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Mammalian mitochondrial transfer RNAs: chromatographic properties, size and origin

Luke Aujame and Karl B.Freeman*

Department of Biochemistry, McMaster University, Hamilton, Ontario, L8S 4J9, Canada

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ABSTRACT

Incubation of isolated rat liver mitochondria with radioactive amino acids resulted in the charging of tRNAs for arginine, asparagine, leucine, lysine, methionine, proline and valine. The aminoacyl-tRNAs were shown to be distinct from their cytosolic counterparts by chromatography on RPC-5. By electrophoresis on urea polyacrylamide slab gels it was found that all these mitochondrial aminoacyl-tRNAs were about 70-76 nucleotides long. The unique mitochondrial asparaginyl- and prolyl-tRNAs, not previously identified in mammalian cells, were shown to hybridize to mtDNA. Mitochondrial leucyl-tRNA separated into 3 peaks on RPC-5 and the first species was shown to be different than a combination of the other two by molecular size and partial RNase T_1 digestion patterns. Each was coded by a separate gene on mtDNA as shown by partial additivity of hybridization. Separate genes for mitochondrial $tRNA_m^{Met}$ and $tRNA_f^{Met}$, separated by RPC-5 chromatography, were also demonstrated. These results bring to 21 the number of individual tRNAs coded by mammalian mtDNA.

INTRODUCTION

Mitochondria contain a partially autonomous protein synthesizing system, the mtDNA genome coding for only a limited number of gene products not all of which have been defined. In animal cells, these transcription products consist of the two large rRNAs (1-4), up to 18 presumptive poly(A)-containing mRNAs (5) which probably code mainly for protein subunits of different respiratory, and energy conservation complexes of the inner mitochondrial membrane (6,7) and no less than 19 tRNAs (8).

In HeLa cells, 17 specific mitochondrial tRNAs have been shown to hybridize to mtDNA (9) but mitochondrial tRNAs for glutamine, histidine, proline and asparagine were not identified. In mitochondria of the yeast, Saccharomyces cerevisiae, only $tRNA^{asn}$ has not been reported (10) and the number of tRNA genes is now thought to be around 26 (11) if not higher (12) of which 22 have so far been identified (10). The number of tRNAs in both mammalian cells and yeast are lower than the 32 tRNAs required

to read all codons if all are used and read according to the wobble hypothesis (13). There have been a number of suggestions to account for the deficit (11,14,15) but it is possible that it might reflect the limitations of current techniques for charging mitochondrial tRNAs. Most studies have used extracted mitochondrial aminoacyl-tRNA synthetases to label preparations of mitochondrial tRNA (9,10,16) and it is possible that either a synthetase or a tRNA could be inactivated in this procedure. Recently we showed that isolated mitochondria could be used to charge specific tRNAs (17,18). In particular, a mitochondrial asparaginyl-tRNA, whose genetic origin was not determined, was isolated from Chinese hamster ovary cells (19). We have now used this approach to study a number of rat liver mitochondrial tRNAs. Aminoacyl-tRNAs were characterized by RPC-5 chromatography and urea polyacrylamide slab gel electrophoresis. Hybridization data indicated that the rat mitochondrial genome codes for $tRNA^{Met}_{m}$, $tRNA^{Met}_{f}$, $tRNA^{Pro}$, $tRNA^{Asn}$ and two isoaccepting species of $tRNA^{Leu}$. This is the first report showing a mitochondrial origin of $tRNA^{Asn}$ and in mammalian cells of $tRNA^{Pro}$.

MATERIALS AND METHODS

Materials

The source of most materials was given previously (18,19). Immobilized RNase (500 units/gm dry weight) and RNase T_1 (500,000 units/ml) were from Worthington Biochemical Corp., Freehold, NJ. L-2,3-^{3}H-arginine (18.1 Ci/mmol), L-^{14}C(U)-arginine (292 mCi/mmol), L-^{14}C(U)-asparagine (200 mCi/mmol), L-3,4-^{3}H(N)-glutamine (21 Ci/mmol), L-3-^{3}H-histidine (10-25 Ci/mmol), L-4,5-^{3}H(N)-lysine (38-63 Ci/mmol), L-^{14}C(U)-lysine (292 mCi/mmol), L-2,3,4,5-^{3}H-proline (111 Ci/mmol), L-^{14}C(U)-proline (255 mCi/mmol), L-2,3,4-^{3}H-valine (22 Ci/mmol) and L-^{14}C(U)-valine (255 mCi/mmol) were all from New England Nuclear, Boston, MA. L-2,3,-^{3}H-asparagine (21.8 Ci/mmol) was from CEA, Saclay, France. Sarcosyl NL-30 was from Geigy Dyestuffs, Geigy Co., Toronto, Ontario.

