# Q/R RNA editing of the AMPA receptor subunit 2 (GRIA2) transcript evolves no later than the appearance of cartilaginous fishes

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**Abstract** **The amino acid, either a glutamine (Q) or an arginine (R), at the Q/R site of the pore-lining segment (M2) of a vertebrate AMPA receptor subunit critically influences the properties of the receptor. The R codon of the mammalian AMPA receptor subunit 2 (GRIA2) transcript is not coded by the chromosomal sequence, but is created by posttranscriptional RNA editing activities. On the other hand, the R codons of some teleost GRIA2 homologs are coded by chromosomal sequences. To elucidate the evolution of the utilization of Q/R RNA editing in modifying vertebrate GRIA2 transcripts, the GRIA2 genes of five fish species and an amphibian were studied. The putative hagfish GRIA2 homolog (hfGRIA2) encodes an R codon, whereas shark and bullfrog GRIA2 genes specify a Q codon at the genomic Q/R site. All gnathostoma GRIA2 genes possess an intron splitting the coding regions of M2 and the third hydrophobic region (M3). The intronic components required for Q/R RNA editing are preserved in all the Q-coding vertebrate GRIA2 genes but are absent from the R-coding GRIA2 genes. Interestingly, the hfGRIA2 is intronless, suggesting that hfGRIA2 is unlikely evolved from a Q/R editing-competent gene. Results of this study suggest that modification of GRIA2 transcripts by Q/R editing is most likely acquired after the separation of the Agnatha and Gnathostome. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

*Key words:* Glutamine/arginine RNA editing; Vertebrate; Hagfish; Zebrafish; GRIA2

## 1. Introduction

AMPA-(α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), kainate- and NMDA-preferring receptors are three pharmacologically defined families of ionotropic glutamate receptors (iGluRs), which mediate fast excitatory synaptic transmission in the vertebrate central nervous system [1]. The AMPA receptors (AMPARs) are assembled from the products of four highly conserved genes, GRIA1–GRIA4 (also known as GluR1–GluR4 or GluRA–GluRD), in homomeric and heteromeric forms [1–3]. The proposed membrane topology of iGluR subunit consists of four hydrophobic domains (M1–M4), of which the second domain (M2) lines the channel and forms a loop with both ends facing the cytoplasmic side [4,5]. The glutamine/arginine (Q/R) site near the carboxyl end of the M2 plays an important role in controlling the calcium permeability and gating properties of AMPARs [5,6]. AMPARs with all component subunits having a Q residue at the Q/R site are permeable to calcium ions and display a rectified current–voltage relationship. On the other hand, AMPARs consisting of at least one subunit with an R residue at the Q/R site are relatively impermeable to calcium ions and display a linear current–voltage relationship [3,5,6].

All the mammalian AMPAR (GRIA1–GRIA4) genes specify a Q codon, either a CAG or a CGG, at the Q/R position. Only the GRIA2 transcript is modified by nuclear adenosine deaminase activities, which convert the CAG (Q) to CIG (R) [7,8]. Modification of the primary GRIA2 transcripts at Q/R site (Q/R editing) depends on a double-stranded RNA structure formed by sequences flanking the adenosine of the Q codon with a downstream complementary sequence of the intron [7,8]. The editing of the GRIA2 transcript is very extensive in that all the GRIA2 mRNA of adult rat brains encode an R at the Q/R site [9]. A genetically altered GRIA2 allele deficient in the Q/R editing exerts a dominant lethal effect, whereas a GRIA2 allele with an exonic R codon shows no obvious abnormality [10,11]. These results demonstrate the importance of the R-bearing GRIA2 subunit and Q/R RNA editing of mammalian GRIA2 transcripts in maintaining normal brain functions.

