P-labeled derivative methods entailing chromatographic fingerprinting and base-specific enzymatic cleavages. The nucleotide sequence of the two hepatoma $tRNA_{Mm\text{-}A\text{-}A}^{Leu}$ species, one of which has U and the other of which has A in position 50 at the tip of the long extra arm, is pG-U-C-A-G-m^2G-A-U-G-(m^2)G-C-(ac^4)C-G-A-G-U-G-G-D-C-ψ-A-A-G-G-C-m_2^2G-C-C-A-G-A-C-U-Mm-A-A-m^1G*-ψ-ψ-C-U-G-G-L-(ψ)U-C-C-G-U or A-A-U-G-G-A-G-m^5C-G-U-G-G-G-T-ψ-C-G-m^1A-A-U-C-C-C-A-C-U-U-C-U-G-A-C-A-C-C-A_{OH}. These are the first leucine tRNA sequences from higher eukaryotes that have been determined. Noteworthy features of the mammalian leucine tRNAs are the presence of ψ in the β region of the D loop and the occurrence of three unknown hypermodified nucleosides (Mm, m^1G*, and L) in positions 35, 38, and 45, respectively. m^1G* was converted to m^1G by treatment with alkali. Sequencing gels indicated that the parent base of the 2′-O-methylated nucleoside Mm may be a pyrimidine, probably a C derivative, as indicated by the chromatographic behavior of nucleotides containing Mm. The presence of a pyrimidine in the wobble position would be consistent with the anticodon sequence Mm-A-A and the leucine codons U-U-G and U-U-A. The occurrence of a hypermodified nucleoside, L, in the first position of the long extra arm appears unusual; thus far the only modified nucleoside found in this position is Um in eukaryotic serine tRNAs. Since all tRNAs with a long extra arm sequenced to date have a pyrimidine in this position, L is likely to be a pyrimidine, probably a U derivative, as inferred from chromatographic data.

The number of mammalian tRNAs sequenced to date is rather small. Only 11 of the ~110 tRNAs whose primary structures have been elucidated are of mammalian origin [see Gauss et al. (1979) for a compilation of sequenced tRNAs]. Although tumor tRNAs have been known for a number of years to exhibit alterations of their column chromatographic elution profiles [e.g., Taylor et al. (1967), Baliga et al. (1969), Gallo & Pestka (1970), and Volkers & Taylor (1971)] as well as their base compositions [e.g., Randerath & Randerath (1973), Randerath, E., et al. (1974), and Chia et al. (1976)] when compared with their normal counterparts, little information on the structure of tRNAs of neoplastic origin is available. So far, only four such tRNAs have been sequenced, i.e., $tRNA_f^{Met}$ (Piper & Clark, 1974), $tRNA_4^{Met}$ (Piper, 1975a), and $tRNA_1^{Val}$ (Piper, 1975b) of mouse myeloma cells and $tRNA^{Asn}$ (Roe et al., 1979) of the Walker 256 carcinosarcoma. The latter tRNA is the only tumor tRNA sequenced to date that was compared directly with its counterpart in normal host tissue, albeit not the tissue of origin of the tumor. This comparison showed a difference in the wobble position of the anticodon, with Q being present in liver and G in tumor $tRNA^{Asn}$ (Roe et al., 1979). In an attempt to elucidate the structural differences between hepatoma and liver tRNAs, we have as a first step recently purified several tRNAs from Morris hepatoma 5123D (Morris & Wagner, 1968) and report here on the sequences of two major leucine tRNAs from this tumor. These are the first leucine tRNAs from a higher eukaryote that have been sequenced.

Experimental Procedures

Materials. Hepatoma 5123D was originally chemically induced in the laboratory of Dr. H. P. Morris (Morris & Wagner, 1968) and serially transplanted intramuscularly in female Buffalo rats. The tumor used in the present work was from generation 123. Tissue was excised quickly from etherized rats immediately after bleeding and stored at −70 °C. BD-cellulose was from Schwarz/Mann, acrylamide and methylenebis(acrylamide) were from Bio-Rad, and poly(A,C,-G,U) was from Miles Laboratories. The sources of materials used for sequence analysis have been indicated previously (Gupta & Randerath, 1979; Randerath et al., 1979, 1980; Gupta et al., 1979).

Crude tRNA. Crude tRNA was isolated from 800 g of tumor by phenol extraction at pH 4.5 (0.14 M NaOAc buffer), followed by adsorption to DEAE-cellulose, essentially as described by Roe (1975). Reextraction of the first phenol phase with NaOAc buffer increased the recovery of tRNA almost twofold. About 650 μg of crude tRNA was obtained per g of tumor.

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Aminoacyl-tRNA Synthetase and Acceptor Assay. Crude aminoacyl-tRNA synthetase was isolated from fresh rat liver by method A of Roe (1975). Aminoacylation was assayed by a scaled-down version of the procedure described by Roe (1975). The standard assay mixture contained the following in a total volume of 200 μL: 100 mM Tris, pH 7.6, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, 2.5 mM ATP, 0.01 mM ^{14}C-labeled amino acid (50 μCi/μmol), 0.05–1 A_{260} unit of partially purified RNA or 0.0015–0.03 A_{260} unit of purified $tRNA^{Leu}$, and a saturating amount of synthetase. Purified $tRNA^{Leu}$ was assayed in the presence of poly(A,C,G,U) as carrier (0.5 A_{260} unit/assay). After incubation for 40 min at 37 °C, the RNA was precipitated with 500 μL of 10% Cl_3-AcOH, filtered, washed with six 2-mL portions of 2% Cl_3-AcOH, and counted.

Purification of Leucine tRNAs. Crude hepatoma 5123D tRNA (12 700 A_{260} units) was fractionated on a BD–cellulose column (2.5 × 93 cm) by a modification of the procedure described by Rogg et al. (1975) for the purification of serine tRNAs from rat liver. The RNA was eluted from the column with a linear gradient (12 L) of NaCl (0.3–1.2 M) in the presence of 10 mM NaOAc, pH 4.5, 10 mM $MgCl_2$, and 1 mM mercaptoethanol, followed by a linear gradient (3 L) of ethanol (0–20%) in NaCl (1.2 M) at a flow rate of 3.5 mL/min. Fractions of 20 mL were collected. Pooled fractions 301–470 (~2500 A_{260} units), which were found to be enriched in $tRNA^{Leu}$ as judged by polyacrylamide gel electrophoresis (see Results), served as the source for the subsequent isolation of leucine tRNAs by polyacrylamide gel electrophoresis.

