#37.

3

RNA editing in mitochondria

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1. Introduction

1.1 Post-transcriptional modification of eukaryotic mRNAs

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→U substitution in the mammalian apolipoprotein B (1, 2), to the multiple C→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 and 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→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 characterized by the insertion and, less frequently, the deletion of uridylate (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 base pair 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 Us 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 kbp circular molecule. However, the bulk of kDNA comprises 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 polyacrylamide gels as compared to agarose gels and is partially responsible for the increased complexity of the polyacrylamide 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 enzyme-digested kDNA, as visualized by electrophoresis in gradient polyacrylamide 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 assays 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 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:

(a) The cells are not pathogenic for humans, which is a major advantage in terms of growing large amounts of cells in the laboratory for the isolation of mitochondria, nucleic acids, and enzymes.

(b) 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.

(c) The cells can be frozen for storage.

(d) Cell concentrations of 4×10^8 cells/ml can be obtained at stationary phase.

(e) 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.

(f) Several recently-isolated strains exist for comparative studies.

(g) 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:

(a) 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).

(b) 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 haemin: add 2 mg haemin (Sigma H-2375) per ml of 50 mM 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.)
- Pyrex bottles, 500 ml (Fisher, #06-414-1C) with screw caps (Fisher, #06-414-2A), for autoclaving solutions
- Brain heart infusion (BHI) medium[a]: dissolve 37 g BHI powder (Difco) per litre of tissue culture grade water; autoclave in Pyrex bottles at 120 °C for 30–40 min[b]; when cool, aseptically add haemin to 10 mg/ml; the medium is stable for several months at 5 °C
- BHI medium containing 20% glycerol, sterilized by autoclaving in Pyrex bottles
- Tissue culture flasks (glass; Corning #25100; 25 cm^2)
- Inverted, phase-contrast microscope
- Freezer vials, sterile, polypropylene .2 ml (Van Waters and Rogers #66008-309)

Method

A. *Maintenance and monitoring of cultures*

1. Inoculate healthy (motile) *L. tarentolae* cells at 0.6–1.5 × 10^6 cells/ml in 5–10 ml BHI medium in 25 cm^2 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 400× or 1000× 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.

B. *Storage and recovery of cells*

1. Dispense 0.5 ml aliquots of healthy log-phase cultures (approximately 10^8 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. Recover live cells from stored cultures by thawing the vials rapidly and inoculating the contents into 2–5 ml of BHI medium. Check the cells by phase-contrast microscopy; they should be motile immediately after thawing.

[a] No antibiotics are necessary if aseptic techniques are used and sterility is preserved.
[b] The medium can be autoclaved directly in glass culture bottles or in glass bottles for storage.

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 microtitre plates or by growth on 0.7–1.0% agar/BHI–haemin 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 (see *Protocol 4*) is an easy method to use for verifying 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 *g* for 10 min at 5 °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 µm Gilson microbiological filter on input and exit lines
- Compressed O_2 (medical grade); required for larger cultures
- Transverse filter system (Millipore Pellicon with 0.45 µm Durapore filter cassette) with 8 litres/min peristaltic pump (Millipore Masterflex XX80 ELO 01)
- Log-phase culture of *L. tarentolae* and BHI medium as in *Protocol 1*

Method

1. Inoculate BHI 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 a cell density of 10^8 cells/ml.
2. Stir the culture at maximum rate at 27 °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, substitute O_2 for air after approximately 50 h. This will allow cells to grow in log-phase up to a cell density of approximately 4×10^8 cells/ml, which is twice the density reached with air alone.

Continued

Protocol 2. *Continued*

4. Harvest the cells when they have reached a density appropriate for the subsequent preparation (see later *Protocols*). To do this, cool the culture by immersing the culture bottle in ice water while stirring the culture. Concentrate the culture to approximately 100–200 ml by filtration using the Millipore Pellicon transverse filter system.
5. Wash the cells by addition of the desired wash medium to the concentrated cell suspension and reconcentrate the cells.
6. Flush the washed cells out of the filter system by a final rinse with wash medium.
7. Concentrate the cells again and then pellet them by centrifugation at 2500 *g* for 10 min at 5 °C.
8. For kDNA isolation, resuspend the cells in SET medium and freeze them at −70 °C (see *Protocol 3*). For isolation of the kinetoplast-mitochondrion fraction, process the cells immediately as described in *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 A+T content (84% A+T versus 55% A+T for minicircle DNA) after its release from the network by digestion with a restriction enzyme that cuts only once or infrequently (38). The complete sequence of the 23 kbp maxicircle of *T. brucei* is known, and 21 kbp of the 30 kbp maxicircle genome of *L. tarentolae* has been sequenced (Genbank entry LEIKPMAX). The structural genes are clustered in ~17 kbp 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 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 G_2 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 *et al.* (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

Equipment and reagents

- SET buffer (0.15 M NaCl, 0.1 M EDTA, 10 mM Tris–HCl, pH 7.5)
- Stationary phase culture of *L. tarentolae*; about 4×10^8 cells/ml for large aerated cultures (see *Protocol 2*) or 1.5–2.0 × 10^8 cells/ml for roller-bottle cultures
- 10 mg/ml pronase (Calbiochem); dissolve the pronase in SET and predigest the solution for 30 min at 37 °C to inactivate nucleases
- 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)
- CsCl (density gradient or optical grade)
- 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)
- 250 ml polycarbonate screw-capped flask
- 10 mg/ml ethidium bromide
- Upper CsCl solution: 37.62 g CsCl in 62 ml of 10/1 TE buffer (refractive index at 25 °C = 1.3705)
- Lower CsCl solution: 29.2 g CsCl in 20 ml of 10/1 buffer plus 0.14 ml of 10 mg/ml ethidium bromide (refractive index at 25 °C = 1.4040); note only the lower CsCl solution contains dye
- Peristaltic pump
- UV microscope
- UV lamp (254 nm)
- Butan-1-ol saturated with water
- 12 ml polystyrene centrifuge tube
- *Sec*-butanol
- Phenol:chloroform (1:1, v/v)
- DAPI (4′, 6′-diamidino-2 phenylindole, Sigma #D1388)
- 2.0 M NaCl

Continued

Protocol 3. *Continued*

Method

1. Harvest the cells from roller cultures by centrifugation (10 min at 2000–3000 *g* at 5 °C) or by filtration for large cultures (see *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 pipetting 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 p.s.i. to shear nuclear DNA selectively and decrease the viscosity. For small volumes (10–100 ml) use a 12 ml syringe fitted with an 18-gauge needle and force the lysate through it 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. at 5 °C. 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 of 10/0.1 TE buffer per 1–2 litre of original culture. Shake the pellets 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 the lower CsCl solution below 24 ml of the upper CsCl solution using a peristaltic pump to give a sharp interface.
8. Layer 6 ml of the resuspended crude kDNA solution (from 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. Allow the rotor to slow down with the 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 the shearing of the lysate was insufficient, some nuclear DNA will also sediment close to the interface.
9. Visualize the kDNA band at the interface with the 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 butan-1-ol.
11. Dialyse the kDNA against 4 litres of 10/0.1 TE buffer.
12. Concentrate the kDNA solution to approximately 400 µl 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 µl kDNA solution to a microfuge tube.