Protein Synthesis by Isolated Mitochondria

Rat liver mitochondria were isolated and incubated as described previously (19-21). Incubations were for 30 min at 3 mg mitochondrial protein/ml and 40 µCi ^{3}H-amino acid/ml each at 10 Ci/mmol. The radioactivity in protein of a 80 µl sample was determined as described previously (21).

Preparation and Characterization of Aminoacyl-tRNAs

The cytosolic fraction of rat liver was prepared by homogenization, centrifugation at 30,000 x g and passage of the supernatant through a Sephadex G-25 column as described previously (17). Labelling and extraction of cytosolic and mitochondrial aminoacyl-tRNA, deamination by nitrous acid treatment, chromatography on RPC-5 and urea polyacrylamide slab gel electrophoresis were as described previously (18,19).

Fingerprint Analysis of Mitochondrial Leucyl-tRNA

^{3}H-leucyl-tRNA$_{1}^{Leu}$ and ^{3}H-leucyl-tRNA$_{2+3}^{Leu}$ were recovered after elution from RPC-5 and precipitated with 2.5 vol 95% (v/v) ethanol at -20°C. Each preparation was deaminated as described previously and reprecipitated. Partial RNase T_1 digestion of each species were performed according to the methods of Donis-Keller et al. (22) or Simoncsits et al. (23) and analysed on 20% polyacrylamide slab gels (22) at 800 V for 10 or 14 h. The gels were 30 cm x 13.5 cm and either 0.075 or 0.15 cm thick. Gels were prepared for fluorography as described by Bonner and Laskey (24), except that RNA was fixed in a 7.5% acetic acid solution for 10 min prior to impregnation with 20% 2,5-diphenyloxazole in dimethylsulfoxide. The soaking times of the impregnation process were 25% as long for the 0.075 cm thick gel.

Isolation of Mitochondrial and Nuclear DNA

MtDNA was purified from a four-times washed mitochondrial fraction lysed in 100 mM NaCl/10 mM EDTA/50 mM Tris-HCl, pH 7.5 and 2% sarkosyl NL/30 at 0°C. The solution was made up to 1 M CsCl and centrifuged for 19 h at 38,000 rpm in a Beckman 65 rotor at 20°C. The nucleic acid pellet was resuspended in a small volume of 10 mM EDTA/2% sarkosyl NL-30/10 mM Tris-HCl, pH 8.0 and layered over an ethidium bromide-CsCl step gradient (25). Centrifugation was in a Beckman SW 50.1 rotor at 35,000 rpm for 5 h at 20°C. The lower band of mtDNA was carefully removed under ultraviolet light and ethidium bromide removed by 6 extractions with isoamyl alcohol. MtDNA was recovered by centrifugation, the pellet resuspended in 1/100 SSC and kept frozen until use. The purity of mtDNA was checked from the pattern of restriction fragments obtained with Eco RI and Hind III endonucleases. The patterns observed were similar to those obtained by others (26-28) indicating that both types of rat liver mtDNA were usually extracted.

Nuclear DNA was recovered from a rat liver nuclear pellet by centrifugation as for mtDNA. About 500 µg of DNA was resuspended in a small volume of 10 mM EDTA/10 mM Tris-HCl, pH 8.0 with a bath-type

sonicator and applied to an ethidium bromide step gradient as above. The nuclear DNA was recovered from the top of the gradient and all further steps were as for mtDNA.

Hybridization

The hybridization of ^{3}H-aminoacyl-tRNA to DNA was by modifications of previous methods of others (10,12). MtDNA was denatured in 1/100 SSC in 0.1 N NaOH for 20 min at 22°C. After rapid cooling on ice, the solution was made up to 6x SSC and pH 7.0. Pre-soaked nitrocellulose filters (Schleicher & Schuell, 0.45 µm) were loaded usually with 10 µg mtDNA under slow suction and washed with 10 ml of 6x SSC, pH 7.0. Loaded filters were dried under vacuum at 80°C for at least 4 h and kept in the dark in a dessicator until use. The same procedure was used for nuclear DNA except that it was further sonicated before loading on filters.

