Human glutamate tRNA forms stable hybrids *in vitro* with 28S ribosomal RNA

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ABSTRACT

A human glutamate tRNA has been shown to form stable hybrids with 28S ribosomal RNA. This tRNA was purified from HeLa cell cytoplasmic RNA by RNA-RNA solution hybridization followed by the isolation of tRNA-28S rRNA complexes by hybridization-selection with ribosomal DNA or by recovery of the 28S peak from formamide-sucrose gradients. The single hybridizing tRNA species was identified as tRNA^{Glu}CUC by sequencing:

pU-C-C-C-U-G-G-U-G-m²G-U-

C-ψ-A-G-U-G-G-D-ψ-A-G-G-A-U-U-C-G-G-C-G-C-U-C-U-C-A-C-C-G-C-C-G-C-G-G-C-m⁵C-m⁵C-G-G-G-Tm-ψ-C-G-A-U-U-C-C-C-G-G-U-C-A-G-G-G-A-A-C-C-A_{OH}.

Computer analysis located a nucleotide sequence near the middle of human 28S rRNA which is complementary to 15-26 nucleotides between residues 20 and 50 of this tRNA. An interaction between this tRNA and 28S rRNA suggests that tRNA^{Glu}CUC may have functions in the cell in addition to translation.

INTRODUCTION

Small RNAs are involved in a number of cellular processes. For example, the small nuclear RNAs are required for the processing of primary mRNA transcripts (1); M1 RNA is the catalytic subunit of RNase P (2); 7SL RNA is involved in protein translocation across the endoplasmic reticulum (3); and adenovirus VAI RNA stimulates translation in the virus-infected cell (4). Although their functions are not understood, 5S and 5.8S rRNA are integral components of the ribosome (5,6).

The most highly modified and complex class of small RNAs is represented by the tRNAs. Over time, certain systems have evolved which have made use of the tRNAs to perform functions other than translation. For example, certain tRNAs base-pair with retrovirus genomic RNA to act as primers for reverse transcriptase (7-9). In addition, tRNA^{Lys} and tRNA^{Arg} are thought to bridge two RNA genomes to promote efficient reverse transcription (10). Recently, a chloroplast tRNA^{Glu} has been shown to be involved in the enzymatic conversion of glutamate to glutamate-1-semialdehyde in the pathway of chlorophyll biosynthesis (11), and a tRNA^{His} has been shown to be an essential component of the ubiquitin- and ATP-dependent protein turnover system of

mammalian cells (12). Although speculative, tRNA^{Lys} has been implicated in the splicing of a mitochondrial mRNA precursor (13,14).

Two studies have revealed that tRNAs form stable base-paired interactions in vitro with ribosomal RNA, indicating perhaps another function. *E. coli* 23S rRNA was found to hybridize with tRNA^{Met} (15), and mouse 28S rRNA was found to hybridize with tRNA^{Pro} (16). Although these appear to represent specific interactions, their significance is not yet understood. This paper describes an interaction between a human glutamate tRNA and 28S rRNA, characterized using in vitro hybridization-selection techniques. The base-pairing interaction is highly specific and has permitted the purification of the tRNA in only a few steps. Because of the unusual nature of this interaction, the identity of this tRNA was sought using RNA sequencing techniques. The complete nucleotide sequence of this tRNA has identified it as tRNA^{Glu}CUC. This represents the first sequence of a human glutamate tRNA, and the first sequence of a eukaryotic tRNA^{Glu}CUC.

MATERIALS AND METHODS

Cells and labeling

HeLa cells (S3 strain) were maintained in suspension culture at densities of 2.5×10^5 cells/ml in Joklik-modified S-MEM (Gibco Diagnostics) supplemented with 3.5% newborn and 3.5% calf serum. For labeling, HeLa cells were grown in spinner culture at a density of 1.5×10^5 cells/ml in medium containing 10% the normal phosphate concentration supplemented with serum as above plus 6 mCi of carrier free (³²P)-phosphoric acid (285 Ci/mg; ICN). After 24 hours the volume was raised with an equal volume of the same medium and after 48 hours the cells were harvested.

