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RNA Editing and the Mitochondrial Cryptogenes of Kinetoplastid Protozoa

Review

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The term "RNA editing" was initially introduced to describe a process resulting in the addition of nongenomically encoded uridine residues (U's) to mitochondrial mRNAs in the kinetoplastid protozoa (Benne et al., 1986), but has recently been used to describe other processes involving modification of RNA in other organisms (Powell et al., 1987; Chen et al., 1987; Thomas et al., 1988). We would like to define RNA editing in a broad sense as any process that results in the production of an RNA molecule which differs in nucleotide sequence in coding regions from the DNA template, provided that the difference is not the result of the removal of introns by a classic splicing mechanism. In this review we will examine RNA editing mainly in kinetoplastids but also in other systems and will critically evaluate the central issues and concepts related to this phenomenon.

The discovery of RNA editing was the direct result of a series of investigations on the genomic organization and transcription of the unusual mitochondrial genome of the kinetoplastid protozoa. Since the initial report, several other examples of kinetoplastid RNA editing have been discovered (Feagin et al., 1987; Feagin et al., 1988a; Shaw et al., 1988; van der Spek et al., 1988; Feagin et al., 1988b; Abraham et al., 1988). To provide a biological perspective on the discovery and role of RNA editing in kinetoplastids, we will first review selected aspects of the molecular biology of kinetoplastid protozoa.

The Mitochondrial DNA of Kinetoplastids Is Present as a Nucleoid Body and Consists of a Single Catenated Network of Minicircles and Maxicircles

Kinetoplastid protozoa, which are also known as trypanosomatids or trypanosomes, consist of eight genera belonging to a family of lower eukaryotic cells (Trypanosomatidae) distinguished by the presence of a single, structurally complex mitochondrion containing an unusual genome known as kinetoplast DNA (kDNA) (see Simpson, 1972, Simpson, 1986, and Simpson, 1987, for reviews). These protozoa are parasitic either in a single invertebrate host (monogenetic) or in both an invertebrate and a vertebrate host (digenetic). An analysis of nuclear small rRNA sequences led to the conclusion that these cells represent a deep branch of the eukaryotic line (Sogin et al., 1989).

The kDNA nucleoid body is found in the mitochondrial matrix at the base of the flagellum and consists of two types of circular DNA molecules, minicircles and maxicircles. The genetic role, if any, of the minicircle DNA is still enigmatic, although small minicircle transcripts have been identified in both T. brucei (Rohrer et al., 1987) and L. tarentolae (Simpson and Simpson, unpublished results).

The Kinetoplastid Mitochondrial Maxicircle Genes

The maxicircle DNA molecules encode an incomplete set of standard mitochondrial genes (Simpson, 1987). The sequence of 21 kb of the 30 kb maxicircle molecule of L. tarentolae is known, as is the sequence of almost the entire 22 kb maxicircle molecule of T. brucei and approximately 6 kb of the 32 kb maxicircle of Crithidia fasciculata. The identified genes of L. tarentolae encode the two small mitochondrial rRNAs and components of the respiratory chain, including cytochrome b (CYb), cytochrome oxidase subunits I, II, and III (COI, COII, and COIII), and NADH dehydrogenase subunits 1, 4, and 5 (ND1, ND4, and ND5) (Figure 1). In addition, there are also four unidentified open reading frames (MURF1–4) (MURF = maxicircle unidentified reading frame, Simpson et al., 1987), one of which (MURF3) has sequence homology with ORF392 from Marchantia polymorpha chloroplast DNA (Ohyama et al., 1986). In comparison, the mitochondrial genomes of mammals and yeast both encode two mitochondrial rRNAs, the structural genes for COI, COII, COIII, and CYb, and at least two subunits of ATP synthetase (6 and 8). Genes for seven subunits of NADH dehydrogenase (ND) are in addition present in the human mitochondrial genome, whereas genes for a small mitochondrial ribosomal subunit protein (Var-1) and a third subunit of ATP synthetase (9) are present in the yeast mitochondrial genome.

A large portion of the maxicircle (13 kb in L. tarentolae and 7 kb in T. brucei) is apparently noncoding, and consists of tandem repeats of varying complexities (Muhich et al., 1985). Since this region varies in size and sequence between species, it has been termed the variable or divergent region; a lack of open reading frames, rapid changes in sequence between species, and a low abundance of steady-state transcripts (de Vries et al., 1988; Tarassoff et al., 1987) make it difficult to assign a genetic function to this portion of the maxicircle.

The maxicircle genes appear normal in terms of conserved functional amino acids, conserved hydropathy patterns, and statistically significant alignments, but several unusual features are apparent: there is an extremely high abundance of cysteine residues in the predicted products, several genes lack ATG initiation codons, three genes (COII, MURF2, and MURF3) encode internal reading frameshifts, and several normally conserved genes such as subunits 6, 8, and 9 of ATP synthetase are apparently absent (de la Cruz et al., 1984; Simpson et al., 1987).

In addition, no mitochondrial tRNA genes have been identified by hybridization studies in either minicircle DNA or maxicircle DNA (Hoeijmakers et al., 1981; Benne et al., 1983; Benne and Sloof, 1987; Suyama, Campbell, Simpson, and Simpson, unpublished data), although an appar-