Reverse transcriptase-PCR surveys of the vertebrate AMPAR subunits have shown that brains of vertebrate express at least one kind of AMPAR subunit bearing an R residue at the Q/R site [12]. These R-bearing AMPAR subunits were considered as GRIA2 homologs. The majority of the vertebrate brains express only one GRIA2 subunit, while brains of some teleost fish express multiple GRIA2 homologs, which may result from a ray-finned fish-specific gene duplication [12–14]. Phylogenetic studies suggest that the GRIA2 genes of jaw vertebrate (Gnathostome) evolve from a common ancestral gene. However, the phylogenetic relationship between the GRIA2 genes of the jawless (Agnatha) and jaw vertebrates remains unclear [12]. Analyses of goldfish (*Carassius auratus*) genes indicate that the Q/R editing of GRIA2 transcripts also operates in teleost fish [14]. One of the four goldfish GRIA2 genes, gfGRIA2a, specifies a Q codon (CAG) at the chromosomal Q/R site. Furthermore, the intron of gfGRIA2a contains an imperfect inverted repeat and the exon-complementary sequence (ECS) which forms perfect base pairing to the exon centered on the editing site [7,14]. The genomic sequences of the remaining three gfGRIA2 genes have not been determined. On the other hand, GRIA2 genes of a more derived teleost, tilapia (*Oreochromis mossambicus*), specify R codons (CGA and AGA) at the chromosomal Q/R site, in-

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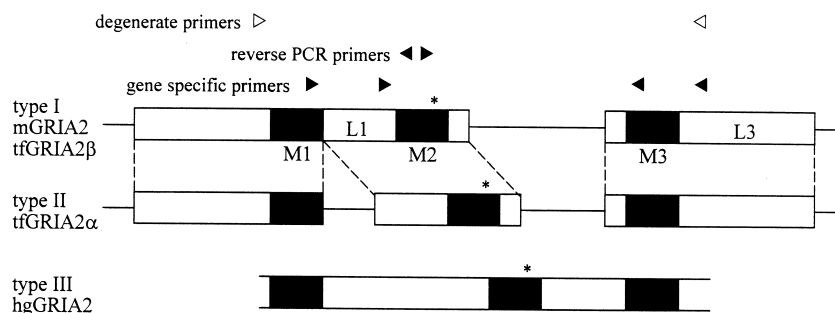*E-mail address:* wychow@life.nthu.edu.tw (W.-Y. Chow).

Fig. 1. Gene structure of the M1-, M2- and M3-coding regions of vertebrate GRIA2. The exons (boxes) were drawn to scale while the introns were not. The sizes of introns are given in Section 2.3. Filled boxes indicate the hydrophobic domains M1–M3. Asterisks mark the Q/R site. Arrowheads indicate the annealing sites and polarities of PCR primers. The gene structures of mGRIA2 and tfGRIA2β were respectively taken from Köhler et al. [15] and Chen et al. [16].

dicating that an exonic Q codon is not universal among teleost GRIA2 genes [13]. In this study, the genomic DNA sequences of the M2- and M3-coding exons of several non-mammalian vertebrate GRIA2 genes were determined to reveal the evolution of Q/R editing of GRIA2 transcripts. Our results show that the GRIA2 genes of shark (skGRIA2) and bullfrog (frGRIA2) specify the Q codon at the chromosomal Q/R site, whereas GRIA2 genes of hagfish (hfGRIA2), an agnathan fish, and some teleost fish specify R codons. Furthermore, only the Q-coding GRIA2 genes have the exon–intron features essential for RNA editing. These results suggest that the Q/R editing of the GRIA2 primary transcripts evolves no later than the appearance of cartilaginous fish.

## 2. Materials and methods

### 2.1. Materials

Shark (*Squalus acanthias*) and hagfish (*Paramyxine yangi*) were respectively obtained from Hsinchu and Geoshi harbors of Taiwan; bullfrog (*Rana catesbeiana*) was purchased from a local market. In this study, liver DNA was used as a source for genomic study. Pufferfish (*Tetraodon fluviatilis*) liver DNA was a gift from Dr. C.J. Huang of Institute of Biological Chemistry, Academia Sinica, Nankang. Genomic (liver) DNA library of zebrafish (*Danio rerio*) Oregon strain was a gift from Dr. S.-P. Huang of Institute of Zoology, Academia Sinica.

