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Role of RNA Structure in RNA Editing

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The coding sequences of some mRNAs and the structures of some tRNAs and rRNAs in a variety of organisms are altered by posttranscriptional reactions termed editing; this can involve the modification or insertion/deletion of nucleosides. In this review, we focus on the role of RNA structure in three types of editing reactions that are known to alter the coding sequences of mRNAs: a cytosine deamination required for apoB-48 synthesis, an adenosine deamination required in some of the mammalian glutamate receptor subunits and also during the life cycle of the hepatitis delta virus, and the uridine insertion and deletion reactions needed to create functional open reading frames in many of the mitochondrial transcripts in the trypanosomatids. Although the contribution of RNA structure to these various types of RNA editing is just beginning to be understood, we attempt to summarize some of the recent advances in this field.

C TO U SUBSTITUTION EDITING IN THE APOLIPOPROTEIN B mRNA

Background

Apolipoprotein B (apoB) is a major protein of plasma lipoproteins and exists in two forms, apoB-100 and apoB-48 (Kane et al. 1980). In mammals, apoB-100 (512 kD) is synthesized in the liver. It is the ligand for the low-density-lipoprotein receptor and is also a component of very low density lipoproteins and intermediate-density lipoproteins (for review, see Chen et al. 1990). In contrast, apoB-48 (241 kD) is synthesized in the small intestine and is present in chylomicron and chylomicron remnants; apoB-48 is also produced by the liver in rodents (mice and rats) (for review, see Chen et al. 1990).

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Both forms of apoB are the product of the same gene. ApoB-48 mRNA is produced from apoB-100 mRNA by RNA editing (Chen et al. 1987; Powell et al. 1987). The editing involves the deamination of cytosine 6666 to a uracil (U), which results in the conversion of a glutamine codon (CAA) to a stop codon (UAA); the size of the resulting truncated open reading frame is 48% of that of apoB-100.

Structural Requirements

Most of the 14-kb apoB mRNA is dispensable for the deamination reaction. The minimal editing cassette consists of a sequence of approximately 26 nucleotides immediately surrounding the editing site that is highly conserved between species and by itself is able to support editing at wild-type levels in vivo when placed in the context of 900 bases of distal apoB sequence (Davies et al. 1989). The 55 nucleotides immediately surrounding the editing site are sufficient for in vitro editing using an S100 extract from rat hepatoma cells, but the 26-nucleotide cassette only supports the in vitro editing at wild-type levels if it is embedded within sufficient bulk RNA (Driscoll et al. 1989). There are three major sequence elements surrounding the editing site that have been identified within the 26-nucleotide cassette: an essential 11-nucleotide sequence 3′ of the editing site, a spacer sequence between the 11-nucleotide block and the editing site, and an enhancer-like sequence immediately upstream of the editing site (Fig. 1) (Chen et al. 1990; Shah et al. 1991; Backus and Smith 1992).

Alteration of an 11-nucleotide block 5 nucleotides downstream from the editing site (6671-6681) drastically affects or abolishes editing both in vitro and in vivo (Backus and Smith 1991; Shah et al. 1991; Driscoll et al. 1993); G6677 is the only nucleotide within this block that could be altered without a deleterious effect (Fig. 1) (Shah et al. 1991). The 11-nucleotide block is perfectly conserved in the five species that have been examined (Teng and Davidson 1992). This 11-nucleotide block has been termed the "mooring sequence," and it has been hypothesized that it functions to position a deaminase over the editing site (Smith et al. 1991). Since editing only takes place on cytidines upstream of the 11-nucleotide block (Backus et al. 1994), it has also been suggested that the block in some manner orients the deaminase on the mRNA.

The space between the editing site and the 11-nucleotide block is important to both in vitro and in vivo editing (Chen et al. 1990; Backus and Smith 1992; Driscoll et al. 1993). There are normally four bases between the editing site and the start of the 11-nucleotide block. Deletion of a single nucleotide from the spacer region is tolerated to some extent, but

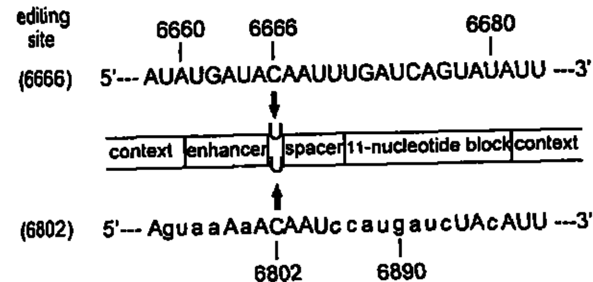

Figure 1 Nucleotide sequence surrounding the editing sites at nucleotides 6666 and 6802 of the human apoB mRNA. The elements that are necessary for the efficient editing of site 6666 are indicated below the sequence by boxes: an 11-nucleotide block, a 4-nucleotide spacer sequence, and an upstream sequence that can enhance the efficiency of the reaction. The efficiency of editing is also influenced by the context of the mRNA in which the three sequence elements are placed. The nucleotides flanking editing site 6802 that differ from the corresponding position around site 6666 are indicated by lowercase letters.

larger deletions severely impair or completely eliminate editing (Backus and Smith 1992; Driscoll et al. 1993). Insertions reduce editing activity, but overall are less detrimental than deletions; a 12-nucleotide spacer sequence only reduced in vitro editing 65-fold (Backus and Smith 1992). Although there is much flexibility in the sequence of the spacer nucleotides, there are also a few substitutions that are detrimental (Chen et al. 1990).

