A-to-I editing sites are a genomically encoded G: Implications for the evolutionary significance and identification of novel editing sites

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ABSTRACT

Ribonucleic acid (RNA) editing can extend transcriptomic and proteomic diversity by changing the identity of a particular codon. Genetic recoding as a result of adenosine-to-inosine (A-to-I) RNA editing can alter highly conserved or invariant coding positions in proteins. Interestingly, examples exist in which A-to-I editing sites in one species are fixed genomically as a G in a closely related species. Phylogenetic analysis indicates that G-to-A mutations at the DNA level may be corrected by post-transcriptional A-to-I RNA editing, while in turn, the edited I (G) may be hardwired into the genome, resulting in an A-to-G mutation. We propose a model in which nuclear A-to-I RNA editing acts as an evolutionary intermediate of genetic variation. We not only provide information on the mechanism behind the evolutionary acquisition of an A-to-I RNA editing site but also demonstrate how to predict nuclear A-to-I editing sites by identifying positions where an RNA editing event would maintain the conservation of a protein relative to its homologs in other species. We identified a novel edited site in the fourth exon of the *cacophony* transcript coding calcium channel $\alpha 1$ and verified it experimentally.

Keywords: A-to-I RNA editing; G-to-A mutation; evolutionary significance; *cacophony*

INTRODUCTION

Ribonucleic acid (RNA) editing is a process resulting in the synthesis of proteins that are not directly encoded in the genome. One type of RNA editing involves the modification of individual adenosine (A) bases to inosine (I) in RNA by adenosine deaminases acting on RNA (ADAR) enzymes (Bass 2002; Maas et al. 2003). Since I acts as guanosine (G) during translation, A-to-I conversion in coding sequences leads to amino acid changes and often entails changes in protein function (Seeburg et al. 1998; Bass 2002; Schmauss and Howe 2002). A-to-I RNA editing is common in animals and is associated with various neurological functions (Seeburg et al. 1998; Schmauss and Howe 2002). *Caenorhabditis elegans*, *Drosophila*, and mouse mutants lacking ADAR enzymes have distinct neurological phenotypes (Higuchi et al. 2000; Palladino et al. 2000; Tonkin et al.

2002; Wang et al. 2000). In addition to amino acid changes, the editing and subsequent destabilization of the RNA duplex present in the 5'- or 3'-untranslated regions (UTRs) could alter the stability, transport, or translation of the mRNA (Knight and Bass 2001; Tonkin and Bass 2003). Moreover, RNA editing could influence alternative splicing decisions (Rueter et al. 1999; Schoft et al. 2007), antagonize RNA interference (RNAi), and redirect microRNA (miRNA) silencing targets (Knight and Bass 2002; Tonkin and Bass 2003; Kawahara et al. 2007).

In animals, genetic recoding by A-to-I RNA editing is most common, altering highly conserved or invariant coding positions in proteins (Reenan 2005). However, some nuclear A-to-I editing sites are shown to be genomically encoded as a G in other species (Grauso et al. 2002; Jin et al. 2007; Ohlson et al. 2007). It is not clear whether this represents the ancestral state, or, whether high levels of editing of an ancestor were "hardwired" into the genome through mis-sense mutation. In this paper, we show that at least some A-to-I RNA editing functions to correct G-to-A mutations, while in turn, the edited I (G) was hardwired into the genome. Based on this model, we predicted a novel A-to-I editing site and verified it experimentally.

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RESULTS AND DISCUSSION

A-to-I editing sites as compensators of G-to-A mutations

In animals, A-to-I RNA editing usually alters highly conserved or invariant coding positions in proteins (Reenan 2005). Interestingly, several examples exist in which an A-to-I editing site represents a genomically encoded G in a related species. For example, site 15 in nAChR $\alpha 6$ is edited in *Bombyx mori* and *Apis mellifera*, while the corresponding site of the $\alpha 6$ ortholog $\alpha 7-2$ in the tobacco budworm *Heliothis virescens* (Lepidoptera) harbors a genomically encoded G (Fig. 1A). Site 3 is edited in *A. mellifera*, while the homologous sites in *Anopheles gambiae*, *H. virescens*, *B. mori*, and *Tribolium castaneum* consist of a genomically encoded G (Fig. 1A). Similarly, the $\alpha 6$ site 2 is edited in *Drosophila* (Grauso et al. 2002) and *Musca domestica*