### 2.2. Screening genomic library

Köhler et al. [15] have reported the gene structure of mouse GRIA2 (mGRIA2). Details of the construction and screening of the tilapia (*Oreochromis mossambicus*) genomic (liver) library have been described by Kung et al. [13]. The gene structure of tilapia GRIA2β (tfGRIA2β) has been reported [13,16]. The genomic DNA containing the M2-coding exon of tfGRIA2α has been amplified by inverse PCR [13]; however, in this study, it was obtained by screening tilapia genomic library using the M1–M3-coding region of tfGluR2α cDNA as a probe. Similarly, the exons of zebrafish GRIA2 (zfGRIA2) genes were identified by screening a genomic library using the M1–M3-coding regions of the two zfGRIA2, zfGRIA2α and zfGRIA2β, cDNA molecules as probes. The DNA fragments containing exons were identified by Southern hybridization, subcloned into pBlueScript II and sequenced as described by Chen et al. [16]. The intron and exon boundaries were assigned by comparing the genomic sequences to the corresponding cDNA sequences by the GAP program of GCG package (University of Wisconsin Genetics Computer Group) and matching the consensus splice donor and splice acceptor sequences.

### 2.3. Genomic PCR and inverse PCR

A schematic representation of the annealing sites of the PCR primers is shown in Fig. 1. Degenerate primers back translated from WMCIVFAYIGL and KQTEIAYGTL [12] were used to amplify the pufferfish (pfGRIA2) and hfGRIA2 gene fragments. Five puffer-

fish and two hagfish AMPAR genes were amplified. The GRIA2 genomic DNAs were further identified by colony hybridization using pfGRIA2 and hfGRIA2 cDNA molecules as probes [12]. Attempts of amplification of frGRIA2 and skGRIA2 gene fragments by the same pair of degenerate primers or by gene-specific primers designed according to the coding sequences of the M1, L1 (the loop sequence between the M1 and M2), M3 and L3 (sequence between the M3 and M4) did not succeed (Fig. 1 and data not shown). Hence, inverse PCR was performed. The sequences of inverse PCR primers used to amplify frGRIA2 genomic DNA were (5′ to 3′) TTAATAG-TCTATGGTTTTCCCTTGTTCC and CCAAACTCATTAGATG-AATCGTTACTTTG; those for amplification of skGRIA2 were CGTATTCTTCGGTGTGCCACTCATACGG and GGACGGGA-AACACAAAGTAACGACCAAAC. For inverse PCR, the liver DNAs were digested by *Sau*3A or *Alu*I, and self-ligated under very low DNA concentration (< 10 pg/μl) to facilitate intra-strand ligation. Expand™ long template PCR system (Boehringer Mannheim) and 200–500 ng of genomic DNA or ligated DNA were used in the PCR. PCR was carried out for 35 cycles of amplification (1 min at 94°C, 1 min at 55°C, and 2–4 min at 72°C). A 10 min extension at 72°C was performed after the last cycle. The amplicons were cloned to pGEM-Teasy vector (Promega) and transformed to *Escherichia coli* XL1-Blue by electroporation. The genomic sequences obtained in this study were deposited to the GenBank under accession numbers of AF350048–AF350056. DNA sequences of the intron downstream to the Q/R site of the 0.6-kb tfGRIA2β [16], 0.45-kb zfGRIA2α and 0.19-kb pfGRIA2 were determined. The intronic sequences of the remaining GRIA2 genes were only partially determined. The sizes of introns of these GRIA2 genes were respectively longer than 10, 1.4, 0.3 and 1.7 kb for tfGRIA2α, zfGRIA2β, skGRIA2 and frGRIA2. The size of mGRIA2 intron is 4.6 kb [15]. Programs included in the GCG were used to scan the intronic sequences for the presence of secondary structures. The mfold version 3.1 [17] was then used to predict secondary structures of RNA sequences transcribed from the exonic and the proximal 360-bp intronic sequences.