The nucleotides immediately 5′ of the editing site are not sufficient nor absolutely required for both in vitro and in vivo editing, but they can function to enhance the efficiency of the reaction (Shah et al. 1991; Backus and Smith 1992). The 5 nucleotides immediately upstream of the editing site (UGAUA) can be entirely substituted with As with no effect on in vitro editing efficiency (Backus and Smith 1992). Some mutations within this same region, however, can either enhance (up to twofold) or decrease (down to fivefold) editing activity both in vitro and in vivo (Shah et al. 1991; Backus and Smith 1992). The effect of the sequence farther upstream seems to be dependent on the nature of the RNA construct used in the assay (Backus and Smith 1991, 1992; Driscoll et al. 1993).

The importance of the three sequence elements to both in vitro and in vivo editing was illustrated when they were translocated adjacent to a C that is normally not edited in apoB (Backus and Smith 1991, 1992; Driscoll et al. 1993) or adjacent to a C within a luciferase mRNA (Driscoll et al. 1993). The 11-nucleotide block alone is able to induce editing at

heterologous sites within apoB. The level of editing, however, does not approach that of site 6666 until the size of the spacer sequence and the sequence of the five bases immediately upstream of the heterologous site match that of the normal editing site (Backus and Smith 1992). Placing these three elements in the context of a luciferase gene induced editing, but the level was only 13% of that of an apoB construct, which suggests that other undefined elements may also be important (Driscoll et al. 1993).

The chicken apoB mRNA differs from the mammalian mRNAs in several positions within the three identified sequence elements (Anant et al. 1995), and it is not edited in chickens, nor is it edited when added to rat enterocyte S100 extracts that support both human and rat apoB editing. Both the 11-nucleotide block and the 3′- and 5′-flanking sequences had to be altered to the mammalian sequence in order to recover in vitro editing of the chicken message; a correct 11-nucleotide block by itself was not sufficient. The recovered editing activity was about ninefold less than that of the rat apoB mRNA (Anant et al. 1995), suggesting that although the identified sequence elements are sufficient to induce editing, other factors are also influencing the efficiency of the reaction.

The context in which the three identified sequence elements are placed has a significant effect on editing (Boström et al. 1990; Backus and Smith 1994). A G-C-rich context can lower or completely inhibit both in vitro and in vivo editing (Boström et al. 1990; Backus and Smith 1994). Having an A-U-rich sequence 3′ of the mooring sequence seems especially important for efficient editing (Backus and Smith 1994).

A functional complementation assay for apoB editing identified a 27-kD protein from rat intestine that was required but not sufficient for in vitro editing (Teng et al. 1993). The protein, designated APOBEC-1 (apolipoprotein B mRNA-editing enzyme catalytic polypeptide #1), is a cytidine deaminase and presumed to be the catalytic subunit of the apoB mRNA-editing enzyme (Navaratnam et al. 1993b). UV cross-linking results suggest that it binds to an A-U-rich region (6656-6661) upstream of the edited C and also to an A-U-rich segment (6678-6683) overlapping the 3′end of the 11-nucleotide block. Because the upstream sequence can be deleted without abolishing editing (Backus and Smith 1991; Shah et al. 1991), the sequence overlapping the 11-nucleotide block was proposed to be the critical region (Navaratnam et al. 1995). The affinity of APOBEC-1 for A-U-rich sequences (Anant et al. 1995) may partially explain the importance to the editing reaction of an A-U-rich sequence 3′ of the 11-nucleotide block. In addition, a 60-kD protein, proposed to play a part in the editing reaction, was shown to cross-link to the 5′end of the 11-nucleotide block (6671-6674 [UGAU]), but its significance has yet to be proven (Navaratnam et al. 1993a).

An additional site (6802) in apoB is edited both in vivo and in vitro (Fig. 1) (Navaratnam et al. 1991). Editing converts a threonine codon (ACA) to an isoleucine codon (AUA). The in vitro editing efficiency of this site was at 10–15% that of site 6666. There is surprisingly little similarity in the sequence of the 11-nucleotide block at the two sites, and the spacer region, a region that was shown to have a relaxed sequence requirement, is identical to site 6666 in three of the four positions (Fig. 1). This again suggests that the sequence elements specifying an editing site have not yet been completely defined. Previously, As had been shown to be able to substitute for the four bases upstream of editing site 6666 (Backus and Smith 1992), and these are also found upstream of site 6802.

The primary sequence seems to be a major factor in identifying the location of an apoB-editing site, but it is not yet clear whether the conserved nucleotide sequence is forming an important secondary structure or whether it is being directly recognized by the editing machinery. It is possible, for example, that the 11-nucleotide block of the second apoB-editing site, although different in primary structure, could be forming a similar secondary structure. Other examples exist of RNAs with different primary structures forming similar secondary and tertiary structures (compare Heus and Pardi 1991 with Cheong et al. 1990; Connell and Yarus 1994). Very little is known about the structure of the apoB RNA surrounding the editing site. A predicted secondary structure puts the editing site in a stem loop (Navaratnam et al. 1993a), but it is not consistent with the mutagenesis results. Chemical probing of this RNA combined with SELEX type experiments (Ellington and Szostak 1990; Tuerk and Gold 1990; see also Baskerville et al., this volume) would be useful in revealing subtle structures that could be required for this reaction.

DOUBLE-STRANDED RNA-SPECIFIC ADENOSINE DEAMINASE

Background

Double-stranded RNA adenosine deaminase (abbreviated dsRAD or DRADA) catalyzes the deamination of A to inosine (I) (Wagner et al. 1989; Polson et al. 1991). This enzyme has been purified from several sources (Hough and Bass 1994; Kim et al. 1994a,b). Since I is treated as G by the translation machinery (Basilo et al. 1962), the deamination reaction effectively represents an A to G transition and therefore has the potential to alter the coding sequence of an mRNA.

