

Determination of relative abundance of splicing variants of *Oreochromis* glutamate receptors by quantitative reverse-transcriptase PCR

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Abstract In this study, the relative abundance of splicing variants of *Oreochromis* non-NMDA subtype glutamate receptors was studied by quantitative reverse-transcriptase PCR (RT-PCR). The relative expression level between the flip and flop transcripts of fGluR2 α determined by quantitative RT-PCR is apparently much higher than that estimated by sequence analysis of the cloned RT-PCR products. Control studies were performed to demonstrate the accuracy of the application of quantitative RT-PCR analysis in studying the relative abundance between the flip and flop transcripts of glutamate receptors.

Key words: Glutamate receptor; Non-NMDA receptor; Quantitative RT-PCR; Splicing variant; *Oreochromis*

1. Introduction

L-Glutamate is the major excitatory neurotransmitter in the central nervous system of vertebrates [1,2]. Mammalian ionotropic glutamate receptors are presently classified into three subtypes according to their preferred agonists. They are *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate (KA)-preferring receptor subtypes [2–4]. The latter two subtypes are also known as non-NMDA receptors. We have reported the cloning of three non-NMDA receptor genes, *fGluR1 α* , *fGluR2 α* , and *fGluR2 β* , of a fresh water fish, *Oreochromis* sp. These genes encode polypeptides with very high amino acid sequence identities (70–85%) to GluR1–GluR4 subunits of the mammalian AMPA receptors [5,6].

Variants of vertebrate glutamate receptor subunits are created by RNA editing and alternative splicing [3–7]. A 38-amino acid segment preceding the last putative transmembrane domain can exist in two sequence forms, termed flip and flop, in each of the four mammalian AMPA receptor subunits. The flip and flop molecules are created by the alternative usage of two adjacent 115-nucleotide exons which are very similar in sequences [7]. Alternative splicing of rat AMPA receptor pre-mRNA at this region is under developmental control and exhibits major influences on the properties of channels [7–9]. The switch from predominantly flip forms before birth to flip plus flop forms after birth was speculated to play a role during synaptogenesis [4,8]. Alternative splicing of C-terminal exons was also reported for mammalian GluR2 and GluR4 subunits [10,11]. So far, no distinctive function is known for the C-terminal variants. C-terminal splicing variants can be either the flip or flop form in mammalian brain [10]. In the brain of mature *Oreochromis*, C-terminal variants of fGluR1 α

and fGluR2 α , designated fGluR1 α c and fGluR2 α c, were found, whereas no C-terminal variant was detected for fGluR2 β [5,6].

The relative expression levels between the flip and flop isoforms of fGluR2 α , fGluR2 α c, and fGluR2 β have been studied by DNA sequence analysis of the cloned RT-PCR products amplified from RNA extracted from the brain of adult *Oreochromis*. Despite a significant portion (26.7%) of the sequenced fGluR2 α c carried the flip exon, no flip form of fGluR2 α was found [5]. This result appeared to be incompatible with the finding that the C-terminal alternative splicing of rat GluR4 is independent of the choice between the flip and flop exons [10]. Here, we report the identification of the flip version of fGluR1 α and fGluR2 α as well as the usage of a quantitative reverse-transcriptase PCR (RT-PCR) assay to directly compare the relative abundance between the flip and flop transcripts of non-NMDA receptor subunits in *Oreochromis*. Our results indicate that the ratio between the flip and flop transcripts of fGluR2 α is approximately the same as that of fGluR2 α c but different from that of fGluR2 β in mature *Oreochromis* brain.