Isolation of cytoplasmic RNA

HeLa cells were washed in Earle's balanced salts and resuspended at 10 times the packed cell volume in RSB (10 mM Tris, pH 7.2, 10 mM NaCl, 1.5 mM MgCl₂). The cells were broken with 20 strokes of a tight Dounce homogenizer after swelling for 20 minutes at 0°C. Nuclei were pelleted by centrifugation for 10 minutes at 600 x g and the cytoplasmic supernatant fraction was made 2% SDS and 2 mM EDTA, and was digested with 200 µg/ml Proteinase K at 37°C for 30 minutes. The digests were then extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (100:99:1) and three times with chloroform/isoamyl alcohol (99:1). The RNA was collected by ethanol precipitation, digested in RSB for 15 minutes with 20 µg/ml iodoacetic acid treated DNase I (17), and the extractions with phenol/chloroform were repeated.

Isolation of nuclear RNA

Nuclear and nucleolar RNA were isolated by modifications of the methods described in (18,19). HeLa cells were washed and resuspended in RSB at 10 ml/g of cells. NP40 was added to 0.5%, the lysate was centrifuged at 600 x g for 2 minutes, 4°C, and the pelleted

nuclei were washed in RSB and resuspended at 10 ml/g of the original cell weight in RSB with 1% Tween 40 and 0.5% sodium deoxycholate. For total nuclear RNA, nuclei were pelleted at 600 x g for 2 minutes, 4°C, resuspended in 5 ml RSB with 20 µg/ml iodoacetic acid treated DNase I, and after 15 minutes SDS was added to 0.5%. For nucleolar RNA, pelleted nuclei were resuspended in four times the packed cell volume of HSB (10 mM Tris, pH 7.4, 0.5 M NaCl, 0.5 mM MgCl₂) plus 20 µg/ml DNase I, and incubated at 37°C for one minute. The sample was layered on a 15-30% (w/w) sucrose gradient made with HSB, and was centrifuged for 15 minutes at 39,500 x g, 5°C, in an SW28.1 rotor. The pelleted nucleoli were resuspended in RSB containing 0.5% SDS. NaCl and EDTA were added to the nuclear and nucleolar fractions to 100 mM and 20 mM, respectively, and each fraction was digested with 200 µg/ml Proteinase K at 37°C. After 30 minutes the samples were extracted and digested with DNase I as described above.

Hybridization-selection

The rDNA clone used for hybridization-selection contained a 7.4 kb EcoRI fragment of human DNA in pBR322 (obtained from N. Arnheim). rDNA-plasmid DNA was sheared by sonication, the DNA was electrophoresed on preparative gels, and 1-2 kb sized DNA was cut out and recovered. The DNA fragments were then covalently bound to a 50 cm² DBM paper prepared by the method of Alwine et al. (20). RNA was 3' end labeled using (5'-³²P) pCp and T4 RNA ligase. The labeled RNA was heated to 90°C in hybridization solution containing 0.75 M NaCl, 75 mM sodium citrate, 25 mM sodium phosphate, pH 6.4, 50% formamide and 0.1% SDS, and was incubated with a DNA-DBM paper in a Parafilm-sealed petri dish at 42°C for 18 hours. The paper was washed in formamide-wash salts (20 mM NaCl, 8 mM sodium citrate, 0.2% SDS, 50% formamide) at 34°C with a change of 8 ml every hour over a 6 hour period, and the hybrid-selected RNAs were eluted twice by heating the paper to 70°C in 2 ml of 100% formamide for one minute. The eluates were combined, adjusted to 0.3 M NaCl, and the RNA was precipitated with ethanol.

Northern hybridization

Polysomal rRNA was glyoxylated according to Thomas (21) and electrophoresed on a 1% agarose gel with 10 mM sodium phosphate buffer, pH 6.8. The RNA was blot transferred to nitrocellulose and hybridized for 36 hours at 42°C in 50% formamide hybridization buffer as described above, containing 30,000 cpm/ml of hybrid-selected gel-purified tRNA^{Glu} which was 3' end-labeled with ³²P-pCp. The nitrocellulose paper was washed in formamide-wash salts for 6 hours at 34°C and exposed to X-ray film.

Solution hybridization and sucrose gradient centrifugation

Cytoplasmic RNA was dissolved in 5 mM sodium phosphate buffer, pH 6.4, at a concentration of 10 mg/ml. An equal volume of hybridization solution was added to give final concentrations of 5 mg/ml cytoplasmic RNA, 0.75 M NaCl, 75 mM sodium citrate, 25

mM sodium phosphate, pH 6.4, 50% formamide, and 0.1% SDS. The solution was heated to 90°C, quick-cooled and hybridized for 24 hours at 42°C. The RNA was layered on 15-30% (w/w) linear sucrose gradients made with formamide-wash salts, and the samples were centrifuged in an SW28 rotor for 20 hours at 82,700 x g, 34°C.