Structural Requirements

The deamination reaction requires a double-stranded RNA substrate (Bass and Weintraub 1988; Wagner et al. 1989). Multiple As are deaminated exclusively within dsRNA (Bass and Weintraub 1988; Wagner et al. 1989), and both inter- and intramolecular helices can be used as substrates (Nishikura et al. 1991). The reaction is not competed by ssRNA, ssDNA, or dsDNA (Bass and Weintraub 1987; Wagner et al. 1989). dsRAD/DRADA modifies dsRNAs of many different sequences, suggesting that the binding to the dsRNA is not sequence-specific (Bass and Weintraub 1988).

The level of editing increases with helix length and is maximal with lengths greater than 100 bp (Nishikura et al. 1991). Up to 50% of the As in both strands of a given RNA can be modified in RNAs >100 bp (Bass and Weintraub 1988; Nishikura et al. 1991). dsRNA regions less than 38 bases are not efficiently modified by dsRAD/DRADA (Nishikura et al. 1991). It has been postulated that fewer deaminations occur in shorter RNAs because the deaminations result in I-U base pairs that destabilize and/or denature the helix, preventing the double-stranded-specific deaminase from acting at subsequent sites (Polson and Bass 1994).

In short dsRNAs (36 bp), not all As are modified to the same extent (Polson and Bass 1994). dsRAD/DRADA has a 5′ nearest neighbor preference (A=U>C>G) (Kimelman and Kirschner 1989; Polson and Bass 1994); an A adjacent to a 5′G, for example, correlates with inefficient deamination, whereas a 5′A or U correlates with efficient modification. With perfectly paired synthetic dsRNA substrates, there does not appear to be a 3′ nearest neighbor preference (Polson and Bass 1994). There is a preference for the modification of those As more 5′ within the helix than 3′, which suggests that the deaminase has a binding polarity. As very close to the termini of the helix are not modified efficiently, but the deamination reaction is not affected by a 3′ or 5′ overhang (Polson and Bass 1994).

Biological Reactions Mediated by dsRAD/DRADA

dsRAD/DRADA is an enzyme that has been in search of a biological substrate since its initial discovery as a double-stranded RNA-unwinding/modification enzyme. It is found throughout the animal kingdom (Bass 1992) and in most mammalian cell lines and tissues (Wagner et al. 1990). Several candidate RNAs that fit the criteria of a dsRAD/DRADA substrate have been proposed, and many of these have already been reviewed (Bass 1992, 1993). We discuss below the RNA structures required for the editing of the mammalian glutamate receptors and the hepatitis delta virus antigenome. Evidence presented, particularly within the last 2 years, has made a compelling argument for these reactions being mediated either by dsRAD/DRADA or a very closely related enzyme.

GLUTAMATE RECEPTOR SUBUNITS

Background

Glutamate is the major excitatory neurotransmitter in the mammalian brain. It gates several different cation-selective channels. The channels can be divided into three pharmacologically distinct classes based on their sensitivity to the glutamate agonists: the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic (AMPA), and kainate (for review, see Sommer and Seeburg 1992).

The non-NMDA glutamate-responsive channels consist of several structurally homologous subunits (for review, see Hollmann and Heinemann 1994). The AMPA receptor comprises four subunits designated GluR-A to GluR-D, whereas subunits designated GluR-5 to GluR-7 are unique to the high-affinity kainate receptor. Transcripts from five of these genes undergo editing at eight sites that results in the channel having either altered ion permeability or kinetic properties (Sommer et al. 1991; Kohler et al. 1993; Lomeli et al. 1994).

Structural Requirements for Editing of the Q/R Site in GluR-B

Q/R site editing occurs in the kainate receptor subunits GluR-5 and GluR-6 as well as in GluR-B, an AMPA receptor subunit. Editing results in a glutamine (Q) codon (CAG) being altered to an arginine (R) codon (CGG) (Sommer et al. 1991). In the rat brain, about 40% of the GluR-5 subunits are edited, 70% of GluR-6, and >99% of GluR-B (Sommer et al. 1991). The structural requirements for Q/R site editing were initially characterized for the GluR-B subunit.

The gene for GluR-B consists of 17 exons spread across 80 kb of DNA, most of which are not required for editing. The Q/R site of GluR-B is located in exon 11, and 85 nucleotides of this exon, together with the proximal 380 nucleotides of intron 11, are able to support editing at a wild-type level (Higuchi et al. 1993; Egebjerg et al. 1994). The three AMPA subunits that are not edited at the Q/R site have a 90–95% identity with the sequence surrounding the editing site of GluR-B, but they do not contain the intron (Higuchi et al. 1993; Egebjerg et al. 1994).

When the intron was placed downstream from the normally unedited GluR-C exon, Q/R site editing was induced (Egebjerg et al. 1994).

The critical intron contains 10 nucleotides of perfect complementarity to the exon sequence encompassing the editing site (Fig. 2). The complementarity is extended to 17 bases if G-U pairs are considered (Higuchi et al. 1993; Egebjerg et al. 1994). This part of the intron complementary to the exon sequence flanking the editing site is termed the editing site complementarity sequence (ECS). Mutations that disrupt the predicted helix also decrease or abolish both in vivo and in vitro editing and can be compensated with secondary mutations that restore the predicted base-pairing (Higuchi et al. 1993; Rueter et al. 1995; Yang et al. 1995). There is also some sequence-specificity, however, in the bases immediately 3′ and 5′ of the editing site, because not all of the compensatory mutants at these positions resulted in wild-type levels of editing.

The intron contains an imperfect inverted repeat that is also essential for both in vivo and in vitro editing (Higuchi et al. 1993; Egebjerg et al. 1994; Rueter et al. 1995; Yang et al. 1995). The units of the repeat are approximately 45 nucleotides in length and have a sequence match of 84%. The units are separated from each other by 130 bases that can be deleted without a decrease in editing efficiency (Higuchi et al. 1993; Rueter et al. 1995; Yang et al. 1995). Compensatory mutations have confirmed part of the predicted helix (Higuchi et al. 1993).