## 3. Results

### 3.1. Cloning of the vertebrate GRIA2 genes

Complementary DNA molecules encoding the hydrophobic domains M1, M2 and M3 of GRIA2 subunits from brains of hagfish (Agnatha), shark (Chondrichthyes), ray-finned fishes (Actinopterygii) and bullfrog (Amphibia) have been reported [12]. All of these vertebrate GRIA2 subunits carry an R residue at the Q/R site and, in general, display high sequence identities (87.5–96.2%) to the mammalian GRIA2. Two paralogous GRIA2 genes are expressed in tilapia and zebrafish brains, whereas only one GRIA2 gene is expressed in brains of the remaining animals [12]. The M1 and M2 of tfGRIA2β, gfGRIA2a and mGRIA2 are coded by a single exon, while the M3 of these GRIA2 genes is coded by a separate exon (type I,

Fig. 2. Sequence alignments of the vertebrate GRIA2 genes. Sequence alignments of (A) the Q-coding GRIA2, (B) the stem loop A of teleost Q-coding GRIA2, (C) the stem loop B of teleost Q-coding GRIA2, and (D) R-coding vertebrate GRIA2. The nucleotide sequences of the stem loops A and B were defined by the secondary structures shown in Fig. 3. Nucleotide sequences were aligned by the Pileup or gap programs of GCG using default settings, and the numbers of gap were manually reduced. Open and filled inverse triangles respectively mark the editing site and the 5′-intron splicing site. The sequences of mGRIA2 and gfGRIA2a were respectively taken from Higuchi et al. [7] and Li et al. [14]. Black-shaded characters represent the consensus sequences (more than 50%) of the vertebrate GRIA2 genes; while the gray-shaded characters show the sequences conserved in either teleost or tetrapod GRIA2 genes.

Fig. 1 [13–16]). To determine the codons at the chromosomal Q/R site, the M2-coding exons of tfGRIA2 and zfGRIA2 genes were obtained by screening genomic libraries (Section 2.2). The organizations of M1-, M2- and M3-coding exons of zfGRIA2α and zfGRIA2β were the same as that of mGRIA2 and tfGRIA2β (type I); however, the organization of the tfGRIA2α differed from that of tfGRIA2β (Fig. 1). An intron splitting the second and the third nucleotides of the codon specifying the last amino acid of M1 was found in the tfGRIA2α gene (type II; Fig. 1). The result of genomic study of tfGRIA2α gene reported here is consistent with that obtained by inverse PCR analysis reported previously [13].

Genomic DNAs of pfGRIA2 and hfGRIA2 were amplified by PCR and those of frGRIA2 and skGRIA2 were obtained by inverse PCR (Section 2.3). The M2- and M3-coding regions of pfGRIA2, frGRIA2 and skGRIA2 genes were also interrupted at the preserved intron insertion site of the type I and type II genes. Although the 5′-boundary of the M1–M2-coding exon was not cloned, the M1- and M2-coding sequences of pfGRIA2, skGRIA2 and frGRIA2 genes were not interrupted, as was observed in the type II gene (data not shown). Hence, the gene structures of pfGRIA2, skGRIA2 and frGRIA2 were very likely the same as that of tfGRIA2β and mGRIA2. Surprisingly, the M1-, M2- and M3-coding regions of the hfGRIA2 were not interrupted (type III, Fig. 1), showing that the gene structures differed between agnathan and gnathostoma GRIA2 genes. These results suggested that type I gene organization was an ancestral type of gnathostoma GRIA2 gene. Consequently, the type II gene, tfGRIA2α, might result from an intron insertion into the M1–M2-coding region.

### 3.2. Q/R editing of the gnathostoma GRIA2 transcript is a plesiomorphic trait

Figure 2 shows the comparisons of vertebrate GRIA2 genomic sequences flanking the Q/R site. The skGRIA2 and frGRIA2 genes encoded the CAG (Q) codon at the Q/R site. Chromosomally encoded R codons were found at the Q/R site of hfGRIA2, pfGRIA2 and zfGRIA2β. Interestingly, the second zfGRIA2 homolog, zfGRIA2α, specified the CAG (Q) codon at the Q/R site, showing a difference between the two paralogous zfGRIA2 genes at the codons of chromosomal Q/R site and their dependency on the RNA editing activities to create an R codon at the Q/R site. The chromosomally encoded Q codon of zfGRIA2α supports the possibility that zfGRIA2α and the gfGRIA2a, which also specifies a Q codon at the chromosomal Q/R site, are orthologous [12,14].

