

tion studies should enable us to determine if Rpm1r and Rpm2p are the only components necessary to support full activity *in vitro*. If they are, studies on the biochemistry and biogenesis of a well-defined yeast mitochondrial RNase P can be initiated. If not, genetic approaches to identify other proteins or RNAs should be possible by starting with the two genes that are now available. Immunoprecipitation of complexes or cross-linking between the known RNA and proteins offer other approaches to identify additional components that could be required for *in vitro* reconstitution or be associated with the enzyme *in vivo*.

Acknowledgment

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[10] RNA Editing in Trypanosomatid Mitochondria

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Introduction

The kinetoplastid protozoa, together with the euglenoids, represent one of the earliest branches of eukaryotes containing mitochondria.¹ There are two major subgroups within the Kinetoplastida: the bodonids—cryptobiids and the trypanosomatids. The former organisms are less investigated than the latter and are not discussed in this chapter. Members of the trypanosomatid genera *Trypanosoma* and *Leishmania* are the causal agents of several important diseases in humans and animals, including visceral and dermal leishmaniasis, South American Chagas disease, and African sleeping sickness. These “digenetic” species have a biphasic life cycle that involves both a vertebrate host and an invertebrate vector. Species which belong to the monogenetic genera *Crithidia*, *Blastocrithidia*, *Leptomonas*, and *Herpetomonas* parasitize only invertebrates. Most species can be grown axenically, and, in some cases, both stages of the life cycle can be maintained.²

The kinetoplastids have a single mitochondrion which contains a large mass of mitochondrial DNA situated in the region adjacent to the basal body of the flagellum. The region with the DNA is called the kinetoplast, and the DNA is called kinetoplast DNA or kDNA. The kDNA is organized into a giant network of approximately 10^4 catenated minicircles, 0.4 to 2.5

¹ L. Simpson and D. A. Maslov, *Curr. Opin. Genet. Dev.* **4**, 887 (1994).

² L. Simpson, *Int. Rev. Cytol.* **32**, 139 (1972).

kb in size, and 20–50 catenated maxicircles, 20 to 36 kb in size.³ The maxicircle is the equivalent of the mitochondrial genome in other eukaryotic organisms, and encodes ribosomal RNAs as well as several components of the electron transport chain. The minicircles encode small 3'-oligouridylated transcripts known as guide RNAs (gRNAs),⁴ which mediate a post-transcriptional process of RNA modification known as RNA editing.^{5–12} The trypanosomatid type of RNA editing involves the insertion and, less frequently, deletion of uridylylate (U) residues and affects coding regions of more than half of the maxicircle mRNA transcripts. Those genes, the transcripts of which are edited, are known as cryptogenes.⁶ Depending on the cryptogene, editing creates initiation and/or termination codons, corrects frameshifts, and, in the case of pan-edited mRNAs, creates an entire reading frame leading in some cases to a doubling in size of the edited transcript. Editing proceeds in a 3' to 5' direction within an editing domain, and in most cases it terminates a few nucleotides upstream of the created or encoded translation initiation codon. It is assumed that only the mature fully edited mRNA is translationally competent; however, a direct proof that fully edited mRNAs are translated is still lacking.

The pattern of inserted and deleted U residues in the mature edited RNA is determined precisely by base-pairing interactions with gRNAs. Genes for gRNAs are present in both the maxicircle and the minicircle DNA molecules.^{4,13,14} The gRNA contains at its 5' end an anchor sequence that is complementary to the cognate mRNA just downstream of the first editing site. In pan-edited transcripts, multiple gRNAs mediate editing in a sequential manner: each downstream gRNA creates the anchor sequence for the adjacent upstream gRNA, thereby determining the overall 3' to 5' polarity of the editing cascade. The central portion of each gRNA contains

³ L. Simpson, *Annu. Rev. Microbiol.* **41**, 363 (1987).

⁴ B. Blum, N. Bakalara, and L. Simpson, *Cell (Cambridge, Mass.)* **60**, 189 (1990).

⁵ R. Benne, J. Van den Burg, J. Brakenhoff, P. Sloof, J. Van Boom, and M. Tromp, *Cell (Cambridge, Mass.)* **46**, 819 (1986).

⁶ L. Simpson and J. Shaw, *Cell (Cambridge, Mass.)* **57**, 355 (1989).

⁷ K. Stuart, *Annu. Rev. Microbiol.* **45**, 327 (1991).

⁸ L. Simpson, D. A. Maslov, and B. Blum, in "RNA Editing—The Alteration of Protein Coding Sequences of RNA" (R. Benne, ed.), p. 53. Ellis Horwood, New York, 1993.

⁹ S. L. Hajduk, M. E. Harris, and V. W. Pollard, *FASEB J.* **7**, 54 (1993).

¹⁰ K. Stuart, in "RNA Editing—The Alteration of Protein Coding Sequences of RNA" (R. Benne, ed.), p. 25. Ellis Horwood, New York, 1993.

¹¹ B. K. Adler and S. L. Hajduk, *Curr. Opin. Genet. Dev.* **4**, 316 (1994).

¹² R. Benne, *Eur. J. Biochem.* **221**, 9 (1994).

¹³ N. R. Sturm and L. Simpson, *Cell (Cambridge, Mass.)* **61**, 879 (1990).

¹⁴ V. W. Pollard, S. P. Rohrer, E. F. Michelotti, K. Hancock, and S. L. Hajduk, *Cell (Cambridge, Mass.)* **63**, 783 (1990).

the guiding nucleotides which, via base-pairing interactions with mRNA (including G/U base pairs), direct the insertions and deletions of U residues. At their 3' ends, gRNAs contain heterogeneously sized oligo(U) tails that are added posttranscriptionally by a mitochondrial terminal uridylyltransferase (TUTase).^{15,16}

Several models have been proposed to explain the mechanism of RNA editing. In the enzyme cascade model,⁴ an endonuclease, a TUTase, and an RNA ligase sequentially interact during each editing cycle and the inserted U residues originate either from UTP or from the 3' end of the gRNA.¹⁷ In the transesterification model,^{18,19} each editing cycle proceeds via two successive transesterification reactions, resulting in the transfer of a U from the 3' end of the gRNA to the editing site, or, in the case of a deletion, from the editing site to the gRNA. Chimeric gRNA/mRNA molecules, which are predicted intermediates of this type of mechanism, have been visualized,¹⁹ but it has not been established that these molecules represent true intermediates of the editing process.

Partially edited RNAs constitute a variable portion of the steady-state RNA. Frequently the junction region between the mature edited 3' sequence and the preedited 5' sequence exhibits unexpected editing patterns. These sequences have been attributed either to misediting caused by the mediation of noncognate gRNAs or to normal intermediates of the editing process.²⁰⁻²³

RNA editing has been studied extensively in three species to date: *Trypanosoma brucei*, *Leishmania tarentolae*, and *Crithidia fasciculata*. The authors have chosen to use *L. tarentolae*, which is a parasite of the gecko, as a model system to study the mechanism of RNA editing for the following reasons: (1) the cells are not pathogenic for humans; (2) the cells grow rapidly (division time 6-9 hr) to a high density (4×10^8 cells/ml) in an inexpensive medium; (3) cell fractionation protocols are well developed, and a large-scale isolation of the intact kinetoplast-mitochondria can be easily achieved; and (4) the informational portion of the mitochondrial genome is completely sequenced and virtually all gRNAs are identified. The major disadvantage of *L. tarentolae* as a model organism is that the

¹⁵ B. Blum and L. Simpson, *Cell (Cambridge, Mass.)* **62**, 391 (1990).

¹⁶ N. Bakalara, A. M. Simpson, and L. Simpson, *J. Biol. Chem.* **264**, 18679 (1989).