There are several additional As near the Q/R site that are edited but with no physiological significance. The editing of a site four bases 3′ of

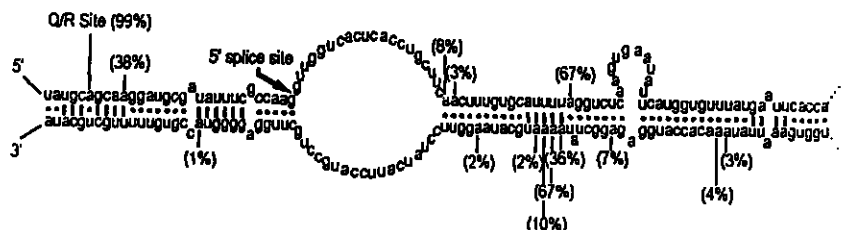

Figure 2 Predicted secondary structure of the GluR-B mRNA surrounding the Q/R editing site (Egebjerg et al. 1994; Bass 1995). Predicted base pairs are indicated by dots, whereas those pairings confirmed by compensatory mutants are indicated by solid lines; there is not yet any other experimental evidence available to support the remaining parts of the structure. The percentage of editing at each position that occurs in the mRNA isolated from rat brain (Rueter et al. 1995) is indicated in parentheses and the location of the 5′ intron splice site is also shown.

the Q/R site results in a glutamine codon (CAA) being converted to a CAG, another glutamine codon (Higuchi et al. 1993; Egebjerg et al. 1994). Numerous As within the intron are also edited in the mRNA isolated from the rat brain (Rueter et al. 1995); there are three major editing sites in addition to nine sites that are not edited in more than 10% of the mRNA pool (Fig. 2). The multiple targeting of As for modification is consistent with the activity of a dsRAD/DRADA-like enzyme.

The 5′ nearest neighbor preference for editing is also in agreement with that determined for dsRAD/DRADA (A=U>C>G) in that a 5′ G is not present in any of the five major editing sites and in only one of the nine minor sites (Fig. 2) (Rueter et al. 1995). A compensatory mutation that preserved the helix but resulted in a G being placed immediately 5′ of the Q/R site also significantly reduced editing (Higuchi et al. 1993).

The similar structural requirements and chemistries (Polson et al. 1991; Melcher et al. 1995; Rueter et al. 1995; Yang et al. 1995) of dsRAD/DRADA-mediated deamination and Q/R site-editing led to the proposal that they are the same reaction (Sommer et al. 1991; Higuchi et al. 1993; Köhler et al. 1993; Kim et al. 1994a; Melcher et al. 1995), but there are also some significant differences. When a crude nuclear extract supporting editing is fractionated on a dsRNA affinity column, the fraction enriched in GluR-B RNA editing eluted at a higher salt concentration than most of the dsRAD/DRADA deaminase activity (Yang et al. 1995). Although in vitro editing of GluR RNA can be competed with various dsRNAs (Melcher et al. 1995; Rueter et al. 1995; Yang et al. 1995), GluR-B RNA competes for its own editing better than synthetic dsRNAs (Rueter et al. 1995), and the dsRAD/DRADA deaminations are not competed by GluR-B RNA (Yang et al. 1995). Furthermore, the preferred sites of in vitro editing in GluR-B RNA are different from those using completely base-paired substrates (GluR-B RNA annealed to its perfect complement) (Yang et al. 1995).

It has been proposed that some of the differences between dsRAD/DRADA-catalyzed deaminations and GluR-B editing could be the result of the editing reaction requiring dsRAD/DRADA to be in a complex with other components (Bass 1995). The tissue-specific and developmental regulation of the editing of the glutamate receptor subunits also points to additional components being required (Egebjerg et al. 1994; Lomeli et al. 1994; Ruano et al. 1995), because dsRAD/DRADA is fairly ubiquitous (Wagner et al. 1990). dsRAD/DRADA purified from *Xenopus* (Hurst et al. 1995) or recombinantly expressed (Dabiri et al. 1996) efficiently deaminates the As within the intron but only edits the Q/R site either at a very low level (Hurst et al. 1995) or not at all (Dabiri et al.

1996). However, with the addition of cofactor protein(s) that is present in both neuronal and nonneuronal cells, the pattern of editing with the recombinant dsRAD/DRADA matches that in vivo (Dabiri et al. 1996). The cofactor appears to restrict access of dsRAD/DRADA to the intronic editing site and to increase site-specific editing at the Q/R site (Dabiri et al. 1996).

Alternatively, the differences between dsRAD/DRADA-catalyzed deaminations and the editing of the Q/R site may suggest that the editing reaction is not catalyzed by dsRAD/DRADA, but instead by a related enzyme. RED1, a dsRNA deaminase that has recently been cloned from a rat brain cDNA library, is by itself very efficiently able to edit the Q/R site in vitro (Melcher et al. 1996). Gene knock-out studies will probably be required to unambiguously identify the enzyme that edits this site in vivo.

Other Physiologically Significant Editing Sites

Editing of the Q/R site in both GluR-5 and GluR-6 also requires that the flanking sequence be base-paired with intronic sequences (Herb et al. 1996). The structure of the mRNA required for the editing of these sites, however, differs from that in GluR-B. Whereas the editing site of GluR-B is in a Watson-Crick base pair (Fig. 1), the editing site in both GluR-5 and GluR-6 is in an asymmetric internal loop, and the ECS is located as far as 1900 nucleotides distal to the site. The editing, however, also appears to be mediated by a dsRAD/DRADA-like activity, because coexpression of GluR-6 with dsRAD/DRADA in HEK 293 cells resulted in deamination.