Q/R editing of mammalian GRIA2 transcripts depends on the intron downstream to the M2-coding exon. The sequence of this intron contains imperfect inverted repeat sequences and ECSs capable of forming perfect complementary pairing with the upstream exonic editing site [5,7,8]. The mfold program was used to predict the secondary structures of the vertebrate GRIA2 transcripts [17]. No stable secondary structure could be predicted from the sequence of the R-coding GRIA2 genes. Furthermore, no significant sequence homology existed among the intronic sequence of the R-coding genes or between the intronic sequences of the R-coding and the Q-coding GRIA2 genes (data not shown). On the other hand, duplex structures involving pairing of intronic and exonic sequences of the primary transcripts of the Q-coding zfGRIA2α and frGRIA2 were predicted (Fig. 3). The secondary structures of gfGRIA2a and zfGRIA2α tran-
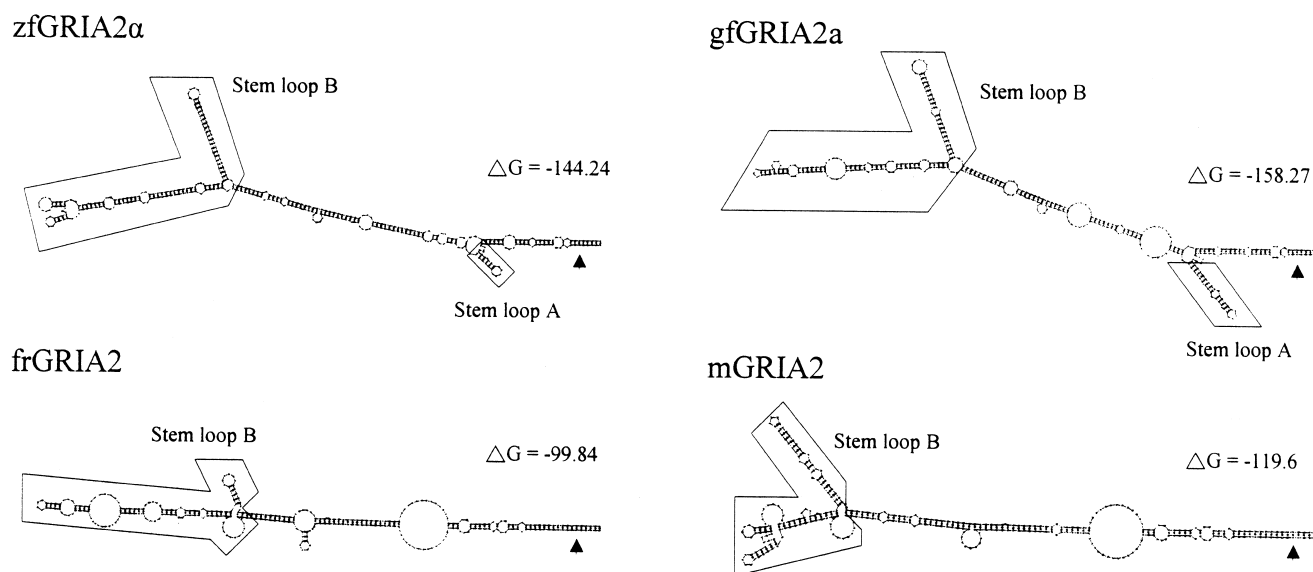
zfGRIA2α

gfGRIA2a



Fig. 3. Secondary structure of the Q-coding GRIA2 transcripts. Arrowheads mark the editing nucleotides.