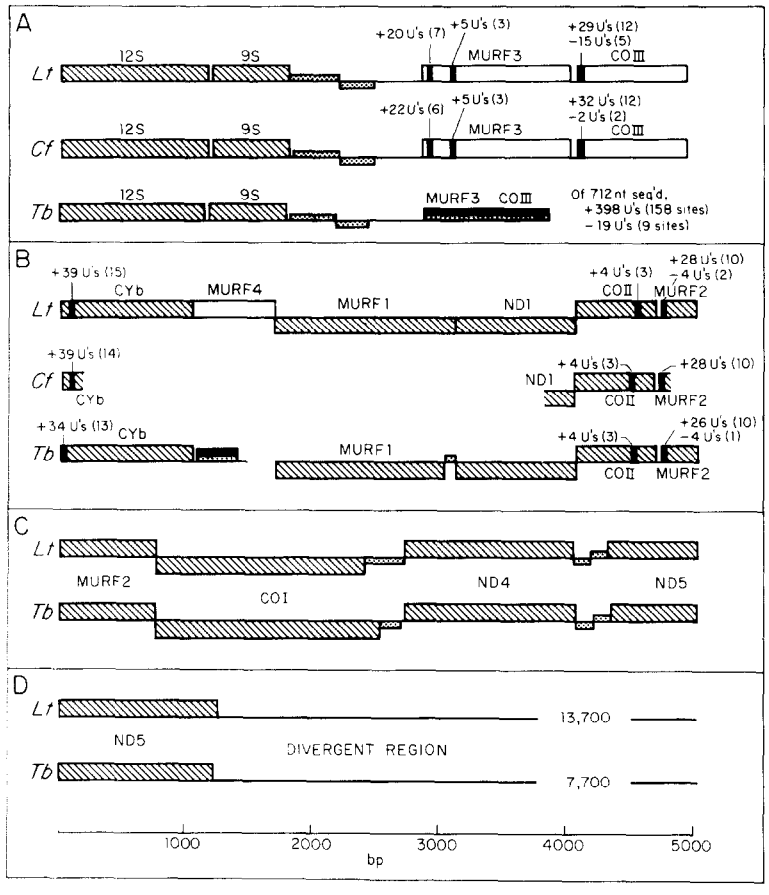

Figure 1. Comparative Maxicircle Genomic Maps—Localization of Edited Regions

Conserved homologous genes from L. tarentolae, C. fasciculata, and T. brucei are indicated by cross-hatching. C-rich intergenic regions are shown by stippling. Genes with no (pre-edited) sequence similarity are indicated by open boxes. Pre-edited regions of cryptogenes are indicated by horizontal black boxes or vertical dark lines, and the number of additions, deletions, and affected sites (in parentheses) is shown for each transcript. A–D, different adjacent regions of the maxicircle genomes.

ently complete set of functional tRNAs exists in the kinetoplastid mitochondrion (Suyama et al., unpublished data). The mitochondrial genomes of mammals and yeast, on the other hand, encode a complete degenerate set of 23–24 tRNAs (Barrell et al., 1980; Bonitz et al., 1980), but another protozoan, Tetrahymena, is thought to encode only 8 tRNAs, with the remainder apparently imported into the organelle (Suyama, 1986; Suyama and Jenney, 1989).

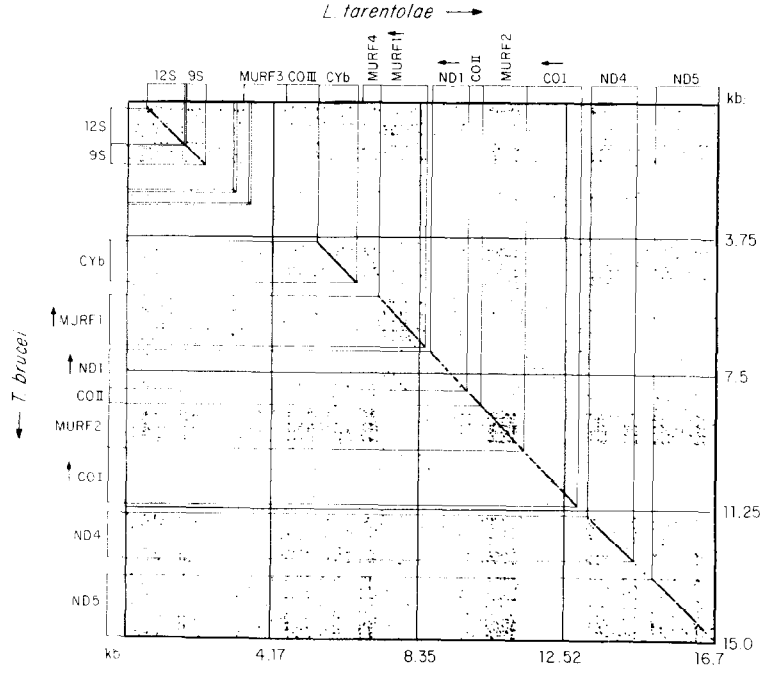

Figure 2. Diagon Dot Matrix of a Comparison of Portions of the L. tarentolae and T. brucei Maxicircle Genomes

A window of 31 nucleotides with a "proportional" match criterion of 21/31 (Staden, 1982) was used. Conserved homologous genes that produce diagonal lines are connected by lines to the axes for ease of visualization. Reprinted from Simpson et al., 1987, with permission.

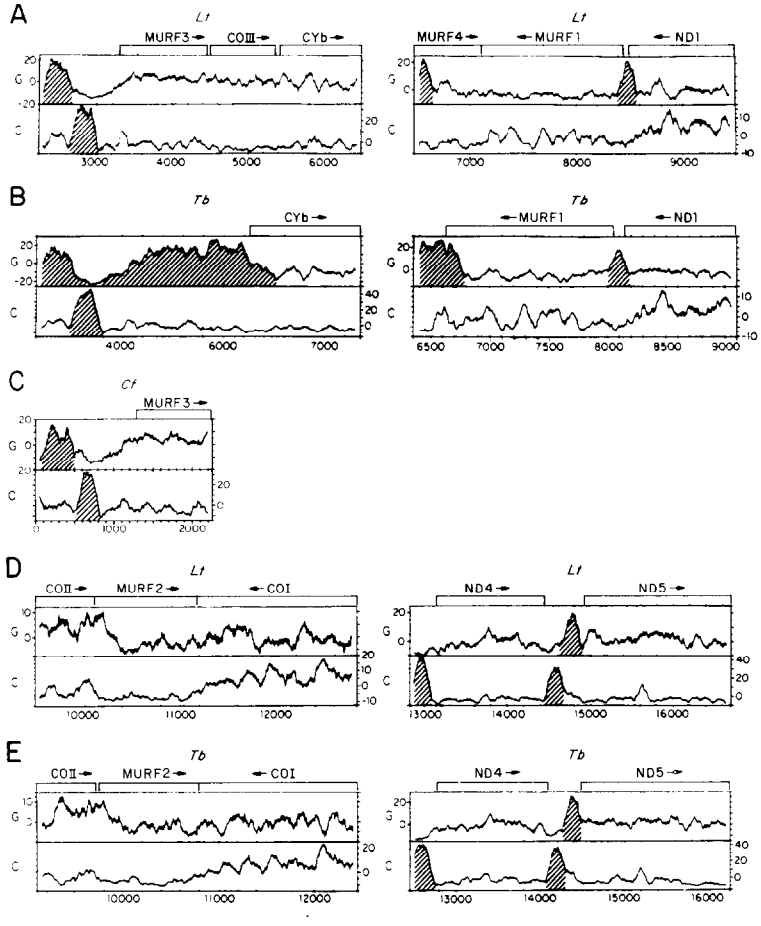

Figure 3. Comparative Maxicircle Localization of GC-rich Intergenic Regions

The distribution of G and C nucleotides in a 100 nucleotide moving window (percent of observed – percent of expected for each nucleotide) for indicated portions of the L. tarentolae (A and D), T. brucei (B and E), and C. fasciculata (C) maxicircle sequences. The G-rich and C-rich regions are shaded. Only a portion of the published C. fasciculata sequence is shown (Sloof et al., 1987). Reprinted in modified form from Simpson et al., 1987, with permission.