2. Materials and methods

2.1. Reverse-transcription PCR assays

Double-stranded cDNA was prepared by oligo(dT)– and random hexamer-primed reversed transcription of poly(A)⁺ RNA extracted from mature *Oreochromis* brain [5]. Forty nanograms of double-stranded cDNA was mixed with 20 pmol of primers in a reaction volume of 20 μ l. PCR [12] was performed in 200 μ M each of the four dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, and 2.5 U of Taq polymerase (Promega). Forty nanograms of salmon sperm DNA was always included in the PCR mixture when plasmid DNA carrying cloned glutamate receptor cDNA was used as the template. Reactions were run for 30 cycles of amplification (1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C) and followed by a 15-min extension at 72°C. Five microliters of the PCR reaction was analyzed by TAE-agarose (2%) gel electrophoresis, and the resultant gel was stained with ethidium bromide. The sequences of PCR primers are as follows (listed from 5' to 3'). (1) For amplification of fGluR1 α , the 5' gene-specific primer FGR8, CCCTGTGACACTATGAAGGTGGGAGGC; the 3' gene-specific primer FGR11; CGTGGGTGAGGATTCGTCCGTCTCTC; the flip exon-specific primer FGR9, GGGAGTGTGGCAGCAAGGACTCCGGAA; the flop exon-specific primer FGR10, AGGGAGAGTGCGGCAGCGGGGGCGGGG. (2) For amplification of fGluR2 α , the 5' gene-specific primer FGR1, GAATGAGTACCTCGAGCAGAGGAAACCC; the 3' gene-specific primer FGR2, CAGACAAGCTTTGGAGCGCATCGCATCCG; the flip exon-specific primer FGR12, AAGGAAGATGTGGCTTCAAGGACTCTA; the flop exon-specific primer FGR13, AGGGAGAATGCGGCAGCGGAGGAGGGG. (3) For amplification of fGluR2 β , the 5' gene-specific primer FGR3, CGAAAACCCTGCGACACCATGAAGGTG; the 3' gene-specific primer FGR4, AATGGCATCCCCAAGGTCATCCCCC; the flip exon-specific primer FGR14, AGGGTGAATGTGGAGCCAAGGACTCTG; the flop exon-specific primer FGR15, AAGGAGAGTGCGGCAGCGGGGGAGGTG. For quantitative

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PCR analysis [13], 20 cycles of PCR were run to ensure that the reaction did not exceed the range of linear amplification. One picomole ^{32}P -labeled 3' gene-specific primer (FGR2, FGR4, FGR11) was added to the standard PCR reaction mixture. The labeled PCR products were separated by gel electrophoresis, excised, and counted in the presence of counting scintillant (Amersham) by a liquid scintillation analyzer (Packard).

2.2. Cloning of RT-PCR products

RT-PCR products were separated by agarose gel electrophoresis, extracted by phenol, and ligated to commercially prepared vectors pMosBlue T (Amersham), pT7Blue T (Novagen), and pGEM-T (Promega) or the *EcoRV* site of pBluescriptII SK⁺ (Stratagene). For cloning the PCR products to the *EcoRV* site, the final 15-min extension at 72°C of standard PCR was omitted to avoid addition of 3'-overhang A [14]. DNA was transformed to *E. coli* XL1-Blue [15].

2.3. Colony hybridization

Colonies were grown at Hybond-N (Amersham) membranes overlaid on the LB medium supplemented with 100 µg/ml ampicillin and subjected to standard lysis, denaturation and neutralization processes [15]. Exon-specific oligonucleotides (FGR9, FGR10, FGR12, FGR13, FGR14, and FGR15) were labeled by [γ - ^{32}P]ATP with T4 DNA polynucleotide kinase (BioLabs) and purified by chroma spin column (Clontech). Prehybridization and hybridization were carried out at room temperature, in 6× SSC (0.9 M NaCl, 90 mM sodium citrate, pH 7.0), 1 mM EDTA, 0.5% SDS, and 0.1% non-fat milk. Filters were washed twice in 2× SSC and 0.1% SDS at room temperature, and finally washed in a solution of 0.1× SSC and 0.1% SDS. For flip-exon- and flop-exon-specific oligonucleotides, the temperatures of the final wash solutions were 45°C and 50°C, respectively.

