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Reprinted from THE JOURNAL OF PARASITOLOGY
Vol. 64, No. 2, April 1978
Made in United States of America

Glucose-Sensitive Culture Strain of *Trypanosoma brucei*

A culture line of strain 367H of *Trypanosoma brucei* was initiated on 12/22/76 with blood trypanosomes from an 8-day-infected X-irradiated (600R) rat by inoculating 5 ml of modified RE I medium of Steiger and Steiger (1976, J Parasitol **62**: 1010–1011) with 0.01 ml infected blood, (Heparin used as anticoagulent). Modification of the medium consisted of the removal of glucose and the use of 5 or 10% dialyzed fetal calf serum. This culture was transferred on 12/26/76 (0.3 ml culture + 5 ml modified RE I medium), and has been passaged continuously at 27 C in rolling tubes every 3 to 5 days since that date.

The original 367H strain of *T. brucei* was received as a frozen stabilate from Dr. L. Jenni of the Swiss Tropical Institute, and is a clone derived from infecting an irradiated mouse with a single metacyclic trypanosome isolated from an infected *Glossina morsitans* fly.

It was found that a minimum inoculum of 1.0–1.4 × 10^6 cells/ml was required to initiate a culture in modified RE I medium. Cultures with lower inoculum concentrations exhibited an extended lag phase or no growth at all. A growth curve performed soon after establishment of the culture strain (1/12/77) showed a generation time of 22.5 hr and a maximum cell density at stationary phase of 9.4 × 10^6/ml. Another growth curve performed 2 months later (3/29/77) showed a generation time of 13 hr and a maximum cell density at stationary phase of 40–50 × 10^6/ml. The cells had obviously adapted to the culture conditions by a decrease in the doubling time and an increase in the maximum cell density reached in stationary phase.

Attempts were made to increase the maximum cell density in modified RE I medium (40–50 × 10^6 cells/ml). Additions of rabbit red blood cell lysate, proline (0.06%), glycerol (0.5%) or a lysate of *Phytomonas davidi* culture forms had no stimulatory effect, nor did the addition of up to 0.1 M HEPES (pH 7.6). Glycerol (0.5%) was routinely incorporated into the modified RE I medium from 3/6/77 on, since it seemed to enhance the survival of the cells in stationary phase somewhat. However, in modified RE I medium (with and without glycerol), it was noted that the cells died rapidly after attainment of stationary phase density. Addition of 0.5% glucose to stationary phase cells was found to enhance the survival of motile nondividing cells. This is consistent with the report that culture forms of *T. brucei* begin metabolizing glucose in stationary phase (Evans and Brown, 1972, J Protozool **19**: 686–690).

Addition of 0.5% glucose to modified RE I medium (with or without glycerol) at the time of inoculation almost completely inhibited cell division. Repression of cell division was also apparent when nondialyzed fetal calf serum was used in place of dialyzed serum, possibly as a result of the glucose present in the nondialyzed serum. This sensitivity to glucose was apparent early in the establishment of this culture strain and has been retained throughout continuous subculture for 9 months. This represents the first case of a glucose-sensitive culture strain of *T. brucei* or of any hemoflagellate. I speculate that this is a variant of *T. brucei* which exhibits the phenomenon of catabolite repression similar to the well studied glucose repression of mitochondrial biosynthetic activities in yeast (Lloyd, 1974, The Mitochondria of Microorganisms. Academic Press, N.Y., p. 196–199). The availability of such a glucose-sensitive culture strain of *T. brucei* may prove important for the study of mitochondrial biogenesis in this hemoflagellate protozoan.

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Cell, Vol. 14, 169-178, May 1978, Copyright © 1978 by MIT

Kinetoplast RNA of Leishmania tarentolae

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Summary

RNA has been isolated from highly purified kinetoplast-mitochondrial fractions of Leishmania tarentolae, and shown to consist of two major species that sediment at 9S and 12S in sucrose and also several additional low molecular weight species which were visualized by gel electrophoresis. The in vivo transcription of 9S and 12S RNAs was inhibited by ethidium bromide and rifampin, and was fairly insensitive to low actinomycin D and camptothecin. The 9S and 12S RNAs were isolated by acrylamide gel electrophoresis or by sedimentation in sucrose. Both RNAs contained approximately 80% A + U and did not contain long stretches of poly(A). The 9S and 12S RNAs were found to hybridize selectively to the maxicircle sequences of the kinetoplast DNA, implying that the maxicircle, and not the minicircle, represents the informational mitochondrial DNA in the kinetoplast.

Introduction

Although there is indirect evidence that the kinetoplast DNA in the hemoflagellate protozoa is essential for the continued synthesis of mitochondrial respiratory proteins (Simpson, 1972), there is yet little direct evidence for in vivo transcriptional activity of this unusual mitochondrial DNA. Presumptive kinetoplast ribosomes have been visualized in Trypanosoma brucei culture forms (Hanas, Linden and Stuart, 1975) by in vivo labeling of nascent polypeptides in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis, but nothing is known about kinetoplast ribosomal RNA, transfer RNA or messenger RNA.

Nichols and Cross (1977) have recently described several presumptive kinetoplast RNA species sedimenting at 6.9S, 10.1S, 11.4S and 14.6S from a mitochondrial fraction of Crithidia fasciculata. The synthesis of these RNAs was inhibited by 10 μg/ml ethidium bromide (EthBr).

In HeLa cells and Xenopus, the identified stable mitochondrial transcripts consist of the large and small ribosomal RNAs (Aloni and Attardi, 1971a; Dawid, 1972a, 1972b), 19-22 transfer RNAs (Wu et al., 1972; Dawid et al., 1976; Angerer et al., 1976) and approximately eight polyadenylated messenger RNAs (Perlman, Abelson and Penman, 1973; Hirsch, Spradling and Penman, 1974; Ojala and Attardi, 1974). Pulse-labeled HeLa mitochondrial RNA apparently is transcribed symmetrically from both mitochondrial DNA strands (Aloni and Attardi, 1971b, 1971c), whereas the stable RNA species are mostly transcripts of the H strand.

We have examined the question of the in vivo transcriptional activity of the kinetoplast DNA of Leishmania tarentolae by isolation of the major stable RNA species from a highly purified kinetoplast fraction (Braly, Simpson and Kretzer, 1974). This paper describes the isolation, physical properties, labeling characteristics and transcriptional origin of the two major kinetoplast RNA species.

Results

Cytoplasmic Ribosomal RNA

Cytoplasmic ribosomes were isolated from L. tarentolae culture forms and subjected to band sedimentation in SDS-sucrose gradients. The rRNA sedimented at 18S and 25S with a shoulder at 14S. There was also a 5S peak and a 10S peak. The 14S peak may represent a breakdown product. The 10S peak may represent contaminating 10S kinetoplast DNA minicircles (Wesley and Simpson, 1973).

