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RNA editing in mitochondria

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1. Introduction

1.1 Post-transcriptional modification of eukaryotic mRNA's

Several examples of specific modifications of nucleotide sequences within coding regions of mRNAs, and, in some cases, within rRNAs, have been observed in recent years. This process, which is known as RNA editing, ranges from the single C to U substitution in the mammalian apolipoprotein B (1,2), to the multiple C to U substitutions found at specific sites in plant mitochondrial mRNAs (3,4), the multiple C insertions found in mitochondrial mRNAs and rRNAs in Physarum polycephalum (5), and the multiple U insertions and deletions found in the mitochondria of kinetoplastid protozoa (6). The use of the term, RNA editing, for these various types of modifications does not necessarily imply that the mechanisms are identical. In fact, the polymerase stuttering-induced addition of extra G residues at specific sites in mRNAs from negative strand RNA viruses is also termed 'RNA editing' (7). The editing occurring in the mammalian apolipoprotein B mRNA appears to be due to a site-specific enzymatic deamination of cytidine (8-13), but nothing is known about the mechanism of the multiple C to U transitions in plant mitochondria. Likewise, nothing is known about the mechanism of C-insertion in Physarum mitochondrial RNAs. The best understood case is kinetoplastid RNA editing which is described below.

1.2 RNA editing in kinetoplastid protozoa

An unusual type of RNA processing occurs in the single mitochondrion of kinetoplastid protozoa. Many of the mRNA transcripts of structural genes encoded in the maxicircle DNA molecules are modified within coding regions in a post-transcriptional process characterised by the insertion and, less frequently, the deletion of uridylyate (U) residues (14). This process, known as RNA editing, involves small RNA molecules, guide RNAs (gRNAs), which specify the sequence information required. Two models have

been proposed for the mechanism of this process (15,16). Both models propose an initial base-pairing interaction between the 5' end of a specific gRNA and the mRNA immediately 3' to the pre-edited region (PER) to form the 'anchor' hybrid. The enzyme cascade model then proposes a specific cleavage at the first mismatched nucleotide of the mRNA, followed by the addition of a U residue to the liberated 3' end, and an RNA ligation to seal the mRNA backbone. Each U residue added extends the initial hybrid between the mRNA and gRNA by forming an additional basepair to either A or G 'guiding' nucleotides of the gRNA. This process is repeated until a complete hybrid between gRNA and mRNA is achieved. The trans-esterification model (16,17) proposes that the cleavage-ligation occurs by means of two successive trans-esterification steps. The first of these involves the 3'-terminal oligo (U)-tail of the gRNA as the source of the U's for transfer. Formation of gRNA/mRNA chimaeric molecules attached within the PER has been observed both in vivo among kinetoplast RNA (kRNA) molecules (16) and in vitro using mitochondrial extracts (18,19). Formation in vitro is dependent on complementary anchor sequences in the gRNA and mRNA (20). However, the available evidence does not allow a distinction to be made between the chimaeric molecules formed by trans-esterification or by cleavage-ligation. The trans-esterification model is attractive due to its close analogy with RNA splicing.

RNA editing is restricted to genes encoded by the mitochondrial genome which consists of a network of catenated DNA minicircles and maxicircles. This network, referred to as kinetoplast DNA (kDNA), is contained within the single mitochondrion which reticulates throughout the entire cell and is located in close proximity to the basal body of the flagellum (21). The genes for the mitochondrial rRNAs and a set of mitochondrial structural genes, several of which undergo RNA editing, are encoded on the maxicircle DNA. The maxicircle DNA of Leishmania tarentolae consists of 20-50 copies of a 30 kb circular molecule. However, the bulk of kDNA is comprised of minicircles, which are present in high copy number (approximately 10^4 molecules per network) and display multiple sequence classes. In L. tarentolae each copy of the DNA minicircle is organized into a conserved region of approximately 170 bp and a variable region of approximately 700 bp which defines the sequence class. Each sequence class encodes a single unique gRNA located approximately 150 bp from the end of the conserved region (22). In other kinetoplastid species, the number of conserved regions varies from one to four. In Trypanosoma

brucei there is a single conserved region, but the variable region encodes three gRNA genes (23). A region of 'bent' DNA of unknown function exists either adjacent to the conserved region in the minicircles of L. tarentolae and T. brucei, or at 90° to the conserved region in the larger minicircles of Crithidia fasciculata. The presence of this bend decreases the electrophoretic mobility of the DNA in poly-acrylamide gels as compared to agarose gels and is partially responsible for the increased complexity of the poly-acrylamide gel profiles of restriction enzyme-digested kDNA (24). The complexity of minicircle DNA varies between kinetoplastid species. Some species, which lack the ability to live in an insect host, have minicircles of a single sequence class. The kDNA of T. brucei contains approximately 300 different minicircle sequence classes; the kDNA of L. tarentolae contains at least 17 different sequence classes in differing abundances (25). The complexity of restriction digested kDNA, as visualized by electrophoresis in gradient poly-acrylamide gels, can be used to classify and type different strains within a species, at least for Leishmania and Trypanosoma cruzi. The term, schizodeme, was coined to indicate organisms with similar kDNA restriction profiles (26,27). The kDNA restriction profile is a very useful molecular marker since strains are frequently mislabelled, even some available through the American Type Culture Collection (28).

In this chapter, procedures are described for the growth of kinetoplastid cells, isolation of the kinetoplast-mitochondrion fraction, isolation of kDNA and kRNA, identification of gRNAs by computer analysis, isolation of gRNAs by hybrid selection and assay for several mitochondrial enzymes possibly involved in RNA editing including the terminal uridylyl transferase (TUTase), an RNA ligase, a cryptic RNase, and a gRNA:mRNA chimaeric-forming activity.

2. Growth and Maintenance of Kinetoplastid Protozoa

2.1 Choice of Species for experimental work

The kinetoplastid protozoa comprise a large group of parasitic flagellated cells with a single multilobular mitochondrion containing the kDNA network of catenated circular DNA molecules (29). The kinetoplastid flagellates belong to 8-10 genera, several of which are digenetic with a life cycle that

involves successive vertebrate (or plant) and invertebrate hosts, and several of which are monogenetic with a life cycle in a single invertebrate host. The monogenetic species such as Crithidia or Leptomonas have simpler nutritional requirements than the digenetic species such as Trypanosoma or Leishmania. In the latter case, the stage of the life cycle in the insect vector is generally easier to culture than the stage in the vertebrate host. Phytomonas is a digenetic species which inhabits a plant host and an insect vector. Unlike the other digenetic species, the stage from the plant host grows readily in simple media and may provide a model system for the study of metabolic changes occurring during the life cycle (F. Opperdoes, personal communication).

RNA editing has been studied in three species to date: T. brucei, L. tarentolae and C. fasciculata (6,14,29-31). The major differences between these protozoa involve the complexity of the minicircle DNA population and the correlated extent of editing of the three cryptogenes - ND7, COIII and MURF4. In addition, regulation of RNA editing has been observed to occur during the life cycle of T. brucei (32,33). The authors have chosen to use the saurian leishmania, L. tarentolae, as a model system to study RNA editing for the following reasons:

- * The cells are not pathogenic for humans, which is a major advantage in terms of growing large amounts of cells in the laboratory for isolation of mitochondria, nucleic acids and enzymes.
- * The cells grow rapidly (6-9 h division time) in brain heart infusion (BHI) medium, an easily prepared rich medium, without the need for serum supplementation.
- * The cells can be frozen for storage.
- * Cell concentrations of 4×10^8 cells/ml can be obtained at stationary phase.
- * The cells can be ruptured, after swelling in hypotonic medium, to yield an intact kinetoplast-mitochondrion fraction which is active in transcription and possibly also in RNA editing.
- * Several recently-isolated strains exist for comparative studies.
- * The minicircle DNA and therefore the gRNA complexity is limited, making it feasible to determine a complete list of gRNAs for all the genes known to be the subjects of RNA editing.

In contrast, the other two protozoa in which RNA editing is known to occur have some disadvantages:

* The procyclic cells of T. brucei require complex media with serum and do not reach high cell densities. The bloodstream cells must be grown either in a rodent host or in a complex medium in which they only reach low cell densities (34).

* The main disadvantage of C. fasciculata is that the cells swell poorly in hypotonic buffers and do not break by the use of shear forces alone. On the other hand, they grow extremely easily and to high densities in BHI medium and also in a defined medium. They also plate on agar surfaces more easily than either L. tarentolae or T. brucei.

The major disadvantage of working with L. tarentolae is that the biology of the parasite within the natural lizard host is essentially unknown. Therefore, one cannot study the vertebrate stage of the life cycle. It is clear, however, that the leishmania which infect lizards form a subgroup of the genus Leishmania (28,35), which also contains the mammalian pathogenic leishmania such as L. major, L. mexicana and L. brasiliensis.

2.2 Growth and Maintenance of L. tarentolae:

Initial stocks of L. tarentolae (UC strain) can be obtained from Dr. L. Simpson, Howard Hughes Medical Institute, UCLA, Los Angeles, CA 90024. Protocol 1 describes the maintenance and storage of stock cultures. To avoid accumulation of genotypic changes by prolonged culture, begin a new stock culture from frozen stocks every few months.

Protocol 1. Growth and storage of L. tarentolae stock cultures

Equipment and reagents

* 2 mg/ml hemin. Add 2 mg hemin (Sigma H-2375) per ml of 50mM NaOH, stir for 30 min and sterilize by filtration through a 0.22 µm membrane filter (Nalge). Store at -20°C.

* Tissue-culture grade water (prepared using Barnstead Nano-pure or Millipore Milli-Q cartridge filtration system) (Barnstead Co.; Millipore Corp.)

* Brain heart infusion (BHI) medium(a). Dissolve 37 g BHI powder (Difco) per litre of tissue culture grade water. Autoclave at 120°C for 30-40 min. When cool, aseptically add hemin to 10 mg/ml. The

medium is stable for several months at 5°C. Medium can be directly autoclaved in glass culture bottles or in glass bottles for storage.

* BHI medium containing 20% glycerol, sterilized by autoclaving.

* Tissue culture flasks (Corning #25100; 25 cm²)

* Freezer vials, sterile, polypropylene, 2 ml (Van Waters and Rogers #66008-309)

* Pyrex bottles, 500 ml (Fisher, #06-414-1C), with screw caps (Fisher, #06-414-2A).