3. Results

3.1. Determination of the relative abundance between flip and flop transcripts by colony hybridization and sequence analyses of RT-PCR products

Gene-specific primers (FGR1–FGR2, FGR3–FGR4, and FGR8–FGR11) which amplified the cDNA sequences spanning from the regions upstream to the flip/flop sequences to the 3'-untranslated regions of fGluR1α, fGluR2α, and fGluR2β were designed. Specific amplification of the desired gene products could be achieved by these primer pairs [5,6]. The amplified products were cloned to various vectors, transformed to *E. coli*, and subjected to DNA sequence and colony hybridization analyses. Table 1 summarizes the frequencies of finding flip sequences of fGluR1α, fGluR2α, and fGluR2β among the cloned RT-PCR products. Plasmid DNA of those rarely occurring colonies showing hybridization to the flip oligonucleotides of fGluR1α and fGluR2α was sequenced and confirmed to carry the flip sequences (data not shown). Results of both analyses suggest that mature fish brain expresses none or very low amounts of the flip version of fGluR1α and fGluR2α while the expression levels of fGluR2β flip and flop forms are nearly equal.

3.2. Detection of the flip transcripts of fGluR1α and fGluR2α by RT-PCR with exon-specific primers

For quantitative measurement of each splicing variant, exon-specific primers derived from the least conserved regions between the flip and flop exons of fGluR1α, fGluR2α, and fGluR2β were designed and used in combination with their cognate 3' gene-specific primers for RT-PCR amplification of a particular isoform. The sequences of the 3' gene-specific primers were derived from the 3'-untranslated region; their distances from the flip/flop sequences varied among the three genes so that the sizes of the resulting RT-PCR products were

different. The absence of product amplified by the flip-specific primers plus flop sequence-carrying plasmid DNA templates or by the flop-specific primers plus flip sequence-carrying plasmid DNA templates indicated that amplification was specific to a particular splicing variant (Fig. 1, lanes 2 and 4). Interestingly, the flip-specific primers designed for fGluR1α and fGluR2α (FGR9 and FGR12) could each amplify two products with expected sizes for the two C-terminal variants from double-stranded cDNA template prepared from mature fish brain (Fig. 2, lane 3). The relative ethidium bromide-staining intensities between the flip and flop RT-PCR products of fGluR1α and fGluR2α are much higher than that estimated by colony hybridization analysis (Fig. 1, lanes 3 and 6; Table 1). Furthermore, the relative intensities between the flip and flop RT-PCR products remain the same for C-terminal variants (Fig. 1, lanes 3 and 6). These results demonstrate that the alternative splicing of exons encoding the C-termini and flip/flop are independent of each other in fGluR1α and fGluR2α of *Oreochromis*. This observation suggests that our original estimation based on the cloned RT-PCR products might be biased.

3.3. Quantitative RT-PCR analysis of the splicing variants of fGluR1α, fGluR2α, and fGluR2β

Quantitative RT-PCR was performed to determine the relative expression levels between splicing variants of fGluR1α, fGluR2α, and fGluR2β. A control reaction suggested that the amplification of glutamate receptor cDNA with the gene-specific primers remained in a linear relationship if the number of PCR cycles was less than 23 (data not shown). This result suggested that there were approximately 3200–51,200 starting copies of templates per 40 ng of cDNA [16]. Thus, a 20-cycle reaction condition was chosen for the subsequent quantitative RT-PCR analysis. The results of quantitative RT-PCR analysis using exon-specific primers and ^{32}P -labeled 3' gene-specific primer show that the flip transcripts of fGluR1α and fGluR2α respectively represent 30.6% and 22.5% of the fGluR1α and fGluR2α transcripts (Table 2). Furthermore, the percent representations of the flip transcripts remain relatively unchanged for the C-terminal variants, fGluR1αc (29.1%) and fGluR2αc (24.2%). These estimations are much higher than that obtained by DNA sequence and colony hybridization analyses (Table 1). A control PCR with mixed flip