Polyacrylamide gels were prepared as described by Peacock & Dingman (1967) and Loening (1967). About 100 A_{260} units of partially purified tRNA was applied to 14 slots of a 15% polyacrylamide slab gel [50 × 30 × 0.2 cm; acrylamide–methylenebis(acrylamide), 30:1] and electrophoresed at 400–500 V until a coelectrophoresed xylene cyanol marker reached the bottom of the gel. The gel and electrophoresis buffer was 90 mM Tris, 90 mM boric acid, and 4 mM EDTA, pH 8.3. For staining, the gel was soaked successively in 1 N acetic acid (7 min), methylene blue solution (0.5 g of methylene blue in 1 L of water; 20 min), and water (2–4 h). Bands containing leucine tRNAs (see Results) were cut from replicate lanes and implanted for reelectrophoresis in a 15% polyacrylamide slab gel containing 8 M urea (pH 8.3) as follows. The excised gel pieces were inserted at 5–8 cm from the bottom edge of the mold. After the gel solution had been poured into the mold to ~5 cm above the gel pieces, trapped air bubbles were allowed to escape by tilting the assembly. The rest of the gel solution was then poured into the mold. The anode compartment was placed at the top of the assembly. The electrophoresis buffer was as described above. The voltage was adjusted to 600–800 V so that the gel remained at 45–50 °C throughout the run. The gel was stained as described above, and the leucine tRNA bands were excised and pooled for extraction of the RNA. Extraction was performed as described by Chia et al. (1973), with the following modifications. The volume of the extraction buffer was reduced to about one-half, and the extracts were concentrated by lyophilization rather than by evaporation at 35 °C. The RNA was precipitated from 1–2 mL of aqueous solution by adding 100 μL of 20% potassium acetate, pH 5.0, and 3.3 mL of acetonitrile–ethanol (4:1) per mL of solution and keeping the sample at −18 °C for several hours. After the precipitate had been washed with ice-cold ethanol and dried, it was taken up in 200 μL of water. Residual insoluble material was removed by centrifugation at 13 000 rpm for 15 min. The concentration of the RNA in the supernatant solution was determined spectrophotometrically. The recovery was ~75 μg of leucine tRNA per gel.

The purity of the RNA preparation was assessed by base analysis and by determining the amino acid acceptance. Base analysis was done according to scaled-down version 1 (Randerath et al., 1980) of the tritium derivative method for base analysis of RNA (Randerath & Randerath, 1971, 1973; Randerath et al., 1972). For the aminoacylation assay, see above.

Sequence Analysis of Leucine tRNAs by the Thin-Layer Readout Procedure. This was done as described by Gupta & Randerath (1979) with minor modifications (Gupta et al., 1980; Randerath et al., 1980). The procedure involved the brief hydrolysis of the RNA (5 μg) in water at 100 °C, 5′-terminal ^{32}P labeling of the fragments, separation of the labeled fragments on polyacrylamide slab gels according to size, contact transfer to a PEI–cellulose thin layer, release of the radioactive 5′ termini by RNAse T_2 digestion of the fragments in situ, and their identification by thin-layer chromatography. The shorter fragments (chain lengths of up to ~20 nucleotides) were separated on 20% gels and the longer fragments on 12% gels.

Sequence Analysis by the Gel Readout Procedure. This was done as described by Gupta et al. (1979, 1980) and Randerath et al. (1980). The procedure involved partial digestions of 5′-terminally ^{32}P-labeled RNA with alkali and RNases T_1, U_2, A, and Phy_1, followed by separation of the fragments by size on a sequencing gel and readout of the sequence from the cleavage patterns displayed by autoradiography.

Analysis of Oligonucleotides in Complete RNase T_1 and A Digests. The procedures (Gupta et al., 1979, 1980; Randerath et al., 1980) entailed the complete digestion of the RNA (10 μg) with RNase T_1 or A plus alkaline phosphatase, 3′-terminal ^3H labeling of the fragments, fingerprinting of the labeled oligonucleotide 3′-dialcohols on PEI–cellulose, determination of molar ratios by direct counting of the labeled compounds, and base as well as 3′- and 5′-terminal analysis of the labeled oligonucleotide derivatives after their isolation from the fingerprint.

Analysis of [^{32}P]pL-U-C-C-G′ and [^{32}P]pL-ψ-C-C-G′. To explore some of the properties of L, we first extracted these oligonucleotides from the RNase T_1 fingerprint with pyridinium formate (Gupta et al., 1976a) and then digested them with nuclease P_1 (Fujimoto et al., 1974) or RNase T_2, followed by digestion with nuclease P_1. In some experiments, the oligonucleotides were preheated in water (10 μL) at 100 °C for 5 min before nuclease P_1 digestion. The oligonucleotides (200–500 cpm) were digested in a volume of 10 μL with nuclease P_1 (0.1 μg/μL) in 20 mM NaOAc, pH 5.0, and 0.1 mM $ZnCl_2$ at 50 °C for 2.5 h. Digestion of the oligonucleotides with RNase T_2 (0.1 unit/μL) was conducted in 10 μL of 20 mM NaOAc, pH 4.5, at 38 °C for 2 h. The samples were then dried in a stream of air. Subsequent nuclease P_1 digestion was done as described above. After addition of a mixture of nonradioactive major nucleoside 5′-monophosphates (~10 nmol of each) as markers, the digests were mapped on PEI–cellulose as described by Gupta et al. (1976b), except that the development in the first dimension was with methanol to the origin, then with 0.4 N acetic acid to 5 cm above the origin, and finally with 1 N formic acid to 2 cm on a Whatman 1 wick attached to the top of the sheet.