Maintenance and Monitoring of Cultures

1. Inoculate healthy (motile) *L. tarentolae* cells at 0.6-1.5 x 10⁶ cells/ml in 5-10 ml BHI medium in 25 cm² tissue culture flasks.
2. Grow the cultures at 27°C.
3. Check the growth of the cultures daily using an inverted, phase-contrast microscope. Cultures can be monitored more accurately for the absence of bacteria by screening wet mount slides under phase contrast microscopy at 400X or 1000X magnification. Leishmania cultures have a characteristic wave-like appearance to the eye that is quite different from the homogeneous turbid appearance of bacterial cultures. Also the smell of the healthy culture is fruity and quite characteristic.
4. Every 2-4 days, aseptically remove most of the culture, leaving about 0.05 - 0.1 ml and add 5 ml of fresh BHI medium.

Storage and Recovery of Cells

1. Dispense 0.5 ml aliquots of healthy log-phase cultures (approximately 10⁸ cells/ml) into sterile 2 ml freezer vials.
2. Add 0.5 ml of BHI medium containing 20% glycerol.
3. Place the vials in a 1 litre beaker filled with cotton wool and put the beaker in a -70° C. freezer. This allows slow freezing of the cells.
4. The next day transfer the vials to liquid nitrogen for long term storage.

5. To recover live cells from stored cultures, thaw the vials rapidly and inoculate the contents into 2-5 ml of BHI medium. Check the cells by phase microscopy; they should be motile immediately after thawing.

a) No antibiotics are necessary if aseptic techniques are used and sterility is preserved.

2.3 Cloning of Kinetoplastid Protozoan Stock Cultures

Most laboratory kinetoplastid stocks are uncloned and may contain several different strains. Soon after receipt, cells should always be cloned either by limiting dilution in microtiter plates or by growth on 0.7-1.0% agar/BHI-hemin plates and selecting single colonies. One should verify the identity of the strain or even the species by a combination of light microscope morphology and molecular characteristics. Schizodeme analysis by comparison of kDNA sequences (Protocol 4) is an easy method to verify the identity of a strain of T. cruzi or Leishmania.

2.4 Growth of Cultures for Preparation of Kinetoplast Components

Cultures up to 1 litre in volume can be grown in 3.8 litre roller bottles using a standard roller bottle culture apparatus at 16 r.p.m. Harvest the cells by centrifugation at 2500 x g for 10 min at 5^o C. Grow larger cultures in a stirrer culture apparatus with forced aeration and harvest the cells by filtration as described in Protocol 2. Cells can also be grown in a microbiological fermentor with aeration and stirring. However, the addition of silicon antifoam (Antifoam B, Sigma) is required to prevent foaming.

Protocol 2. Growth and harvesting of large-scale cultures

Equipment and Reagents

* Stirrer culture apparatus (Bellco 15 litre or 36 litre bottles, #1964-15000 or 36000, with overhead stirrer #7664-00110 and stainless steel impeller, #1964-60015).

* Air pump or source of compressed air fitted with a 0.2 um Gilson microbiological filter on input and exit lines.

* Compressed O₂ (medical grade)

* Transverse filter system (Millipore Pellicon system with 0.45 um Durapore filter cassette) with 8 l/min peristaltic pump (Millipore Masterflex XX80 ELO 01).

* Log-phase culture of L. tarentolae grown as in Protocol 1.

* BHI medium (Protocol 1)

Method

1. Inoculate BHI-hemin medium with L. tarentolae at approximately 1.2×10^6 cells/ml using a log-phase culture. For 15 litre bottles use 180 ml of culture at cell density of 10^8 cells/ml.

2. Stir the culture at maximum rate at 27^o C. Blow filtered air into the bottle (but not through the medium) at 6-8 litres/min. Filter the exhaust air into a hood exhaust.

3. If larger cell yields are desired, after approximately 50 h, substitute O₂ for air. This will allow cells to grow at log phase rate up to a cell density of approximately 4×10^8 cells/ml, which is twice the density reached with air alone.

4. Harvest the cells when they have reached a density appropriate for the subsequent preparation (see later Protocols). Cool the culture by immersing the bottle in ice water while stirring. Concentrate the culture to approximately 100-200 ml by filtration using the Millipore Pellicon transverse filter system. The cells can be washed by addition of the desired wash medium to the concentrated cell suspension and reconcentrating. Cells within the filter system are flushed out by a final rinse with the wash medium. After concentration, the cells are pelleted by centrifugation at 2500 x g for 10 min at 5^o C. For kDNA isolation, the cells are resuspended in SET medium and frozen at -70^o C (See Protocol 3). For isolation of the kinetoplast-mitochondrion fraction, cells are immediately processed (see Protocol 6).

3. Kinetoplast DNA

3.1 Introduction

Kinetoplast DNA is present in the form of a single giant network per cell composed of approximately 10^4 catenated minicircles and 20-50 maxicircles (21). The kDNA network has a sedimentation coefficient of 4000 S units and is relatively resistant to shear forces due to its compactness (36,37). It is easily isolated from a sheared total cell lysate by sedimentation through CsCl (27). The maxicircle DNA represents 5% of the kDNA and can be isolated on the basis of its relatively higher AT content (84% A+T versus 55% A+T for minicircle DNA) after release from the network by digestion with a restriction enzyme that cuts only once or infrequently (38). The complete sequence of the 23 kb maxicircle of T. brucei is known and 21 kb of the 30 kb maxicircle genome of L. tarentolae has been sequenced (Genbank entry LEIKPMAX). The structural genes are clustered in ~17 kb and the remainder, which is known as the divergent region, represents tandem repeats of differing complexities.

Minicircles and maxicircles replicate once per cell cycle in an S phase which is synchronous with the nuclear DNA S phase. Minicircles are randomly decatenated, replicate outside the network and are recatenated at two sites at the ends of the kDNA nucleoid body (39). Replicated minicircles are 'nicked' or 'gapped', and are simultaneously covalently closed early in the G2 phase of the cell cycle. Maxicircle DNA replicates by the rolling circle mechanism and a low concentration of linearized maxicircle intermediate molecules can be found free of the network (40). Therefore, to maximize yields and to obtain covalently closed circular DNA molecules, stationary phase cells must be used for kDNA isolation.

The complexity of the minicircle DNA population is characteristic of each kinetoplastid species. Furthermore, in Leishmania and T. cruzi, a specific sequence heterogeneity within the minicircle population defines each particular strain or schizodeme. Tibayrenc and Ayala (41) have suggested that the genetic reason for this heterogeneity is the long-term genetic isolation of each schizodeme or clonal line without gene exchange. In the case of T. cruzi, they have coined the term 'clonet' (Tibayrenc and Ayala, personal communication) to describe these lines of parasites which have been clonally derived and isolated for perhaps millions of years. In any case, the term 'schizodeme' is an appropriate operational term to describe T. cruzi or Leishmania strains that differ in kDNA minicircle sequences.

3.2 Isolation of kDNA

Protocol 3 describes the isolation of kDNA from stationary phase cultures of L. tarentolae.

Protocol 3. Isolation of kDNA

- * SET buffer (0.15 M NaCl, 0.1 M EDTA, 0.01M Tris HCl, pH 7.5).
- * Stationary phase culture of L. tarentolae; about 4×10^8 cells/ml for large aerated cultures (Protocol 2) or $1.5\text{-}2.0 \times 10^8$ cells/ml for roller bottle cultures.
- * 10 mg/ml pronase (Cal Biochem). Dissolve pronase in SET and predigest the solution for 30 min at 37°C to inactivate nucleases.
- * CsCl (density gradient or optical grade)
- * Butanol saturated with water.
- * 10/0.1 TE buffer (10 mM Tris HCl, pH 7.9, 0.1 mM EDTA)
- * 10/1 TE buffer (10 mM Tris HCl, pH 7.9, 1 mM EDTA)
- * 30% Sarkosyl. Stir 30 g of sodium sarcosinate in 60 ml of water at 60°C . Add water to 100 ml. Store the solution in aliquots at 20°C .
- * Hypodermic syringe (12 ml.) fitted with an 18 gauge needle or
- * Dispensing pressure vessel (Millipore XX67 OOP 05 or 10, for the 5 litre or 10 litre sizes, respectively). This has an 18 gauge needle fitted via a luer lock adapter to one outlet and a pressure release valve attached to another outlet. Use a quick release tube adaptor to attach the vessel to a compressed air supply.
- * Beckman ultracentrifuge, SW28 rotor and 36 ml polyallomer tubes (or equivalent)
- * 10 mg/ml ethidium bromide
- * Upper CsCl solution (37.62 g CsCl in 62 ml of TE buffer; refractive index at 25°C = 1.3705). TE buffer is 10/1.
- * Lower CsCl solution (29.2 g CsCl in 20 ml of TE buffer plus 0.14 ml of 10 mg/ml ethidium bromide; refractive index at 25°C = 1.4040). Note only the lower CsCl has dye.
- * Peristaltic pump
- * UV lamp (254 nm)

* DAPI (4',6'-diamidino-2 phenylindole, Sigma # D1388)

* 2 M NaCl

* N-butanol, saturated with water

Method

1. Harvest the cells from roller cultures by centrifugation (10 min at 2000-3000 x g) or by filtration for large cultures (Protocol 2)
2. Wash the cells by resuspending them in at least 50 vol of SET- buffer and repeating step 1.
3. Resuspend the cells at 1.2×10^9 cells/ml by stirring and pipeting in SET buffer ensuring there are no clumps. Add 0.02 vol of 10 mg/ml pronase and 0.1 vol of 30% sarkosyl. Incubate at 60° C for 1-3 h with occasional shaking until the lysate clarifies.
4. Pass the viscous lysate through an 18 gauge syringe needle at 25 psi to shear nuclear DNA selectively and decrease the viscosity. For small volumes (10-100 ml) use a 12 ml syringe with this gauge needle and force the lysate through by hand. For large volumes, use a dispensing pressure vessel with the pressure supplied by compressed air.
5. Centrifuge the lysate for 1.5 h in an SW28 Beckman rotor at 22 000 r.p.m. This pellets the crude network DNA.
6. Add 1-2 ml of 10/0.1 TE buffer to each gelatinous pellet. Resuspend each pellet by shaking and pour the pellets from all the tubes into a 250 ml polycarbonate screw cap flask. Use 6 ml TE per 1-2 l of original culture. Shake vigorously for 30 min to dissolve the crude network DNA. The crude network DNA can be stored at 5° C.
7. Prepare CsCl step gradients in SW28 polyallomer tubes. Slowly introduce 6 ml of lower CsCl solution below 24 ml of upper CsCl solution using a peristaltic pump to give a sharp interface.
8. Layer 6 ml of the resuspended crude kDNA solution (step 6) on each gradient and centrifuge the gradients for 15 min at 20 000 r.p.m. at 20° C in an SW28 rotor (brake on). The kDNA networks will sediment to the interface between the lower and upper CsCl solutions while nuclear DNA, RNA and

protein will remain in the upper CsCl solution. If shearing of lysate was insufficient, some nuclear DNA will also sediment close to the interface.

