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Identification and characterization of the RNA-binding protein Rbfox3 in zebrafish embryo

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ABSTRACT

Rbfox3, an RNA-binding fox protein, binds to the antibody to pan-neuronal marker, neuronal nuclei (NeuN). Rbfox3 is expressed in neural tissues across a wide range of species including mammals, birds, and amphibians. However, the molecular identity of Rbfox3 in the zebrafish is largely unknown. In this study, we cloned two zebrafish Rbfox3 genes, *Rbfox3a* and *Rbfox3b*. We also cloned the Rbfox3-d31 isoform, which excludes a 93-nucleotide alternative exon within the RNA-recognition motif in both, Rbfox3a and Rbfox3b. Multiple protein sequence alignment revealed that the amino acid sequence for residues 1–20 of the zebrafish Rbfox3, which is the epitope region of NeuN antibody, was different from that of other species. Therefore, NeuN antibody lost its function as a neuronal marker antibody in zebrafish. Reverse transcriptase-polymerase chain reaction showed that both Rbfox3-d31 transcripts were abundant in the early blastula stage, after which they dramatically reduced, suggesting that these isoforms exist mainly as maternal transcripts. In contrast, full-length Rbfox3 transcripts were detected from the 24 h post-fertilization embryo, expression was also maintained at a constant level. Furthermore, full-length Rbfox3-expressing cells were located within the central nervous system during later stages of the zebrafish embryo. Our study provides insight into the molecular structure of zebrafish Rbfox3 as a step towards genetic association studies investigating the developmental role of Rbfox3.

1. Introduction

Antibodies to several mature neuronal protein markers are currently used for immunohistochemical staining of cells or tissue sections to investigate the molecular properties of the neuron, including neuronal nuclei (NeuN), microtubule-associated protein 2 (MAP2), class III beta tubulin (Tuj1), neurofilament (NF)-M and NF-H, neuron-specific enolase (NSE), as well as post-synaptic density (PSD)-93 and PSD95 [1]. Since NeuN was first reported in 1992 by Mullen et al. NeuN antibody has been widely used as a reliable marker to detect postmitotic neurons in neural tissues with the exception of cerebellar Purkinje cells, retinal photoreceptor cells, olfactory bulb mitral cells, and dopaminergic neurons in the substantia nigra [1–3]. Among several advantages for use as a pan-neuronal marker, NeuN antibody alone is primarily located in the nucleus.

Although the identity of the antigen binding to NeuN antibody was not fully understood until 2009, NeuN antibody is widely used in research as well as diagnostic histophathology [4–16]. Our previous report identifying Rbfox3 as the antigen for NeuN antibody set precedence to investigating the functional importance of Rbfox3 in neural tissues [17]. Rbfox3, the third member of the RNA-binding fox family of proteins, plays a role in alternative pre-mRNA splicing, which is indeed essential for neuronal differentiation during chick embryo development as well as normal synaptic function in the mouse brain [18,19]. Although NeuN antibody is widely used in a wide range of species including birds, salamander, rat, pig, mouse, human, ferret, and chicken to detect postmitotic neurons, the use of NeuN antibody in the zebrafish has not yet been reported. The zebrafish (*Danio rerio*) is now recognized as an excellent vertebrate

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A

Rbfox3a

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  1 ATGGCTCAGC CGTACGCCCC AGCTCAGTAT CCTCCTCCTC CACCTCAAAA CGGGCTGCCT
 61 GGCGAATACA CCCACCCTGG GCAGGACTAC ACAGGGCCCA GTCCGGTCCC TGAGCATGCG
121 GCGGCCCTCA CCATCTACAC ACCAACACAG ACACACAGCG AGCCGCCTGG CACCGACAGC
181 AGCACACCAT CCCTCACAGG GACCAGCAGC TTAACACAGG CGGACGATGT GGTACAGACA
241 GACGGATCTC AGCAGCTTCA GCTCCAGCCA TCAGACTCTT CGGAGAAGCA GCAGCCCAAA
301 CGGTTACACG TCTCCAACAT TCCCTTCCGC TTCCGAGACC CAGACCTCCG GCAAATGTTC
361 GGGCAATTTG GGAAAATTTT GGATGTGGAA ATTATTTTTA ACGAGCGAGG CTCTAAGGGT
421 TTTGGTTTTG TAACTCTTGA AACAAGTGCA GATGCAGACC GTGCACGGGA GAAATTAAAC
481 GGTACAATCG TAGAGGGACG CAAAATTGAG GTAAATAATG CAACGGCACG AGTAATGACA
541 AACAAAAAAG TGGCCAACCC CTATACAAAT GGCTGGAAGC TGAATCCAGT GGTGGGAGCT
601 GTCTATGGTC CTGAATTTTA TGCAGTGACA GGCTTCCCCT ATCCAACCAC AGGGGCGACG
661 GTGGCTTACA GGGGCGCCCA CTTGAGAGGC AGAGGTCGTG CCGTCTATAA TACGTTCCGT
721 GCTGCGCCTC CACCCCCTCC CATTCCCGCC TACGGAGCTG TGGTTTACCA AGATGGCTTC
781 TACGGTGCTG AGATCTATGG TGGCTATGCA GCATACAGAT ATGCCCAACC AGCCGCAGCA
841 GCCGCAGCCG CCTACAGTGA CAGCTATGGC AGAGTCTATG CAACAGCAGA CCCTTATCAC
901 CACACGATTG GACCAGCAGC CACGTACAGC GTGGGCACTA TG
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B

