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Kinetoplastid mitochondria contain functional tRNAs which are encoded in nuclear DNA and also contain small minicircle and maxicircle transcripts of unknown function

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ABSTRACT

The mitochondrion of *Leishmania tarentolae* contains approximately 35−40 tRNAs, many of which comigrate with cytoplasmic tRNAs. Both mitochondrial (KtRNA) and cytoplasmic (CtRNA) tRNAs are functional, as they could be acylated either by mitochondrial or cytoplasmic synthetase extracts. There are two methionyl tRNA species in the cytoplasmic and mitochondrial fractions, one of which is unique to each fraction, indicating that the KtRNA fraction is free of CtRNA contamination. Leucyl and glycyl tRNAs were identified by hybridization with a genomic clone from *Trypanosoma brucei*. KtRNA hybridizes with nuclear chromosomes, but not with minicircle or maxicircle DNA. KtRNA isolated by DEAE chromatography or agarose gel electrophoresis contains additional small RNAs which hybridize with both minicircle and maxicircle DNA. These transcripts do not migrate like tRNAs in acrylamide gels and their function is unknown. We suggest that most if not all mitochondrial tRNAs in *L. tarentolae* are nuclear-encoded and imported into the mitochondrion.

INTRODUCTION

The kinetoplast DNA of the kinetoplastid protozoa consists of 20−50 catenated maxicircle DNA molecules of 20−36 kb and a network of approximately 10^4 catenated minicircle DNA molecules varying in size in different species from 465 to 2500 bp (1,2). The minicircle molecules are composed of a conserved region and a variable or unique region, and the molecules within a single network consist of a variable number of different sequence classes. Minicircle transcripts of unknown function have been described (3): RNAs approximately 240 nt in size which hybridized to the conserved region of the minicircle DNA were isolated from both procyclic and blood stream *Trypanosoma brucei*. The function of these minicircle-specific transcripts is unknown; suggested functions include the encoding of a putative structural gene, the priming of minicircle DNA replication, and acting as a structural determinant of the kDNA nucleoid body (3).

The maxicircle DNA is the functional equivalent of the animal or fungal mitochondrial genomes, since this DNA encodes several standard mitochondrial respiratory chain proteins−three subunits of cytochrome oxidase (COI, COII, COIII), three subunits of NADH dehydrogenase (ND1−3), apocytochrome b (Cyb), four unidentified open reading frames (MURF1−4) and two small rRNAs (9S and 12S RNAs) (4,5). At least five of the 13 maxicircle genes are 'cryptogenes', the transcripts of which are edited to produce translatable sequences by specific addition and deletion of uridine residues (See ref. 6 for terminology and review of RNA editing).

The question of the existence of maxicircle or minicircle-encoded tRNAs is unresolved (7−9). We have approached this question by direct isolation and characterization of functional mitochondrial tRNAs from *Leishmania tarentolae* and determination of their

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transcriptional origin. In the course of this work we found that the mitochondrial tRNA fraction is contaminated with non-tRNA transcripts of unknown function derived from both minicircle and maxicircle DNA. The purified mitochondrial tRNAs, on the other hand, showed no hybridization to minicircle or maxicircle DNA and did hybridize with nuclear DNA, suggesting a nuclear transcriptional origin.

MATERIALS AND METHODS

Cell culture

L. tarentolae (UC strain) cells were grown in Difco Brain-Heart Infusion medium at 27°C as described previously (9). Log phase cells were used for synthetase isolations and isolation of tRNAs.

Isolation of kinetoplast-mitochondrial fraction

This was performed by isopycnic flotation in Renografin density gradients after hypotonic cell rupture through a #27 needle at 100 psi as described previously (11−12). The final mitochondrial fraction was washed several times at 4°C in 0.25 M sucrose, 0.01 M Tris HCl (pH 7.9), 3 mM EDTA to attempt to remove any contaminating cytoplasmic ribosomes.

Isolation of tRNA and aminoacyl tRNA synthetases

Mitochondrial RNA was isolated from a purified kinetoplast-mitochondrial fraction by SDS lysis, phenol-chloroform extraction and ethanol precipitation as described (12). The kinetoplast tRNA (KtRNA) was isolated either by electrophoresis in 10% acrylamide-7M urea or by DEAE chromatography on a Nucleogen 500/10 HPLC column using a salt gradient of 0.24 M KCl to 1.0 M KCl for elution. The buffer for the DEAE chromatography was 0.02 M potassium phosphate, pH 6.8, 5 M urea. The tRNA was eluted in the first two peaks. Cytoplasmic tRNA (CtRNA) was isolated from the supernate of a hypotonic cell lysate after addition of 0.25 M sucrose and removal of the kinetoplast fraction by low speed centrifugation. The CtRNA was recovered and purified in an identical manner to the KtRNA.

The DEAE tRNA fractions were in some cases further purified by electrophoresis in 10% acrylamide-7M urea; the main ethidium bromide-staining tRNA fraction was eluted and labeled KF1 for the KtRNA fraction and CF1 for the CtRNA fraction.

Crude aminoacyl synthetase fractions were isolated from cytosolic and mitochondrial fractions by standard methods (13). The cytoplasmic enzyme was isolated by precipitation with 5% streptomycin, 40−70% ammonium sulfate fractionation, and hydroxyapatite fractionation (150 mM KCl eluted fraction), and concentration. The mitochondrial enzyme was isolated by solubilization of a purified mitochondrial fraction by sonication or homogenization with 0.5% Triton X-100. The clarified supernate was used as the synthetase extract.

[^{32}P]pCp 3' end labeling of tRNAs

This was performed as described (14), omitting the BSA in the reaction mix and the desalting of the sample on Sephadex after the reaction (16−20 hr at 4°C). Yeast tRNA (5−10 μg) was added as a carrier for ethanol precipitation.

Charging and deamination of tRNAs

Charging of tRNAs with the following labeled amino acids was performed as described (13,15): Tran-[^{35}S]methionine (1173 C/mM) (ICN), L-[^{35}S]methionine (1100 C/mM) (Amersham), [^{3}H]glycine (20 C/mM) (ICN), L-[3,4,5-^{3}H(N)]leucine (143 C/mM) (NEN), [^{3}H]tryptophan (20 C/mM) (NEN). Two charging reaction mixes were used with identical results: (1) 50 mM Tris-HCl (pH 7.65), 10 mM KCl, 5 mM Mg acetate, 1 mM DTT,

2 mM ATP, 5−15 μg tRNA, 10 μc labeled amino acid, 2 μM 19 unlabeled amino acids (minus the labeled amino acid), 10 units enzyme. (2) 100 mM Na cacodylate buffer (pH 7.6), 10 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 2 μM 19 amino acids, 10 units enzyme, 10−30 μg tRNA. The reaction mix (100 μl) was incubated 30 min at 25° or 37°C, extracted with 100 μl water saturated phenol, and the tRNA precipitated with ethanol and the pellet resuspended in 5 mM Na acetate (pH 4.5). Heterologous synthetases tested for activity included crude *E. coli* synthetase (Sigma A-3646), and crude Baker's yeast synthetase (Sigma A-3778). Only the latter had significant activity with *Leishmania* tRNA. Crude homologous synthetases were isolated as described above.

Deamination of the charged tRNA with sodium nitrite was necessary to maintain the stability of the aminoacyl group at the pH of gel electrophoresis and allow the visualization of the charged tRNA by autoradiography after electroblotting the gel onto a nylon filter. The charged tRNA in 10 mM Na acetate (pH 4.5) was mixed with 1/2 vol $NaNO_2$ (saturated at 0°C) and 1/200 vol glacial acetic acid (16,17). After 15 min at 25°C, the tRNA was precipitated with 0.5 M NaCl and two vol ethanol and the pellet washed with 70% ethanol, dried and resuspended in formamide gel loading buffer.

