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Uridine Insertion/Deletion RNA Editing as a Paradigm for Site-specific Modifications of RNA Molecules

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A SENSE OF WONDERMENT AT THE VARIETY, complexity, and beauty of natural phenomena is, I believe, the defining characteristic of our species. I wonder at the very existence of the universe, at physical laws and what they mean, and at the marvelous emergent properties in the complexities of living systems. In particular, the trypanosomatid protists caught my imagination many years ago and never let go. Wonder after wonder emerged from the study of these creatures, with the most striking being RNA editing. Uridine insertion/deletion RNA editing was discovered by Benne et al (1986). Four non-encoded U's were found in the mRNA at the site of an evolutionarily conserved frameshift in the cytochrome oxidase subunit II gene which was encoded in the maxicircle mitochondrial DNA of trypanosome protists. Subsequent examples encompassed different trypanosome species and genes and included multiple U-insertions and U-deletions (Feagin et al. 1988; Shaw et al. 1988, 1989; Van der Spek et al. 1988), including the dramatic case of the pan-edited genes in which hundreds of U's were inserted and deleted at hundreds of sites throughout the gene (Bhat et al. 1990; Koslowsky et al. 1990; Maslov et al. 1992). The mechanism of editing began to be revealed upon discovery of short guide RNAs (gRNAs) encoded both in the maxicircle DNA and in the thousands of catenated minicircle molecules; the gRNAs were perfectly complementary to completely edited mRNAs (Blum et al. 1990; Blum and Simpson 1990; Pollard et al. 1990; Sturm and Simpson 1990). Based on