(Deacutis et al. 2007), while the homologous $\alpha 6$ sites in other species consist of genomically encoded Gs (Fig. 1A). Two editing sites (Fig. 1A, sites 5,12) in nAChR $\alpha 6$ are conserved in four orders of insect, represented by *Drosophila melanogaster*, *B. mori*, *T. castaneum*, and *A. mellifera*. This is considered the most highly conserved RNA editing event yet reported in invertebrates. These two homologous $\alpha 6$ sites have a genomically encoded G in *Pediculus humanus* (Phthiraptera) (Fig. 1A). The situation is similar for the I/M editing site of GABAA receptor subunit $\alpha 3$, where the frog and pufferfish have a genomically encoded methionine at the equivalent position, while more evolved species have an I codon in the same position (Ohlson et al. 2007). The situation is also similar for the Q/R editing site of the glutamate receptor subunit gene B (GluR-B); the hagfish, a jawless fish, and teleosts have an R codon at this position, while species that appeared after the cartilaginous fish have a Q codon in the same position (Wu et al. 1996; Kung et al. 2001).

Although we do not know the generality of this phenomenon, it led us to determine whether the genomically encoded G represents the ancestral state, or whether high levels of editing of an ancestor were hardwired into the genome through missense mutations.

FIGURE 1. Position of the A-to-I edited site in the fifth exon of nAChR $\alpha 6$ and evolutionary conservation. (A) Alignment of the homologous exon 5 genomic nucleotide of nAChR subunit $\alpha 6$ genes from *D. melanogaster* (Dme), *M. domestica* (Mdo), *A. gambiae* (Aga), *H. virescens* (Hvi), *B. mori* (Bmo), *T. castaneum* (Tca), *A. mellifera* (Ame), and *P. humanus* (Phu). RNA editing of the nAChR subunit $\alpha 6$ genes from *D. melanogaster*, *M. domestica*, *H. virescens*, *A. gambiae*, *T. castaneum*, and *A. mellifera* has been described previously (Grauso et al. 2002; Jones et al. 2005, 2006; Deacutis et al. 2007; Jin et al. 2007). (B) Alignment of the amino acids of the homologous exon 5 of the nAChR subunit $\alpha 6$ genes. The editing sites from positions 1–16 and amino acids are shaded in red. Those sites that are G constitutively are shaded in green at the editing sites. (C) Evolutionary tree showing the phylogenetic relationships among the species. Residues altered by RNA A-to-I editing are enclosed in squares.

G-to-A mutations may be corrected by A-to-I RNA editing

The evolutionary history of the editing of nAChR $\alpha 6$ is charted roughly in insects. Figure 1 shows that site 2 is edited in the nAChR $\alpha 6$ of *D. melanogaster* and *M. domestica*, while the homologous sites in *A. gambiae*, *H. virescens*, *T. castaneum*, *B. mori*, and *P. humanus* have a genomically encoded G. Correspondingly, these orthologs encode V at the position of this I/V editing site. Phylogenetic analysis indicates that G represents the ancestral state at this site, at least before the radiation of the Drosophilidae and Culicidae (spanning ~200 million years). We also analyzed these orthologs from 12 *Drosophila* species (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis*, *D. virilis*, and *D. grimshawi*) and found that the corresponding position encodes an A in these orthologs. This indicates that G-to-A mutations

occurred at least before the radiation of the Drosophilidae and Culicidae.

Similarly, site 8 is edited in *M. domestica*, while the position of this editing site in *D. melanogaster*, *A. gambiae*, *H. virescens*, *T. castaneum*, *B. mori*, and *P. humanus* contains a genomically encoded G (Fig. 1A). Again, phylogenetic analysis indicates that a G represents the ancestral state at this site, at least before the radiation of the Drosophilidae and Culicidae. At the divergence of the Muscidae and Drosophilidae, the G was converted into an A in the Muscidae, while a G remained in drosophilids. Analysis of these orthologs from 12 *Drosophila* species (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis*, *D. virilis* and *D. grimshawi*) showed that they possess a G. Compared with site 2, the conversion at site 8 is confined to a smaller phyletic branch.