Cytoplasmic rRNA runs in 3.5% acrylamide as three major bands and several minor bands (Figure 1a), indicating that some breakdown has occurred. Several low molecular weight bands are separated in 10% acrylamide (Figure 1a), three of which do not co-migrate with E. coli 4S or 5S RNA. These bands will be designated the "5.8S," "5.9S" and "6.2S" RNAs.

Total RNA Isolation from Purified Kinetoplast Fraction

A highly purified kinetoplast mitochondrion fraction was isolated from late log-phase cells by isopycnic centrifugation in Renografin density gradients (Braly et al., 1974), and total RNA was isolated by SDS lysis and phenol deproteinization. Band sedimentation of this preparation in a sucrose gradient yielded three major peaks, sedimenting at 4-5S, 9S and 12S.

The 9S peak was contaminated with free 10S K-DNA minicircles, which are known to be released to a small extent upon lysis of the kinetoplast (Wesley and Simpson, 1973). The extent of this contamination was evidenced by the gel electrophoresis pattern of ^{32}P-labeled K-RNA before and after DNAase treatment, shown in Figure 1d, slots 1 and 2. Identification of the position of free monomeric minicircles in this gel was achieved by co-running ^{32}P-labeled, closed monomeric minicircles in slot 3. It is clear that the use of gel electrophoresis for RNA isolation eliminates the necessity for

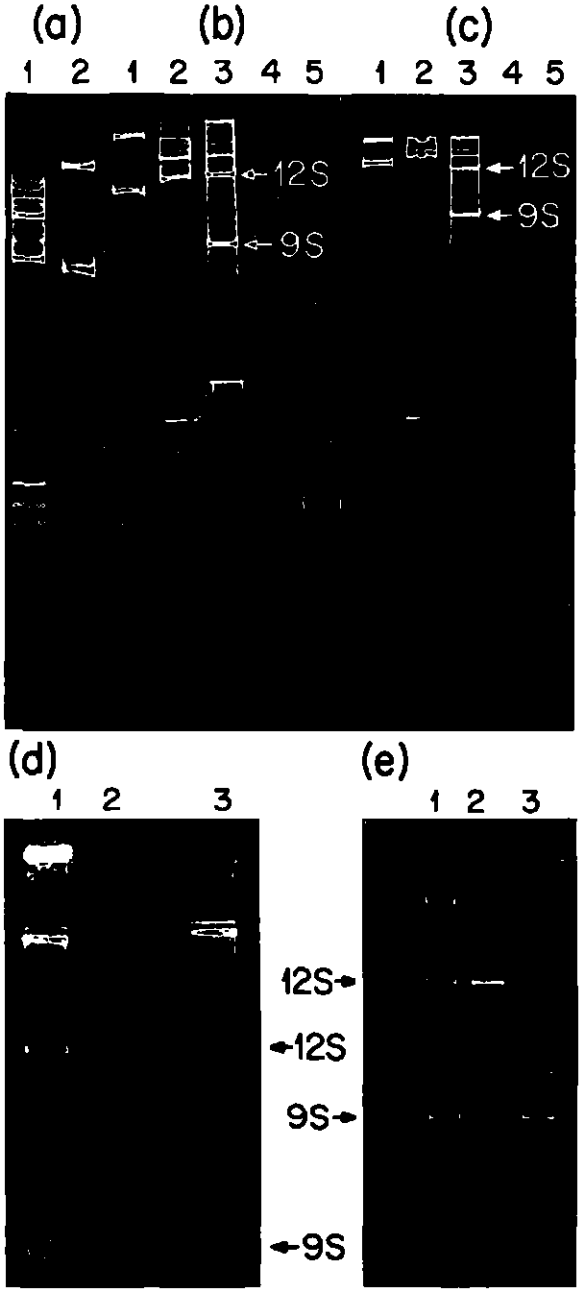


Figure 1. Acrylamide Gel Electrophoresis of Cytoplasmic Ribosomal RNA and Kinetoplast RNA

(a) EthBr-stained 3.5–10% acrylamide gel: slot (1) cytoplasmic rRNA; (2) E. coli rRNA.

(b) EthBr-stained 4–10% acrylamide gel: slot (1) E. coli rRNA; (2) cytoplasmic rRNA; (3) K-RNA DNAase-treated; (4) E. coli 4S RNA; (5) E. coli 5S RNA.

(c) EthBr-stained 5–10% acrylamide gel: slot (1) E. coli rRNA; (2) cytoplasmic rRNA; (3) K-RNA, DNAase-treated; (4) E. coli 4S RNA; (5) E. coli 5S RNA.

(d) Autoradiograph of a 3–4% acrylamide gel of long-term in vivo ^{32}P-labeled K-RNA and K-DNA: slot (1) K-RNA before DNAase treatment; (2) K-RNA after DNAase treatment; (3) covalently closed monomeric minicircles isolated from sonicated DNA networks by alkaline sedimentation.

(e) EthBr-stained 3–5% acrylamide gel: slot (1) K-RNA; (2) purified 12S K-RNA; (3) purified 9S K-RNA.

prior DNAase treatment.

The electrophoretic mobilities of the 9S and 12S RNAs in acrylamide were strongly affected by the gel concentration. In 4% acrylamide at 25°C, the 9S RNA ran slower than 16S E. coli rRNA (Figure 1b), while in 5% acrylamide, the 9S RNA ran faster than the 16S RNA (Figure 1c).

Several additional low molecular weight RNA species were seen in 10% acrylamide gels of DNAase-treated RNA isolated from the Renografin kinetoplast fraction (Figures 1b and 1c). These RNAs co-migrated with E. coli 4S RNA and 5S, 5.8S, 5.9S and 6.2S RNA species that were isolated from L. tarentolae cytoplasmic ribosomes as shown above. It is therefore probable that they were derived at least in part from cytoplasmic contamination of the kinetoplast fraction. In most kinetoplast RNA preparations, these small RNA species were present in lower concentrations than the 9S and 12S RNAs.

Control experiments were performed to show that the 9S and 12S K-RNAs were not artifacts of the organelle isolation procedure. First, a kinetoplast-enriched fraction was isolated by differential centrifugation in 0.25 M sucrose, and total RNA was isolated and shown to contain 9S and 12S RNA in addition to cytoplasmic rRNA species. This eliminated the possibility that exposure to Renografin gave rise to the 9S and 12S RNAs. In addition, the presence of 9S and 12S RNAs was demonstrated in total cell ^{32}P-labeled RNA by gel electrophoresis.

There was no evidence of heterogeneity of the 9S and 12S RNA bands in nondenaturing acrylamide gels. The 9S and 12S bands were completely susceptible to RNAase treatment and alkali digestion (data not shown).