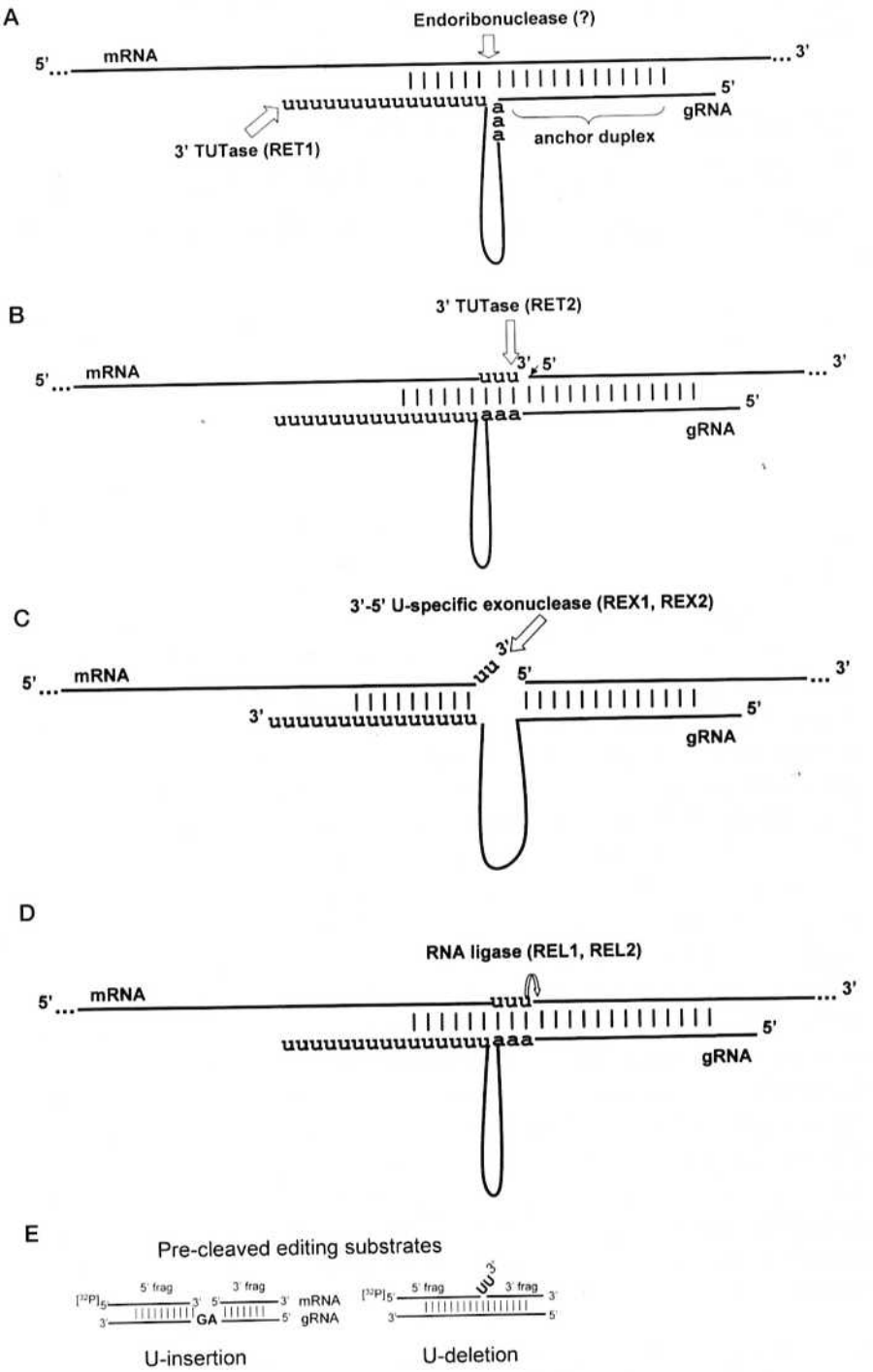

Figure 1. *(See facing page for legend.)*

the presence of a short complementary sequence (anchor sequence) just downstream of the 3′-most editing site, a model was proposed in which there is an initial endonucleolytic cleavage of the pre-edited mRNA at the first mRNA/gRNA mismatch followed by either an addition of U's to the 3′ end of the 5′ mRNA fragment, which then can base-pair with the guiding nucleotides in the gRNA, or a deletion of unpaired U's from the 3′ end of the 5′ fragment, and finally a religation of the mRNA fragments (Fig. 1A–D) (Blum et al. 1990). The enzymatic machinery was proposed to translocate to the next upstream mismatch, and the entire cycle would be repeated. The observed overall 3′ to 5′ polarity of editing in multiple gRNA-mediated editing domains was neatly explained by the creation of the upstream anchor sequences by downstream editing (Maslov and Simpson 1992).

This model has been subsequently experimentally confirmed in essentially all details (Seiwert and Stuart 1994; Byrne et al. 1996; Cruz-Reyés and Sollner-Webb 1996; Seiwert et al. 1996), with the exception that there appear to be separate but interconnected enzymatic pathways for U-insertion and U-deletion sites (Cruz-Reyes et al. 1998a,b, 2002). In the last few years, many of the proteins involved in this process have been identified, and progress has been made toward understanding structural and enzymatic details (Stuart et al. 2002; Simpson et al. 2003, 2004; Worthey et al. 2003). Despite this progress (or perhaps as a result of this progress), I still maintain a child-like fascination with this very successful but apparently unique evolutionary adaptation for gene regulation: How did it evolve, why is it still maintained, and why is it restricted to trypanosomes?

Figure 1. Mechanism of RNA editing. (**A–D**). Model for U-insertion editing and U-deletion editing. The arrows indicate enzymatic activities participating in the reaction, with the name of the enzyme (if known) in parentheses. The gRNA/mRNA anchor duplex is indicated, as is a putative duplex formed by the gRNA 3′ oligo U tail and the GA-rich pre-edited region of the mRNA (Blum and Simpson 1990; Leung and Koslowsky 1999, 2001a,b). (**A**) The initial annealing of the pre-edited mRNA and the cognate gRNA, and the initial cleavage at the first mismatch. (**B**) 3′ addition of U's to the 5′ cleavage fragment, which base-pair with three guiding A's in the gRNA (U-insertion). (**C**) Trimming of 3′ non-base-paired U's from 5′ cleavage fragment (U-deletion). (**D**) Ligation of the 5′ and 3′ cleavage fragments to extend the anchor duplex by three base pairs (U-insertion). Ligation of the two fragments in U-deletion editing is not shown. (**E**) RNA substrates for the precleaved in vitro editing assay. In the +2 U-insertion substrate, two guiding nucleotides for the insertion of two U's are in bold caps. In the −2 U-deletion substrate, the two U's to be deleted are shown.

In this chapter I review recent advances in our knowledge of the biochemistry and molecular mechanisms of this process and also speculate on the biological significance of this and related phenomena.

IN VITRO EDITING ASSAYS

In the case of the African trypanosome, *Trypanosoma brucei*, a gRNA-directed in vitro editing assay at a single site has been utilized extensively (Cruz-Reyes and Sollner-Webb 1996; Seiwert et al. 1996). The RNA substrate, transcribed in vitro using phage T7 RNA polymerase, usually contains the first and second editing sites of the ATPase subunit 6 (A6) pre-edited mRNA. The reaction is "full round" in that there is a site-directed cleavage followed by either U-addition or U-deletion and RNA ligation, depending on the specific gRNA sequence. However, there is little or no processivity with regard to adjacent upstream editing sites. In addition, there are no reports of processivity involving overlapping gRNAs.