Comparison of Mitochondrial Genomes of Three Kinetoplastid Species

A comparison of the informational maxicircle DNA sequences from L. tarentolae and T. brucei (Simpson et al., 1987) and a partial sequence from C. fasciculata (Sloof et al., 1987) revealed that the T. brucei maxicircle lacks sequences homologous to the COIII, MURF3, and ORF12 (=MURF4) genes (Figures 1 and 2). In place of these genes, shorter GC-rich sequences are substituted (Figure 3). All other maxicircle genes in T. brucei and L. tarentolae are present at equivalent relative locations and with identical polarities. The C. fasciculata sequence clearly belongs to the L. tarentolae class, in that homologous genes for MURF3 and COIII are found immediately upstream of the CYb gene. The other significant observation was that the internal frameshifts present in the COII and MURF3 genes are conserved in the homologous C. fasciculata (Benne et al., 1986; van der Spek et al., 1988) and T. brucei (Payne et al., 1985) gene sequences and therefore are not likely to be due to DNA sequencing errors.

Discovery of RNA Editing

The presence of a conserved frameshift in the COII genes from all three species was intriguing and prompted the investigation of the COII transcripts. It was found that there were four extra uridine nucleotides at this position in the mRNA sequence (Benne et al., 1986) which eliminated the −1 frameshift encoded by the gene. Hybridization studies indicated that a DNA template encoding the modified sequence was not present in the mitochondrial or nuclear genomes of these organisms (Benne et al., 1986). Soon thereafter, additional examples of RNA editing were reported, including 34 added U's in the 5′ end of the CYb mRNA of T. brucei (Feagin et al., 1987), 39 added U's in the CYb mRNAs of L. tarentolae and C. fasciculata (Feagin et al., 1988a), and multiple added U's in mRNAs for three other maxicircle genes of all three species (Feagin and Stuart, 1988; Shaw et al., 1988; van der Spek et al., 1988). The deletion of genome-encoded U's was also found to occur in several mRNAs (Shaw et al., 1988). The importance of comparative studies was underscored by the observation that the new methionine initiation codons and the N-terminal amino acid sequences created by this process were almost completely conserved in the three species, which are thought to have been separated by at least 100 million years (Lake et al., 1988).

A dramatic confirmation of the existence of this phenomenon was provided by the discovery that the missing COIII gene in T. brucei was actually present in an abbreviated, encrypted form and that RNA editing acted as a decoding device to expand the DNA sequence into a translatable mRNA (Feagin et al., 1988a). In this case, a transcript encoding the COIII protein was identified in T. brucei, although a DNA sequence that exactly matched

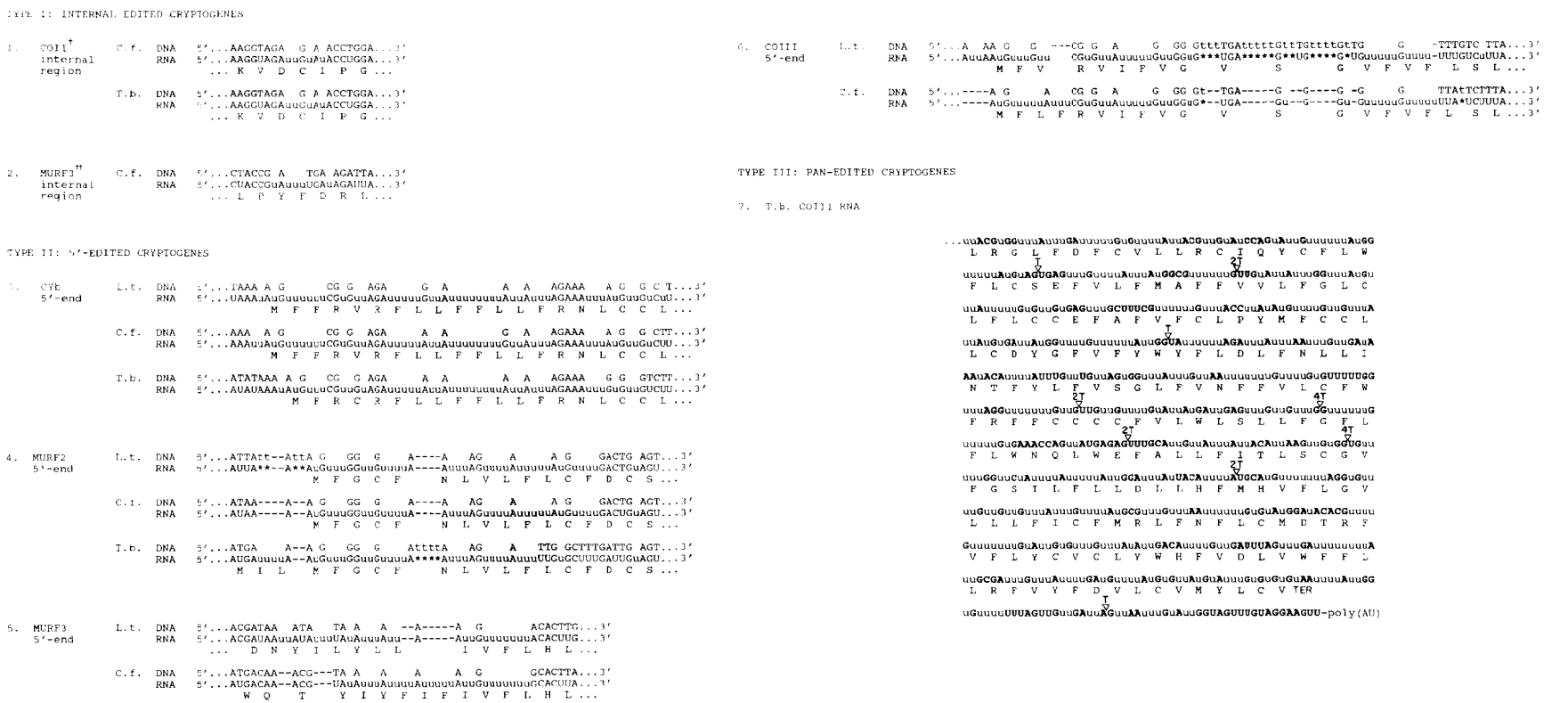

Figure 4. Summary of All Reported Kinetoplastid Edited Sequences

Added U's are indicated as u, T's deleted from the DNA sequence as *, and gaps introduced for the purpose of aligning sequences from different species as —. For the T. brucei COIII DNA sequence, deletions of T's are indicated by open arrowheads. +, the L. tarentolae MURF3 edited region is identical. ++, the L. tarentolae COII edited region is identical (Shaw et al., unpublished data). References: 1. Benne et al., 1986. 2. van der Spek et al., 1988. 3. Feagin et al., 1987; Feagin et al., 1988a. 4. Shaw et al., 1988. 5. Shaw et al., 1988; van der Spek et al., 1988. 6. Shaw et al., 1988. 7. Feagin et al., 1988b.