In Vivo Labeling of the 9S and 12S RNA

Long-term labeling of cells with ^{32}Pi or ^{3}H-uridine led to the labeling of both 9S and 12S RNA to nearly identical levels as shown in the autoradiograph in Figure 1d, and the sucrose gradients of ^{3}H-uridine labeled K-RNA in Figure 2. Pulse-labeling cells with ^{32}Pi for 1 hr led to a slightly higher (1.28X) relative specific activity of the 12S species as compared with the 9S species (Figure 2a).

The effect of various antibiotics on the in vivo pulse-labeling of the kinetoplast RNA was studied by prelabeling cells with ^{3}H-uridine for several generations and then pulse labeling cells with ^{32}Pi for 1 hr in the presence of the antibiotics. Sedimentation profiles of the isolated kinetoplast RNA (DNAase-treated) are shown in Figure 2. The graphs were normalized with respect to the size of the ^{3}H-uridine-labeled peaks, so that the relative specific activities can be read off the graphs. The presence of 1 μg/ml ethidium bromide (Figure 2b) inhibited the labeling of the 9S by 74% and the 12S by 65% (Table 1). The presence of 2 μg/ml ethidium

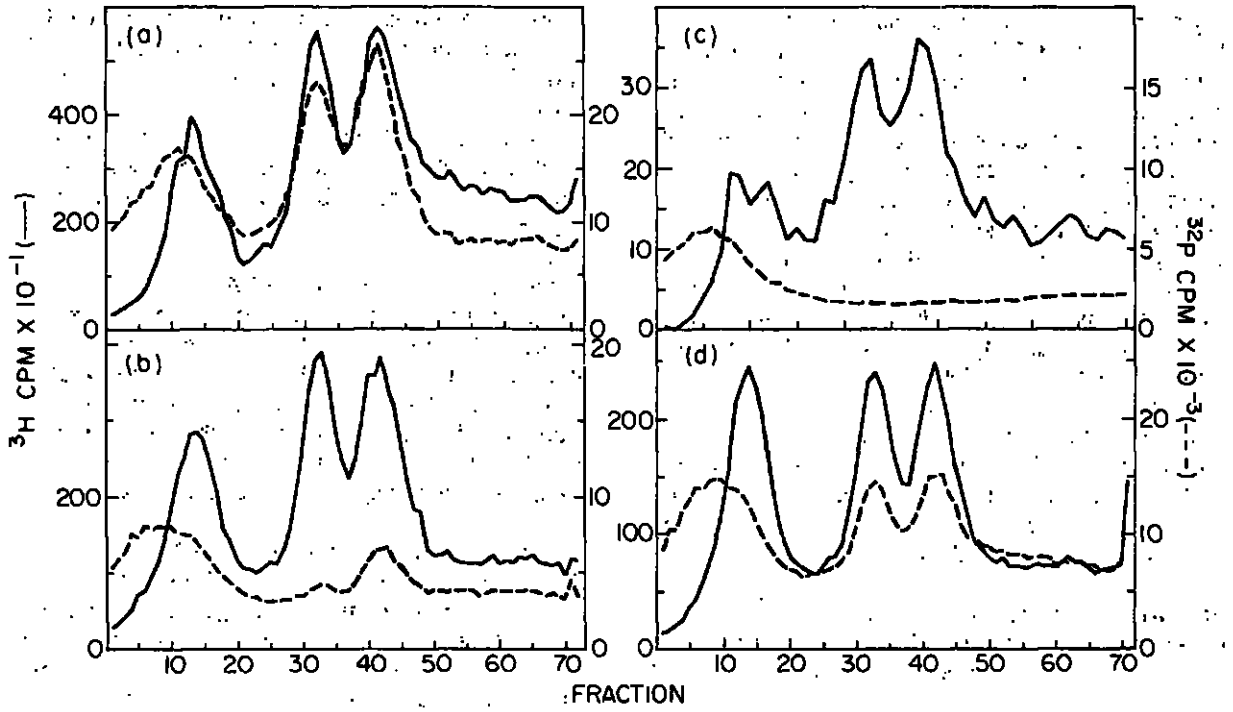


Figure 2. Effect of Antibiotics in in Vivo Labeling of 9S and 12S RNA

Cells in Neo YE medium were prelabeled for 3 days with 5 mCi ^{3}H-uridine per liter and then pulse-labeled for 1 hr with 10 mCi ^{32}Pi per liter (50 × 10^8 cells per ml) in the absence and presence of antibiotics. The antibiotics were added 10 min (EthBr) and 20 min (actinomycin D + camptothecin) before the addition of label. The kinetoplast fractions were isolated by the Renografin method, and the K-RNA was extracted, DNAase-treated and sedimented through 5 ml 5–20% sucrose gradients. Conditions: SW65 rotor, 5 hr, 60,000 rpm, 5°C. The fractions were collected on filter discs, TCA-processed and counted. (a) Control, no antibiotics; (b) 1 μg/ml EthBr; (c) 2 μg/ml EthBr; (d) 0.1 μg/ml actinomycin D plus 20 μg/ml camptothecin..

Table 1. Effect of Antibiotics on Labeling of 9S and 12S K-RNA

Antibiotic	Concentration	% Inhibition 9S RNA	% Inhibition 12S RNA	Experiment
Rifampin	10 μg/ml	0	0	1[a]
	100	89	57	1
	100	87	85	2[a]
	200	100	96	2
Ethidium Bromide	1 μg/ml	74	67	3[b]
	2	100	100	3[b]
Actinomycin D	0.1 μg/ml	27	42	3[b]
+ Camptothecin	20			

[a] Experiments 1 and 2: Late log-phase cells were preincubated with the antibiotic for 20–30 min and were then pulse-labeled with ^{32}Pi for 1 hr. The kinetoplast fraction was isolated, and the K-RNA was separated by electrophoresis in 3–5% acrylamide gels. The gels were stained with ethidium bromide and photographed, and then dried for autoradiography. The control cells were not treated with the antibiotics.
[b] Experiment 3: Cells were labeled for 2 days with ^{3}H-uridine, and then preincubated with the antibiotics and pulse-labeled with ^{32}Pi for 1 hr. The kinetoplast fraction was isolated, and the K-RNA was treated with DNAase and sedimented in sucrose gradients.

bromide (Figure 2c) completely inhibited the labeling of the 9S and 12S RNA, but had no effect on total cell RNA synthesis (data not shown). The presence of 0.1 μg/ml actinomycin D and 20 μg/ml camptothecin inhibited the labeling of the 9S by 27% and the 12S by 42% (Figure 2d).