Chemical and Enzymatic Studies on the Hypermodified Nucleotides. Spots of pm^{1}G*p, pLp, and pMm-Ap were cut from six replicate ammonium formate chromatograms (Gupta

& Randerath, 1979) and soaked in methanol for 7 min. After application of a mixture of the four major nucleoside 3′,5′-diphosphates (5–10 nmol of each) to the spots to serve as carriers, the compounds were eluted with 2 M LiCl and desalted (Randerath et al., 1979, 1980). Aliquots (15 μL, 200–300 cpm) of the pooled eluates of each compound were treated with 0.2 N HCl at 25 °C for 17 h or with 10% piperidine at 95 °C for 2 h. Before chromatography, HCl or piperidine was removed by evaporation in a stream of air, the residues were dissolved in water, and the drying operating was repeated. Aliquots as described above were also treated with periodate and borohydride under conditions similar to those described for tritium base analysis of RNA (Randerath & Randerath, 1971, 1973; Randerath et al., 1972). Concentrations of $NaIO_4$ and KBH_4 were 4 and 20 mM, respectively, and incubation was at 25 °C for 2 h each. Excess borohydride was removed by treatment with acetic acid. After addition of a mixture of the four major nucleoside 3′,5′-diphosphates to the dried residues of the reaction mixtures, the samples were chromatographed in the ammonium formate and ammonium sulfate systems of the thin-layer readout procedure (Gupta & Randerath, 1979) alongside untreated aliquots of the original eluates. Aliquots of the eluted compounds (700–800 cpm) were also digested with nuclease P_1 and mapped in the presence of nonradioactive nucleoside 5′-monophosphates as described above for [^{32}P]pL-U-C-C-G′ and [^{32}P]pL-ψ-C-C-G′. Nuclease P_1 digests of ^{32}P-labeled pNp compounds released from position 38 (see Figure 1) were, in addition, chromatographed on PEI–cellulose in 0.5 M LiCl and in 1 M ammonium formate, pH 3.5.

Results

Purification of tRNA^{Leu}. tRNA^{Leu} was isolated from Morris hepatoma 5123D by a three-step procedure involving phenol extraction of crude tRNA at pH 4.5, BD–cellulose column chromatography, and polyacrylamide gel electrophoresis. The two major tRNA^{Leu} species, which are the subject of this paper, were found to comigrate with a major tRNA^{Ser} species (E. Randerath, A. S. Gopalakrishnan, R. J. Rhines, R. C. Gupta, and K. Randerath, unpublished results) on BD–cellulose. They were separated from tRNA^{Ser} and from other tRNAs by polyacrylamide gel electrophoresis at pH 8.3 in the absence of urea. As expected from the presence of long extra arms in leucine and serine tRNAs, these RNAs migrated more slowly on the gel than all other tRNAs present in the column fraction. The two leucine tRNAs traveled as a single band just ahead of tRNA^{Ser}. Further purification on a denaturing polyacrylamide gel gave one major and one minor band, with the minor band migrating slightly ahead of the major one. Only the major band was studied in the present experiments. Recoveries for the various purification steps were as follows. About 12 700 A_{260} units of crude tRNA and ~2500 A_{260} units of chromatographically purified tRNA were obtained from 800 g of tumor tissue. Polyacrylamide gel electrophoresis of 100 A_{260} units of partially purified tRNA first on a nondenaturing gel and then on a denaturing gel at pH 8.3 yielded 1.87 A_{260} units (~75 μg) of highly purified tRNA^{Leu}, which accepted ~1200 pmol of [^{14}C]leucine/A_{260} unit when charged by a crude preparation of rat liver synthetase and did not accept measurable amounts of [^{14}C]serine or [^{14}C]phenylalanine.

Base Composition of tRNA^{Leu}. Tritium base analysis (Randerath & Randerath, 1971, 1973; Randerath et al., 1972, 1980) showed the highly purified tRNA^{Leu} to contain the following modified nucleosides (in moles per mole of RNA): ψ, 3.8; m^2G, 1.6; m^1G, 0.9; m_2^2G, 0.9; m^1A, 1.0; m^5C, 1.1; D, 1.1; T, 1.0; ac^4C, 0.7; m^3C, 0.1; I, 0.05. This result suggests that the preparation was slightly contaminated (~5%) with tRNA^{Ser} (Ginsberg et al., 1971) contributing m^3C (2 mol/mol of tRNA^{Ser}) and I (1 mol/mol of tRNA^{Ser}).

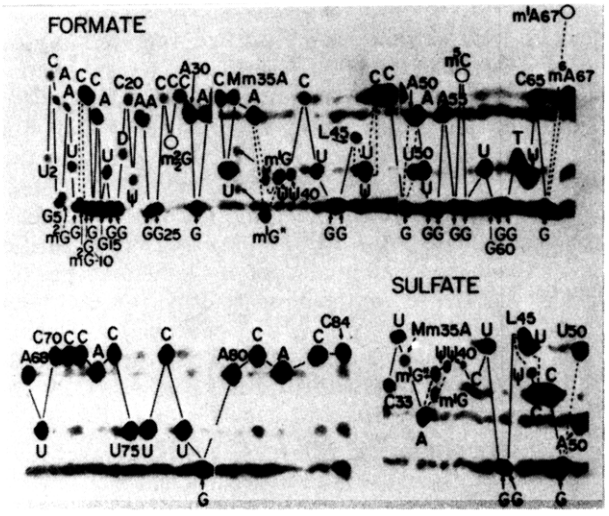

FIGURE 1: PEI–cellulose thin-layer readouts (Gupta & Randerath, 1979) of positions 2–84 of Morris hepatoma 5123D leucine tRNAs (anticodon Mm-A-A). The autoradiograms display the 5′ termini of ^{32}P-labeled fragments of decreasing chain length (from left to right), obtained by controlled hydrolysis and ^{32}P labeling. The 5′ termini were released as 5′-^{32}P-labeled nucleoside 3′,5′-diphosphates by in situ RNase T_2 treatment, after the fragments had been separated by polyacrylamide gel electrophoresis and contact transferred to PEI–cellulose thin layers. The formate readout was derived from five separate chromatograms encompassing positions 2–32, 33–48, 49–67, 68–79, and 80–84, respectively. pm_2^2Gp, pm^5Cp, and pm^1Ap, represented by circles, were visualized after prolonged film exposure (not shown) because the corresponding labeled fragments were obtained in low yields. Broken lines indicate two termini (see teext). C11-C12, A22-A23, and G43-G44 gave two spots each and G60-G61-G62 gave three spots upon shorter film exposure (not shown). C in position 28 could be read from the ammonium sulfate chromatogram (not shown) and was confirmed by the occurrence of C-C-A-G in the complete RNase T_1 digest (Figure 2A).