Continued

Protocol 3. *Continued*

13. Extract the kDNA by vortexing with an equal vol. of phenol:chloroform (1:1). Separate the phases by centrifugation at 10 000 *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 °C overnight.
16. Recover the precipitated kDNA by centrifugation at 12 000 *g* for 14 min at 5 °C in a microcentrifuge.
17. Resuspend the kDNA pellet at 1 mg/ml in 10/1 TE buffer.
18. Check the integrity of the network DNA by diluting a sample ten-fold in 10/1 TE buffer and adding DAPI to 1 μg/ml final concentration. Observe the stained kDNA using an UV microscope at 1000 × magnification. 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 °C.

Using the procedure described in *Protocol 3*, the yield of kDNA is 0.5–1.0 mg/litre of culture. *Figure 1* shows the kDNA isolated from *L. tarentolae* as in *Protocol 3*. If desired, nuclear DNA can be isolated from the crude DNA preparation (step 6). Add 2 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*III, *Msp*I, *Taq*I, and *Rsa*I 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 supplier's instructions)
- Polyacrylamide gel (1 mm thick, 4.5–10% linear gradient of acrylamide, 4% stack) in TBE buffer (see Volume I, Chapter 1, *Protocol 8* or Volume I, Chapter 4, *Protocol 11*); use a standard gradient former and pump and add 15% glycerol to the 10% acrylamide solution when forming the gradient

Continued

Protocol 4. *Continued*

- 10 mg/ml ethidium bromide
- 50% methanol, 10% trichloroacetic acid
- 5% acetic acid, 10% methanol
- Concentrated NH_4OH
- Silver nitrate solution (1 g in 10 ml water)
- 0.01% citric acid containing 0.45 ml of formaldehyde per 300 ml; make this solution fresh
- 20% ethanol, 5% acetic acid

Method

1. Digest 4 µg 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 Volume I, Chapter 1, *Protocol 8* or Volume I, Chapter 4, *Protocol 11*.
3. Visualize the DNA bands by staining the gel with either ethidium bromide (0.5 µg/ml final concentration) or silver . For ethidium bromide staining see ref. 42. For silver staining, see steps below[a].
4. Fix the gel in 200 ml of 50% methanol, 10% trichloroacetic acid for 30 min.
5. Wash the gel twice in 250 ml 5% acetic acid, 10% ethanol for 20 min each time, then wash twice in 250 ml 10% ethanol for 10 min each.
6. Mix 40 ml of 0.1 M NaOH, 3 ml of concentrated NH_4OH, and 10 ml of silver nitrate solution. Stir the mixture until the black precipitate dissolves. Add water to 200 ml.
7. Stain the gel in the silver solution (step 6) for 40 min.
8. Wash the gel with 300 ml of water for 5 min.
9. Develop the gel with 300 ml of freshly made 0.01% citric acid containing 0.45 ml formaldehyde. Stop the development in 20% ethanol, 5% acetic acid for 10 min.
10. Wash the gel three times in 250 ml of 20% ethanol for 30 min each time.

[a] Based on refs 43, 44.

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. *Figure 2* shows the schizodeme analysis of *T. cruzi* strains.

3.4 Isolation of maxicircle DNA

In all kinetoplastid species analysed, maxicircle DNA, which ranges in size from 23 to 36 kbp, 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 kbp in size.

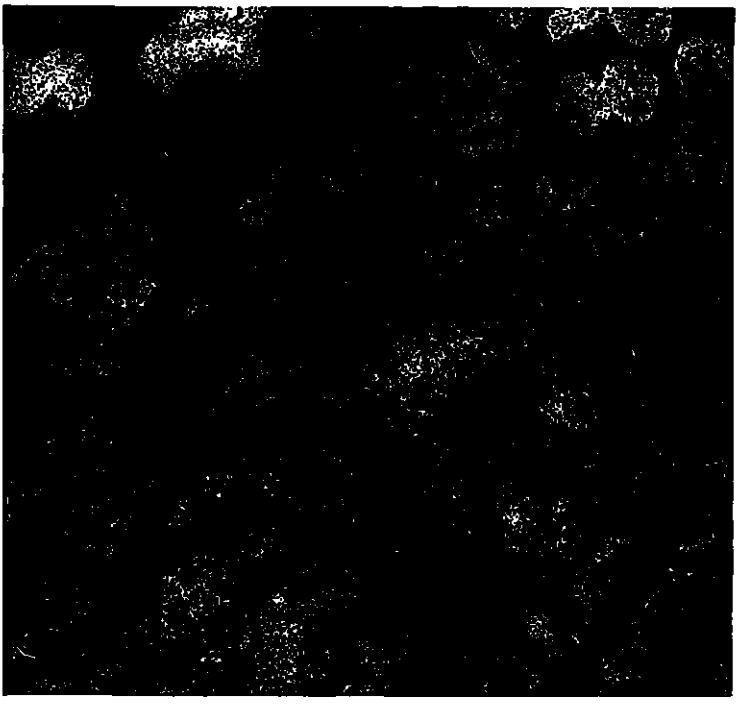


Figure 1. Kinetoplast network DNA from *L. tarentolae*. Isolated kDNA stained with DAPI and visualized at 1000× magnification with UV illumination (see *Protocol 3*). These cup-like structures represent monomolecular sheets of catenated minicircles and maxicircles.

Protocol 5. Isolation of maxicircle DNA

Equipment and reagents

- kDNA, 10/1 TE buffer, *sec*-butanol, 2.0 M NaCl, and UV lamp (254 nm) 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 (Volume I, Chapter 1, *Protocol 6* or Volume I, Chapter 4, *Protocol 12*)
- CsCl (optical grade)
- 0.5 mg/ml Hoechst dye L33258 (Sigma, B 2883)
- Beckman ultracentrifuge, 50 and 50.2 or Ti60 rotors and tubes (or equivalent)
- Propan-2-ol

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 h at 37 °C.