The effect of the antibiotic rifampin on the labeling of the 9S and 12S RNA was studied by a different procedure which eliminated the necessity for double labeling and DNAase treatment. Late log-phase cells were pulse-labeled with ^{32}Pi for 1 hr in the absence and presence of 10, 100 and 200

μg/ml rifampin. The kinetoplast fraction was isolated, and the K-RNA was extracted and electrophoresed through a 3–5% acrylamide gel. The gel was stained with EthBr and photographed, then dried and subjected to autoradiography (Figure 3). Densitometry tracings of the negative and the autoradiograph allowed a comparison of the relative specific activities of each RNA band. It is clear that 10 μg/ml rifampin had little or no effect on the synthesis of 9S and 12S RNA, whereas 200 μg/ml rifampin almost completely inhibited synthesis of both species (Table 1). Rifampin at 200 μg/ml had no effect on total cell RNA synthesis in a 1 hr pulse (data not shown).

An identical protocol was used to study the antibiotic sensitivity of the synthesis of the low molecular weight RNAs found in the kinetoplast fraction. The labeling of these RNAs in a 1 hr pulse with ^{32}Pi was unaffected by 2 μg/ml EthBr.

Preparative Isolation of 9S and 12S RNA

Isolation of 9S and 12S RNA was achieved by several methods: sucrose band sedimentation of DNAase-treated kinetoplast RNA; preparative disc gel electrophoresis of untreated kinetoplast RNA; and slab gel electrophoresis of untreated kinetoplast RNA.

Elution of RNA from EthBr-stained bands in acrylamide slab gels yielded 9S and 12S RNA of high purity, as shown by the analytical slab gel in Figure 1e.

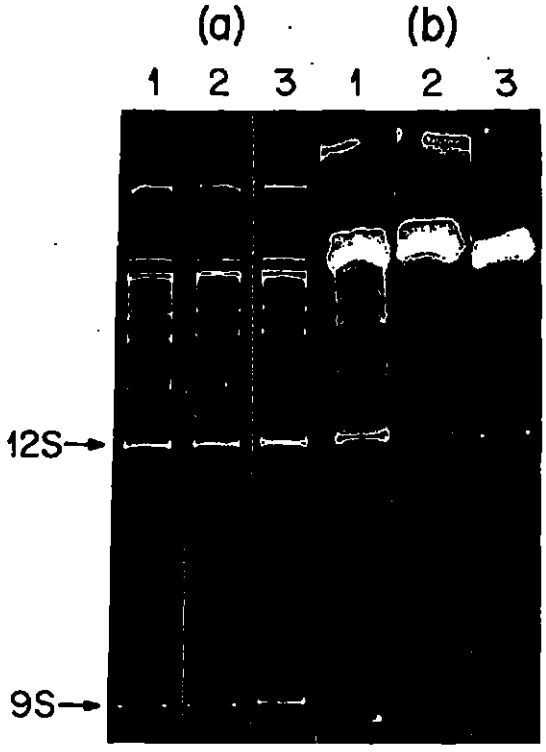


Figure 3. Effect of Rifampin on Synthesis of 9S and 12S RNA

Cells were labeled for 1 hr with ^{32}Pi in the absence and presence of rifampin, and the K-RNA was extracted from the purified kinetoplast fraction and run in a 3–5% acrylamide slab gel. Rifampin was added to the culture 20 min before addition of label. Conditions: 75 V, 17 hr, 25°C, 1 mm × 14 cm × 13 cm. (a) EthBr-stained gel: slot (1) control, no antibiotic; (2) 100 μg/ml rifampin; (3) 200 μg/ml rifampin. (b) Autoradiograph: slot (1) control; (2) 100 μg/ml rifampin; (3) 200 μg/ml rifampin.

Base Ratios of 9S and 12S RNA

Table 2 presents base ratio data for long-term ^{32}P-labeled 9S and 12S RNA. Both species are unusually high in A and U. The 12S RNA preparation was isolated by a single preparative sucrose gradient and had approximately 40% contamination with 9S RNA; the 9S RNA preparation was homogeneous by acrylamide gel electrophoresis.

Molecular Weights of 9S and 12S RNA

The molecular weights of purified 9S and 12S RNA were measured by agarose slab gel electrophoresis in the presence of the strong denaturing agent, methylmercury hydroxide. The gels were stained with EthBr and photographed. E. coli 4S, 5S, 16S and 23S RNAs, chicken 18S and 28S RNAs, and also rabbit globin mRNA (obtained from A. Tobin) were co-run as molecular weight standards. The 9S and 12S RNAs ran as homogeneous bands with calculated sizes of 518 ± 43(5) and 1022 ± 100(5) nucleotides, respectively (Figure 4).

Absence of Poly(A) in 9S and 12S RNA

Purified ^{32}P-labeled 9S and 12S RNAs were heated to 100°C and then self-annealed in 2 X SSC for 24 hr at 66°C. The annealed samples were then tested for RNAase A + T_1 resistance. Approximately 6.3%

Table 2. Base Ratios of ^{32}P-Labeled 9S and 12S RNA[b]

	9S RNA	12S RNA
A	34.7 ± 1.5%[a]	37.3 ± 1.2%
U	42.0 ± 2.0%	42.0 ± 2.0%
C	9.0 ± 1.0%	8.0 ± 1.7%
G	14.3 ± 0.6%	12.7 ± 3.1%
C + G	23.3 ± 0.6%	20.7 ± 3.1%

[a] Mean ± standard deviation [two runs with solvent (1) and one run with solvent (2)].

[b] The separated ^{32}P-labeled RNAs were ethanol-precipitated with 1 mg unlabeled E. coli tRNA as carrier. The pellets were hydrolyzed in 0.3 N KOH for 17.5 hr at 37°C, after which the solutions were neutralized with perchloric acid and the precipitate of Na perchlorate was removed by centrifugation. The solutions were then neutralized by extraction with alamine. One-dimensional chromatography was performed on PEI thin-layer plates using two different solvent systems: (1) 1 M acetic acid: 1 M LiCl (25:1), (2) 1 M acetic acid for 4 cm, followed by 0.3 M LiCl for 15 cm. The nucleotide spots were cut out and counted in Aquasol in a scintillation counter.

(9S) and 6.0% (12S) of the self-annealed RNAs were resistant to the RNAase treatment.

The RNAase resistance of denatured ^{32}P-labeled 9S and 12S RNA was measured by heating the RNA to 100°C in 2 X SSC or in dilute buffers, cooling and digesting with RNAase A + T_1. RNAase resistance values of 2.3 and 1.4% were obtained for the 9S and 12S, respectively, implying, in the absence of foldback secondary structure, maximum polyadenylate sequence lengths of 12 nucleotides for the 9S RNA and 14 nucleotides for the 12S RNA. In agreement with these results, only ~4% of the purified ^{32}P-labeled 9S and 12S RNAs bound to oligo(dT)-cellulose under poly(A) binding conditions. Isolation of 9S and 12S RNA by the use of phenol-chloroform-isoamyl alcohol instead of phenol alone did not change the oligo(dT)-cellulose binding characteristics.

