CHAPTER SIX

Strategies of Kinetoplastid Cryptogene Discovery and Analysis

Dmitri A. Maslov* *and* Larry Simpson†

Contents

Abstract

The experimental approach to revealing the genetic information hidden in kinetoplastid cryptogenes and expressed through the posttranscriptional mRNA processing of U-insertion/deletion editing proceeds in reverse to the informational flow of the RNA editing process itself. While the editing integrates the informational content of maxicircle-encoded cryptogenes with that of minicircle-encoded gRNAs to produce functional edited mRNAs, the cryptogene analysis utilizes a comparison of the mature mRNA sequence with the cryptogene sequence to deduce the locations of edited sites and editing patterns, and a comparison of that mRNA sequence with the minicircle (or minicircle equivalent) sequences to identify the corresponding guide RNAs. Although a "direct" approach (prediction of a fully edited sequence pattern based on the analysis of cryptogene and minicircle sequences) seems to be theoretically possible, it proved to be not practically feasible. The major steps of the procedures utilized to decipher editing in a broad range of kinetoplastid species are presented in this chapter.

* Department of Biology, University of California, Riverside, California
† Department of Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine at UCLA, University of California, Los Angeles, California

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1. Introduction

Unicellular protists united in the class Kinetoplastea are characterized by the presence of a massive body of DNA, termed the "kinetoplast," situated near the basal body of the cell's flagellar apparatus (Vickerman, 1976). The group is well known for including a score of important human pathogens, as well as for several molecular and cellular mechanisms that are not observed in most eukaryotic systems (Campbell *et al.*, 2003; Lukeš *et al.*, 2005). This list includes the kinetoplastid U insertion/deletion RNA editing, which stands apart from other types of editing not only because of its unique guide RNA-based mechanism, but also by the pronounced genomic manifestations and genetic consequences of the process. While base-substitution/modification editing, as a rule, involves a relatively small alteration of an already-functional mRNA sequence, the insertion/deletion type of editing essentially creates a translationally competent template out of a preedited transcript that does not encode a functional product (Feagin *et al.*, 1988b; Shaw *et al.*, 1988). Accordingly, the sites of potential editing in kinetoplastid mitochondrial genomes become apparent by inspection of the reading frames displaying various defects (frameshifts or missing initiation codons), which could be corrected by U insertions/deletions at the mRNA level. Historically, this approach led to the original discovery of RNA editing in cytochrome *c* oxidase subunit II (COII) mRNA, wherein a –1 frameshift was corrected by the insertion of four Us (Benne *et al.*, 1986). The mechanism of editing involves an enzymatic cascade (Blum *et al.*, 1990); current information on this can be found in several recent reviews (Panigrahi *et al.*, 2006; Simpson *et al.*, 2004; Stuart *et al.*, 2005) as well as in other chapters of this book.

The trypanosomatids represent a subgroup of the kinetoplastids in which the original discovery of RNA editing was made and with which most subsequent work has been done. In these cells the organization of the mitochondrial genome is highly conserved. It consists of a network of interlocked minicircles and maxicircles (Simpson, 1972). The latter contain a set of genes usually found in mitochondria, including the genes with edited transcripts. The information for editing is provided by small (<50 nt) guide RNAs, most of which are encoded in minicircles (Blum *et al.*, 1990; Pollard *et al.*, 1990; Sturm and Simpson, 1990b). These guide RNAs are targeted to their respective editing sites by means of a short (10–15 nt) region of complementarity between the 5′ end of the gRNA (the anchor region) and an mRNA sequence immediately downstream of a preedited region. Upon completion of editing, the entire gRNA becomes complementary to a respective segment of the mRNA. In addition

to canonical G-C and A-U base pairs, G-U base pairs frequently occur in the mRNA–gRNA duplex. A single gRNA mediates editing of only a few editing sites. Longer preedited regions in the mRNA are edited by a sequential involvement of two or more gRNAs. The process starts at the 3′ end of a preedited transcript and gradually moves upstream, with the anchors for each upstream gRNA created by the editing mediated by an adjacent downstream gRNA (Maslov and Simpson, 1992).