Hybridizations were in 2x SSC/0.1% sodium dodecyl sulfate/10 mM sodium acetate, pH 5.0 with either 30% or 50% formamide for 18 h at 33°C in a final volume of 0.3 or 0.5 ml. Blank filters were added to all incubations. After hybridization, the RNA was recovered, filters washed with incubation buffer and left to sit for 30 min at 33°C in the incubation buffer. The filters were then washed thoroughly with 2x SSC, pH 5.0 and incubated in 2x SSC, pH 5.0 with 25 units of RNase T_1/ml at 33°C. After 30 min, the filters were washed again, left in 2x SSC, pH 5.0 for up to 1 h, before final washing under slow suction with 10 ml 2x SSC, pH 5.0 on both sides, drying and counting for radioactivity at 5% efficiency. This procedure gave blanks usually not exceeding 40 cpm. In the case of mitochondrial leucyl-tRNA, where 25 µg mtDNA/filter was used, the RNase T_1 digestion step was omitted. Previous controls indicated that this omission did not affect either background radioactivity or hybridization levels.

Controls with ^{3}H-labelled adenovirus-5 DNA indicated 95-100% retention of DNA after loading and hybridization under the conditions used. As well there was no significant deacylation of ^{3}H-aminoacyl-tRNAs during incubation as determined by acid-precipitable radioactivity.

RESULTS

Charging of tRNAs by Isolated Mitochondria

HeLa cell mitochondrial aminoacyl-tRNA synthetase preparations charge mitochondrial tRNA with all amino acids except asparagine, glutamine, histidine and proline (9). The ability of isolated rat liver mitochondria

to incorporate these amino acids into proteins and into aminoacyl-tRNAs was examined. Incorporation of leucine and methionine as well as the three amino acids whose tRNAs have been suggested to be imported into mitochondria of Tetrahymena pyriformis (14) was also examined. As shown in Table 1, isolated mitochondria incorporated all these amino acids except glutamine into protein. In all cases incorporation was completely inhibited by Tevenel, a known inhibitor of mitochondrial protein synthesis (21). Polyacrylamide slab gel electrophoresis revealed that all the amino acids tested except histidine and glutamine were incorporated into proteins characteristic of mitochondrially-synthesized proteins (29, Aujame and Freeman, unpublished results). Using amino acids of the highest specific radioactivity available, tRNAs for all amino acids except histidine and glutamine could be charged and isolated confirming the protein synthesis results. Generally the extent of charging paralleled the level of incorporation of each amino acid into protein.

Table 1: Incorporation ^{3}H-amino acids into Protein by Isolated Rat Liver Mitochondria

Amino Acid	Incorporation (% Leucine)
Leucine	100
Methionine	32
Proline	20
Lysine	7.3
Arginine	7.3
Valine	5.6
Asparagine	1.8
Histidine	0.5
Glutamine	0.09

Incorporation is expressed as a percentage of ^{3}H-leucine incorporation which was 31,000 cpm/mg protein after a 30 min incubation at 30°C in the presence of 40 µCi ^{3}H-amino acid/ml each at 10 Ci/mmol as described in the Methods Procedures. Zero time controls or incubations in the presence of Tevenel were about 300-400 cpm/mg protein in most cases.

Chromatographic Analysis of Mitochondrial Aminoacyl-tRNAs

In order to distinguish the mitochondrial and cytosolic aminoacyl-tRNAs, they were co-chromatographed on RPC-5 as shown in Figure 1 for methionyl-, leucyl-,arginyl-, asparaginyl-, prolyl- and lysyl-tRNA. In all cases the elution profile of the mitochondrial aminoacyl-tRNA differed