Continued

Protocol 5. *Continued*

2. Monitor the extent of release of the 30 kbp maxicircle DNA by running 10 µl samples on a 0.7% agarose gel (Volume I, Chapter 1, *Protocol 6* or Volume I, Chapter 4, *Protocol 12*).
3. Mix the 2 ml digested kDNA with 10/1 TE buffer to 12 ml final volume. Add 18.5 g CsCl and stir until dissolved.
4. Add 0.5 mg/ml Hoechst 33258 dye dropwise with mixing to approximately 1 µg dye per µg 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 °C by adding either more CsCl or more 10/1 TE buffer.
6. Centrifuge the mixture at 40 000 r.p.m. in the Beckmann 50.2 or Ti60 rotor for 48 h at 40 000 r.p.m. at 25 °C to establish the density gradient.
7. Visualize the blue-fluorescent DNA bands with 3000 A° UV illumination and recover the minor upper band. The lower band contains the undigested kDNA networks and released minicircle DNA.
8. Re-adjust the refractive index of the upper band to 1.3950–1.3935, and centrifuge 6.5 ml per tube in the Beckmann 50 rotor at 39 000 r.p.m. for 48 h at 25 °C. Again recover the upper band. This rotor provides a better separation and the second centrifugation completely removes any contaminating minicircle or kDNA.
9. Remove the Hoechst dye by extraction with an equal vol. of propan-2-ol.
10. Dialyse the maxicircle DNA against 10/1 TE buffer and concentrate the DNA using *sec*-butanol (see *Protocol 3*, step 12).
11. Recover the DNA by ethanol precipitation (see *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 cretenated disc 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*. The use of

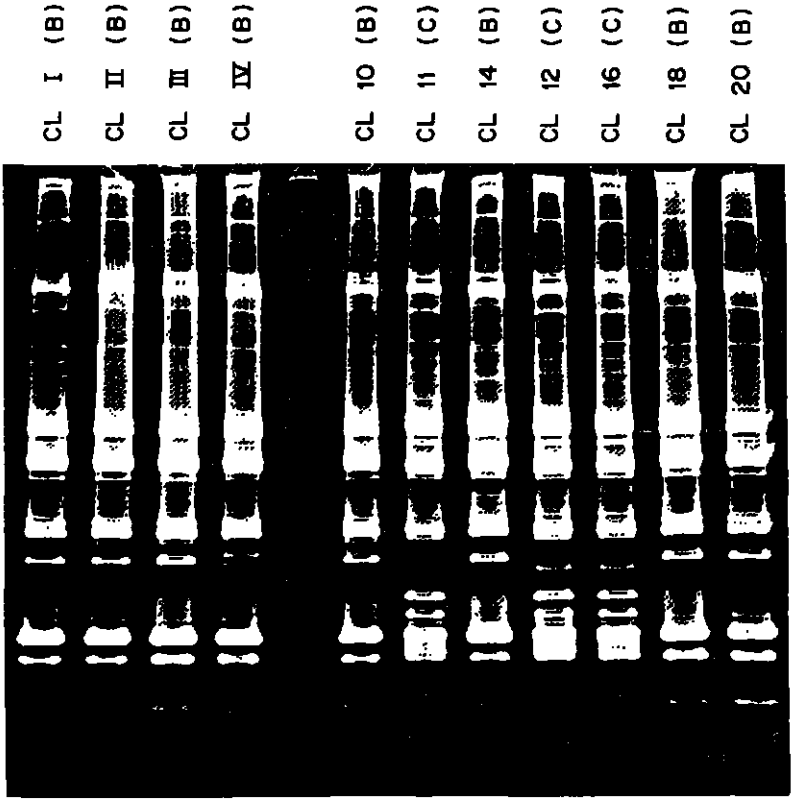


Figure 2. Schizodeme analysis. Acrylamide gradient gel profiles of *Eco*R1 digests of kDNA from several *T. cruzi* CL strain cultures and clones. I, CL cells kept in culture for 2 years (1978–80) and harvested in the stationary phase: II, CL cells harvested in log phase; III, CL cells kept at −70 °C 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, are clones from the parental CL strain. Zymodeme groups (B, C) are given in parentheses. Note the presence of two schizodemes in the stock CL strain. Reprinted from ref. 26 with permission.

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) (see *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, H_2O to 1 litre)
- DTE buffer; a 1:100 dilution of 10/1 TE buffer (see *Protocol 3*)

Continued

Protocol 6. *Continued*

- STM buffer (85.6 g sucrose, 100 ml of 0.2 M Tris–HCl, pH 7.9, 3 ml of 1.0 M $MgCl_2$, H_2O to 1 litre)
- 60 ml plastic syringe fitted with a 26-gauge needle and a piston driven by compressed air at 100 p.s.i., or a dispensing pressure vessel (see *Protocol 3*) fitted with a 26-gauge needle
- Beckman ultracentrifuge and SW28 tubes, Sorvall RC-5 centrifuge and SS34 or GS3 rotors, plus appropriate tubes (or equivalents)
- 1.75 M sucrose
- SET buffer, DAPI, and UV microscope as in *Protocol 3*
- *L. tarentolae* cells harvested at late log-phase ($50–150 \times 10^6$ cells/ml) from either roller cultures (1 litre) or aerated stirred cultures (13 litre) as described in *Protocol 2*
- DNase I (Sigma, electrophoretically pure), 2 mg/ml in 10 mm Tris–HCl, pH 7.4, 10 mM $CaCl_2$, 50% glycerol

For Renografin gradients

- 76% Renografin (Squibb Pharmaceutical Co.: 66% diatrizoate meglumine and 10% diatrizoate sodium)
- 76% RSE buffer (8.56 g sucrose, 50 µl of 0.2 M EDTA, 76% Renografin to 100 ml)
- 20% RSTE buffer (26.3 ml of 76% Renografin, 8.56 g sucrose, 10 ml of 0.2 M Tris–HCl, pH 7.9, 50 µl of 0.2 M EDTA, H_2O 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 µl of 0.2 M EDTA, H_2O to 100 ml); adjust the density to 1.26 g/ml
- 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 the tubes at −20 °C. Thaw the tubes overnight at 4 °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

For Percoll gradients

- 93% Percoll (Pharmacia) in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.9, 0.1 mM EDTA, 0.01 mM 2-mercaptoethanol; similar solutions containing 52.5% Percoll and 16% Percoll
- 16–52.5% Percoll gradients formed as for Renografin gradients (see above) using 16% Percoll and 52.5% Percoll solutions

Method

1. Wash the cells by resuspending them in 50–100 vol. of ice-cold SET buffer and centrifuging at 4000 *g* for 10 min at 5 °C. Repeat this washing. Alternatively, add SET to the cell filtrate when the Millipore Pellicon system (see *Protocol 2*) is used.
2. Thoroughly resuspend the cells in *X* vol. of cold DTE 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× 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 DTE buffer; 5–10 min on ice is generally sufficient for complete swelling.