9. Visualize the kDNA band at the interface with the short wavelength UV lamp. Seal the top of the tube with a rubber stopper fitted with a needle attached to a 20 ml syringe. Puncture the bottom of the tube and use the syringe to collect the kDNA band into a 12 ml polystyrene centrifuge tube.

10. Remove the ethidium bromide by extracting twice with an equal vol of water-saturated N-butanol.

11. Dialyze the kDNA against 4 litres of 10/0.1 TE buffer.

12. Concentrate the kDNA solution to approximately 400 ul by several extractions with sec-butanol. Use an equal vol of sec-butanol for each extraction and the volume will be halved each time. Centrifuge for 2 min in a clinical centrifuge to break the emulsion. Transfer the 400 ul to a microfuge tube.

13. Extract the kDNA by vortexing with an equal vol of phenol:chloroform (1:1). Separate the phases by centrifugation at 10 000 x g for 1 min. Excess centrifugation will result in loss of network DNA at the interface.

14. Remove the aqueous (upper) phase and remove traces of phenol from it by vortexing it 2-4 times with an equal vol of water-saturated ether. Allow the phases to separate by standing and then aspirate off the ether.

15. Add 0.01 vol of 2 M NaCl and 2 vol of ethanol. Incubate in dry ice-alcohol bath for 15 min or at -20^o C overnight.

16. Recover the precipitated kDNA by centrifugation at 12 000 x g for 15 min at 5^o C in a microfuge.

17. Resuspend the kDNA pellet in 10/1 TE buffer at 1 mg/ml.

18. Check the integrity of the network DNA by diluting a sample ten-fold and adding DAPI to 1 ug/ml final concentration. Observe the stained kDNA using a UV microscope at 1000 x magnification (Fig. 1).

The size and shape of the liberated kDNA networks are distinctive for each species of kinetoplastid. If the shearing (Step 4) was too harsh, the networks will be fragmented. L. tarentolae networks often break into half- or quarter-sized networks. C. fasciculata networks are more stable to shear forces.

19. Store the kDNA in aliquots at -20^o C

Using the procedure described in Protocol 3, the yield of kDNA is 0.5-1.0 mg/litre of culture. If desired, nuclear DNA can be isolated from the crude DNA preparation (Step 6). Add two vol of cold (-20° C) ethanol and spool the nuclear DNA onto a glass rod. Dissolve the DNA in 10 ml of 10/1 TE buffer. Deproteinize it by phenol extraction and recover the DNA by ethanol-precipitation as described in Protocol 3, steps 13 - 16.

3.3 Schizodeme-typing of Kinetoplastid Protozoan Strains

Schizodeme typing is used to verify the strain of Protozoan used. It relies on the use of specific restriction enzymes to cut the kDNA minicircles and maxicircles. For L. tarentolae, Hae 3, Msp 1, Taq 1 and Rsa 1 are suitable enzymes which liberate most of the minicircles and have multiple sites in some of the minicircle DNA sequence classes. The procedure is described in Protocol 4.

Protocol 4. Schizodeme-typing of strains

Equipment and Reagents

- * kDNA prepared as described in Protocol 3.
- * Appropriate restriction enzymes and buffers (see suppliers instructions)
- * Polyacrylamide gel (1 mm thick, 4.5-10% linear gradient of acrylamide, 4% stack) in TBE buffer (See chapter X, Protocol X). Use a standard gradient former and pump and add 15% glycerol to the 10% acrylamide solution when forming the gradient.
- * 10 mg/ml ethidium bromide.
- * Concentrated NH₄OH
- * Silver nitrate (1 g in 10 ml water)

Method

1. Digest 4 ug aliquots of the kDNA with several different restriction enzymes.
2. Separate the restricted kDNA on the 4.5-10% polyacrylamide gradient gel using TBE buffer in the electrode reservoirs as described in chapter X, Protocol X.

3. Visualize the DNA bands by staining the gel with either ethidium bromide (0.5 ug/ml final concentration) (42) or silver.

4. Silver staining (43,44): Fix the gel in 200 ml 50% methanol, 10% trichloroacetic acid for 30 min. Wash twice in 250 ml 5% acetic acid-10% ethanol for 20 min each. Wash twice in 250 ml 10% ethanol for 10 min each. Mix 40 ml 0.1 M NaOH, 3 ml concentrated NH₄OH, 10 ml silver nitrate solution, and stir until black precipitate dissolves. Add water to 200 ml. Stain gel in this solution for 40 min. Wash gel with 300 ml water for 5 min. Develop gel with 300 ml freshly made 0.01% citric acid containing 0.45 ml formaldehyde. Stop development in 20% ethanol, 5% acetic acid for 10 min. Wash gel three times in 250 ml 20% ethanol for 30 min each time.

The complex restriction pattern obtained, which is mainly due to the digested minicircle DNA, is characteristic of a particular schizodeme. Maxicircle DNA can be seen as minor high molecular weight bands. Patterns of several *T. cruzi* schizodemes are shown in refs. 26, 27 and 45-47 (Fig. 2).

3.4 Isolation of Maxicircle DNA

In all kinetoplastid species analyzed, maxicircle DNA, which ranges in size from 23-36 kb, has a relatively high A+T content as compared to minicircle DNA. This property can be exploited to allow the separation of maxicircle DNA on Hoechst 33258-CsCl density gradients (38). The Hoechst dye binds preferentially to A+T-rich sequences, thereby decreasing the buoyant density. The technique is described in Protocol 5. The linearized maxicircle DNA prepared by this method from *L. tarentolae* is 30 kb in size.

Protocol 5. Isolation of maxicircle DNA

Equipment and Reagents

* kDNA prepared as described in Protocol 3

* Appropriate restriction enzymes and buffers (as described in supplier's instructions). Use an enzyme that cuts maxicircle DNA once or infrequently. Eco R1 is suitable for *L. tarentolae* kDNA.

* 0.7% agarose gel in TBE buffer (Chapter X, Protocol X)

- * CsCl (optical grade)
- * 0.5 mg/ml Hoechst dye L33258 (Sigma, B 2883)
- * 10/1 TE buffer ([Protocol 3](#))
- * Beckman ultracentrifuge, 50 and 50.2 rotors and tubes (or equivalent).
- * UV light (254 nm)
- * Sec-propanol and N-butanol
- * Reagents for ethanol precipitation of DNA ([Protocol 3](#))

Method

1. Dilute 1 mg of kDNA into 2 ml of Eco R1 restriction enzyme buffer. Add 1000 units of Eco R1 and incubate the mixture for 3 hr at 37^o C.
2. Monitor the extent of release of the 30 kb maxicircle DNA by running 10 ul samples on a 0.7% agarose gel. ([Chapter X, Protocol X](#)).
3. Mix the 2 ml digested kDNA with 10/1 TE buffer to 12 ml. Add 18.5 g CsCl and stir until dissolved.
4. Add 0.5 mg/ml Hoechst 33258 dye dropwise with mixing to approximately 1 ug dye per ug DNA. Stop the addition if the solution becomes cloudy since this can result in the precipitation of the DNA.
5. Adjust the density of the solution to a refractive index of 1.3950 at 25^o C by adding either more CsCl or 10/1 TE buffer.
6. Centrifuge at 40 000 r.p.m. in the Beckmann 50.2 or Ti60 rotor for 48 h at 40 000 r.p.m. to establish the density gradient.
7. Visualize the blue-fluorescing DNA bands with 3000 A^o UV illumination and recover the minor upper band. The lower band contains the undigested kDNA networks and released minicircle DNA. Re-adjust refractive index of the upper band to 1.3935, and centrifuge 6.5 ml per tube in the #50 rotor at 39 000 r.p.m. for 48 h. Again recover the upper band. This rotor provides a better separation and the second centrifugation completely removes any contaminating minicircle or kDNA.
8. Remove the Hoechst dye by extraction with an equal vol of isopropanol.

9. Dialyze the maxicircle DNA against 10/1 TE buffer and concentrate the DNA using sec-butanol (Protocol 3, Step 12).

10. Recover the DNA by ethanol precipitation (Protocol 3, steps 15 and 16).

4. Isolation of the Kinetoplast-mitochondrion

Kinetoplastids contain a single complex mitochondrion per cell. The portion of the mitochondrion that contains the kDNA nucleoid body is called the kinetoplast. Most kinetoplastid cells are very resistant to shear forces in isotonic media. However, in hypotonic media, most swell and consequently can be ruptured by shear forces, thereby releasing the swollen kinetoplast-mitochondrion (48). Due to the multilobular nature of the single mitochondrion, isolation of the entire organelle is probably not possible. When the swollen cell is ruptured, the single mitochondrion breaks at the narrow portion adjacent to the nucleus. After resealing, however, the mitochondrion portion containing the kDNA can be isolated by its relatively high buoyant density (1.2 g/ml) (49). Often the kinetoplast remains associated with the basal body of the flagellum, indicating some type of attachment, which is also apparent *in vivo*. The kinetoplast portion shrinks to a crenated disk upon addition of 0.25 M sucrose and is resistant to exogenous DNase I. Digestion with DNase I to remove nuclear DNA followed by density gradient centrifugation are then used to recover the kinetoplast-mitochondrion fraction as described in Protocol 6 (Fig. 3).

The use of Renografin gradients gives better results than sucrose or Percoll gradients, and flotation is better than sedimentation from the top. However mitochondria from Percoll gradients are more active in transcription assays (50) (Protocol 11) than are mitochondria from Renografin gradients.

