Evolutionary Aspects of RNA Editing

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"Complex, statistically improbable things are by their nature more difficult to explain than simple, statistically probable things."

Richard Dawkins

Abstract RNA editing is the sequence alteration of RNA molecules by nucleotide insertion/deletion or conversion mechanisms. In this chapter, I describe how the different forms of RNA editing may have evolved from pre-existing activities. It appears that repeated and widespread independent evolution of RNA editing occurred. The diversity in origins seems to be mirrored in the range of possible functions of editing:

(1) Multiple proteins could be encoded by one gene. Different editing patterns would generate several proteins from one gene. Conversion editing in vertebrate mRNAs seems to be an instance of such an adaptive function. (2) RNA editing could provide organisms with an extra level of regulation of gene expression, and

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indications for this function are seen in most RNA editing forms. (3) Editing could serve as a defence against viruses and transposons. This could be another role of editing of vertebrate mRNAs. (4) Editing might counteract mutations which have occurred in the genome. These could occur particularly in organellar genomes, when selective pressures are absent. This role may be the raison d'être of mitochondrial tRNA editing. (5) RNA editing could offer the possibility to retain 'difficult' coding sequences, and such a function might be performed by mitochondrial RNA editing in myxomycetes. (6) Last but not least, RNA editing could speed up evolution by creating higher amounts of genetic variation over a shorter period of time. For its function, this model relies heavily on an analogy with splicing, where the possibility of domain shuffling has been invoked as a functional advantage.

All these explanations seem not to suffice for kinetoplastid panediting, the most complex and extensive form of RNA editing known. In this case, I propose that the original advantage was found in the gene fragmentation it entails, protecting against loss of temporarily non-expressed mt genes during periods of intense intraspecific competition. Present-day kinetoplastid editing, however, reflects the effects of a long history of opposing selective forces obscuring its evolutionary origin.

1 Introduction

RNA editing, the sequence alteration of RNA molecules which was first discovered in the mitochondria (mt) of trypanosomes in 1986 (Benne et al. 1986), has been found in many different organisms. The evolutionary histories and possible roles of the different forms of RNA editing seem to be as diverse. Here, I will deal with the evolutionary aspects of most forms of RNA editing, going from relatively simple forms and simple evolutionary explanations to the most complex, kinetoplastid panediting (see chapter by Torsten Ochsenreiter and Steve Hajduk, and chapter by Jason Carnes and Ken Stuart, this volume). Darwinian evolution can be seen as the continuous interplay between chance, generating diversity, and selection, restricting it. The relative contribution of either to the development of a present-day biological process can vary. By looking at the different RNA editing forms in this context, I hope to highlight the great diversity mentioned above.

2 Evolutionary Models

In 1993, Patrick Covello and Michael Gray (Covello and Gray 1993) published a three-step model explaining the evolution of all RNA editing forms. The first step is the acquisition of RNA editing activity by pre-existing enzymes. Next, mutation at 'editable' nucleotide positions in the genome occurs and, finally, editing becomes essential for survival upon fixation by genetic drift. Only in this last step of their proposed mechanism does natural selection come into play: the RNA editing activity is maintained because, to make functional RNAs, it has become indispensable.

Thus, the 'chance' contribution in their model is dominant. This is clear from the fact that the organism with RNA editing capacity does not have an adaptive advantage in comparison to the 'original' organism. Aptly, the model is sometimes referred to as the 'they got stuck with it' hypothesis. All other models invoke evolutionary advantage(s) of the RNA editing capacity as the reason for its retention in present-day organisms. Two types of advantage can be distinguished, though the distinction is artificial and far from absolute. Direct adaptive advantages refer to changes in the organism which immediately bestow improved fitness (e.g. a change in a receptor protein so that a parasite can no longer enter the host). Indirect adaptive advantages, on the other hand, are changes in the organism which do not necessarily lead to improved fitness as such, though preferably they should not compromise individual fitness, but which lead to higher 'evolvability'. 'Evolvability' refers to changes in the organism that, though possibly without any impact whatsoever on the organism's direct survival, allow descendants of the organism to spread more successfully. A few examples will highlight the salient points of these kinds of genetic changes.

To explain the widespread occurrence of splicing in eukaryotes, it has been proposed that (one of) its evolutionary benefit(s) lies in the fact that it allows functional protein domains to be exchanged, making very rapid modular protein evolution possible. The development of sexual exchange of genetic material in all its different forms opened up far more efficient methods of recombination. This gives rise to much more complex samples of genetic combinations for natural selection to choose from (Hoekstra 2005). Thus, one could divide the proposed adaptive functions of RNA editing into two groups, based on whether a direct survival benefit or a higher level of 'evolvability' predominates. It should be stressed again that the distinction is artificial: examples of RNA editing evolution 'having a bit of both' will highlight this further. The following list of proposed advantages for RNA editing starts with direct benefits and leads on to 'evolvability' advantages.

1. Multiple proteins could be encoded by one gene. Different editing patterns (including absence of editing) would allow the generation of several proteins from only one gene. Especially organisms in which genome space is limited, such as viruses (although the recent discovery of the 1.2-Mb Mimivirus gives pause for thought; Raoult et al. 2004), would be expected to make use of the possibilities offered by RNA editing in this respect.
2. RNA editing could provide organisms with an extra level of regulation of gene expression. As editing is an essential step in the maturation of the RNAs which are subject to this process, it is hard to imagine the organisms not developing mechanisms to regulate gene expression at this point.
3. RNA editing might function as an efficient defence against viruses and transposons (Grivell 1993). Genetic parasites would be made harmless when their RNAs would be rendered meaningless by editing.
4. Another advantage of the RNA editing potential could be that it offers the possibility to retain 'difficult' coding sequences. It is imaginable that a relatively unsophisticated replication or transcription machinery would encounter problems

with certain sequences. These sequences could then be generated by RNA editing (Grivell 1993).
5. Editing is sometimes seen as counteracting mutations which have occurred in the genome. These could occur especially in organellar genomes, accumulating during periods when organellar function is not, or only partially, needed, and selective pressures thus absent (see, e.g. Cavalier-Smith 1997).
6. It has been proposed that RNA editing can speed up evolution by creating higher amounts of genetic variation over a shorter period of time. Indeed, edited sequences seem to have a higher rate of molecular evolution (Landweber and Gilbert 1993). This model relies heavily on an analogy with splicing, where the possibility of domain shuffling (cf. above) has been invoked as functional advantage. This would strongly favour the retention of the splicing machinery.
7. For kinetoplastid panediting, it has been proposed that the advantage is provided by the gene fragmentation it entails, protecting against loss of temporarily non-expressed mt genes during periods of intense intraspecies competition (Speijer 2006 and see below). This would force organisms to retain genes which are not under selective pressure during prolonged periods of growth and division, thus retaining a higher 'ecological' flexibility which would, e.g. enable them to evolve complex (parasitic) lifecycles.

Regarding the concept of evolvability, it should be stressed that the apparent foresight of the examples mentioned is indeed only 'apparent'. If a mechanism giving rise to an increase in genetic flexibility would be really detrimental to the organism, severely hampering its chances to generate offspring, then all putative benefits for future generations would not lead to its retention in the species. Such a mechanism should at least be (almost) neutral for the organism to give rise to a line in which 'evolution speeds up'. Let us look at most of the known editing mechanisms in the light of all these different proposals to explain their occurrence, starting with the most 'simple'.

3 RNA Editing in Paramyxoviruses

RNA editing in paramyxoviruses is the co-transcriptional process leading to extra inserted G residues in the viral P mRNA, which are derived from the action of a stuttering polymerase (Vidal et al. 1990). This type of editing falls into the category of co-transcriptional RNA alteration processes which historically have been considered forms of editing (Hausmann et al. 1999). Another example of co-transcriptional viral editing is found in the insertion of an A residue in the G(lyco)P(rotein) mRNA of Ebola viruses (Volchkov et al. 1995), switching expression from the encoded (non-structural) secreted glycoprotein to that of the structural virion glycoprotein. This editing also seems to be due to a stuttering polymerase. These editing capabilities are retained by the organisms in order to make multiple proteins with clearly different functions in the lifecycle of these viruses. As a further bonus, the expression of the various P or GP gene products, the relative proportions of which seem to be critical, can thus be tightly regulated.

