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Two tyrosine hydroxylase genes in teleosts

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Abstract

We report the finding of two non-allelic genes encoding tyrosine hydroxylase (TH) from the diploid teleost barramundi *Lates calcarifer*. Barramundi TH1 is the homologue of the higher vertebrate TH genes and encodes a protein of 489 amino acids that shares 90% sequence identity to the THs of other teleost species. A second non-allelic tyrosine hydroxylase gene (TH2) encodes a protein of 472 amino acids and shares 62% identity with TH1 and the vertebrate THs. TH1 mRNA is found in the brain and kidney of barramundi while TH2 mRNA is found only in brain. The TH2 gene is also present in the genomes of the pufferfish *Takifugu* and zebrafish *Danio*. Estimates of the rates of nucleotide substitution suggest the teleost TH2 genes are selectively constrained although not to the degree seen in the TH1 genes. Differential regulation of the two TH genes is, however, indicated by differences in transcript distribution, the nature of the Ca^{2+}-responsive elements found in the proximal promoter region and the lack of recognised phosphorylation sites in TH2. Preservation of two apparently functional TH genes in phylogenetically distant teleost species is consistent with the notion of partitioning of function between duplicate genes.

Keywords: Tyrosine hydroxylase; Teleost; Gene duplication

1. Introduction

The aromatic amino acid hydroxylases (AAAHs) are a small family of mixed function oxidases that serve as the rate-limiting enzymes in the hydroxylation of their respective amino acids using the reduced cofactor tetrahydrobiopterin and molecular oxygen. Tyrosine hydroxylase (TH, EC 1.14.16.2), phenylalanine hydroxylase (PAH, EC 1.14.16.1) and tryptophan hydroxylase (TPH, EC 1.14.16.4) have a common structure comprising a highly conserved C-terminal catalytic domain and an enzyme-specific N-terminal regulatory domain that has a role in ligand affinity [1,2]. The finding of genes encoding TH, TPH and PAH in the nematode *Caenorhabditis* (GenBank U80836, NP_495863, NP_495584) suggests the multigene family evolved early in the radiation of the Metazoa. Most likely, TH and TPH evolved from PAH as the latter is the only AAAH found in prokaryotes. Patton et al. [3] proposed that while the AAAH gene family arose as separate tandem duplications on a single chromosome in primitive Metazoa, the present distribution of the AAAH genes on the human chromosome 11/12 paralogous region reflects a series of gene loss events subsequent to two rounds of genome duplication that accompanied the rise of the vertebrates (the 2R hypothesis) [4,5]. Such gene loss events probably reflect the need for maintenance of gene dosage requirements for these essential enzymes. In teleosts, the number and distribution of AAAHs may be further influenced by an additional genome duplication event early in Actinopterygii (ray-finned fish), subsequent to the divergence from Sarcopterygii (lobe-finned fish and tetrapods) that is thought to be impetus for the rapid and widespread radiation of this

Abbreviations: TH, tyrosine hydroxylase; PAH, phenylalanine hydroxylase; TPH, tryptophan hydroxylase; AAAH, aromatic amino acid hydroxylase; PCR, polymerase chain reaction; RT, reverse transcriptase; NTP, nucleotide triphosphate; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; UTR, untranslated region; IGF, insulin-like growth factor; CRE, cAMP-response element; K_a, rate of replacement site substitution; K_s, rate of synonymous site substitution; Mya, million years ago

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Class [6–9]. This latter event may account for a larger number of duplicate genes observed in diploid teleost species than have been found in mammals.

One corollary of the hypotheses of Patton et al. [3] and of Meyer and Schartl [7] is that duplicate genes for the AAAHs may exist in some vertebrate groups. Indeed Walther et al. [10] reported a second neuronal TPH gene (TPH2) in mice, rat and humans and have since isolated the TPH2 mRNA from chicken, zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes*) (GenBank NP_001001301, AAT 38216, AAT39423). In our investigation of the gene structure–function relationships of the AAAHs in teleosts, we discovered two non-allelic TH genes in the diploid teleost barramundi (*Lates calcarifer*). This paper reports the characterisation of the duplicate TH genes in barramundi. The existence of two TH genes in teleosts is confirmed through our mining the *Fugu* (*Takifugu*) and *Danio* genome databases and the recent deposit of the duplicate pufferfish, zebrafish and *Tetraodon* TH2 genes in GenBank (CAG04159, AAT39425, NP_001001829). Our findings suggest that teleosts have two functional non-allelic TH genes that are expressed in the brain but are regulated differentially at the transcriptional and post-translational levels. The presence and the differential regulation of two TH genes in teleosts suggest the preservation of duplicate copies may have been facilitated by partition of function between the two TH genes and their enzyme products.