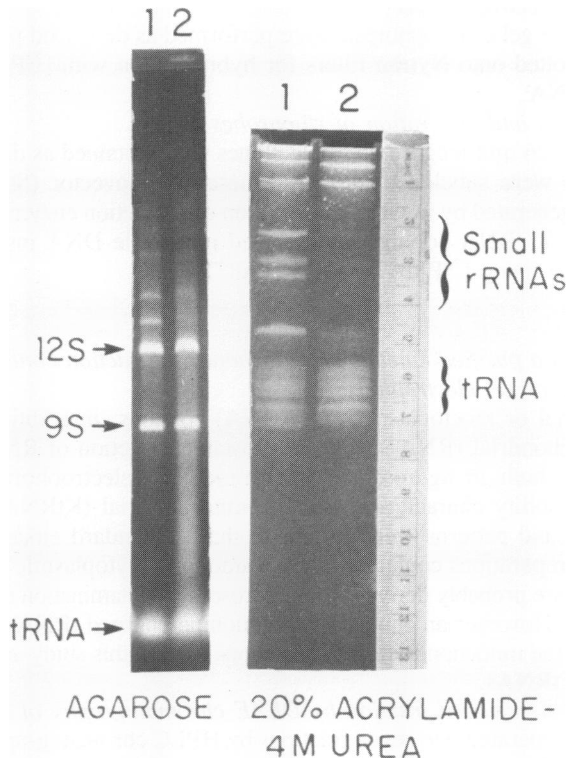


Figure 1. Gel profiles of two different preparations of KRNA, showing the presence of a tRNA region. A. Agarose gel of two kRNA preparations. B. 10% acrylamide-7M urea gel of same two KRNA preparations. The locations of the tRNA region, the 9S and 12S rRNAs, and the small cytoplasmic rRNA contaminants are indicated.

Acrylamide Gel electrophoresis and electroblotting
The first dimension of the 2D gel (18) consisted of electrophoresis in 10% acrylamide-7M urea for 48 hr at 200 V (50 cm×18 cm gel). The tRNA region was cut out and layered onto a 20% acrylamide-8M urea gel (38 cm×18 cm) and electrophoresed for 72 hr at 250 V. A marker lane in the 20% gel contained the original tRNA. The buffer for both gels was 1× TBE (90 mM Tris-borate, pH 8.2, 2.5 mM EDTA). After electrophoresis, the gel was stained for 30 min in 0.5 μg/ml ethidium bromide, destained in water, and photographed with 300 nm UV transillumination. The gel was electroblotted in a Hoeffer Model TE50 apparatus onto Nytran filter (Schleicher and Schuell). The buffer was 12 mM Tris HCl, pH 7.5, 6 mM Na Acetate, 0.5 mM EDTA. The gel was blotted at 500 mA for 15 hr with cooling. The tRNA was crosslinked to the filter by exposure to 254 nm UV light for 2 min and the filter washed for 30 min at 65°C in 1× SSC-0.1% SDS. The blot was prehybridized, hybridized and washed at high stringency (45°C, 0.1× SSC, 1% SDS) as described previously (19). Autoradiography of electroblotted acrylamide gels of labeled charged tRNAs (^{35}S or ^{3}H) was enhanced by treatment of the filter with Autofluor (National Diagnostics), drying under a lamp, and exposure to pre-flashed X-Ray film at −80°C.
Chromosome gel electrophoresis
OFAGE and CHEF gel electrophoresis were performed as described previously (20−22). The gels were blotted onto Nytran filters for hybridization with [^{32}P]pCp-3′end-labeled KtRNA and CtRNA.
Minicircle plasmids and generation of riboprobes
The pLt19 and pLt26 unit length minicircle clones were obtained as described previously (23). The inserts were subcloned into the Bluescript ribovector (Stratagene). Labeled riboprobes were generated by in vitro transcription of restriction enzyme truncated plasmid DNA with T7 or T3 RNA polymerase. Labeled minicircle DNA inserts were obtained by nick translation using [^{32}P]dATP and [^{32}P]dCTP.

RESULTS

RNA isolated from a purified kinetoplast-mitochondrion fraction contains a fraction that migrates as tRNA in gel electrophoresis
Total mitochondrial or kinetoplast RNA (KRNA) contains, in addition to the abundant 9S and 12S mitochondrial rRNA species, a substantial fraction of RNA that migrates in the tRNA region both in agarose and acrylamide gel electrophoresis (Fig. 1). The electrophoretic mobility characteristics of the mitochondrial (KtRNA) and cytoplasmic (CtRNA) tRNA band patterns were similar to that of standard eukaryotic tRNAs.

Some KRNA preparations contained a small amount of cytoplasmic small rRNAs (Fig. 1) (12,24), which are probably derived from microsomal contamination of the mitochondrial fractions (Fig. 1). However an analysis of methionine-charged tRNAs (presented below) demonstrated that the mitochondrial tRNA fractions used in this study are not contaminated with cytoplasmic tRNAs.
Isolation of a tRNA-enriched fraction by DEAE chromatography of kinetoplast RNA
Total KRNA was separated into seven fractions by HPLC chromatography on a Nucleogen DEAE 500−10 column (Fig. 2B). A CtRNA preparation showed a similar separation pattern (Fig. 2A). Peaks 1 and 2 from both RNA preparations contained molecules which comigrated in agarose gels with tRNAs from *E. coli* and yeast, peaks 3 represented 5S RNA, peaks 4 and 5 represented small cytoplasmic rRNAs in the 5.8−6.2S range (12),

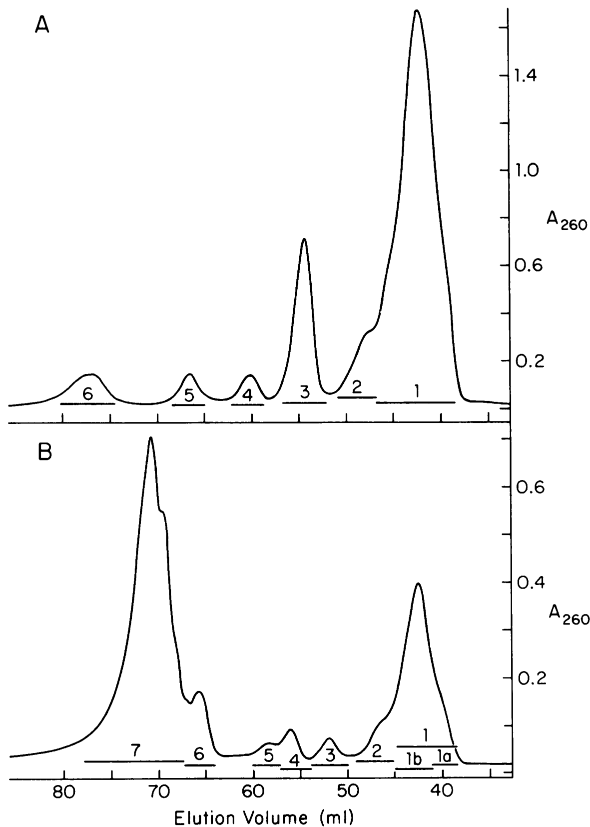


Figure 2. Chromatography of CtRNA (A) and KRNA (B) on a Nucleogen HPLC DEAE 500−10 column. The numbered peaks were collected; fractions 1 and 2 were rechromatographed to decrease cross contamination.

and peak 6 in the cytoplasmic tRNA fractionation represented cytoplasmic rRNA contamination, and peaks 6 and 7 in the kinetoplast RNA fractionation represented 9S and 12S mitochondrial rRNAs (12).