Sequence Analysis of the Two Leucine tRNAs. The sequences of the leucine tRNAs were deduced by a combination of the thin-layer readout sequencing procedure (Gupta & Randerath, 1979; Gupta et al., 1980; Randerath et al., 1980) with gel readout sequencing (Gupa & Randerath, 1977a,b; Donis-Keller et al., 1977; Simoncsits et al., 1977; Lockard et al., 1978; Gupta et al., 1979, 1980; Randerath et al., 1979, 1980) and "postlabeling" analysis of the fragments in complete RNase T_1 and A digests (Gupta et al., 1976a,b, 1979, 1980; Randerath et al., 1980). The analysis required a total of ~30 μg of the highly purified tRNA.

Figure 1 shows the PEI–cellulose thin-layer readout (Gupta & Randerath, 1979) of the 5′-terminal nucleotides of RNA fragments, which had been separated by size on a polyacrylamide gel and contact transferred to a PEI–cellulose thin layer, in ammonium formate (positions 2–84) and ammonium sulfate (positions 33–50) solvents. Results obtained in these two systems were in agreement with each other. Distinct single 5′ termini were released from most fragments, but two nucleotides were released from positions 10 (pm^2Gp + pGp), 38 (pm^1G*p + pm^1Gp), 46 (pUp + pψp), 50 (pAp + pUp), and 67 (pm^1Ap + pm^6Ap), as indicated by broken lines in Figure 1. Two additional weak spots were obtained from position 35 (pMm-Ap) in the formate system (Figure 1, arrows) but not in the sulfate system, in which they cochromatographed with a radioactive background close to pUp and pAp. The presence of A and U in position 50 showed that the tRNA^{Leu} prepa-

ration consisted of two major species. The release of two terminal nucleotides from positions 10, 38, and 46 indicated partial modification of the RNAs or, in the case of position 38, possibly the loss of a substituent during the isolation of the RNA (see below), while m^6A in position 67 was due to extensive conversion of m^1A to m^6A under the experimental conditions. Instead of ac^4C, the thin-layer readouts showed C in position 12. pm_2^2Gp (position 27), pm^5Cp (position 57), and pm^1Ap (position 67), indicated by circles in Figure 1, could be detected on autoradiograms after prolonged exposure (not shown). As shown by base analysis of the RNA and its enzymatic digestion products (see below), the RNA contains 1 mol each of these nucleotides. The thin-layer readout showed three nucleotides whose chromatographic behavior did not correspond to that of known nucleotides (Gupta & Randerath, 1979). They have been designated as Mm, m^1G^*, and L, occupying positions 35, 38, and 45, respectively. A gap between fragments 35 and 37 on the original gel ladder was due to ribose methylation of nucleotide 35, see below. The two minor components released from fragment 35 (Figure 1) may be precursors of Mm. A36 was identified by fingerprint analysis and the gel readout procedure (see below).

The modified nucleosides identified on the thin-layer readouts were also obtained as ^3H-labeled nucleoside trialcohols by mapping on cellulose thin layers for base analysis (see above), except for Mm, m^1G^*, and L. The absence of a spot corresponding to Mm was expected, since ribose methylation makes this compound resistant to periodate oxidation.

m^1G^* was completely recovered as m^1G, as 1 mol of the nucleoside trialcohol of m^1G was obtained per mol of RNA. The identity of this nucleoside trialcohol was confirmed by cochromatography on cellulose and silica gel thin layers (Randerath, K., et al., 1974; solvents E and F) with chemically synthesized (Randerath et al., 1972) m^1G'.[1] pm^1Gp (Figure 1) may be due either to incomplete modification or to the experimental manipulations because of the alkali lability of m^1G^*.

To find out whether the nucleoside trialcohol of L might have been obscured by its cochromatography on cellulose with one of the major nucleoside trialcohols, we rechromatographed these compounds by two-dimensional thin-layer chromatography on silica gel (Randerath, K., et al., 1974), but no additional compound was detected. The trialcohol of L may have remained at the origin of the cellulose map, as found for other nucleoside trialcohols such as the derivative of Q (our unpublished experiments), or it may have traveled with the solvent front(s), where it would have been obscured by radioactive background material. This is under further investigation. Further properties of Mm, m^1G^*, and L will be presented under Further Analysis of the Hypermodified Nucleotides.

Sequencing gels (not shown) enabled us to read 64 positions of the RNA chain; this included five modified nucleotides (m^2G6, m^2G10, $\psi 21$, $\psi 40$, and $\psi 46$), which were read as the corresponding major nucleotides, and the 5′-terminal G. Ribose methylation in position 35 was indicated by the lack of a band of an intensity comparable to that of the neighboring bands in the partial alkaline digest. A very weak fragment of chain length 35 was detected, however, in both the partial alkaline and RNase A digests, suggesting a partial lack of ribose methylation. RNase A cleavage suggests that M may be a pyrimidine, in agreement with the leucine codons U-U-A and U-U-G. RNase U_2 cleavage showed anticodon positions

[1] Abbreviations used: m^1G', m^5C-G′, etc., nucleoside trialcohol of m^1G and oligonucleotide 3′-dialcohol of m^5C-G, respectively, etc.