this RNA could not be found. However, the COIII transcript sequence could be aligned with a region of the maxicircle genome immediately upstream of the CYb gene, provided that multiple U's were added to and deleted from the RNA: in the 712 nucleotide sequence obtained thus far, 398 U's are added at 158 sites and 19 U's are deleted at 9 sites. The relative location of the DNA sequence in the genome was identical to that of the COIII gene sequence in L. tarentolae and C. fasciculata. This was interpreted as an example of massive RNA editing in which more than 50% of the nucleotides in the edited mRNA were contributed by this process, resulting in an RNA sequence that had no obvious similarity with the DNA sequence.

Summary of Editing Events in Kinetoplastids

In the interest of clarity, we would like to introduce some new terminology which is generally applicable to discussions of RNA editing. First, we shall call the incomplete genes whose transcripts are edited to yield translatable sequences, "cryptogenes." The region of the cryptogene or the putative primary, unedited RNA that can be aligned (via the addition and deletion of U or T residues) with its corresponding edited sequence will be referred to as a "pre-edited" sequence. At least three major types of kinetoplastid cryptogenes have been identified, based on the pattern of editing events found in the transcripts:

Type I: internal-edited cryptogenes
Type II: 5′ edited cryptogenes
Type III: pan-edited cryptogenes

Type I cryptogenes (e.g., COII in all three species) yield RNA molecules that are edited at internal positions within protein coding regions (Figure 4–1). Type II cryptogenes (e.g., CYb in all three species) yield transcripts that are edited at the 5′ ends within protein-coding regions (Figure 4–3). Some genes have both Type I and Type II cryptogene properties, as, for example, the C. fasciculata and L. tarentolae MURF3 genes (Figure 4–5). Finally, Type III cryptogenes (e.g., T. brucei COIII) yield transcripts that are extensively edited (pan-edited) over their entire length to generate RNA nucleotide sequences that encode completely new proteins (Figure 4–7). The use of this terminology is not meant to imply that these divisions represent three distinct types of editing. In fact, as new examples of RNA editing are described, we may find a continuum of cryptogene types. All edited and pre-edited regions described to date are presented in Figure 4 and the equivalent maxicircle genomic locations are shown in Figure 1. CYb, MURF2, COIII, and COII transcripts are edited in all three kinetoplastid species, and MURF3 transcripts are edited in two species. It should be noted that the T. brucei MURF3 and MURF4 genes appear on the basis of preliminary RNA sequences (Feagin et al., 1988a; Feagin and Stuart, personal communication) to represent Type III pan-edited cryptogenes also.

At the present time, pan-edited cryptogenes have not been identified in the L. tarentolae or C. fasciculata maxicircles. There are, however, several intergenic regions in the maxicircle DNAs of all three species (shaded regions in Figure 3 and Figure 1) which produce G-rich transcripts of heterogeneous size (Jasmer et al., 1987). Although these regions do not exhibit sequence similarity (Figure 2), there is nevertheless a conservation of the relative location of regions of G versus C strand bias (Figure 3). We speculate that these sequences may represent cryptogenes that could give rise to pan-edited transcripts. The low abundance transcripts of the (AT-rich) divergent region, which are heterogeneous in size (Tarassoff et al., 1987; de Vries et al., 1988), represent additional candidates for pan-editing events. The 9S and 12S rRNAs have not been examined for edited regions, except for the 5′ ends, which were found to be unedited by primer extension sequencing (Simpson et al., 1985; Shonekan and Simpson, unpublished data).

U residues also occur in the poly(A) tails of maxicircle transcripts (Figure 5). This type of modification is apparently less precise, since different cDNAs for the same gene have poly(AU) tails that differ in sequence. The introduction of U residues in poly(A) tails may be coordinated with upstream editing of coding regions since RNAs lacking U's in the tail generally lack upstream editing events, and vice-versa (van der Spek et al., 1988). Editing of the 3′ untranslated region of T. brucei COIII transcripts has also been reported, with different patterns of editing in cDNAs from different stocks of parasites (Feagin et al., 1988a).

Unedited and Partially Edited Transcripts

The mitochondrial steady-state RNA population contains both completely edited, completely unedited, and partially edited forms of each gene transcript. Unedited transcripts were detected in RNA sequencing experiments as specific extension products of predicted sizes which disappeared when primers specific for edited RNAs were used (Feagin et al., 1987; Shaw et al., 1988). Both unedited and edited forms of transcripts were also detected in Northern blot analysis using specific oligonucleotides as probes (Benne et al., 1986; Feagin et al., 1987; Feagin et al., 1988a; van der Spek et al., 1988). In addition, the amount of edited transcripts in the steady-state RNA population varies from gene to gene. Primer extension assays have been used to measure the ratio of edited to unedited RNA for several genes, and these studies indicate that, in L. tarentolae, 89% of the COII and 36% of the MURF3 transcripts are edited (Shaw, Campbell, and Simpson, unpublished data).

Analysis of cDNA sequences has identified three types of partially edited forms of transcripts for several genes (Figure 6). Several T. brucei CYb cDNAs and one T. brucei COIII cDNA have been identified that contain edited 3′ sequences but terminate at a 5′ editing site in a long stretch of U residues. In most cases, the 5′ run of U's is longer than the number of added residues present in the mature transcript. For example, in one CYb cDNA (Figure 6–1), the 5′ terminus has 36 U's although the fully edited CYb transcript has only 8 added U's at this position (Feagin et al., 1987). However these types of intermediates have thus far been found only in T. brucei. Although analysis of C. fasciculata MURF3 editing events identified a high propor-

Species/Gene	Sequence 5′->3′
Cf MURF3[a]	uuuuuuuuuuA$_n$
Cf MURF3[a]	uuuuuuuuuuuuuuuuA$_n$
Cf MURF3[a]	uuuuuuuuuuuuuuuuAuA$_n$
Tb COIII[b]	uuuuuuuAAAAAAAAAAAAAuuuuAAAAAAuuuA$_n$
Tb COIII[c]	uuuuuuAuA$_n$
Cf COIII[d]	uuA$_n$
Cf COIII[d]	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAuA$_n$
Cf COII[d]	uuuAuuuuuA$_n$
Lm ND1[e]	AAuAAuuuuuuuuuuuuAAAAAAAAAAAAAAAAAAAAAAAAAuuuuuAAAA- uuAAAuAuuAAAAuuAAAAAAAAAAAAAAAAAAAAAAuAuAAAAAAAAAuuAAuAu

Cf-Crithidia fasciculata, Tb-Trypanosoma brucei, Lm-Leishmania major.
[a]Van der Spek et al., 1988.
[b]Benne et al., 1986.
[c]Feagin et al., 1988.
[d]Feagin et al., 1988.
[e]Benne et al., 1989.
Campbell et al., 1989.