RNA was recovered from KRNA peaks 1 and 2, 3′ end-labeled with [^{32}P]pCp and electrophoresed on a two dimensional (2D) acrylamide gel. As shown in Fig. 3A and 3B, approximately 35−40 spots can be visualized using DEAE peak 1 RNA, and a subset of 10−15 spots using DEAE peak 2 RNA. The same pattern of 35−40 spots can be visualized by staining with ethidium bromide (data not shown), indicating that end-labeling with T4 RNA ligase is not selective for a subset of RNAs in this fraction. Therefore, these RNAs, which migrate like tRNAs in this gel system, clearly represent the major RNA species in the DEAE peaks 1 and 2.

The DEAE peaks 1 and 2 contain a minor fraction of RNAs that hybridize with both minicircle and maxicircle DNA

Hybridization of 3′ end-labeled EKiERNA recovered from DEAE peaks 1 and 2 to Southern blots of digested maxicircle DNA (which has approximately 50% minicircle DNA contamination) was performed (Fig. 4). Peak 1 contains RNA molecules which hybridize

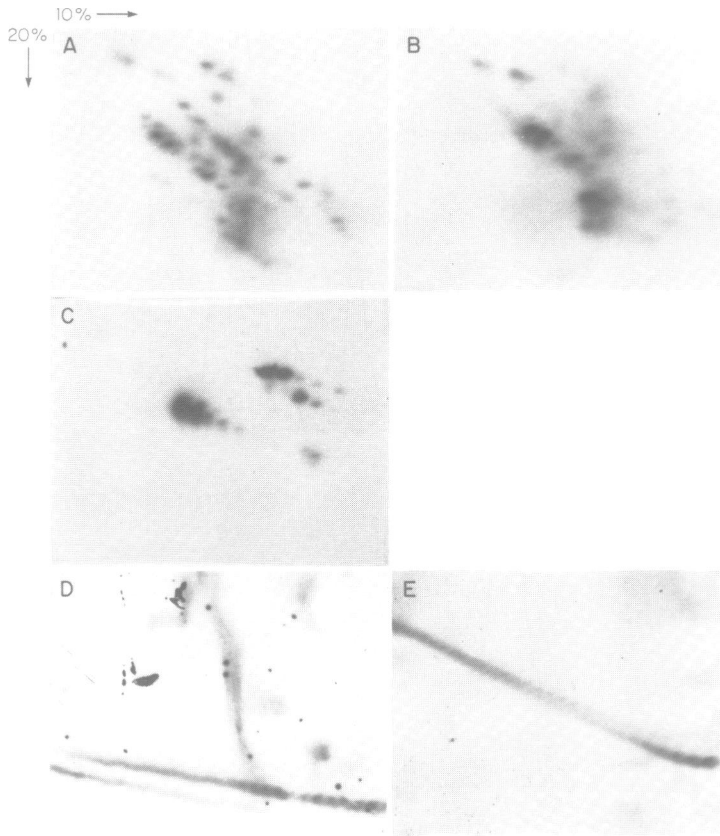


Figure 3. Two dimensional acrylamide-7 M urea gel electrophoresis of kinetoplast RNAs isolated by DEAE HPLC. The samples were run first in 10% acrylamide and then in 20% acrylamide. A. 3'-[^{32}P]pCp end-labeled RNA from DEAE fraction 1. B. 3'-[^{32}P]pCp end-labeled RNA from DEAE fraction 2. C. Unlabled kinetoplast RNA from DEAE fraction 1 was electroblotted and the blot hybridized with a nick-translated *T. brucei* probe, pTtRNA1, that contains glycine and leucine tRNA genes. D. An identical blot of unlabeled KtRNA as in C was hybridized with nick-translated pLt26 cloned minicircle insert DNA. E. An identical blot of unlabeled KtRNA as in C was hybridized with nick-translated pLt150 cloned maxicircle insert DNA.

mainly to the major unit length linearized minicircle DNA fragment, and peak 2 contains RNA molecules which hybridize both to unit length minicircle DNA and to a minor shorter minicircle fragment, and also to several maxicircle fragments. The transcriptional origin of the maxicircle-hybridizing RNAs was further analyzed by Southern blot hybridization to several digests of cloned and uncloned maxicircle DNA (data not shown). The results indicate that the maxicircle-specific RNAs in DEAE peak 2 derive from two regions of the maxicircle molecule: the non-coding region between the 9S rRNA gene and the MURF3 gene, and a poorly defined region containing the COII, MURF2, COI and ND4 genes, including the non-coding intergenic region between ND4 and COI (4,5). We show below that the tRNAs, which are the major components of DEAE peaks 1 and 2, do not hybridize with maxicircle or minicircle DNA but rather with nuclear DNA. This suggests that the

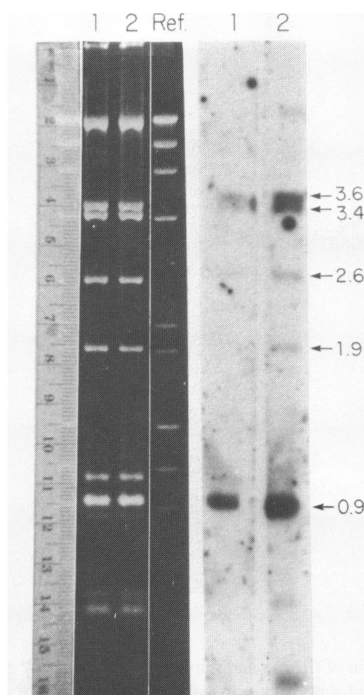


Figure 4. Presence of minicircle and maxicircle transcripts in the KRNA DEAE fractions 1 and 2. Lanes 1 and 2, digests of a maxicircle-enriched KDNA fraction with MspI and HaeIII. Note the 0.9 kb linearized minicircle DNA. Reference DNA fragments, Lambda DNA/HindIII and OX174 DNA/HaeIII. Autoradiograph lanes 1 and 2, blots 1 and 2 were hybridized with DEAE fraction 1 and 2, respectively. The sizes of the labeled fragments are indicated on the right side in kb.

RNAs in DEAE peaks 1 and 2 which hybridize with minicircle and maxicircle DNA are minor components of these fractions.

Identification of minicircle-specific transcripts in DEAE KtRNA as a family of at least 20 closely migrating bands in acrylamide

The KtRNA fraction isolated by DEAE chromatography (or total KRNA) was electrophoresed in acrylamide-urea and blotted onto nylon filters. Detection of a heterogeneous minicircle-specific minor RNA component which migrates ahead of the mitochondrial tRNAs but which is not abundant enough to visualize by staining with ethidium bromide was accomplished by hybridization with nick-translated cloned minicircle probes (Fig. 5). A family of 20–24 closely spaced bands was apparent. Cross-linking the filter by short wavelength UV irradiation was required to maintain the RNA on the filter during hybridization and washing. This necessity for UV cross-linking apparently explains why previous attempts in our laboratory failed to visualize these small minicircle transcripts. These minicircle transcripts copurify with tRNAs in DEAE chromatography in peaks 1 and 2 and account for the minicircle DNA hybridization described above in Fig. 4.

As shown in Fig. 5, the same RNA bands were recognized by either of two cloned

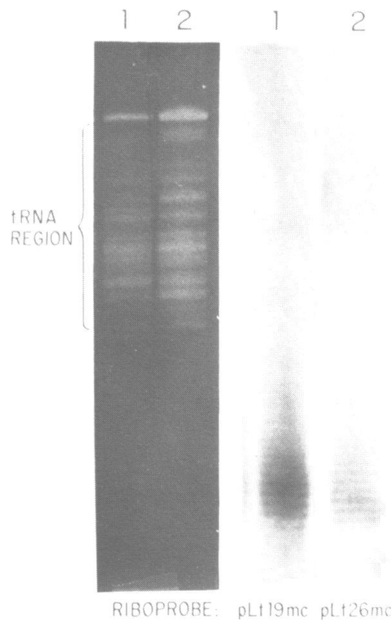


Figure 5. High resolution separation of tRNAs and minicircle transcripts by electrophoresis in 20% acrylamide-7 M urea. The blots were hybridized with nick-translated pLt19 and pLt26 cloned minicircle insert DNA.

minicircle probes (pLt19, pLt26), which represent different minicircle sequence classes and possess a conserved region of approximately 120 bp and a region unique to each sequence class (22). This suggests that transcription of the conserved region gives rise to this family of RNAs.