The experimental and computational tools for the analysis of editing at the nucleotide sequence level were developed in the 1990s during the period of intensive investigation of RNA editing in the human pathogenic species, *Trypanosoma brucei* and *Trypanosoma cruzi*, and the nonpathogenic model organisms, such as *Leishmania tarentolae* and *Crithidia fasciculata*. Technically, the work consists of cloning and analysis of gene and mRNA sequences. Only limited attempts were made to develop a computational approach to predicting cryptogene editing patterns (Von Haeseler *et al.*, 1992). Most of the experimental methods employed are standard and have been described in detail previously, as were the methods of growing the trypanosomatid cultures, subcellular fractionation and isolation of the kinetoplast–mitochondrial fraction, and isolation of kinetoplast DNA (Simpson *et al.*, 1994, 1996). The most important computational methods employed include analysis of the DNA sequence for potential sites of editing and a search for a guide RNA gene by a complementary sequence match with an edited mRNA sequence. Most of the remaining kinetoplastids, collectively referred to as bodonids, remain uninvestigated, and their study may provide some important insight into the origin and evolution of this phenomenon. Thus, investigations of *Trypanoplasma borreli* and *Bodo saltans* have revealed that the kinetoplast DNA overall genomic organization, gene order, occurrence of preediting regions, and even structural features of guide RNAs are different from trypanosomatids (Blom *et al.*, 1998, 2000; Lukeš *et al.*, 1994; Maslov and Simpson, 1994; Yasuhira and Simpson, 1996). The main goal of this chapter is to complement earlier publications by describing general strategies applicable to analysis of RNA editing in diverse kinetoplastid species including bodonids.

2. Cell Growth

Besides trypanosomatids, only a few other members of the Kinetoplastea, such as a blood fish parasite, *T. borreli*, are cultivable axenically (Maslov *et al.*, 1993). Most bodonids are phagotrophic and need feeder bacteria for propagation in culture, as described below for *B. saltans* (Blom *et al.*, 2000) (J. Lukeš, personal communication).

2.1. Cultivation of *T. borreli*

1. Per 1 liter of the LIT medium, combine

Liver infusion	5 g
Tryptone	5 g
Na_2HPO_4	8 g
NaCl	4 g
KCl	0.4 g

Bring up the volume to 900 ml with distilled water, adjust the pH to 7.0, and autoclave. Add D-glucose to the final concentration of 2 g/liter (using 200 g/liter stock solution sterilized by filtration), hemin to 10 μg/ml (2 mg/ml stock solution in 0.5 *N* NaOH, sterilized by filtration), fetal bovine serum to 10% (sterile serum must be heat inactivated at 56° for 45–60 min).

2. Cells are grown at 15° in stationary T-flasks or in Erlenmeyer flasks with a very slow agitation. Transfers are made weekly by a 10-fold dilution with fresh medium. Cell density at the stationary phase is 7–9 $\times$ 10^6 cells/ml.

2.2. Cultivation of *B. saltans*

1. Per 1 liter of the 100-fold concentrated medium combine

Bactopeptone	9 g
Yeast extract	1 g

Bring the volume to 1 liter with distilled water and autoclave.

2. Maintain a culture of *Bodo* spp. in a 100-ml Erlenmeyer flask with a "seed" medium prepared by autoclaving two or three seeds of wheat in 50 ml of tap water (not distilled water). Keep this at room temperature and transfer biweekly by adding several milliliters of an old culture to a fresh medium. If a culture is kept at 16–17°, transfers can be done once a month.
3. Feeder bacteria (*Alcaligenes* spp.) are maintained using standard techniques in an LB medium (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) at 37°. To grow a large-scale *Bodo* culture, prepare a 5-ml feeder bacteria culture grown overnight.
4. Fill a 4-liter Erlenmeyer flask with 2–2.5 liters of tap water (not distilled water) and boil for 30 min. After cooling to room temperature, add 25 ml of the 100-fold concentrated medium stock solution and 0.3 ml of a stationary culture of the feeder bacteria. Leave this at room temperature for 24 h until a slightly whitish color appears. Transfer the flask to 16–17°, add 5 ml of a *Bodo* culture from the "seed" medium, and stir vigorously on a magnetic stirrer. After 4–6 days, the culture should become more transparent (most of the bacteria will be consumed) and the *Bodo* cell density should reach 10^7 cells/ml.