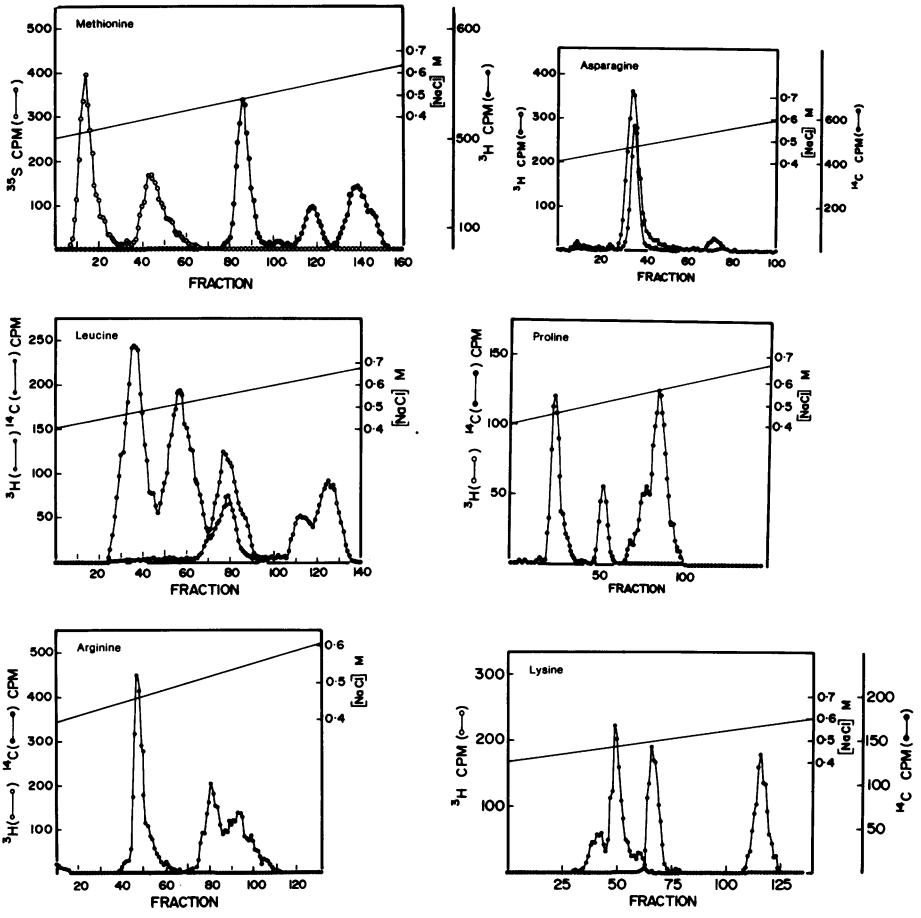

Figure 1. RPC-5 chromatographic profiles of rat liver mitochondrial aminoacyl-tRNAs co-chromatographed with cytosolic aminoacyl-tRNAs. ^{3}H-Mitochondrial and ^{14}C-cytosolic rat liver aminoacyl-tRNAs were prepared as described in the methods. Elution gradients were 0.4 to 0.7 M NaCl except for leucyl-tRNA where the gradient was 0.45 to 0.7 M NaCl. Except for mitochondrial methionyl- and leucyl-. tRNAs, mitochondria were incubated in the presence of 0.1 units of immobilized RNase/ml (19). The running temperature was 22°C except for asparaginyl-tRNA when it was 18°C. o-o, Mitochondrial aminoacyl-tRNA; ●-●, cytosolic aminoacyl-tRNA.

from its cytosolic counterpart.

Mitochondrial methionyl-tRNA separated into two broad peaks eluting at salt concentrations lower than the three cytosolic species. Analysis of the mitochondrial peaks for terminal methionyl-adenosine or formylmethionyl-adenosine as described previously (17) demonstrated that the first peak consisted of methionyl-tRNA ($tRNA_m^{Met}$) while the second peak was formylmethionyl-tRNA ($tRNA_f^{Met}$). The same order of elution was found for mouse liver mitochondrial methionyl-tRNAs by Wallace and Freeman (17).

Mitochondrial leucyl-tRNA appeared as three peaks eluting after the bulk of the cytosolic species which separated into three major peaks with shoulders on either side of the first peak. The mitochondrial elution profile was similar to that obtained with Syrian hamster mitochondrial leucyl-tRNA (18). Mitochondrial lysyl-tRNA separated into three peaks and prolyl-tRNA into two peaks while only one peak was observed for each of arginyl- and asparaginyl-tRNA. A greater separation of mitochondrial from cytosolic asparaginyl-tRNA was obtained at a slightly higher temperature but the peaks were broader. In these experiments all the mitochondrial aminoacyl-tRNAs except those for leucine and methionine were extracted after incubation of isolated mitochondria with immobilized RNase (19). If the digestion step was not included, the proportion of contaminating labelled cytosolic aminoacyl-tRNA was significant, as shown in Figure 2A for mitochondrial valyl-tRNA. Comparison of Figures 2A and 2B indicates that RNase digestion removed all cytosolic contamination without affecting the mitochondrial valyl-tRNA.

Since mammalian mitochondrial asparaginyl-tRNA and prolyl-tRNA had not

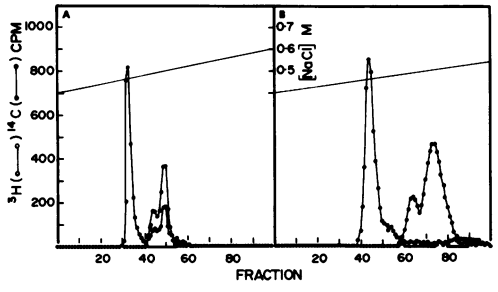

Figure 2. RPC-5 chromatography of mitochondrial (o-o) and cytosolic (•-•) valyl-tRNA.
Mitochondria were incubated with ^{3}H-valine in the absence of (A) or presence (B) of 0.1 units of immobilized RNase/ml and ^{3}H-valyl-tRNA isolated as described previously (19). RPC-5 chromatography of mitochondrial ^{3}H- and cytosolic ^{14}C-valyl-tRNAs were as described in the methods.

been reported previously, these aminoacyl-tRNAs were deacylated and the amino acids identified by paper chromatography (19). Only the amino acids used for charging were recovered (results not shown).