The direction of transcription of the major minicircle transcripts was examined by probing Northern blots of KRNA with strand-specific riboprobes generated from pLt19 minicircle DNA cloned into a Bluescript vector (Fig. 6). RNAs hybridizing to the T7 riboprobe are clearly more abundant than RNAs hybridizing to the T3 riboprobe. The direction of transcription of the more abundant RNAs is from the conserved 12mer origin of replication sequence to the bend region adjacent to the conserved region (22). Similar results were obtained for the pLt26 minicircle clone (data not shown). The hybridization of the complementary riboprobe may be due to non-specific *in vitro* transcription from the Bluescript template which contains a 3′ protruding end generated by truncation with SacI; extraneous transcripts from such templates can contain sequences complementary to the expected transcript. However it is also possible that a low level of minicircle transcription off the complementary DNA strand occurs and this must be analyzed further.

The small minicircle and maxicircle transcripts do not migrate like tRNAs in 2D acrylamide gels

Hybridization of a blot of a 2D acrylamide-urea gel of DEAE KtRNA (or total KRNA) with a nick-translated minicircle DNA probe gave rise to two diagonal lines of closely migrating RNA spots of varying intensity, as shown in Fig. 3D. The migration of these RNAs in this two dimensional gel is quite dissimilar to that of tRNAs in the same gel,

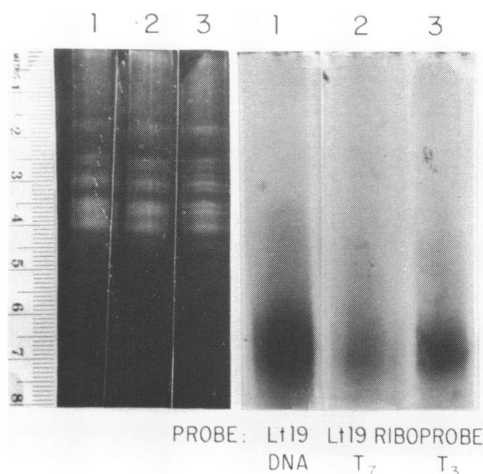


Figure 6. Hybridization of labeled pLt19 minicircle strand-specific riboprobes to total kinetoplast RNA. Electrophoresis conditions: 20% acrylamide-8M urea. The gel was stained with ethidium bromide (A) and then electroblotted onto Nytran filters (B). Lanes 1 and 4, probe is pLt19 nick-translated insert DNA; lanes 2 and 5, probe is pLt19 riboprobe generated with T7 RNA polymerase; lanes 3 and 6, probe is pLt19 riboprobe generated with T3 RNA polymerase.

which migrate at unique positions on both sides of the diagonal probably due to varying secondary structures and sizes (Fig. 3A,B). This suggests that the minicircle transcripts possess less secondary structure than the tRNAs.

A similar pattern of closely spaced RNA spots running along a diagonal was obtained by hybridization of an identical blot of KtRNA with a cloned maxicircle DNA fragment (pLt150 insert, nt 11,962−13,734 in LEIKPMAX, the *L. tarentolae* maxicircle sequence in Genbank) (Fig. 3E). Other maxicircle fragments (pLt120 insert, nt 1−6558 in LEIKPMAX, or Sau3A fragment 4 of the pLt120 insert, nt 1582−2368 in LEIKPMAX) (Fig. 7) gave the same type of pattern.

It can be seen from the two dimensional gels that a minor fraction of the minicircle and, especially, maxicircle RNAs actually extends through the tRNA region in a 10% acrylamide-urea gel, resulting in a minor contamination of acrylamide gel-purified KtRNA with non-tRNA maxicircle and minicircle transcripts.

Many of the KtRNA spots on two dimensional acrylamide gel electrophoresis comigrate with CtRNA spots

As shown above in Fig. 3 and in Fig. 7, 2D acrylamide gel electrophoresis resolved both CtRNA and KtRNA into approximately 35−40 spots. Some spots were well separated, but many were clustered and both RNA preparations exhibited areas of smears in staining as well as in radioactivity; whether these areas represent unresolved tRNA molecules or some other heterogeneous small RNA species such as the minicircle- and maxicircle-specific transcripts described above has not yet been determined.

Many of the KtRNA spots appear to comigrate with CtRNA spots. In order to determine the number of KtRNA spots that comigrate with CtRNA spots, unlabeled CtRNA was mixed with [^{32}P]pCp 3′end-labeled KtRNA and corun in a 2D acrylamide gel (Fig. 8).

Figure 7. Hybridization of 2D gel of KtRNA with cloned maxicircle DNA. The gel was stained with ethidium bromide and electroblotted onto Nytran filter. The blot was UV cross-linked and probed with Sau3A fragment 4 of the pLt120 region of the maxicircle DNA.

An unequivocal interpretation of comigrating and unique spots was made difficult the fact that the mobility of several of the tRNAs was changed by 3′-end labeling with [^{32}P]pCp (data not shown). However, we tentatively conclude from these experiments that approximately 10 KtRNA spots are unique and approximately 10 CtRNA spots are unique, and that the remainder comigrate. Comigration suggests that these tRNA species are identical in sequence, but this conclusion must of course be confirmed by direct sequence analysis.

Isolation of cytoplasmic and mitochondrial amino acyl tRNA synthetases from L. tarentolae

One of the functional criteria of a tRNA molecule is the ability to accept amino acids. Crude aminoacyl tRNA synthetase preparations were isolated from cytosol and mitochondrial fractions and used to charge both CtRNA and KtRNA with several radioactive amino acids. Table 1 presents the maximum charging activities obtained with [^{35}S]methionine, [^{3}H]leucine, [^{3}H]glycine and [^{3}H]tryptophan. CtRNA was acylated well with either the cytoplasmic or mitochondrial enzyme preparation; KtRNA was also charged with either cytoplasmic or mitochondrial enzymes, but the extent of amino acid acceptance was generally lower. In the case of [^{35}S]methionine, a commercial yeast synthetase preparation was also somewhat active in charging KtRNA, but an *E. coli* synthetase fraction was inactive.

tRNAs charged with [^{35}S]methionine, [^{3}H]leucine and [^{3}H]tryptophan can be identified by gel electrophoresis in both CtRNA and KtRNA preparations

After charging with [^{35}S]methionine, two labeled tRNA bands were seen with both CtRNA and KtRNA (Fig. 9A,B), one of which was unique to each tRNA preparation. Charging of both CtRNA and KtRNA was accomplished with either the yeast, cytoplasmic or mitochondrial synthetase preparations with no effect on the patterns observed. The two CtRNA labeled species could be identified as ethidium bromide-stained spots in a 2D gel (Fig. 9C).