Table 1
Percent representations of flip and flop isoforms of *Oreochromis* non-NMDA receptors as estimated by DNA sequencing and colony hybridization analyses

	DNA sequencing ^a		Colony hybridization ^b	
	flip	flop	flip	flop
fGluR1α	0	100	1.5	98.5
fGluR1αc	0	100	0	100
fGluR2α	0	100	3.2	96.8
fGluR2αc	26.7	73.3	3.4	96.6
fGluR2β	42.8	57.2	51.6	48.4

^aEighteen RT-PCR products of fGluR1α and fGluR1αc were cloned to pGEM-T or pT7BlueT vector and were sequenced. The results of fGluR2α, fGluR2αc, and fGluR2β were taken from Kung et al. [5].

^bRT-PCR products amplified by the gene-specific primers were cloned to pMOSBlueT or pBluescript IISK⁺ vector and were hybridized to the cognate exon-specific primers used for quantitative RT-PCR analysis. Numbers of colony picked for hybridization analysis are 205, 125, 31, 174, and 93 for fGluR1α, fGluR1αc, fGluR2α, fGluR2αc, and fGluR2β, respectively.

and flop plasmid DNA as templates (total amount of 0.1 pg plasmid DNA, equivalent to a total of 25,000 molecules) in ratios ranging from 0.01 to 10 demonstrates that the relative yields of the flip and flop PCR products are proportional to the ratios of added templates (Fig. 2). Therefore, there is no obvious difference between the efficiencies of PCR amplifications by the flip- and flop-specific primers.

3.4. Reliability of using exon-specific primer in quantitative PCR

Since discrepancies were observed between the results obtained by quantitative PCR analysis and that obtained by colony hybridization, the reliability of the quantitative RT-PCR needs to be further investigated. Amplification reactions by the exon-specific primers are rather specific because the sizes of major RT-PCR products match the expected sizes (Fig. 1). The concentration of primer is another important factor that may influence the yield of a PCR product. Although sequences of the exon-specific primers were derived from the most divergent region, significant degree of homologies between primers and other non-NMDA receptor subunits were unavoidable. The sequences of the exon-specific primers of fGluR2 β , FGR14 and FGR15, share 77–96% homologies with the corresponding exons of various fish non-NMDA receptor subunits (data not shown). To test if competition between the target cDNA and other related sequences present in the cDNA, i.e., cDNAs of other non-NMDA receptor subunits, exists, competition experiments were performed. One picogram each (at least 10 times the amount of a specific template) of the plasmid DNA encoding either the flip or flop form fGluR1 α , fGluR2 α , and fGluR3 α (Hsu and Chow, in preparation) was added and competed for the cDNA of fGluR2 β in the quantitative RT-PCR mixture. The results show that efficiencies of RT-PCR amplifications of fGluR2 β flip and fGluR2 β flop were not affected by the presence of competitor plasmid DNA (data not shown).

To test the reliability of the ratios obtained by quantitative RT-PCR further, a two-round PCR analysis was performed. The cDNA was first amplified by the gene-specific primers for 10 cycles and the resultant products were diluted 20-fold for a second amplification by the exon-specific primers and labeled 3' gene-specific primers. The yields of the flip and flop RT-PCR products obtained by two-round RT-PCR analysis

Table 2

Percentages of flip and flop isoforms of *Oreochromis* non-NMDA receptors as estimated by quantitative RT-PCR analysis^a

	One-round RT-PCR ^b		Two-round RT-PCR ^c	
	flip	flop	flip	flop
GluR1 α	30.6	69.4	29.8	71.2
GluR1 α c	29.1	70.9	30.1	69.9
GluR2 α	22.5	77.5	24.2	75.8
GluR2 α c	24.2	75.8	23.1	76.9
GluR2 β	52.4	47.6	54.5	45.5

The percentage was calculated as the radioactivity of either the flip or the flop RT-PCR product over total radioactivity.