Rbfox3b

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  1 ATGGCCCAGA CGTACACCAC GCAGTACGCT CATCCCTCAC AGAACGGCAT CCCGGCTGAG
 61 TTCACCGCAC TCCCCTCACA AGACTACACA GGACAGAGCC GCGTCCCGGA CCATGGCCTG
121 ACGCTCTACA CACCTGCACA GACTCACAGC GACCTGAACA ACACAGACAG CCAAACGCCA
181 GCCATCAGCA CTGGCTCCAA CACAGCGCCG ACGGAAGACG TGACGCAAAC GGACGTGTTG
241 ATTTCAGAGT CCACAGAAAA GCAGCAGCCC AAGCGGCTAC ACGTCTCCAA CATCCCCTTC
301 CGCTTCCGGG ACCCTGATTT ACGGCAGATG TTTGGGCAAT TCGGGAAGAT CTTAGACGTG
361 GAGATTATCT TTAATGAAAG AGGATCAAAG GGCTTTGGCT TTGTAACTTT TGAAACGAGT
421 GCAGATGCAG ATCGCGCACG GGAGAAACTA AACGGTACAA TCGTAGAAGG ACGTAAAATC
481 GAGGTGAACA ACGCCACGGC GAGAGTGATG ACGAATAAGA AAGTAGTAAA CCCGTACACA
541 AACAGCTGGA AGCTCAACCC TGTTGTAGGA GCCGTTTACG CACCAGAGCT CTACGCAGTG
601 ACGGGTTTTC CGTATCCCGC AGCAGGAGCG ACGGTGGCCT ACAGAGGGGC TCATCTGAGG
661 GGCAGAGGTC GTGCCGTTTA TAACACCTTC CGGACGGCTC CTCCTCCTCC ACCAATCCCA
721 GCGTACGGAG CTGTGGTGTA TCAGGACGGC TTCTACGGCG CAGAGATCTA CGGTGGATAT
781 GCGGCGTACA GATATACTCA GCCTGCGACA GCTACAGCGT ACAGCGACAG CTATGGAAGA
841 GTCTATGCAA CCACTGACCC TTATCACCAC AGCATCGGCC CGGCAGCAGC GTACAGCGTC
901 GGCACCATGG CGAGTCTGTA CAGAGGAGGC TGCAGCCGCT TCACGCCCTA CTAG
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Fig. 1. cDNA sequences of two zebrafish Rbfox3 genes. (A, B) Nucleotide sequence of zebrafish Rbfox3 cDNA. The red nucleotide sequence indicates RRM. The underlined red nucleotide sequence indicates the 93-nucleotide alternative exon within RRM. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

model system to study the functions of specific developmental genes. However, to utilize the zebrafish model to understand the biological function of Rbfox3, it is necessary to first determine the molecular identity of Rbfox3 in zebrafish.

In this study, we cloned *Rbfox3* from the zebrafish embryo using reverse transcriptase-polymerase chain reaction (RT-PCR) and analyzed its expression pattern during various developmental stages of the zebrafish embryo. We found that two Rbfox3 genes (*Rbfox3a* and *Rbfox3b*) were specifically expressed in the central nervous system (CNS), similar to that in other species. These results provide the first clue that Rbfox3 could be used as a neuronal marker in zebrafish.

2. Materials and methods

2.1. Cell culture, transfection, and MG132 treatment

Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, NY, USA) at 37 °C in an atmosphere containing 5% CO_2. Transfection was performed with the PolyMAG Magnetofection kit (Chemicell GmbH, Germany) according to the manufacturer instructions. A specific proteasome inhibitor MG132 was purchased from Merck Millipore.

2.2. Plasmid constructs

The expression construct encoding mouse myc-Rbfox3 in the pCS3+MT plasmid was obtained from cDNA of mouse brain by PCR amplification. The following PCR primers were used: 5′-ggg ccc agg cct ATG GCC CAG CCC TAC CCC CCT GCC CAG TAC-3′ and 5′-ccc ggg ctc gag TCA CAT GGT TCC GAT GCT GTA GGT TGC TGT-3′. Underlined letters represent adapter sequences including the StuI/XhoI restriction sites. The expression constructs encoding myc-Rbfox3a, myc-Rbfox3a (d31), myc-Rbfox3b, and myc-Rbfox3b (d31) in the pCS3+MT plasmid were obtained from cDNA of zebrafish embryo by PCR amplification. The following PCR primers were used: for Rbfox3a 5′-ggg ccc gaa ttc a ATG GCT CAG CCG TAC GCC CCA GCT CAG TAT-3′ and 5′-ccc ggg ctc gag CAT AGT GCC CAC GCT GTA CGT GGC TGC TGC-3′; for Rbfox3b 5′- ggg ccc gaa ttc a ATG GCC CAG ACG TAC ACC ACG CAG TAC GCT -3′ and 5′-ccc ggg ctc gag CTA GTA GGG CGT GAA GCG GCT GCA GCC TCC-3′. Underlined letters represent adapter sequences including the EcoRI/XhoI restriction sites. The specificity of the products generated by each set of primers was confirmed by sequencing analysis. The primers 5′- TCT ACG ATT GTA CCG T-3′ and 5′-ACG GTA CAA TCG TAG A-3′ for rapid amplification of cDNA ends (5′-RACE) and 3′-RACE, respectively, were designed to obtain full-length Rbfox3 using the GeneRacer Kit (ThermoFisher Scientific).

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