4 tRNA Editing in Mitochondria

The mt tRNA editing examples (see also chapter by Juan Alfonzo, this volume) seem to be quite compatible with the Covello and Gray model. Evolutionary fixation of editing has been nicely described for marsupial mt tRNA editing in Börner and Pääbo (1996). First, a T to C transition in the gene for $tRNA^{Asp}$ occurred. It changed the anticodon of this tRNA, allowing recognition of GGY codons and a concomitant shift in charging from aspartic acid to glycine. This turned out to be not lethal to the organism, presumably because a pre-existing deaminase activity converted this nt in the tRNA back into U, and because the presence of multiple copies of the non-mutated tRNA in the mt genome allowed this activity to become more efficient. This resulted in an editing activity changing about 50% of the mutated tRNA back into the original $tRNA^{Asp}$. A situation in which two of the GGN glycine codons were recognized by two different tRNAs was the result: GGY codons were decoded by the unedited $tRNA^{Asp}$ and the original $tRNA^{Gly}$. Restoration of the original situation became impossible because of a second mutation making the RNA editing process indispensable. This mutation occurred in the $tRNA^{Gly}$ gene, limiting the $tRNA^{Gly}$'s decoding capacity to the two GGR codons, in the process making both forms (edited and unedited) of $tRNA^{Asp}$ essential for survival. In plant organellar editing, a similar situation exists: in this case, the abundance of editing sites makes it impossible to revert to a situation where loss of editing activity would not be lethal. The mt tRNA editing activity in marsupials has not been shown to have any function apart from the repair of a genomic mutation at the level of the RNA. An overview of mt tRNA editing is given in Table 1.

All instances of tRNA editing seem to have no function apart from 'repair' of the encoded tRNAs which have accumulated mutations impairing proper functioning of the tRNA in translation and, thus, seem to conform to the original Covello and Gray model. tRNA editing independently evolved many times in the mitochondria of diverse eukaryotes, co-opting mostly different pre-existing enzymatic activities seemingly without any direct benefit to the organism. The widespread occurrence and independent evolution of organellar RNA editing is illustrated in Fig. 1.

5 RNA Editing in Plant Organelles

Plant pyrimidine (C to U and U to C) editing is very widespread in plant organelles, especially in mitochondria (see chapter by Mizuki Takenaka and colleagues, and chapter by Masahiro Sugiura, this volume, and below). As the raison d'être for the existence of RNA editing in plant organelles, the 'one gene, multiple proteins' explanation has often been invoked. Is this correct? There are several observations which make it unlikely.

First of all, the large majority of plant editing events changes transcripts 'back' into the form which is conserved over distantly related species, giving the impression these

Table 1 tRNA editing in mitochondria

Organism	Site	Pre-existing enzyme	Remarks	Ref.
Marsupials	Anticodon	Deaminase	See text	Börner and Pääbo (1996)
Leishmania tarentolae	Anticodon	Deaminase	*Imported* $tRNA^{Trp}$	Kapushoc et al. (2000)
Acanthamoeba castellanii	5′ acceptor stem	?	12 tRNAs	Lonergan and Gray (1993)
Chytridiomycete fungi	5′ acceptor stem	?	Evolved at least twice	Laforest et al. (1997, 2004)
Naegleria Gruberi	5′ acceptor stem	?	Lobose amoeba (see Fig. 1); predicted only	
Land snails	3′ acceptor stem	Poly(A) polymerase[a]		Yokobori and Pääbo (1995a)
Squids	3′ acceptor stem	Poly(A) polymerase[a]		Tomita et al. (1996)
Gallus gallus	3′ acceptor stem	Poly(A) polymerase[a]		Yokobori and Pääbo (1997)
Ornithorhyncus anatinus	3′ acceptor stem	CCase[b]		Yokobori and Pääbo (1995b)
Lithobius forficatus	3′ acceptor stem	RdRp?[c]	22 cases predicted, eight confirmed	Lavrov et al. (2000)
Seculamonas ecuadoriensis	3′ acceptor stem	RdRp?[c]	Jakobid (see Fig. 1)	Leigh and Lang (2004)

[a] Examples of the very efficient use of animal mt genome coding capacity (e.g. in *G. gallus*, mt $tRNA^{Tyr}$ and $tRNA^{Cys}$ overlap by a single G residue; see also below), closely resembling 3′ polyadenylation of some vertebrate mt mRNAs where UAA stop codons result from A addition(s) at the 3′ end (Ojala et al. 1981)

[b] (modified?) tRNA nucleotidyl transferase, involved in 3′ CCA addition to tRNAs

[c] RNA-*dependent* RNA polymerase. N.B. poly(A)polymerase and CCase are template-independent

organelles use editing only as a repair mechanism and that plants would be better off starting out with the ‘correct’ DNA sequence (Tsudzuki et al. 2001). Pyrimidine interconversions observed in plant mitochondria do give rise to different transcripts associated with polysomes in the process of being translated (Lu and Hanson 1996). However, studies with antibodies against the unedited ATP6 protein showed translation products from unedited mRNAs to be very unstable (Lu and Hanson 1994). Even when products of unedited transcripts can be detected, they are absent from complexes they should be part of, despite the fact that, compared to the proteins as encoded by edited transcripts, only minor changes are present. This is illustrated by the product of unedited RPS12 RNA, which is not detectable in plant mitoribosomes (Hanson 1996). The role of proteins encoded by (partially) unedited messengers, if any, thus remains unclear. Nevertheless, it seems likely that plants do use at least some of the editing instances to control organellar gene expression. An example is found in the tobacco plastid ndhD transcript in which the initiation codon is generated by a C to U conversion (Hirose and Sugiura 2001). The transcript is (partially) edited in leaves

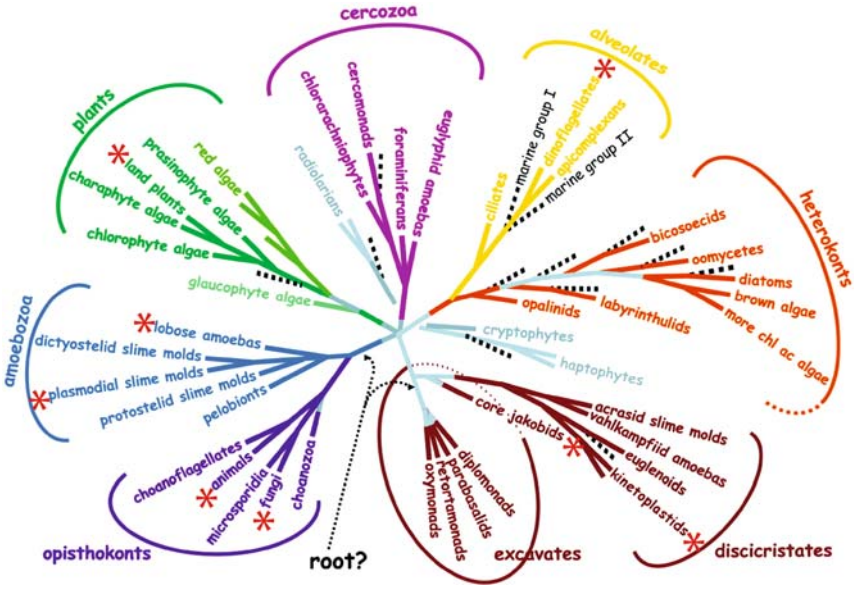


Fig. 1 Repeated and widespread independent evolution of organellar RNA editing in eukaryotes. The unrooted tree has been adapted from Baldauf (2003). *Red asterisks* indicate the acquisition of RNA editing in the clade. Comparative studies demonstrate multiple independent instances of organellar RNA editing acquisition also within clades: so far, it has been observed in fungi, animals, plasmodial slime moulds, and kinetoplastids (see text)

containing active NADH dehydrogenase and not edited in root, which does not make the active complex. This regulation could be achieved by, for example, controlling the availability of one of the hundreds of pentatricopeptide repeat (PPR) motif-containing proteins implicated in the specification of editing sites (Shikania 2006). It should be noted that the *Arabidopsis* transcript has four additional editing sites which all have to be converted before the transcript encodes the correct protein, and that wheat, rice and maize all encode the start AUG (rather than ACG in tobacco) directly in the plastid genome (Tsudzuki et al. 2001). Again, these observations seem to be in agreement with an editing mechanism that simply 'took over' by chance without any direct benefit to the organism, editing only subsequently being used as an extra level of gene expression control and, even then, only in a minority of cases.