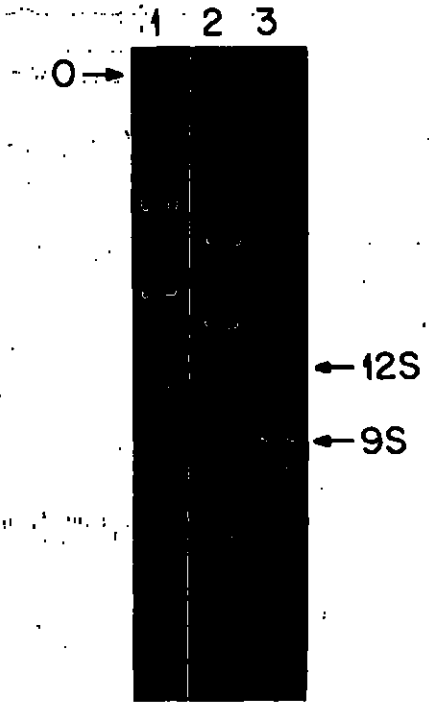


Figure 4. Methylmercury Hydroxide-Agarose Gel Electrophoresis of K-RNA

Slot (1) chicken 18S and 28S rRNA; (2) E. coli 4S, 5S, 16S and 23S RNAs; (3) K-RNA. Rabbit globin mRNA was not included in this gel as a reference.

Hybridization of K-RNA

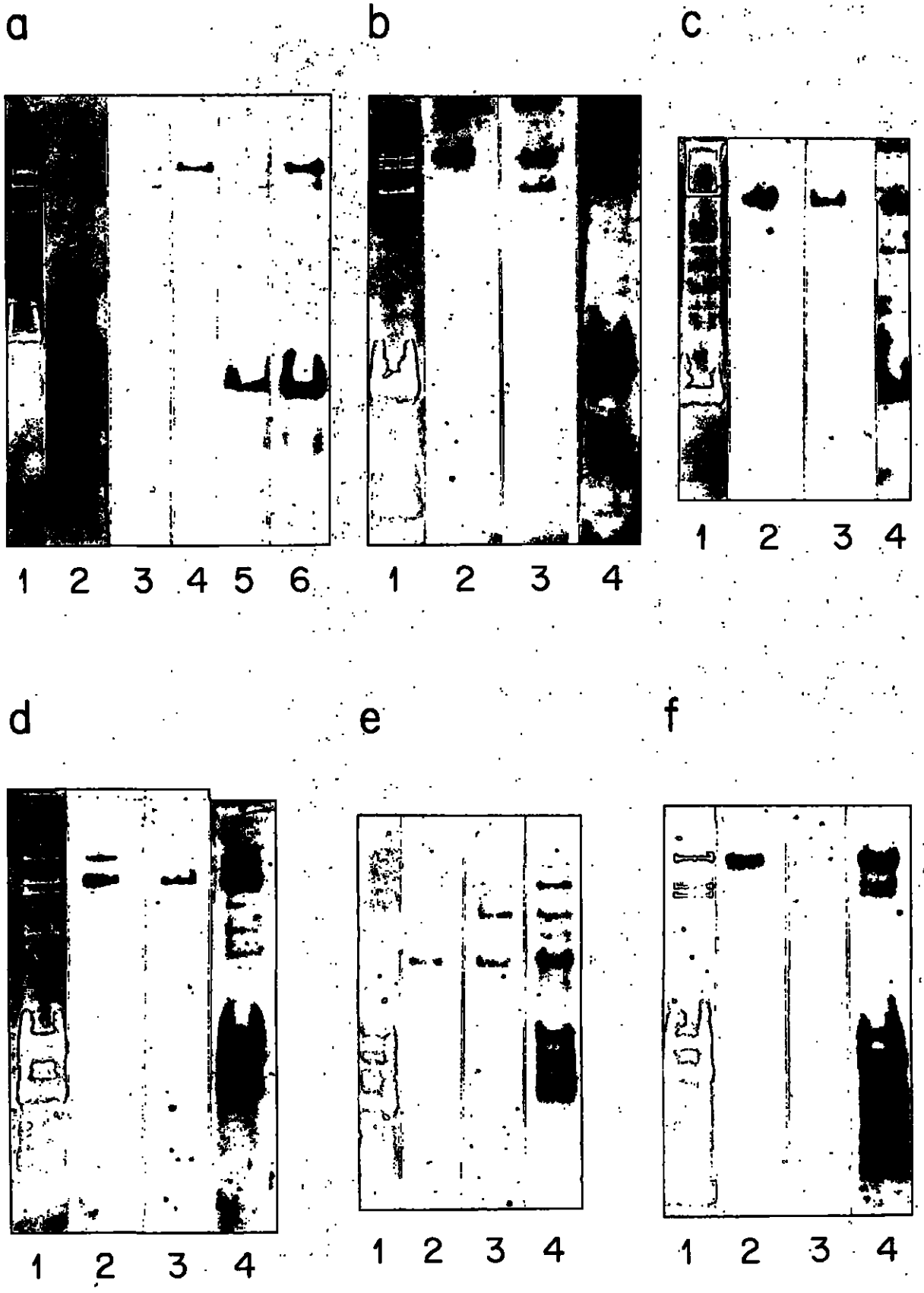
The homologies of 9S and 12S K-RNAs were tested by hybridization. Specificity of hybridization to K-DNA was shown by hybridizing long-term ^{32}P-labeled 9S and 12S RNA to a sonicated fraction of total K-DNA on filters. Specific hybridization to K-DNA was also demonstrated by DNA excess hybridization in solution. At least 48 and 44% of the 9S and 12S RNA, respectively, could be driven into an RNAase-resistant hybrid by annealing with a sonicated fraction of total K-DNA (0.1–0.3 μg 9S and 12S RNA, and 50 μg K-DNA in 100 μl 2 X SSC for 23 hr at 47°C).

The use of Southern's (1975) transfer technique combined with in vitro ^{32}P-labeling of the RNA to high specific activities allowed a more precise localization of the transcriptional origin of the 9S and 12S RNA. Long-term ^{3}H-labeled K-DNA was digested with several different restriction nucleases, and the fragments were separated by electrophoresis in 1% agarose (Figure 5). As demonstrated elsewhere (Simpson and Hyman, 1976; L. Simpson and B. Hyman, manuscript in preparation), the high molecular weight upper bands in each case represent maxicircle fragments, and the several lower bands represent minicircle fragments, which are overloaded and poorly resolved in this gel system. The gels were stained with ethidium bromide and photographed, and the fragment patterns were denatured and transferred to Millipore filters. In vitro ^{32}P-labeled 9S and 12S RNAs were allowed to hybridize to the Millipore filters. After hybridization, the filters were RNAse-treated and subjected to autoradiography to detect RNA/DNA hybrids. Following autoradiography, the filters were subjected to fluorography as a control for the retention of low molecular weight ^{3}H-DNA.