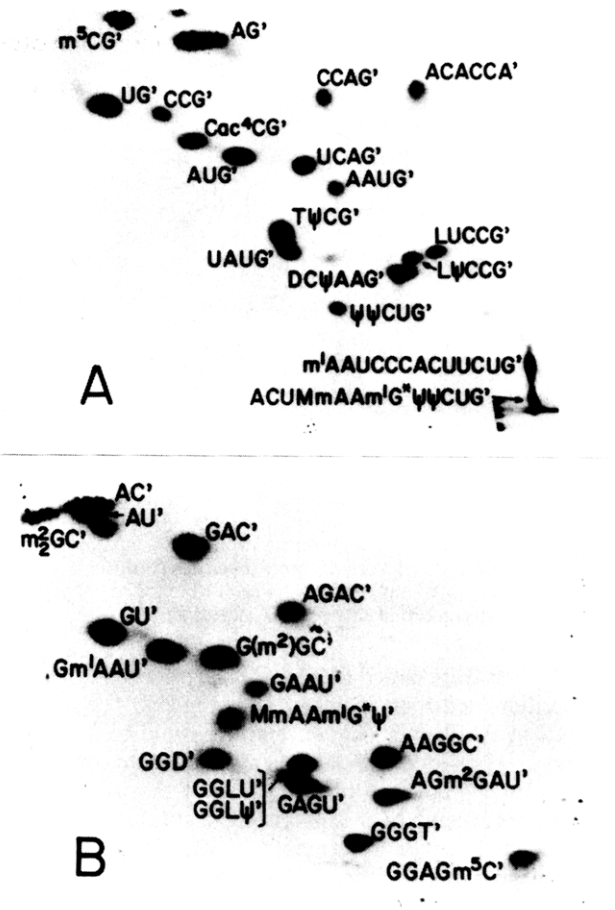

FIGURE 2: PEI–cellulose maps of 3′-terminally ^3H-labeled oligonucleotide dialcohols obtained by digestion with RNase T_1 (A) and RNase A (B) of Morris hepatoma 5123D leucine tRNAs (anticodon Mm-A-A), followed by chemical ^3H labeling. First dimension (LiCl gradient), from right to left; second dimension (ammonium formate, pH 2.6, gradient), from bottom to top. Detection by fluorography (Randerath, 1970). m^5C-G′, A-C′, etc. are oligonucleotide 3′-dialcohol derivatives of m^5CpG, ApC, etc. The origins are not shown because this part of the chromatogram had been cut off after predevelopment for the removal of radioactive background (Gupta et al., 1979). The spot below A-U′ is a mixture of the dialcohols of G-C and G-m^5C from contaminating $tRNA^{Ser}$, which contains 3 mol of the former and 1 mol of the latter.

36 and 37 to be occupied by A. Bands of chain length 50 were observed in the RNase U_2, Phy_1, and A tracks, indicating that position 50 was occupied by two nucleotides, A and U, in agreement with the results of thin-layer readout analysis (Figure 1) and ^3H fingerprinting (Figure 2A). Twenty-one positions could not be identified by the gel readout procedure because of the absence of enzymatic cleavage products (D19, C26, m_2^2G27, C33, m^1G^*38, $\psi 39$, L45, T63, and $\psi 64$), ribose methylation (Mm35), band compression (positions 57–59 and 70–74), or incomplete resolution (positions 83–85). All of these positions, except 35, could be read from the thin-layer readout chromatograms.

Figure 2 depicts fluorograms of fractionations on PEI–cellulose thin layers of oligonucleotide 3′-dialcohols obtained by complete digestion of $tRNA^{Leu}$ with RNase T_1 (Figure 2A) and RNase (Figure 2B) plus alkaline phosphatase and subsequent 3′-terminal ^3H labeling of the oligonucleotides. All labeled compounds were well resolved in both maps except for the pairs A-C′/A-U′, G-m^2G-C′/G-G-C′, and G-G-L-U′/G-G-L-ψ′ (Figure 2B). The dinucleotides were resolved by rechromatography (Gupta et al., 1979), while the tri- and tetranucleotides were analyzed directly. The oligonucleotide

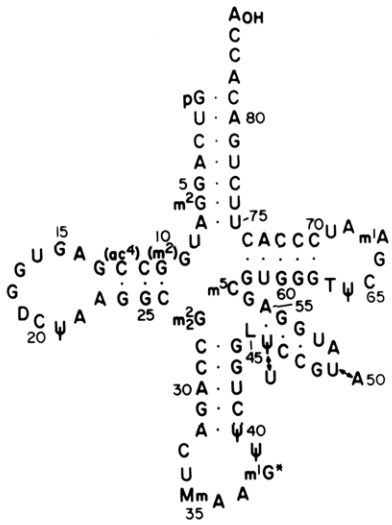

FIGURE 3: The primary structures of two Morris hepatoma 5123D leucine tRNAs in the cloverleaf form. The tRNAs differ in position 50, where both U (0.6 mol) and A (0.4 mol) were found. Mm, m^{1}G*, and L are hypermodified nucleosides (see text).

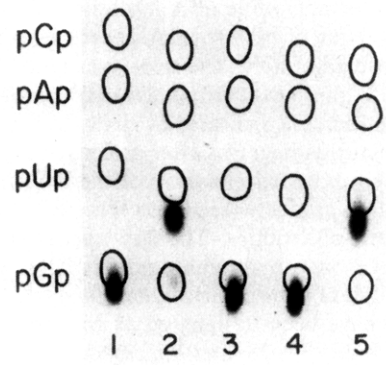

FIGURE 4: Stability of ^{32}P-labeled pm^{1}G*p against acid, alkali, and periodate–borohydride. pm^{1}G*p, extracted from thin-layer readout chromatograms with 2 M LiCl solution and desalted, was treated with HCl (lane 1), piperidine (lane 2), and periodate–borohydride (lane 3) as described in the text. Chromatography was on PEI–cellulose in an ammonium formate system (Gupta & Randerath, 1979) in the presence of nonradioactive pCp, pAp, pUp, and pGp. In lanes 4 and 5, authentic radioactive pm^{1}G*p and pm^{1}Gp, respectively, were chromatographed. Detection was by autoradiography.

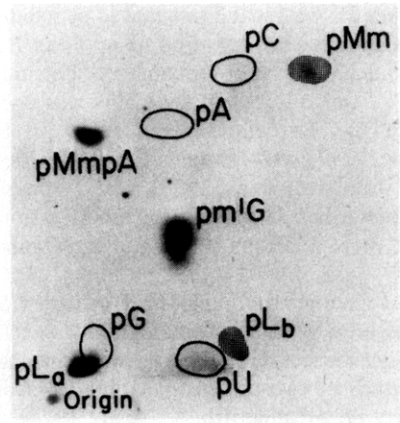

FIGURE 5: Two-dimensional separation of 5′-monophosphate nucleotides obtained by enzymatic digestions of ^{32}P-labeled pMmpAp, pm^{1}G*p, and pLp. pMmpA, pm^{1}G, and pL$_a$, respectively, were obtained by incubation of these compounds with nuclease P$_1$. pMm was obtained by treating pMmpA with snake venom phosphodiesterase. pL$_b$ was released from [^{32}P]pL-U-C-C-G′ and [^{32}P]pL-ψ-C-C-G′ by nuclease P$_1$ treatment. pMm and pL$_b$ were chromatographed separately; the stippled areas indicate their positions. For chromatographic conditions, see the text.