For the preparative isolation of tRNA^{Glu} for sequencing, up to 30 mg RNA was layered on a sucrose gradient made with formamide-wash salts and was centrifuged as above at 34°C. Fractions containing the 28S rRNA peak were pooled and ethanol precipitated. RNA pellets were redissolved in TE (10 mM Tris, pH 7.2, 1 mM EDTA), ³²P-tRNA^{Glu} marker (4,000 cpm) was added, and the sample was denatured at 90°C. The solution was then layered over several 15-30% (w/w) linear sucrose gradients made with TE, 0.1% SDS and centrifuged in an SW28 rotor for 27 hours at 82,700 x g, 22°C. 1.5 ml fractions were collected and the region corresponding to the ³²P-tRNA^{Glu} peak was pooled and ethanol precipitated. Up to 100 µg of tRNA/lane was electrophoresed on a 3 mm, 7 M urea 10% polyacrylamide gel. Autoradiography was used to identify the labeled RNA bands, and the tRNA^{Glu} was recovered by electroelution.

3' end labeling and analysis

tRNA^{Glu} was 3' end-labeled using (5'-³²P) pCp (2900 Ci/mmol; NEN) and T4 RNA ligase (P-L Biochemicals) at 1.5 units/µg RNA for 16 hours at 4°C. 3' end-labeled tRNA^{Glu} was digested to completion with RNase T2 in a reaction containing 50 mM ammonium acetate, pH 4.5, and carrier yeast tRNA. 3' monophosphate ribonucleotides were added to the T2-digested RNA and the mixture was separated by two-dimensional ascending paper chromatography (22).

5' end labeling and analysis

Unlabeled tRNA^{Glu} was dephosphorylated with 200 units of bacterial alkaline phosphatase (BRL) in a reaction mixture containing 50 mM Tris, pH 8.0, 0.1% SDS by incubation for 45 minutes at 65°C. The tRNA was extracted five times with Tris-saturated phenol, once with phenol/chloroform/isoamyl alcohol, three times with ether, and was precipitated with 2.5 volumes ethanol.

The dephosphorylated RNA was dissolved in 1.7 mM spermidine, heated to 90°C and quick-cooled. The RNA was 5' end-labeled by adding 35 µCi (21 pmols) of (γ-³²P) ATP, 16 units of polynucleotide kinase (P-L Biochemicals), and 5X kinase buffer to make final concentrations of 50 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 0.75 mM spermidine. The mixture was incubated at 37°C for 40 minutes, and SDS and EDTA were added to final concentrations of 1% and 20 mM to stop the reaction. The sample was heated to 100°C, quick-cooled, and was electrophoresed for 17 hours at 1,500 volts. The labeled RNAs were cut out of the gel, electroeluted, and ethanol precipitated.

5' end-labeled tRNA^{Glu} was digested with 1.5 units of RNase P1 (P-L Biochemicals) in 20 mM ammonium acetate, pH 5.3, 10 mM ZnCl₂, 0.1 mg/ml yeast tRNA for 5 hours at

37°C. 5' monophosphate ribonucleotides were added to the digested tRNA and the nucleotides were separated by two-dimensional chromatography on a 20 cm X 20 cm thin-layer cellulose plate (23).

Sequencing

3' or 5' end-labeled tRNA^{Glu} was dissolved in 0.5 mM spermidine, heated to 100°C, quick-cooled, and aliquots of 10,000 cpm were digested using the protocol supplied by P-L Biochemicals. Digestions were carried out with the following enzymes: RNase T1, RNase U2, RNase Phy M, and RNase B. cereus. Digestion with RNase CL3 was in 7 M urea, 10 mM sodium phosphate, pH 6.5, 20 mM KCl, 0.01 mM EDTA, and 0.3 mg/ml yeast tRNA for 12 minutes at 37°C. In all samples the final concentration of spermidine was 0.1 mM. The digested samples were electrophoresed over 0.75 mm x 40 cm, 7 M urea 20% polyacrylamide gels at 1,700 volts, and exposed to X-ray film overnight.

Modified base analysis

Uniformly ³²P-labeled tRNA^{Glu} was digested to completion with RNase T2 in 50 mM ammonium acetate, pH 4.5, and carrier yeast tRNA. The sample was chromatographed following the protocol of Nishimura (24) on a two-dimensional thin layer cellulose system using isobutyric acid : 0.5 M NH₄OH (5:3) in the first dimension and isopropanol : HCl : HOH (14:3:3) in the second dimension.