scripts were very similar to each other, but these structures differed slightly from those of the mGRIA2 and frGRIA2 transcripts (Fig. 3). The tetrapod GRIA2 transcripts lack the small stem loop A which is present in the teleost GRIA2 transcripts (Fig. 3). The distal intronic sequences, beyond 360 nucleotides downstream of the splice donor site, were highly diverged (< 33% homologies) among the Q-coding GRIA2 genes (data not shown). On the other hand, 48–80% sequence homologies existed among the proximal 360-bp intronic sequences, which included the inverted repeats, stem loops and ECS of the Q-coding GRIA2 genes (Fig. 2A and data not shown). Results of site-directed mutagenesis and deletion analyses have shown that the stem loop B of the mGRIA2 can be deleted without perturbing the Q/R editing, whereas destabilizing the pairing between the inverted repeats results in significant losses of editing [7,8]. Consistently, sequences of the inverted repeats were much more conserved than those of the stem loop B among the Q-coding GRIA2 genes (Table 1). Consistent with taxonomic relationships, the sequences of stem loops A and B were well conserved between gfGRIA2a and zfGRIA2α (74.7%), but diverged from the stem loop sequences of tetrapod (36.6–44.1%; Fig. 2B,C, Table 1).

The intronic sequence of the skGRIA2 was incomplete. Nevertheless, the proximal intronic sequence of skGRIA2 dis-

played significant degrees of sequence homologies to the Q-coding GRIA2 introns (Table 1). Unexpectedly, the homologies between the inverted repeat sequences of skGRIA2 and tetrapod GRIA2 were higher than those between skGRIA2 and the Q-coding teleost GRIA2. Furthermore, the skGRIA2 lacked the sequence corresponding to the stem loop A found in the teleost GRIA2, implying that the primary transcript of skGRIA2 might form a secondary structure similar to that of the mGRIA2 (Figs. 2A and 3).

Downstream of the imperfect inverted repeats of zfGRIA2α, an 8-nucleotide element (ECS) formed a perfect complementarity with the editing nucleotide and flanking exonic sequence (Figs. 2A and 3). The ECS found in the zfGRIA2α and gfGRIA2a were 8-nucleotide, whereas the ECS found in the mGRIA2 and frGRIA2 were 10-nucleotide (Fig. 2A, [7,14]). The exonic sequences flanking the Q/R site are very conserved (27 out 29 nucleotides) among the Q-coding genes but are more diverged (20 conserved nucleotides out of 29) among the R-coding genes (Fig. 2). Furthermore, in contrast to the exclusive use of CAG trinucleotide at the Q/R site of the Q-coding GRIA2 genes, two kinds of R codons (CGA and AGA) were found in the R-coding GRIA2 genes. This observation showed that the exonic sequence flanking the Q/R site was no longer highly selected for the R-coding GRIA2 genes.

Table 1
Pairwise sequence comparisons[a] among intronic sequences downstream of the M2-coding exon of vertebrate Q-coding GRIA2 genes

|          | Homology (%) | | | | |
|          | gfGRIA2a | zfGRIA2α | skGRIA2 | frGRIA2 | mGRIA2 |
|----------|----------|----------|---------|---------|--------|
| gfGRIA2a | –        | 85.3     | 58.2    | 65.4    | 64.0   |
| zfGRIA2α | 74.7     | –        | 64.2    | 65.4    | 64.6   |
| skGRIA2  | n.d.     | n.d.     | –       | 70.1    | 71.6   |
| frGRIA2  | 44.1     | 42.7     | n.d.    | –       | 70.1   |
| mGRIA2   | 41.2     | 36.6     | n.d.    | 41.6    | –      |

[a]Homologies were calculated on the basis of sequence alignments generated by the GAP program of GCG. Variable gap-extension and gap-creation penalty values were used to enhance the alignments. The sequence homologies of the stem loop B are shown below the diagonal and those of the inverted repeats (the intronic sequences omitting the stem loop B sequence) are shown above the diagonal. Only half of the inverted repeat sequence of skGRIA2 was available for the comparisons. The homology between the stem loop B sequence of skGRIA2 and the other GRIA2 genes were not determined (n.d.).