Identification of [^{3}H]leucine-charged tRNAs in the CtRNA and KtRNA preparations was accomplished in an identical manner. At least four tRNA^{leu} species could be

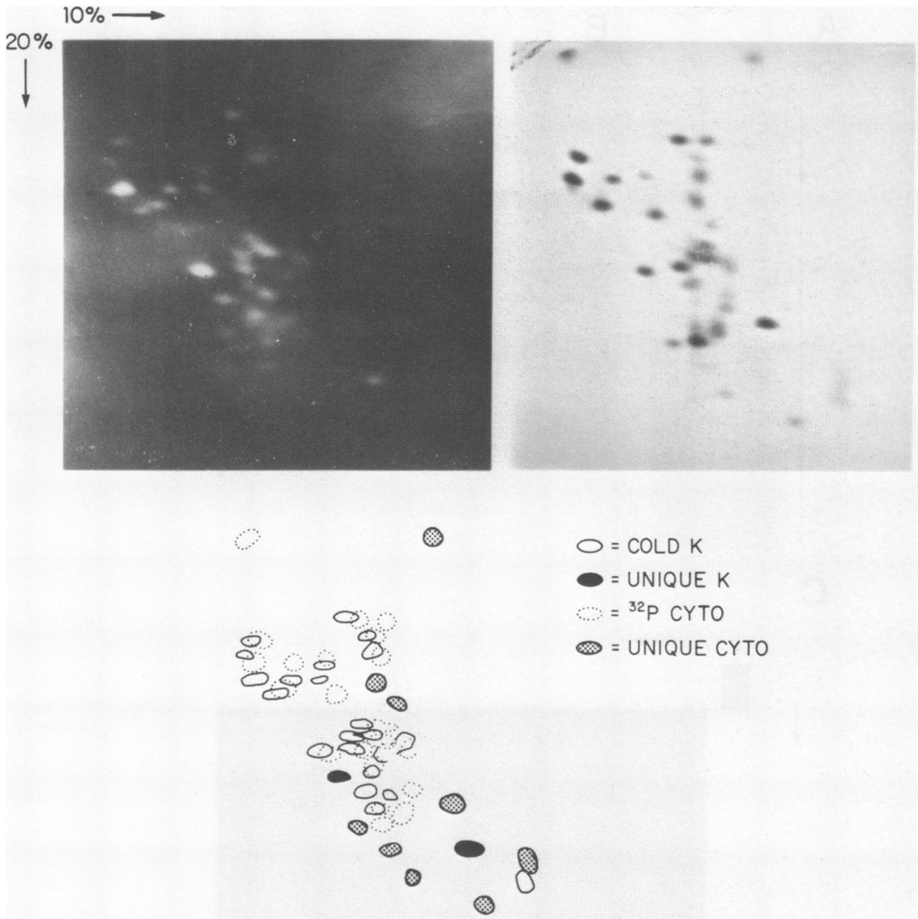
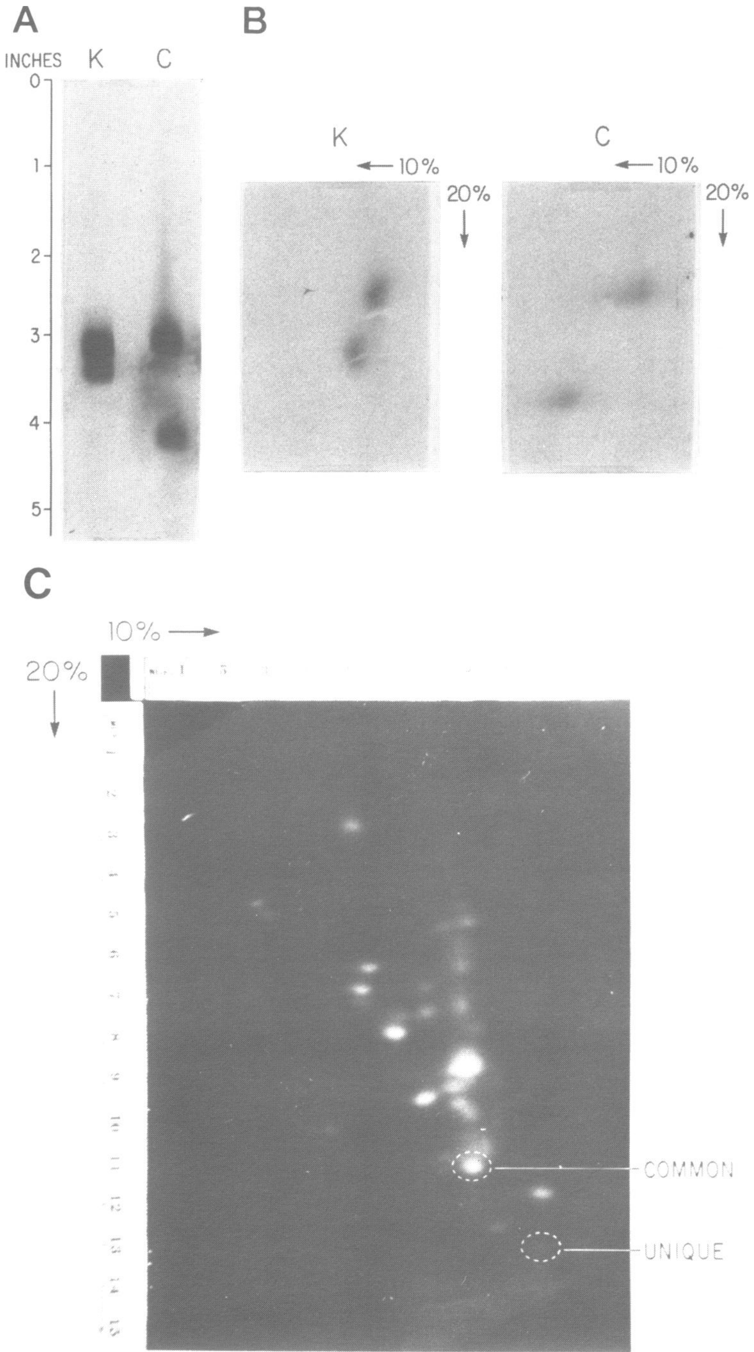


Figure 8. 2D gel electrophoresis of mixtures of labeled and unlabeled CtRNA and KtRNA. Unlabeled CtRNA corun with [^{32}P]pCp-labeled KtRNA. Below the gels is shown a diagram of the unique and comigrating tRNA spots, as determined by superimposition of the autoradiograph and the photograph of the stained gel.

Table 1. Charging of *L. tarentolae* cytoplasmic and mitochondrial tRNA with homologous cytoplasmic and mitochondrial amino acyl tRNA synthetase preparations.

tRNA	Enzyme	35S-Met	3H-Leu	3H-Tryp	3H-Gly
Cyto	Cyto	39000^{+}	68000	6900	12800
Cyto	Mito	46600	21000	9700	ND
Mito	Cyto	4300	15700	5200	10800*
Mito	Mito	9300	600	4400	ND

$^{+}$ Expressed as CPM/μg tRNA.
* Activity was measured with total KRNA. The amount of tRNA was estimated to be 1/3 of total.
ND: Not determined.


A
INCHES
K
C
0
1
2
3
4
5
B
K
C
10%
20%
C
10%
20%
COMMON
UNIQUE

visualized in CtRNA (Fig. 10A,B) and two $tRNA^{leu}$ species in KtRNA (Fig. 10C,D).

A single [^{3}H]tryptophan-charged tRNA species was visualized in a one dimensional gel for both KtRNA and CtRNA (data not shown).

Nuclear transcriptional origin of mitochondrial tRNAs

It was shown above that hybridization evidence suggests an absence of maxicircle-encoded mitochondrial tRNA genes. This conclusion was complicated by the fact that DEAE or agarose gel-purified KtRNA preparations are contaminated with a low abundance of small transcripts of unknown function that are derived from both minicircle and maxicircle DNA. Additional evidence for the nuclear origin of most if not all of the mitochondrial tRNAs is provided by hybridization of acrylamide-purified KtRNA (fraction KF1 RNA) to the same set of nuclear DNA restriction fragments as acrylamide-gel purified CtRNA (fraction CF1 RNA) (Fig. 11A and 11B, lanes N). The hybridization of the KF1 fraction to maxicircle and minicircle DNA (lanes M and K) is due to contamination of the KF1 fraction with the more rapidly migrating minicircle and maxicircle-specific RNAs.