Melting temperature determination of tRNA^{Glu}-28S hybrids

³²P-pCp 3' end-labeled cytoplasmic RNA was hybridized to a 1 cm² rDNA-DBM paper, washed, and placed in a vial that was temperature regulated in a waterbath. At 5°C increments from 25°C to 100°C, an aliquot of elution buffer (0.1 M NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 20 µg/ml yeast tRNA) was added to the paper. After the temperature equilibrated, the elution buffer was removed, and the RNA was ethanol precipitated and electrophoresed over a 7 M urea 10% polyacrylamide gel. Eluted RNAs were visualized by autoradiography.

RESULTS

Glutamate tRNA hybridizes with 28S ribosomal RNA

To identify RNAs capable of interacting with ribosomal RNA, samples of ³²P-labeled HeLa cell cytoplasmic, nuclear, and nucleolar RNA were hybridized to an excess of unlabeled polysomal RNA, and RNA-RNA hybrids were recovered by hybridization-selection with cloned ribosomal DNA. The clone used was a 7.4 kb EcoRI fragment of human DNA in pBR322 containing 18S, 5.8S, and 28S rRNA-coding regions together with internal transcribed spacer sequences. Figure 1, lanes A, B, and C show the ³²P-labeled starting samples of cytoplasmic, nuclear, and nucleolar RNA, and lanes D, E, and F show the labeled RNAs selected by hybridization. These experiments show two prominent, low molecular weight RNAs selected by the hybridization procedures: 5.8S rRNA, which

Figure 1. tRNA^{Glu} can be hybrid-selected from cytoplasmic RNA.

Three fractions of 3' end-labeled HeLa cell RNA (cytoplasmic RNA, lane A; nuclear RNA, lane B; and nucleolar RNA, lane C) were resolved on a 7M urea 10% polyacrylamide gel. Approximately 30 μ g of each fraction was hybridized to an rDNA-DBM paper, and the selected RNAs were electrophoresed on a second polyacrylamide gel. Scanning densitometry of the autoradiograph of the gel showed that 90% of the tRNA^{Glu} (tGLU) was localized in the cytoplasm (lane D), 10% was localized in the nuclear fraction (lane E), and none was detected in the nucleolar fraction (lane F).

Figure 2. tRNA^{Glu} hybridizes to rRNA.

15 μ g of 3' end-labeled HeLa tRNA was hybridized to an rDNA-DBM paper in the absence (lane A) or presence (lane B) of 10 μ g of unlabeled cytoplasmic RNA. The selected RNAs were electrophoresed on a 7M urea 10% polyacrylamide gel and exposed to X-ray film. This deliberately overexposed autoradiograph shows that tRNA^{Glu} (tGLU) does not hybridize to the cloned rDNA. Lane C contains marker cytoplasmic RNA.

hybridizes directly to the cloned DNA (lanes D and E), and a 4S RNA, which was subsequently identified (see below) as a glutamate tRNA (tGLU). Scanning densitometry of the autoradiograph showed that 90% of the hybridizing tRNA was present in the cytoplasmic fraction, that 10% was present in the nuclear fraction, and that none was detectable in the nucleolar fraction. Since tRNA genes are not present in eukaryotic rDNA clusters, it was thought that this tRNA^{Glu} was being selected by hybridization to the ribosomal RNA which in turn was hybridizing to the rDNA clone.

To determine whether tRNA^{Glu} was being hybrid-selected directly or indirectly by the ribosomal DNA clone, HeLa cell tRNA prepared from the 4S region of a 10% polyacrylamide gel was ³²P-pCp labeled and divided equally between two hybridization solutions, each containing an rDNA-DBM paper. Unlabeled HeLa cytoplasmic RNA was then added to only one solution as a source of ribosomal RNA. After hybridization the papers were washed together, eluted separately, and the RNAs were analyzed by electrophoresis. An autoradiograph of the gel is shown in Figure 2. Labeled tRNA^{Glu} (tGLU) was hybrid-selected only when the cold carrier cytoplasmic RNA was present in the hybridization mixture (lane B), and was not selected in the absence of this added RNA (lane A). Thus, the tRNA^{Glu} in lane B hybridized to the hybrid-selected rRNA and not to the rDNA.