Figure 5. Summary of cDNA Sequences Showing the Presence of U's in the 3′ Poly(A) Tails of Maxicircle mRNAs

It is not known if the L. major ND1 gene is edited in coding regions also, since the genomic sequence is not known.

tion of cDNAs truncated within editing regions, the 5′ ends of these cDNAs do not possess runs of U's in excess of those present in the mature edited RNA (van der Spek et al., 1988). A second type of intermediate was characterized for the C. fasciculata MURF3 editing events. In this case, 1 cDNA out of 11 was edited at an internal site but was not edited at all in the 5′ region (Figure 6–6). Seven of the 11 cDNAs were fully edited in both the 5′ and internal regions. It is possible that the 5′ edited sequences reported for both the C. fasciculata MURF3 mRNA and the L. tarentolae MURF3 mRNA represent partially edited sequences, as the created N-terminal amino acid sequences are only similar in the 3′ edited region (Figure 4).

A third type of partially edited RNA was found in the case of the T. brucei COIII pan-edited cryptogene (Abraham et al., 1988). Edited antisense probes detected a smear of partially edited COIII transcripts on Northern blots which ranged in size from 450 to 1200 nucleotides,

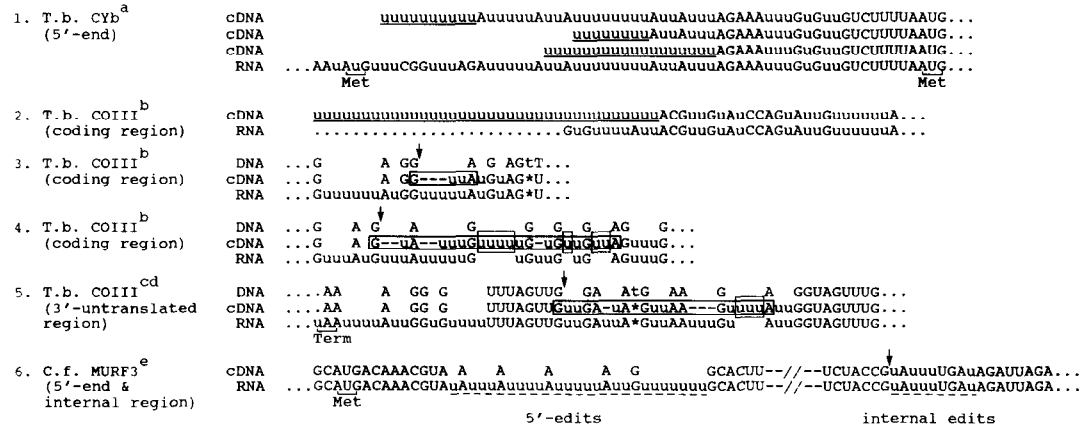

Figure 6. Summary of All Reported cDNA Sequences Showing Partially Edited mRNAs

5′ poly(U) sequences in 1 and 2 are indicated by underlining. Junction regions are indicated in 3, 4, and 5 by boxes. Arrows mark the 5′ unedited/3′ edited borders. Extra U's at normally nonedited sites are indicated in 3, 4, and 5 by larger boxes. Deleted T residues are shown as * . The 5′ edited region and the internal-edited region are indicated in 6 by dashed underlines. References: 1. Feagin et al., 1987. 2, 3, and 4. Abraham et al., 1988. 5. Benne et al., 1986. 6. van der Spek et al., 1988.

the length of the fully edited transcript. Analysis of cDNAs identified COIII transcripts that contained progressively more editing events moving in a 3′ to 5′ direction. PCR amplification, using combinations of unedited and edited oligonucleotide primers, confirmed the presence of 5′ unedited/3′ edited molecules, but did not detect 5′ edited/3′ unedited molecules. The sequences at the unedited/edited junctions in these molecules are complex and can cover up to six editing sites (see boxed regions in Figure 6). Moreover, the sequences at the junctions often differ from the sequences found in the DNA or the fully edited RNA at these positions. In several cases the number of U's at an editing site differs from that found in the mature transcript, and in one case, two U's are added at a site that is not edited in the mature RNA. If we assume that the pan-editing of Type III cryptogene transcripts is mechanistically identical to the editing of Type I and Type II cryptogene transcripts, then these results suggest that kinetoplastid editing in general may occur in a 3′ to 5′ direction. However, this model assumes that partially edited cDNAs represent editing intermediates, but a precursor–product relationship between unedited, partially edited, and fully edited RNA has not yet been demonstrated.

RNA Editing as a Translational Control Mechanism

RNA editing events in all three kinetoplastid species generate translatable transcripts. Editing of Type I COII and MURF3 transcripts allows translational readthrough of genomically encoded frameshifts. In several species, the Type II cryptogenes, CYb, MURF2, and COIII, lack ATG codons for the initiation of translation. RNA editing events in the 5′ ends of these transcripts create in-frame AUG codons and thereby provide a translational control mechanism for the production of mitochondrial proteins. However, noncanonical initiation codons may also be used to translate some maxicircle mRNAs, since transcripts of several genes lack AUG initiation codons. In some cases, the 5′ ends of transcripts are edited but an in-frame AUG is not generated (e.g., L. tarentolae and C. fasciculata MURF3), while in other cases, transcripts that do not contain AUG initiation codons are not edited at all (L. tarentolae MURF4 and MURF1, C. fasciculata ND1, and T. brucei ND1 and MURF1) (Shaw et al., 1988; van der Spek et al., 1988; Feagin and Stuart, personal communication). Two additional functions of RNA editing are the creation of new N-terminal amino acids in Type II editing which may be important for structure and/or function of the proteins, and, of course, the creation of an entire protein coding sequence in Type III editing. The known editing events generate codons for all of the amino acids but lysine, with certain hydrophobic amino acids represented more frequently: phenylalanine, leucine, cysteine, and valine. As would be expected, U-rich codons are preferentially used. Finally, the production of translatable MURF3 mRNA in C. fasciculata may be negatively controlled since an in-frame ATG encoded by the MURF3 cryptogene is moved out of frame by U addition in the 5′ region of the transcript (van der Spek et al., 1988).