5. The cells are pelleted by centrifugation at 4000×*g* for 10 min. The pellet is resuspended in 200 ml of fresh medium and the suspension is left at 4° in a cylinder to allow most of the remaining feeder bacteria to sediment.

3. DNA Isolation

The massive kinetoplast DNA networks of trypanosomatids are isolated by velocity sedimentation (Simpson and da Silva, 1971; Simpson *et al.*, 1996). In general, this method is not applicable to other groups of kinetoplastids since many of them have a noncatenated structure of kinetoplast DNA (Blom *et al.*, 2000; Lukeš *et al.*, 1998, 2002; Maslov and Simpson, 1994; Štolba *et al.*, 2001). In these cases, an equilibrium density centrifugation in CsCl gradients supplemented with ethidium bromide can be used to separate covalently closed molecules of kinetoplast–mitochondrial DNA from linear fragments of the nuclear chromosomal DNA (Maslov and Simpson, 1994). Alternatively, these components can be separated by the difference in their buoyant densities using CsCl-Hoechst 33258 gradients (Blom *et al.*, 2000; Maslov and Simpson, 1994; Simpson, 1979). The same procedure allows for the removal of DNA of the feeder bacteria used to propagate the phagotrophic kinetoplastids (Blom *et al.*, 2000). The starting material for both procedures is a total cell DNA preparation isolated by a standard detergent-phenol protocol or by a commercial kit.

3.1. Ethidium bromide method

1. Total cell DNA from 10–15 × 10^9 cells in 60 ml TE (10 m*M* Tris–HCl, pH 8.0, 1 m*M* EDTA) is combined with 63.8 g CsCl and 3.42 ml of 10 mg/ml ethidium bromide. The refractive index ($n_D^{25^\circ}$) is adjusted to 1.3890.
2. The solution is centrifuged in two tubes in a Beckman type 50.2 Ti or equivalent fixed angle rotor at 45,000 rpm (RCF_{avg} = 184,000×*g*) for 40 h at 20°.
3. The bands are detected by UV and the lower band is recovered by side puncture of the tube. To achieve a higher purity of the isolated covalently closed DNA, the volume of the recovered material is brought up to 9.5 ml with CsCl-ethidium bromide, the $n_D^{25^\circ}$ is adjusted to 1.3850, and the solution is centrifuged in a single tube in a Beckman type 50 Ti or equivalent fixed angle rotor at 40,000 rpm (RCF_{avg} = 106,000×*g*) for 40 h. The lower band is recovered as above.
4. The DNA of the lower band is extracted twice with an equal volume of isoamyl alcohol. The aqueous phase is diluted 3-fold with the TE buffer. DNA is precipitated by addition of three volumes of ethanol.

3.2. Hoechst 33258 method

1. The DNA in 52 ml of TE is mixed with 74 g CsCl and 4 ml of a 0.5 mg/ml solution of the dye. The refractive index is adjusted to 1.3950.
2. The material is centrifuged in two tubes in a Beckman VTi 50 or a similar vertical rotor at 40,000 rpm (132,000×*g*) for 40 h. A fixed angle rotor can also be used.
3. The upper band is collected and, after adjusting the refractive index to 1.3935 and the volume to 9.5 ml using a CsCl-Hoechst solution, the centrifugation is repeated in a Beckman Type 50 Ti rotor at 39,000 rpm (100,000×*g*) for 40 h.
4. The DNA of the upper band is extracted three times with isopropanol (readjusting the volume of the aqueous phase after each extraction to the initial value with TE), diluted 3-fold with TE, and precipitated with three volumes of ethanol.