To determine which of the rRNAs being selected by the rDNA clone was responsible for hybrid-selecting tRNA^{Glu}, a similar hybridization experiment was carried out using a subcloned Bam HI fragment of rDNA containing only 28S rRNA sequence. The results obtained with the subclone were identical to those obtained with the larger 7.4 kb DNA, indicating that tRNA^{Glu} was hybridizing to 28S rRNA (data not shown). To confirm this, Northern analysis was carried out in which purified tRNA^{Glu} was hybridized to 18S and 28S rRNAs. Two samples containing glyoxylated HeLa cell polysomal RNA were electrophoresed on an agarose gel along with DNA markers (Figure 3). The lambda DNA marker lane (A) and one lane of polysomal RNA (B) were stained with acridine orange. The second lane of polysomal RNA was transferred to nitrocellulose and the blot was hybridized with ³²P-labeled, hybrid-selected gel-purified tRNA^{Glu}. The autoradiograph (lane C) shows that the labeled tRNA^{Glu} hybridized to 28S, but not to 18S rRNA.

Sucrose gradient centrifugation was also used to demonstrate specific hybridization between tRNA^{Glu} and 28S rRNA. Uniformly ³²P-labeled cytoplasmic RNA was incubated in hybridization salts at 42°C. After hybridization, the solution was layered on a sucrose gradient made with formamide-wash salts and was centrifuged at 34°C to duplicate the wash conditions. The amount of tRNA^{Glu} in the sucrose gradient fractions around the 28S peak (Figure 4A) was quantitated by collecting the RNA from each fraction and analyzing it by electrophoresis. After autoradiography (Figure 4B), the relative amount of tRNA^{Glu} (tGLU) in each lane was determined by scanning densitometry. These values

Figure 3. Northern hybridization of polysomal RNA with purified tRNA^{Glu}.

20 μ g of glyoxylated HeLa cell polysomal RNA was electrophoresed on a 1% agarose gel and stained with acridine orange (lane B). A duplicate lane of unstained RNA was blot transferred to nitrocellulose in 20X SSC, hybridized with 125,000 cpm of 32 P-pCp 3' end-labeled tRNA^{Glu}, and exposed to X-ray film (lane C). Lane A contains Hind III digested lambda DNA marker.

show that the amount of tRNA^{Glu} in each fraction is proportional to the amount of 28S rRNA (Figure 4A).

Stability of the glutamate tRNA-28S rRNA hybrids

The stability of the interaction between tRNA^{Glu} and 28S rRNA was determined from the melting temperature of tRNA^{Glu}-28S hybrids. 32 P-labeled HeLa cytoplasmic RNA hybridized to an rDNA-DBM paper was washed and then eluted in 0.1 M NaCl by raising the temperature from 25°C to 100°C in a stepwise manner. After temperature equilibration, the eluate was removed and the process was repeated at each temperature increment. The samples were electrophoresed and the amount of tRNA^{Glu} (tGLU) at each step was visualized by autoradiography (Figure 5A). These results show that tRNA^{Glu}-28S hybrids melt between 65°C and 80°C. The melting temperature (T_m) for the tRNA^{Glu}-28S

rRNA hybrids, determined by scanning densitometry of the gel lanes, was found to be 70°C (Figure 5B). 5.8S rRNA can be seen (Figure 5A) melting from the DNA over the temperature range of 65°-100°C. It should be noted that 5.8S-28S hybrids are actually less stable than tRNA^{Glu}-28S rRNA hybrids, and as a result, any 5.8S which hybridized to 28S rRNA was lost during the washing procedure (see Discussion).

Sequence analysis of tRNA^{Glu}

tRNA^{Glu} was purified by hybridization-selection and was 3' end-labeled with ³²P-pCp or 5' end-labeled with ³²P-ATP. The labeled RNA was partially digested with RNases T1, U2, Phy M, B. cereus, or CL3 and analyzed on sequencing gels. The 5' terminal nucleotide was determined by digestion with nuclease P1 followed by two-dimensional thin-layer chromatography, and the 3' terminal nucleotide was determined by digestion with RNase T2 followed by two-dimensional paper chromatography. The results of these procedures gave rise to the sequence shown in Figure 6.

Modified bases were identified by two-dimensional thin-layer chromatography of uniformly ³²P-labeled tRNA^{Glu} digested with RNase T2. The major bases were identified as ψp (2 moles), m⁵Cp (2 moles), m²Gp (1 mole), pUp (1 mole), and Tmψp (1 mole). Dp could not be positively identified by chromatography because it migrated between Tmψp and Up, but was assigned to position 20 (Figure 6) because Phy M and B. cereus

Figure 4. Sucrose density gradient resolution of tRNA^{Glu}-28S rRNA hybrids.

