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Characterization of two paralogous muscleblind-like genes from the tiger pufferfish (*Takifugu rubripes*)

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Abstract

Muscleblind-like (Mbnl) proteins are required for terminal muscle differentiation in mammals. In this study we have identified two *mbnl* paralogues from the tiger pufferfish, *tmbnl2a* and *tmbnl3*, which are the first examples of non-mammalian *mbnl* genes. *Tmbnl2a* and *tmbnl3* were found in regions of conserved synteny and had a high degree of global conservation with their mammalian homologues. Phylogenetic analysis showed that the *T. rubripes* genome contains one *mbnl3* gene and two copies of *mbnl1* and *mbnl2*. Moreover, the *mbnl1* and *mbnl3* paralogues are derived from duplication of a common ancestral gene. The average rates of synonymous substitutions between *T. rubripes*, mouse and human *mbnl2* and *mbnl3* genes were much higher than the corresponding rates of non-synonymous mutations, suggesting that Mbnl2 and Mbnl3 are subjected to strong purifying selection. Quantitation of *tmbnl2a* and *tmbnl3* transcripts by real-time PCR revealed that these two paralogues are differentially expressed in fast and slow myotomal muscle, heart, liver, skin, brain and testes. *Tmbnl2a* was expressed at similar levels in all tissues examined, as was the mouse orthologue. *Tmbnl3* was expressed at higher levels than *tmbnl2a*, with a ubiquitous tissue distribution. Expression of *tmbnl3* remained high in adult pufferfish muscle whereas the mouse orthologue was down-regulated in adults, perhaps reflecting the indeterminate and determinate growth patterns of these taxa, respectively.
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1. Introduction

Myogenesis is a complex process that involves the commitment of proliferating myoblasts, irreversible cell cycle withdrawal, terminal differentiation into myocytes with a contractile phenotype and fusion to form multinucleated myotubes that mature into functional muscle (Andres and Walsh, 1996). These distinct events require the coordinate expression of a large number of genes, including the MyoD family of muscle regulatory factors, Id proteins, paired box proteins and the MEF2 gene family (reviewed in (Brand-Saberi, 2005)). In contrast to what is observed in mammals (Rowe and Goldspink, 1969), muscle fibre recruitment in teleosts is not limited to early stages of ontogeny. In teleosts, fast muscle fibres are continuously produced throughout juvenile and adult stages, continuing to longer body lengths in species that reach a large maximum size (Johnston, 2006). New myotubes formed during post-embryonic development arise from a population of myogenic progenitor cells equivalent to the muscle satellite cells present in mammals (Johnston, 2006; Koumans and Akster, 1995). Our understanding of the complex genetic networks involved in myotube production and control of muscle fibre number is still rather limited.

One gene that has recently been implicated in mammalian muscle differentiation is the muscleblind-like gene (*Mbnl*) 3 (Squillace et al., 2002), which is a homologue of the *Drosophila* muscleblind (*mbl*) gene required for terminal differentiation of visceral and somatic musculature (Artero et al., 1998). *Mbnl3* was found to be an inhibitor of the myogenic programme, as shown by loss and gain of function *in vitro* assays (Squillace et al., 2002). Expression levels of this negative regulator of muscle differentiation decreased upon differentiation of mouse myoblasts (Squillace et al., 2002). *Mbnl3* is one of three *Mbnl* genes present in the human genome, all of which have been associated with

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myotonic dystrophy (DM), a multisystemic disorder caused by two microsatellite expansions (Miller et al., 2000; Fardaei et al., 2002). In addition to direct RNA binding to expansion repeats (Miller et al., 2000; Fardaei et al., 2002), Mbnl proteins were found to be regulators of alternative splicing of pre-mRNAs that display an abnormal splicing pattern in DM tissues (Ho et al., 2004).

The tiger pufferfish (*Takifugu rubripes*) is a commercially important species in Japan and North Korea, where its flesh is considered a delicacy. Since the numbers of wild tiger pufferfish have been declining, aquaculture production of this fish on the Southern coast of Japan has become increasingly important during the last decade (Miyadai et al., 2001). *T. rubripes* is an attractive model for identification of genes involved in muscle development and growth in teleosts because its compact genome has been sequenced to draft level (Aparicio et al., 2002). Since *Mbnl* genes had not been previously identified in fish and *mbnl3* has been shown to be a key myogenic gene in mammals, the aim of the present study was to clone and characterize *mbnl3* from *T. rubripes* in order to gain some insight into its potential role in myogenesis in teleosts.