Protocol 6. Isolation of kinetoplast-mitochondrion fraction

Equipment and Reagents

* STE buffer (85.6 g sucrose, 100 ml of 0.2 M Tris-HCl, pH 7.9, 10 ml of 0.2 M EDTA and H₂O to one litre)

- * STM buffer (85.6 g sucrose, 100 ml of 0.2 M Tris HCl, pH 7.9, 3 ml of 1.0 M MgCl₂ and H₂O to one litre)
- * 76% Renografin (Squibb Pharmaceutical Co.; 66% diatrizoate meglumine and 10% diatrizoate sodium).
- * 76% RSE buffer (8.56 g sucrose, 50 ul of 0.2 M EDTA and 76% Renografin to 100 ml)
- * 20% RSE buffer (26.3 ml of 76% Renografin, 8.56 g sucrose, 10ml of 0.2 M Tris HCl, pH 7.9, 50 ul of 0.2 M EDTA and water to 100 ml, adjust the density to 1.14 g/ml).
- * 35% RSTE buffer (46.1 ml of 76% Renografin 8.56 g sucrose, 10 ml of 0.2 M Tris HCl, pH 7.9, 50 ul of 0.2 M EDTA and water to 100 ml; adjust the density to 1.26 g/ml)
- * 60 ml plastic syringe fitted with a 26 gauge needle and a piston driven by compressed air at 100 psi, or dispensing pressure vessel (see [Protocol 3](#)) fitted with a 26 gauge needle.
- * Beckman ultracentrifuge, Sorvall RC-5 centrifuge or equivalent and SS34 or GS3 rotors plus appropriate tubes.
- * 1.75 M sucrose
- * SET buffer, DAPI and UV microscope ([Protocol 3](#))
- * L. tarentolae cells harvested at late log phase (50-150 x 10⁶ cells/ml) from either roller cultures (1 litre) or aerated stirred cultures (13 litre) as described in [Protocol 2](#).
- * 20-36% RSE gradients. Layer 16 ml of 35% RSTE buffer in a series of SW28 tubes. Gently overlay with 16 ml of 20% RSTE. Freeze tubes at -20^o C. Thaw the tubes overnight at 4^o C before using. The gradient is created by the freezing and thawing. The advantage is that many tubes can be easily prepared and stored frozen until use.
- * DNase I (Sigma, electrophoretically pure; 2 mg/ml in 10 mM Tris-HCl, pH 7.4, 10 mM CaCl₂, 50% glycerol)
- * 93% Percoll (Pharmacia) in 0.25 M sucrose, 10 mM Tris HCl, pH 7.9, 0.1 mM EDTA, 0.01 mM 2-mercaptoethanol.
- * 52.5% Percoll in same medium as above.
- * 16% Percoll in same medium as above.

Method - Renografin gradients

1. Wash the cells by resuspending them in 50-100 vol of ice cold SET buffer and centrifuging at 4000 x g for 10 min. Repeat this washing. Alternatively, add SET to the cell filtrate when the Millipore Pellicon system (Protocol 2) is used.
2. Thoroughly resuspend the cells in X vol of cold 10/0.01 TE buffer and leave them on ice. X is calculated by dividing the total cell number by the factor, 1.2×10^9 .
3. Monitor the cells for swelling by phase contrast microscopy. At 1000 x magnification the swollen kinetoplast-mitochondrion is readily seen. It often 'pops' out of the cell and remains attached by the flagellum. Be careful not to induce rupture by shearing when applying the coverslip. If insufficient swelling has occurred, add more 10/0.01 TE buffer; 5-10 min on ice is generally sufficient for complete swelling.
4. Break the cells by passage through a 26 gauge needle. For 10-100 ml of cell suspension, use a 60 ml syringe with air driven piston. For 100 ml - 3 litre volumes, use the dispensing pressure vessel. When the needle becomes clogged, close the shutoff valve and replace the needle. Monitor the extent of breakage by phase contrast microscopy at 400 x magnification. There should be no more than one intact cell every 3-4 fields. If there are more intact cells, they will contaminate the final fraction, as visualized by the presence of cytosolic rRNA.
5. Add 0.125 vol of 1.75 M sucrose to the lysate directly after rupture. This causes the intact kinetoplast-mitochondrion to shrink back into a highly refractile crenated disc.
6. Centrifuge the lysate at 5°C for 10 min in the SS34 rotor at 11 500 r.p.m. or in the GS3 rotor for 15 min at 9 000 r.p.m.
7. Aspirate the supernatant carefully since the pellet is loosely packed. Resuspend the pellet in STM buffer (50 ml for each 2 litres of the lysate in step 4).
8. Add 0.005 vol of 2 mg/ml DNase I and incubate for 1 h on ice to digest the nuclear DNA which would otherwise cause aggregation of the material in the density gradient step.
9. Add an equal vol of STE buffer to stop the reaction.

10. Centrifuge the solution in the SS34 rotor for 10 min at 11 500 r.p.m. or in the GS3 rotor for 15 min at 9 000 r.p.m.
11. Aspirate the supernatant; the pellet should be well packed if the DNase digestion was successful.
12. Add cold 76% RSE buffer to the pellets (4 ml of 76% RSE per litre of original culture). Vortex well.
13. Layer 4-5 ml of the mixture beneath each 20-35% RSE gradient using a 12 ml syringe with polyethylene tubing attached to an 18 gauge needle. If the first drop of lysate floats, add more 76% RSE buffer to the lysate until the drop remains at bottom of gradient.
14. Centrifuge the gradients for 2 h at 24 000 r.p.m. at 5^o C in a Beckmann SW28 rotor.
15. Visualize the kinetoplast-mitochondrion band just above the 76%-35% RSE interface by side illumination. Puncture the side of the tube and remove the band using a syringe and needle.
16. Dilute the mixture with 2 vol of STE buffer and centrifuge the preparation in an SS34 rotor for 15 min at 11 500 r.p.m. A sample of the pellet can be resuspended in STE for visualization by phase microscopy at 1000 x to check the purity. The kDNA can be visualized by staining with 1 ug/ml DAPI and observing by phase contrast and UV microscopy.
17. Wash the pellet by resuspending it in 50 vol of STE buffer and centrifuging as in step 16. A final wash with STM is used prior to kRNA isolation. STM causes aggregation of the mitochondria, so microscopy is performed prior to this wash.
18. Resuspend the mitochondrial pellet in an appropriate medium (see later protocols) and store it frozen at -70^o C in small aliquots.
 - A. A quantitative estimate of the contamination can be obtained by measuring the relative amount of cytosolic rRNA in the final preparation of kRNA (Protocol 7) using gel electrophoresis in 1% agarose gels in TBE.
 - B. The RSE gradient procedure (step 13) is the rate-limiting step in large scale preparations. The lysate from up to two litres of culture can be loaded on each Renografin gradient and 6 gradients can be run in each SW28 rotor.

Method - Percoll gradients

1. Follow above steps 1-11.
2. Resuspend pellet in 93% Percoll (use 4 ml Percoll for pellet from 1 litre culture).
3. Layer under 16-52.5% Percoll gradient (use 2 gradients for material from 1 litre culture). Centrifuge 45 min at 24 000 r.p.m. in Beckmann SW28 rotor.
4. Remove kinetoplast band by side puncture. Dilute with 200 ml of STE. Recover kinetoplast-mitochondrion fraction by centrifugation at 12 000 x g for 30 min at 5^o C. Wash one time and resuspend in STE.

5. Isolation of transcripts from the mitochondrial genome

5.1 Introduction and Strategy

Isolation of intact RNA (kRNA) from the purified kinetoplast-mitochondrion fraction (Protocol 6) is easily accomplished due to the lack of nuclease activity in this fraction (49). The major steady-state components of the kRNA are the 9S and 12S mitochondrial rRNAs and tRNAs (51). All the mitochondrial tRNAs appear to be nuclear-encoded and must be transported into the mitochondrion by a mechanism as yet undetermined (52,53). Un-edited transcripts of maxicircle structural genes can be detected by Northern blot hybridization using specific oligonucleotide probes. Edited transcripts can be detected using genomic DNA probes if the editing is not extensive. However, mature pan-edited mRNAs, such as the L. tarentolae RPS12 RNA (54), can be detected only by knowledge of the edited sequence. In this case, primary transcripts containing pre-edited regions can be monitored using genomic DNA probes. Furthermore, one can take advantage of the 3' - 5' polarity of editing to detect partially-edited mRNAs which still contain genomic 5' sequences, by selective PCR amplification using an oligo (T) 3' primer for the first strand synthesis and a genomic 5' primer for the subsequent PCR (54,55). The ratio of pre-edited transcripts to mature edited mRNA varies from gene to gene (56). Partially-edited mRNAs are usually less abundant than edited or pre-edited RNAs and can only be detected by selective PCR amplification in L. tarentolae.

The gRNA transcripts which are involved in RNA editing comigrate with tRNA in agarose gels and migrate ahead of tRNA in polyacrylamide gels (52). The 3' oligo (U)-tails of gRNAs vary in length from 5-28 nt and are responsible for the characteristic family of electrophoretic bands each differing by a single nt (57). The abundance of gRNAs is relatively low compared to mitochondrial tRNAs and so they can not be visualized in gels stained with ethidium bromide unless 4-5 ug of kRNA is used per lane.

5.2 Isolation of kinetoplast RNA

Protocol 7 describes the isolation of kRNA from the kinetoplast-mitochondrion fraction prepared by Renografin gradient centrifugation (Protocol 6).

Protocol 7. Isolation of kinetoplast RNA

Equipment and Reagents

- * TMN buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 5 mM NaCl)
- * DNase I (RNase-free; BRL, #80475A)
- * Phenol:chloroform (1:1, v/v)
- * 10 mg/ml ethidium bromide
- * Kinetoplast-mitochondrion fraction. Prepare by Renografin gradient centrifugation (see Protocol 6) and resuspend the mitochondria from 1-2 litres of original cell culture in 5-10 ml of TMN buffer. May be frozen at -70^o C until use.
- * 1% agarose gel in TBE buffer (Chapter X, Protocol X).
- * 10% SDS

Method

1. If necessary, quickly thaw the kinetoplast-mitochondrion fraction, add 0.01 vol of 10% SDS and incubate the mixture on ice for 5 min.
2. Extract the mixture by vortexing with an equal vol phenol:chloroform (1:1) as described in Protocol 3, Step 13.

3. Re-extract the interface with one vol of water and pool this with the first upper phase.
4. Ethanol-precipitate the nucleic acids, (Protocol 3, steps 15 and 16).
5. Resuspend the nucleic acids in TMN buffer using 1.0 ml TMN per 2-3 litres of original cell culture.
6. Add 0.005 vol of 2 mg/ml DNase I and digest the mixture for 30 min at 37^o C.
7. Extract the solution with phenol:chloroform and precipitate the kRNA with ethanol as described in steps 2-4.
8. Wash the pellet in 70% ethanol. Resuspend it in water (1 ml per 4 litres of original culture yields approximately 2.0 ug RNA/ul).
9. Monitor the purity by electrophoresing 2-5 ug of the sample in a 1.5% agarose gel in TBE buffer, and staining the gel with ethidium bromide (0.5 ug/ml). Any contaminating cytosolic rRNA will be apparent as three bands migrating above the mitochondrial 12S rRNA band (the cytosolic large rRNA has a break and gives rise to two bands in gel electrophoresis). Also visible are the 9S rRNA and the tRNA bands (51).
10. Store the kRNA in aliquots at -20^o C.

5.3 Northern blot analysis of kRNA

Electrophorese the kRNA (Protocol 7) in a formaldehyde-agarose gel and blot the gel onto a nylon filter. Hybridize the filter with an appropriate radiolabelled probe complementary to the kRNA sequences under investigation. For internal size standards, strip and reprobe the kRNA blot with a nick-translated pLt120 probe, which represents a 6.6 kb fragment of the maxicircle DNA containing the 9S and 12S rRNA genes, and the ND7, COIII and Cyt 6 genes (58). The resulting bands in the autoradiograph should be 320 nt, 610 nt, 1100 nt and 1200 nt in size.