Although the G-to-A mutation probably occurred against negative selective pressure, subsequent A-to-I RNA editing counteracted this conversion, maintaining similarity at the protein level to reduce negative selective pressure. Therefore, the A-to-I RNA editing of this site must have evolved immediately after the G-to-A conversion event. Consistently, the converted A was located within a secondary structure. Subsequently, RNA editing at this position may have evolved to be dispensable and has consequently been abolished due to loss of the corresponding secondary structure, such as in *A. gambiae* (Fig. 1A). Unlike other A-to-I editing sites, these editing sites represent an atypical A-to-I editing event, which increases the conservation of a protein relative to homologs in other species.

However, this occurrence of A-to-I RNA editing cannot be fully explained in terms of correcting G-to-A mutations. Mutational data show that the AMPA receptor subunit of GluR-B in its unedited Q/R site form is not essential for brain development and function (Kask et al. 1998); however, this is the only editing site with nearly 100% frequency throughout development in rodents. Unlike the case of GluR-B, most genes are partially edited or edited in a tissue-dependent manner. This implies that a more complex editing mechanism has evolved in the nuclear genome, one that plays a role in regulating gene expression, in addition to neutralizing G-to-A mutations. The evolutionary conservation of the spatial and developmental regulation of RNA editing also suggests that the extent of editing may modulate biological function. Much evidence indicates that the unedited RNA was important biologically (Vissel et al. 2001). Moreover, an early gene might be edited at an optimal, yet intermediate, level. Over time, the subsequent mutations in double-stranded RNA (dsRNA) regions may have allowed higher levels of editing, even 100% editing, to achieve the maximum compensatory level. However, such 100% editing is rarely found at all developmental stages, and perhaps partial RNA editing may be sufficient to correct G-to-A mutations.

Edited nucleotides may be hardwired into the genome

Site 15 is edited in the nAChR $\alpha 6$ of *B. mori* and *A. mellifera*, while this site in the $\alpha 6$ ortholog $\alpha 7-2$ in the tobacco budworm *H. virescens* has a genomically encoded G (Fig. 1A). Phylogenetic analysis indicates that A represents the ancestral state at this site, which gained A-to-I RNA editing during evolution. A-to-G conversion tends to occur because of the low negative selective pressure at the protein and functional levels; consequently, edited nucleotides may be hardwired into the genome. This hypothesis appears reasonable because eliminating the requirement for editing by engineering a constitutive R at the GluR-B gene Q/R site had no obvious effects in mice (Kask et al. 1998) and was able to rescue the lethal phenotype observed in ADAR2 knockout mice (Higuchi et al. 2000).

The implications of A-to-I RNA editing as an evolutionary intermediate of genetic variation

Based on the comparative data presented above, we proposed a model for the evolutionary mechanism of A-to-I RNA editing (Fig. 2). The first consideration of this model is that G represents the ancestral state and was converted into A during evolution. A-to-I RNA editing occurred immediately after the G-to-A conversion event, maintaining similarity at the protein level to reduce negative selective pressure (Fig. 2A). In this case, the G-to-A mutation at the DNA level may be corrected by post-transcriptional A-to-I RNA editing. The second consideration of this model is that A represented the ancestral state, which gained A-to-I RNA editing during evolution. A-to-G conversion tended to occur because of low negative selective pressure, so that edited nucleotides might be hardwired

FIGURE 2. Model of the evolutionary mechanism of nuclear A-to-I RNA editing as an evolutionary intermediate of genetic variation. (A) G represents the ancestral state, which was converted to A during evolution. A-to-I RNA editing occurred immediately after the G-to-A conversion event, maintaining similarity at the protein level to reduce negative selective pressure. A-to-I RNA editing might be lost in the subsequent evolutionary step. (B) A represents the ancestral state, which gained A-to-I RNA editing during evolution. A-to-G conversion tended to occur because of low negative selective pressure, and thus the edited nucleotides might be hardwired into the genome. G → A and A → G denote G-to-A and A-to-G mutations, respectively, and A/G indicates A-to-I RNA editing.

into the genome, resulting in an A-to-G mutation at the DNA level (Fig. 2B). In any case, nuclear A-to-I RNA editing might act as an evolutionary intermediate of A-to-G or G-to-A mutation, maintaining conservation at the protein and functional levels during evolution despite sequence divergence at the DNA level.