Exon-specific primers and ³²P-labeled 3' gene-specific primers were used for quantitative PCR analysis. Average values of at least four experiments are shown.

The two-round reaction refers to a first 5-cycle (fGluR1 α and GluR2 α) or 10-cycle (fGluR2 β) amplification by gene-specific primers and subsequently amplified by exon-specific primers and labeled 3' gene-specific primers.

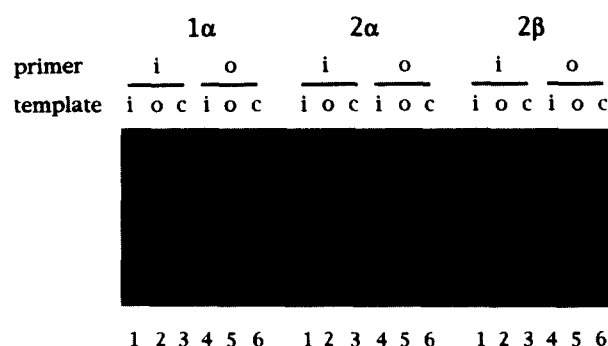


Fig. 1. Results of PCR analysis by exon-specific primers. PCR was performed by the indicated exon-specific primer and cognate 3' gene specific primer. Templates for PCR are 0.1 pg of each plasmid DNA carrying *Oreochromis* non-NMDA receptor cDNA mixed with 40 ng of salmon sperm DNA (lanes 1, 2, 4, 5) and 40 ng of cDNA prepared from brain mRNA (lanes 3, 6). i, flip; o, flop; c, cDNA.

are comparable to that obtained by one-round RT-PCR analysis (Table 2).

4. Discussion

The relative gene expression levels between the flip and flop variants of rat and fish non-NMDA receptor subunits have been studied by in situ hybridization [7] and by sequence analysis of cloned RT-PCR products [5], respectively. Here, we report the usage of quantitative RT-PCR with exon-specific primers in the analysis of relative abundance between transcripts of splicing variants. Although analysis by in situ hybridization is advantageous in revealing variation in regional distribution of a specific mRNA, this method is less accurate and is more time-consuming than quantitative RT-PCR. Furthermore, quantitative RT-PCR analysis measures the amounts of PCR products directly, thus preventing bias resulting from differential cloning efficiency. This is exemplified by the different representations between the flip and flop transcripts of fGluR1 α and fGluR2 α as obtained by quantitative RT-PCR and colony hybridization methods (Tables 1 and 2). Direct analysis of the RT-PCR product by primer extension in the presence of a chain-terminator has been performed to analyze the extent of RNA editing of rat non-NMDA receptors [17]. The sequence difference between the flip and flop transcripts is more prominent than that between the edited and unedited transcripts; consequently, a one-step amplification with an exon-specific primer is sufficiently specific to amplify a particular splicing variant. The specificity of amplifications achieved by the exon-specific primers designed for this study is clearly demonstrated by the amplification of products of expected lengths (Fig. 1). The mixed templates (Fig. 2) and competition experiments (data not shown) suggest that the discrepancy between results obtained by different methods employed in this study unlikely originates from non-linear and differential amplification efficiency of the flip and flop transcripts. Consistent results obtained by one- and two-round RT-PCR amplifications further supports the accuracy of applying quantitative RT-PCR with exon-specific primers in comparing the expression levels between splicing variants of non-NMDA glutamate receptors.