4. Search for Cryptogenes

The discovery of editing by Benne and coworkers in the insect trypanosomatid, *C. fasciculata* (Benne *et al.*, 1986) was followed by additional cases of frameshift repair or other types of a limited-scale editing in *L. tarentolae* and *T. brucei* (Feagin *et al.*, 1988b; Shaw *et al.*, 1988, 1989; Simpson and Shaw, 1989). The genomic organization of the maxicircle DNA was similar in both cases, with the exception that a few seemingly noncoding G-rich regions were present in *T. brucei* in place of some genes with 5′ or internal editing in *L. tarentolae* (Simpson *et al.*, 1987). It was shown shortly thereafter that one of these regions in *T. brucei* represented a cryptogene that encoded an extensively edited (pan-edited) mRNA for NADH dehydrogenase subunit 7 (Feagin *et al.*, 1988a). Additional examples of pan-editing followed, including transcripts of the six conserved intergenic G-rich regions found in all trypanosomatid species (Corell *et al.*, 1994; Maslov *et al.*, 1992; Read *et al.*, 1992; Souza *et al.*, 1993; Thiemann *et al.*, 1994). The pan-editing process was asymmetrical, with U insertions more frequent than deletions, and thus the preedited mRNAs were largely devoid of genomically encoded U nucleotides, with most of the Us present in the mature transcripts acquired through editing. This explains why gene sequences for such extensively edited mRNAs appeared as G-rich (and T-poor) islands. This feature of preedited regions was particularly noticeable because maxicircle coding sequences that did not require editing (unedited or never edited) were highly T-rich (Simpson *et al.*, 1987). This bias is related to the highly hydrophobic nature of the

corresponding polypeptides, with a frequent occurrence of Phe, Leu, Ile, and Val amino acids, which are usually encoded by the U-rich codons.

Sequence analysis of maxicircles, which represent a minor component of the kinetoplast DNA, is facilitated by their isolation using the CsCl-Hoechst procedure as described above (Simpson, 1979; Simpson *et al.*, 1996). Once the more abundant minicircle component is removed, cloning of the maxicircle component is accomplished by a shotgun approach or by cloning of restriction fragments. For closely related species, an overlapping set of maxicircle fragments can be amplified using conserved primers derived from the known sequences (Simpson *et al.*, 1998). In bodonids, identification of the maxicircles or their equivalents is not straightforward, because the mitochondrial genome organization can be completely different in these organisms. Successful approaches involved screening of a mitochondrial genomic library with a conserved trypanosomatid COI gene probe (Maslov and Simpson, 1994) or amplification of a COI or COII gene region with universally conserved sequence primers followed by library screening (Blom *et al.*, 1998; Lukeš *et al.*, 1994).

The G-rich regions can be found using a computer program that plots a distribution of nucleotide frequency in a sliding window along the DNA molecule. One of the best tools for this purpose is the GCG program WINDOW, which shows the difference between the observed and expected nucleotide frequency and, therefore, accounts for the compositional bias of the DNA. The observed G-rich regions (or C-rich regions for genes with an opposite strandedness) in all species correspond to the conserved pan-edited cryptogenes ND8 (G1), ND9 (G2), G3, G4, ND3 (G5), and RPS12 (G6), and in *T. brucei* to COIII, A6, and ND7. Additional G-rich regions observed in some but not all trypanosomatid species represent extensively edited gene segments, such as A6, which is 5′ pan-edited in *Leishmania*, but only moderately edited in the 5′ region in several other species (Maslov *et al.*, 1994).