The "enzyme" used was either the ~20S glycerol gradient fraction of a clarified mitochondrial lysate or equivalent column-fractionated lysate fractions (see below). The maximum yield of edited RNA was very low (~1–2%). Modification of the gRNA sequence led to a substantial enhancement of the in vitro U-deletion reaction efficiency to ~60% conversion of input into edited product (Cruz-Reyes et al. 2001), but no equivalent enhancement of the U-insertion reaction has been reported. Increasing the "tether" duplex (i.e., the duplex produced by annealing the gRNA with the pre-edited region upstream of the editing site) did increase U-insertion activity somewhat while it decreased U-deletion activity. A substantial increase in the efficiency of full round in vitro editing was obtained in the *Leishmania* system by providing the gRNA in *cis* at the 3′ end of the mRNA and stabilizing the tether duplex (Kapushoc and Simpson 1999).

In the case of the lizard parasite, *Leishmania tarentolae*, initial reports indicated that the efficiency of full round editing was extremely low, forcing the use of RT-PCR to amplify the signal (Byrne et al. 1996). Using this method, there appeared to be a background of gRNA-independent U-insertion in addition to the precise gRNA-mediated insertions. However, Pai et al. (2003) utilized in vitro selection-amplification to obtain modified mRNA and gRNA sequences that mediated efficient full cycle editing by column fractionated mitochondrial extract, suggesting that the rate-limiting step is the specific editing RNA substrate.

A major technological improvement was the "pre-cleaved" assay developed by Igo et al. (2002), which bypasses the requirement for the initial nuclease cleavage by providing two pre-edited mRNA cleavage

fragments bridged by a cognate gRNA; the gRNA either contains guiding nucleotides and mediates U-insertions, or it lacks guiding nucleotides and mediates U-deletions (Fig. 1E). The yield of edited products is more than 50% of the input. This assay has been extensively used to examine the editing reaction using both *T. brucei* and *L. tarentolae* enzyme fractions.

THE ~20S RNA LIGASE-CONTAINING COMPLEX

The RNA ligase-containing core-editing complex from trypanosomatid mitochondria was first identified as a single auto-adenylated high-molecular-weight band in a native gel (Peris et al. 1997; Rusché et al. 1997), with the REL1 and REL2 ligases (Sabatini and Hajduk 1995) representing the adenylated components. This complex has been labeled the L-complex or "editosome" in different labs (Simpson et al. 2004). We argue for the former operational nomenclature, reserving the latter for the yet poorly characterized RNA-mediated super complex (see below). The L-complex, which sediments in glycerol gradients at approximately 20–25S, has been reported to contain from 7 (Rusché et al. 1997) to over 20 polypeptides (Panigrahi et al. 2003), but there is currently a general consensus that there are ~16 polypeptide components (Aphasizhev et al. 2003c) which are in approximately equimolar stoichiometry. However, the relative amounts of different components vary with the isolation technique, suggesting a somewhat loose or dynamic interaction. RNase predigestion of the mitochondrial lysate prior to gradient separation of the L-complex had no effect on the S value or polypeptide composition of the native gel band, indicating that the complex is stabilized entirely by protein–protein interactions (Aphasizhev et al. 2003c).

The cleanest method to isolate the L-complex uses epitope tagging of component proteins which, when expressed and targeted to the mitochondrion, allow affinity isolation of the complex from isolated mitochondrial fractions. Several different TAP-tagged L-complex proteins were expressed in *L. tarentolae* and integrated into the L-complex, and each yielded approximately the same polypeptide profile (Fig. 2) (Aphasizhev et al. 2003c). The presence of both the tagged and the endogenous proteins in the pull-down material suggested a dimeric organization of the L-complex, but this remains to be firmly established.