Does RNA editing affect genetic variation? Comparative analysis indicated that the fifth exon of the nAChR $\alpha 6$ encodes 61 amino acids, of which 50 (85%) are conserved in all eight species. Sixteen edited sites exist, resulting in 12 amino acid changes, of which only five of 12 (42%) are conserved in all eight species. This suggests that the edited sites accumulate mutations nearly twice as rapidly as the unedited sites. Moreover, the prevalence of A/G and C/T(U) substitutions in the genetic variation might be explained by the fact that the most prevalent changes of RNA editing in the nuclei of higher eukaryotes are hydrolytic deaminations, in which the genomically encoded C or A is converted into U and I, respectively (Brennicke et al. 1999). Therefore, nuclear A-to-I RNA editing not only extends the sequence diversity at the RNA and protein levels but is also a novel source of genetic variation, at least in some genome sequences.

Detection and verification of a novel A-to-I editing site

Since nuclear A-to-I RNA editing tends to increase protein conservation across species by “correcting” codons that specify less-conserved amino acids in some cases, this principle can be used to predict A-to-I editing sites by identifying positions where an RNA editing event would increase the conservation of a protein relative to homologs from other species. One of the most promising predicted structures suitable for A-to-I editing was found in the fourth exon of the *cacophony* (*cac*) transcript coding the calcium channel $\alpha 1$ subunit. A-to-I editing has been identified at 12 positions (Smith et al. 1996; Keegan et al. 2005), while A-to-I editing sites in the fourth exon have not been studied. This region mainly encodes transmembrane segment S4 and the preceding extracellular loop in the first repeat

domain of this channel (Fig. 3A). Phylogenetic analysis indicated that G represented the ancestral state at this site. Until the radiation of the *Sophophora* and *Drosophila* groups (spanning ~41 million years), G was mutated to A at this site in the *Drosophila* groups. Therefore, the I/M sites of *cac* in *D. mojavensis*, *D. virilis*, and *D. grimshawi* have a genomically encoded M, while the species that appeared after the *Drosophila* group have an I at the same position. The homologous site is also a genomically

FIGURE 3. Position of the edited site in *cac* and the evolutionary analysis. (A) The protein sequence of *cac* consists of four repeating transmembrane domains (TM1–TM4), each including six transmembrane segments (S1–S6) (Catterall 2000; Keegan et al. 2005). Shading represents the plasma membrane. Both the N and C termini of the $\alpha 1$ subunit are intracellular, and the four repeats are linked by three major intracellular loops. The edited site (shown by the “*”) was located within the extracellular loop between transmembrane segments S3 and S4 of the first repeating domain. (B) The I/M sites of *cac* in *D. mojavensis* (Dmo), *D. virilis* (Dvi), and *D. grimshawi* (Dgr) have a genomically encoded methionine, while *D. melanogaster* (Dme), *D. simulans* (Dsi), *D. sechellia* (Dse), *D. yakuba* (Dya), *D. erecta* (Der), *D. ananassae* (Dan), *D. pseudoobscura* (Dps), *D. persimilis* (Dpe), and *D. willistoni* (Dwi) have an isoleucine. Shading represents conserved nucleotide and amino acid sequences across *Drosophila* species. (C) Evolutionary tree showing the phylogenetic relationships among 12 *Drosophila* species, one mosquito species (*A. gambiae*), and the silkworm (*B. mori*).

encoded G in *A. gambiae* and *B. mori*. The situation is similar for sites 2 and 5 of nAChR $\alpha 6$ (Fig. 1A), although this conservation is confined to a younger phyletic branch. Therefore, we predicted that A-to-I editing occurred at this site. In parallel, we predicted a weak stem-loop structure suitable for A-to-I editing in the encoded sequences using the program mFold (Zuker 2003).

To validate the predicted editing site of the calcium channel $\alpha 1$ gene experimentally, we isolated total RNA from an adult *D. melanogaster* and compared the sequence determined after RT-PCR with the genomic sequence. An adenosine was present in the genomic sequence of exon 4 at the position in which a G and A mixed signal was present in the cDNA sequence (Fig. 4). At this position, an isoleucine (I) AUA codon is changed to an AUI and read as a methionine (M) codon (AUG), since inosine is read as a guanosine. RNA editing of the I/M site was confirmed for the other *Drosophila* species: *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, and *D. ananassae*. To investigate if editing at the I/M site of *cac* changes during development, total RNA was extracted from fruit fly embryos, larvae, pupae, and adults. The extent of editing was determined by sequencing individual clones derived from the RT-PCR products. As a result, the I/M-site in the *cacophony* tran-

script shows an increase in RNA editing through development. This trend is similar to that at other editing sites and is consistent with increasing levels of ADAR expression through development, which is most evident in adult flies (Keegan et al. 2005). Since we have insufficient editing data, we cannot determine the generality of this rule; however, its importance lies in the fact that the resulting editing occurs in the coding region of a gene. We will be able to make broader generalizations, particularly for these atypical editing sites, after considering the other main criteria regarding editing probability, such as dsRNA regions.