¹⁷ B. Sollner-Webb, *Curr. Opin. Cell Biol.* **3**, 1056 (1991).

¹⁸ T. R. Cech, *Cell (Cambridge, Mass.)* **64**, 667 (1991).

¹⁹ B. Blum and L. Simpson, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11944 (1992).

²⁰ N. R. Sturm, D. A. Maslov, B. Blum, and L. Simpson, *Cell (Cambridge, Mass.)* **70**, 469 (1992).

²¹ C. J. Decker and B. Sollner-Webb, *Cell (Cambridge, Mass.)* **61**, 1001 (1990).

²² L. K. Read, R. A. Corell, and K. Stuart, *Nucleic Acids Res.* **20**, 2341 (1992).

²³ D. J. Koslowsky, G. J. Bhat, L. K. Read, and K. Stuart, *Cell (Cambridge, Mass.)* **67**, 537 (1991).

biology of the parasite within the natural host is poorly known, and an *in vitro* system which simulates the natural life cycle differentiation of the parasite is lacking.

This chapter describes a series of methods relevant to the study of RNA editing in *L. tarentolae*. However, with minor modifications, most procedures may be applied to the study of RNA editing in other trypanosomatid species.

Cell Culture

Leishmania tarentolae cells are grown in brain–heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) supplemented with 10 $\mu\text{g}/\text{ml}$ hemin. T-flasks may be used for small-scale cultures (3–10 ml), and 3.8-liter bottles, using a roller bottle culture apparatus maintained at approximately 16 rpm, can be used to grow cultures up to 1 liter. To avoid cultivation-induced changes, new cultures should be started from a frozen stock every few weeks. To freeze cells, aliquots of log-phase cultures (5×10^7 to 10^8 cells/ml) are transferred into sterile freezer vials, and an equal volume of 20% glycerol in BHI is added. The cells are slowly frozen overnight at -80° and then transferred to liquid nitrogen for long-term storage.

Isolation of Kinetoplast DNA

The kDNA network has a sedimentation coefficient of approximately 4000 S and is relatively resistant to shear forces owing to its compact shape.^{24,25} Network DNA can be isolated from a sheared total cell lysate by sedimentation through CsCl.^{24,25} The maxicircle DNA, which represents approximately 5% of the kDNA and is also catenated into the network, 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.²⁶ The complete sequence of the 23 kb maxicircle of *T. brucei* is known^{27,28}

²⁴ L. Simpson and J. Berliner, *J. Protozool.* **21**, 382 (1974).

²⁵ L. Simpson and A. Simpson, *J. Protozool.* **21**, 774 (1974).

²⁶ L. Simpson, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1585 (1979).

²⁷ P. Sloof, A. De Haan, W. Eier, M. Van Iersel, E. Boel, H. Van Steeg, and R. Benne, *Mol. Biochem. Parasitol.* **56**, 289 (1992).

²⁸ P. J. Myler, D. Glick, J. E. Feagin, T. H. Morales, and K. D. Stuart, *Nucleic Acids Res.* **21**, 687 (1993).

and 21 kb of the 30-kb maxicircle genome of *L. tarentolae* has been sequenced.²⁹ The structural genes are clustered in approximately 17 kb, and the remainder, which is known as the divergent region, represents tandem repeats of varying complexity.³⁰

The following protocol describes the isolation of kDNA from stationary phase cultures of *L. tarentolae*.

1. The cells are harvested by centrifugation (10 min at 2000–3000 *g* at 4°) or by filtration for large-scale cultures. A Pellicon transverse filter system (Millipore, Bedford, MA) is used to concentrate 5–20 liters of culture.

2. The cells are washed by resuspension in 50 volumes SET buffer (0.15 *M* NaCl, 0.1 *M* EDTA, 10 *mM* Tris-HCl, pH 7.5).

3. The cells are suspended at 1.2×10^9 cells/ml in SET buffer. Add pronase to 0.2 mg/ml and sarkosyl to 3% (w/v). Incubate at 60° for 1–3 hr.

4. The viscous lysate is passed through an 18-gauge syringe needle at 25 psi to shear nuclear DNA and reduce the viscosity. For small volumes (10–100 ml) use a 12-ml syringe and force the lysate through the needle by hand. For larger volumes, use a dispensing pressure vessel (Millipore XX67 OOP 05 or 10) with the pressure supplied by compressed air. Use a quick-release tube adaptor to attach the vessel to the tank with compressed air.

5. The lysate is centrifuged for 1.5 hr in an SW28 Beckman rotor at 22,000 rpm (Beckman Instruments, Columbia, MD). This pellets the network DNA.

6. Suspend each pellet in 1–2 ml TE buffer (10 *mM* Tris-HCl, 1 *mM* EDTA, pH 7.9) by shaking. Pour into a flask for suspension. Bring up the total volume with TE buffer to 6 ml per each 1–2 liters of original culture. Agitate 30 min to dissolve the network DNA.

7. Prepare CsCl step gradients in SW28 polyallomer tubes by slowly introducing 6 ml of the lower CsCl solution under 24 ml of the upper CsCl solution using a peristaltic pump. The upper CsCl solution contains 37.62 g CsCl in 62 ml TE buffer (refractive index at 25° 1.3705). The lower CsCl solution contains 29.2 g CsCl in 20 ml TE buffer plus 0.14 ml of 10 mg/ml ethidium bromide (refractive index at 25° 1.4040). Only the lower CsCl solution contains dye.

8. Layer 6 ml suspended crude kDNA solution on top of each gradient and centrifuge the gradients 15 min at 20,000 rpm at 20° in an SW28 rotor. The kDNA networks will sediment to the interface between the lower and

²⁹ V. de la Cruz, N. Neckelmann, and L. Simpson, *J. Biol. Chem.* **259**, 15136 (1984).

³⁰ M. Muhich, N. Neckelmann, and L. Simpson, *Nucleic Acids Res.* **13**, 3241 (1985).

upper CsCl solutions, whereas 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 a UV source (302 nm). Collect the kDNA band by side puncture.

10. Remove the ethidium bromide by extracting twice with an equal volume of water-saturated *n*-butanol.

11. Dialyze the kDNA against 4 liters TE buffer overnight at 4°.

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

13. Extract the kDNA by vortexing with an equal volume of phenol–chloroform (1:1, v/v). 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 by 2–4 extractions with an equal volume of water-saturated ether.

15. Add 0.1 volume of 2 *M* NaCl and 2 volumes ethanol. Incubate in dry ice–ethanol bath for 15 min or at –20° overnight.

16. Recover the precipitated kDNA by centrifugation at 12,000 *g* for 15 min at 4° in a microcentrifuge.

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

18. Check the integrity of the network DNA by diluting a sample 10-fold and adding DAPI (4',6'-diamidino-2-phenylindole, Sigma, St. Louis, MO) to 1 μ g/ml. Observe the stained kDNA using a UV microscope at 1000 \times magnification. The size and shape of the kDNA networks are distinctive for each kinetoplastid species. If shearing was too harsh, the networks will be fragmented. *Leishmania tarentolae* networks often break into half- or quarter-sized networks. *Crithidia fasciculata* networks are more resistant to shear forces. *Trypanosoma brucei* networks are smaller than those from *C. fasciculata* or *L. tarentolae*.

19. Store the kDNA in aliquots at –20°. Using the procedure described, the yield of kDNA is 0.5–1.0 mg/liter of culture. If desired, nuclear DNA can be isolated from the crude DNA preparation. Add 2 volumes of cold (–20°) ethanol and spool the nuclear DNA onto a glass rod. Dissolve the DNA in 10 ml TE buffer. Deproteinize by phenol extraction and recover the DNA by ethanol precipitation. This can be used directly for restriction enzyme digestion, or the contaminating RNA can be removed by digestion with RNase A or by ethidium bromide–CsCl isopycnic centrifugation.