5.4 Hybrid selection of guide RNAs

A hybrid selection procedure can be employed (57) to isolate specific gRNAs. The method makes use of synthetic oligonucleotides which contain a reactive amino group at the 5' end. The amino group is covalently reacted with the hydroxyl groups of agarose in the presence of a strong reducing agent. This

step immobilizes the oligonucleotides to the agarose which can then be used for affinity purification of specific RNA species by column or batch procedures. Protocol 8 is a modification of the filter hybridization method of Wood et al. (59) adapted for the hybrid selection of specific RNA species complementary to the oligonucleotides coupled to the agarose beads. A stringent wash removes non-specifically bound RNAs before the selected RNAs are eluted by using a low salt buffer at an elevated temperature.

Protocol 8. Hybrid selection of guide RNAs

Equipment and Reagents

- * Synthetic DNA oligonucleotide, 100 ug; 20-30 nt complementary to the gRNA to be isolated. At the last step of the synthesis, couple the reagent, Aminolink 2 (Applied Biosystems) to the 5' end, following the supplier's instructions.
- * kRNA from 5 liters of a log phase Leishmania culture at a cell density of $0.7 - 1.5 \times 10^8$ cells/ml is used for the hybrid selection of each gRNA (see Protocol 7).
- * 10% SDS stock solution
- * ImmunoPure Ag/Ab Immobilization kit (Pierce 44890) together with immobilization buffer as provided by the manufacturer
- * Centricon 10 centrifugal microconcentrator (Amicon 4205)
- * 50 ml centrifuge tubes with plug seal caps (Corning 25331-50)
- * 20 x SSC buffer stock - (3.0 M NaCl, 0.3 M trisodium citrate, pH 7.0 with NaOH)
- * 5.0 M tetramethylammonium chloride stock solution (Fischer #04640-500; adjust the density to a refractive index of 1.421 at 25^o C.
- * 1M Tris-HCl, adjust pH to 8.0 using HCl
- * 0.5 M EDTA, pH 8.0

* TMA buffer (3.0 M tetramethylammonium chloride, 50 mM Tris-HCl, pH 8.0, 2mM EDTA, 1% SDS).
Make this up using 5.0 M tetramethylammonium chloride, 10% SDS, 1 M Tris-HCl, pH 8.0 and 0.5 M EDTA.

* 6 x SSC buffer and 1 x SSC buffer (prepared using 20 x SSC buffer stock)

* 0.1 x SSC buffer containing 0.1% SDS (prepared using 20 x SSC buffer stock and 10% SDS)

* Hybridization buffer (1.0 M NaCl, 0.166 M HEPES-KOH, pH7.5, 1 mM EDTA, 0.1% SDS)

* Wash buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

* 2 ml agarose beads together with the appropriate chromatography column (as provided by the ImmunoPure Ag/Ab Immobilization kit, Pierce 44890).

* Carrier nucleic acid (5'-amino oligonucleotide, not complementary to the gRNA to be hybrid selected).
5'-amino linked oligonucleotides are convenient carriers because they do not interfere with the 5' labelling reaction which follows the hybrid selection step. Circular DNA molecules or glycogen may work as well.

Method

1. Attach 100 ug of the aminolink 2-oligonucleotide to 2 ml of agarose beads in 2 ml of the recommended immobilization buffer.
2. At the end of the required incubation period pack the agarose into the column and wash it twice with 5ml wash buffer and store the washed column at 4^o C. until required.
3. For hybrid selection of gRNA, equilibrate the column in hybridization buffer.
4. Load approximately 500 ug of kRNA in 1 ml hybridization buffer onto the column.
5. Seal the column with the provided cap and stopper; incubate it for 18 h at 37^o C.
6. Drain the column and add 5 ml of ice cold 6xSSC.
7. Repeat step 6 twice, then incubate column at 4^o C for 2 h with gentle agitation by placing the column into a 50 ml centrifuge tube (Corning) and attaching it horizontally to a rotating platform which is set to 50 r.p.m.
8. Drain the column and equilibrate it with 5 ml of TMA buffer at room temperature.

9. Repeat step 8 and place the sealed column into a 50 ml centrifuge tube. Seal the centrifuge tube and place it into a 37^o C waterbath for 30 min.
10. Drain the column, then add 5 ml TMA buffer (preheated at 60^o C). Again seal column into a 50 ml centrifuge tube and place into a 60^o C waterbath for 30 min.
11. Wash the column successively with 10 ml of TMA buffer at room temperature, 10 ml of ice cold 6 x SSC buffer and 10 ml of ice cold 1 x SSC buffer.
12. Elute the gRNA at 70 C for 20 min using 2 ml of 0.1 x SSC, 0.1% SDS, pre-heated to 70^o C. This is done by placing the column into a sealed 50 ml centrifuge tube and immersing it into a 70^o C waterbath. Repeat this twice with fresh buffer.
13. Concentrate the pooled eluate with the Centricon 10 centrifugal microconcentrator following the manufacturer's instructions.
14. Store the hybrid-selected gRNA at - 20^o C.
 - a) Note that the exact washing temperature must be varied according to the size of the hybrid (59). Highly stringent washing occurs at T_m - 4^o C. The melting temperature (T_m) of a hybrid in TMA buffer is solely dependent on its length and can be derived from Fig. 3 in (59).
 - b) Do not heat the columns containing the agarose beads to more than 70^o C. Above this temperature melting of agarose could occur.

5.5 PCR amplification of partially-edited mRNAs and gRNA:mRNA chimaeric molecules

Partially edited RNAs can be amplified by use of an oligo-(dT) 3' primer complementary to the oligo (U)-tail of the gRNA, for the reverse transcriptase step (60) (Fig. 4). This is followed by the addition of a 5' primer consisting of a genomic sequence complementary to the unedited 5' sequence of the mRNA and Taq polymerase for the PCR amplification cycles. In a modified procedure, it is possible to select for partially-edited molecules covering a limited region of the mRNA by choosing the appropriate set of primers (55,61). In this case, the 3' primer should cover the edited sequence while the 5' primer should cover the upstream pre-edited sequence. PCR amplification selects for RNA molecules partially-edited in

the sequence between the two primers. This approach allows for fine analysis of partially-edited molecules at single sites.

In vivo chimaeric gRNA:mRNA molecules have been detected in kRNA by Northern blot hybridization and by PCR amplification using a 3' primer complementary to mRNA downstream of a PER (16). The polymerisation of cDNA using reverse transcriptase is followed by the addition of a 5' primer with the 5' terminal sequence from the corresponding gRNA. PCR amplification reveals the presence of chimaeric molecules. The existence of such chimaeras is indicative for the trans-esterification model. However, they could also arise as byproducts of a cleavage-ligation process.

Detailed procedures for PCR will be found in Chapter X (Protocols X, X) and in ref. (62). PCR products both from partially edited and chimaeric molecules may be checked by gel electrophoresis and their nucleotide sequences are determined.

5.6 Synthesis of gRNAs by transcription in vitro

Using bacteriophage promoters and purified phage RNA polymerase, gRNAs may be transcribed in vitro. For instance, a T7 phage promoter sequence can be incorporated into the 5' PCR primer for gRNA amplification (Section 5.5). The amplified DNA is then transcribed using T7 RNA polymerase. The transcription products will have heterogenous 3' termini due both to the propensity of the polymerase to add an extra non-encoded A or C and to premature termination (63), and so the desired species must be purified by gel electrophoresis. Such transcripts have been used to study the formation in vitro of gRNA:mRNA chimaeric molecules in a mitochondrial extract (18-20). Procedures for the transcription of RNA in vitro using phage promoters and polymerases will be found in Chapter X, (Protocol X) and Y (Protocol Y).

6. Run-On transcription in isolated kinetoplast mitochondria

Kinetoplast mitochondria isolated by the procedure described in Protocol 6 are active in run-on transcription whereby RNA chains initiated in vivo are completed in vitro. As explained in section 4, mitochondria prepared in Percoll gradients are generally more active in run-on transcription than those prepared using Renografin gradients. Nevertheless, mitochondria prepared by the latter procedures are adequate for studying run-on transcription. Run-on transcription may also be studied in mitochondrial extracts, prepared as described in Section 8.

Protocol 9 describes run-on transcription using intact mitochondria. Using this method, the rate of incorporation of [³²P]GTP increases for 10-15 min and then decreases.

Protocol 9. Run-on transcription in isolated mitochondria

Reagents

- * 5X STE (Protocol 6)
- * Mitochondrial fraction isolated as described in Protocol 6, resuspended at 2-3 ug/ul in STE buffer for immediate use or stored frozen in STE with 8% DMSO at -70^o C. Run-on transcription activity is stable for several weeks in frozen mitochondria.
- * 10 mM ATP
- * 10 mM CTP
- * 10 mM UTP
- * [α -³²P]GTP (New England Nuclear; 3000 Ci/mmol, 10 mCi/ml)
- * 30 mM potassium phosphate (pH 6.8)
- * 2 M KCl
- * 0.25 M Hepes-KOH, pH 7.5
- * 250 mM 2-mercaptoethanol
- * DE81 filter discs (Whatman, 2.5 cm diameter)
- * 0.5 M sodium phosphate buffer (pH 6.8), 0.5% sodium pyrophosphate.

Method

1. In a microcentrifuge tube on ice mix:

30 mM potassium phosphate	5 ul
2 M KCl	1.5 ul
0.25 M Hepes-KOH, pH 7.6	1 ul
250 mM 2-mercaptoethanol	1 ul
STE buffer	2.5 ul
[alpha- ³² P]GTP	1 ul
10 mM ATP	5 ul
10 mM CTP	5 ul
10 mM UTP	5 ul
Mitochondrial fraction	10 ul
H ₂ O to	50 ul

2. Incubate for 15 min at 27° C.

3. Remove 10-20 ul aliquots and spot onto DE81 filter discs.

4. Dry the discs and then wash them for 30 min in 100 ml of 0.5 M potassium phosphate buffer (pH 6.8), 0.5% sodium pyrophosphate. Repeat washing three times.

5. Replace the washing solution with 100 ml of ethanol and wash the discs as in step 4.

6. Dry the discs and count the retained radioactivity in a scintillation counter.

The run-on transcripts, synthesised by the method given in [Protocol 9](#) migrate in agarose gels as a smear, suggesting that they represent nascent RNAs elongated in vitro during the labelling period (50). The labelled RNAs hybridize to all regions of the maxicircle, even to the divergent region which shows very low levels of steady-state transcripts, and to minicircle DNA.