Urea Polyacrylamide Slab Gel Electrophoresis of Aminoacyl-tRNAs

We had previously shown that the size of deaminated aminoacyl-tRNAs (HNO_2-aminoacyl-tRNA) could be determined by electrophoresis in the urea polyacrylamide slab gel system of Maniatis et al. (30). Results of this type of analysis, using yeast HNO_2-^{3}H-leucyl-tRNA as a marker of 85 nucleotides (slot a), are shown in Figure 3. All mitochondrial HNO_2-aminoacyl-tRNAs had faster mobilities than their cytosolic counterparts, the mobilities corresponding to a length of 76 nucleotides or slightly shorter. The only exception was HNO_2-^{3}H-lysyl-tRNA whose apparent length was about 70 nucleotides as seen in Figure 3A, slot i. HNO_2-^{3}H-leucyl-tRNA in Figure 3A, slot c was the only mitochondrial tRNA to separate into two bands, both of which ran faster than all but one of the cytosolic species.

The faster electrophoretic mobility of the mitochondrial aminoacyl-tRNAs confirms that they are different from their cytosolic counterparts as shown by RPC-5 chromatography. Further the results support our suggestion (18) that the mitochondrial tRNAs are of sufficient length to fit the "clover-leaf" model structure. As pointed out previously (18) the gel system does not entirely differentiate on the basis of size; a slight base composition difference can account for variations in mobility as is evident from the separation of the yeast leucyl-tRNAs (Figure 3, slot a) which are the same length (31,32). Thus the slightly higher mobility of many mitochondrial tRNAs including $tRNA^{Lys}$ does not necessarily reflect a smaller size compared to their cytosolic counterparts.

Fingerprint Analysis of Mitochondrial Leucyl-tRNAs

The electrophoretic separation of mitochondrial $tRNAs^{Leu}$ suggested that the isoaccepting species had distinct sequences. This was examined by obtaining sequence information applying the new RNA sequencing methodology to HNO_2-leucyl-tRNAs (22,23). Radioactive leucine at the 3'-end of the tRNA served as a specific marker of each fragment and deamination insured stability of the leucyl-oligonucleotide bond during electrophoresis. Leucyl-tRNAs were separated by chromatography on RPC-5 and analyzed separately. The first peak to elute ($tRNA_1^{Leu}$) had the faster electrophoretic mobility while the other two peaks ($tRNA_2^{Leu}$ and $tRNA_3^{Leu}$) ran as a single band of slower mobility. Figure 4 shows the partial RNase T_1