Uniformly ³²P-labeled cytoplasmic RNA was hybridized for 24 hours at 42°C, layered on a 15-30% sucrose gradient in formamide-wash salts, and centrifuged at 82,700 x g for 20 hours, 34°C. (A) Fractions of 1.5 ml were collected and aliquots from each were counted (open circles). Unlabeled cytoplasmic RNA was processed identically except that the absorbance at 260 nm of each fraction was plotted (closed circles). (B) Aliquots from the sucrose gradient fractions around the 28S peak were precipitated with ethanol, and the RNA was recovered, heat denatured, and electrophoresed on a 7M urea 10% polyacrylamide gel. The tRNA^{Glu} (tGLU) released from 28S rRNA was quantitated by scanning densitometry, as shown by the triangles in (A).

B

Figure 5. Thermal stability of tRNA^{Glu}-28S rRNA hybrids.

³²P-labeled cytoplasmic RNA hybridized to an rDNA paper was eluted in stepwise 5°C increments from 25° to 100°C in buffer containing 0.1M NaCl. (A) The eluted RNAs were recovered at each step and resolved by electrophoresis. The autoradiograph shows that the majority of the tRNA^{Glu} melts from rRNA between 65°C and 80°C. The 5.8S rRNA melting at the higher temperatures was hybridized directly to DNA on the filter. Lane M contains cytoplasmic marker RNAs. (B) The relative amount of tRNA^{Glu} in each gel lane was determined by scanning densitometry, and the percent released at each temperature increment was calculated. The graph indicates a melting temperature of 70°C.

ribonucleases were blocked at this site, and because doublets (25) were produced only in the partial alkaline hydrolysis lane starting at that position. The assignment of ψ p to positions 13, 20:A, and 55 was determined by the characteristic resistance to RNase Phy M but not to the *B. cereus* enzyme (26,27) and by the lack of cleavage with RNase CL3. Tm was assigned to position 54 because of the absence of a band at this site in the alkaline hydrolysis ladder (25,26), the absence of Tp but the presence of Tm ψ p in the

Under the hybridization-selection conditions used in these experiments tRNA^{GluCUC} is essentially pure, and this has permitted its unambiguous identification by sequence analysis. The tRNA was also identified by aminoacylation (32) and therefore is likely to

Figure 7. Base-pairing models for a possible tRNA^{Glu}-28S rRNA interaction.

(A) Complementary nucleotide sequences located by computer analysis forming the most stable tRNA^{Glu}-28S rRNA base-paired structure. (B) Additional base-pairing identified by inspection of the flanking regions in tRNA^{Glu} and 28S rRNA on both sides of the computer-generated site. Four published 28S rRNA sequences were searched and found to contain regions of complementarity: 1) human (33), 2) mouse (34,35) (2039-2054 in A, and 2039-2067 in B), 3) rat (36) (2126-2141 in A, and 2126-2154 in B), and 4) rat (37) (2113-2128 in A, and 2113-2141 in B). The rat 28S sequence of Hadjiolov et al. (37) is noted to contain a C, equivalent to position 2275 (asterisk) in human 28S rRNA, which would produce an additional mismatched pair. Mouse and rat 28S sequences contain C's equivalent to positions 2280 and 2281 (double asterisk) in human 28S rRNA. The 28S sequence shown is that of human.

be functional in vivo. tRNA^{Glu} has been isolated by hybridization-selection not only from HeLa cells, but also from human placenta, mouse Friend cells, and mouse liver (32). However, polysomal RNA isolated from HeLa cells under non-denaturing conditions showed

Table 1. Calculated and observed stabilities for several tRNA-RNA interactions.

tRNA	base-pairs	ΔG kcal	% GC	Na ⁺ mM	calc	T _m °C obs
tRNA ^{Glu} (A)	15/16	-49.1	81	100	69.9	70
tRNA ^{Glu} (B)	26/31	-51.8	--	---	---	
tRNA _f ^{Met}	16/17	-46.6	71	600	81.3	75
a _t RNA ^{Pro}	19/19	-61.7	68	10	57.4	76
b _t RNA ^{Pro}	18/18	-60.9	72	10	56.9	
a _t RNA ^{Trp}	17/17	-48.5	65	50	63.4	76
b _t RNA ^{Trp}	18/18	-50.6	61	50	64.0	

Free energy values were calculated for the two tRNA^{Glu}-28S rRNA base-pairing models (Figure 7, A and B) and for the tRNA_f^{Met}-23S rRNA interaction (15). The free energies for the interactions between the primer tRNA^{Pro} and tRNA^{Trp} and their respective retroviral RNA genomes were calculated for (a) experimentally determined (41) and (b) hypothetical (53) base-pairing models. T_m's were calculated (calc) using formulas described in the text and values given in the columns headed by base-pairs, Na⁺, and %GC. Observed melting temperatures (obs) were obtained from the literature: tRNA_f^{Met} (15), tRNA^{Pro} (41), and tRNA^{Trp} (10).

no enrichment for tRNA^{Glu} (32) suggesting that it might not interact with 28S in the cell; or that it does so only transiently or in a cell type not yet examined.