One can also compare the overall evolution of genomic architecture in organelles of different eukaryotes to see what can be learned about the evolution of editing in different organelles. This comparison was performed by Lynch et al. (2006). They show quite convincingly that the genomic development of mitochondria (and chloroplasts) can be understood as the evolutionary result of an interplay of two *non-adaptive forces only*: random genetic drift and mutation pressure. Comparing the mt mutation rate (measured as silent site divergence rate) between different phylogenetic groups, they found that mammalian mitochondria have the highest and plant mitochondria by far the lowest mutation rate (i.e. a hundredfold lower). The mutation rates in the

mitochondria of unicellular organisms and plant chloroplasts fall between these two extremes. It is clear from their analysis that genome size, amount of intergenic DNA, amount of introns and, last but not least, amount of editing in organelles are *inversely* related to the local mutation rate. The highly reliable plant mt replication allows these organelles to retain large mt genomes with rampant editing without direct evolutionary benefits or even a (slight) selective disadvantage: 'restorative' mutations, rendering editing superfluous, occur four times as fast as the neutral rate would predict (Shields and Wolfe 1997). Mammalian mitochondria, on the other hand, with their much higher mutation rate, have to counteract this error rate by reducing their mt DNA to the absolute minimum, with very limited editing only if allowing further reduction of overall genome size (see above). Superficially, this might seem to contradict the 'non-adaptive' Covello and Gray model, because one might conclude that as a genomic repair mechanism, editing should be most abundant in organelles with the highest mutation rate. This is, however, not correct. The generation of mutations is not 'limiting' (the overwhelming majority of non-silent mutations simply die out) but the possibility of retaining mutations in the absence of an adaptive advantage is. The mutation rate thus limits the amount of non-beneficial 'weight' a genome can carry (compare, e.g. the viral error catastrophe concept). The observed editing patterns thus seem to be in agreement with a non-adaptive model, with the amount of pyrimidine interconversion and tRNA editing observed in chloroplasts and animal, unicellular and plant mitochondria being determined by the relative fidelity of the operating replication system.

Although Lynch et al. (2006) do not mention this possibility, it is tempting to extend their argument: in mt systems where the amount of editing is much higher than expected on the basis of the local mutation rate, real adaptive advantages must (have) be(en) present. Assuming a 'normal' mutation rate for the mitochondria of unicellular organisms, only a few cases of mt editing seem to be so massive that this could be indicative of real adaptive advantages: possibly, mixed substitution editing in dinoflagellates (Lin et al. 2002), mixed editing in myxomycetes (see chapter by Jonatha Gott and Amy Rhee, this volume) and, especially, U-insertion/deletion editing in kinetoplastids. Before discussing the latter two in more detail, examples of less extensive editing should be looked at. These forms of non-organellar conversion editing have clear adaptive advantages: anti-viral properties and multiple protein production.

6 RNA Editing in Cytoplasm and Nucleus

The instances of conversion editing in vertebrate mRNAs do demonstrate contributions beyond 'genome repair' of RNA editing. Cases of RNA editing which have been reported include C to U editing in the mammalian nuclear apolipoprotein B (apoB) mRNA (Davidson 1993), in the Neurofibromatosis type-1 (NF1) mRNA (Skuse et al. 1996) as well as in several viral RNA and DNA molecules (e.g. in HIV; see Turelli and Trono 2005), and A to I editing in mRNAs encoding vertebrate and insect neurotransmitter receptor subunits (Sommer et al. 1991; Palladino

et al. 2000) as well as in viral antigenomic RNAs of human hepatitis delta virus (Polson et al. 1996).

In apoB mRNA, a glutamine codon (CAA) is changed into a stop codon (UAA). The edited mRNA encodes the 48-kDa N-terminal part of the 100-kDa apoB protein encoded by the unedited mRNA. The editing activity is tissue-specific, normally occurring only in the intestine (and in the liver of rodents), and physiologically highly relevant because while both proteins bind lipids to form lipoprotein particles, the ones containing the 48-kDa apoB protein (chylomycrons) are involved in the transport of dietary fat, whereas 100-kDa apoB protein is the major protein component of the low-density lipoprotein particles, the 'bad' cholesterol (Davidson 1993). NF1 mRNA editing is found in certain human tumours. The C to U conversion changes an arginine codon (CGA) into a stop codon (UGA). The truncated product presumably loses its GTPase activity and so does not convert the proto-oncogene product RAS into the inactive (GDP-bound) form. Thus, editing would be selected for during, and contribute to, tumorigenesis. These two instances of C to U conversion are catalyzed by the cytidine deaminase APOBEC-1, the founding member of a large family of C-deaminases with different specificities, which also contains activation-induced deaminase, AID, and 10 other (predicted) APOBEC homologues in humans (Turelli and Trono 2005). AID is involved in class switch recombination and somatic hypermutation (though presumably not as an RNA deaminase), processes leading to the production of high-affinity antibodies by B-cells. At least some of the other APOBEC enzymes are also involved in immune reactions but, in their case, mostly by direct C-deaminase activity on (intermediate) DNA and RNA forms of human viruses, such as HIV (Bishop et al. 2004). However, not all anti-viral activity of the protein family is due to the deaminase activity. As a countermeasure, the HIV-encoded Vif protein specifically targets APOBEC3G and APOBEC3F for proteosomal destruction. Comparing evolution of the family between rodents and humans, diversification seems to have been rapid, especially in humans. This could be related to differences in the ongoing arms' race between viruses and retrotransposons, and their human or rodent hosts (Turelli and Trono 2005). All these homologous zinc-containing proteins most likely have evolved from an ancestor involved in pyrimidine metabolism (Gerber and Keller 2001) which also gave rise to the family of A to I deminases mentioned above (the ADAR enzymes; see also chapter by Michael Jantsch and Marie Öhman, this volume).

In the case of A-deaminase activity, the evolution and functional diversification are strikingly similar to what we observed in the C-deaminase family. A to I editing is found in mRNAs encoding vertebrate neurotransmitter receptor subunits (Sommer et al. 1991). It has been reported for rodents and humans in mRNAs encoding subunits of the glutamate receptor, mediating fast excitatory responses in the central nervous system. These receptors function as glutamate-gated cation channels which open in response to L-glutamate binding. There are three types, called AMPA, NMDA and KA responsive channels (based on agonist sensitivity; see Seeburg 1996). The different sensitivities result from the hetero- or homomeric composition of these multimeric channels, which are built from combinations of gene products encoded by the GluR gene family. The variety in response is further increased by A to I editing, as initially discovered by Seeburg and colleagues (Sommer et al. 1991): e.g. in GluR-B (and in

GluR-5 and 6) mRNA, a CAG codon in exon 11 is changed into a CIG codon which is read as CGG by the translational machinery. This changes the encoded glutamine (Q) into arginine (R) at these so-called Q/R sites. Editing efficiencies differ for the different transcripts (from almost 100% change in GluR-B to 40% in GluR-5 mRNAs; Smith and Snowden 1996). In GluR-B, C and D mRNAs, which all encode subunits of AMPA-sensitive channels, an additional A to I editing site is found in exon 13 changing AGA into IGA (GGA). This arginine to glycine codon change is known as the R/G site. In the GluR-6 transcript, the so-called I/V and Y/C sites also result from editing. The channels are profoundly influenced by these changes: e.g. the Q/R editing places an arginine in a transmembrane domain affecting calcium permeability. A to I editing was also found in the serotonin 2c receptor mRNA. Other human targets of ADARs have recently been identified: although none of these transcripts encode receptors, half of them were strongly expressed in the CNS (Levanon et al. 2005). All the endogenous A to I targets so far identified in rodents, squid, teleost fish (Kung et al. 2001) and *Drosophila* encode proteins functioning in the nervous system. As editing diversity combines with gene (family) diversity, an enormous potential of different specificities and responses could be envisaged, which is of course of great value during evolution, development and functioning of the animal brain. Similarly to the APOBEC family, however, endogenous transcripts are not the only targets. It has been suggested that ADARs also evolved for their protective function against viruses (Scott 1995). That they recognize viral targets is illustrated by the fact that A to I conversion occurs in the human hepatitis delta virus in 20–50% of the viral antigenomic RNAs at the so-called amber/W site, although in this case the virus is not the hapless victim of deaminase activity. Editing changes a stop codon (UAG) into a tryptophane codon (UIG), resulting in a 19 amino acid extension of the reading frame. The shorter protein (hepatitis delta antigen) encoded by the mRNAs transcribed from the unedited template is involved in viral replication, while the longer version is needed for packaging and inhibits replication (Bass 2002). The longer protein actively inhibits editing activity in a negative feedback loop, so that replication will not be inhibited too strongly. From the fact that the respective deaminase activities are involved in the arms' race with pathogens and are used in, e.g. GluR and ApoB editing, giving rise to protein products with physiologically relevant differences, it seems logical to conclude that natural selection actively favoured those organisms which gained (diversification of) deaminase editing capacity.