Isolation of Maxicircle DNA

In all kinetoplastid species analyzed, maxicircle DNA 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.²⁶ The Hoechst dye binds preferentially to A+T-rich sequences, thereby decreasing the buoyant density. The maxicircle DNA from *L. tarentolae* is 30 kb in size and can be linearized at the single *EcoRI* site.

1. Dilute 1 mg kDNA into 2 ml *EcoRI* restriction endonuclease buffer. Add 1000 units *EcoRI* and incubate mixture 3 hr at 37°.

2. Monitor the extent of release of the 30-kb maxicircle DNA by running 10- μ l samples on a 0.7% agarose gel.

3. Add TE buffer to 12 ml. Add 18.5 g CsCl.

4. Add 0.5 mg/ml Hoechst 33258 dye (Sigma) dropwise with mixing to approximately 1 μ g dye per microgram DNA. Stop the addition if the solution becomes cloudy since this can result in the precipitation of the DNA.

5. Adjust density of the solution to a refractive index of 1.3950 at 25°.

6. Centrifuge in a Beckmann 50.2 or Ti60 rotor 48 hr at 40,000 rpm at 25°.

7. Visualize DNA bands with 302 nm UV illumination and recover the minor upper band. The lower band contains undigested kDNA networks and released minicircle DNA. Adjust the refractive index of the upper band to 1.3935, and centrifuge 6.5 ml per tube in a 50 rotor (Beckman) at 39,000 rpm for 48 hr at 25°. Recover the upper band. The second centrifugation is required to remove contaminating minicircle or kDNA.

8. Remove the dye by extraction with an equal volume of 2-propanol.

9. Dialyze the maxicircle DNA against TE buffer and concentrate by *sec*-butanol extraction.

10. Recover DNA by ethanol precipitation.

Isolation of Kinetoplast–Mitochondrial Fraction

Most kinetoplastid cells are generally quite resistant to rupture by shear forces in isotonic media. The method described here employs hypotonic conditions to allow efficient cell breakage by shear forces, thereby releasing a swollen kinetoplast–mitochondrion. Because of the complex, multilobular structure of the single mitochondrion, the isolation of intact mitochondria is most likely not possible. However, mitochondrial fragments apparently reseal effectively, and, in particular, the resealed portion containing the kinetoplast DNA can be isolated by its relatively high isopycnic buoyant

density in Renografin density gradients.³¹ There is no evidence that this portion of the mitochondrion differs enzymatically from the remainder of the mitochondrion.

1. Grow *L. tarentolae* cells to mid or late log phase ($1-2 \times 10^8$ cells/ml). Harvest the cells by centrifugation (4000 g for 10 min at 4°). Wash cells twice with a 50- to 100-fold excess of ice-cold 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1 M EDTA (SET).

2. Suspend the cells without clumps in cold 1 mM Tris-HCl (pH 7.9 at 4°), 1 mM EDTA (DTE). The volume in ml of DTE is determined by dividing the total cell number by 1.2×10^9 . Monitor swelling of the cells by phase-contrast microscopy at a magnification of 400–1000×. If insufficient swelling has occurred, add more DTE. Leaving the cells on ice for 5–10 min is generally adequate for complete swelling.

3. Rupture the cells by passing through a 26-gauge needle connected to a dispensing pressure vessel (Millipore) driven by compressed air at 100 psi. Monitor the extent of breakage by phase-contrast microscopy. Small volumes can be processed by hand using a 1–10 ml syringe. Immediately add 0.125 volume of 60% (w/v) sucrose to the lysate. This causes the swollen kinetoplast-mitochondria to shrink into crescent-shaped refractile disks.

4. Centrifuge the lysate at 4° for 10 min at 16,000 g. Discard the supernatant and suspend the loosely packed pellet in cold 0.25 M sucrose, 20 mM Tris-HCl (pH 7.9), 3 mM MgCl₂ (STM), using one-sixth the volume of the lysate obtained in step 3. Add 0.005 volume of 2 mg/ml DNase I (Sigma) and incubate on ice 1 hr, in order to digest nuclear DNA, which would interfere with the fractionation procedures.

5. Add an equal volume of cold 8.56% sucrose, 10 mM Tris-HCl (pH 7.9), 2 mM EDTA (STE) and centrifuge at 4° for 10 min at 16,000 g. If the DNase digestion was successful, the pellet should now be well packed.

6. To the pellet add cold 76% Renografin (Squibb Diagnostics, Princeton, NJ: 66% diatrizoate meglumine, 10% diatrizoate sodium), 0.25 M sucrose, 0.1 mM EDTA (RSE). Use 4 ml of 76% RSE per liter of original culture. Vortex and let sit on ice for a few minutes to remove all air bubbles.

7. Layer 4–5 ml of the mixture underneath a 20–35% RSE gradient, using a syringe attached to an 18-gauge needle and polyethylene tubing. If the first drop of mixture floats rather than remaining at the bottom of the gradient, add more 76% RSE to increase the density. The gradients are prepared in 38-ml Beckman SW28 ultracentrifuge tubes by layering 16 ml of 20% Renografin, 8.65% sucrose, 20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, density 1.14 g/ml (20% RSTE), over 16 ml of 35% RSTE (density

³¹ P. Braly, L. Simpson, and F. Kretzer, *J. Protozool.* **21**, 782 (1974).

1.26 g/ml). The tubes are frozen at -20° and thawed overnight at 4° before used to establish the gradients.

8. Centrifuge the gradients at 4° for 2 hr at 24,000 rpm in a Beckman SW28 rotor. Visualize the kinetoplast-mitochondrial band at a density of approximately 1.22 g/ml by side illumination. Puncture the tube with a syringe needle, and collect the material.

9. Dilute the suspension with 2 volumes cold STE and centrifuge at 4° for 15 min at 16,000 g. Wash the mitochondrial fraction once with at least 50 pellet volumes cold STE.

10. For kRNA isolation, wash the pellet once with 50 volumes cold STM, and suspend the material in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM NaCl (TMN), using 5 ml per liter of original culture.

11. For the preparation of mitochondrial extracts, the pellet from step 9 is suspended at a concentration of approximately 5 mg/ml in 20 mM HEPES-KOH (pH 7.5), 20 mM KCl, 1 mM EDTA, 9–20% (v/v) glycerol (K buffer). The suspension is either used directly or stored frozen at -80° .

Purification of Kinetoplast RNA

Isolation of intact kRNA from a purified kinetoplast-mitochondrial preparation is readily accomplished due to the low ribonuclease activity of this fraction.³² More complex purification procedures using chaotropic salts are usually not necessary.

1. To the mitochondrial suspension in TMN add 20% sodium dodecyl sulfate (SDS) to a final concentration of 0.1% and leave the mixture on ice 5 min.

2. Perform an extraction with an equal volume of phenol-chloroform. Transfer the aqueous phase to a fresh tube, reextract the interface with water, and pool the aqueous phases.

3. Adjust the NaCl concentration to 0.2 M and add 2 volumes ethanol. Incubate at -20° for at least 1 hr, and then recover the nucleic acids by centrifugation at 4° for 30 min at 12,000 g. Wash the pellet twice with 70% (v/v) ethanol.

4. Dissolve the nucleic acids in 1 ml TMN per each 2–3 liters original cell culture. Add 0.005 volume DNase I (RNase-free) and incubate at 37° for 30 min.

5. Extract with phenol-chloroform and precipitate the kRNA with ethanol as described in steps 2 and 3. Wash the pellet three times with 70% ethanol.

³² L. Simpson and A. Simpson, *Cell (Cambridge, Mass.)* **14**, 169 (1978).