2. Materials and methods

2.1. Isolation and characterisation of genes encoding barramundi aromatic amino acid hydroxylases

Construction of the λEMBL3 genomic library containing Sau3A-partially digested barramundi DNA and isolation of the genomic region encompassing IGF-II has been described elsewhere [11]. To clone the barramundi TH1 gene, λ clones containing the genomic region extending 15 kb 5′ of the barramundi IGF-II gene was subjected to digestion using either *Hin*dIII, *Bam*HI or *Eco*RI with the resultant fragments subcloned into pUC18 (Pharmacia). The sequence of the overlapping fragments spanning the 15-kb region was determined for both strands using an ABI 373 automatic sequencer. The use of specific primers facilitated the sequencing through large plasmid subclones or from the λ clones. Sequence contigs were generated manually after alignment of overlapping sequences using the Pustell sequence analysis package (IBI).

A second non-allelic TH gene was cloned in a serendipitous manner. Degenerate primers anchored in exons 3 and 10 were used in RT-PCR experiments to target the barramundi PAH gene (cycle conditions and primer sequences are detailed below). A single fragment was subsequently cloned, sequenced and confirmed as the PAH gene. The cDNA fragment was used to screen the barramundi genomic library under conditions of high stringency [11]. A region encompassing TH2 was isolated from barramundi PAH λ genomic clones during PCR experiments targeting PAH with the degenerate primers used to obtain the cDNA. Two PCR products of high concentration were resolved on a 1% agarose gel, one a 1700-bp genomic PAH fragment and a second fragment of 400 bp that contained exon 3 of TH2. The subcloning and sequencing strategies used to characterise the genomic region encompassing barramundi PAH and TH2 were the same as for the TH-IGF-II region.

Pair-wise comparisons of the teleost TH coding sequences were undertaken using the DNA matrix algorithm from the Pustell sequence analysis package. Searches for potential transcription factor binding sites in the promoters of the teleost TH genes were undertaken using the Signal Scan (ver 4.05) algorithm [12] available on-line (http://bimas.dcrt.nih.gov/molbio/signal/).

2.2. Total RNA preparation

Brain, gill, intestine, liver, heart, muscle, and kidney tissues were collected from 6-month-old (juvenile) barramundi specimens and snap frozen in liquid N_2. Larvae were also collected and snap frozen. Total mRNA was isolated by guanidium–phenol/chloroform extraction and ethanol precipitated [13]. Single-stranded cDNAs were then synthesised from 5 μg of total mRNA using oligo-dT priming and Superscript II reverse transcriptase (Gibco-BRL) at 42 °C for 120 min. One tenth of these reaction products were used as templates in PCR reactions to assess patterns of mRNA expression.

2.3. mRNA expression in barramundi tissues

Oligonucleotide primer pairs specific for the barramundi genes were constructed as follows: TH1: 5′ -GCATTCG-CAGAGCAGCATCCG-3′ and 5′ -GTGAAAACTGGGC-AAAGGTG-3′ spanning exons 1 to 9, TH2: 5′ -CGGGGAGGAAGTCCAAGAACAG-3′ and 5′ -AGCGA-GGCAAGTCCAATCTC- 3′ spanning exons 2 to 9, and for PAH the degenerate primers (5′ -TATMAACCTSACMCA-MATYGA- 3′ and 5′ -CCRAACTCCACRGTRAACCAG-3′) were used spanning exons 3 to 11. PCR amplifications using cDNA from the barramundi tissues detailed above were performed in a final volume of 50 μl containing 100 μM of each primer, 100 μM dNTPs, 1.5 mM MgCl2, 1 U Taq DNA polymerase and the appropriate PCR buffer from Roche Molecular Biochemicals. Cycle conditions for the TH PCR reactions were 1× [94 °C, 2 min]; 35× [94 °C, 40 sec; 55 °C, 1 min; 72 °C, 2 min], 1× [72 °C, 7 min]; for the PAH PCR reaction the annealing temperature was 50 °C. PCR products were resolved on agarose gels and blotted onto Hybond N+ membranes (Amersham). Filters were hybridised to ^{32}P-labelled probes specific for the target gene at high stringency for two hours at 65 °C in hybridizing