7 RNA Editing in Myxomycetes

Returning to extensive organellar editing, adaptive advantages, if any, are less clear in this case. In the myxomycete *Physarum polycephalum*, four different forms of editing are operational in the mitochondrion, even working on one transcript: cytochrome c oxidase subunit I (cox1) mRNA undergoes 59 C insertions, one U insertion, three different dinucleotide (CU, GU and UA) insertions, and four C to U base conversions. As seen in this example, editing of mt RNAs in this organism

predominantly concerns insertion of single C residues, while less frequently, single Us or dinucleotides (only 19 reported instances, so far) are inserted (Gott et al. 1993; Miller et al. 1993; Visomirski-Robic and Gott 1995; Byrne and Gott 2004). The insertion of single residues at 'regularly' spaced intervals in this and other transcripts results in the removal of multiple gene-encoded frameshifts, making this form of editing a crucial step in gene expression, like its kinetoplastid counterpart (see below). Different from kinetoplastids, however, insertion of residues has also been observed in mt tRNAs and rRNAs (Miller et al. 1993). No consensus sequences defining the hundreds of insertion sites seem to be present but they usually follow a purine/pyrimidine dinucleotide, and are found mostly in third codon positions. Myxomycete insertional editing is distinct from uridine insertion/deletion editing in kinetoplastids: the edited sites have dissimilar patterns, the identity of nucleotides involved is different, myxomycetes apparently lack gRNA-like template molecules, and most importantly, all insertional editing seems to be co-transcriptional. This was shown by analysis of mRNAs obtained under conditions of stalled RNA polymerization, due to limiting concentrations of nucleotides added to isolated mitochondria (Visomirski-Robic and Gott 1997; Byrne and Gott 2004).

A phylogenetic analysis of cox1 genes and transcripts in different myxomycetes allows a reconstruction of the most likely evolutionary history of insertional editing in myxomycetes (Horton and Landweber 2000). Presumably, this form of editing started with an ancestral U-addition capacity only, followed by the acquisition of (other factors allowing) C addition and dinucleotide addition respectively. This is not unlike the situation in kinetoplastids, where apart from the TUTase for U addition or the U-exonuclease for U deletion, the religation of the 5′ and 3′ parts of the mRNA is also catalyzed by different, insertion- and deletion-specific ligases. A basic operating mechanism is thus extended to enhance its versatility. The C to U conversion editing, which edits mRNAs post-transcriptionally and thus can be separated from insertional editing activity in *P. polycephalum* extracts (Visomirski-Robic and Gott 1997), clearly evolved independently. The complexity of editing in *P. polycephalum* is illustrated not only by the example of the cox1 transcript given above but also by the fact that even inserted C residues could subsequently be converted into U residues (Byrne and Gott 2004). It goes without saying that all the different editing mechanisms are indispensable for the generation of the 'correct' mt mRNAs. The sheer abundance of editing sites (on average, one in every 25 bases is edited), however, seems to demand additional explanation. Without more information about the evolution and specific mechanisms of RNA editing in this lineage, such an explanation is not easy. It is conceivable that myxomycetal insertion editing is the only example of editing evolved specifically to counteract the effects of a relatively 'deletion-prone' mt polymerase in these organisms. Possibly, the replication machinery encounters problems with certain sequences leading to frequent deletions in the mtDNA. These sequences could then be generated co-transcriptionally (compare model 4 above). In the discussion of pyrimidine interconversion editing in plant organelles, the work by Lynch et al. (2006) was quoted: overall genomic architecture is linked to respective mutation rate. Could this insertional editing be an instance of coevolution with the idiosyncrasies of a specific mt polymerase? The

insertional editing level possibly is so high because it has to counteract a relatively high rate of *very specific* mutations only: single nucleotide (and very rarely, double) deletions. This model can explain the following aspects.

1. In most cases, editing follows a purine/pyrimidine dinucleotide sequence: this should be part of the sequence forcing the mt polymerase to make occasional deletions.
2. It fits with the strong preference for *insertion* at the third position of the encoding triplet in mRNAs: only in that position is the identity of the inserted nucleotide in most cases irrelevant to the protein encoded. Thus, third position deletions have a much better change of survival if a 'blind' insertion mechanism is present at the transcriptional level. This also explains the surprising difference with pyrimidine interconversions in plant mitochondria, which occur *least* in the third position. In this case, editing of a first or second position is actively maintained by selective pressure and becomes unnecessary only in the case of a 'restorative' mutation, while third position *conversion* can 'come and go as it pleases', being neutral.
3. The replacement of the ancestral U-insertion mechanism by the very extensive C-insertion mechanism also makes some sense: the identity of the inserted nucleotide is not important, allowing C insertion to take over, possibly as a result of genetic drift only.
4. This type of insertion editing is really different from that observed in kinetoplastids, where transcripts are either not, only very locally, or completely changed by U insertion. If the editing sites in myxomycetes are due only to deletions resulting from random occurrence of certain sequences in the DNA, then one would expect editing sites to be 'sprinkled' rather regularly all over their mt genomes. This is indeed observed. The appearance of 'evenly' spaced editing sites again results from the strong preference for their occurrence in third codon positions, with the minimum distance of nine nucleotides possibly due to processivity demands of the RNA polymerase.

However, some questions remain. If the model of Lynch et al. is correct, then despite being relatively 'deletion-prone', the specific mutation rate of mt DNA polymerase from *P. polycephalum* must be relatively low to sustain a genome with such an abundance of RNA editing. Alternatively, myxomycetal insertion editing confers a real adaptive advantage, which has not been identified yet. In the light of this hypothesis, the characterization of mt DNA polymerase from *P. polycephalum* should have high priority.

8 RNA Editing in Kinetoplastids

8.1 Introduction

RNA editing was discovered in trypanosomatids, parasitic unicellular organisms belonging to the kinetoplastid order, in 1986 (Benne et al. 1986), and in this instance defined as the process of post-transcriptional sequence alteration via the

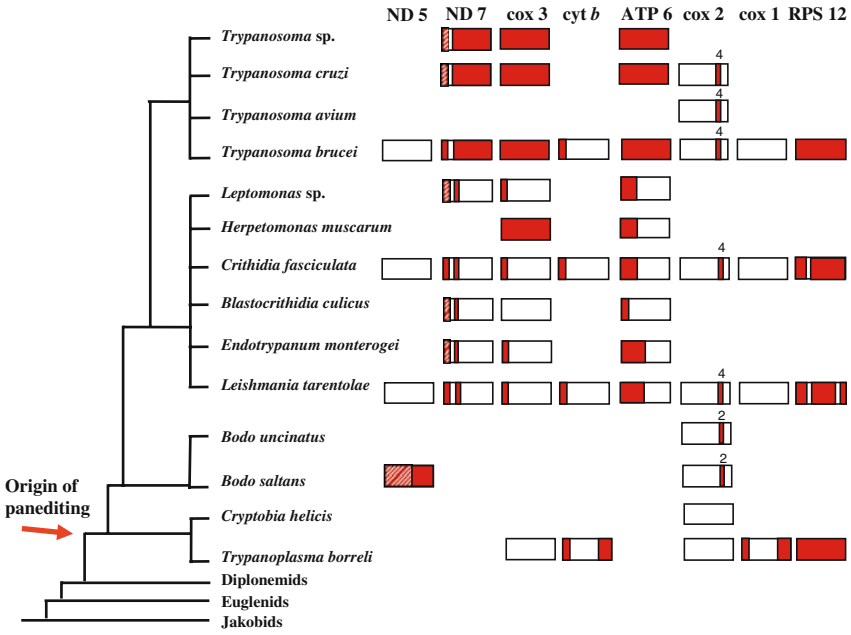


Fig. 2 Evolution of uridine insertion and deletion editing in kinetoplastids. An updated consensus phylogenetic tree of kinetoplastids is given (based on Maslov and Simpson 1994; Arts and Benne 1996; Blom et al. 1998; Simpson et al. 2002). On the *right*, editing patterns of eight mRNAs are shown (not drawn to scale) as far as they are known for each species. The *boxes* indicate (the regions of) the transcripts which are edited (*red*) or unedited (*white*). *Hatched boxes* indicate that the editing status of the segment is not known. Cox II editing has been observed with insertion of either two or four residues, as indicated above the boxes. The inferred stage at which panediting originated is indicated by a *red arrow*

insertion and deletion of uridylate residues at specific sites of mt RNAs. mt RNAs are encoded by the kinetoplast DNA (kDNA) which, in most cases, contains several thousand small minicircles and a few dozen maxicircles (Benne 1993; Simpson et al. 2000; Madison-Antenucci et al. 2002). The maxicircles encode subunits of the respiratory chain complexes and the ribosomal RNAs, while the minicircles encode the so-called guide (g) RNAs containing information for the editing of mt mRNAs encoded by the maxicircles (Blum et al. 1990). The large majority of the maxicircle genes encode RNAs which, without editing, are not translationally competent because they encode frameshifted proteins and/or lack translation initiation codons. A number of transcripts are edited only at the 5′ termini, which results in extra codons for N-terminal amino acids, including an in-frame AUG start codon. Examples of 5′ editing are found in, e.g. cyt*b* mRNA (see, e.g. Shaw et al. 1988; van der Spek et al. 1990) in *Trypanosoma brucei*, *Crithidia fasciculata* and *L. tarentolae* (see Fig. 2). Editing can be so extensive that certain genes are not recognizable as such in the maxicircle of certain trypanosomatids. A good example is the cox3 gene, which can easily be identified in *C. fasciculata* and *L. tarentolae*

(the transcripts of which are subject only to 5′ editing). The *T. brucei* cox3 'gene' appears to be absent and can be recognized only after editing over the complete length of the transcript (Feagin et al. 1988). While the G, A and C nucleotides (the GAC sequence) are encoded by the maxicircles, the U sequence is generated by editing: 547 Us are inserted and 41 Us are deleted at 223 sites. More than half of all nucleotides in this transcript are derived from editing. Because these genes can not be identified on the basis of their GAC sequence alone, they are referred to as cryptogenes while this form of extensive editing has been christened panediting. Panediting is common in *T. brucei*, and nine transcripts are edited in this fashion. From the analyses of cDNAs of several partially edited transcripts and RT-PCR experiments with combinations of oligos recognizing edited and unedited sequences, an overall 3′ to 5′ polarity of the editing process could be deduced (Feagin et al. 1988; Koslowsky et al. 1990; van der Spek et al. 1991). In this volume, the precise molecular mechanisms of the editing process are described in the chapter by Jason Carnes and Ken Stuart (also see Cruz-Reyes et al. 2002; Madison-Antenucci et al. 2002 and references therein; Schnaufer et al. 2003).