There is the tacit assumption in this discussion, of course, that there is mitochondrial protein synthesis in kinetoplastids and that edited RNAs are translated. Proof for the translation of edited mRNAs must come from amino acid sequences of the corresponding protein products and this has not yet been done in any case. In fact, mitochondrial protein synthesis itself has never been rigorously demonstrated in any kinetoplastid (Benne et al., 1983; Spithill et al., 1981). Although it is clear that the 9S and 12S rRNAs represent highly diverged homologs of standard rRNAs (Eperon et al., 1983; de la Cruz et al., 1985a; de la Cruz et al., 1985b; Sloof et al., 1985), no mitochondrial ribosomal particle containing these RNAs has ever been isolated. Evidence for the existence of mitochondrial protein synthesis mainly involves the observed transcription of maxicircle DNA, the precise editing of some transcripts to yield translatable mRNAs, and the presence of a set of mitochondrial tRNAs. In addition, there is Western blot evidence for translation of the L. tarentolae COII mRNA, using an antibody generated against a synthetic peptide synthesized from the predicted COII COOH-terminal sequence (Shaw, Campbell, and Simpson, unpublished data).

Developmental Regulation of RNA Editing in Kinetoplastids

The African pathogenic trypanosomes undergo a biphasic life cycle involving the periodic repression (bloodstream forms) and derepression (procyclic forms) of mitochondrial respiratory activity. Changes in the abundance of certain cryptogene RNAs are correlated with these different life cycle stages. In T. brucei, editing of COII and CYb cryptogene transcripts appears to be developmentally regulated in that only unedited RNAs are found in slender bloodstream forms that lack cytochromes, whereas both edited and unedited RNAs are found in stumpy bloodstream forms and procyclic insect forms. Editing of the MURF2 and COIII transcripts, however, occurs during both lifecycle stages. These results suggest that RNA editing is involved in regulating the expression of some maxicircle genes during the lifecycle of the parasite.

Evidence for the Absence of Edited DNA or RNA Templates

The most startling aspect of the phenomenon of kinetoplastid RNA editing is the apparent absence of a conventional DNA or RNA template encoding the fully edited region. The evidence is mainly derived from hybridization studies using oligonucleotide probes complementary to either the edited or the unedited sequence for each cryptogene. These experiments did not detect edited templates in the nuclear or mitochondrial genomes of these cells. Moreover, edited templates could not be detected by several alternative methods, including direct sequencing of uncloned maxicircle DNA (Shaw et al., 1988) and PCR amplification of total kDNA using primers flanking the edited regions (Sturm, Shonekan, and Simpson, unpublished data).

It has been suggested that edited RNAs might self-replicate (Maizels and Weiner, 1988). If it can be shown by kinetic experiments that the partially edited molecules

represent true editing intermediates, then the existence of a continuum of such molecules is strong theoretical evidence against an RNA template hypothesis since separate templates would be required for each type of intermediate. However, the partially edited molecules could also possibly represent bizzare recombinants between edited and nonedited RNA and not necessarily editing intermediates. The fully edited antisense RNAs predicted by the Weiner model have not been detected by Northern hybridization using sense edited oligonucleotide probes (Feagin et al., 1988a) or by PCR experiments designed to amplify antisense edited RNAs (Sturm, Shonekan, Balakara, and Simpson, unpublished data). However, PCR amplification studies have provided preliminary evidence for the presence of antisense *unedited* RNA for the L. tarentolae MURF2 gene (Sturm and Simpson, unpublished data), the origin and significance of which is not understood.

Hypotheses for the Apparent Template-Independence of Kinetoplastid RNA Editing

Several hypotheses have been suggested to account for the apparent template-independence of kinetoplastid RNA editing:

—The basic "pseudogene" hypothesis maintains that the cloned and sequenced maxicircle molecules are actually pseudogenes, and that edited versions of the cryptogenes are present on one or a few copies of the 50 maxicircle genomes present in each organelle (Benne, 1985). As discussed above, there is no evidence for this type of maxicircle heterogeneity or for the presence of single copies of edited genes in mitochondrial or nuclear DNA.

—A variant of the pseudogene hypothesis states that there are small edited RNAs which are transcribed from small edited DNA sequences scattered throughout the genome (mitochondrial or nuclear) which could give rise to mature edited RNA molecules by a process of *trans*-splicing, such as that which occurs for the cytosolic mRNA population in these cells. This model has been tested with negative results (Shaw et al., 1988) by hybridization of Northern blots containing total mitochondrial RNA with labeled antisense edited oligomers. In addition, the presence of small edited sequences of 20 bp or greater in mitochondrial or nuclear DNA would have been detected by oligonucleotide probes used in the DNA hybridization experiments described above.

—A third hypothesis suggests that the edited template is somehow refractive to hybridization, perhaps as a result of nucleotide modifications, or that the template is easily lost during standard purification procedures (Downs and Roth, 1987). For example, a template that is tightly bound to protein might be resistant to proteases or deproteinization procedures. This hypothesis, however, is difficult to test experimentally.

—Some investigators have suggested that cryptic signals sequence might become exposed in incompletely edited molecules and that these molecules could then proceed to become fully and precisely edited species (Stuart, 1989). This argument does not address the issue of what determines that a pre-edited region is to be edited in the first place, nor the issue of the multiplicity of signals that would be required to generate the multiplicity of editing patterns.

—The information determining the final edited sequences may be encoded in the DNA sequences of the pre-edited or flanking regions of the cryptogenes in the form of secondary structures or primary sequence motifs, which could somehow guide a multienzyme editing complex in making the precise additions and deletions of uridines. However, a detailed analysis of these regions did not reveal any structures or sequences that might function in this manner (Shaw et al., 1988). Nevertheless, this hypothesis must remain as a viable one.

—The edited RNA might self-replicate and produce an edited antisense RNA, which could then act as a template for editing by a mechanism involving direct base-pairing, or by a process involving asymmetric replication, such as that which occurs in certain bacteriophage (Maizels and Weiner, 1988). As stated previously, there is no evidence for the presence of edited antisense RNA in the mitochondrial compartment. Furthermore, the existence of a pre-edited DNA sequence identical to the edited RNA sequence except for the presence and absence of T's at precise sites argues against a self-replicating RNA model, since there would be no reason to maintain pre-edited sequences in the DNA.