6. Dissolve the kRNA in water at a concentration of approximately 2 mg/ml. Store the RNA as frozen aliquots.

Cloning of Edited and Partially Edited mRNA Sequences

Preparations of steady-state kinetoplast RNA contain a mixture of preedited, partially edited, and fully edited transcripts in a ratio that varies for different cryptogenes as well as for different species and life cycle stages. The sequences of editing intermediates and fully edited mRNAs can be determined by reverse transcriptase (RT)-PCR (polymerase chain reaction) followed by cloning and sequencing.³³ Obtaining a sequence of fully edited mRNA may be a nontrivial task for some genes since most clones will contain partially edited or incorrectly edited (misedited) sequences. Often no single clone that corresponds to a fully edited mRNA can be obtained, and the mature editing pattern can only be deduced as a consensus edited sequence from the clones aligned according to the overall 3' to 5' progression of editing.^{20,34} Because of this polarity, there will be more clones with 3' mature editing than with 5' editing, thus affecting the reliability of the consensus sequence at the 5' end.

The following strategy has been successfully employed to investigate the editing of many cryptogene-derived mRNAs. Putative cryptogenes are identified by defects in the reading frame or by a purine-rich region, which is typical of pan-editing.^{35,36} The GCG (Genetics Computer Group, Madison, WI) programs Window and StatPlot can be used to locate (G+A)-rich regions in the DNA sequence. Synthesis of cDNA is performed using an oligo(dT) primer or a primer specific for the genomic sequence downstream of the preedited region. The latter may be preferable, since it results in a more specific amplification. In *L. tarentolae*, the 3'-most editing site is separated from the poly(A) tail by 20–40 nucleotides of unedited sequence. Therefore, if the 3' boundary of the putative cryptogene is well defined, an oligonucleotide complementary to the sequence just downstream of this boundary may be selected as a primer.

There are several options for choosing a 5' primer. For small cryptogenes, a sequence upstream of the preedited region may be chosen, thus yielding preedited, partially edited, and fully edited molecules in the same PCR. A stepwise approach may be more effective for large cryptogenes. Partially edited molecules are amplified with a 5' primer selected from an

³³ D. A. Maslov, N. R. Sturm, B. M. Niner, E. S. Gruszynski, M. Peris, and L. Simpson, *Mol. Cell. Biol.* **12**, 56 (1992).

³⁴ J. Abraham, J. Feagin, and K. Stuart, *Cell (Cambridge, Mass.)* **55**, 267 (1988).

³⁵ L. Simpson, N. Neckelmann, V. de la Cruz, A. Simpson, J. Feagin, D. Jasmer, and K. Stuart, *J. Biol. Chem.* **262**, 6182 (1987).

³⁶ J. E. Feagin, J. Abraham, and K. Stuart, *Cell (Cambridge, Mass.)* **53**, 413 (1988).

internal sequence of the purine-rich region. A consensus edited sequence for the 3' end of mRNA is deduced from these clones. Another PCR with an edited sequence-specific 3'-primer and an upstream genomic 5'-primer would extend the defined edited sequence further upstream. Information about the 5'-most editing sites is obtained by directly sequencing the 5' end of the mRNA or by taking a RACE (rapid amplification of cDNA ends)-PCR approach.

The overall procedure is as follows:

1. Anneal 10–50 pmol of the oligo(dT) primer or the downstream genomic primer to 2.5–5 μg kinetoplast or total cell RNA in 50 μl of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 at 65° for 10 min, followed by incubation on ice for 10 min. Add 5 μl of 0.1 M dithiothreitol (DTT) and 5 μl dNTP solution (10 mM of each deoxynucleoside triphosphate). Add 2 μl of 200 units/ μl SuperScript II RNase H⁻ reverse transcriptase (GIBCO-BRL, Grand Island, NY). Incubate at 37° for 30 min. Add another aliquot of enzyme and incubate at 45° for another 30 min. Inactivate the enzyme by incubation at 95° for 5 min.

2. Set up a 50- μl PCR reaction containing 5 μl of the above mixture, 20 pmol of each primer, 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl_2 , 25 mM KCl, 0.05% Tween 20, 0.1 mg/ml nuclease-free bovine serum albumin (BSA), 0.25 mM each dNTP, and 5 units *Taq* DNA polymerase. Denature at 95° for 5 min. Perform 5 PCR cycles at 95° for 30 sec, 45° for 30 sec, and 65° for 1 min. Perform 30 cycles at 95° for 30 sec, 50° for 30 sec, and 72° for 1 min.

3. Gel purification of the PCR product of the expected size may be required, as the reaction usually generates some spurious products, especially if oligo(dT) was used to prime the cDNA synthesis. To improve the specificity, a single nucleotide-anchored 3' primer may be used for PCR. Because *Taq* DNA polymerase adds single, nonencoded A residues to the 3' ends, the purified PCR products can be cloned directly into vectors containing overhanging T residues (e.g., pT7Blue, Novagen, Madison, WI).

Construction of Guide RNA Libraries

Cloning of gRNAs is based on a RACE-PCR procedure that involves synthesis of cDNA, ligation of an anchor oligonucleotide, and PCR amplification³⁷ (Fig. 1). To monitor some critical steps of the procedure, cDNA synthesis is performed using 5'-labeled oligonucleotides. Because cloning of PCR products based on the single 3'-overhanging A residues is not very efficient, we have usually employed the CloneAmp system (GIBCO-BRL),

³⁷ O. H. Thiemann, D. A. Maslov, and L. Simpson, *EMBO J.* **13**, 5689 (1994).

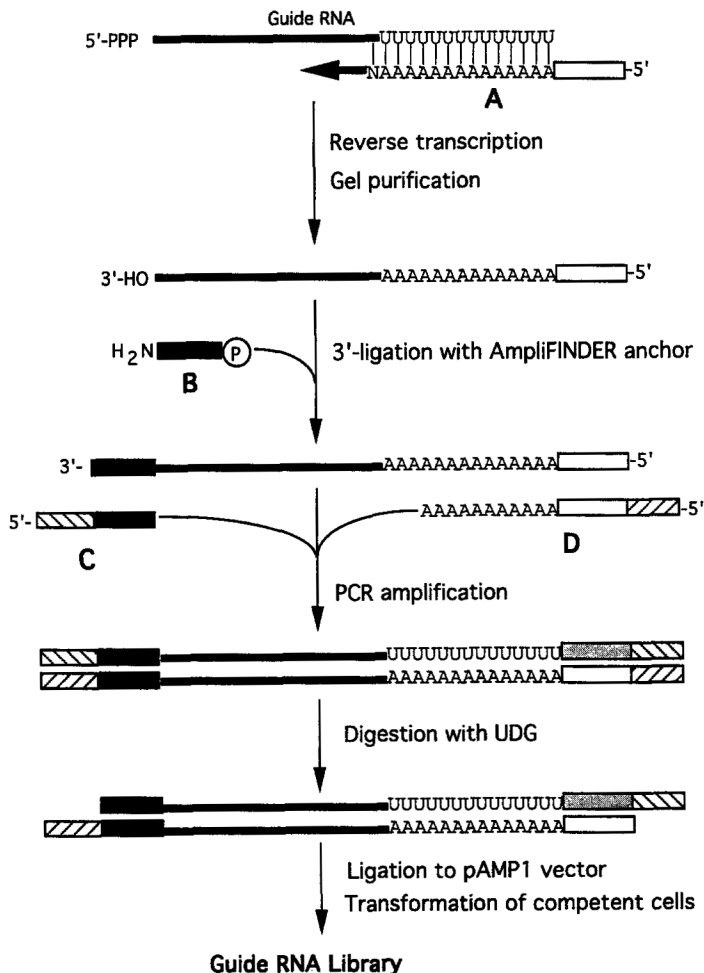


FIG. 1. Construction of gRNA library. Thick black lines represent the encoded part of a guide RNA and the corresponding part of a cDNA. Open boxes in oligonucleotides A and D represent an adaptor sequence added for the purpose of increasing the melting temperature. The black box in oligonucleotide C represents a sequence complementary to the AmpliFINDER anchor (oligonucleotide B). Note that both oligonucleotides A and B contain an *Eco*RI site (see text for the structure of the oligonucleotides) that can be used for cloning in an appropriate vector instead of the CloneAmp system. Hatched areas of oligonucleotides C and D represent sequences that contain deoxyuridines instead of thymidines.