8.2 Evolution and Age of Kinetoplastid RNA Editing

Information regarding the evolutionary history of the RNA editing process can be obtained from studies with the bodonid species *Trypanoplasma borreli*, which demonstrated the existence of the U-insertion/deletion type of RNA editing also in this organism (Lukeš et al. 1994). This indicates that the process is at least 500–700 million years old, given the estimated time of divergence of the two kinetoplastid suborders (Fernandes et al. 1993). The flagellates (kinetoplastids, diplonemids and euglenoids) seem to form one of the earliest diverging eukaryotic lineages which have mitochondria. Research in *Euglena gracilis* and *Diplonema papillatum* has not yet revealed evidence of the existence of mt RNA editing in the non-kinetoplastid flagellates (Marande et al. 2005). Although this research has been far from extensive, most likely U-insertion/deletion editing evolved only in the kinetoplastid lineage (see Fig. 2).

Panediting occurs not only in *T. brucei* but also in cultivated strains of *L. tarentolae* and *C. fasciculata*, although clearly less frequently. In these organisms, the cox3 and ND7 genes are much less cryptic, the transcripts requiring only limited editing. Interestingly, the potential to edit transcripts encoding proteins which are not necessary during prolonged culture has been lost. When freshly isolated from the host, *L. tarentolae* (LEM 125 strain) does show extensive editing of six transcripts (Maslov et al. 1992; Thiemann et al. 1994). Extensive editing has further been observed in the trypanosomatids *Trypanosoma cruzi* and *Trypanosoma* species E1-CP, infecting fish, and *Herpetomonas muscarum*. The bodonid *T. borreli* also displays extensive editing in the RPS12 RNA; by contrast, cox3 RNA, edited in all trypanosomatids studied so far, is completely unedited in this organism (Maslov and Simpson 1994). Combining the kinetoplastid evolutionary tree with the differences in editing patterns observed (Fig. 2; Landweber and Gilbert 1994;

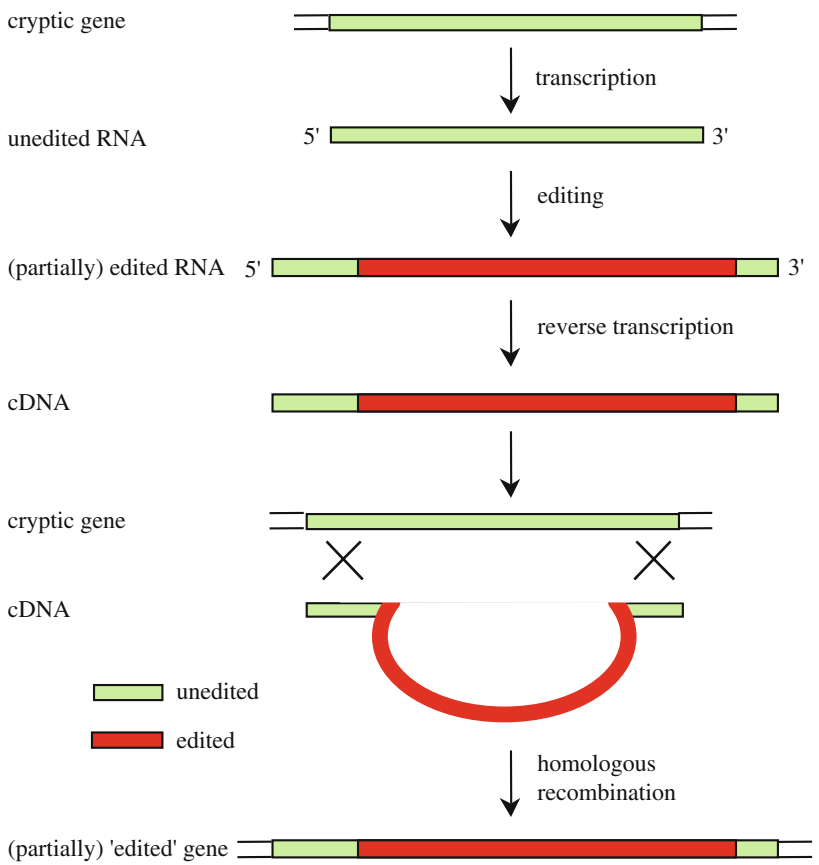


Fig. 3 Model explaining the loss of RNA editing via recombination. A cryptic gene is converted into a (partially) 'edited' gene via homologous recombination with a (partially) 'edited' cDNA. The transcript of the new gene no longer requires extensive editing; 'unedited' sequences are in *green*, 'edited' sequences in *red* (see text for details; taken from Arts and Benne 1996)

Arts and Benne 1996; Blom et al. 1998; Simpson et al. 2002), one can draw conclusions regarding the evolution of kinetoplastid RNA editing. Surprisingly, the distribution of RNA editing patterns in different kinetoplastids clearly indicates panediting to be an ancient trait. Presumably, panediting very quickly followed the evolution of editing itself in the kinetoplastid lineage. One can conclude also that editing becomes progressively less extensive, at times even completely disappearing: e.g. loss of cox3 editing in *Blastocrithidia culicis*. This resembles the current situation in plant organelles where local mutations which do away with the need for editing occur four times faster than the neutral rate (Shields and Wolfe 1997). A possible mechanism for the loss of editing in kinetoplastids was given by Landweber (1992). The model (illustrated in Fig. 3) is based on reverse transcription of (almost completely) edited RNA followed by homologous recombination of the

cDNA with the mt DNA. The 5′ and 3′ homology requirements of the cDNA would lead to the occasional retention of the need for 5′ editing of the ‘new transcript encoded by the mt DNA following recombination. This is indeed what one observes in, e.g. cox3 in *Endotrypanum monterogei*, *L. tarentolae*, *C. fasciculata* and *Leptomonas* species (compare Figs. 2 and 3). Not surprisingly in the light of its immense complexity and energetic costs, panediting at the current level seems disadvantageous and to be disappearing. One of the driving forces for this could be the loss of gRNA genes. As mentioned above, cultivated strains of *L. tarentolae* clearly show less editing potential upon prolonged culture. In the absence of selective pressures on (some aspects of) mt activity, loss of guide RNAs and loss of heterogeneity of the minicircles seem to occur quite rapidly due to large-scale deletions in, and asymmetrical divisions of, kDNA.

8.3 How Did Kinetoplastid RNA (Pan) Editing Evolve?

Can the Covello and Gray (1993) model account completely for the evolution of kinetoplastid RNA editing? Several considerations make this highly unlikely. As described above, the last step in their model consists of the fixation of RNA editing by genetic drift. The term ‘genetic drift’ is used here to describe a chance process by which an altered form replaces the original *without* a selective advantage. In the case of kinetoplastid editing, this is rather difficult to conceive (although genetic drift in relatively small populations should not be underestimated): not only all the copies of the mt genome at the time of origin (present-day organisms such as *T. brucei* have 50 maxicircles) but also the population of organisms as a whole have to be replaced without any selective advantage whatsoever, and this hundreds of times! This point will be dealt with below. Natural selection plays a role only in the final step of the Covello and Gray model: the RNA editing activity is maintained because it is required to make functional RNAs. Although this model could indeed apply to rare isolated instances of editing, it is hard to see how it explains the observed rapid acquisition of multiple editing sites in panediting requiring myriads of gRNAs. As stated above in the context of the evolution of mt genomic architecture, the sheer bulk of editing tells us that real adaptive advantages must (have) be(en) present and that the ‘they got stuck with it’ hypothesis clearly misses a description of the selective pressure(s) responsible for an *active* increase of the editing potential. The distribution of editing patterns excludes the development of panediting as a way to counteract specific mistakes of the kinetoplastid mt DNA polymerase. With regard to the models describing selective advantages, the following can be stated. Multiple proteins could indeed be encoded by one (crypto)gene but, firstly, translatable open reading frames seem to result only from complete editing (see below) and, secondly, no experimental evidence for this model has as yet been reported. Possibly, un- or partially edited transcripts are occasionally used to generate proteins, but this would clearly be a ‘late’ use of a mechanism rapidly evolving for other reasons. It is also true that editing provides

the kinetoplastids with some extra level of regulation of gene expression (e.g. compare editing patterns during different lifecycle stages). However, this can hardly be used to explain the emergence of this highly elaborate system. Both models apply only to simple editing events and can not explain extensive editing. Alternatively, RNA editing could counteract mutations accumulating during periods when mt function is not needed (such as during anaerobiosis with glycosomal activity for energy generation in free-living bodonid ancestors; Cavalier-Smith 1997). The problem with this latter model is clearly that kDNA with multiple gRNAs would become even more mutation-prone because more coding capacity is needed for a given message. To make matters worse, during these periods of absence of selective pressure on mt activity, gRNA loss could be enormous. An alternative explanation was needed (also see Simpson et al. 2002) and has been proposed by Speijer (2006). This model will now be discussed.