Site of interaction in 28S rRNA

A human (33), mouse (34,35), and two rat (36,37) 28S rRNA sequences were searched for complementarity to tRNA^{Glu} using an International Biotechnologies, Inc. forward homology matrix sequence analysis program. This analysis revealed that regions of complementarity of greater than 12 base-pairs in length do not exist in any of the four rRNA sequences; however, a 16 nucleotide region that contains one mismatch was identified in the human, the mouse, and in one of the rat (36) sequences (Figure 7A). The rat sequence of Hadjiolov et al. (37) contains a second mismatch which corresponds to position 2275 (asterisk) in human 28S rRNA (position 2052 in mouse).

Further inspection of this region revealed the possibility for the formation of additional base-pairs, to position 20 of tRNA^{Glu} (Figure 7B). Using thermodynamic parameters for calculating RNA-RNA base-pairing interactions (38-40) ΔG values of -49.1 kcal (Figure 7A) and -51.8 kcal (Figure 7B) were obtained (Table 1) for the two model structures (A and B) involving human 28S. Thermodynamic values were also computed for the interaction between tRNA_f^{Met} and 23S rRNA (15) and the interaction of two primer tRNAs (mouse tRNA^{Pro}, 41; and avian tRNA^{Trp}, 10) with their respective retrovirus

genomes (Table 1). The values obtained for tRNA^{Glu} are within the range of those seen for the other interactions.

The expected T_m for each of the interactions was calculated using a formula for DNA-DNA duplexes (42). Comparisons between RNA-RNA and DNA-DNA duplexes (43,44) show that the T_m can be 7°C to 15°C higher for RNA duplexes, depending on the GC composition. The upper value of 15°C was chosen for these calculations because of the relatively high GC content (61-81%) of the RNA duplexes (Table 1). With these assumptions, the calculated T_m for tRNA^{Glu} is almost identical to the observed T_m and predicts that the 15 base-pair model would be stable up to 70°C. It should be noted, however, that these calculations do not take into consideration the mismatched pair within the tRNA^{Glu}-28S site, which would lower the T_m , nor the additional base-pairs that flank this site, which would raise the T_m . Calculated T_m 's for the other tRNAs range from 19.1°C below to 6.3°C above the observed T_m 's.

DISCUSSION

In vitro hybridization-selection has provided evidence for Watson-Crick base-pairing between human 28S rRNA and tRNA^{Glu}CUC. Base-pairing interactions have also been observed for other tRNAs, for example, tRNA^{Met} with 23S rRNA (15), tRNA^{Pro} with 28S rRNA (16), and primer tRNAs with retrovirus genomic RNA (8,10). tRNA^{Glu} was shown to hybridize to 28S rRNA using three independent approaches: hybridization-selection, Northern hybridization, and solution hybridization followed by sucrose gradient centrifugation under partially denaturing conditions. While these experiments showed tRNA^{Glu} hybridizing preferentially to 28S, we cannot rule out the possibility of a less-efficient, less-stable hybridization to 18S rRNA (45).

Since tRNA^{Glu} was the only tRNA hybridizing with 28S under these conditions, it proved to be relatively simple to purify for sequencing. Human tRNA^{Glu}CUC was found to have the same nucleotide sequence as a rat tRNA^{Glu}CUC gene sequence which has a C at position 4 (46), and our analysis of mouse tRNA^{Glu}CUC suggests that it, too, has the same sequence (32). The thymidine found in the T-ψ-C loop of most tRNAs is further modified to 2'-O-methylated T (Tm) in human tRNA^{Glu}CUC. The only other tRNAs known to contain Tm are rat tRNA^{Glu}UUC (28) and the tRNA^{Lys} isoacceptors of rabbit and mouse (47), rat (48), and Drosophila (49). Based on this information, it is likely that both isoacceptors of tRNA^{Glu} have Tm at position 54, as with the different isoacceptors of the tRNA^{Lys} family.