Continued

Protocol 6. *Continued*

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 shut-off valve and replace the needle. Monitor the extent of breakage by phase-contrast microscopy at 400 × 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[a].
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 cretenated 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 9000 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 will otherwise cause aggregation of the material in the density gradients (steps 13–15).
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 9000 r.p.m. at 5 °C.
11. Aspirate the supernatant; the pellet should be well packed if the DNase digestion was successful.

    Now proceed to separate the kinetoplast-mitochondrion fraction using either Renografin or Percoll gradients.

A. *For Renografin gradients*

1. Add cold 76% RSE buffer to the pellets (4 ml of 76% RSE per litre of original culture). Vortex well.
2. Layer 4–5 ml of the mixture beneath each 20–35% RSE gradient [b] 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 lysate remains at bottom of gradient.
3. Centrifuge the gradients for 2 h at 24 000 r.p.m. at 5 °C in a Beckman SW28 rotor.
4. Visualize the kinetoplast-mitochondrion band just above the 76%–35% RSE interface by side illumination with a UV lamp. Puncture the side of the tube and remove the band using a syringe and needle.
5. 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. at 5 °C.
6. Check the purity of the preparation at this point, if desired; in the next step, the use of STM buffer will cause aggregation of the mitochondria. To check the purity, resuspend a sample of the pellet in STE buffer and

Continued

Protocol 6. *Continued*

check the purity by phase microscopy at 1000× magnification. Visualize the kDNA by staining with 1 µl DAPI and observing by phase-contrast and UV microscopy.

7. Wash the pellet by resuspending it in 50 vol. of STE buffer and centrifuging as in step 5A. Finally wash the pellet with STM buffer prior to kRNA isolation.
8. Resuspend the mitochondrial pellet in an appropriate medium (see *Protocols 7, 9, 11, 15*). Use it immediately or store it frozen at −70 °C in small aliquots.

B. *For Percoll gradients*

1. Resuspend the pellet in 93% Percoll (use 4 ml of Percoll for the pellet from a 1 litre culture).
2. Layer the mixture under 16–52.5% Percoll gradient (use 2 gradients for material from 1 litre culture). Centrifuge for 45 min at 24 000 r.p.m. in a Beckman SW28 rotor at 5 °C.
3. Locate and remove the kinetoplast band (see step 4A).
4. Dilute the kinetoplast band with 200 ml of STE buffer. Recover kinetoplast-mitochondrion fraction by centrifugation at 12 000 *g* for 30 min at 5 °C. Wash the preparation once in STE buffer and resuspend it in STE buffer.
5. Store the fraction as for Renografin gradients (step 8A).

[a] A quantitative estimate of the contamination can be obtained by measuring the relative amount of cytosolic rRNA in the final preparation of kRNA (see *Protocol 7*). The cell lysate can be used to prepare cytosolic rRNA substrate for *Protocol 12*.
[b] The RSE gradient procedure 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 six gradients can be run in the SW28 rotor.

Figure 3 illustrates the use of Renografin gradients to prepare the kinetoplast-mitochondrion fraction from *L. tarentolae*.

5. Isolation of transcripts from the mitochondrial genome

5.1 Introduction and strategy

Isolation of intact RNA (kRNA) from the purified kinetoplast-mitochondrion fraction (prepared as in *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). Unedited transcripts of maxicircle structural genes can be detected by Northern blot

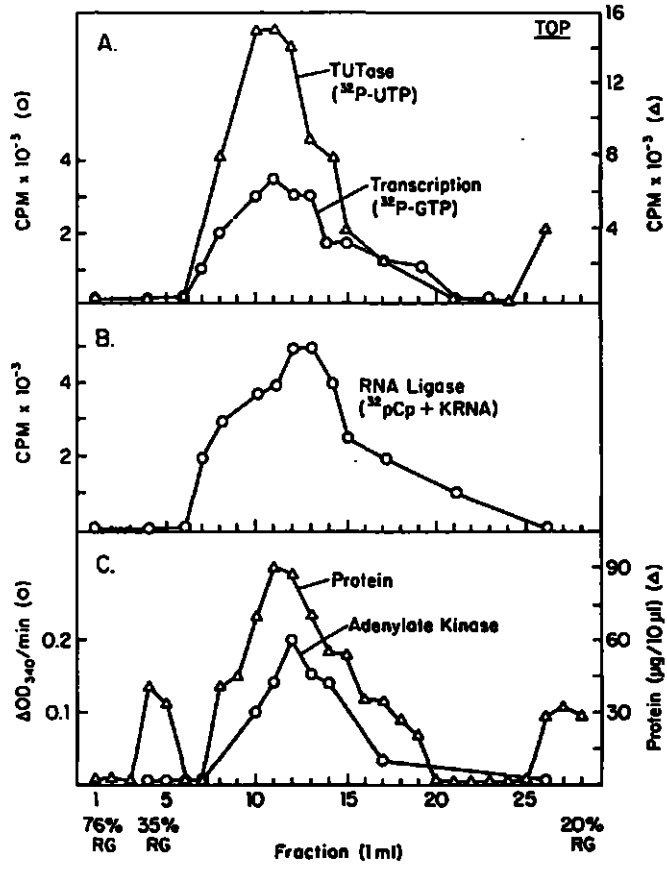


Figure 3. Renografin isopycnic density gradient fractionation of the kinetoplast-mitochondrion fraction from *L. tarentolae*. Gradients were fractionated and fractions washed with STE buffer (*Protocol 6*). Aliquots were assayed for (A) TUTase (*Protocol 12*) and run-on transcription (*Protocol 9*) activities; (B) RNA ligase activity (*Protocol 13*); (C) adenylate kinase activity and protein. Adenylate kinase is a marker enzyme for the mitochondrial inner membrane space. RG, Renografin. Reprinted from ref. 50 with permission.

hybridizaton 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(dT) 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 by detected by selective PCR amplification in *L. tarentolae*.

The gRNA transcripts which are involved in RNA editing co-migrate 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 chracteristic family of electrophoretic bands each differing by a single nucleotide (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 μg 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 (see *Protocol 6*).

Protocol 7. Isolation of kinetoplast RNA

Equipment and reagents

- TMN buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM NaCl)
- 2 mg/ml DNase I (RNase-free; BRL, #80475A)
- Phenol:chloroform (1:1, v/v)
- 10 mg/ml ethidium bromide
- Kinetoplast-mitochondrion fraction: prepared by Renografin gradient centrifugation (see *Protocol 6*) and with the mitochondria from 1–2 litres of original cell culture resuspended in 5–10 ml of TMN buffer; may be frozen at −70 °C until use
- 1% agarose gel in TBE buffer (Volume I, Chapter 1, *Protocol 6* or Volume I, Chapter 4, *Protocol 12*)
- 10% SDS

Method

1. Quickly thaw the kinetoplast-mitochondrion fraction (if necessary). 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. of phenol:chloroform (1:1) as described in *Protocol 3*, step 13. Remove the aqueous (upper) phase to a fresh tube.
3. Re-extract the interface with one vol. of water and pool this with the upper phase.
4. Ethanol-precipitate the nucleic acids, (see *Protocol 3*, steps 15 and 16).
5. Resuspend the nucleic acids in TMN buffer using 1 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 °C.
7. Extract the solution with phenol:chloroform and precipitate the kRNA with ethanol as described in steps 2–4.