8.4 A Possible Explanation for the Spread of Kinetoplastid Panediting

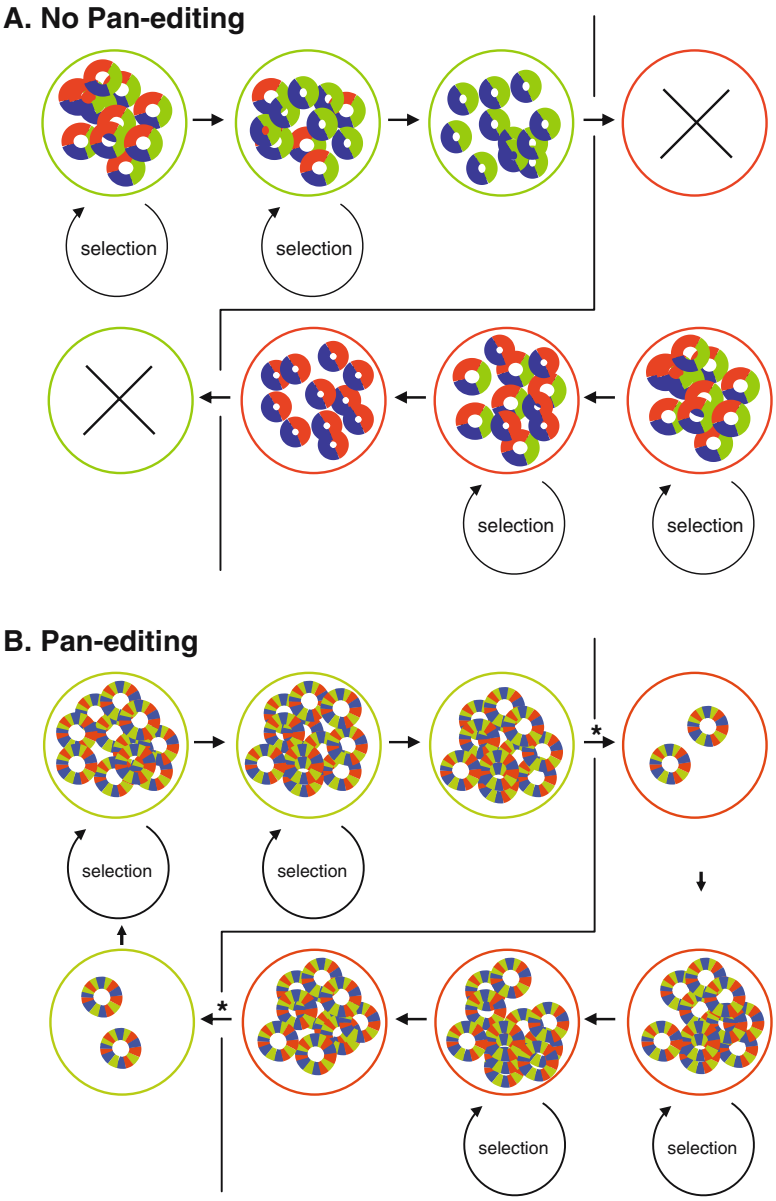
The vast majority of present-day kinetoplastids have a parasitic lifestyle. This means that these organisms grow and divide under varying conditions during their lifecycle (alternating between free-living and host or between different hosts). To understand the emergence of (pan)editing, an example of mt functioning during the lifecycle of trypanosomatids – in this case, *T. brucei* – is described in some detail.

T. brucei infects the mammalian bloodstream and can be taken up by Tsetse flies. It completes its developmental cycle in the mouthparts of the insect and is transmitted to the mammalian host during blood intake by the insect. In the Tsetse fly, a respiratory chain starting with complex II is operational. This implies complete editing of the transcripts for the mt-encoded subunits of complex III and IV which have to be edited (cyt*b* and cox2 and 3 respectively). The transcript of the ATP6 gene product encoding a subunit of the F_oF_1-ATPase should, of course, also be fully edited in the fly. In the long, slender mammalian bloodstream form, the respiratory chain is absent because all energy is generated from glycolysis alone. The F_oF_1-ATPase is still present in the mt inner membrane in this form but, rather than generating ATP from the proton gradient, it generates a proton gradient from ATP, presumably to make protein import into the mt matrix possible under these conditions (Nolan and Voorheis 1992). When the rapidly dividing, long slender form changes into the non-dividing short stumpy form, complex I is formed in the mitochondrion. The mt messengers for ND3, 7, 8 and 9 have to be panedited during this stage. Under these circumstances, ATP is presumably generated by the F_oF_1-ATPase, using the proton gradient generated by complex I (Bienen et al. 1991). When taken up by the fly, only the stumpy form can start the whole cycle again, possibly due to the fact that its metabolic circuitry more closely resembles the one needed in the fly. Although repressed, mt function is clearly not absent in bloodstream parasites. A very extensive description of the energy metabolism during the different lifecycle stages can be found in Hannaert et al. (2003).

In summary, cyt*b*, cox2 and 3 are needed in the insect stage, the ND subunits in one of the bloodstream stages and ATP6 probably in all lifecycle stages. Massive editing occurs in most of the transcripts needed. *T. brucei*, with its highly complex lifecycle, is the trypanosome species with the most abundant panediting of all organisms studied so far. Whether these two facts – complexity of lifecycle and high levels of panediting – are somehow interconnected will be discussed in the following.

The mass of kDNA encoding all these transcripts and their cognate gRNAs amounts to 10^{10} kDa (40% of the total cellular DNA), and is organized in a large network of thousands of catenated circles. Like all mt DNAs, kDNA is far less stable than chromosomal DNA. Mutations, large-scale deletions and even total loss of all kDNA, e.g. due to asymmetric divisions of heteroplasmic populations, have been observed frequently. This gave rise to the hypothesis that the kDNA network present in trypanosomatids evolved to counter these kinds of rapid mitotic segregations (Borst 1991). In some cases, so-called dyskinetoplastic strains (like *Trypanosoma equiperdum* ATCC 30023) can even survive in specific hosts. This poses a very challenging problem to parasites, because it is likely that individuals missing large parts of their kDNA (except for the relatively small part which is still needed) could easily out-compete normal individuals in the host (see Fig. 4A). In this case, the parasites would effectively die with their host. In the light of these considerations, it could be envisaged that increasing the amount of editing (i.e. splitting genes in a cryptogene containing the GAC sequence and multiple gRNA genes containing the information for the U sequence, which are then shuffled in such a way that parts encoding the complete message are found in many different locations) would be strongly selected for. Individual trypanosomes can no longer have large deletions in their kDNA because of the concomitant loss of essential gRNAs which have to be retained in the host (whether insect or mammal, in the case of *T. brucei*).