7. Identification of gRNAs by computer-assisted sequence comparison

In the editing of mRNAs, gRNA molecules specify the editing by forming duplexes with the edited mRNA (15). Such duplexes display G-U basepairs in addition to the canonical G-C and A-U base pairing. A search for gRNAs can be carried out with the help of a computer (see also ref. 64). The edited

mRNA sequences are compared with the kinetoplast genome (consisting of the maxicircle and the known minicircle DNA sequences) taking into account such non-canonical base pairing. Prior to the computer search, the sequence of all or part of the edited mRNA must be obtained by direct RNA sequencing. Ref. 65 gives details of RNA sequencing methods. Protocol 10 describes the computer analysis.

A modified local homology alignment program (BESTFIT, University of Wisconsin Genetics Computer group package, version 6) is used in the search for gRNA sequences. The scoring matrix is altered so as to score positively for complementary base pairs (including G-U) rather than for matches. Moreover, it is possible to apply different weights for each possible base pair.

Protocol 10. Identification of gRNA sequences by computer analysis

Preparation of the files

1. Download the kDNA genomic sequences from the Genbank database.
2. Reformat the files to GCG format.
3. Create a GCG sequence file with the determined edited mRNA sequence (use SEQED, UWGCG).
4. Reverse the mRNA sequence, using REVERSE (reverse only, UWGCG)

Modification of the scoring matrix

5. Edit the scoring matrix SWGAPDNA.CMP (FETCH this file) using an appropriate editor to score for base pairing instead of matches. The following table SWGAPDNA.CMP is an example of weighted scores:

	A	C	G	T	U	..
-0.9	0.01	-0.9	0.5	0.5	A	
	-0.9	1.0	-0.9	-0.9	C	
		-0.9	-0.9	0.25	G	
			-0.9	-0.9	T	
				-0.9	U	

Note: This table does not "punish" for C-A base pairing; C-A pairs have been found in putative gRNA/mRNA duplexes from C. fasciculata (66).

Running the program

6. Type: BESTFIT/DATA1= SWGAPDNA.CMP/PAI=0.01

7. Select a high value for gap weight (*100*) and gap weight length (*2.00*) to avoid any alignments with gaps.

8. In order to find the gRNA sequences corresponding to an edited mRNA sequence, it may be necessary to vary the size and borders of the input mRNA sequence (e.g. at the sites of two overlapping gRNA's). Known gRNAs help to define the borders for the search of additional gRNAs. The smaller the size of the gRNA:mRNA duplex, the more difficult it becomes to find the corresponding gRNA. (reference to NAR Waterman paper)

In the case of L. tarentolae, each minicircle encodes a single gRNA located 150 nt from the end of the conserved region (22). The DNA sequence of this region can be tested for the presence of gRNA sequences against known edited mRNA sequences. In the case of T. brucei, each minicircle encodes at least three gRNAs located between 18 mer inverted repeats (23). One could locate these conserved repeats and computer search the intervening DNA sequences.

Search for edited mRNA

In some cases putative gRNA sequences (for instance, as indicated by the presence of a 3' oligo (U)-tail) have been found prior to the identification of the corresponding edited mRNA (52). In this case the method described in Protocol 9 can be used to search for the corresponding mRNA. However, when genomic kDNA is scanned for the presence of the corresponding mRNA, the search is complicated by the fact that pre-edited mRNA does not yet contain the U residues to be inserted or deleted.

8. Enzymatic activities in the kinetoplast-mitochondrion fraction which are involved in RNA editing

8.1 Introduction

Several enzymatic activities which are thought to be involved in editing maxicircle transcripts have been identified in purified kinetoplast-mitochondrion fractions. These include a terminal uridylyl transferase (TuTase) (50), an RNA ligase (50), a site-specific cryptic RNase (67), and a gRNA:mRNA chimaeric-forming activity (18-20) (Fig. 3).

The TUTase adds U residues to the 3' hydroxyl group of RNA molecules with no apparent sequence specificity. The role of the TUTase is thought to be the re-addition of U residues to the 3' oligo (U)-tail of the gRNA which has been used as the source of UMP for transfer to the editing site of the mRNA in two successive trans-esterifications.

The function of the RNA ligase in the mitochondrion is unclear; in the original enzyme cascade model (15) for RNA editing, a ligase is required for joining together the cleaved mRNA fragments after addition of UMPs to the 3' hydroxyl group by the TUTase, but there is no requirement for an RNA ligase in the trans-esterification model (16).

A cryptic RNase in the kinetoplast-mitochondrion fraction can be activated by addition of heparin or by pre-digestion of the extract with Proteinase K or pronase (67). The authors have suggested that the cleavage activity involved in the activation is actually a site-specific hydrolysis catalyzed by the same enzyme which normally trans-esterifies gRNA and the mRNA, and that this hydrolysis is induced by inhibiting or destroying the TUTase. It is also possible that this activity is an enzyme involved in RNA turnover.

According to the trans-esterification model of RNA editing (16,17), uridine residues are directly transferred from the oligo-(U) 3' tail of the gRNA into the mRNA via a gRNA/mRNA chimaeric intermediate. Such chimaeric molecules have been found in steady state mitochondrial RNA from L. tarentolae (16) and T. brucei (68-70). In L. tarentolae, these in vivo chimaeras generally consist of gRNA covalently linked via the 3' oligo-[U] sequence to the corresponding mRNA at a normal editing site, with the downstream editing sites being fully edited. Recently, mitochondrial extracts have been prepared from Trypanosoma brucei (18,19) which show chimaera-forming activities in vitro. The in vitro-synthesized chimaeric molecules in Leishmania tarentolae (20) are similar but distinct from the ones observed in vivo

in that attachment also occurs predominantly at editing sites, but no editing could be detected downstream of the attachment site.

8.2 Preparation of mitochondrial extracts

Several of the activities involved in mitochondrial RNA editing mentioned in Section 8.1 may be assayed in either intact isolated mitochondria or various mitochondrial extracts. [Protocol 11](#) describes the preparation of detergent lysates of kinetoplast mitochondria isolated in Renografin gradients. These are the TL, TS and S-100 extracts.

Protocol 11. Preparation of Triton X-100 lysates

Equipment and Reagents

* Kinetoplast-mitochondrion fraction isolated by flotation in renografin density gradients (see [Protocol 6](#)).

Resuspend the washed mitochondrial pellet at 5 mg protein/ml in 20 mM Hepes-KOH, pH 7.5, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol. It may be stored frozen in 200 ul aliquots at -70^o C.

* 10% Triton X-100 (Pierce)

* Pellet pestle mixer (Kontes Glass Co #749520, motor driven)

Method:

1. If necessary, thaw the mitochondrial fraction in ice.
2. Add 6 ul of 10% Triton X-100 to 200 ul of the mitochondrial fraction in a microcentrifuge tube. Mix gently.
3. Homogenise the mitochondria for 15 sec at 5^o C using the pellet pestle mixer. This homogenate is the TL extract.
4. Centrifuge the TL extract for 30 min at 12 000 x g in a microcentrifuge (to obtain the TS extract) or for 1 h at 100 000 x g (to obtain the S-100 extract). In each case, carefully remove and retain the supernatant.
6. If necessary, store the extracts in aliquots at -70^o C.

8.3 Terminyl uridylyl transferase (TUTase)

The kinetoplast-mitochondrion fraction isolated from Renografin gradients contains a TUTase. This activity, which adds multiple uridine residues to the 3' hydroxyl group of RNA molecules, can be solubilized by homogenization with 0.3% Triton X-100. The solubilized TUTase activity may be assayed by the incorporation of UMP residues from UTP. Since it only requires one nucleoside triphosphate in addition to UTP, TUTase may be assayed in the absence of the run on transcription activity, which would also result in nucleotide incorporation. TUTase activity may be studied in either intact mitochondria or mitochondrial extracts. To assay TUTase activity in extracts, substrate RNAs must be provided by adding cytosolic RNA from L. tarentolae. This has five small rRNA components (215 nt, 195 nt, 175 nt, 140 nt and 110 nt), that migrate in polyacrylamide gels between the 9S rRNA and the tRNA regions (51).

No substrate RNA need be added with intact mitochondria, in which endogeneous mitochondrial RNAs are labelled with [³²P]UTP, including the 9S and 12S rRNAs and the gRNAs. The mitochondrial tRNAs do not label well with this enzyme activity.

Heparin (5 ug/ml) inhibits the TUTase activity of extracts, but does not affect the endogenous TUTase activity in isolated intact kinetoplast mitochondria probably due to the lack of penetration through the mitochondrial membrane.

Protocol 12 describes the assay of TUTase in mitochondrial extracts and the preparation of the cytosolic RNA substrate. Under these conditions, the incorporation of UTP occurs linearly for at least 30 min at 27°C. To assay TUTase in intact mitochondria, Protocol 12 should be modified by deleting the addition of rRNA substrate.

Protocol 12. Assay of terminal uridylyl transferase (TUTase) in isolated mitochondrial extracts

Reagents

- * Mitochondrial extract (Protocol 10) (2-3 ug protein/ul)
- * 0.25 M Hepes-KOH, pH 7.5
- * 2 M KCL

- * 60 mM Mg acetate
- * 10 mM ATP
- * 1 M DTT
- * [α - 32 P]UTP (New England Nuclear; 800 Ci/mmol, 10 uCi/ul)
- * 30 mM KPO₄ (pH 7.0)
- * Template RNA (0.5-1.2 ug RNA/ul)
- * 0.5 M NaPO₄ buffer (pH 6.8), 0.5% sodium pyrophosphate with and without 0.1% SDS
- * DE81 filter discs (Whatman, 2.5 cm. diameter)
- * TMN buffer (10 mM Tris HCl, pH 7.5, 10 mM MgCl₂, 5 mM NaCl)

Method:

A. Preparation of cytosolic RNA substrate

1. Follow Protocol 6 until step four in Methods.
2. Centrifuge cell lysate at 16 000 x g for 10 min at 5^o C.
3. Remove supernate and add 0.01 vol 10% SDS.
4. Deproteinize with equal vol of phenol:chloroform (1:1) as described in Protocol 3, step 13. Re-extract phenol interface with one vol of water and pool with the first upper phase.
5. Ethanol-precipitate the nucleic acids (Protocol 3, steps 15, 16).
6. Resuspend pellets in TMN buffer (1 ml TMN per 2-3 litres of original cell culture). Add 0.005 vol 2 mg/ml DNase I and digest for 30 min at 37^oC.
7. Extract with phenol:chloroform and ethanol-precipitate the RNA. Wash the pellet three times with 70% ethanol and spinvac dry. Resuspend RNA from four litres of original culture in 1 ml redistilled sterile water and store at -20^o C.