CONCLUSIONS

In conclusion, we found several atypical nuclear editing events that generated a conserved codon identity at the RNA level, but not at the DNA level. G-to-A mutations at the DNA level may be corrected by post-transcriptional A-to-I RNA editing, while in turn, the edited I (G) may also be hardwired into the genome, resulting in an A-to-G mutation. Therefore, nuclear A-to-I RNA editing might act as an evolutionary intermediate of genetic variation. We proposed a model for the evolutionary mechanism of nuclear A-to-I RNA editing, and based on this model, we identified a novel edited site in the fourth exon of the *cacophony* transcript coding the calcium channel $\alpha 1$ and verified it experimentally.

MATERIALS AND METHODS

Materials

Total RNA was isolated from *D. melanogaster* at different developmental stages using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Genomic DNA was isolated using the Universal Genomic DNA Extraction Kit (TaKaRa). Plasmid DNA was purified using a Qiagen plasmid isolation kit.

Gene analysis

The sequences of the nAChR subunit $\alpha 6$ genes from *D. melanogaster*, *M. domestica*, *H. virescens*, *B. mori*, *A. gambiae*, *T. castaneum*, and *A. mellifera* have been described previously (Grauso et al. 2002; Jones et al. 2005; Deacutis et al. 2007; Jin et al. 2007). The sequences of the nAChR subunit $\alpha 6$ genes from the human body louse, *P. humanus*, were assembled from individual raw sequence reads available from the NCBI trace archives. The sequences of the *cac* genes for the other *Drosophila* species (*D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis*, *D. virilis*, and *D. grimshawi*), *A. gambiae*, and *B. mori* were assembled from individual raw sequence reads available from the NCBI trace archives. The sequence of the *cac* gene for *D. simulans* was determined using PCR and sequencing. The nucleotide and amino acid sequences of each alternative exon from each species were aligned using the program ClustalW (EMBL-EBI).

FIGURE 4. A-to-I editing site of the calcium channel $\alpha 1$ gene. (A) Reverse transcriptase reactions were performed with oligo(dT)₁₅ on poly(A)⁺ RNA from *D. melanogaster* embryos, larvae, pupae, and adults. The RT-PCR and PCR products corresponding to the mRNAs were purified for direct sequencing. The editing sites (arrow) show a mixed A/G or pure G signal, while the DNA sequence contains adenosine signals only. (B) RT-PCR products from the embryos, larvae, pupae, and adults were cloned, and relative editing levels were analyzed by sequencing. The number (n) of clones analyzed for each sample is indicated.

Analysis of RNA editing

RNA editing was analyzed using total RNA from *D. melanogaster* as the template for RT-PCR, which was performed using primer pairs for the *cac* gene in different species. The primers used to amplify the RT-PCR products were 5'-GTGTAGAAGCGTCGCTCAAGATCC-3' (forward) and 5'-ACTAGGAATTCCAGACACT-3' (reverse). The PCR products were sequenced directly after gel purification. Primers for the *cac* gene exon 4 used to amplify genomic DNA from the same tissues used for RNA isolation were 5'-CCATAGTTTACATTGCTCT-3' (forward) and 5'-ACTAGGAATTCCAGACACT-3' (reverse). The genomic PCR amplification product was sequenced directly to demonstrate that the genomic products give a pure A signal at editing sites, ruling out a polymorphism. In addition, the RT-PCR products were cloned into the pGEM-T Easy vector (Promega), and ~20 cDNA clones were sequenced for every stage to determine the relative A-to-G abundance. RNA editing of the I/M site was confirmed using RT-PCR analysis from adult total RNA and direct sequencing analysis for the other *Drosophila* species: *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, and *D. ananassae*.

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