The same relative gene order is maintained in all investigated trypanosomatids with functional mitochondria, including the reduced mitochondrial genome of *Phytomonas serpens,* which has deletions of all cytochrome *c* oxidase and cytochrome *b* subunits (Arts *et al.*, 1993; Nawathean and Maslov, 2000; Simpson *et al.*, 1987). Thus, the identity of the cryptogene in question can be deduced from its position in relation to neighbors, even when the entire coding sequence is cryptic. However, this is not the case in the investigated bodonids, which showed a different gene order (Blom *et al.*, 1998; Lukeš *et al.*, 1994; Maslov and Simpson, 1994). Another difference with trypanosomatids was the presence of 3′-editing domains in some genes, a phenomenon that appears to contradict the theory of a gradual replacement of pan-edited cryptogenes by retroposition of partially edited mRNA copies (Maslov *et al.*, 1994; Simpson and Maslov, 1994).

5. Elucidation of Edited mRNA Sequences

Once a putative preedited region is identified by inspection of a DNA sequence, the further analysis of editing requires cloning and sequence analysis of the transcripts. In most cases, this work involves a straightforward reverse-transcription PCR using oligonucleotide primers annealing upstream and downstream of the putative editing site(s). The products obtained usually represent a mixed population of molecules that have been edited to various degrees usually in a 3′ to 5′ gradient. A combination of two criteria is then used to derive the mature edited pattern: it usually represents a consensus of individual editing patterns and it contains an apparently functional reading frame. These criteria are not absolute. Thus, an editing consensus might be impossible to derive from the data obtained, while a few clones may exist that appear to represent a fully edited mRNA satisfying the functionality criterion. Yet, even this criterion may be difficult to apply in the absence of a clear homology between the derived polypeptide sequence and proteins from other organisms.

The effort required for elucidation of a mature edited sequence depends on the ratio of preedited and edited transcripts, which varies greatly for different genes, developmental stages, or species. In extreme cases, hundreds of cDNA clones have to be analyzed before a mature editing pattern will start to emerge, especially during analysis of long pan-edited transcripts with abundant partially edited intermediates. Since editing proceeds in a general 3′-to-5′ direction, such partially edited molecules are edited at the 3′ end and preedited at the 5′ end. In order to enrich a collection of cDNA molecules with edited products, the 3′ edited sequence found in the partially edited clones can be used to design an edited downstream PCR primer, which can be applied in combination with the same 5′ primer. An additional caveat of this analysis is the presence of so-called junction regions between the 5′ preedited and 3′ edited sequences (Decker and Sollner-Webb, 1990; Sturm and Simpson, 1990a). Junction regions are also edited, although their editing patterns are diverse and do not match the final edited pattern. These regions represent sites of active editing, and their origin reflects the mechanism of the process, which can utilize noncognate guide RNAs with a successive reediting by correct guide RNAs [“misediting by misguiding” model (Sturm *et al.*, 1992)] and involves multiple rounds of editing and reediting until the best gRNA–mRNA match is achieved [“dynamic mRNA–guide RNA interactions” model (Koslowsky *et al.*, 1991)]. Moreover, some sections of extensively edited mRNA molecules may deviate from the majority of other edited molecules, which is apparently a consequence of reediting of the correctly edited sequence by a noncognate guide RNA.

6. Search for Guide RNA Genes

The last stage in cryptogene analysis is a search and assignment of guide RNAs, which becomes possible only after a fully edited mRNA sequence has been determined. A local similarity search algorithm (e.g., the BESTFIT program of the GCG package with a modified weight matrix) is employed to find a match between the mRNA and a minicircle segment or a putative gRNA transcript (Simpson *et al.*, 1994).