KtRNA also hybridizes with nuclear chromosomes separated by orthogonal field gel electrophoresis (Fig. 12). Hybridization was to two megabase chromosome bands (arrows), with an estimated size of 1.5–2.5 Mb. Labeled CtRNA hybridized to the same two large chromosome bands as KtRNA, but in addition showed some weak hybridization with two smaller chromosomes (arrows), with estimated sizes of 600 kb and 800 kb.

Heterologous hybridization of L. tarentolae tRNAs with a Trypanosoma brucei genomic clone

Additional evidence for a nuclear transcriptional origin of specific mitochondrial tRNAs is provided by the use of a genomic clone (pTtRNA1) from *T. brucei*, which has been shown by sequence analysis to contain at least two tRNA genes, $tRNA^{gly}$ and $tRNA^{leu}$ (Campbell, Suyama and Simpson, unpublished results). This probe gave strong hybridization signals with *L. tarentolae* KtRNA and CtRNA (but not with yeast tRNA) in Northern blot analysis. The cloned insert DNA was subdivided into four restriction fragments, which were used separately as hybridization probes (Fig. 13). Fragment I-S, which only contains the *T. brucei* $tRNA^{leu}$ gene, hybridized with two RNA bands in both KtRNA (M) and CtRNA (C), whereas fragment H-A hybridized with several additional more rapidly migrating bands. The latter RNA bands must therefore be due to hybridization with an additional yet unidentified tRNA sequence in the S-A region. The second band (b) in the KtRNA lane does not appear to comigrate with the second band in the CtRNA lane. Fragment E-H, which only contains the *T. brucei* $tRNA^{gly}$ gene, hybridized with at least four bands (c,y,x,e) in the KtRNA lane, but with only two major bands (a,c) and one minor band (d) in the CtRNA lane. Fragment A-B showed no hybridization to tRNAs (data not shown).

The one dimensional gel localizations of the $tRNA^{leu}$ isoacceptor species in both KtRNA and CtRNA are consistent with the 2D localizations of tRNAs hybridizing to the pTtRNA1 probe in Fig. 3C and with the localizations of the [^{3}H]leucine-charged tRNAs in the 2D gel in Fig. 10.

Figure 9. Acrylamide gel electrophoresis of [^{35}S]methionine-charged tRNA species from KtRNA and CtRNA. The KtRNA and CtRNA were both charged with [^{35}S]methionine using the cytoplasmic synthetase preparation. Identical results were obtained using the mitochondrial or the yeast synthetase preparations. A. One dimensional gel electrophoresis in 10% acrylamide-7 M urea. B. 2D gel electrophoresis in 10% and 20% acrylamide-7 M urea. C. 2D gel electrophoresis of unlabeled CtRNA showing the identification of the two $tRNA^{met}$ ethidium bromide-stained spots. The gels in A and B were electroblotted onto Nytran filters for autoradiography.

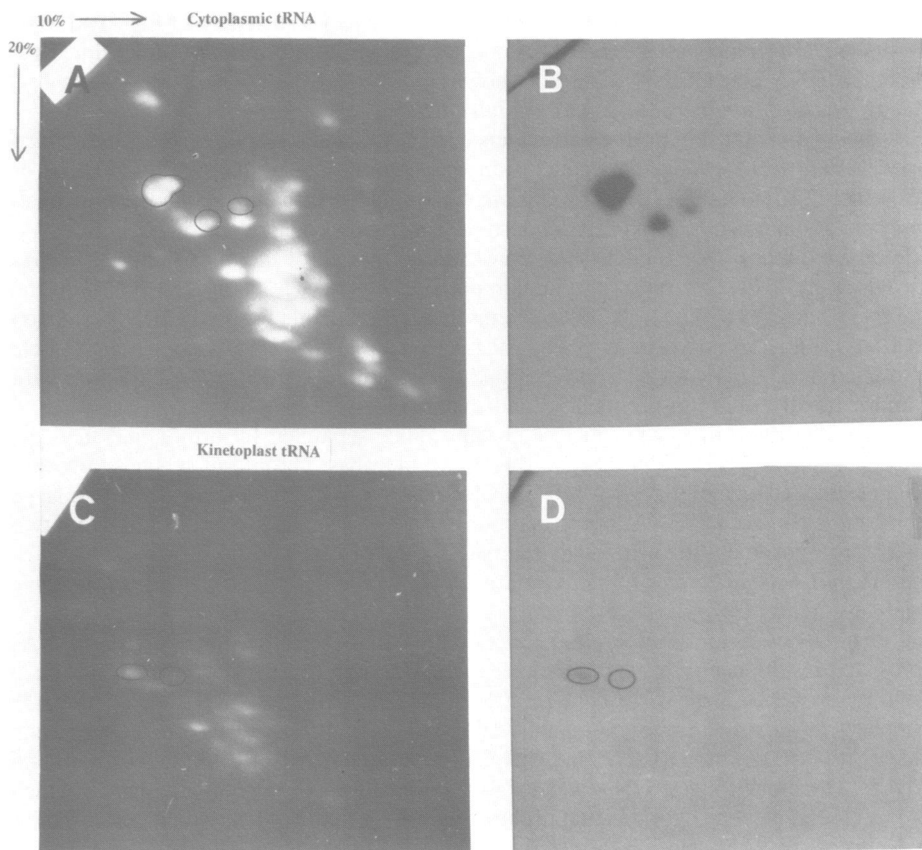


Figure 10. 2D acrylamide gel electrophoresis of [^{3}H]leucine-charged tRNA species from CtRNA and KtRNA. A, B, CtRNA charged with [^{3}H]leucine was separated and the gel electroblotted and autoradiographed. The localizations of the multiple $tRNA^{leu}$ species are indicated by circling of the stained spots in A. C, D, KtRNA charged with [^{3}H]leucine. The localizations of two $tRNA^{leu}$ species are indicated by circling of the stained spots in C.

DISCUSSION

We conclude that there is no evidence for a maxicircle or minicircle transcriptional origin of mitochondrial tRNA in *L. tarentolae*, and that a nuclear DNA origin is clearly suggested by the data. This conclusion however must await the cloning and sequencing of all KtRNA species and comparison with nuclear DNA clones, since the existence of RNA editing of several maxicircle mRNAs in these cells has been established (6) and this may provide a mechanism for post-transcriptional modification of maxicircle transcripts to produce tRNAs which do not even hybridize with the mitochondrial DNA template. There is no evidence for or against this possibility at the present time.

The mitochondrial tRNA fraction contains functional tRNAs, most of which are similar, if not identical, to cytoplasmic tRNAs. Several mitochondrial tRNAs are clearly