Computer analysis located a potential site of interaction for tRNA^{Glu} near the middle of human, mouse, and rat 28S rRNA. The proposed site of interaction in 28S involves a region which lies immediately adjacent, on the 3' side, to a conserved region of secondary structure (33). This site of interaction was shown to have the GC content

and a sufficient number of base-pairs to account for the thermal stability of tRNA^{Glu}-28S hybrids. 28S would base-pair to the central portion of tRNA^{Glu} from position 20 in the D-loop to position 50 in the T-ψ-C stem. Yang and Hwang (45) have reported that a tRNA^{Glu}-28S complex formed under their hybridization conditions could prime the reverse transcription of rRNA. If the tRNA^{Glu}-28S complex identified by us also has priming activity, additional base-pairing of tRNA^{Glu} to 28S might have to take place toward the 3' end of tRNA^{Glu}.

Two sequence regions (residues 39-54 and 63-75) mapped in tRNA^{Pro} by ribonuclease digestion have been postulated to base-pair with 28S rRNA (45). We performed a computer sequence analysis to compare mouse tRNA^{Pro} (50) with mouse 28S rRNA (34,35) to determine whether the complementary sites of interaction in 28S could be identified. The analysis found two regions in 28S rRNA (1172-1183 and 1238-1259) complementary to the two tRNA regions: 35-56 (with four mismatches) and 63-74 (with no mismatches). tRNA^{Pro} was not observed in the hybrid selections reported here because tRNA^{Pro}-28S hybrids reportedly melt at 60°-65°C in 0.1 M salt (16), in contrast to tRNA^{Glu}-28S hybrids which melt at 65°-80°C (Figure 5). Our hybridization and washing conditions, which were more stringent than those used by Yang et al. (16), probably resulted in the loss of tRNA^{Pro}. Likewise, 5.8S rRNA did not remain hybridized to 28S (Figure 4B), due probably to its lower melting temperature (52°C in 50 mM salt) (51). What is more difficult to reconcile is that Yang et al. (16) and Yang and Hwang (45) saw tRNA^{Glu} hybridized to mouse 18S and human 28S, but not to mouse 28S rRNA.

Although the significance of the tRNA^{Glu}-28S rRNA interaction is not at this time understood, tRNAs are known to have unusual properties. The fact that only two tRNAs have been discovered to be primers for retroviruses in mouse (tRNA^{Pro}, 50; and tRNA^{Lys}, 52) and only one tRNA in chicken (tRNA^{Trp}, 8) may imply that there is something unique about these tRNAs. Since it is not known why or how these specific tRNAs obtained this function, the observation by Yang et al. (16) that tRNA^{Pro} hybridizes to mouse 28S rRNA and tRNA^{Trp} hybridizes to chicken 28S rRNA may provide a link between 28S rRNA and primer tRNA function.

All mammalian retroviruses examined thus far use tRNA^{Pro} as their primer tRNA, except for the mouse mammary tumor virus and the human lymphadenopathy associated virus (AIDS virus) which use tRNA^{Lys} (53-55). The aminoacylation data of Yang et al. (16) which suggested that tRNA^{Glu} hybridized with 28S rRNA in humans, and the analysis here which demonstrates that this is the only tRNA species which hybridizes to human 28S rRNA, suggests that tRNA^{Glu} might serve as a primer tRNA for human retroviruses. Evidence in support of this idea has recently been obtained which shows that the primer binding site of an endogenous human retrovirus-like sequence (retrotransposon) matches a tRNA^{Glu}CUC rat gene sequence (56). The rat tRNA gene sequence has now been shown

by us to be identical to human tRNA^{Glu}CUC. This endogenous retrovirus-like sequence has a tRNA primer binding site with an 18 base-pair homology with the 3' end of tRNA^{Glu}.

Endogenous retrovirus-like sequences have primer tRNA binding sites which differ from those found in functional retroviruses: tRNA^{Phe} in mouse and hamster (57), and tRNA^{His} in human (58). Some of these endogenous retrovirus-like sequences are actively transcribed in early embryos and in transformed cell lines (57,59). For example, human fetal thymus cells produce reverse transcriptase and retrovirus-like particles when co-cultured with mitomycin C treated human B-cells (60). The expression of these particles has been associated with the reverse transcription of cellular RNAs which are subsequently reinserted back into the genome (59,61). Further studies will be required to determine if these particles are functional, and if tRNA^{Glu} acts as a tRNA primer for retrovirus-like cDNA transcripts. The interaction of tRNA^{Glu} with 28S rRNA and the possibility for interaction with retrovirus-like transcripts may suggest additional functions for tRNA^{Glu} besides its role in translation.

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