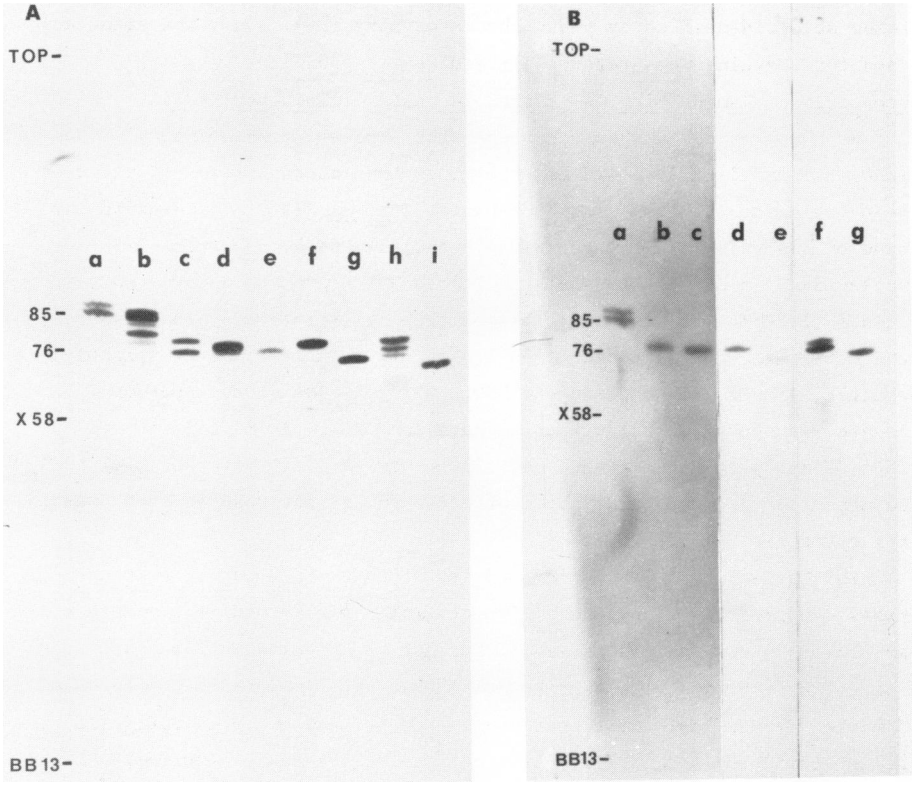

Figure 3. Slab gel electrophoretic analysis of cytosolic and mitochondrial HNO_2-^{3}H-aminoacyl-tRNAs.
Cytosolic and mitochondrial ^{3}H-aminoacyl-tRNAs were deaminated, resuspended in 98% formamide, brought to boiling for 2 min and cooled rapidly before applying to the gels. Electrophoresis was for 5.5 h at 10V/cm as described previously (18,19). The marker dyes were X, xylene cyanol ff and BB, Bromophenol Blue. The numbers refer to nucleotide length. In A and B slot a is yeast cytosolic HNO_2-^{3}H-leucyl-tRNA. The cellular origin and amino acid of the rat liver HNO_2-^{3}H-aminoacyl-tRNAs for each of the other slots are:
A. b, cytosolic, leucine; c, mitochondria, leucine, d, cytosol, methionine; e, mitochondria, methionine; f, cytosol, valine, g, mitochondria, valine; h, cytosol, lysine; i, mitochondria, lysine.
B. b, cytosol, asparagine, c, mitochondria, asparagine, d, cytosol, proline, e, mitochondria, proline, f, cytosol, arginine,; g, mitochondria, arginine.

digests of HNO_2-^{3}H-leucyl-$tRNA_1^{Leu}$ in slots a and b and HNO_2-^{3}H-leucyl-$tRNA_{2+3}^{Leu}$ in slots c and d analysed on 20% polyacrylamide slab gels.
The assumption was made that $tRNA_2^{Leu}$ and $tRNA_3^{Leu}$ differed only in their

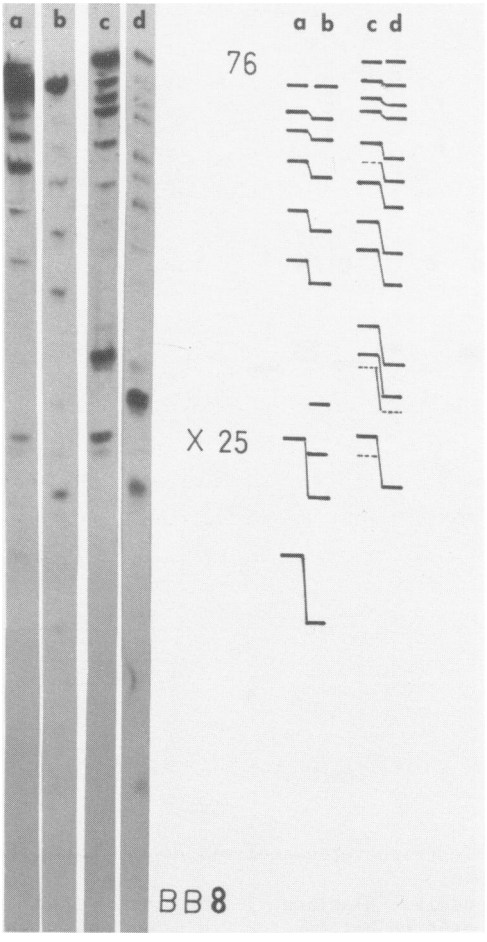

Figure 4. Fluorograms of partial RNase T_1 digests of mitochondrial HNO_2-^{3}H-leucyl-tRNAs.
Leucyl-$tRNA_1^{Leu}$ (slots a and b) are leucyl-$tRNA_{2+3}^{Leu}$ (slots c and d) were separated by chromatography on RPC-5, deaminated and partially digested with RNase T_1 as described in the methods. Samples in slot a and c were run on the same 0.05 cm thick gel for 14 h. Samples in slot b and d were run on a 0.075 cm thick gel for 10 h. Partial digests were performed as described by Donis-Keller et al. (22) except for slot d where the method of Simoncsits et al. (23) was used. Samples of approximately 8,000 cpm were used and exposure was for up to 45 days. A diagramatic representation of the gel patterns is shown to the right since fainter bands were not reproduced clearly. Bands which are either not present in one of the gels or could represent artifacts are indicated by dashed lines.