which yields a large number of clones. The PCR primers are synthesized with 5'-end adaptors containing deoxyuridines (dU) instead of thymidines. Digestion of the PCR products with uracil DNA glycosylase generates protruding single stranded 5' ends suitable for efficient cloning in the pAMP1 vector. The method has been used successfully to obtain gRNA libraries from *T. cruzi*,^{37a} *C. fasciculata*,^{37b} *Trypanoplasma borreli* (S. Yasuhira, D. Maslov, and L. Simpson, unpublished results, 1995), as well as from two strains of *L. tarentolae*.³⁷

1. Combine 1 μl of a mixture containing 10 pmol of each of the three oligonucleotides CGCGGATCC(A)₁₄C, CGCGGATCC(A)₁₄T, and CGCGGATCC(A)₁₄G (oligonucleotide A, Fig. 1), 2 μl of [γ -³²P]ATP (6000 Ci/mmol, 10 $\mu\text{Ci}/\mu\text{l}$), 1 μl of 10 \times buffer (0.5 M Tris-HCl, pH 7.6, 0.1 M MgCl₂, 50 mM DTT, 1 mM spermidine, 1 mM EDTA), 1 μl of 10 units/ μl T4 polynucleotide kinase (GIBCO-BRL), and 5 μl double-distilled water. Incubate at 37° for 30 min. Purify the labeled oligonucleotides using NucTrap columns (Stratagene, La Jolla, CA). Add 2 volumes of 7.5 M ammonium acetate and precipitate the oligonucleotides with 3 volumes of ethanol. Suspend the pellet in 20 μl water.

2. Incubate 4 μl of the labeled oligonucleotides with 10–20 μg of kRNA or gel-purified guide RNA (usually less than 1 μg) in 38 μl of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂ at 65° for 5 min. Add 5 μl of 0.1 M DTT and 5 μl of dNTP solution (10 mM of each), and anneal for 10 min on ice. Add 2 μl of 200 units/ μl SuperScript II RNase H⁻ reverse transcriptase (GIBCO-BRL) and incubate at 16° for 30 min. Add another 1- μl aliquot of enzyme and incubate at 37° for 1 hr.

3. Hydrolyze the RNA by the addition of 50 μl of 0.4 M NaOH, 30 mM EDTA for 1 hr at 65°. Neutralize the solution by adding 12 μl of 3 M sodium acetate (pH 5.2). Precipitate the cDNA by adding 0.3 ml of ethanol. Suspend the pellet in 12 μl water.

4. Fractionate the cDNA on a 0.4 mm 8% (w/v) polyacrylamide–7.5 M urea sequencing gel. Visualize the cDNA bands by exposing the wet gel at 4°. This is a critical step of the procedure, since a failure to remove unreacted primers from the cDNA results in the addition of anchor oligonucleotides to the primers as well as to the cDNA, and causes a subsequent contamination of the library with clones containing anchored primers. Elute the cDNA overnight by diffusion. Precipitate the cDNA by the addition of 50 μg glycogen and 3 volumes ethanol. Suspend the final pellet in 12 μl water.

^{37a} H. Avila and L. Simpson, *RNA*, in press (1995).

^{37b} S. Yasuhira and L. Simpson, *RNA* **1**, 634 (1995).

5. Ligate the 3'-end-blocked anchor oligonucleotide B (Fig. 1), CAC-GAATTCACTATCGATTCTGGAACCTTCAGAGG (AmpliFINDER anchor, provided with 5'-AmpliFINDER RACE kit, Clontech Laboratories, Palo Alto, CA), by incubating 2.5 μ l of the gel-purified cDNA, 2 μ l of 2 pmol/ μ l oligonucleotide, 5 μ l of 2 \times ligation buffer (provided by the manufacturer), and 0.5 μ l of 20 units/ μ l T4 RNA ligase at room temperature for 16–24 hr. In an alternative approach, a homopolymer tail is added to the 3' ends of the cDNA. The reaction is performed with terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN) by incubating 25 units enzyme and 1 μ M dGTP for 30 min at 37°. If this method is chosen, the sequence of the PCR primers must be modified accordingly.

6. To PCR-amplify the anchor-ligated cDNA, combine 2 μ l of the 10-fold diluted ligation product, 1 μ l (200 pmol) of each of the two oligonucleotides (CADU)₄GTTCCAGAATCGATAGTGAAT (oligonucleotide C, Fig. 1) and (CdUA)₄CGCGGATCC(A)₁₁ (oligonucleotide D, Fig. 1) in a 50- μ l PCR reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM MgCl₂, 0.2 mM each dNTP, and 5 U *Taq* polymerase. Denature at 95° for 30 sec and amplify for 30 cycles at 94° for 5 sec, 60° for 10 sec, and 72° for 30 sec.

7. Analyze and, if desired, purify the cDNA further by using a 4% (w/v) NuSieve agarose gel or an 8% nondenaturing polyacrylamide gel. In addition to the expected PCR products (120–150 bp), a faster migrating product is usually observed, which results from amplification of carried-over cDNA synthesis primers.

8. To clone the cDNA, perform uracil DNA glycosylase treatment, ligation to pAMP1 vector (GIBCO-BRL), and transformation of DH5 α Library Efficient competent cells (GIBCO-BRL).

Mitochondrial Extract Preparation and Fractionation by Glycerol Gradient Sedimentation

It is essential to first obtain crude kinetoplast-mitochondrial lysates, using the purified mitochondrial fraction obtained as described previously. This is most conveniently achieved by breaking the mitochondrial membranes either by sonication or by the addition of detergents. The following protocol describes the preparation of extracts containing Triton X-100,¹⁶ and subsequent fractionation of the clarified extracts by glycerol gradient sedimentation.³⁸

1. If necessary, thaw on ice a 0.2- to 0.3-ml aliquot of kinetoplast-

³⁸ M. Peris, G. C. Frech, A. M. Simpson, F. Bringaud, E. Byrne, A. Bakker, and L. Simpson, *EMBO J.* **13**, 1664 (1994).

mitochondrial fraction isolated as described above. Add 10% (v/v) Triton X-100 to a final concentration of 0.3% and mix gently.

2. Place the tube in an ice-water bath and homogenize the mixture for 15 sec using a motor-driven pellet pestle mixer (Kontes Glass Co., Vineland, NJ, Cat. No. 749520). This yields the TL (Triton lysate) extract.

3. Centrifuge the TL extract at 4° for 30 min at 12,000 *g* to obtain the clarified TS (Triton supernatant) extract, or at 4° for 1 hr at 100,000 *g* to obtain the S-100 extract. Store the extracts at -80° or use them directly for glycerol gradient sedimentation and/or various assays as described below.