Editing also results in a specific arginine (R) codon (AGA) being changed to a glycine (G) codon (GGA) in the GluR-B, GluR-C, and GluR-D subunits (Lomeli et al. 1994). The R/G site is similar to the Q/R sites in that editing is dependent on a distal intron that contains an analogous ECS. It differs from the Q/R sites in that the distal regions of the intron, rather than being a prerequisite for editing, act more as enhancers of the activity (Lomeli et al. 1994). The site in all three subunits also differs from the Q/R sites in that the edited A is mismatched opposite a C. Both RED1 and dsRAD/DRADA are able to edit this site in vitro (Melcher et al. 1996).

In addition to the Q/R site, the GluR-6 subunit is also edited at two other physiologically significant sites that result in an isoleucine codon (AUU) being altered to a valine codon (GUU) and a tyrosine codon (UAC) being converted to a cysteine codon (UGC) (Köhler et al. 1993).

Little is known about the structural requirements of these editing sites; it seems likely, however, that they will also involve a dsRNA deaminase. The additional structural information obtained from these sites will be particularly useful in understanding the role of RNA structure in the developmental and tissue-specific regulation of the editing of the glutamate receptor subunits.

HEPATITIS DELTA VIRUS

Background

Hepatitis delta virus (HDV) is a subviral satellite of hepatitis B (Kuo et al. 1989). It has a single-stranded circular 1.7-kb genome that has significant intramolecular complementarity; 70% can form base pairs in an unbranched rod structure. Hepatitis delta antigen (HDAg) is the only protein encoded by the genome.

HDAg exists as both 24-kD and 27-kD products; the 24-kD protein is necessary for replication (Kuo et al. 1989), whereas the 27-kD protein inhibits replication (Chao et al. 1990) and is necessary for packaging of the genome (Chang et al. 1991). The size heterogeneity results from a UAG to UGG change that converts a stop codon to a tryptophan codon (referred to as the amber/W site [Tang et al. 1994]). Editing results in a 19-amino-acid extension being added to the carboxyl terminus of the protein (Zheng et al. 1992).

There has been considerable confusion in this field as to whether the editing is the result of an A to G transition on the antigenomic strand, or the result of the replication of a U to C transition on the genomic RNA (Casey et al. 1992; Zheng et al. 1992; Casey and Gerin 1995; Wu et al. 1995). The general consensus reached during the last year, however, is that the editing is a result of the deamination of an A on the antigenomic strand (Casey and Gerin 1995; Wu et al. 1995; Polson et al. 1996).

Structural Requirements

The structural requirements for the deamination reaction at the amber/W site are strongly suggestive that a dsRAD/DRADA-like enzyme is involved. A 10-bp duplex structure surrounding the amber/W site is predicted (Casey et al. 1992), and compensatory mutations confirmed that the helix is required for editing both in vivo (Casey et al. 1992) and in vitro (Polson et al. 1996).

There are additional As near the amber/W site that are also deaminated in antigenomic RNA isolated from HDV after successive passages

in woodchucks (Netter et al. 1995). This editing results in an isoleucine codon (AUA) being converted to a valine codon (GUA or GUG) and, as such, represents a fairly conservative change. The 5′-flanking nucleotide of the amber/W site, as well as that of the two adjacent editing sites, is a U. The As in the vicinity of the amber/W site that were not edited have either a 5′-flanking G or a C; these results have to be interpreted cautiously because these As are within the protein's coding sequence and as such could also be under strong selective pressures not to be edited. The data, however, are in agreement with the 5′ nearest-neighbor preference of dsRAD/DRADA (A=U>C>G). Also in agreement, a mutation that created a potential editing site with a 5′-flanking G was not edited (Casey et al. 1992).

The amber/W site is edited in vitro using dsRAD/DRADA purified from *Xenopus laevis* eggs (Polson et al. 1996). dsRAD/DRADA on average only modified 3 As in an antigenome that has approximately 340; this was done under conditions that deaminate more than 50% of the As in an 800-bp completely duplexed RNA. The secondary structure of the HDV RNA has been proposed to provide additional selectivity to the reaction.

Editing of the amber/W site also requires some structural features that cannot be explained by the known substrate specificity of dsRAD/DRADA. Like the R/G site, an A-C mismatch at the editing site is required for efficient editing (Casey et al. 1992). In addition, although there has not been a 3′ preference described for dsRAD/DRADA, the base immediately 3′ of the amber/W site has a sequence-specific role (Casey et al. 1992), and the same effect was seen with the Q/R site of GluR-B (Higuchi et al. 1993). It is possible that additional selectivity of a dsRAD/DRADA-catalyzed reaction may be provided by the bulges and internal loops that interrupt the double-stranded region of the GluR-B RNA; the selectivity rules were determined using artificial perfectly paired dsRNAs that did not have these additional structural features (Hurst et al. 1995). Understanding the role of these additional structural determinants is one of the new frontiers in this field.

URIDINE INSERTION/DELETION RNA EDITING IN KINETOPLASTID MITOCHONDRIA

Background

In the mitochondrion of the parasitic trypanosomatid protozoa, the transcripts of approximately 12 of the 20 genes encoded in the maxicircle molecule are edited to varying extents by the insertion and occasional

deletion of uridine (U) residues (Simpson et al. 1993; Benne 1994; Simpson and Emerson 1996). The editing overcomes frameshifts and creates continuous open reading frames, and frequently creates AUG methionine translation initiation codons. The genes whose transcripts are edited are termed cryptogenes. The transcripts of certain cryptogenes in some species are "pan-edited" by the addition and deletion of hundreds of Us at hundreds of sites. A similar type of editing has also been detected in the mitochondrion of the related kinetoplastid protozoan, *Trypanoplasma borelli* (Lukes et al. 1994; Maslov and Simpson 1994).