As shown in Figure 5, the 9S and 12S RNAs hybridized solely to the network "upper bands" in all cases examined. In the case of the Hpa II networks, the 9S RNA hybridized to upper bands 1 and 2, whereas the 12S RNA hybridized mainly to upper band 1. In the Hae III experiment, the 9S RNA hybridized solely to upper band 1, whereas the 12S RNA hybridized mainly to upper band 1, but also to upper bands 2 and 3 to lesser extents. Selective hybridization of both 9S and 12S RNAs to network upper bands was observed also in the Sal I and Alu I experiments, but in neither case were any differences apparent in the hybridization of the 9S and 12S RNAs. Also shown in Figure 5 are the results of hybridization of ^{32}P 9S and 12S RNAs to the gel profiles of two double digestions of networks—Hpa II + Hae III and Hpa II + Alu I. In both cases, several additional lower molecular weight hybridizable bands are apparent resulting from the cleavage of one or more of the Hpa II upper bands.

In all cases, the fluorographic controls demonstrated that little ^{3}H-labeled minicircle DNA was lost from the filters during the hybridization. Due to the lack of resolution of minicircle fragments in this gel system, however, any loss of minor bands would not have been visualized.

The low molecular weight RNAs found in the purified kinetoplast fraction were preparatively separated by gel electrophoresis in 10% acrylam-


a
b
c
1 2 3 4 5 6
1 2 3 4
1 2 3 4
d
e
f
1 2 3 4
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ide. The 5S, 5.8S and 6.2S RNAs were labeled in vitro with ^{32}P by 5′ kinasing of alkali-cleaved fragments and were allowed to hybridize to various DNAs bound to filters. These RNAs hybridized specifically to L. tarentolae nuclear DNA rather than to K-DNA (data not shown).

Cross-Species Hybridization of 9S and 12S RNA

L. tarentolae 9S and 12S K-RNAs, labeled in vitro with ^{32}P by 5′ kinasing of alkali-cleaved fragments, were found to hybridize to a presumptive maxicircle-derived high molecular weight upper band of Hae III-digested Phytomonas davidi K-DNA (Cheng and Simpson, 1978), although to a lesser extent than to the homologous K-DNA, as shown in Figure 6.

Discussion

The development of a procedure to obtain a highly purified kinetoplast fraction from L. tarentolae culture forms has led directly to the isolation of several stable kinetoplast RNAs. The 9S and 12S species represent the major stable RNA species which are localized specifically in the kinetoplast fraction. The 5S, 5.8S, 5.9S and 6.2S RNA species present in lower concentrations in the kinetoplast fraction appear to be derived from cytoplasmic ribosome contamination, since these species were visualized in RNA isolated from purified cytoplasmic ribosomes. A 4S RNA fraction was also present in the kinetoplast fraction.

The molecular sizes of the Leishmania 9S and 12S RNAs are 518 ± 43(5) nucleotides and 1022 ± 100(5) nucleotides as measured in denaturing gels. Little, if any, poly(A) sequences could be detected by oligo(dT)-cellulose binding and RNAase A + T_1 digestion. Both RNAs contain approximately 80% A + U. The high A + U content of the 9S and 12S RNAs probably accounts for the observed unusual dependence of electrophoretic mobility on gel concentration. Similar electrophoretic behavior in nondenaturing gels has been observed with the "10.5S" and "13S" mitochondrial rRNAs from Drosophila, which also contain approximately 80% A + U (Klukas and Dawid, 1976).

The in vivo synthesis of the 9S and 12S RNAs is sensitive to low levels of EthBr, which is a known inhibitor of mitochondrial transcription in HeLa cells (Perlman et al., 1973) and is relatively insensitive to actinomycin D plus camptothecin, which

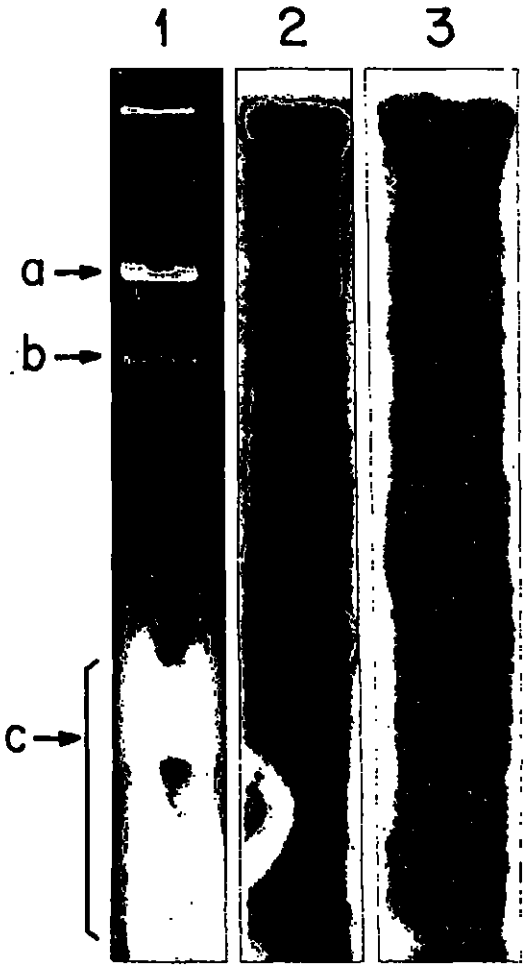

Figure 6. Cross-Species Hybridization of L. tarentolae 9S and 12S K-RNA

Hybridization of in vitro ^{32}P-labeled L. tarentolae 9S and 12S RNA to Southern transfers of agarose gel profiles of Hae III-digested Phytomonas davidi kinetoplast DNA on Millipore filters. P. davidi K-DNA was isolated and restricted as described elsewhere (Cheng and Simpson, 1978). The gel was 1% agarose in TBE buffer. Transfer and hybridization are described in Experimental Procedures. Slot (1) EthBr-stained agarose gel of Hae III-digested P. davidi K-DNA; (2) autoradiogram of 9S RNA hybrid; (3) autoradiogram of 12S RNA hybrid. The maxicircle fragment bands are labeled (a and b), and the minicircle fragment bands are labeled (c).

Figure 5. Homology of 9S and 12S RNA

Hybridization of in vitro ^{32}P-labeled, purified 9S and 12S RNAs to Southern transfers of agarose gel profiles of restricted ^{3}H network DNA on Millipore filters. Restriction, gel electrophoresis, transfer and hybridization are described in Experimental Procedures.

(a) Hpa II: slot (1) EthBr-stained gel; (2) fluorogram of Millipore filter directly after transfer; (3) autoradiogram of 9S RNA hybrid; (4) autoradiogram of 12S RNA hybrid; (5 and 6) fluorograms of some filters shown in (3 and 4).