Proteins from such isolations were subjected to mass spectrometry for gene identification (Panigrahi et al. 2003; Worthey et al. 2003; Simpson et al. 2004). Several sequence motifs could be identified that suggested biological roles for specific proteins, but as yet only a few have been expressed and characterized. These include the REL1 and REL2 RNA

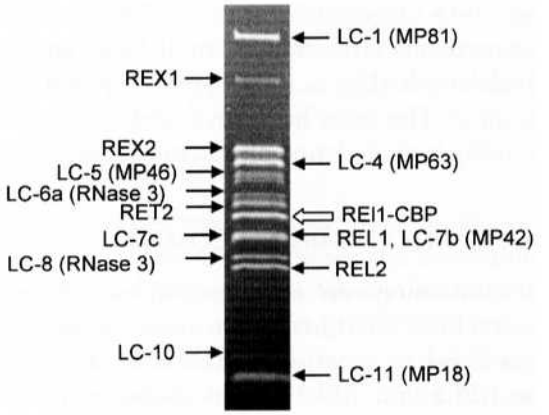

Figure 2. SDS gel of TAP-isolated L-complex from *L. tarentolae*. Stained with Sypro Ruby. Bands are indicated by both the LC and MP nomenclatures, and those with known enzymatic activity are indicated. The CBP-tagged REL1 protein used for the pull-down is indicated by an open arrow. (Modified, with permission, from Aphasizhev et al. 2003c [©MacMillan].)

ligases (McManus et al. 2001; Rusché et al. 2001; Schnaufer et al. 2001), the RET2 3′ TUTase (Aphasizhev et al. 2003c; Ernst et al. 2003), and the REX1 and REX2 3′–5′ exonucleases (Kang et al. 2005). In addition, there are several proteins with RNase III motifs, several with single-strand-binding motifs, and a group of proteins with zinc finger motifs (Simpson et al. 2004).

EXPRESSION AND CHARACTERIZATION OF THE REL1 AND REL2 RNA LIGASES, THE REX1 EXONUCLEASE, AND THE RET2 3′ TUTASE

L. tarentolae REL1 and REL2 and *T. brucei* REL1 were expressed in insect cells using the baculovirus system (Gao et al. 2005). The recombinant proteins were enzymatically active and showed similar K_m values for the RNA substrate in the 100 nM range. RNAi down-regulation of REL1 in *T. brucei* was lethal but did not affect the stability of the L-complex, whereas down-regulation of REL2 had no phenotype. The REL1-depleted L-complex had reduced activity for both U-insertion and U-deletion editing of a precleaved RNA substrate in vitro (Gao and Simpson 2003).

The *L. tarentolae* LC-2 (MP100) (Kang et al. 2005) and the *T. brucei* MP100 (LC-2) and MP99 (LC-3) (K. Rogers and L. Simpson, unpubl.) proteins were also expressed in insect cells. All recombinant proteins showed 3′ to 5′ exonuclease activities specific for single-stranded 3′ oligo U overhangs. These were therefore renamed RNA-editing exonuclease 1 and 2

into the L-complex. This complementation assay, combined with mutagenesis of the recombinant protein, allowed the localization of the region of REL1 that interacts with the L-complex to within the nonenzymatic carboxy-terminal 90 amino acids (Gao et al. 2005). The assay should be useful for investigation of the role of specific motifs in these proteins.

THE ENDONUCLEASE INVOLVED IN THE INITIAL CLEAVAGE OF THE MESSENGER RNA AT THE EDITING SITE

Piller et al. (1997) identified an RNA cleavage activity that co-sedimented with the L-complex and had the characteristics predicted for an editing nuclease, but this activity has not yet been localized to any specific L-complex protein. Cruz-Reyes et al. (1998b) showed that cleavage at U-insertion and U-deletion sites may involve different enzymes, since the former is inhibited by ATP and ADP whereas the latter is stimulated by ATP and ADP.

No cleavage activity could be detected in *L. tarentolae* 20S fractions by direct analysis (Alfonzo and L. Simpson, unpublished results). However, Pai et al. (2003) could directly detect specific cleavage activity in *Leishmania* fractions using an mRNA/gRNA substrate derived by in vitro selection.

The most likely candidates for the nuclease(s) are one or more of the proteins containing RNase III motifs—LC-6A (MP61), LC-8 (MP44), MP67, and MP90—but recent work indicates that the zinc finger-containing protein, MP42 (LC-7B), exhibits exonuclease and endonuclease activities (Brecht et al. 2005), so this remains an open question.