modified bases rather than in nucleotide sequence. If this were true then the number of bands obtained from RNase T_1 digests should be equivalent to that expected from one species. About 15 G residues would be expected for a tRNA with a G + C content of 43% as in rat liver mitochondrial tRNAs (33). Since no bands are seen below a length of 20-25 nucleotides, the number of bands (9 for $tRNA_1^{Leu}$ and up to 11 for $tRNA_{2+3}^{Leu}$) indicates that the tRNAs had been cleaved at 11 G residues and that the equivalent of only one species was present in $tRNA_{2+3}^{Leu}$. It is not known why rapidly migrating bands were not present. The band patterns of $tRNA_1^{Leu}$ and of $tRNA_{2+3}^{Leu}$ are nevertheless quite distinct. The tRNAs consequently differ in their sequences and must be coded by two different genes.

Hybridization of Mitochondrial Aminoacyl-tRNAs to mtDNA

The hybridization curves for methionyl-, leucyl-,prolyl- and asparaginyl-tRNA to mtDNA are shown in Figure 5. Assuming complete hybridization the saturation levels indicated that most aminoacyl-tRNAs had a low specific activity. In the case of leucyl-tRNA, up to 25 µg of mtDNA per filter had

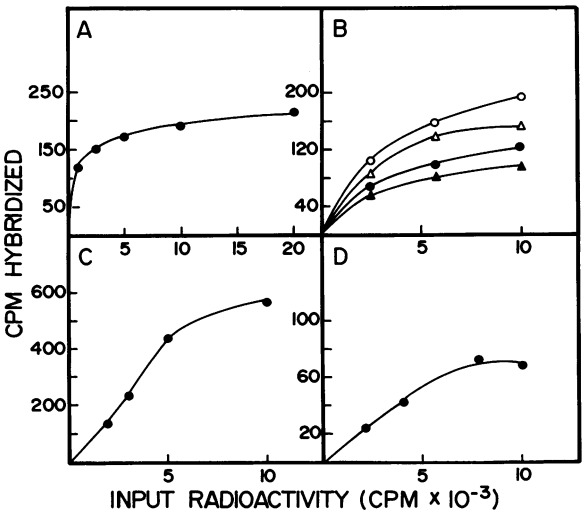

Figure 5. Hybridization curves of mitochondrial aminoacyl-tRNAs to mtDNA. Isolation of aminoacyl-tRNAs and hybridization conditions were as described in the methods. A, ^{35}S-methionyl-tRNA; B, ^{3}H-leucyl-tRNAs, o-o total unfractionated ^{3}H-leucyl-tRNA, ●-●, HNO_2-^{3}H-leucyl-$tRNA_1^{Leu}$, ▲-▲, HNO_2-^{3}H-leucyl-$tRNA_{2+3}^{Leu}$, Δ-Δ, HNO_2-^{3}H-leucyl-$tRNA_1^{Leu}$ + HNO_2-^{3}H-leucyl-$tRNA_{2+3}^{Leu}$; C,^{3}H-prolyl-tRNA; D, ^{3}H-asparaginyl-tRNA.

to be used to obtain levels of hybridized radioactivity significantly above background. The mitochondrial origin of the tRNAs was further corroborated by the lack of hybridization to nuclear DNA and by the lack of hybridization of cytosolic aminoacyl-tRNAs to mtDNA, as summarized in Table 2.

Nass and Buck (16) had already shown that rat liver mtDNA codes for leucyl-tRNA. To confirm the existence of at least two genes for mitochondrial leucyl-tRNA, suggested by the RNase T_1 digest results, leucyl-$tRNA_1^{Leu}$ and leucyl-$tRNA_{2+3}^{Leu}$ were hybridized to mtDNA, separately or together. As shown in Figure 5 there was partial additivity of hybridized radioactivity. With methionyl-tRNA, a similar experiment summarized in Table 2 showed complete additivity indicating that there is at least one gene for each of $tRNA_m^{Met}$ and $tRNA_f^{Met}$.