B. TUTase Assay

1. In a microcentrifuge tube on ice, mix:

0.25 M Hepes-KOH, pH 7.5	5 ul
2 M KCL	1.5 ul

60 mM Mg acetate	5 ul
ATP	5 ul
[alpha- ³² P]UTP	1 ul
Mitochondrial extract	10 ul
Cytosolic RNA substrate	1 ul
H ₂ O to	50 ul

2. Incubate the mixture for 40 min at 27^o C.
3. Stop the reaction by adding 50 ul of 0.5 M sodium phosphate buffer (pH 6.8), 0.5% sodium pyrophosphate, 0.1% SDS.
4. Remove 80 ul aliquots onto DE81 filter discs.
5. Process the discs and determine the retained radioactivity as described in Protocol 11, steps 4-6.

8.5 RNA ligase

An RNA ligase activity was first observed in total cell extracts from T. brucei (71). The relationship of the total cell RNA ligase activity to the mitochondrial activity described in L. tarentolae is not clear. The L. tarentolae RNA ligase activity cosediments with the kinetoplast-mitochondrion fraction (50). It is solubilised in Triton X-100 and thus can be assayed in mitochondrial extracts. Incubation of cytosolic RNA with mitochondrial TL extract and [alpha-³²P]UTP under the conditions required for TUTase in the presence of ATP and Mg²⁺ ions yields a 180 nt labelled RNA product whose relative electrophoretic mobility varies with the gel concentration, a behaviour characteristic of circular RNA molecules. RNA ligase is quantitatively assayed by the addition of [³²P]pCp to the 3' termini of RNA molecules as described in Protocol 13.

Protocol 13. Assay of RNA ligase in mitochondrial extracts

Reagents

* Mitochondrial extract (TL, TS or S100, Protocol 10)

- * Cytosolic rRNA substrate (500 ug/ml) prepared as described in Protocol 11
- * 10 mM ATP
- * 1 M MgCl₂
- * 0.25 M HEPES-KOH, pH 7.9
- * 0.1 M DTT
- * [alpha-³²P]pCp (New England Nuclear; 3000 Ci/mimol, 10 mCi/ml)
- * DMSO
- * RNA guard (Pharmacia, #27-0815-01, 33 000 u/ml)

Method

1. In a microcentrifuge tube on ice, mix:

Cytosolic RNA substrate	1-5 ul
MgCl ₂	2 ul
DTT	3.3 ul
HEPES-KOH, pH 7.9	20 ul
ATP	1 ul
DMSO	10 ul
[alpha- ³² P]pCp	2 ul
RNA guard	1 ul
Mitochondrial extract	10 ul
H ₂ O to	50 ul

2. Incubate the mixture at 4°C for 15 h.

3. Measure addition of [³²P]pCp to RNA substrate by spotting on DE81 filter disks and processing as described in Protocol 11, steps 4-6.

8.6 Cryptic RNase

A sequence - or structure - specific cryptic RNase activity can be detected in mitochondrial extracts (TL, TS or S-100 extracts) (67). This cryptic RNase can be activated either by addition of heparin (5 ug/ml) or by predigestion of the lysate with Proteinase K.

Protocol 14 describes the activation of the cryptic RNase and its subsequent assay. The RNA substrate used for this assay is a 200 nt RNA synthesised by in vitro transcription from a T7 bacteriophage promoter using T7 RNA polymerase. The template DNA consists of the 22 nt PER of the cytochrome b gene together with 56 nt of 5' flanking sequence, 186 nt of 3' flanking sequence and 73 nt of Bluescript vector sequence at the 5' end. Cleavage by the cryptic RNase occurs at one major site and four minor sites within the PER.

Protocol 14. Assay for a sequence - or structure specific cryptic RNase activity in mitochondrial extracts

Equipment and reagents

- * Mitochondrial extract (TL, TS or S100, Protocol 10)
- * Heparin (Sigma)
- * Proteinase K (BRL). Dissolve the Proteinase K at 10 mg/ml in 50 mM Tris-HCl, pH 8.0, 1 mM CaCl₂.
- * Tris-HCl, pH 7.5
- * MgCl₂
- * ATP
- * 10X buffer: 30 mM MgCl₂, 100 mM Tris HCl, pH 7.5, 50 ug/ml heparin
- * pNB2 plasmid - a 198 bp AccI/RsaI restriction fragment from the pLt120 maxicircle region containing the 5' end of cytochrome b gene was cloned into the SmaI site of pBluescript SK(-) vector (Stratagene).
- * pNB2 RNA substrate (10⁸ cpm/ug). Transcribe BamHI-digested pNB2 plasmid DNA in vitro using T7 RNA polymerase and [alpha-³²P]UTP as described in Chapter X (Protocol X)
- * Phenol: chloroform (1:1, u/u)
- * 8% polyacrylamide - 7.0 M urea denaturing (sequencing) gel, loading and electrophoresis buffers and equipment (see Chapter X, Protocol X)

Method

Activation of the cryptic RNase

1. Either add Proteinase K (100 ug/ml final concentration) to the mitochondrial extract and incubate the extract for 5 min at 37^o C or add heparin to 5 ug/ml. Activation by protease pre-digestion and heparin are synergistic and together result in better cleavage.

2. In a microcentrifuge tube on ice, mix:

10 X buffer	5 ul
ATP	5 ul
[³² P]-labeled substrate RNA (10 ⁴ cpm)	1 ul
Activated mitochondrial extract	10 ul
H ₂ O to	50 ul

3. Incubate the mixture for 1 h at 27^o C.

4. Phenol-extract the RNA and recover the RNA by ethanol-precipitation as described in Protocol 3, steps 13-16.

5. Add gel loading buffer (10 ul of 10 M urea) and analyse the cleavage products by gel electrophoresis and autoradiography as described in Chapter X, Protocol X.

8.7 gRNA:mRNA chimaeric-forming activity

In order to study the first step of the proposed trans-esterification model for RNA editing (16,17), the authors developed a cell free system using an extract from mitochondria prepared by sonication followed by salt extraction. Chimaeric formation is monitored by the covalent transfer of uniformly [³²P]-labelled gRNA to a higher molecular weight nonradioactive test mRNA by gel electrophoresis. Both gRNA and mRNA are synthesized by T7 transcription from PCR-derived DNA templates (63). Sequence analysis of the reaction products by selective PCR amplification and cloning revealed gRNA/mRNA chimaeric molecules as the most prominent products (20). This in vitro system has been used to confirm the importance of the anchor sequence between the gRNA and mRNA, and should be useful to develop conditions for complete in vitro editing (20).

Protocol 15. gRNA:mRNA chimaeric-forming activity

Equipment and Reagents

- * Centricon 10 centrifugal microconcentrator (Amicon 4205)
- * Mitochondrial fraction from one liter of Leishmania tarentolae isolated according to Protocol 6. Keep kinetoplasts at -70^o C until extraction is performed.
- * [α -³²P]UTP uniformly labeled gRNA (T7 transcribed, purified by gel electrophoresis)
- * mRNA containing pre-edited region plus flanking (anchor sequence) region (T7 transcribed and purified by gel electrophoresis)
- * 1 M HEPES-NaOH, pH 7.9
- * 0.5 M EDTA, adjust pH to 8.0 with NaOH
- * Glycerol, ultrapure (BRL 5514UA)
- * 2 M NaCl
- * 1 M KCl
- * 100 mM MgCl₂
- * 13% (w/v) polyethylene glycol 8000 (Sigma #P-2139)
- * N-laurylsarcosine (Sigma #L-5125)
- * 20 mM ATP (diluted in H₂O, Pharmacia #27-2056-01)
- * 100 mM dithiothreitol (DTT)
- * 100 mM PMSF (Phenylmethylsulfonyl, Sigma #P-7626) in isopropanol
- * Human placental ribonuclease inhibitor (BRL #5518SA)
- * 20 mg/ml Proteinase K (BRL #5530UA) stock
- * Hypotonic buffer (10 mM HEPES-NaOH, pH 7.9, 0.5 mM EDTA)
- * Extract buffer (20 mM HEPES-NaOH, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, prepared freshly from stock solutions)
- * 2x salt extraction buffer (40 mM HEPES pH 7.9, 50% [v/v] glycerol, 0.84 M NaCl, 0.4 mM EDTA, 1 mM PMSF, 1 mM DTT, prepared freshly from stock solutions)
- * Annealing buffer (20 mM HEPES-NaOH, pH 7.9, 100 mM KCl, 1 mM EDTA)

* Reaction buffer (8% [w/v] PEG 8000, 12.5 mM MgCl₂, 2.5 mM ATP, RNase inhibitor [1 unit/ul])

* Proteinase K solution (0.25% [w/v] N-laurylsarcosine, 25 mM EDTA, 0.25 units/ml proteinase K)

Method

1. The mitochondrial fraction from one litre of cell culture is resuspended in 2 ml of ice cold hypotonic solution. Keep on ice for 10 min.
2. The swollen mitochondria are disrupted by sonication (Braunsonic #1510, 100 watts, micro tip, three 20 sec periods, 5°C).
3. Immediately add 2 ml of 2 x salt extraction buffer and gently agitate the solution with a small magnetic stirrer for 30 min on ice.
4. Clarify the extract by a 30 min spin at 50 000 x g, 4°C.
5. Concentrate the extract at 4°C on two Centricon 10 microconcentrators to an approximate vol of 100 ul each.
6. Change buffer at 4°C using the same microconcentrators by applying twice 2 ml of extract buffer to each vial.
7. The pooled extracts (approximately 200 ul) are aliquoted and quick frozen in dry ice/ethanol. Stored at -80°C, chimaeric-forming activity is maintained for up to 3 month.
8. Anneal equimolar amounts of the test RNA molecules ([³²P]UTP-labeled gRNA and cold mRNA) in 2.5 ul annealing buffer. Denature 3 min at 70°C prior to annealing at 37°C and 25°C for 10 min each.
9. Add 8 ul of reaction buffer and 15 ul of thawed mitochondrial extract. Chimaeric formation is observed upon incubation for 15 to 120 min at 27°C.
10. The reaction is stopped with 100 ul proteinase K solution at 37°C for 20 min.
11. The RNA is recovered by phenol extraction and ethanol precipitation. The resulting RNA products were analysed on denaturing acrylamide-urea gels by autoradiography.

a) RNA products may also be analysed by sequence determination following reverse transcription, PCR amplification and cloning. In order to specifically amplify exogenous RNA, a 'tag' sequence may be added to the test RNA.