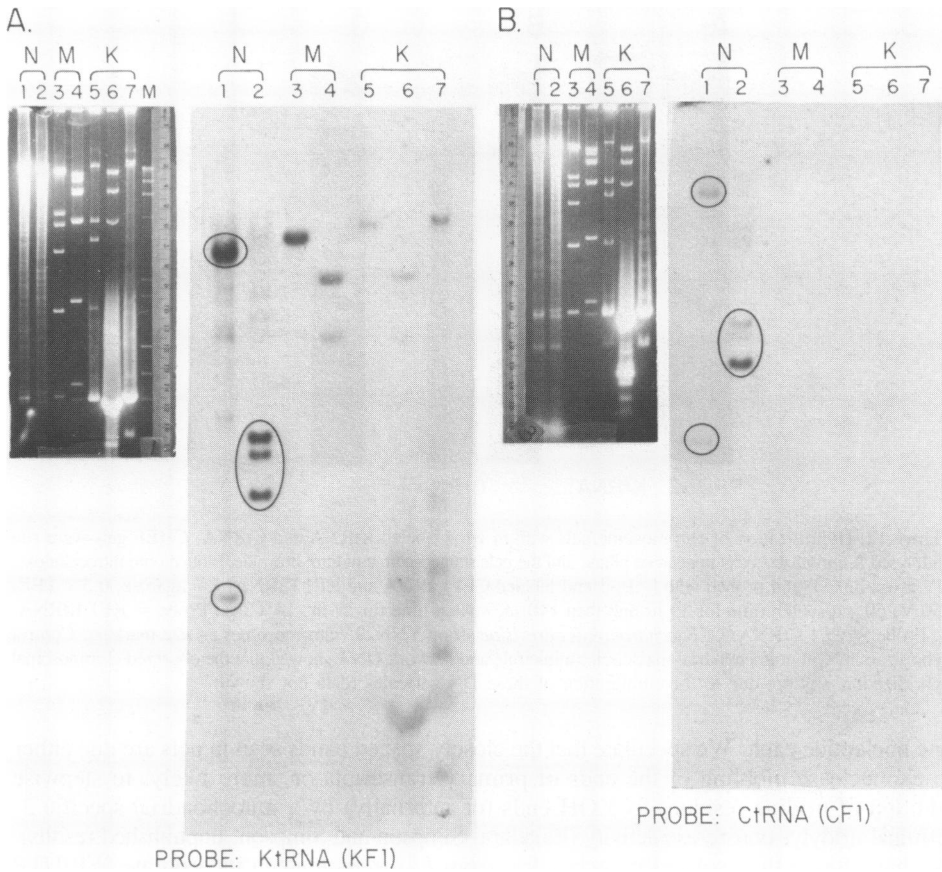


Figure 11. Nuclear DNA transcriptional origin of KtRNA. The DNA used for each digestion is indicated above the lanes: N= nuclear DNA, M= maxicircle DNA, K= kinetoplast DNA. Digestions: lane 1, EcoRI; 2, HindIII; 3, HindIII; 4, MspI; 5, HindIII; 6, MspI; 7, EcoRI. A. Probe was [^{32}P]-pCp-labeled KF1 KtRNA. B. Probe was [^{32}P]pCp-labeled CF1 CtRNA.

mitochondrial-specific, as determined by unique 2D gel migration properties, but these particular tRNAs have not yet been correlated with specific amino acid charging.

We have also shown that a low abundance family of small RNAs, approximately 80 nt in size, which are transcriptionally derived from minicircle and maxicircle DNA comigrate with mitochondrial tRNAs in agarose gel electrophoresis and coelute with tRNAs in DEAE chromatography. These RNAs can be separated from tRNAs by a more rapid electrophoretic migration behavior in 10–20% acrylamide-7M urea gels. A striking difference in electrophoretic migration is also apparent in 2D acrylamide-urea gels, in which the kDNA transcripts migrate as a series of approximately 20 closely spaced spots along diagonals, unlike the tRNAs which migrate off the diagonal probably as a result of RNA secondary structures. The patterns of spots suggest a family of molecules that differ by

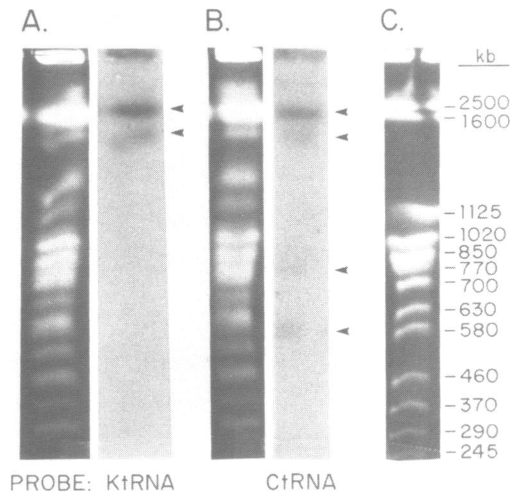


Figure 12. Hybridization of chromosome gels with *in vitro* labeled KtRNA and CtRNA. CHEF gels were run with lysed *L. tarentolae* cells in agarose plugs, and the gels stained with ethidium bromide, blotted onto nitrocellulose, UV-cross-linked, and probed with [^{32}P]-3'end-labeled CF1 CtRNA and KF1 KtRNA. 1% agarose, 0.5× TBE, 150 V, 60 sec switch time for 19 hr and then 140 sec switch time for 27 hr, 14°C. A. Probe = KF1 KtRNA. B. Probe = CF1 CtRNA. C. *Saccharomyces cerevisiae* strain YNN295 chromosomes as size markers. Control hybridizations with mitochondrial maxicircle, minicircle and network DNA showed that the observed chromosomal hybridization was not due to the comigration of these DNA species (data not shown).

one nucleotide each. We speculate that the closely spaced bands seen in gels are due either to exonuclease nibbling of the ends of primary transcripts or, more likely, to stepwise addition of uridine residues to 3'OH ends (or internally) by a mitochondrial-specific 3' terminal uridylyl transferase activity (Bakalara, Simpson and Simpson, unpublished results). We have found that isolated mitochondria from *L. tarentolae* can incorporate [^{32}P]UTP into endogeneous maxicircle and minicircle transcripts as a result of this activity (Bakalara et al, unpublished results), and it is likely that this also occurs *in vivo*, giving rise to the observed family of minicircle-specific transcripts.

The minicircle transcripts appear to be derived from the conserved region of the molecule, and the maxicircle transcripts are derived from at least two regions of the maxicircle, but the determination of the actual transcriptional origin in each case awaits cloning and sequencing of cDNAs. The functions of the minicircle transcripts and the maxicircle transcripts are unknown. They may represent RNA primers involved in DNA replication, RNA processing products or intermediates. A further analysis of these unusual RNA molecules may lead to a deeper understanding of both minicircle and maxicircle transcription and function.

Although an extensive purification of the mitochondrial and cytoplasmic aminoacyl synthetases was not attempted in the present work, it was found that both CtRNA and KtRNA could be charged with amino acids using the crude enzyme preparations derived from either cellular compartment. Zaitseva and Volfson (25) have previously reported the isolation of valyl-tRNA synthetases from mitochondria and cytoplasm of *C. oncopelti* and have shown an absence of any significant differences in chromatographic properties and

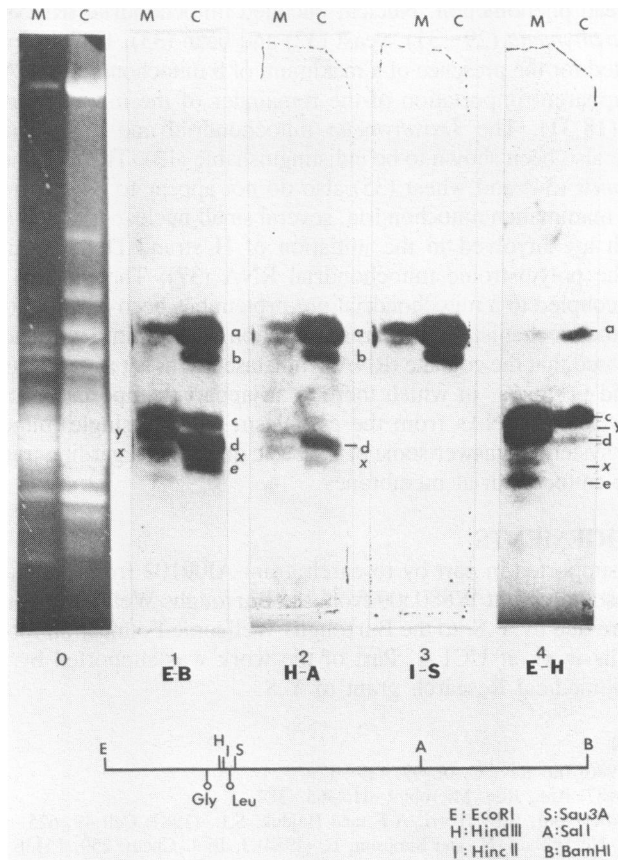


Figure 13. Northern blot hybridization of KtRNA (M) and CtRNA (C) with restriction fragments from a cloned fragment of *T. brucei* genomic DNA containing several tRNA genes (pTtRNA1). tRNAs for glycine and leucine have been identified from the sequence as indicated (Campbell, Suyama and Simpson, unpublished results). Lanes 0, stained gel tRNA pattern. The probe fragments were gel-isolated and labeled with [^{32}P] by nick translation.