There are two approaches to this task. The first approach works well with trypanosomatids. It is based on the observation that most guide RNAs are encoded in minicircles, and the position and polarity of the gRNA coding regions with respect to the minicircle CSB-3 region and the bent region are well conserved in each investigated species (Simpson, 1997). A minicircle library is constructed to include the entire anticipated complexity of the heterogeneous minicircle population. If possible, a rational negative selection and sequencing strategy is then developed to analyze most or all minicircle classes present without sequencing multiple reiterative clones. Thus, in various species of *Leishmania*, a single gRNA gene is localized at 150 bp from the CSB-3 region of the 600–900 bp minicircles (Sturm and Simpson, 1991). Full-length minicircle libraries were constructed by using single-cut restriction enzymes or incomplete digestions with multiple cutters (Gao *et al.*, 2001; Maslov and Simpson, 1992). Clones with minicircle inserts are identified by hybridization with a CSB-3 probe. At first, positive clones are selected and sequenced at random and several minicircle classes are identified after the first round. For the second round of library screening, oligonucleotide probes are designed to represent each sequenced minicircle class, and a negative screening is performed to identify novel minicircle classes. In *T. cruzi*, minicircles have a tetrameric structure with a single gRNA gene per a 370-bp repeated unit flanked by CSB-3 sequences. A library of individual units was constructed instead of full-length minicircles to facilitate the acquisition of gRNA gene sequences (Avila and Simpson, 1995). Random cloning and analysis of full length minicircles were used in *T. brucei*, where it was not possible to devise a negative selection method (Hong and Simpson, 2003).

When the genomic localization of gRNA genes in unknown, as is the case in most kinetoplastids besides the Trypanosomatidae, guide RNA libraries are employed instead of minicircle libraries (Simpson *et al.*, 1996). The method includes isolation of an RNA fraction enriched with gRNA by gel electrophoresis with subsequent amplification by 3′ and 5′ RACE procedures (Avila and Simpson, 1995; Simpson *et al.*, 1996; Yasuhira and Simpson, 1996).

These transcripts (<50 nt) are smaller than tRNAs and migrate ahead of these molecules in polyacrylamide-urea gels. However, they are relatively

low in abundance and their presence in gels is inconspicuous after staining with ethidium bromide. In order to visualize gRNA molecules, a method of selective capping gRNAs with [α-^{32}P]GTP and vaccinia virus guanylyltransferase can be used. The method utilizes the fact that gRNAs possess 5′-triphosphate groups that are a substrate for the transferase. Purified mitochondrial RNA or total cell RNA can be used as a starting material.

1. Denature total cell RNA (10 μg) or mitochondrial RNA (1 μg) in 15 μl of water at 50° for 3 min. Place on ice.
2. To the RNA sample add
   2 μl of [α-^{32}P]GTP (800 Ci/mmol)
   2 μl 10× reaction buffer (600 m*M* Tris–HCl, pH 8.0, 60 m*M* $MgCl_2$, 100 m*M* DTT, supplied by the enzyme manufacturer)
   1 μl (5 U) vaccinia virus capping enzyme (available from Ambion)
   1 μl RNasin (20 U, optional)
3. Incubate at 37° for 30 min.
4. Add an equal volume of the standard formamide denaturing dye.
5. Resolve the RNA in 10% polyacrylamide 8 *M* urea gel.
6. Detect the labeled RNA by autoradiography.

Once the presence and gel position of gRNAs are determined, preparative isolation of gRNA is performed using at least 100 μg of total cell RNA or an equivalent amount of purified mitochondrial RNA. To facilitate detection of the gRNA bands in the gel, the content of the above GTP-labeling reaction can be added to unlabeled RNA prior to gel fractionation. The radioactive band is detected by autoradiography and the RNA is eluted. The rest of the procedure involves an oligo(A)-primed cDNA synthesis, a specific ligation of a 3′-modified "anchor" oligonucleotide to 3′ ends of the cDNA, PCR amplification of the products, cloning, and sequencing. This was described in detail previously (Simpson *et al.*, 1996). For construction of a gRNA library in *T. borreli,* the cDNA synthesis step was performed after 3′ C-tailing the gel-isolated RNA with CTP and poly(A)-polymerase due to relatively short oligo(U)-tails of gRNAs in this organism (Yasuhira and Simpson, 1996). The procedure can be further modified to be used with commercially available 5′ and 3′ RACE systems.

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