—We would like to suggest an alternative RNA/RNA heteroduplex model based on our preliminary evidence for the presence of antisense unedited RNA for the L. tarentolae MURF2 gene. Ignoring the problem for the moment of the origin of this species, we propose that antisense unedited RNA might hybridize with preexisting sense edited RNA, forming a heteroduplex with mismatch loops at edited sites. This heteroduplex then might associate with the hypothetical editing complex and somehow guide the precise modification of a separate sense unedited RNA. This model does not address the question of the mechanism involved in transfer of the information from the heteroduplex to the editing machinery. However, the model does maintain the necessity for a pre-edited DNA sequence as well as a preexisting edited RNA sequence.

What Do We Really Know about the Mechanism of RNA Editing?

The cumulative frequency distribution pattern of U additions (and deletions) for all edited cryptogenes is shown in Figure 7. Most edits involve the addition of single U's 3′ of purines, with decreasing numbers of multiple additions up to 8-mers. U deletions occur 3′ of purines and only occur in transcripts that also contain U additions, suggesting that the process of U deletion is mechanistically related to the process of U addition.

RNA editing is probably a posttranscriptional process. The evidence for posttranscriptional editing includes the fact that poly(A) tails contain U's which most likely are inserted by the same mechanism that adds U's to coding regions, and polyadenylation is known to be a posttranscriptional enzymatic process (Humphrey et al., 1987; Conway and Wickens, 1987; Zarkower and Wickens, 1987). The fact that completely unedited and partially

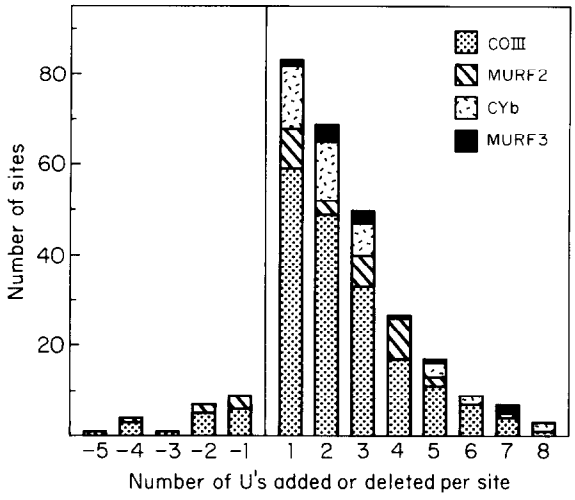

Figure 7. Cumulative Frequency Distributions of U Additions and Deletions for All Known Maxicircle Edited Regions

The number of sites containing different numbers of U's added or deleted is indicated for each cryptogene. Data from all three species are summed.

edited RNAs exist and that the ratio of edited/unedited RNAs varies from gene to gene also suggests a posttranscriptional process.

The evidence for the involvement of a multienzyme complex in editing includes the apparent 3′ to 5′ polarity, which suggests the progressive movement of a complex along the primary transcript, and the fact that the junction regions of some partially edited molecules extend over several editing sites, which suggests that the complex is quite large. The existence of edited RNAs truncated at editing sites and the presence of excess U's in junction regions of partially edited RNAs even at sites not edited in the mature RNA suggest the involvement of a riboendonuclease that lacks specificity and a terminal transferase U-addition enzyme that adds multiple U's to the released termini. The polarity of editing also suggests that editing does not occur on the ribosome, as the ribosome must bind to the initiation codon which is created by editing at the termination of the process. The following hypothetical scenario is suggested by these facts: A specific riboendonuclease recognizes some aspect of the edited region and produces multiple random cleavages within a pre-edited sequence covering up to six editing sites (and in the poly(A) tail), a uridylyl transferase then adds multiple U's to the termini of the cleaved fragments, an exonuclease trims the excess U's to exactly the required number (or deletes encoded U's), and an RNA ligase rejoins the modified regions.

Two Possible Candidates for Editing Enzymes

Purified mitochondria from L. tarentolae have been found to contain two enzyme activities (Bakalara, Simpson, and Simpson, unpublished data) similar to those previously reported in total cell extracts of T. brucei (White and Borst, 1987), that are possible candidates for participation in editing reactions: a 3′ terminal uridylyl transferase (TUTase) and an RNA ligase. These activities comigrate with the kinetoplast-mitochondrion fraction in isopycnic Renografin (Braly et al., 1974) and Percoll density gradients, and can be released by Triton lysis of the organelle (Bakalara et al., unpublished data). The TUTase activity adds several U's to the 3′OH of both endogeneous mitochondrial RNAs in intact mitochondria and exogeneous RNA molecules in Triton lysates of mitochondria (Bakalara et al., unpublished data). Although this activity could not directly produce the subclass of truncated 5′poly(U)-partially edited molecules (Figures 6-1 and 6-2), it is possible that the 5′ poly(U) tails on these cDNAs are artifacts of cloning and are not true intermediates since they were not observed on truncated MURF3 cDNAs (van der Spek et al., 1988). Work is in progress to determine if these two enzymes are involved in the editing of maxicircle transcripts and are present in the form of editing complexes.

Are There Other Examples of RNA Editing?

In the case of the kinetoplastids, the absence of recognizable genes for ATP synthetase subunits in the mitochondrial genome is intriguing, and we speculate that it may in part be due to as yet undescribed events of pan-editing of G-rich RNAs transcribed from GC-rich intergenic regions. Although there is evidence that most kinetoplastid mitochondrial tRNAs are transcribed from nuclear genes (Suyama, Campbell, Simpson, and Simpson, unpublished data), the possibility remains that a small subset of tRNAs is produced by extensive editing of mitochondrial transcripts. Finally, editing of small minicircle DNA transcripts (Rohrer et al., 1985; Simpson and Simpson, unpublished data) to produce translatable or structural RNAs that have not yet been identified may occur, and this may finally provide a rationale for the existence of this unusual DNA.

In the acellular slime mold Physarum polycephalum, a mitochondrial cDNA has been sequenced that has homology with the N-terminal portion of the alpha subunit of ATP synthetase (D. Miller, personal communication). The homologous mitochondrial DNA sequence contained more than 50 [-1] frameshifts in the translated amino acid sequence. By comparing the cDNA to the mitochondrial DNA sequence, it was found that the 26 frameshifts covered by the cDNA were in each case corrected by the insertion of a single C residue (D. Miller, personal communication). The insertion sites are fairly uniformly spaced at 24 + 8 nucleotides between sites. This appears to represent another example of RNA editing, but there are several obvious differences in that C addition occurs instead of U addition, and the additions are uniformly spaced and limited to single nucleotides.