Presence of toxic gene products or unclonable sequences in the inserts has been suggested to influence the cloning efficiency of certain DNA molecules [19]. Cloning the RT-PCR

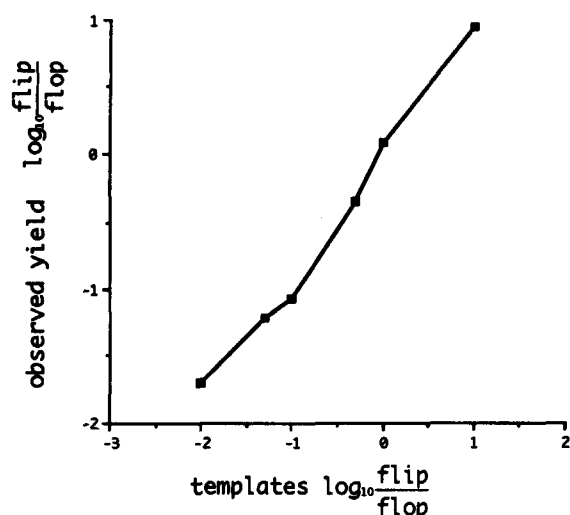


Fig. 2. Ratios of PCR products forms as a function of the ratios of the input flip and flop cDNA templates. Approximately 0.1 pg DNA of plasmids carrying flip and flop sequences of fGluR1 α were mixed in ratios of 0.001 : 0.1 (0.01), 0.005 : 0.1 (0.05), 0.01 : 0.1 (0.1), 0.05 : 0.1 (0.5), 0.05 : 0.05 (1), 0.1 : 0.01 (10). Each quantitative PCR mixture including 40 ng of salmon sperm DNA, labeled 3' gene-specific primer (FGR11), and either one of the exon-specific primers (FGR9 or FGR10). The ratios observed are expressed as the yield of PCR product by FGR9 over that by FGR10.

products into different vectors (pGEM-T, pT7 Blue T, pMos-Blue T, pBluescriptII SK⁺) had no significant influence on the representation of flip form fGluR1 α (Table 1). Since the RT-PCR products may be inserted in two orientations and with different reading frames relative to the *lacZ* gene, the failure to clone the fGluR1 α flip form is unlikely a result of a toxic gene product. Consistently, we experienced great difficulty in cloning a longer cDNA fragment carrying the flip form fGluR1 α (unpublished results). Examination of the sequences of flip-form PCR products revealed no obvious secondary structure. Transformation efficiencies of the cloned fGluR1 α flip and flop sequences into isogenic *recB* or *recA* strains were the same (data not shown). Thus, the low representation of the flip sequences of fGluR1 α and fGluR2 α cannot be explained by the presence of unclonable sequences. The reason why flip sequences of fGluR1 α , fGluR1 α c and fGluR2 α are under-represented in the cloned populations remains unclear.

Similar to the GluR4 of rat [10], choice of the flip or flop exons of fGluR1 α and fGluR2 α is independent of C-terminal alternative splicing because the relative abundance between the flip and flop transcripts of the C-terminal variants are the same (Table 2). Detection of the flip transcripts of fGluR1 α and fGluR2 α by RT-PCR analysis with the flip exon-specific primers indicates continuous expression of flip transcripts in the mature fish brain and is consistent with the findings in adult rat brain [7,8]. Expression of the edited form (R-form) of rat GluR2 exhibits a dominant influence on the divalent ion permeability of AMPA receptors [18]. The

homologs of rat GluR2, fGluR2 α and fGluR2 β , encode polypeptides that are 89% identical between each other and contain an arginine codon at the Q/R site. Thus, the physiological functions of fGluR2 α and fGluR2 β are likely to be similar. Difference in the expression levels of splicing variants implies that fGluR2 α and fGluR2 β may not be functionally equivalent. The expression level of the flip fGluR2 β transcript is slightly higher than that of the flop transcript in mature fish brain, whereas the expression level of the flip fGluR2 α is 2-fold lower than that of the flop form. Results obtained by quantitative PCR analysis, however, are unable to reveal whether this difference is cell-specific or widely distributed in fish brain. Further studies by in situ hybridization are required.

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