4. Prepare linear 10–30% glycerol gradients containing 20 mM HEPES-KOH (pH 7.5), 20 mM KCl, 1 mM EDTA, in 13.2 ml ultracentrifuge tubes (Beckman, Cat. No. 344059 or 331372), by using a standard gradient mixer or a gradient master (Biocomp Instruments, Frederickton, New Brunswick). Layer up to 0.2 ml TS or S-100 extract (containing a glycerol concentration of 9% or less) on top of a gradient. Concurrently, overlay equivalent gradients with S-value standards, such as alcohol dehydrogenase (7.6 S, Sigma), catalase (11.5 S, Pharmacia, Piscataway, NJ) thyroglobulin (19.3 S, Pharmacia), and *Escherichia coli* small (30 S) ribosomal subunits.

5. Centrifuge the gradients at 4° for 14 hr at 33,000 rpm in a Beckman SW-41 rotor.

6. Collect 16–20 fractions by using a density gradient fractionator (Instrumentation Specialties Co., Lincoln, NE). The gradient fractions may be used directly for various assays, or their volume and composition may first be modified by using Centricon centrifugal microconcentrators (Amicon, Danvers, MA) or microdialysis cassettes (Pierce, Rockford, IL).

Enzymatic Activities Possibly Involved in RNA Editing

Several enzymatic activities have been identified from mitochondrial extracts of *L. tarentolae* that are possibly involved in the editing of maxicircle transcripts. These include a terminal uridylyltransferase (TUTase) activity,¹⁶ an RNA ligase activity,¹⁶ and a site-specific endonuclease activity.^{39,40} All of these activities can be solubilized with Triton X-100 and are detectable in the TL, TS, and S-100 extracts of purified kinetoplast-mitochondrial preparations.

An internal U-incorporation activity, which has several characteristics of an *in vitro* RNA editing activity, has also been identified in *L. tarentolae* mitochondrial extracts.⁴¹ Uridine residues are incorporated into the preedited region of several synthetic mitochondrial transcripts. This activity is selectively inhibited by predigestion of the extract with micrococcal

³⁹ A. M. Simpson, N. Bakalara, and L. Simpson, *J. Biol. Chem.* **267**, 6782 (1992).

⁴⁰ M. Harris, C. Decker, B. Sollner-Webb, and S. Hajduk, *Mol. Cell. Biol.* **12**, 2591 (1992).

⁴¹ G. C. Frech, N. Bakalara, L. Simpson, and A. M. Simpson, *EMBO J.* **14**, 178 (1995).

nuclease, suggesting the involvement of some type of endogenous RNA species. However, no direct evidence was obtained for the involvement of endogenous gRNAs, and exogenous synthetic gRNA was found to inhibit the internal U incorporation. This activity sediments in glycerol gradients at approximately 25 S and the ribonucleoprotein complexes which mediate this activity have operationally been termed the G complexes.³⁸

Terminal Uridyltransferase

The mitochondrial TUTase activity transfers UMP from UTP to the 3'-OH of RNA molecules. It is stimulated by at least one additional nucleoside triphosphate (NTP) but, unlike transcription, does not require all four NTPs to be present. Therefore, by omitting at least one nucleoside triphosphate from the reaction, the TUTase activity can be conveniently assayed in the absence of run-on transcription activity. The TUTase is relatively indiscriminate in terms of the RNA substrate, although certain RNA species, including tRNAs, are less efficiently labeled. The TUTase assay is as follows.¹⁶

1. Mix approximately 1 μg substrate RNA with mitochondrial extract in a 50- μl reaction containing 5 mM HEPES-KOH (pH 7.5), 60 mM KCl, 3 mM potassium phosphate (pH 7.5), 6 mM magnesium acetate, 20 mM DTT, 1 mM ATP, 1 mM GTP, and 10 μCi [α -³²P]UTP (800 Ci/mmol). Incubate the mixture at 27° for at least 30 min.

2. For a quantitative determination of TUTase activity, stop the reaction by adding an equal volume of 0.5 M sodium phosphate (pH 6.8), 0.5% (w/v) sodium pyrophosphate, 0.1% (w/v) SDS, and transfer aliquots onto DE-81 filter disks. Dry the disks before washing them three times in excess 0.5 M sodium phosphate (pH 6.8), 0.5% sodium pyrophosphate, and once in ethanol. Count the radioactivity retained on the dried disks in a liquid scintillation counter.

3. For an additional qualitative assessment of TUTase activity, an aliquot of the reaction mixture may be extracted with phenol-chloroform and the labeled RNAs analyzed by gel electrophoresis and subsequent autoradiography or PhosphorImager analysis.

RNA Ligase

The *L. tarentolae* mitochondrial RNA ligase activity¹⁶ catalyzes the intra- or intermolecular ligation of a donor containing a 5'-phosphate with an acceptor containing a 3'-OH. The activity can be quantitatively assayed by addition of [³²P]pCp to the 3' termini of RNA molecules. However, not all RNA molecules are equally well modified by using this assay. If total

kinetoplast RNA is used as a substrate, the tRNAs appear to incorporate most of the radioactivity.

1. Mix 0.5–2.5 μg kRNA with 10 μl mitochondrial extract in a 50- μl reaction containing 0.1 M HEPES–KOH (pH 7.9), 20 mM MgCl_2 , 7 mM DTT, 0.2 mM ATP, 20% (v/v) dimethyl sulfoxide (DMSO), 1 unit RNase inhibitor, and 10 μCi [^{32}P]pCp (3000 Ci/mmol). Incubate the mixture at 27° for 2 hr, or at 4° overnight.

2. For a quantitative determination of ligase activity, transfer aliquots of the reaction onto DE-81 filter disks and process them as described above in step 2 of the TUTase reaction.

3. In addition, an aliquot of the reaction may be extracted with phenol–chloroform and then analyzed by gel electrophoresis and subsequent autoradiography or PhosphorImager analysis.

Cryptic RNase

A sequence- or structure-specific cryptic RNase activity can be detected in mitochondrial extracts (TL, TS, or S-100 extracts).³⁹ This cryptic RNase can be activated either by the addition of heparin (5 $\mu\text{g}/\text{ml}$) or by predigestion of the lysate with proteinase K. The RNA substrate used for this assay is a 280-nucleotide RNA synthesized by *in vitro* transcription from a recombinant plasmid using T7 RNA polymerase. The template DNA consists of the 22-nucleotide preedited region of the cytochrome *b* gene together with 56 nucleotides of 5' flanking sequence, 129 nucleotides of 3' flanking sequence, and 73 nucleotides of Bluescript vector sequence at the 5' end. Cleavage by the cryptic RNase occurs at one major site and four minor sites within the preedited region.

1. Prepare uniformly ^{32}P -labeled synthetic RNA substrate [10^8 counts/min (cpm)/ μg] by *in vitro* transcription from the recombinant plasmid or from PCR templates amplified with upstream primers containing the T7 phage RNA polymerase promoter.

2. Either add proteinase K (0.1 mg/ml final concentration) to the mitochondrial extract and incubate the extract 5 min at 37°, or add heparin (Sigma) to 5 $\mu\text{g}/\text{ml}$. Activations by protease predigestion and heparin are synergistic, and together result in better cleavage.

3. Mix approximately 10^4 cpm RNA substrate together with 10 μl activated mitochondrial extract (from step 2) in a 50- μl reaction containing 10 mM Tris–HCl (pH 7.5), 3 mM MgCl_2 , 1 mM ATP, and 5 $\mu\text{g}/\text{ml}$ heparin.

4. Incubate the mixture at 27° for 1 hr and extract with phenol–chloroform (1:1, v/v) followed by ethanol precipitation.

5. Analyze the cleavage products on an analytical polyacrylamide–7.5 M urea gel followed by autoradiography or PhosphorImager analysis.