Continued

Protocol 7. *Continued*

8. Wash the pellet in 70% ethanol. Resuspend it in water (1 ml per 4 litres of original culture yields approximately 2 µg RNA/µl).
9. Monitor the purity of the RNA by electrophoresing 2–5 µg in a 1.5% agarose gel in TBE buffer, and staining the gel with ethidium bromide (0.5 µg/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 should be the 9S rRNA and the tRNA bands (51).
10. Store the kRNA in aliquots at −20 °C.

5.3 Northern blot analysis of kRNA

Northern blot analysis is described in Volume I, Chapter 2, Section 3.6 and Volume I, Chapter 4, Section 7.5.

Electrophorese the kRNA (see *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 kbp 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). 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 µg; 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 litres of a log-phase *Leishmania* culture at a cell density of 0.7–1.5 × 10^6 cells/ml for the hybrid selection of each gRNA (see *Protocol 7*)
- 10% SDS

Continued

Protocol 8. *Continued*

- ImmunoPure Ag/Ab immobilization kit (Pierce 44890) together with immobilization buffer as provided by the manufacturer
- Centricon 10 centrifugal microconcentrater (Amicon 4205)
- 50 ml centrifuge tubes with plug seal caps (Corning 25331-50)
- Rotating platform stirrer
- 20 × 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 °C
- 1.0 M Tris–HCl, adjust the 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, 2 mM EDTA, 1% SDS)
- 6 × SSC buffer and 1 × SSC buffer (prepared using 20 × SSC buffer stock)
- 0.1 × SSC buffer, 0.1% SDS (prepared using 20 × SSC buffer stock and 10% SDS)
- Hybridization buffer (1.0 M NaCl, 0.166 M Hepes–KOH, pH 7.5, 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 µg 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 5 ml of wash buffer and store the washed column at 4 °C until required.
3. For hybrid selection of gRNA, equilibrate the column in hybridization buffer.
4. Load approximately 500 µg of kRNA in 1 ml hybridization buffer onto the column.
5. Seal the column with the cap and stopper provided; incubate it for 18 h at 37 °C.
6. Drain the column and add 5 ml of ice-cold 6 × SSC buffer.
7. Repeat step 6 twice, then incubate the column at 4 °C for 2 h with gentle agitation by placing the column into a 50 ml centrifuge tube and rotating it horizontally on a rotating platform stirrer which is set to 50 r.p.m.
8. Drain the column and equilibrate it with 5 ml of TMA 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 °C water bath for 30 min.
10. Drain the column, then add 5 ml TMA buffer (preheated at 60 °C). Again seal the column into a 50 ml centrifuge tube and place into a 60 °C water bath for 30 min[a].

Continued

Protocol 8. *Continued*

11. Wash the column successively with 10 ml of TMA buffer at room temperature, 10 ml of ice-cold 6 × SSC buffer, and 10 ml of ice-cold 1 × SSC buffer.
12. Elute the gRNA at 70 °C for 20 min using 2 ml of 0.1 × SSC buffer, 0.1% SDS, preheated to 70 °C. This is done by placing the column into a sealed 50 ml centrifuge tube and immersing it into a 70 °C water bath[b]. Collect the column eluate. Repeat this twice with fresh buffer.
13. Add 2 µg of carrier nucleic acid. Concentrate the pooled eluate with the Centricon 10 centrifugal microconcentrator following the manufacturer's instructions.
14. Store the hybrid-selected gRNA at −20 °C.

[a] Note that the exact washing temperature must be varied according to the size of the hybrid (59). Highly stringent washing occurs at $T_m - 4$ °C. The T_m of a hybrid in TMA buffer is solely dependent on its length and can be calculated from ref. 59.
[b] Do not heat the columns containing the agarose beads to more than 70 °C. The agarose could melt above this temperature.

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

Partially-edited RNAs can be amplified by the use of an oligo(dT) 3′ primer complementary to the oligo(U)-tail of the gRNA, for the reverse transcriptase step (60). 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 polymerization 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 by-products of a cleavage–ligation process.

Detailed procedures for PCR will be found in Volume I, Chapter 2, Section 3.4 and Volume I, Chapter 3, Section 2.2.3 and in ref. 62. PCR products both from partially-edited and chimaeric molecules may be checked by gel electrophoresis and their nucleotide sequences determined. *Figure 4* shows partially-edited sequences in the maxicircle G-rich region 6 of *L. tarentolae*.

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 (see 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 Volume 1, Chapter 1 (*Protocol 4*) and Chapter 2, this volume (*Protocol 4*).

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.

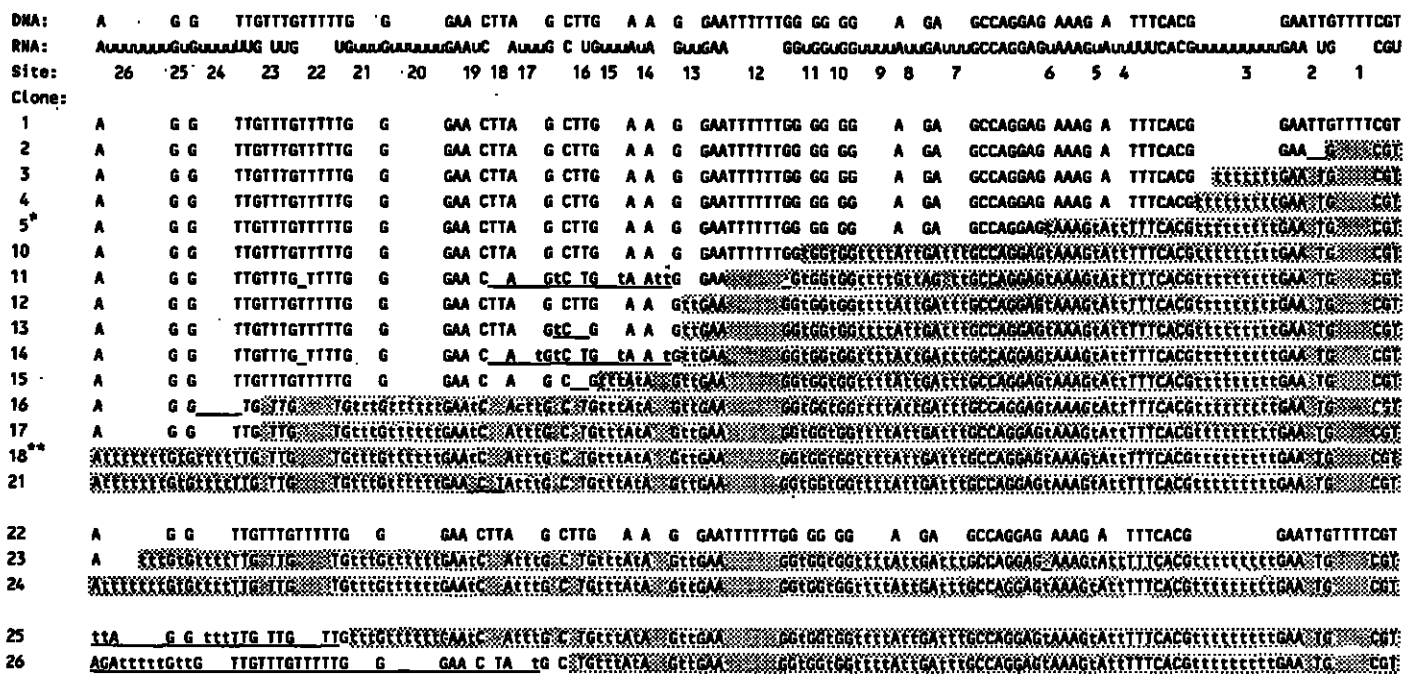
Protocol 9 describes run-on transcription using intact mitochondria. Using this method, the rate of incorporation of [α-^{32}P]GTP increases for 10–15 min and then decreases.