References

1. Hodges, P. E., Navaratnam, N., Greeve, J. C. & Scott, J. (1991) *Nucleic Acids Res.* **19**, 1197-1201.
2. Lau, P. P., Xiong, W., Zhu, H. -J., Chen, S. -H. & Chan, L. (1991) *J. Biol. Chem.* **266**, 20550-20554.
3. Gualberto, J. M., Lamattina, L., Bonnard, G., Weil, J. H. & Greinenberger, J. M. (1989) *Nature* **341**, 660-666.
4. Covello, P. S. & Gray, M. W. (1989) *Nature* **341**, 662-666.
5. Mahendran, R., Spottswood, M. R. & Miller, D. L. (1991) *Nature* **349**, 434-438.
6. Simpson, L. & Shaw, J. (1989) *Cell* **57**, 355-366.
7. Vidal, S., Curran, J. & Kolakofsky, D. (1990) *EMBO J.* **9**, 2017-2022.
8. Driscoll, D., Wynne, J., Wallis, S. & Scott, J. (1989) *Cell* **58**, 519-525.
9. Chen, S. -H., Li, X., Liao, W. S. L., Wu, J. H. & Chan, L. (1990) *J. Biol. Chem.* **265**, 6811-6816.
10. Lau, P. P., Chen, S. -H., Wang, J. C. & Chan, L. (1990) *Nucl. Acids Res.* **18**, 5817-5821.
11. Smith, H. C., Kuo, S. -R., Backus, J. W., Harris, S. G., Sparks, C. E. & Sparks, J. D. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1489-1493.

12. Greeve, J., Navaratnam, N. & Scott, J. (1991) *Nucleic Acids Res.* **19**, 3569-3576.
13. Backus, J. W. & Smith, H. C. (1991) *Nucleic Acids Res.* **19**, 6781-6786.
14. Simpson, L. (1990) *Science* **250**, 512-513.
15. Blum, B., Bakalara, N. & Simpson, L. (1990) *Cell* **60**, 189-198.
16. Blum, B., Sturm, N. R., Simpson, A. M. & Simpson, L. (1991) *Cell* **65**, 543-550.
17. Cech, T. R. (1991) *Cell* **64**, 667-669.
18. Koslowsky, D. J., Göringer, H. U., Morales, T. H. & Stuart, K. (1992) *Nature* **356**, 807-809.
19. Harris, M. E. & Hajduk, S. L. (1992) *Cell* **68**, 1091-1099.
20. Blum, B. & Simpson, L. (1992) *Proc. Natl. Acad. Sci. USA* (in press)
21. Simpson, L. (1987) *Ann. Rev. Microbiol.* **41**, 363-382.
22. Sturm, N. R. & Simpson, L. (1991) *Nucleic Acids Res.* **19**, 6277-6281.
23. Pollard, V. W., Rohrer, S. P., Michelotti, E. F., Hancock, K. & Hajduk, S. L. (1990) *Cell* **63**, 783-790.
24. Kidane, G., Hughes, D. & Simpson, L. (1984) *Gene*. **27**, 265-277.

25. Maslov, D. A. & Simpson, L. (1992) *Cell* (in press)
26. Morel, C., Chiari, E., Camargo, E., Mattei, D., Romanha, A. & Simpson, L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6810-6814.
27. Sturm, N. R., Degrave, W., Morel, C. & Simpson, L. (1989) *Mol. Biochem. Parasitol.* **33**, 205-214.
28. Gomez-Eichelmann, M. C., Holz, G., Jr., Beach, D., Simpson, A. M. & Simpson, L. (1988) *Mol. Biochem. Parasitology.* **27**, 143-158.
29. Simpson, L. (1972) *Int Rev. Cytol.* **32**, 139-207.
30. Stuart, K. (1991) *Annu. Rev. Microbiol.* **45**, 327-344.
31. Benne, R. (1989) *Biochim. Biophys. Acta* **1007**, 131-139.
32. Feagin, J. & Stuart, K. (1988) *Mol. Cell Biol.* **8**, 1259-1265.
33. Feagin, J., Jasmer, D. & Stuart, K. (1987) *Cell* **49**, 337-345.
34. Duszenko, M., Ferguson, M., Lamont, G., Rifkin, M. & Cross, G. (1985) *J. Exp. Med.* **162**, 1256-1263.
35. Briones, M. R. S., Nelson, K., Beverley, S. M., Affonso, H. T., Camargo, E. P. & Floeter-Winter, L. M. (1992) *Mol. Biochem. Parasitol.* **53**, 121-128.
36. Simpson, L. & Berliner, J. (1974) *J. Protozool.* **21**, 382-393.

37. Simpson, L. & Simpson, A. (1974) *J. Protozool.* **21**, 774-781.
38. Simpson, L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1585-1588.
39. Ryan, K. A., Shapiro, T. A., Rauch, C. A. & Englund, P. T. (1988) *Ann. Rev. Microbiol.* **42**, 339-358.
40. Hajduk, S., Klein, V. & Englund, P. (1984) *Cell* **36**, 463-492.
41. Tibayrenc, M., Ward, P., Moya, A. & Ayala, F. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 115-119.
42. Brunk, C. & Simpson, L. (1977) *Anal. Biochem.* **82**, 455-462.
43. Goncalves, A. M., Nehme, N. S. & Morel, C. M. (1984) in *Genes and Antigens of Parasites*, ed. Morel, C. M. (Fundacao Oswaldo Cruz, Rio de Janeiro), pp. 95-110.
44. Beidler, J. L., Hilliard, P.R. & Rill, R. L. (1982) *Anal. Biochem.* **126**, 374-380.
45. Lopes, U., Momen, H., Grimaldi, G., Marzochi, M., Pacheco, R. & Morel, C. (1984) *J. Parasitol.* **70**, 89-98.
46. Morel, C. & Simpson, L. (1980) *Am. J. Trop. Med. Hyg.* **29**, 1070-1074.
47. Avila, H., Goncalves, A. M., Nehme, N. S., Morel, C. M. & Simpson, L. (1990) *Mol. Biochem. Parasitol.* **42**, 175-188.

48. Simpson, L. (1968) *J. Protozool.* **15**, 132-136.
49. Braly, P., Simpson, L. & Kretzer, F. (1974) *J. Protozool.* **21**, 782-790.
50. Bakalara, N., Simpson, A. M. & Simpson, L. (1989) *J. Biol. Chem.* **264**, 18679-18686.
51. Simpson, L. & Simpson, A. (1978) *Cell* **14**, 169-178.
52. Simpson, A. M., Suyama, Y., Dewes, H., Campbell, D. & Simpson, L. (1989) *Nucl. Acids Res.* **17**, 5427-5445.
53. Hancock, K. & Hajduk, S. L. (1990) *J. Biol. Chem.* **265**, 19208-19215.
54. Maslov, D. A., Sturm, N. R., Niner, B. M., Gruszynski, E. S., Peris, M. & Simpson, L. (1992) *Mol. Cell. Biol.* **12**, 56-67.
55. Sturm, N. R. & Simpson, L. (1990) *Cell* **61**, 871-878.
56. Shaw, J., Campbell, D. & Simpson, L. (1989) *Proc. Natl. Acad. Sci.* **86**, 6220-6224.
57. Blum, B. & Simpson, L. (1990) *Cell* **62**, 391-397.
58. Masuda, H., Simpson, L., Rosenblatt, H. & Simpson, A. (1979) *Gene.* **6**, 51-73.
59. Wood, W., Gitschier, J., Lasky, L. & Lawn, R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1585-1588.
60. Abraham, J., Feagin, J. & Stuart, K. (1988) *Cell* **55**, 267-272.

61. Decker, C. J. & Sollner-Webb, B. (1990) *Cell* **61**, 1001-1011.
62. (1991) *PCR A Practical Approach* (IRL Press, New York),
63. Milligan, J. F., Groebe, D. R., Witherell, G. W. & Uhlenbeck, O. C. (1987) *Nucl. Acids Res.* **15**, 8783-8798.
64. Von Haeseler, A., Blum, B., Simpson, L., Sturm, N. & Waterman, M. S. (1992) *Nucleic Acids Res.* **20**, 2717-2724.
65. Shaw, J., Feagin, J. E., Stuart, K. & Simpson, L. (1988) *Cell* **53**, 401-411.
66. Van der Spek, H., Arts, G. -J., Zwaal, R. R., Van den Burg, J., Sloof, P. & Benne, R. (1991) *EMBO J.* **10**, 1217-1224.
67. Simpson, A. M., Bakalara, N. & Simpson, L. (1992) *J. Biol. Chem.* **267**, 6782-6788.
68. Koslowsky, D. J., Bhat, G. J., Read, L. K. & Stuart, K. (1991) *Cell* **67**, 537-546.
69. Read, L. K., Myler, P. J. & Stuart, K. (1992) *J. Biol. Chem.* **267**, 1123-1128.
70. Read, L. K., Corell, R. A. & Stuart, K. (1992) *Nucleic Acids Res.* **20**, 2341-2347.
71. White, T. & Borst, P. (1987) *Nucl. Acids Res.* **15**, 3275-3290.

Figure Legends:

Figure 1. Kinetoplast network DNA from L. tarentolae. Isolated kDNA stained with DAPI and visualized at 1000 X magnification with UV illumination. These cup-like structures represent monomolecular sheets of catenated minicircles and maxicircles.

Figure 2. Schizodeme analysis. Acrylamide gradient gel profiles of EcoRI digests of kDNA from several T. cruzi CL strain cultures and clones. I, CL cells kept in culture for 2 years (1978-80) and harvested in stationary phase; II, CL cells harvested in log phase; III, CL cells kept at -70°C for 2 years (1978-80); IV, CL cells kept in mice for 2 years (1978-80) and grown for 10 passages in culture; CL 10,11,14,12,16,18 and 20, clones from parental CL strain. Zymodeme groups are given in parentheses. Note presence of two schizodemes in stock CL strain. Reprinted from ref. 26 with permission.

Figure 3. Renografin isopycnic density gradient fractionation of mitochondrial fraction from L. tarentolae. Gradients were fractionated and fractions washed with STE buffer. Aliquots were assayed for TUTase and run-on transcription activities (A), RNA ligase activity (B), and adenylate kinase activity and protein (C). Adenylate kinase is a marker enzyme for the mitochondrial inner membrane space. Reprinted from ref. 50 with permission.

Figure 4. Partially edited maxicircle G-rich region 6 (=RPS12) cDNA sequences from L. tarentolae. kRNA was amplified using a 3'-oligo(T) primer and a 5'-genomic primer. The PCR products were cloned and the clones sequenced. Sequences are aligned so as to show the 3'-to-5' progression of expected editing events. Unexpected editing events are underlined. Pattern 5 was seen in 5 clones, pattern 18 was seen in 3 clones. The unedited domain-connection-sequences are boxed. (A) 3'-terminal portions of the cDNA sequences. (B) 5'-terminal portions of the sequences. Clones 1-126 are not shown since their sequences, like clone 17, are unedited in this region. Reprinted from ref. 54 with permission.