3′-dialcohols were eluted from the fingerprints and their base compositions and 3′ and 5′ termini determined. The sequences of most of the shorter oligonucleotides could be determined this way. The sequences of the longer oligonucleotides were derived by combination of the results of thin-layer and gel readout sequencing of the intact RNA. Molar ratios of oligonucleotides were in agreement with the readout data. The main purpose of these experiments was a quantitative determination (1) of those modified nucleosides that gave weak spots on thin-layer readout chromatograms (m_2^2G27, m^{5}C57, and m^{1}A67), (2) of partially modified nucleosides (m^2G/G10, ac^{4}C/C12, and ψ/U46), and (3) of the relative proportion of the two leucine tRNA species (reflected by the U50/A50 ratio). Furthermore, these experiments served to determine the 3′ terminus of the RNA and to provide fragments for some studies on the properties of L (see below). The molar ratios of m_2^2G-C′, m^{5}C-G′, and G-m^{1}A-A-U′ were found to be 1.0, indicating that the RNAs were fully modified in positions 27, 57, and 67. The molar ratios for the partial modifications were as follows: m^{2}G/G10, 0.7:0.3; ac^{4}C/C12, 0.7:0.3; ψ/U46, 0.5:0.5. These values are in reasonable agreement with the base composition data. ^{3}H fingerprint analysis also indicated that the leucine tRNA preparation consisted of a mixture of two RNAs. Thus, the molar ratios for U50-A-U-G′ and A50-A-U-G′ (Figure 2A) as well as for A51-U′ (resulting from cleavage at U50) and G-A50-A-U′ (Figure 2B) were 0.6 and 0.4, respectively, indicating that the tRNA preparation contained ~60% tRNA$_{U50}^{Leu}$ and 40% tRNA$_{A50}^{Leu}$. 3′-Terminal analysis of A-C-A-C-C-A′ (Figure 2A) gave the 3′ terminus of the RNAs.

The above data enabled us to deduce the nucleotide sequences of the leucine tRNAs, as shown in Figure 3.

Further Analysis of the Hypermodified Nucleotides. The behavior of pMm-Ap, pm^{1}G*p, and pLp, isolated from thin-layer readout chromatograms against treatments with acid, alkali, and periodate–borohydride, was studied (for conditions, see Experimental Procedures). The reaction mixtures were analyzed by thin-layer chromatography in the ammonium formate and ammonium sulfate systems alongside aliquots of the original compounds as controls. The only reaction observed was the conversion of pm^{1}G*p by alkali, and to a small extent also by acid, to a new compound that moved faster than pm^{1}G*p in the ammonium formate system (Figure 4) and that had the same chromatographic properties as pm^{1}Gp in this system (Figure 4, lane 5), as well as in ammonium sulfate (not shown). The conversion of pm^{1}G*p to pm^{1}Gp at alkaline pH is consistent with the recovery of m^{1}G* as the nucleoside trialcohol of m^{1}G in the ^{3}H-labeled derivative procedure for base analysis; most of the conversion of m^{1}G* to m^{1}G probably occurred during the enzymatic digestion of the RNA at alkaline pH.

To remove the 3′-phosphomonoester groups (Fujimoto et al., 1974), we treated pMm-Ap, pm^{1}G*p, and pLp with nuclease P$_1$ and chromatographed the digests two-dimensionally on PEI–cellulose thin layers to resolve the resulting 5′-monophosphate derivatives (Gupta et al., 1976b) (see Figure 5). Incubation of pMm-Ap with nuclease P$_1$ removed only the 3′-terminal phosphate without cleaving the phosphodiester

bond, yielding pMm-A (Figure 5). The enzyme thus appeared to be inhibited by the base modification of M, because 2′-O-methylated dinucleotides are usually susceptible to cleavage by nuclease P_1 (Gupta et al., 1976c). However, snake venom phosphodiesterase treatment converted pMm-A to pMm (Figure 5), which was found to behave like a derivative of C [see Gupta et al. (1976b)].

Incubation with nuclease P_1 at pH 5.0 converted pm^{1}G*p quantitatively to pm^{1}G, which cochromatographed on the nucleoside monophosphate map (Gupta et al., 1976b), as well as in 0.5 M LiCl and in 1 M ammonium formate, pH 3.5, with authentic pm^{1}G prepared by incubation of pm^{1}Gp with nuclease P_1. The conversion of m^{1}G* to m^{1}G by nuclease P_1 appears to be due to a contaminating enzyme activity present in the nuclease preparation since pm^{1}G*p remained intact after incubation at pH 5.0 in the absence of enzyme.

Nuclease P_1 treatment of pLp isolated from thin-layer readout chromatograms (Figure 1) resulted in the quantitative formation of a compound, pL_a, which migrated close to pG on the nucleoside monophosphate map (Figure 5); pLp itself remained at the origin of the chromatogram. However, when [^{32}P]pL-U-C-C-G′ and [^{32}P]pL-ψ-C-C-G′ isolated from a RNase T_1 fingerprint (Figure 2A) and 5′-^{32}P labeled, were treated with nuclease P_1, a faster moving compound, pL_b (Figure 5), was the major product and only a trace of pL_a was obtained. Complete digestion of the oligonucleotides first with RNase T_2 and then with nuclease P_1 also resulted mainly in the formation of pL_b. However, if the oligonucleotides were heated in neutral aqueous solution (5 min, 100 °C) prior to nuclease P_1 digestion, the major reaction product was pL_a.