Guide RNA–Messenger RNA Chimera-Forming Activity

To study the first step of the proposed transesterification model for RNA editing, cell-free systems were developed using mitochondrial extracts from both *T. brucei*^{42,43} and *L. tarentolae*.¹⁹ Chimera formation is monitored by following the covalent transfer of uniformly ³²P-labeled gRNA to a higher molecular weight nonradioactive test mRNA by gel electrophoresis. Both gRNA and mRNA are synthesized by *in vitro* transcription from recombinant plasmids or PCR-derived DNA templates. Sequence analysis of the reaction products by selective PCR amplification and cloning revealed gRNA/mRNA chimeric molecules as the most prominent products.¹⁹ This *in vitro* system has been used to confirm the importance of the anchor sequence between the gRNA and mRNA.¹⁹

1. Suspend the mitochondrial fraction from 1 liter of cell culture in 2 ml ice-cold 10 mM HEPES–NaOH (pH 7.9), 0.5 mM EDTA. Keep the mixture on ice 10 min.

2. Disrupt the swollen mitochondria by sonication at 100 W for three 20-sec periods at 4° (Braunsonic No. 1510, microtip; Branson Ultrasonics, Danbury, CT).

3. Immediately add 2 ml freshly prepared 40 mM HEPES–NaOH (pH 7.9), 50% glycerol, 0.84 M NaCl, 1 mM DTT, 0.4 mM EDTA, 1 mM PMSF, and gently agitate the solution with a small magnetic stirrer for 30 min on ice.

4. Clarify the extract by centrifugation at 50,000 g for 30 min at 4°.

5. Concentrate the extract at 4° to approximately 0.1 ml by using two Centricon 10 centrifugal microconcentrators (Amicon). Exchange the buffer by adding twice to each microconcentrator 2 ml freshly prepared 20 mM HEPES–NaOH (pH 7.9), 20% glycerol, 0.1 M KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, and recentrifuging.

6. Quick-freeze the pooled extracts (~0.2 ml) in dry ice/ethanol. When samples are stored at –80°, chimera-forming activity is maintained for up to 3 months.

7. Anneal equimolar amounts of the test RNA molecules ([³²P]UTP-labeled gRNA and cold mRNA) in 2.5 μl of 20 mM HEPES–NaOH (pH 7.9), 0.1 M KCl, 1 mM EDTA. Denature 3 min at 70° prior to annealing at 37° and 25° for 10 min each.

8. Add 8 μl of 8% (w/v) polyethylene glycol (PEG) 8000, 12.5 mM MgCl₂, 2.5 mM ATP, 1 unit/μl RNase inhibitor, and 15 μl thawed mitochon-

⁴² M. E. Harris and S. L. Hajduk, *Cell (Cambridge, Mass.)* **68**, 1091 (1992).

⁴³ D. J. Koslowsky, H. U. Göringer, T. H. Morales, and K. Stuart, *Nature (London)* **356**, 807 (1992).

drial extract (from step 6). Chimera formation is observed on incubation for 15 to 120 min at 27°.

9. Stop the reaction by adding 0.1 ml of 0.25% *N*-laurylsarcosine, 25 mM EDTA, 0.25 mg/ml proteinase K, and incubating at 37° for 20 min. Extract the mixture with phenol–chloroform (1:1, v/v) and recover the RNA by ethanol precipitation.

10. Analyze the resulting RNA products on an analytical polyacrylamide–7.5 *M* urea gel followed by autoradiography or PhosphorImager analysis. RNA products may also be analyzed by sequence determination following reverse transcription, PCR amplification, and cloning. To amplify exogenous RNA specifically, a tag sequence may be added to the test RNA.

Internal Uridine-Incorporation Activity

Incubation of certain synthetic preedited mRNAs with mitochondrial extracts in the presence of [α -³²P]UTP leads to the internal incorporation of U residues, in addition to the addition of U residues to the 3' termini caused by the TUTase activity.⁴¹ This internal U-incorporation activity requires ATP and is stimulated by spermidine. In synthetic preedited transcripts derived from the cytochrome *b* mRNA and the NADH dehydrogenase subunit 7 mRNA, which are both edited *in vivo*, the preedited regions were identified as the predominate areas of *in vitro* internal U incorporation. To differentiate between internal U incorporation and 3' addition of U residues, an assay is used (Fig. 2) that involves cleavage of the processed RNA by RNase H in conjunction with specific oligodeoxynucleotides and analysis of the heterogeneously sized cleavage products by gel electrophoresis followed by autoradiography or PhosphorImager analysis.

1. Prepare synthetic RNA substrates by *in vitro* transcription from recombinant, transcription-competent plasmids, or from PCR templates amplified with an upstream primer containing the T7 phage RNA polymerase promoter.

2. Mix approximately 1 μ g synthetic transcript with mitochondrial extract in a 50- μ l reaction containing 5 mM HEPES (pH 7.5), 60 mM KCl, 3 mM potassium phosphate (pH 7.5), 6 mM magnesium acetate, 20 mM DTT, 2 mM spermidine, 1 mM ATP, 1 mM GTP, 1 μ M unlabeled UTP, and 25 μ Ci [α -³²P]UTP (800 Ci/mmol). The optimal amount of mitochondrial extract should be determined by titration, since it varies with different mitochondrial preparations.

3. Incubate the mixture at 27° for 40–100 min and extract with phenol–chloroform.

4. Separate the RNA on a preparative polyacrylamide–7.5 *M* urea gel. Excise the full-length ethidium-stained RNA band (including material of

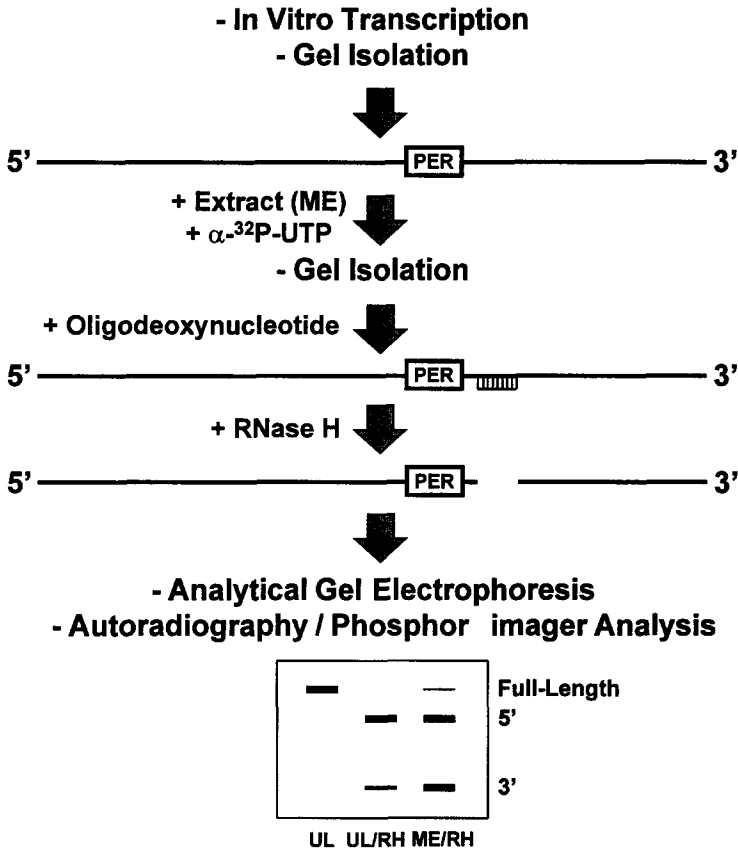


FIG. 2. Diagram of an *in vitro* assay to detect internal U incorporations into synthetic mRNA transcripts. ME, Mitochondrial extract; PER, preedited region; UL/RH, uniformly labeled RNA digested with RNase H; ME/RH, RNA labeled by incubation with mitochondrial extract and [α -³²P]UTP, and subsequently digested with RNase H.

retarded mobility containing potential U incorporations) and elute the RNA in 0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1% SDS, 0.1 mM EDTA, followed by phenol-chloroform extraction and ethanol precipitation.