Table 2: Hybridization of Mitochondrial and Cytosolic Aminoacyl-tRNAs to mtDNA and Nuclear DNA

Source	tRNA	Input aminoacyl-tRNA (cpm)	mtDNA 10µg (cpm)	mtDNA 25 µg (cpm)	Nuclear DNA 100 µg (cpm)	Blank Filter (cpm)
Mitochondria	Methionine					
	Total	10,000			-10	93
		10,000	198			45
	$tRNA_m^{Met}$	10,000[a]	120			
	$tRNA_f^{Met}$	10,000[a]	118			
	$tRNA_m^{Met}$ + $tRNA_f^{Met}$	10,000[a]	222			
Mitochondria	Leucine					
	Total	5,000			6	26
		5,000	48	160		15
		10,000	75	194		29
	$tRNA_1^{Leu}$	5,000[abc]		104		19
		10,000[abc]		126		22
	$tRNA_{2+3}^{Leu}$	5,000[abc]		83		5
		10,000[abc]		101		14
	$tRNA_1^{Leu}$ + $tRNA_{2+3}^{Leu}$	5,000[abc]		141		15
		10,000[abc]		151		25
Mitochondria	Asparagine	8,000	71			35
		6,600			8	40
Cytosol	Asparagine	8,000	-4		28	29
Mitochondria	Proline	2,000	140			40
		8,000	431			65
Cytosol	Proline	2,000			21	64
		1,000	2			25

Individual aminoacyl-tRNAs were either hybridized directly or separated by chromatography on RPC-5 ([a]) and in some cases the aminoacyl moiety deaminated with nitrous acid ([b]) before hybridization. Conditions of hybridization were as described in the experimental procedures. In some cases RNase T_1 digestion was omitted following hybridization ([c]). Levels of hybridization are expressed as cpm bound per filter with the cpm of the blank filters subtracted.

DISCUSSION

The major aim of this study was to determine whether mammalian mtDNA codes for tRNAs corresponding to each of the common amino acids. The identification of mitochondrial $tRNA^{Asn}$ and $tRNA^{Pro}$ and the demonstration that they hybridize to mtDNA, together with the 16 tRNAs for different amino acids previously shown to hybridize to mammalian mtDNA (9), bring this number to 18. The detection of the two new aminoacyl-tRNAs possibly reflects the advantage of using isolated mitochondria rather than extracted aminoacyl-tRNA synthetases and tRNAs (9,34) to charge tRNAs. The demonstration of a mitochondrial $tRNA^{Asn}$ together with tRNAs for the 19 other amino acids found in yeast (10) makes it probable that the mitchondrial genome codes for tRNAs for all amino acids in all organisms studied so far, with the possible exception of T. pyriformis (14).

Neither histidinyl-tRNA nor glutaminyl-tRNA could be recovered from isolated mitochondria incubated in the presence of the corresponding radioactive amino acid and the incorporation of these amino acids into protein was very low. A very low but significant incorporation of glutamine into protein by HeLa mitochondria, either isolated (35) or in whole cells (15) has been observed. Transport and/or internal pool effects (36) could explain all of these results. However, it may be that in mammalian mitochondria the process of aminoacylation of $tRNA^{Gln}$ is similar to that found in yeast mitochondria (37) in which this tRNA is first aminoacylated with glutamate followed by amidation to form glutaminyl-$tRNA^{Gln}$.

In addition to tRNAs for each common amino acid, isoaccepting species are necessary to read all codons during initiation and propagation in protein synthesis. The observation that $tRNA_m^{Met}$ and $tRNA_f^{Met}$ are transcribed from distinct sequences in rat liver mtDNA extends the results obtained with HeLa cells in which hybridization of $tRNA_m^{Met}$ to mtDNA was not examined (9) and confirms those in yeast (38). Both the hybridization and RNase T_1 digest results indicate there are two isoaccepting $tRNAs^{Leu}$ coded for by two distinct genes. The hybridization was partly additive suggesting that the two genes might have common sequences or overlap in part. The presence of two genes for $tRNA^{Leu}$ seems to be a general feature of mtDNA including that from T. pyriformis (39), Locusta migratoria (40), Neurospora crassa (41) and possibly yeast (10,12,42,43). The other tRNAs characterized here probably consist of species coded by one gene each as in most cases only one peak on RPC-5 and one electrophoretic band were

observed. The results reported here add 4 tRNAs ($tRNA^{Asn}$, $tRNA^{Pro}$, $tRNA_m^{Met}$ and a $tRNA^{Leu}$) to the 17 found previously to hybridize to mammalian mtDNA. Angerer et al. (8) found 19 sites for tRNAs on HeLa mtDNA by electron miscroscopy of hybridized ferritin-labelled aminoacyl-tRNAs whereas Dawid et al. (44) with a similar technique found 21-22 sites on Xenopus laevis mtDNA. If tRNAs for glutamine and histidine are eventually found, it would bring the numer of tRNAs coded by mammalian mtDNA to 23. Further definition of the number of tRNAs coded by mammalian mtDNA must involve hybridization of individual tRNAs to restriction fragments of mtDNA (cf. 11,26,27) and perhaps eventually genetic studies.

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*To whom correspondence should be addressed.

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