Discussion

To define tRNA alterations in cancer cells and their functional significance, one needs to compare in detail the distribution and the structure of individual tRNAs in normal and neoplastic cells, because the multiple functions of tRNA in protein synthesis as well as in regulation and control processes are likely to depend on specific structural features of the tRNA molecules. In this paper we report the initial results of a project aimed at the eventual elucidation of structural differences between Morris hepatoma and liver tRNAs. This study was prompted by our earlier findings of base composition differences between Morris hepatoma and rat liver cytoplasmic (Randerath, E., et al., 1974) and mitochondrial (Chia et al., 1976) tRNA preparations.

Detailed structural investigations of mammalian tRNA, including those of neoplastic origin, have only recently become feasible with the development of highly sensitive radioactive derivative ("postlabeling") methods in this laboratory (Randerath, K., et al., 1974; Gupta et al., 1976a; Gupta & Randerath, 1977a,b, 1979; Randerath et al., 1979, 1980) as well as in other laboratories (Donis-Keller et al., 1977; Simoncsits et al., 1977; Lockard et al., 1978; Stanley & Vassilenko, 1978; Peattie, 1979). Thus, in the present work, only ~30 μg of highly purified tRNA^{Leu}, corresponding to ~12 g of tumor tissue, was needed to deduce the sequence and to explore some properties of three unknown nucleotides of the leucine tRNAs.

The combination of gel electrophoretic purification methods with postlabeling methods for sequence analysis appeared ideal, since the amount of RNA from a single gel was more than sufficient for the entire analysis. The BD–cellulose purification step (Gillam et al., 1967; Rogg et al., 1975) before gel electrophoresis served primarily to separate isoaccepting leucine tRNAs from each other.

Among the postlabeling methods used, the thin-layer readout procedure (Gupta & Randerath, 1979) proved particularly useful. The major advantages of this procedure are (1) the direct chromatographic identification of modified nucleotides in the RNA chain and (2) the identification of nucleotides in base-paired regions that resist the enzymatic hydrolysis employed in the gel readout procedure but are cleaved by the chemical hydrolysis employed in the thin-layer readout procedure. While 69% of the sequence could be read correctly from sequencing gels, the thin-layer readout procedure yielded 95% of the sequence and, most importantly, made possible the rapid and direct chromatographic detection and location in the sequence of three unknown nucleotides, which would not have been achieved on sequencing gels or by base analysis. The gel readout procedure and the analysis of RNA fragments served mainly the following purposes: (1) to identify positions that could not be identified on the thin-layer readouts (5′ and 3′ terminus, ac^{4}C12, and A36), (2) to confirm the presence of three modified nucleotides (m_2^2G27, m^{5}C57, and m^{1}A67) that were only weakly labeled by the [γ-^{32}P]ATP–polynucleotide kinase reaction, (3) to assay quantitatively the partial modifications (m^{2}G/G10, ac^{4}C/C12, and ψ/U46), and (4) to determine the relative amounts of the two tRNAs (ratio of U50/A50).

Although the structure of Mm, the nucleotide in the first position of the anticodon triplet of the tRNAs, is not yet known, aminoacylation experiments and structural features clearly identify the tRNAs as leucine tRNAs. Only phenylalanine tRNAs also have N-A-A anticodons, with N being G or Gm. While all eukaryotic phenylalanine tRNAs sequenced to date contain 5 nucleotides in the extra arm, the RNAs described here have a long extra arm of 13 nucleotides. Among the sequenced eukaryotic tRNAs, only yeast (Kowalski et al., 1971; Chang et al., 1971, 1973; Randerath et al., 1975, 1979) and *Torulopsis utilis* (Murasugi & Takemura, 1978) leucine tRNAs have this number of nucleotides in the extra arm. Furthermore, our experiments indicated that Mm is a pyrimidine, suggesting that these tRNAs recognize the leucine codons U-U-A and/or U-U-G. Finally, amino acid acceptor assays showed that the RNAs could be charged with leucine but not with phenylalanine.

Taking into account an overall recovery of the gel purification steps of ~50%, we estimate that the two leucine tRNAs represent at least 0.74% of the total tumor tRNA population. This is a minimum estimate because the unavoidable losses during column chromatography were not considered in this calculation. The leucine tRNAs thus are major isoacceptors.

It is not yet possible to compare the structures of the two leucine tRNAs with other leucine tRNAs from higher eukaryotes because no other such tRNA sequence has yet been published. However, the hepatoma tRNAs can be compared with leucine tRNAs from lower eukaryotes, such as two leucine tRNAs from baker's yeast [anticodons m^{5}C-A-A (Kowalski et al., 1971; Chang et al., 1971, 1973) and U-A-G (Randerath et al., 1975, 1979)] and a leucine tRNA from *T. utilis* [anticodon Cm-A-A (Murasugi & Takemura, 1978)]. The sequence homology between the hepatoma tRNAs and these tRNAs is 50% if differences in posttranscriptional modifications are included in the comparison and 56% if they are excluded, indicating that a substantial portion of the sequence has been conserved during the evolution from lower to higher eukaryotes. Extended homologous sequences are present in the D stem, the D loop, and the T loop, while the aminoacyl stem and the extra arm exhibit the greatest sequence differences, suggesting that these parts of the molecule may have been adapted to new functions during the evolution of higher eukaryotes.

While the two hepatoma leucine tRNAs exhibit no unusual features in terms of the invariant and semiinvariant nucleosides [Rich & RajBhandary, 1976; see also Gauss et al. (1979)], there are some noteworthy structural features which will be discussed below.

In tRNAs sequenced to date, m^2G6 has been found only in mammalian methionine (Petrissant & Boisnard, 1974; Piper, 1975a) and glycine (Gupta et al., 1979, 1980) tRNAs. Since silkworm glycine tRNAs have G6 (Garel & Keith, 1977; Zuniga & Steitz, 1977; Kawakami et al., 1978), the methylation of G6 may be restricted to mammalian (or vertebrate) tRNAs. The mammalian leucine tRNAs differ from all other known leucine tRNAs in lacking the Gm modification in position 17, perhaps indicating a tumor-specific undermethylation (Randerath, E., et al., 1974). The occurrence of ψ in position 21 is unusual; no sequenced tRNA has ψ in the α or β region (Rich & RajBhandary, 1976) of the D loop.