THE RET1 3′-TERMINAL URIDYLYL TRANSFERASE ADDS U'S TO THE GUIDE RNA 3′ END AND IS PRESENT IN A SEPARATE COMPLEX

In addition to the 3′ U's that are added to the cleaved pre-mRNA and subsequently inserted, 3′ U's are also added to the gRNAs (see Fig. 1). These do not appear to be incorporated into editing sites but may be required for the interaction of the gRNA with the cleaved mRNA fragment. Since there are two 3′ TUTase enzymes, RET1 and RET2, the question arises as to the specific role of each enzyme. The RET1 3′ TUTase was expressed in active form in *E. coli* and characterized in detail (Aphasizhev et al. 2002; Aphasizheva et al. 2004). The recombinant enzyme added multiple U's to the 3′ end of RNA substrates. During the course of the biochemical isolation of this enzyme from *L. tarentolae* mitochondria, a

minor peak of activity was noted in an ion exchange fractionation (Aphasizhev et al. 2002). This peak contained L-complex material, as shown by migration of an autoadenylated band in a native gel, and in addition contained a ~700-kD band that reacted with anti-RET1 antibody. Native recombinant RET1 was tetrameric, but in mitochondrial lysates a number of higher-molecular-weight RET1-containing bands were observed, including one corresponding to the 700-kD complex which survives ion exchange chromatography. The latter was operationally named the RET1 complex.

THE MRP RNA-BINDING RNA CHAPERON COMPLEX

The MRP1 and MRP2 mitochondrial RNA-binding proteins have been identified in several trypanosomatid species (Koller et al. 1997; Lambert et al. 1999; Blom et al. 2001; Aphasizhev et al. 2003b). These proteins bound single-stranded and double-stranded RNA in the nanomolar range and could stimulate RNA annealing 20- to 70-fold (Muller et al. 2001; Aphasizhev et al. 2003b). The proteins were present in mitochondrial lysates as a stable heterotetramer. RNAi down-regulation of expression of MRP1 and/or MRP2 has been reported (Vondruskova et al. 2004). Loss of MRP1 produced little effect on cell growth, but loss of MRP2 was lethal (Vondruskova et al. 2004). Differential effects were observed on the extent of editing of different mRNAs, leading to the suggestion that these proteins play a regulatory role in the editing of specific transcripts.

RNA-DEPENDENT INTERACTIONS OF THE L-COMPLEX: THE RET1 COMPLEX AND THE MRP COMPLEX

The MRP RNP complex was shown to interact with both the L-complex and the RET1 complex in an RNA-dependent manner (Aphasizhev et al. 2003b). *L. tarentolae* were transfected with MRP1-TAP, and mitochondrial lysates were analyzed by gradient sedimentation, autoadenylation, and western analysis. Substoichiometric amounts of REL1, REL2, and RET1 were detected in the 20–30S region together with the MRP RNP complex. Pretreatment of the lysate with RNase removed the co-sedimenting MRP complex, suggesting that RNA linkers were required for the maintenance of this interaction.

A substoichiometric amount of RET1 was also immunologically detected in TAP-isolated ~20S L-complex from *L. tarentolae* (Aphasizhev et al. 2003c). Predigestion of the lysate with RNase led to a loss of this

material, indicating that the linkages between RET1 and the L-complex contained RNA. There was no effect of RNase on the S value or polypeptide composition of the L-complex eluted from the native gel.

From these data we proposed that the functional editing supercomplex consists of at least three independent entities, the L-complex, the MRP complex, and the RET1 complex, interacting via RNA linkers, as diagrammed in Figure 3 (Simpson et al. 2004). The nature of these linkers is not yet established, but the most likely candidates are gRNAs and the annealed cognate mRNAs. Additional factors such as the mHel61 RNA helicase (Missel and Goringer 1994; Missel et al. 1997) and other proteins (Madison-Antenucci et al. 1998; Vanhamme et al. 1998; Pelletier and