5. Anneal the gel-purified RNA to 0.1–0.2 μ g DNA oligomer, and incubate 1 hr at 37° in a 20- μ l reaction containing 50 mM Tris (pH 7.5), 0.1 M KCl, 10 mM MgCl₂, 0.1 mM DTT, 10 μ g/ml BSA, and 0.2–0.3 units RNase H (Pharmacia).

6. Visualize the digestion products containing incorporated label by autoradiography or PhosphorImager analysis of an analytical polyacrylamide-7.5 M urea gel.

Identification of Ribonucleoprotein Complexes Possibly Involved in RNA Editing

RNA processing reactions in eukaryotes occur within large ribonucleoprotein (RNP) complexes. This appears to also be the case with RNA editing in trypanosomatids.^{38,44-47} Mitochondrial RNP complexes can be identified by sedimentation in glycerol gradients and by gel electrophoresis under native conditions. When *L. tarentolae* mitochondrial extract is incubated under TUTase conditions in the presence of [α -³²P]UTP and the mixture subsequently electrophoretically separated, several labeled complexes of different gel mobilities can be identified, which we have termed the T-complexes. These complexes sediment in a glycerol gradient at 10-13 S. All of these complexes contain RNA that is accessible to labeling by the TUTase, and some or all of them may be involved in RNA editing. The most intensely labeled T-complex (T-IV) was shown to contain grRNA, and mRNA fragments are found associated with all of the T-complexes. Another class of RNP complexes (G-complexes) comigrate with the internal U-incorporation activity at approximately 25 S.

Identification of [α -³²P]UTP-Labeled T-Complexes

1. Label TS mitochondrial extract by incubation for 40 min at 27° in 5 mM HEPES-KOH (pH 7.5), 60 mM KCl, 3 mM potassium phosphate (pH 7.5), 6 mM magnesium acetate, 20 mM DTT, 1 mM ATP, 1 mM GTP, and 0.2 mCi/ml [α -³²P]UTP. Note that CTP is omitted to avoid the occurrence of run-on transcription, which requires the presence of all four nucleoside triphosphates.

2. Pour a 4-16% polyacrylamide gradient gel containing 40 mM Tris-acetate (pH 8.0), 1 mM EDTA (1× TAE buffer), 0.1% (v/v) Tween 20, and a stabilizing gradient of 10-30% glycerol. Overlay with *n*-butanol and allow the gel to polymerize. After drainage of the *n*-butanol, add more 4%

⁴⁴ V. W. Pollard, M. E. Harris, and S. L. Hajduk, *EMBO J.* **11**, 4429 (1992).

⁴⁵ H. U. Göringer, D. J. Koslowsky, T. H. Morales, and K. Stuart, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1776 (1994).

⁴⁶ J. Köller, G. Nörskau, A. S. Paul, K. Stuart, and H. U. Göringer, *Nucleic Acids Res.* **22**, 1988 (1994).

⁴⁷ L. K. Read, H. U. Göringer, and K. Stuart, *Mol. Cell. Biol.* **14**, 2629 (1994).

gel solution and insert the comb 0.5 cm from the already polymerized gradient gel. Allow to polymerize.

3. Before loading the gel, add glycerol to the samples to a final concentration of at least 10%. The following molecular weight markers may be used: thyroglobulin (669 kDa, Pharmacia), ferritin (443 kDa, Pharmacia), and β -amylase (200 kDa, Sigma). Run the gel at 4° in 1× TAE buffer.

4. Stain the gel with rapid Coomassie stain (Diversified Biotech, Boston, MA) to identify the positions of the marker proteins. Visualize the labeled complexes by autoradiography or PhosphorImager analysis.

Detection of Specific T-Complexes Containing Terminal Uridyltransferase Activity

Two specific T-complexes containing TUTase activity can be identified by performing a native gel *in situ* TUTase assay.³⁸ The endogenous RNAs in the remainder of the T-complexes presumably are labeled by interaction with these complexes.

1. Run an aliquot of unlabeled TS mitochondrial extract in a native gel as described above. Excise the gel lane (8 cm long) and transfer it to a 15-ml centrifuge tube.

2. Add 3 ml TUTase labeling mix containing 5 mM HEPES-KOH (pH 7.5), 60 mM KCl, 3 mM potassium phosphate (pH 7.5), 6 mM magnesium acetate, 20 mM DTT, 1 mM ATP, 1 mM GTP, and 0.2 mCi/ml [α -³²P]UTP. Incubate on a shaker 1 hr at room temperature.

3. Wash the gel slice with copious amounts of water to remove most of the unincorporated [α -³²P]UTP.

4. Realign the gel slice next to the original gel, which should contain a lane of endogenously labeled extract for comparison. Dry the gel under reduced pressure before autoradiography or PhosphorImager analysis.

Discussion

We have emphasized experimental protocols involving *L. tarentolae*, but similar protocols have been described for *T. brucei*. The major difference in RNA editing in *T. brucei* is the high gRNA complexity and the resulting presence of redundant gRNAs of different sequences overlapping in regions other than the anchor sequence.⁴⁸ In addition, editing in *T. brucei* appears to be regulated during the complex biphasic life cycle, which involves

⁴⁸ R. A. Corell, J. E. Feagin, G. R. Riley, T. Strickland, J. A. Guderian, P. J. Myler, and K. Stuart, *Nucleic Acids Res.* **21**, 4313 (1993).

periods of mitochondrial repression and derepression (see Ref. 10 for review). The mechanism of this transcript-specific regulation is not known.

A *T. brucei* mitochondrial TUTase,⁴⁴ an RNA ligase,^{44,49} a preedited RNA site-specific ribonuclease,⁴⁰ and a gRNA/mRNA chimera-forming activity have been described.^{42,43} In addition, mitochondrial RNP complexes containing these activities have been partially characterized, and a small set of proteins have been shown to interact with gRNAs.⁴⁴⁻⁴⁷

A breakthrough in the analysis of gRNA-mediated RNA editing was reported by Seiwert and Stuart.⁵⁰ They showed that a mitochondrial extract in the presence of exogenous synthetic gRNA could direct the deletion of U residues from the first editing site of the preedited ATPase subunit 6 (=MURF4) transcript and that this deletion activity was mediated by base pairing with guiding nucleotides in the gRNA. However, U additions were not observed in this system, suggesting that the mechanism for U deletions may differ from that for U additions.

It was shown that a trypanosomatid-like RNA editing also occurs in the cryptobid kinetoplastid *Trypanoplasma boreli*.^{51,52} In this organism, however, the mitochondrial genome is not composed of a network of catenated mini- and maxicircles, but rather contains 80-kb circular molecules, which are the maxicircle equivalents, and 200-kb circular molecules with tandem repeats of gRNA-like genes. An analysis of this type of editing system is in progress.

It is clear that, except for the investigation of editing in the cryptobids and possibly in other lower kinetoplastids, the field has progressed past a purely descriptive stage and is entering a more biochemical stage. We hope that the procedures described in this chapter will aid in investigating the molecular and biochemical mechanisms involved in this type of RNA editing.

⁴⁹ T. White and P. Borst, *Nucleic Acids Res.* **15**, 3275 (1987).

⁵⁰ S. D. Seiwert and K. Stuart, *Science* **266**, 114 (1994).

⁵¹ D. A. Maslov and L. Simpson, *Mol. Cell. Biol.* **14**, 8174 (1994).

⁵² J. Lukes, G. J. Arts, J. Van den Burg, A. De Haan, F. Opperdoes, P. Sloof, and R. Benne, *EMBO J.* **13**, 5086 (1994).