Editing proceeds 3′ to 5′, giving rise to a variety of incompletely edited RNAs in steady-state mitochondrial RNA (Abraham et al. 1988; Sturm and Simpson 1990a; Maslov and Simpson 1992). Frequently, incorrectly edited or "misedited" sequences occur at the junction regions between fully edited and fully unedited regions of the transcripts, and these are apparently subsequently corrected, at least in some cases, by further 3′ to 5′ editing (Decker and Sollner-Webb 1990; Sturm and Simpson 1990a).

Guide RNAs

The U insertion/deletion type of editing is mediated by small 3′-oligo-uridylated RNA molecules termed guide RNAs or gRNAs, which are complementary to short sections or blocks of the edited transcripts, if G-U base pairs are allowed (Blum et al. 1990; Pollard et al. 1990). The editing information is contained in A and G guiding nucleotides, which can form base pairs with the inserted U residues. Each gRNA mediates the editing of a block of the pre-edited mRNA, and adjacent blocks overlap with each other; the 3′ to 5′ polarity is determined by the creation of upstream anchor sequences by downstream editing (Maslov and Simpson 1992). The gRNAs possess a non-encoded 3′ oligo[U] tail approximately 15 nucleotides long (Blum and Simpson 1990). It was proposed on the basis of RNA folding (Blum and Simpson 1990) that the initial hybrid structure formed between the pre-edited mRNA and the cognate gRNA involved not only a duplex anchor region, but also a duplex region produced by base-pairing of the 3′ oligo[U] tail with the purine-rich pre-edited region itself, with a resultant internal single-stranded loop containing the guiding region of the gRNA (Fig. 3). However, there is as yet no structural evidence for this hypothesis.

Guide RNAs are not free in the mitochondrial extracts, but are bound to one or more proteins, forming ribonucleoprotein complexes (Göringer et al. 1994; Köller et al. 1994; Peris et al. 1994; Read et al. 1994; Shu et

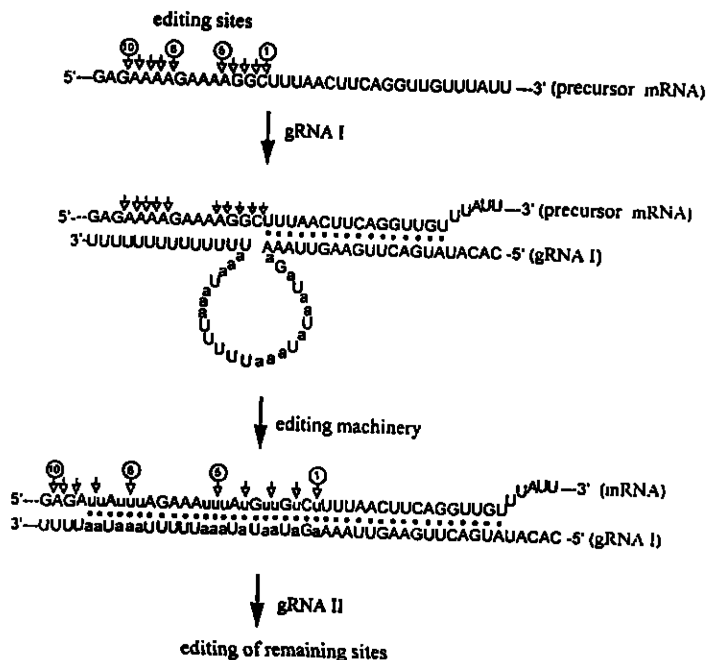

Figure 3 Proposed interaction of precursor Cyb mRNA from *L. tarentolae* and cognate gCyb-I gRNA. The gRNA is shown base-paired to the mRNA just 3′ of the first editing site. This pairing is supported both by phylogenetic data and by in vitro studies. The helix can potentially be extended through an interaction of the poly U tail with the purine-rich sequence flanking the editing sites. As a result of this interaction, the guiding nucleotides (*lowercase*) would be present in some type of internal loop structure. Editing by the first gRNA creates an anchor-binding site for a second gRNA that can mediate the editing at the remaining sites.

al. 1995) that, as monomers, sediment at approximately 10S and have been visualized in the electron microscope. These so-called "T-IV-complexes" (Peris et al. 1994) also exhibit 3′-terminal uridylyl transferase (TUTase) activity. Several proteins that can be UV cross-linked to gRNA have been identified from *Leishmania tarentolae* (Bringaud et al. 1995; Byrne et al. 1995), *Trypanosoma brucei* (Göringer et al. 1994; Köller et al. 1994; Read et al. 1994), and *Crithidia fasciculata* mitochondrial extracts (Leegwater et al. 1995).

Peris et al. (1997) have recently described a novel class of heterodisperse complexes that sediment from 10S to 30–40S in glycerol gradients and apparently represent complexes of T-IV subunits with mRNA. A 70-kD polypeptide was identified that was a component of both the T-IV and the heterodisperse gRNA-containing complexes.

The secondary structures at physiological conditions of four different gRNAs from *T. brucei* have been analyzed (Schmid et al. 1995). The use of structure-sensitive chemical and enzymatic probes showed that the four molecules, despite differences in primary sequences, folded into similar structures consisting of two imperfect hairpin loops of low thermodynamic stability. Both the 5′ and 3′ termini appeared to be single-stranded. It was proposed that these gRNA structures represent a mechanism to minimize stability in order to optimize the annealing reaction with the pre-mRNAs, while at the same time to permit the assembly together with other components into the editing complexes.