Protocol 9. Run-on transcription in isolated mitochondria

Reagents

- 5 × STE buffer (see *Protocol 6*)
- Mitochondrial fraction isolated by Renografin or Percoll gradients as described in *Protocol 6*, resuspended at 2–3 μg/μl in STE buffer for immediate use or stored frozen in STE with 8% DMSO at −70 °C; run-on transcription activity is stable for several weeks in frozen mitochondria
- 10 mM ATP
- 10 mM CTP
- 10 mM UTP
- [α-^{32}P]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 7.6
- 0.25 M 2-mercaptoethanol
- DE81 filter discs (Whatman, 2.5 cm diameter)
- 0.5 M sodium phosphate buffer, pH 6.8, 0.5% sodium pyrophosphate

Continued



Identical clones:
* - 6, 7, 8, 9
** - 19, 20

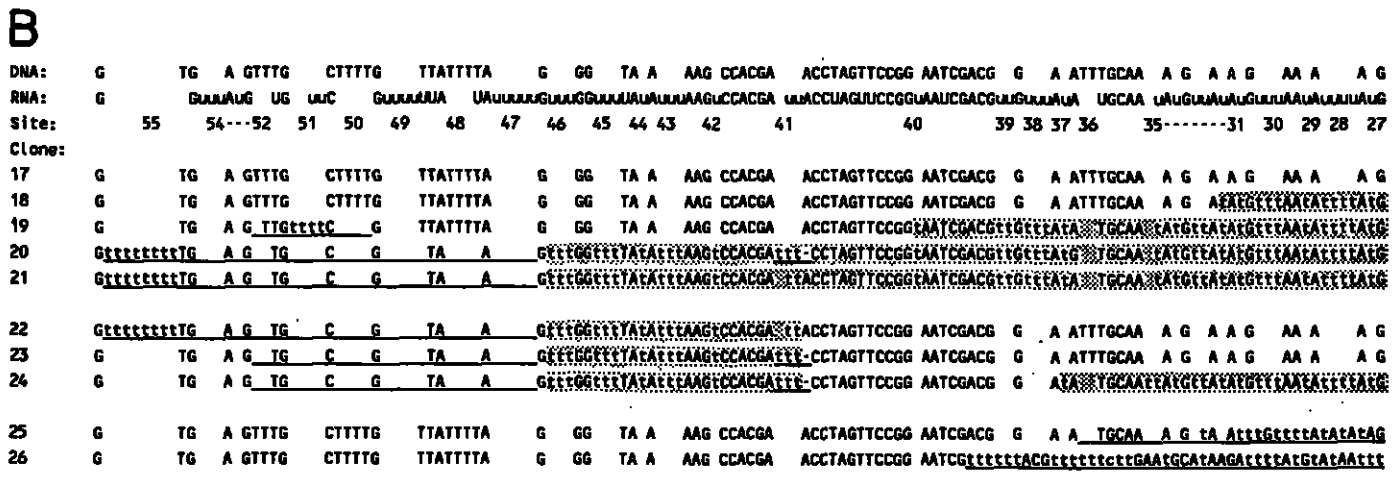


Figure 4. Partially edited maxicircle G-rich region 6 (=RPS12) cDNA sequences from *L. tarentolae*. kRNA was amplified using a 3′ oligo(T) primer and a 5′ genomic primer. The PCR products were cloned and the clones sequenced. Sequences are aligned to show the 3′ → 5′ progression of expected editing events. 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 shaded. (A) 3′ terminal portions of the cDNA sequences. (B) 5′ terminal portions of the sequences. Clones 1–16 are not shown since their sequences, like clone 17, are unedited in this region. Reprinted from ref. 54 with permission.

Protocol 9. *Continued*

Method

1. In a microcentrifuge tube on ice mix:
   - 30 mM potassium phosphate, pH 6.8 — 5 µl
   - 2 M KCl — 1.5 µl
   - 0.25 M Hepes-KOH, pH 7.6 — 1 µl
   - 0.25 M 2-mercaptoethanol — 1 µl
   - 5 × STE buffer — 2.5 µl
   - [α-^{32}P]GTP — 1 µl
   - 10 mM ATP — 5 µl
   - 10 mM CTP — 5 µl
   - 10 mM UTP — 5 µl
   - mitochondrial fraction — 10 µl
   - H_2O — to 50 µl final volume
2. Incubate the mixture for 15 min at 27 °C.
3. Remove 10–20 µl aliquots and spot them 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 the washing three times.
5. Wash the discs with 100 ml of ethanol as in step 4.
6. Dry the discs and count the retained radioactivity in a scintillation counter.

The run-on transcripts, synthesized 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 base pairs 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. Reference 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

A. *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 (see SEQED, UWGCG).
4. Reverse the mRNA sequence, using REVERSE (reverse only, UWGCG).

B. *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).

C. *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 gRNAs). 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 (64). 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 18mer inverted repeats (23). One could locate these conserved repeats and computer search the intervening DNA sequences.

Continued

Protocol 10. *Continued*

D. *Search for edited mRNA*

9. 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 chimaera-forming activity (18–20) (*Figure 3*).