(b) Hae III: slot (1) EthBr-stained gel; (2) autoradiogram of 9S RNA hybrid; (3) 12S RNA hybrid; (4) fluorogram of filter in (3).

(c) Sal I: slot (1) EthBr-stained gel. (2) 9S RNA hybrid; (3) 12S RNA hybrid; (4) fluorogram of filter in (3).

(d) Alu I: slot (1) EthBr-stained gel; (2) 9S RNA hybrid; (3) 12S RNA hybrid; (4) fluorogram of filter in (2).

(e) Hpa II + Alu I: slot (1) EthBr-stained; (2) 9S RNA; (3) 12S RNA; (4) fluorogram of filter in (2).

(f) Hpa II + Hae III: slot (1) EthBr-stained; (2) 9S RNA hybrid; (3) 12S RNA hybrid; (4) fluorogram of filter in (2).

are known inhibitors of high molecular weight nuclear RNA synthesis in HeLa cells (Attardi and Attardi, 1971; Abelson and Penman, 1972). The synthesis of the 9S and 12S RNAs is also sensitive to rifampin, a known inhibitor of RNA chain initiation in procaryotes (Riva and Silvestri, 1972). Both the EthBr and the rifampin sensitivities are consistent with a mitochondrial DNA transcriptional origin for the 9S and 12S RNAs. The lack of sensitivity to EthBr of the synthesis of the small RNAs present in the kinetoplast fraction is consistent with a probable origin of these RNAs from contaminating cytoribosomes in the kinetoplast fraction; a nuclear DNA transcriptional origin of the 5S, 5.8S and 6.2S RNAs was confirmed by direct hybridization.

We show elsewhere (Simpson and Hyman, 1976; L. Simpson and B. Hyman, manuscript in preparation) that the mitochondrial DNA of L. tarentolae consists of two different molecular species—the thousands of covalently closed minicircles and the larger molecules, which have been termed maxicircles by Kleisen et al. (1976b). A minicircle transcriptional origin of the 9S and 12S RNAs is rendered improbable both by our hybridization results and by the unusually high A + U content of these RNAs; the minicircle sequence contains 56% A + T and is approximately the same size as the 12S RNA molecule which contains 80% A + U. Furthermore, as in the case of all other hemoflagellates that have been examined (Riou and Yot, 1975; Kleisen, Borst and Weijers, 1976a), there is a marked sequence microheterogeneity within the population of minicircles in L. tarentolae (Price, DiMaio and Englund, 1976; Simpson and Hyman, 1976; L. Simpson and B. Hyman, manuscript in preparation).

We previously reported preliminary results that indicated a minicircle origin for the 9S and 12S RNAs of L. tarentolae (Simpson, 1973). These results were in error, possibly due to contamination of the minicircle preparations with maxicircle fragments. Our present hybridization data clearly indicate that the 9S and 12S RNAs are transcribed from the high molecular weight restriction fragments liberated from kinetoplast DNA networks by Hpa II, Hae III, Sal I or Alu I. We present evidence elsewhere that these fragments are cleavage products of the maxicircle component of the kinetoplast DNA network. We conclude that the 9S and 12S kinetoplast RNAs are transcribed in an EthBr-sensitive and rifampin-sensitive process from the kinetoplast DNA maxicircle.

We have shown elsewhere (Cheng and Simpson, 1978) that two RNAs with gel mobilities identical to the L. tarentolae 9S and 12S RNAs can be isolated from a kinetoplast fraction of Phytomonas davidi, suggesting that the occurrence of 9S and 12S kinetoplast RNA is a general phenomenon among the lower kinetoplastida protozoa. Furthermore, the evolutionary conservation of the maxicircle sequence implied by the cross-species hybridization of L. tarentolae 9S and 12S K-RNAs to a presumptive maxicircle-derived fragment of Phytomonas davidi K-DNA is consistent with the concept that the maxicircle represents the informational mitochondrial DNA in the kinetoplast. Previous studies (Steinert et al., 1973, 1976; Chance, 1976) have shown that there is no sequence homology between minicircles from different species and little sequence homology between minicircles from different strains of the same species. The gene product, if any, of the minicircle component of the K-DNA remains an open question.

Experimental Procedures

Cell Culture

L. tarentolae cells (clonal strain Lt-C-1) were grown continuously as described previously (Simpson and Braly, 1970). It is of some interest that the log-phase division time of this strain is now 6–9 hr as compared to the previously reported 10–12 hr. There is no indication of contamination with another species; the decrease in division time is probably a result of cell change during continuous subculture.

Isolation of Kinetoplast Fraction

This was performed as described previously (Braly et al., 1974) with the following modifications which improved the yield and the reproducibility. Large-scale cell rupture was accomplished by use of a Millipore Pressure Tank with an attached Luer-lock adaptor for a #26 disposable needle. Washed cells were resuspended in 1 mM Tris-HCl (pH 7.9 at 4°C), 1 mM EDTA at a cell concentration of 1.2×10^9 cells per ml and passed through the #26 needle at 100 lb/in² air pressure. Sucrose was immediately added to 0.25 M. Following centrifugation at 16,000 × g for 10 min, the crude kinetoplast fraction was treated with DNAase I (10 μg/ml; Worthington, E.P.) in 0.25 M sucrose, 0.02 M Tris HCl (pH 7.9), 5 mM $MgCl_2$, 0.3 mM $CaCl_2$ for 30–60 min at 5°C. The fraction was then washed by centrifugation in 0.25 M sucrose, 0.02 M Tris-HCl (pH 7.9), 2 mM EDTA, and resuspended in 76% Renografin (Meglumine diatrizoate 76%; E.D. Squibb and Sons), 0.1 mM EDTA, 0.25 M sucrose for layering underneath a 32 ml linear 20–35% (1.15–1.25 g/ml) Renografin containing 0.25 M sucrose, 0.02 M Tris-HCl (pH 7.9) and 0.1 mM EDTA.

The gradients were centrifuged for 2 hr at 24,000 rpm at 4°C in the SW27 rotor, and the kinetoplast band was recovered. The kinetoplast fraction was washed twice in 0.25 M sucrose, 0.02 M Tris-HCl (pH 7.9), 2 mM $MgCl_2$, and the pellet was resuspended in the medium for RNA isolation, usually 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$.

Isolation of Cytoplasmic Ribosomes

Cells were harvested, washed and resuspended in 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 25 mM KCl, and passed through a French pressure cell at 7000 lb/in². After clarification at 16,000 × g for 20 min, Triton X-100 was added to 0.5%, and the ribosomes were pelleted at 100,000 g for 2 hr in the #50 rotor. Pellets were resuspended in 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$.

Isolation of Kinetoplast RNA

Several different lysis media were tried with identical results: 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$; 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl; 10 mM Tris-HCl, 10 mM $MgCl_2$, 150 mM NaCl.