Some of the gRNAs are located on the maxicircle DNA (Blum et al. 1990), but the majority are located on the minicircle molecules (Pollard et al. 1990; Sturm and Simpson 1990b, 1991; Pollard and Hajduk 1991; Riley et al. 1994). The complexity of the gRNA repertoire varies between species from as low as 20–30 different gRNAs in the old UC laboratory strain of *L. tarentolae* (Thiemann et al. 1994), to 60–80 different gRNAs in the recently isolated LEM125 strain of *L. tarentolae* (Thiemann et al. 1994), to as high as an estimated 200–300 different gRNAs in *T. brucei*. "Redundant" gRNAs exist in some species which encode the same editing sequence information, but differ in sequence, as a result of allowing G-U base-pairing between the gRNA and the mRNA (Corell et al. 1993; Thiemann et al. 1994; Avila and Simpson 1995).

gRNA/mRNA Chimeric Molecules

Chimeric gRNA/mRNA molecules can be detected in steady-state mitochondrial RNA in vivo by amplification using downstream edited and upstream unedited polymerase chain reaction (PCR) primers or can be produced in vitro by addition of gRNA and pre-edited mRNA to mitochondrial extracts (Blum et al. 1991; Blum and Simpson 1992; Harris and Hajduk 1992; Koslowsky et al. 1992; Read et al. 1992). The in vivo chimeras represent molecules in which the gRNAs are covalently attached to the mRNAs at editing sites and the downstream editing sites are fully edited. The in vitro chimeras represent molecules in which the downstream sites are usually unedited. A requirement for the presence of a complementary anchor sequence for the in vitro formation of chimeras was demonstrated by showing that mutations in the mRNA anchor inhibited chimera formation, which was restored by complementary mutations in the synthetic cognate gRNA (Blum and Simpson 1992).

The secondary structures of three ATPase 6 gRNA/mRNA chimeric molecules from *T. brucei* were determined by the use of chemical and

enzymatic probes (Schmid et al. 1996). All three molecules exhibited a bipartite domain structure consisting of a gRNA/pre-mRNA interaction hairpin and an independently folding mRNA stem/loop. Additional higher-order structural features were detected by optical melting analysis, with four defined melting transitions between 10°C and 90°C.

It is not known if chimeras are intermediates or nonfunctional byproducts of the editing reactions. However, evidence from an in vitro system from *T. brucei* in which U deletions are directed by added synthetic gRNA suggests that chimeras are formed as a consequence of the destabilization of the double-stranded structure formed by basepairing of the oligo[U] tail with the pre-edited sequence of the mRNA (Seiwert et al. 1996). Use of synthetic gRNAs with longer oligo[U] tails resulted in an inhibition of formation of chimeras but had no effect on the appearance of the final product with the expected site-1 deletions.

In Vitro RNA Editing Systems

Several in vitro systems have been reported that show possible RNA editing-like activities in mitochondrial extracts. In the case of *T. brucei*, U-deletion and U-insertion activities were described in which the number of Us deleted at site 1 or inserted at site 2 of the A6 (=MURF4) pre-edited mRNA is determined by the number of guiding nucleotides in added synthetic cognate gRNA (Corell et al. 1996; Cruz-Reyes and Sollner-Webb 1996; Kable et al. 1996; Seiwert et al. 1996). This system has enabled a direct visualization of 5′- or 3′-end-labeled putative intermediate molecules by gel electrophoresis.

In the case of *L. tarentolae*, two different but probably related activities have been reported for two pre-edited mRNAs, cytochrome b (Cyb) and NADH dehydrogenase subunit 7 (ND7) (block II): a gRNA-independent activity in which a variable number of Us are inserted at multiple sites within the pre-edited region (Frech et al. 1995; Byrne et al. 1996; Frech and Simpson 1996; Connell et al. 1997), and a gRNA-dependent activity in which the number of Us inserted at a specific editing site is determined by the number of guiding nucleotides in the added synthetic gRNA (Byrne et al. 1996; Connell et al. 1997). The gRNA-independent activity within the pre-edited region was detected by an RNase H digestion assay (Frech et al. 1995), and within specific sites of the pre-edited region by sensitive direct and indirect primer extension assays (Byrne et al. 1996; Connell et al. 1997). The gRNA-dependent activity from *L. tarentolae* mitochondria could only be assayed by the primer extension reaction due to the low efficiency of this process in vitro (Byrne et al. 1996; Connell et al. 1997). Both the gRNA-dependent U-deletion activity from *T. brucei* and the gRNA-independent U-insertion activity from *L. tarentolae* sedimented in glycerol gradients at approximately 20S (Peris et al. 1994, 1997; Corell et al. 1996).

Models for U-insertion/deletion Editing

In the enzyme cascade model (Blum et al. 1990), there is an initial cleavage at the first mismatched base of the pre-edited mRNA adjacent to the gRNA/mRNA anchor duplex, followed by the 3′ addition of a U residue from UTP to the 5′ mRNA cleavage fragment, and re-ligation of the mRNA. A variant of this model is one in which two successive cleavage-ligations lead to the transfer of a U residue from the 3′ end of the gRNA to the editing site (Sollner-Webb 1991). Another model invokes a transfer of U residues from the 3′ end of the gRNA to the editing site by two successive *trans*-esterifications, such as occur in RNA splicing (Blum et al. 1991; Cech 1991). Several enzyme activities have been identified in purified mitochondrial extracts which are candidates for involvement in editing in terms of the enzyme cascade model: a 3′-terminal uridylyl transferase (Bakalara et al. 1989), an RNA ligase which involves the adenylation of two proteins of approximately 50 kD and 45 kD (Bakalara et al. 1989; Sabatini and Hajduk 1995; Peris et al. 1997), an RNA helicase (Missel et al. 1995), and an endoribonuclease that cleaves within the pre-edited region (Harris et al. 1992; Simpson et al. 1992; Cruz-Reyes and Sollner-Webb 1996). A gRNA-mRNA chimeric-forming activity has also been reported (Blum and Simpson 1992; Harris and Hajduk 1992; Koslowsky et al. 1992; Rusche et al. 1995). The TUTase, RNA ligase, RNA helicase, and endonuclease activities from *T. brucei* mitochondrial extracts were found to cosediment at approximately 20S in glycerol gradients (Piller et al. 1995b; Rusche et al. 1995), together with the gRNA-mediated U-deletion activity (Corell et al. 1996). The RNA ligase and gRNA-independent U-insertion activities in mitochondrial extracts from *L. tarentolae* were also found to cosediment at 20–25S in glycerol gradients (Peris et al. 1997).