The hypermodified nucleoside Mm in position 35 apparently has not been found in any tRNA sequenced to date. As indicated by gel readout sequencing, M may be a pyrimidine; the chromatographic behavior of pMm on the nucleoside monophosphate map (Figure 5) suggests that Mm is a derivative of C rather than U. Thus, in terms of codon recognition, the two mammalian leucine tRNAs would be related to yeast $tRNA_{m^5C\text{-}A\text{-}A}^{Leu}$ and *T. utilis* $tRNA_{Cm\text{-}A\text{-}A}^{Leu}$ and one might then speculate that Mm might be m^5Cm. However, the presence on the thin-layer readout chromatogram (Figure 1, arrows) of two minor components in addition to pMm-Ap, which may represent precursors of Mm, would argue against Mm being m^5Cm. The structure of Mm is under current investigation.

The mammalian leucine tRNAs have m^1G^*, an alkali-labile derivative of m^1G, in position 38, while yeast and *T. utilis* leucine tRNAs were reported to have m^1G in this position. Our data are consistent with the substituent of m^1G^* being an acyl group. The nuclease P_1 preparation contained an activity removing the group from pm^1G^* to yield pm^1G (see Results). This activity may be a nonspecific esterase, because digestion of the leucine tRNAs, which contain ac^4C in position 12, to nucleosides by successive treatments with nuclease P_1 and alkaline phosphatase and subsequent tritium base analysis failed to yield ^3H-labeled ac^4C' (unpublished experiments), whereas under standard conditions, which do not specify the use of nuclease P_1 for the digestion of the RNA (Randerath & Randerath, 1971, 1973; Randerath et al., 1972, 1980), ^3H-labeled ac^4C' was obtained. An unknown, possibly acylated (Blank & Söll, 1971) guanosine derivative adjacent to the 3′ end of the anticodon was also found by Dube et al. (1970) and by Blank & Söll (1971) in *Escherichia coli* leucine tRNAs. Tritium base analysis of such RNAs was shown to yield 1 mol of m^1G' per mol of tRNA (Randerath et al., 1972). The mammalian and *E. coli* leucine tRNAs thus conceivably contain the same derivative or structurally related derivatives of m^1G adjacent to the 3′ end of the anticodon. The question arises whether leucine tRNAs from lower eukaryotes also have an alkali-labile derivative of m^1G rather than m^1G in this position. For example, the occurrence of m^1G in yeast $tRNA_{U\text{-}A\text{-}G}^{Leu}$ (Randerath et al., 1975) was deduced by tritium base analysis and tritium sequencing methods, which would have yielded m^1G' from m^1G^* or a structurally related compound.

The presence of a hypermodified nucleoside, L, in position 45, the first position of the long extra arm, is unusual as no other tRNA has been found to contain a hypermodified nucleoside in this position. The only modified nucleoside known to occur in this position is Um in eukaryotic serine tRNAs (Zachau et al., 1966; Ginsberg et al., 1971; Rogg et al., 1975; Piper, 1978). Yeast and *T. utilis* leucine tRNAs have unmodified U in this position, which always appears to contain a pyrimidine in tRNAs having a long extra arm. The chromatographic behavior of pL_b (Figure 5) suggests that L may be a U derivative. The relationship between pL_a and pL_b (Figure 5) is not yet clear; heating of the tRNAs or of pL-U(ψ)-C-C-G′ in aqueous solution, followed by nuclease P_1 or RNase T_2 plus nuclease P_1 digestion, gave rise to the slow moving compound pL_a (Figure 5), suggesting heat lability of the parent nucleoside in position 45. Structural analysis of this nucleoside will be required to explain our observations.

The finding of the sequence ψ-ψ in positions 39 and 40 appears noteworthy, because no known eukaryotic tRNA has this sequence [see Gauss et al. (1979)] and pseudouridine residues located in the anticodon loop and/or stem of various prokaryotic tRNAs have been shown to play regulatory roles (Lewis & Ames, 1972; Cortese et al., 1974; Turnbough et al., 1979). Thus, $tRNA^{His}$, isolated from a mutant of *Salmonella typhimurium* (*his*T), in which histidine biosynthesis is not repressed when histidine is added to the culture medium, contains the sequence U-U in this particular location, while $tRNA^{His}$ from wild-type cells has ψ-ψ (Singer et al., 1972). Similarly, the leucine biosynthetic enzymes were found to be derepressed in the *his*T mutant (Singer et al., 1972); $tRNA^{Leu}$ from this mutant was shown to contain two U residues in the anticodon region, whereas wild-type $tRNA^{Leu}$ contained two ψ residues (Allaudeen et al., 1972). Since the regulation of a large number of amino acid biosynthetic pathways is altered in *his*T mutants (Turnbough et al., 1979), it is tempting to speculate that the ψ residues in the anticodon region of the mammalian tRNAs may also be involved in regulatory functions.

The question of whether the occurrence of both A and U in position 50 is due to a tumor-specific mutation is under current investigation. It is of interest that among autologous tRNA isoacceptors having a long extra arm variations of the extra arm sequence appear quite common [see Gauss et al. (1979)] and that in the case of the genes for two *E. coli* tyrosine tRNAs exhibiting such variations the sequences both upstream and downstream of the mature tRNA sequences as well as the overall structural organization of the gene clusters coding for two tyrosine tRNAs are quite different (Rossi & Landy, 1979). The two tyrosine tRNAs are also identical, except for two adjacent nucleotides at the tip of the variable loop, i.e., a location corresponding to position 50 of the hepatoma leucine tRNAs. The genetic organization as well as the functional significance of the two closely related mammalian $tRNA^{Leu}$ species, which, in analogy to silkworm $tRNA^{Ala}$ (Sprague et al., 1977; Garel et al., 1977), may reflect specific functional adaptations, deserves further investigation.

Additional studies, currently in progress, will be aimed at elucidating the structures of the hypermodified nucleosides by mass spectrometry (McCloskey & Nishimura, 1977) and at comparing the tumor leucine tRNAs with the corresponding liver tRNA(s).

Added in Proof

After submission of this paper Pirtle et al. (1980) reported the sequence of a different mammalian leucine tRNA (anticodon IAG) from beef liver.

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