The TUTase adds U residues to the 3′ hydroxyl group of RNA molecules with no apparent sequence specifity. The role 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 the 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 the addition of heparin or by predigestion 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 catalysed 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 *T. brucei* (18, 19) which show chimaera-forming activities *in vitro*. For *L. tarentolae* the chimaeric molecules synthesized *in vitro* are similar but distinct from the ones observed *in vivo* (20), 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 (see *Protocol 6*). 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 µl aliquots at −70 °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 µl of 10% Triton X-100 to 200 µl of the mitochondrial fraction in a microcentrifuge tube. Mix gently.
3. Homogenize the mitochondria for 15 sec at 5 °C using the pellet pestle mixer. This homogenate is the TL extract.
4. Centrifuge the TL extract for 30 min at 12 000 *g* at 5 °C in a microcentrifuge (to obtain the TS extract) or for 1 h at 100 000 *g* at 5 °C (to obtain the S-100 extract). In each case, carefully remove and retain the supernatant.
5. If necessary, store the extracts in aliquots at −70 °C.

8.3 Terminal 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 endogenous mitochondrial RNAs are labelled with [α-^{32}P]UTP, including the 9S and 12S rRNAs and the gRNAs. The mitochondrial tRNAs do not label well with this enzyme activity.

Heparin (5 μg/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 omitting the addition of the cytosolic RNA substrate.

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

Equipment and reagents

- Cell lysate prepared as in *Protocol 6*, steps 1–4
- Mitochondrial extract (TL, TS or S-100, see *Protocol 11*) (2–3 μg protein/μl)
- 0.25 M Hepes–KOH, pH 7.5, 2 M KCl, 10 mM ATP, and DE81 discs as in *Protocol 9*
- 60 mM magnesium acetate
- 1.0 M DTT
- [α-^{32}P]UTP (New England Nuclear; 800 Ci/mmol, 10 μCi/μl)
- 30 mM potassium phosphate, pH 7.0
- 0.5 M sodium phosphate buffer, pH 6.8, 0.5% sodium pyrophosphate with and without 0.1% SDS
- TMN buffer, 10% SDS, 2 mg/ml DNase I solutions as in *Protocol 7*
- 2.0 M NaCl and phenol:chloroform as in *Protocol 3*
- 10 mM GTP

Method

A. *Preparation of cytosolic RNA substrate*

1. Centrifuge the cell lysate (prepared as per *Protocol 6*, steps 1–4) at 16 000 *g* for 10 min at 5 °C.
2. Remove the supernatant and add 0.01 vol. of 10% SDS.
3. Deproteinize with an equal vol. of phenol:chloroform (1:1) as described in *Protocol 3*, step 13. Remove the upper (aqueous) phase to a fresh tube. Re-extract the phenol layer and interface with one vol. of water and pool it with the first upper phase.

Continued

Protocol 12. *Continued*

4. Ethanol-precipitate the nucleic acids (see *Protocol 3*, steps 15, 16).
5. Resuspend the pellet in TMN buffer (1 ml TMN per 2–3 litres of original cell culture). Add 0.005 vol. of 2 mg/ml DNase I and incubate the mixture for 30 min at 37 °C.
6. Extract with phenol:chloroform and ethanol precipitate the RNA (steps 4, 5). Wash the pellet three times with 70% ethanol and spinvac dry.
7. Resuspend the RNA from four litres of original culture in 1 ml redistilled sterile water (0.5–1.2 μg RNA/μl) and store it at −20 °C.

B. *TUTase assay*

1. In a microcentrifuge tube on ice, mix:

● 1 M DTT	1 μl
● 30 mM potassium phosphate, pH 7.0	5 μl
● 10 mM GTP	5 μl
● 0.25 M Hepes–KOH, pH 7.5	5 μl
● 2 M KCl	1.5 μl
● 60 mM magnesium acetate	5 μl
● 10 mM ATP	5 μl
● [α-^{32}P]UTP	1 μl
● mitochondrial extract	10 μl
● cytosolic RNA substrate (prepared as above)	1 μl
● H_2O	to 50 μl final volume

2. Incubate the mixture for 40 min at 27 °C.
3. Stop the reaction by adding 50 μl of 0.5 M sodium phosphate buffer, pH 6.8, 0.5% sodium pyrophosphate, 0.1% SDS.
4. Remove 80 μl aliquots onto DE81 filter discs.
5. Process the discs and determine the retained radioactivity as described in *Protocol 9*, steps 4–6.

8.4 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 co-sediments with the kinetoplast-mitochondrion fraction (50). It is solubilized in Triton X-100 and thus can be assayed in mitochondrial extracts. Incubation of cytosolic RNA with mitochondrial TL extract and [α-^{32}P]UTP under the conditions required for TUTase in the presence of ATP and Mg^{2+} 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 [α-^{32}P]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 S-100; see *Protocol 11*)
- Cytosolic RNA substrate (500 µg/ml) prepared as described in *Protocol 12*
- 10 mM ATP
- 1.0 M $MgCl_2$
- 0.25 M Hepes–KOH, pH 7.9
- 0.1 M DTT
- [α-^{32}P]pCp (New England Nuclear; 3000 Ci/mimol, 10 mCi/ml)
- DMSO
- RNAguard (Pharmacia, #27-0815-01, 33 µ/ml)
- DE81 discs and 0.5 M sodium phosphate buffer, pH 6.8, with 0.5% sodium pyrophosphate (see *Protocol 9*)

Method

1. In a microcentrifuge tube on ice, mix:

• cytosolic RNA substrate	1–5 µl
• 1.0 M $MgCl_2$	2 µl
• 0.1 M DTT	3.3 µl
• 0.25 M Hepes–KOH, pH 7.9	20 µl
• 10 mM ATP	1 µl
• DMSO	10 µl
• [α-^{32}P]pCp	2 µl
• RNAguard	1 µl
• mitochondrial extract	10 µl
• H_2O	to 50 µl final volume

2. Incubate the mixture at 4 °C for 15 h.
3. Measure the addition of [α-^{32}P]pCp to the RNA substrate by spotting aliquots of the mixture on DE81 filter discs and processing them as described in *Protocol 9*, steps 4–6.