Direct analysis of putative editing intermediates in the *T. brucei* U-deletion and U-insertion system has led to the conclusion that the original enzyme cascade model (Blum et al. 1990) is essentially correct and that the Us are derived from UTP rather than from the 3′ end of the gRNA. A similar conclusion was reached by Byrne et al. (1996) in the *L. tarentolae* ND7 U-insertion system using an indirect primer extension analysis.

The Importance of Being Double-stranded

As discussed above, the primary interaction of the cognate gRNA with the pre-edited mRNA has been postulated to involve a double-stranded region produced by base-pairing of the 5′end of the gRNA with the mRNA sequence just downstream from the pre-edited region, which has been termed the 3′anchor. The 3′anchor duplex is essential for the initiation of both gRNA-dependent U-insertion and U-deletion editing in vitro, since mutations in the mRNA which abolish base-pairing inhibit these activities, and complementary mutations in the added gRNA restore them (Byrne et al. 1996; Seiwert et al. 1996).

The 3′anchor duplex was also shown to be required for formation of gRNA-mRNA chimeric molecules in vitro (Blum and Simpson 1992), and the presence of a stable duplex between an extended gRNA 3′oligo[U] tail and a purine-rich pre-edited sequence just upstream of the editing site being assayed was shown to inhibit this process (Seiwert et al. 1996), suggesting, as discussed above, that these molecules may be aberrant by-products of a cleavage-ligation mechanism of U-insertion/deletion.

The gRNA-independent U-insertion activity using Cyb pre-edited mRNA in *L. tarentolae* mitochondrial extracts also requires a specific secondary structure (Connell et al. 1997). The activity required the presence of an extended 5′end of the pre-edited mRNA and was inhibited by truncation of the 5′end to the natural 5′end of the mature edited Cyb mRNA. Structural analysis provided evidence that the role of the upstream sequence could be to stabilize a helix. Deletion of this upstream sequence led to inhibition of the U-insertion activity, which was restored by addition of a 5-bp G-C duplex; disruption of this helix again inhibited the activity. It was speculated that the intramolecular helix formed by the mRNA could be mimicking the intermolecular 3′anchor helix formed by the gRNA interacting with the mRNA. There is, however, no evidence that the upstream mRNA helix is present in vivo, and therefore there is no evidence that the gRNA-independent U-insertion activity observed in vitro is biologically meaningful. Similar results were obtained using synthetic ND7 pre-edited mRNA substrates (Byrne et al. 1996).

The creation of a recognition element for the assembly of the U-insertion machinery may represent an important role of the gRNA in the gRNA-mediated reaction. The recognition element could be the double-stranded RNA formed by the gRNA-mRNA interaction. The demonstration that the addition of cognate gRNA to the pre-edited mRNA results in a specific cleavage at the first mismatched base suggests that formation of the duplex creates a recognition site for an endonuclease (Cruz-Reyes and Sollner-Webb 1996; Seiwert et al. 1996). Other parts of the mRNA may also be involved in editing-site recognition. It has previously been proposed that the presence of the editing sites within a single-stranded loop is important for an endonucleolytic cleavage hypothesized by the enzyme cascade model to be part of the editing reaction (Harris et al. 1992; Simpson et al. 1992). Only limited evidence, however, has been provided in support of this hypothesis (Piller et al. 1995a,b).

COMMON THREADS

Although deaminations are involved in both the C to U substitution occurring in apoB editing and the A to I substitutions occurring in glutamate receptor editing, the structural requirements for the two editing reactions are different, and the catalytic mechanism and RNA-binding sites of the two deaminases are also different. This suggests that these editing reactions represent convergent evolution and chemical determinism rather than being the result of a common evolutionary root.

In comparing the adenosine deaminase and the U-insertion/deletion classes of editing reactions, double-stranded RNA appears to be a recognition element in both cases. Even though the chemistries of the two reaction classes are obviously different, the RNA structures that determine an editing site could still prove to be similar. However, a better characterization of the kinetoplastid editing reaction must be accomplished in order to evaluate this possibility.

A variety of other RNA editing systems exist which have not been discussed in this review because of the absence of evidence on the role of RNA structure. For example, in the plant mitochondrial (Covello and Gray 1989; Gualberto et al. 1989; Hiesel et al. 1989; Araya et al. 1994) and chloroplast (Hoch et al. 1991; Maier et al. 1992; Bock et al. 1994; Ruf et al. 1994) C to U cytidine deaminase editing, the possibility of the existence of guide RNAs that confer site specificity on this reaction has been discussed, but no evidence has yet been presented (Bock and Maliga 1995). The extensive C-insertion editing in *Physarum* mitochondria (Mahendran et al. 1991, 1994; Gott et al. 1993; Rundquist and Gott 1995; Visomirski-Robic and Gott 1995) is still a complete mystery, both in terms of mechanism and determination of site specificity. Finally, a large number of posttranscriptional modification reactions, the mechanisms of which are just starting to be characterized, are known to occur with both rRNA and tRNA, and to distinguish between these reactions and the RNA editing reactions is more a matter of

semantics than of biological reality. It is clear that the study of the role of RNA structure in RNA modification reactions in general is just beginning and should lead to interesting new horizons in molecular biology.

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