2. Materials and methods

2.1. Identification of Takifugu mbnl genes in silico

The third *T. rubripes* genomic sequence assembly (http://www.ensembl.org/) was screened for *mbnl3* by TBLASTN similarity search using the corresponding human protein sequence as probe. The Ensembl predictions of *mbnl* genes were refined with the gene structure prediction software Genebuilder, available from the National Institute for Biomedical Technologies, Italy (http://l25.itba.mi.cnr.it/~webgene/genebuilder.html).

2.2. RNA extraction and cDNA synthesis

Samples of fast myotomal muscle were dissected from two juvenile (136 and 159 g) and two adult (1.32 and 1.35 kg) *T. rubripes* specimens, and stored in RNAlater (Ambion, Cambridgeshire, UK) for subsequent RNA extraction. Total RNA was isolated with Tri reagent (Sigma, Dorset, UK), according to the manufacturer's instructions. First-strand cDNA was synthesised using the RETROscript kit (Ambion), by the recommended method. A 1:1 mixture of random decamers and oligo$(dT)_{18}$ was used as first-strand primers for cDNA synthesis.

2.3. cDNA cloning of Takifugu mbnl cDNAs

Computational gene predictions obtained *in silico* were used to design specific primers for amplification of the full-length coding sequences of *mbnl* genes. Amplification by polymerase chain reaction (PCR), cloning and sequencing of PCR products were performed as previously described (Fernandes et al., 2005). Complete coding sequences were obtained for the genes on scaffolds 118 and 364 using the primer sets Fwd1 (5′-ATGGCCGTCAACATGACTATG-3′)/Rev1 (5′-TCAGCACAGCGTCACCACAC-3′) and Fwd2 (5′-ATGGCTCTAAATATTGCATCG-3′)/Rev2 (5′-TCAGGACCTACCAAGACTGC-3′), respectively.

2.4. Sequence analyses

Gene structures of *mbnl2a* and *mbnl3* were determined using Spidey (http://www.ncbi.nlm.nih.gov/spidey/) by comparison between experimental cDNA sequences and the corresponding genomic regions obtained from the current genome assembly at Ensembl. Synteny analysis between *T. rubripes mbnl* genes and their human and mouse orthologues was performed with BioMart (http://www.ensembl.org/Multi/martview). *Mbnl* nucleotide sequences were translated using DNAman (Lynnon Biosoft, Quebec, Canada) and the putative proteins aligned with BioEdit (Hall, 1999). Average synonymous and non-synonymous substitutions, insertions and deletions in the coding regions of *T. rubripes* and mouse *mbnl2* and *mbnl3* were estimated with the method of Nei and Gojobori (1986) using the software SNAP (Korber, 2001). A one-tailed Fisher's exact test of positive selection based on the comparison of the numbers of synonymous and non-synonymous substitutions between sequences was performed in MEGA 3.1 (Kumar et al., 2001).

2.5. Phylogenetic analysis

The following Mbnl protein sequences were obtained from the Uniprot database: human MBNL2 (Q8TD82) and MBNL3 (Q9NUK0), mouse Mbnl2 (Q8C181) and Mbnl3 (Q8R003). In addition to these mammalian Mbnl sequences, the current genome assemblies of the tiger pufferfish, green-spotted pufferfish (*Tetraodon nigroviridis*), zebrafish (*Danio rerio*), stickleback (*Gasterosteus aculeatus*) and medaka (*Oryzias latipes*) were screened for *mbnl* sequences. Only the tiger pufferfish and stickleback genomes contained reliable predictions of full-length coding sequences of *mbnl* genes. The Mbl sequence from *Drosophila melanogaster* (O16011) was used as outgroup to root the tree. Bayesian inference of phylogeny through Markov chain Monte Carlo simulations was performed with Mr Bayes (http://mrbayes.csit.fsu.edu/) using an average mixed amino acid model of protein evolution. Convergence was achieved after 50,000 generations and Bayesian posterior probabilities were based on the following 450,000 generations (4500 trees). For comparison purposes a maximum parsimony analysis was also performed on the same alignment using MEGA 3.1 (Kumar et al., 2001). The reliability of the inferred tree was tested using a bootstrap test of phylogeny with 10,000 replicates.

2.6. Quantitative real-time PCR

Quantitation of *mbnl2a* and *mbnl3* expression in fast and slow myotomal muscle, heart, liver, skin, brain and testes of *T. rubripes* by real-time PCR was performed essentially as reported by Fernandes et al. (2006), following total RNA extraction and reverse transcription as described above. A 193 bp amplicon of *mbnl2a* was obtained using the primer pair

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