The first lysis medium was used for most of the work.

K-RNA was isolated from the kinetoplast fraction by SDS lysis, deproteinization with phenol, 0.1% 8-hydroxyquinoline or with phenol-chloroform-isoamyl alcohol (24:24:1 v/v) and ethanol precipitation.

In several large isolations (>10 cell culture), the pellets were resuspended in 0.01 M Tris-HCl (pH 7.9), 1 mM EDTA, and the contaminating kinetoplast networks were selectively removed by centrifugation for 2 hr at 22,000 rpm in the SW27 rotor. The RNA was then recovered from the supernatant by ethanol precipitation.

DNAase treatment of K-RNA was performed in 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ with 40 μg/ml DNAase I [Worthington, E.P.; rendered RNAase-free by the method of Zimmerman and Sandeen (1976)] for 30 min at 30°C. The RNA was phenol-extracted and ethanol-precipitated.

Band Sedimentation in Sucrose Gradients

Band sedimentation of K-RNA was performed in linear 5-20% sucrose gradients, 0.2 M NaCl, 0.05 M Na phosphate (pH 7.2) for 24 hr at 26,000 rpm in the SW27 rotor or for 3-5 hr at 60,000 rpm in the SW65 rotor at 5°C.

Acrylamide Slab Gel Electrophoresis

The vertical slab gel dimensions were 18 × 15 × 3 mm or 14 × 13 × 1 mm. The acrylamide stock solution was 9.5 g acrylamide, 0.5 g bisacrylamide in 100 ml. The electrophoresis buffer was 0.09 M Tris-borate, 2.5 mM EDTA (pH 8.3) (TBE).

Gels were stained in 1 μg/ml ethidium bromide in TBE for 30 min and photographed by 3000 Å transillumination (Brunk and Simpson, 1977) on Kodak Royal Pan 4 × 5 sheet film. Samples (5 μl) were mixed with 1/3 vol of 50% glycerol, 10% sarkosyl, 0.025% bromophenol blue, 0.025% xylene cyanole CFF.

Slab gels of ^{32}P-labeled RNA were dried on 3MM filter paper and covered with Kodak RP/54 or SB/54 X-ray film for autoradiography.

Elution of RNA from acrylamide gels was carried out by the method of Maxam and Gilbert (1977).

Slab Gel Electrophoresis with Methylmercury Hydroxide

This was performed exactly as described by Bailey and Davidson (1976), using 5 mM methylmercury hydroxide in 1.5% agarose in a 2 mm slab gel which was run at 50 V for 5 hr at 25°C. The reference RNAs used were as follows: E. coli 5S RNA, 40,680 daltons (Brownlee, Sanger and Barrell, 1968); E. coli 16S RNA, 0.56 × 10^6 daltons, and E. coli 23S RNA, 1.07 × 10^6 daltons (Kurland, 1960; Stanley and Bock, 1965); chicken 18S RNA, 0.70 × 10^6 daltons, and chicken 28S RNA, 1.58 × 10^6 daltons (Rabbitts and Work, 1971); rabbit globin mRNA, (α) 202,000 daltons and (β) 227,000 daltons (Gould and Hamlyn, 1973).

Radioactive Labeling of Kinetoplast RNA

In Vivo

All pulse-labeling experiments were performed with late log-phase cells (50-150 × 10^6 daltons).

To label with ^{32}PI, cells were grown in the low phosphate Neo YE medium of Simpson and Berliner (1974). For continuous labeling, 10-30 μCi ^{32}PI per ml (ICN) were used.

For long-term labeling with ^{3}H-uridine, 5 mCi ^{3}H-uridine (20 Ci/mM) were added per liter of Neo YE culture.

In Vitro

Purified 9S and 12S RNAs (1-2 μg) were labeled in vitro by the 5' end kinasing method of Maxam and Gilbert (1977) using T4 polynucleotide kinase (New England Biolab). The RNA was prepared for labeling by liberation of internal 5'-OH ends by mild alkaline hydrolysis (Maizels, 1976) [15 min at 75°C in 0.05 M Tris-HCl at (pH 9.5)].

γ-^{32}P-ATP was synthesized from HCl-free, carrier-free ^{32}Pi (ICN) and ATP by the exchange reaction of Maxam and Gilbert (1977).

Oligo(dT)-Cellulose Chromatography

Labeled RNA was diluted into the binding buffer [0.5 M NaCl, 0.01 M Tris-HCl (pH 7.5)] and applied to a 1 ml oligo(dT)-cellulose column (Collaborative Research, type T_3). The column was then rinsed with binding buffer and eluted with 0.01 M Tris (pH 7.5). The unbound RNA and the bound RNA were precipitated with an equal volume of 10% TCA, and collected on Millipore HA filters for scintillation counting.

Hybridization of ^{32}P-RNA to Gel Patterns of Restricted Networks

Closed long-term in vivo ^{3}H-labeled kinetoplast DNA (99,000 cpm/μg) networks were digested with the restriction nucleases Hpa II, Hae III, Sal I and Alu I (New England Biolabs) as described elsewhere (L. Simpson and B. Hyman, manuscript in preparation).

The restricted DNA samples were run in 1% agarose 2 mm slab gels for 3 hr at 50 V in TBE buffer. The gels were stained with 1 μg/ml ethidium bromide and photographed using 3000 Å transillumination, and the slots of interest were sliced out. The gel strips were denatured and neutralized, and the fragments were transferred to Millipore filters as described by Southern (1975). Hybridization was performed by moistening each filter with 130 μl of the ^{32}P-RNA in 1 × SSC (500,000 cpm), immersing the wet filter in Nujol oil and incubating at 47°C for 2 days. The filters were then extracted with chloroform, 6 × SSC and 2 × SSC, and were then treated with RNAase A and T1 (20 μg/ml and 20 units per ml in 2 × SSC) for 30 min at 25°C. The filters were than rinsed well with 2 × SSC, blotted dry and covered with Kodak SB/54 X-ray film to detect the ^{32}P hybrids. The filters were then impregnated with 20% PPO in toluene and covered with preflashed Kodak SB/54 film to detect ^{3}H-DNA (Bonner and Laskey, 1974; Laskey and Mills, 1975). The fluorograms were exposed at −70°C.

Several different hybridization protocols were examined (2 × SSC at 60°C; 4 × SSC in 50% formamide at 37°C; 2 × SSC at 47°C). The best results were obtained using 2 × SSC at 47°C.

Acknowledgments

David Masterman assisted in the ribosome studies, and Carl West assisted in the initial RNA isolation studies. This research was supported in part by grants from the National Institute of Allergies and Infectious Diseases and from the University of California, and by a Biomedical Science Support Grant.

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Received November 17, 1977; revised March 3, 1978

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