Fig. 4 Schematic representation of a panediting evolution model. Individual kinetoplastids are represented by single multicoloured circles of mt DNA with two or three genes only: a 'green' gene required for growth in the 'green' environment (*large green circle*; e.g. the mammal in the case of *T. brucei*), a 'red' gene required for growth in the 'red' environment (*large red circle*; e.g. the fly in the case of *T. brucei*) and a 'blue' gene required in both. The *arrows* underneath the environments indicate multiple replication rounds selecting the most rapidly multiplying individuals. Reduced generation times result from loss of mt DNA segments (note reduction in circle size) not under selective pressure in the respective environments. *Crosses* indicate absence of kinetoplastids still able to switch to the other environment. **A** Potential loss of host infectivity in parasites with mt genomes under limited selection. **B** Panediting: retention of host infectivity in parasites as a result of extensive gene fragmentation and scattering of gene fragments over the mt genome. Editing leads to gene fragmentation because it reduces the size of the 'gene' to the GAC sequence only, with the 'U sequence' encoded by multiple gRNAs placed in between other gene fragments. This in turn will make large-scale deletions result in loss of viability because the production of proteins which *are* under selective pressure will now also be compromised. Genome size is not affected by multiple replication rounds and, though small-scale mutations may still abound, some organisms will be able to pass the selective barrier (indicated by *). Adapted from Speijer (2006)


A. No Pan-editing
selection
selection
selection
selection
B. Pan-editing
selection
selection
*
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The essentials of this model are shown in Fig. 4A and B. To explain the central idea in the most general way: panediting evolved as a mechanism to minimize the danger of irreversible loss of mt genes encoding proteins necessary only during part of the kinetoplastid lifecycle. Loss of these genes from the mt DNA would be rampant because of the 'replication advantage' of deletion of large parts of the kinetoplast DNA during intraspecific competition in every 'new' environment making different demands on the mitochondrion. Editing is an effective way of making such large-scale deletions improbable, because (in order of relative importance):

1. It allows the intermixing of 'gene fragments' (multiple gRNAs and the cryptogene) in such a way that large-scale deletions will result in loss of gene fragments necessary for different lifecycles, and thus give rise to a non-viable organism,
2. It makes the presence of *all* the gRNAs (containing the information for the U sequence) necessary for the expression of such a 'gene', and
3. It *reduces* the local size of the original gene to that of the cryptogene (encoding the GAC sequence only).

Ad 1. The best candidates for gene fragmentation are the genes encoding proteins which are 'always' needed, rather than those needed only in some environments. The local presence of an essential 'part' of these genes will protect the mt DNA in that region against loss during strong intraspecific competition in *all* environments. That would mean that the mRNAs encoding a mitoribosomal protein such as RPS12 or, in the case of *T. brucei*, ATP6 should be the ones to be extensively edited. This is exactly what is observed (see Fig. 2). Indeed, RPS12 is the only gene found so far which encodes a transcript extensively edited in both kinetoplastid suborders, trypanosomatids and bodonids. Of course, also transcripts such as cox3 or ND7 could be in need of panediting as a result of the kind of evolutionary pressures envisaged in this model, because their fragmentation would protect the kDNA in those lifecycles in which they are essential (see above). In essence, the fragmentation of mt genes and the 'random' mixing of the resulting fragments can be seen as an extreme form of protective genetic linkage.

Ad 2. The different gRNAs all contain a part of the information needed for the production of the complete protein, making every single one of them indispensable. This is nicely illustrated by the fact that panediting has 3′ to 5′ directionality with the information of almost every single gRNA being crucial for the generation of the anchor sequence for the next, culminating in the final generation of an in-frame start codon (see Fig. 5D). Thus, every loss of a gRNA leads to the loss of a functional transcript, making gRNA genes perfectly suitable in rendering large-scale mt DNA deletions impossible.

Ad 3. A puzzling aspect of the kinetoplastid RNA editing process is the asymmetric distribution of U insertions and deletions. In *T. brucei*, we find a total of 2,965 instances of U insertions in the nine panedited transcripts, compared to only 318 U deletions, with insertion always far in the majority in every single panedited transcript. This is also found in the three transcripts with limited editing, having 64 insertions in total with only four deletions. The fact that insertions far outnumber deletions can be explained by the current model, as this distribution translates into a strong overall *reduction* of local 'gene' size in the case of cryptogenes, with a concomitant increase of small parts of the gene distributed over the mt genome in the form of gRNA genes.

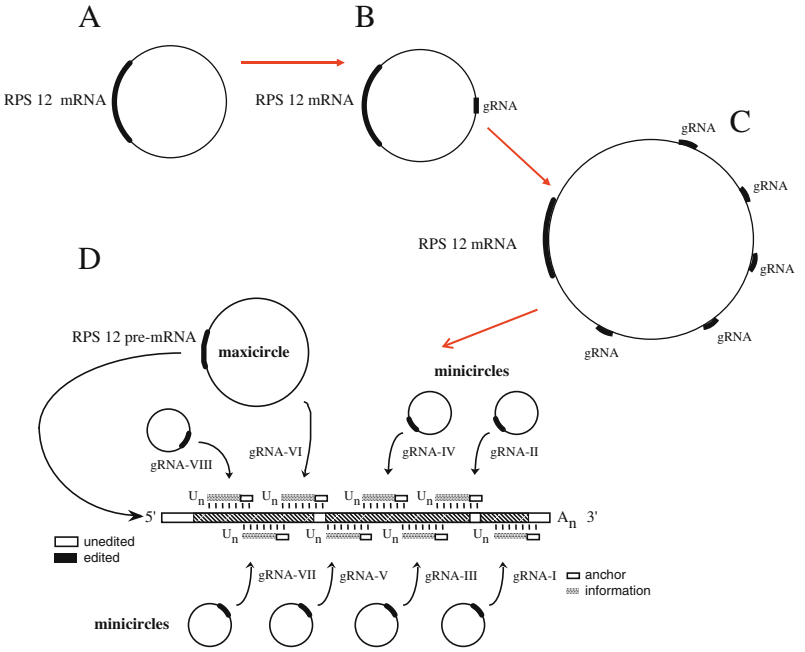

Fig. 5 Hypothetical stages in the evolution of panediting. Starting from the situation without the need for editing of the transcript (**A**), a single necessary gRNA is first encoded on the same DNA molecule (**B**). More gRNAs spread over the mt genome, possibly with a concomitant increase in size (**C**). Finally, minicircles encode most of the different gRNAs which are needed to end up with the fully edited mRNA (**D**). In **D**, the RPS12 mRNA transcribed from the maxicircle of *L. tarentolae* is depicted to illustrate the current situation and the sequential action of gRNAs in panediting. *Red arrows* indicate possible developments during evolution. The three editing domains are *hatched*. The eight gRNAs involved in editing of the transcript and their genomic origin are indicated. Anchor regions are *white*, informational regions *grey*. Each of the three domains is edited separately with a 3′ to 5′ polarity. For further details, see the text (adapted and extended from Sloof and Benne 1997)

Whether the reduction in size (from a complete gene to its GAC 'skeleton') of genes which are not protected by selective pressure under certain conditions (such as cox3 in the *T. brucei* bloodstream form) significantly reduces their chance to be lost from the local population as a result of strong intraspecific competition remains a matter of debate. It should be stressed that these aspects (the requirement that *all* cognate gRNAs are present and the fact that insertions far outnumber deletions) do not make any sense in models where editing is invoked as an extra level of expression control or as a way of making more proteins from a 'single gene'.

Further observations seem to support the model. Extensive editing is indeed most common in kinetoplastid organisms which live under high evolutionary pressure, like members of the genus *Trypanosoma*. The role of gRNAs as 'checkpoints' for mt kDNA integrity, i.e. ensuring that large-scale deletions are not viable, could also be performed by evenly distributed tRNA genes (compare, e.g. their distribution in the circular human mt DNA; Attardi 1985). They are essential in

every lifecycle, similarly to the two mt rRNAs, 9S and 12S, and the mt-encoded ribosomal protein RPS12. In *T. brucei*, *L. tarentolae* and *C. fasciculata*, however, tRNAs are not encoded by the kinetoplast DNA and are imported from the cytoplasm (Hancock and Hajduk 1990; Schneider et al. 1994). This could be the case for all kinetoplastid protozoa; to date, information regarding the genome location of mt tRNAs in the other flagellates is lacking. Mt tRNA import thus also seems to be a rather ancient trait, implying that tRNAs were not there to play a role in supplying 'checkpoints for kDNA integrity'. Could large-scale kDNA deletions really make such a difference in speed of multiplication of the individual organism? If we look, e.g. at the rapidly dividing, long slender form of *T. brucei*, only the parts encoding the ATP6 protein, the rRNAs of 9 and 12S, the RPS12 protein and the mt origins of replication seem to be under selective pressure. This makes up less than 30% of the coding capacity. A reduction of ~70% of the ~40% share of all DNA in the organism (kDNA is really huge, which is why it was the first extranuclear DNA discovered; Ziemann 1898) would mean ~30% reduction of the overall 'replication load'. Clearly, in a rapidly dividing population without panediting, trypanosomes retaining their capacity for switching between lifecycles would be at a serious disadvantage. Presumably, when panediting evolved, the kDNA, though large, was not that massive: paradoxically, editing, selected to counteract the temporary selective disadvantage of 'useless' kDNA, seems to have increased kDNA size.