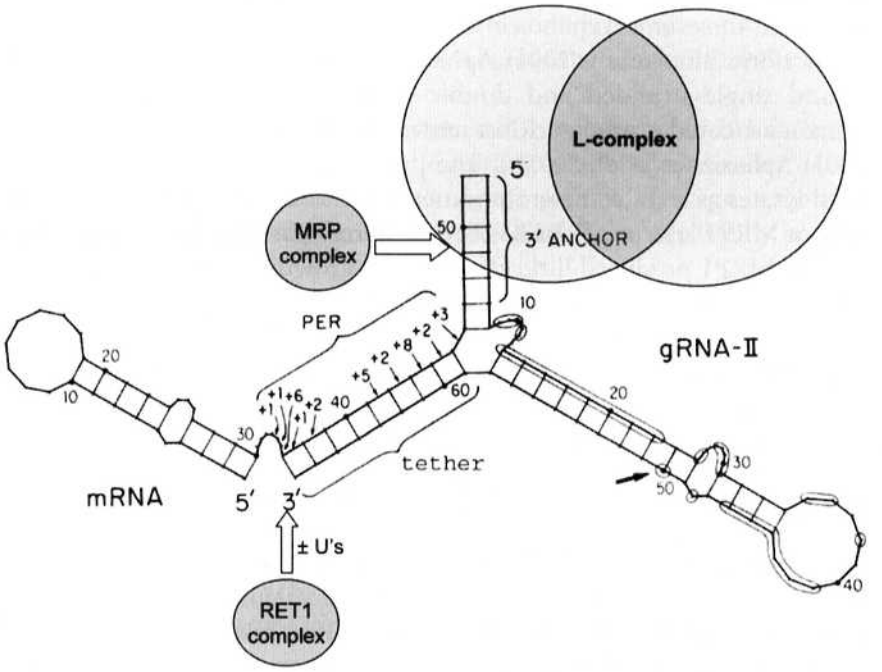

Figure 3. Model of organization of editing RNP supercomplex. The RET1 complex is involved with maintaining the oligo U tail of the gRNAs, and the MRP complex is tentatively shown as involved with catalyzing the annealing of the mRNA and gRNA. The cytochrome pre-edited mRNA is shown annealed with the Cyb gRNA-II. The folding was made using MFold. (PER) Pre-edited region. The number of U's inserted at each editing site are indicated, as is the 5′ anchor helix. The black arrow indicates the start of the oligo U tail. Guiding nucleotides in the gRNA are circled. The mRNA/gRNA fold is reprinted, with permission, from Blum and Simpson (1990 [©Elsevier]).

Read 2003) found to be associated with gRNAs and mRNAs may also be associated with the functional editing machinery, but this remains to be investigated.

SUMMARY AND CONCLUSIONS

A great deal of progress has been made on the molecular mechanism of U-insertion/deletion RNA editing in the last few years, but much remains to be clarified. Much progress has been made in the biochemical dissection of the editing apparatus by combining the power of affinity tagging with mass spectrometry and the availability of the genome databases, but the precise biological roles and interactions of the variety of proteins in the L-complex are still largely unknown. In addition, the nature and composition of the RET1 complex which is responsible for maintaining the length of the 3′ oligo U tails of the gRNAs are still obscure. Furthermore, the mechanism determining processivity of editing has not been defined, either processivity within a gRNA-mediated block from site to site or within a multiple gRNA-mediated domain from block to overlapping block. A beginning has been made on reconstruction of editing activities using recombinant proteins, but progress is dependent on developing more efficient assays for partial reactions of editing.

SPECULATIONS: URIDINE INSERTION/DELETION RNA EDITING AS A PARADIGM FOR SITE-SPECIFIC MODIFICATIONS OF RNA MOLECULES IN GENERAL

The term, RNA editing, was first used for the U-insertion/deletion editing of mRNAs in trypanosomatid mitochondria (Benne et al. 1986) but was subsequently used to describe a variety of post- and co-transcriptional RNA modifications such as the site-specific cytidine deamination in the apoB mRNA in mammals (Bostrom et al. 1989) and the adenosine deaminations in certain nervous system-related mRNAs in mammals (Sommer et al. 1991) and insects (Palladino et al. 2000), the co-transcriptional addition of G residues in negative-strand RNA viruses (Vidal et al. 1990) and the apparently co-transcriptional insertion of multiple cytidine residues in *Physarum* mitochondrial RNAs (Mahendran et al. 1991; Byrne and Gott 2002). Always lurking in the historical darkness was the specter of the many highly conserved but functionally enigmatic nucleotide modifications found in eukaryotic and archaeal ribosomal RNAs and tRNAs (Decatur and Fournier 2002).

majority of cases involves simple base-pairing by *trans*- or *cis*-acting molecules. True, there are some cases in which nucleic-acid-binding proteins alone determine site specificity, but these are becoming a real minority. The existence of indiscriminate RNA and DNA editing merely strengthens the argument that specific editing requires base-pairing.

In any case, since language is neither true nor false but simply a device to communicate more effectively, it may help in the understanding of all these diverse phenomena to use a common designation based on a common mechanism of site determination.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the National Institutes of Health (AI09102). I thank Dr. Guanghan Gao for a critical review of the paper, and I thank all the postdoctoral fellows and graduate students in my laboratory over the years who have contributed to the research reviewed.

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