8.5 Cryptic RNase

A sequence- or structure-specific cryptic RNase activity can be detected in mitochondrial extracts (TL, TS, or S-100 extracts, see *Protocol 11*). This cryptic RNase can be activated either by the addition of heparin (5 µg/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 synthesized 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 S-100, see *Protocol 11*)
- Heparin (Sigma)
- Proteinase K (BRL): dissolve the proteinase K at 10 mg/ml in 50 mM Tris–HCl, pH 8.0, 1 mm $CaCl_2$
- 10 mM ATP
- 10× buffer (30 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.5, 50 µg/ml heparin)
- pNB2 plasmid [a 198 bp *AccI/RsaI* restriction fragment from the pLt120 maxicircle region containing the 5′-end of cytochrome *b* gene cloned into the *Sma*I site of pBluescript SK(−) vector from Stratagene]
- pNB2 RNA substrate (10^8 c.p.m./µg): transcribe *Bam*HI-digested pNB2 plasmid DNA *in vitro* using T7 RNA polymerase and [α-^{32}P]UTP as described in Volume I, Chapter 1 (*Protocol 4*) or Chapter 2, this volume (*Protocol 4*)
- Phenol:chloroform (1:1, v/v) and 2 M NaCl as *Protocol 3*
- 8% polyacrylamide/7 M urea denaturing (sequencing) gel, loading and electrophoresis buffers and equipment (see Volume I, Chapter 1, *Protocol 8* or Volume I, Chapter 4, *Protocol 11*)

Method

Activation of the cryptic RNase

1. Either add proteinase K (100 µg/ml final concentration) to the mitochondrial extract and incubate the extract for 5 min at 37 °C or add heparin to 5 µg/ml. Activation by protease predigestion and heparin are synergistic and together result in better cleavage.
2. In a microcentrifuge tube on ice, mix:
   - 10× buffer 5 µl
   - 10 mM ATP 5 µl
   - ^{32}P-labelled pNB2 RNA substrate (10^4 c.p.m.) 1 µl
   - activated mitochondrial extract (from step 1) 10 µl
   - H_2O to 50 µl final volume
3. Incubate the mixture for 1 h at 27 °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 µl of 10 M urea) and analyse the cleavage products by gel electrophoresis and autoradiography as described in Volume I, Chapter 1, *Protocol 8* or Volume I, Chapter 4, *Protocol 11*.

8.6 gRNA:mRNA chimaera-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. Chimaera formation is monitored by the covalent transfer of uniformly-labelled ^{32}P-gRNA

to a higher molecular weight non-radioactive test mRNA by gel electrophoresis. Both the ^{32}P-gRNA and the test 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 for developing conditions for complete *in vitro* editing (20). The system is described in *Protocol 15*.

Protocol 15. gRNA:mRNA chimaera-forming activity

Equipment and reagents

- Centricon 10 centrifugal microconcentrators (Amicon 4205)
- Kinetoplast-mitochondrion fraction isolated from one litre of *L. tarentolae* according to *Protocol 6*. Keep the kinetoplast-mitochondrion fraction at −70 °C until extraction is performed
- Uniformly-labelled ^{32}P-gRNA prepared by *in vitro* transcription with [α-^{32}P]UTP and T7 RNA polymerase (Volume I, Chapter 1, *Protocol 4* or Chapter 2, this volume, *Protocol 4*) and isolated by gel electrophoresis (Volume I, Chapter 1, *Protocol 8* or Volume I, Chapter 4, *Protocol 11*)
- Test mRNA containing the PER plus *flanking (anchor sequence) region*; prepare this by *in vitro* transcription with T7 RNA polymerase followed by gel electrophoresis (see above)
- 1.0 M Hepes–NaOH, pH 7.9
- 0.5 M EDTA (adjust the pH to 8.0 with NaOH)
- Glycerol (ultrapure; BRL 5514UA)
- 2.0 M NaCl
- 1.0 M KCl
- 0.1 M $MgCl_2$
- 13% (w/v) polyethylene glycol 8000 (PEG 8000; Sigma #P-2139)
- *N*-laurylsarcosine (Sigma #L-5125)
- 20 mM ATP (Pharmacia #27-2056-01)
- 0.1 M DTT
- 0.1 M PMSF (Sigma #P-7626) in *iso*-propanol
- Human placental RNase 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 fresh from stock solutions)
- 2 × salt extraction buffer (40 mM Hepes, pH 7.9, 50% glycerol, 0.84 M NaCl, 0.4 mM EDTA, 1 mM PMSF, 1 mM DTT, prepared fresh from stock solutions)
- Annealing buffer (20 mM Hepes–NaOH, pH 7.9, 0.1 M KCl, 1 mM EDTA)
- Reaction buffer (8% PEG 8000, 12.5 mM $MgCl_2$, 2.5 mM ATP, 1 unit/µl RNase inhibitor)
- Proteinase K solution (0.25% *N*-laurylsarcosine, 25 mM EDTA, 0.25 mg/ml proteinase K)
- Sonicator (Braunsonic #4510 with microtip; Braun)
- Materials and equipment for polyacrylamide/urea gel electrophoresis as described in Volume I, Chapter 1, *Protocol 8* or Volume I, Chapter 4, *Protocol 11*

Continued

Protocol 15. *Continued*

Method

1. Resuspend the kinetoplast-mitochondrion fraction from one litre of cell culture in 2 ml of ice-cold hypotonic buffer. Keep this on ice for 10 min to allow the mitochondria to swell.
2. Disrupt the swollen mitochondria by sonication at 5 °C using three 20 sec. periods at 100 watts.
3. Immediately add 2 ml of 2 × salt extraction buffer and gently agitate the solution with a small magnetic stirrer for 30 min on ice.
4. Clarify the extract by centrifuging at 50 000 *g* for 30 min at 4 °C.
5. Concentrate the extract at 4 °C on two Centricon 10 microconcentrators to an approximate vol. of 100 µl each.
6. Change the buffer at 4 °C using the same microconcentrators by applying two 2 ml lots of extract buffer to each.
7. Quick freeze 200 µl aliquots of the pooled extracts in dry ice/ethanol and store them at −70 °C. Chimaera-forming activity is maintained for up to 3 months.
8. Anneal equimolar amounts of the ^{32}P-labelled gRNA and the test mRNA in 2.5 µl of annealing buffer. Denature the RNA for 3 min at 70 °C then anneal them at 37 °C and 25 °C for 10 min each.
9. Add 8 µl of reaction buffer and 15 µl of thawed mitochondrial extract (from step 7) to the annealed RNAs. Incubate for 15–120 min at 27 °C.
10. Stop the reaction with 100 µl of proteinase K solution and incubate at 37 °C for 20 min.
11. Recover the RNA by phenol extraction and ethanol precipitation as described in *Protocol 3*, steps 13–16.
12. Analyse the resulting RNA chimaeric products on denaturing acrylamide/urea gels followed by autoradiography (Volume I, Chapter 1, *Protocol 8*, or Volume I, Chapter 4, *Protocol 11*).

The RNA products from *Protocol 15* may also be analysed by sequence determination following RT–PCR and cloning. In order to amplify specifically the exogenous RNA, a 'tag' sequence may be added to the test RNA. Further details of RT–PCR can be found in Volume I, Chapter 2, Section 3.4 and Volume I, Chapter 3, Section 2.2.3

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