Another interesting feature can be observed when we examine the known subunits of complex I. *T. brucei* capable of multiplying in the Tsetse fly can come only from the short stumpy population in the mammalian host. This population has an operational complex I for which at least six, and possibly eight mt-encoded subunits are necessary (ND3–5, ND7–9 and CR3 and 4), 4–6 of which are extensively edited (Feagin 2000; see also above). In terms of the model, this means that the fly will take up a population still able to synthesize a functional complex I, and therefore most likely possessing a full complement of genetic information. This in turn relies heavily on the integrity of the kDNA. Thus, at least some individuals will still be able to generate a completely functional respiratory chain starting with complex II in the insect. At first glance, the model would seem somewhat counterintuitive here: a gene such as cox3 is 'chopped into little pieces' to make it *less* likely of being lost during rapid growth under non-selective conditions. Many trypanosomes will indeed have lost the capability to synthesize a functional COX III protein during growth in the mammalian host but a sample taken up by the fly will have a much higher chance of still containing a few individuals which have retained this ability, in contrast to the situation without panediting in which they would have been outcompeted.

8.5 The Appearance of gRNAs

How did the gRNAs evolve? Several proposals have been made. The most simple model proposes that they originated by recruitment of random sequences (Simpson and Maslov 1999). Another model is based on kinetoplastid cox2 gRNA, the only

gRNA sequence known so far which works (only) in cis because it is located in the 3′ untranslated region of the cox2 mRNA. As this interaction needs only a very small anchor region and the transcription of the 'gRNA' is linked to its target, it has been proposed that all gRNAs started as intramolecular guides (Golden and Hajduk 2005). Yet other workers interpret them as the indirect product of mt DNA recombination, presumably coupled with the emergence of minicircles (Horton and Landweber 2002). As a result of recombination, a (small) circle encoding part of an mRNA with both an origin of replication and sequences allowing transcription is envisaged. It would be easier to choose between all these models if we knew the copy number of the mt DNA circles at the time gRNA genes started to evolve. The problem with the intramolecular gRNA model is the fact that the sequence 'comes out of nowhere', that there is only one example and that cox2 gRNA probably evolved when panediting already existed (as can be deduced from Fig. 2). The most parsimonious scenario is that as a result of recombinations, (parts of) complementary strands of certain protein coding genes had the possibility to mutate, giving rise to the forerunners of gRNA genes. Being part of 'normal' circles, replication and transcription of such sequences, possibly as parts of polycistronic messengers, would have been ensured.

8.6 Discussion

This model for the acquisition of panediting in kinetoplastids also helps to explain some other general features of kinetoplastids. The kinetoplastids have many biochemical aspects which are absolutely unique. They infect a wide range of hosts due to their rapid speciation and metabolic flexibility. As shown in Fig. 4, every time the kinetoplastids have to readjust to a different environment (indicated with an * in Fig. 4B), only a few of them in the sample still have all the genetic information necessary. However, this does not mean that they have not changed genetically. Evolutionarily speaking, we are looking at a founder effect with every switch between hosts. Again taking *T. brucei* as (the most extreme) example: from a rapidly dividing population of slender forms under strong selective pressure resulting from the sequential outgrowth of clonal populations with ever changing variant surface glycoproteins (VSGs; see Borst and Rudenko 1994; Vanhamme et al. 2001), a tiny sample is taken up by the fly and, from this sample, only a part will be able to thrive in the insect. This would ensure rapid speciation and the development of unexpected, 'weird' biochemical properties. This recurring founder effect could also help explain the acquisition of (limited) RNA editing in the first place: the 'genetic drift' phase of the Covello and Gray model clearly becomes less unlikely. Of course, mt functioning is integrated in the overall cellular metabolism. Its rapid evolution could accommodate large changes in kinetoplastid metabolism, such as the development of 'turbo-type' glycolysis and the unique glycosome in which it occurs (Hannaert et al. 2003). RNA editing has not been found in the diplonemids, the kinetoplastids sister group, but there has been a recent report of the cox1 gene in parasitic *D. papillatum* being split

up in ~250-bp fragments: again, this points to the possibility that spreading genes throughout the mt DNA is essential for different kinds of parasites (Marande et al. 2005). Why do diplonemids and, for that matter, other parasites possibly confronted with the problem of rapid mt DNA loss not all end up with panediting? There seem to be several possibilities. First of all, only the kinetoplastids appear to have acquired limited RNA editing in the first place. Secondly, only organisms which really compete severely with each other in at least one of their lifecycle stages would need to fragment their genes in mt DNA. Thirdly, the kinetoplastid lineage could already have lost all its tRNA genes to the nucleus, making it more vulnerable to large-scale mt deletions. Editing and tRNA import from the nucleus in kinetoplastids could be linked, but we do not know which triggered which. Finally, it could be that kDNA was already disproportionally large at the time editing evolved, thus laying a larger claim on the organisms' resources than in parasitic organisms with a relatively small mt genome. Another question is related to the fact that *T. brucei* is something of an exception with its (even for kinetoplastids) highly elaborate lifecycle. Could the ancestors in which panediting has supposedly evolved really have encountered such differing demands regarding mt contributions to their overall metabolism? An important point: the discussion of the model has been illustrated with examples taken from the parasitic lifestyle of the kinetoplastids, but any free-living ancestor in a periodically changing environment in which the organism uses highly differential mt functioning (Cavalier-Smith 1997) would be subject to the same kind of selective pressures, favouring fragmentation of mt genes *as long as strong intraspecific competition would occur*.

It is not easy to critically evaluate this model for the evolution of panediting. The present-day situation for (most of) the kinetoplastids does not reflect the evolutionary pressures faced by their 'panediting ancestor'. This is strongly reflected by the fact that panediting is becoming ever less extensive in the kinetoplastid lineage. We can, however, reconstruct the most likely evolutionary history in terms of the proposed theory (see Fig. 5) and assess whether this is supported by extensive studies of other kinetoplastids. Starting from the situation with a single necessary gRNA probably encoded on the same DNA molecule, an increasing number of essential gRNAs would have spread over the mt genome, possibly with a concomitant increase in size. The large increase in coding capacity needed and the intricate regulation of transcription necessary could have been reasons for the emergence of minicircles encoding most of the different gRNAs needed to generate fully edited mRNAs. The presence of several thousand small minicircles of hundreds of different sequence classes, over and above the 50 maxicircles, could have made replication and equal distribution upon division so complex that this, in turn, lead to the evolution of the kDNA network to combat minicircle loss (Borst 1991). Both minicircles and networks thus presumably evolved later, in response to coding capacity demands of the panediting mechanism (Blom et al. 2000), reflecting its drawbacks. Although minicircles are thus seen as a later development, the intermingling of gRNAs on minicircles in the trypanosomatid order is again strongest in *T. brucei* (encoding 2–5 gRNA genes; Hong and Simpson 2003). The 180-kb gRNA encoding circles found in the early-diverging cryptobiid kinetoplastid,

T. borreli (Yasuhari and Simpson 1996), come closer to the hypothetical ancestral state illustrated in Fig. 5.

What other predictions does the model make? One important 'in vivo' prediction of the model involves the correlation between the complexity of the lifecycle of a kinetoplastid and the amount of panediting. Such a prediction is fraught with difficulties, however, because the present-day level will also reflect the complexity the organisms' ancestors encountered during their evolutionary history. Extensive studies of lifecycle complexity, evolutionary history and amount of mt panediting have to be combined to check the model in this respect. Did panediting really evolve as the unlikely keeper of the kinetoplast genome? Let's wait for what the lab has to tell us!

9 Concluding Remarks

Seeing the diversity in evolutionary histories and uses of RNA editing, both in organisms displaying as well as in mechanisms used in RNA editing, one could wonder whether there are also general patterns. First of all, nature loves complexity. Every additional level of complexity in an organism gives selection extra possibilities to shift into new and unexpected directions (in itself a form of 'evolvability'). Secondly, one should never underestimate the power of genetic drift. Crucial in this respect is the population size, small populations going into unexpected directions much more easily than our intuition would allow for. Within this context, the possible occurrence of RNA editing in bacteria could be discussed: maybe their larger effective population sizes would exclude this. Higher-order processes, such as sexual propagation (with its concomitant creation of a species genepool), could very well be the original legacy of small population sizes. Thirdly, panediting in kinetoplastids nicely illustrates that evolution is 'trying to hit a moving target' (and that the 'trying' is part of the 'moving'). Last but not least, panediting in kinetoplastids also reflects 'conflicting' tendencies in evolutionary innovations: it speeds up evolution but, at the same time, makes gene loss much less likely due to the extreme genetic linkage it entails. Again, parallels with sex are obvious: this opens up new possibilities by rapidly forming myriads of combinations from the genepool but is also a conservative force because 'any … enterprising new evolutionary direction is held in check by the swamping effect of sexual mixing' (see Dawkins 2004). RNA editing thus illustrates, in its microcosmos, the great capability of very simple mechanisms to generate all the unpredictable wonders of molecular biology. To paraphrase Charles Darwin: 'there is a wonder in this view of RNA editing, with its several powers, having been originally started with few forms; and from so simple a beginning endless forms most beautiful and most wonderful have been, and are being evolved' (Darwin 1859).

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