Km values between these enzymes. They have also shown that the mitochondrial valyl tRNA is identical in sequence with the cytoplasmic valyl tRNA (26–28).

Specific charging of the *L. tarentolae* cytoplasmic and mitochondrial methionine, leucine and tryptophan tRNAs has been demonstrated and the charged species localized in one dimensional and 2D gel electrophoresis. In the case of tRNA^{met}, the absence of the unique cytoplasmic tRNA^{met} in the KtRNA fraction indicates that the KtRNA fraction is not contaminated with cytoplasmic tRNA, and therefore the presence of comigrating tRNAs in both fractions suggests that a substantial number of mitochondrial and cytoplasmic tRNAs are similar if not identical in sequence. This conclusion is consistent with the apparent nuclear transcriptional origin of most if not all of the mitochondrial tRNAs, as determined by hybridization analysis.

The presence of nuclear-encoded small molecular weight RNAs in mitochondria is proving

to be a widespread phenomenon. Nuclear-encoded mitochondrial tRNAs have also been described in *Tetrahymena* (29–31), yeast (32) and bean (33). In *Tetrahymena*, evidence has been presented for the presence of a maximum of 8 mitochondrial DNA-encoded tRNA genes and the apparent importation of the remainder of the mitochondrial tRNAs from the cytoplasm (18,31). The *Tetrahymena* mitochondrial and cytoplasmic valyl tRNA synthetases have also been shown to be indistinguishable (13). The mitochondrial genomes of *Chlamydomonas* (34) and wheat (35) also do not appear to encode a complete set of tRNA genes. In mammalian mitochondria, several small nuclear-encoded RNAs have been described which are involved in the initiation of H strand DNA replication (36) and processing of the polycistronic mitochondrial RNA (37). The in vitro transport of an oligonucleotide coupled to a mitochondrial pre-protein has been reported (38), but nothing is known about the mechanism of *in vivo* importation of RNA into mitochondria, although it has been proposed that the cognate tRNA synthetase might act as a cotransporter (13,39). The kinetoplastid protozoa, in which there is an apparent importation of a complete or nearly complete set of tRNAs from the cytoplasm into the single mitochondrion, may provide a model system to answer some of the basic questions regarding transport of nucleic acids across the mitochondrial membranes.

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REFERENCES

1. Simpson, L. (1986) Int. Rev. Cytol. 99, 119–179.
2. Simpson, L. (1987) Ann. Rev. Microbiol. 41, 363–382.
3. Rohrer, S.P., Michelotti, E.F., Torri, A.F. and Hajduk, S.L. (1987) Cell 49, 625–632.
4. de la Cruz, V., Neckelman, N. and Simpson, L. (1984) J. Biol. Chem. 259, 15136–15147.
5. Simpson, L., Neckelman, N., de la Cruz, V., Simpson, A., Feagin, J., Jasmer, D. and Stuart, K. (1987) J. Biol. Chem. 262, 6182–6196.
6. Simpson, L. and Shaw, J. (1989) Cell, 57, 355–366.
7. Benne, R., Agostinelli, M., De Vries, B., Van den Burg, J., Klaver, B. and Borst, P. (1983) In Mitochondria 1983:Nucleo-Mitochondrial interactions, R.J. Schweyen, K. Wolf and F. Kaudewitz, eds. (Berlin: De Gruyter), pp. 258–302.
8. Benne, R. and Sloof, P. (1987) BioSystems 21, 51–68.
9. Hoeijmakers, J., Snijders, A., Janssen, J. and Borst, P. (1981) Plasmid 5, 329–350.
10. Simpson, L. and Braly, P. (1970) J. Protozool. 17, 511–517.
11. Braly, P., Simpson, L. and Kretzer, F. (1974) J. Protozool. 21, 782–790.
12. Simpson, L. and Simpson, A. (1978) Cell 14, 169–178.
13. Suyama, Y. and Hamada, J. (1978) Arch. Biochem. Biophys. 191, 437–443.
14. England, T., Bruce, A. and Uhlenbeck, O. (1980) Methods in Enzymol. 65, 65–74.
15. Svensson, I., Isaksson, L. and Henningsson, A. (1971) Biochim. Biophys. Acta 238, 331–337.
16. Herve, G. and Chapville, F. (1965) J. Mol. Biol. 13, 757–766.
17. Aujame, L., Wallace, B. and Freeman, K. (1978) Biochim. Biophys. Acta 518, 308–320.
18. Suyama, Y. (1986) Curr. Genet. 10, 411–420.
19. Masuda, H., Simpson, L., Rosenblatt, H. and Simpson, A. (1979) Gene 6, 51–73.
20. Carle, G. and Olson, M. (1985) Proc. Natl. Acad. Sci. USA 82, 3756–3760.
21. Chu, G., Vollrath, D. and Davis, R. (1986) Science 234, 1582–1585.
22. Gomez-Eichelmann, M.C., Holz, Jr., G., Beach, D., Simpson, A.M. and Simpson, L. (1988) Mol. Biochem. Parasitology 27, 143–158.

23. Kidane, G., Hughes, D. and Simpson, L. (1984) Gene 27, 265–277.
24. White, T., Rudenko, G. and Borst, P. (1986) Nucl. Acids Res. 14, 9471–9489.
25. Zaitseva, G. and Volfson, A. (1985) Biochemistry (Russian) 50, 432–438.
26. Zaitseva, G. and Entelis, N. (1985) Biochemistry (Russian) 50, 833–838.
27. Zaitseva, G., Mett, I., Maslov, D., Lunina, L. and Kolesnikov, A. (1979) Biokhimiya 44, 2073–2082.
28. Entelis, K., Maslov, D., Bolshakova, E. and Zaitseva, G. (1987) Doklady 297, 1498–1501.
29. Chiu, N., Chiu, A. and Suyama, Y. (1975) J. Mol. Biol. 99, 37–50.
30. Suyama, Y. (1967) Biochemistry 6, 2829–2839.
31. Suyama, Y. (1982) In 'Mitochondrial Genes' (P. Slonimski, P. Borst and G. Attardi, eds.) Cold Spring Harbor Laboratory New York. , 449–455.
32. Martin, R., Schneller, J., Stahl, A. and Dirheimer, G. (1979) Biochem. 18, 4600–4605.
33. Marechal-Drouard, L., Weil, J. and Guillemaut, P. (1988) Nucl. Acids Res. 16, 4777–4788.
34. Boer, P.H. and Gray, M.W. (1988) Cell 55, 399–411.
35. Joyce, P., Spencer, D., Bonen, L. and Gray, M. (1988) Plant Mol. Biol. 10, 251–262.
36. Chang, D. and Clayton, D. (1989) Cell 56, 131–139.
37. Doersen, C.J., Guerrier-Takada, C., Altman, S. and Attardi, G. (1985) J. Biol. Chem. 260, 5942–5949.
38. Vestweber, D. and Schatz, G. (1989) Nature 338, 170–172.
39. Suyama, Y. and Hamada, J. (1976) In Genetics and Biogenesis of Chloroplasts and Mitochondria (Th. Bucher, W. Neupert, W. Sebald and S. Werner, eds.), Elsevier Biomedical Press, Amsterdam, Netherlands., 763–770.

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