## 4. Discussion

The genomic analysis shows that the R (CGG) codon at the Q/R site of skGRIA2 and frGRIA2 transcripts is posttranscriptionally created, whereas R (CGA and AGA) codons of hfGRIA2 and some teleost GRIA2 genes are chromosomally encoded. A recent phylogenetic study suggests that gnathostoma GRIA2 genes are evolved from a common ancestral gene [12]. However, the phylogenetic relationship between the hfGRIA2 and gnathostoma GRIA2 genes has not been fully elucidated. This study revealed that the gene structure of the hfGRIA2 differed from that of the gnathostoma GRIA2 genes (Fig. 1). A second hagfish AMPAR subunit, hfGRIA1, was also intronless in the M2–M3-coding region (Chen, unpublished result). Since all the agnathan AMPAR genes (GRIA1 and GRIA2) are intronless and all gnathostoma AMPAR genes (GRIA1-GRIA4) possess one intron at the M2–M3-coding region, a splitting of the M2–M3-coding region might occur at the ancestral gnathostoma AMPAR gene.

No invertebrate iGluR subunit has been reported to carry an R residue at the site homologous to the Q/R site of vertebrate iGluRs and the majority of the vertebrate AMPAR genes are Q-coding at the chromosomal Q/R site, suggesting that the ancestral vertebrate AMPAR gene is unlikely an R-coding gene. The R-coding AMPAR genes can evolve either by a chromosomal mutation at the sequence specifies the Q/R codon or by acquiring the intronic modules for RNA editing. When did the Q-coding vertebrate GRIA2 gene acquire the ability of Q/R converting by RNA editing? The presence of Q-coding GRIA2 genes in various classes of Gnathostome and especially in the shark indicate that Q/R editing of the GRIA2 transcripts evolves as early as the appearance of cartilaginous fish. The lack of intron within the M2–M3-coding region of hfGRIA2 implies that this gene is unlikely evolved from a Q/R editing-competent gene that needs intronic components to guide RNA editing [5,7]. Hence, the utilization of the Q/R editing machinery in controlling the properties of AMPARs may evolve after the divergence between Agnatha and Gnathostome. Consequently, the chromosomal R codon of hfGRIA2 may result from point mutations.

Mutations are also responsible for the generation of R-coding teleost GRIA2 genes; however, the teleost GRIA2 genes are derived from editing-competent genes. This possibility is supported by the presence of Q-coding GRIA2 genes in the more primitive teleost fishes, zebrafish and goldfish. The coexistence of Q- and R-coding GRIA2 genes in zebrafish further strengthens the argument that the ancestral teleost GRIA2 gene carries an exonic Q codon and is editing-competent. A mutation of the second nucleotide of the Q (CAG) codon might occur after the duplication of the GRIA2 gene in the more primitive teleost lineages. Afterwards, a second mutation or a gene conversion event might change the Q-coding GRIA2 to an R-coding gene in the more derived teleost lineages. Alternatively, the Q-coding gene might be lost in the common ancestor of more derived fishes, and the R-coding GRIA2 genes of the highly derived fishes, i.e. tilapia, were duplicated from the remaining R-coding GRIA2 gene. The latter possibility, however, is not supported by the analysis of the C-terminal protein–protein interacting domains of tele-

ost GRIA2 subunits [12]. Interestingly, none of the R codon of the currently known R-coding GRIA2 genes is CGG (Fig. 2D), which is assumed to be the result of single mutational conversion of CAG codon. It is yet to reveal a selection advantage to have a codon preference of C/AGA for the R residue at Q/R site.

As suggested by the apparently normal phenotype of mice bearing an exonic R codon [11], the replacement of the Q-coding by R-coding GRIA2 genes in the teleost fishes can be selectively neutral. On the basis of the importance of the intronic components in Q/R editing, it is not surprising that the intronic and exonic sequences previously defined as essential for the Q/R RNA editing are highly conserved ($>58\%$ homologies) among the gnathostoma Q-coding GRIA2 genes (Table 1). Hence, the Q codon at the Q/R site, the flanking exonic sequence, and the downstream intronic sequence are co-selected. The silent substitutions of exonic sequences and the highly diverged intronic sequences of the four teleost R-coding GRIA2 genes reflect an alleviation of the selection pressure for maintaining the structure for Q/R editing (Fig. 2D).

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