Another possible example of RNA editing is a tissue-specific posttranscriptional, non genome-encoded C to U conversion in mammalian apolipoprotein B mRNA (Powell et al., 1987; Chen et al., 1987; Thomas et al., 1988; Tennyson et al., 1989a; Tennyson et al., 1989b). This modification changes a glutamine codon (CAA) into a translational stop codon (UAA) and thereby generates a shortened protein product, apo-B48. This situation, however, may repre-

sent a more traditional modification of an existing residue, similar to the type of posttranscriptional nucleotide modification that occurs in tRNA biosynthesis (Elliot and Trewyn, 1984).

In this regard, an unwinding/modifying activity was recently described in Xenopus which produces covalent modifications in double-stranded RNA by conversion of adenosine to inosine (Bass and Weintraub, 1988), and it was suggested that this type of activity could be responsible for the nucleotide modifications observed in the apo-B situation. Another example of a posttranscriptional change in RNA that could be produced by an unwinding/modifying activity is the so-called "biased hypermutation" phenomenon in the RNA genome of a defective measles virus (Cattaneo et al., 1988; Bass et al., 1989; Lamb and Dreyfuss, 1989), in which 132 of 266 A residues in the matrix gene were found to be mutated to G residues.

Late transcripts of Vaccinia virus contain a fairly discrete length (35 nucleotides) of 5′ poly(A) sequence that is not encoded in the viral genome (Schwer and Stunnenberg, 1988). The mechanism in this case appears to involve a slippage or stuttering of the RNA polymerase at an encoded T residue. A similar cotranscriptional RNA modification phenomenon appears to be occurring in negative strand RNA viruses, both in the synthesis of 3′ poly(A) tails by transcriptional stuttering on a terminal homopolymer stretch of U residues, and within coding regions. The latter was first described in the paramyxovirus, SV5, in which the presence of two nontemplated G residues in a subpopulation of mRNAs for the P gene overcomes a frameshift and joins two open reading frames to produce the P protein (Thomas et al., 1988). The mRNAs lacking these extra G residues code for the V protein which is amino coterminal with the P protein. Since the nonencoded G's are added after four encoded G's, a stuttering mechanism is also thought to be responsible for this phenomenon. However, out of 22 cDNAs, 10 were found to be modified by the addition of 2 G residues at the same location and the remainder were not modified, which suggests that the mechanism may be precise in this case (Thomas et al., 1988). A similar phenomenon was described for the case of measles virus, and it was suggested that similar events might occur in other paramyxoviruses (Cattaneo et al., 1989). The main difference in the measles virus situation is that the G addition is imprecise with respect to the number of G's added (Cattaneo et al., 1989).

Another mechanism for modification of RNA sequences is the "jumping RNA transcription" model developed to explain unusual features of coronavirus mRNAs (Makino et al., 1988a). A free leader RNA molecule is synthesized, dissociates from template RNA and then reassociates with template at downstream initiation sites to act as a primer for transcription. A defective-interfering mouse hepatitis viral RNA comprised of three non-contiguous genomic regions fused into a single open reading frame appears to have been formed by this discontinuous type of RNA synthesis (Makino et al., 1988b).

Finally, the generation of TAA termination codons by polyadenylation of processed mammalian mitochondrial DNA transcripts could also be classified as RNA editing, since translatable mRNAs are produced as a result of RNA modifications (Attardi and Schatz, 1989).

Evolutionary Implications and Speculations

Mitochondrial cryptogenes and the accompanying RNA editing decoding mechanism are present throughout the Trypanosomatidae family of kinetoplastid protozoa, occurring in the primitive monogenetic genus, Crithidia, as well as in the more recently evolved digenetic genus, Trypanosoma (Lake et al., 1988), and may also occur in the slime mold, Physarum. The paramyxoviral type of RNA editing also involves a modification of the primary sequence of coding regions of mRNA molecules, but clearly involves a different mechanism and has a different evolutionary origin, as does the coronaviral type of RNA editing. Assuming tentatively that the RNA editing phenomena in kinetoplastids and Physarum are mechanistically similar and are derived from a common ancestor of these primitive lower eukaryotes, this argues for an ancient origin of this process and therefore a possibly wider distribution in present day organisms. In general, RNA editing has a clear selective advantage in evolution in addition to a possible role in regulation of translation, in that genetic information can be maintained at the RNA level in the face of sequence drift and even sequence compression at the genomic level. The presence of abundant noncoding DNA sequences and pseudogenes is a hallmark of the genomic organization of higher eukaryotic cells, and the existence of a process such as the kinetoplastid type of RNA editing might make it worthwhile to reevaluate the significance of some of these sequences.

The most extreme form of kinetoplastid RNA editing, pan-editing, has thus far been found only in transcripts of three genes in T. brucei. In an attempt to determine whether pan-edited genes exist in other kinetoplastid species, we screened kDNAs from a variety of species and genera with restriction fragments containing the unedited portions of the L. tarentolae COIII gene (Simpson et al., 1987; de la Cruz, Shaw, and Simpson, unpublished data). The results indicated that COIII genes cannot be detected in the mitochondrial genomes of two Herpetomonas species. This led to the tentative conclusion that pan-edited cryptogenes may also be present in Herpetomonas. This conclusion is consistent with previous suggestions that Herpetomonas is an evolutionary precursor of Trypanosoma, since Herpetomonas sp. are one of the two monogenetic kinetoplastids that undergo morphogenetic changes resembling those occurring in the digenetic Trypanosoma (Hoare, 1972).

Conclusions

RNA editing in the broad sense involves the modification of the primary sequences of coding regions of mRNAs and provides yet another method of modulating genetic activity. There appear to be different mechanisms for RNA editing in different systems. The kinetoplastid and perhaps the Physarum type of editing involves the precise addition and deletion of nucleotides within coding regions of mRNA molecules and differs from the paramyxoviral type

of editing, which involves a cotranscriptional stuttering of the RNA polymerase on homopolymer stretches. Both types differ from the mammalian apolipoprotein B type of editing which is thought to involve a chemical nucleotide modification. Since the kinetoplastid type of editing is probably of ancient origin, RNA editing activities are likely to be found in other organisms also. The major puzzle that the discovery of this phenomenon in the kinetoplastids has presented is the mechanistic nature of the information for the precise modifications of coding sequences of mRNAs in the apparent absence of templates encoding this information. The question of what encodes the specificity of edited RNA has direct relevance to the basic tenets of the central dogma of information transfer in molecular genetics, and it is likely that the solution to this problem may prove to be of general biological significance.

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