行政院國家科學委員會補助專題研究計畫成果報告

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計畫主持人:郭欽明
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計畫參與人員:張百恩、謝淑玲

Molecular cloning of full-length cDNA encoding delta-9 desaturase through PCR strategies and its genomic organization and expression in grass carp (*Ctenopharyngodon idella*)

Bei-En Chang, Shu-Ling Hsieh, Ching-Ming Kuo*
Institute of Fisheries Science, National Taiwan University, Taipei, Taiwan, Republic of China

中文摘要

細胞膜不飽和脂肪酸在生理調節上扮演重要之角色，且不飽和脂肪酸乃經脫飽和酵素之催化而合成。本文報導應用 PCR 及 RACE 技術所獲得草魚 Δ^9-脫飽和酵素 cDNA 全長之序列及表現。此一 cDNA 含有全長 2420 bps，包含有一段轉譯讀窗（open reading frame），相當於 324 胺基酸序列和哺乳類相似度極高，經由北方點墨氏（Northern blot）及 RT-PCR 的方法分析，結果顯示在肝臟有較高量的表現，而在腦部的表現量則較少，更進一步的利用 genomic DNA library 來了解此基因結構，分析顯示此基因含有六個表現序列（exons）和五個插入序列（introns），此區域包含 8.5kb。最後一個表現序列含有 1382 bps 之 3' 端未轉譯區(3' untranslated region)。雖然此基因的原始序列以及全體基因體的結構在魚和哺乳動物之間是極為保守的，但是牠們的基因表現調節確有不同之處,值得深入探討。

關鍵詞: 酵素, 脂肪酸, RACE

ABSTRACT

Desaturases are enzymes that catalyze double bond formation in fatty acids, which is a critical step in the synthesis of unsaturated fatty acids in organisms. Desaturase cDNA has been cloned from various species. Here we report the cloning of a full-length cDNA of Δ^9-*desaturase* from grass carp (*Ctenopharyngodon idella*), using a combination of PCR techniques: reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The resolved cDNA encompasses 2420 bp, containing an open reading frame corresponding to 324 amino acids. The deduced amino acid sequence shares high homology with those of mammalian desaturases. Northern blot and RT-PCR analyses demonstrated that the transcript shows high abundance in liver tissue but low abundance in brain tissue. Furthermore, the structure of the gene has been resolved by screening its cognate genomic DNA library. The analysis shows that this gene is composed of six exons and five introns, encompassing a region of 8.5 kb. In particular, the last exon contains a length of the 3' untranslated region as long as 1382 bp. Although the primary sequence and the genomic organization are phylogenetically conserved between fish and mammals, the regulation of the gene expression appears to be divergent among species.

Key Words: enzyme, fatty acid, RACE

for the unsaturation of fatty acids by introduction of double bonds into the fatty acids, and the importance of desaturases is manifested by their functional roles implicated in a variety of biological processes, including low-temperature adaptation (Tiku et al., 1996), signal transduction (Gyorfy et al., 1997A), cell-membrane fluidity (Gyorfy et al., 1997B), cell apoptosis (de Vries et al., 1997), lipid metabolism (de Antueno et al., 1993), obesity (Legrand and Hermier, 1992; Kouba et al., 1993; Jones et al., 1996), and malignant cell growth (Zoeller and Wood, 1985; Borgeson et al., 1989; Li et al., 1994).

Although numerous studies concerning desaturases have been performed in vertebrates, particularly in mammals, very little is known about the distribution of the transcripts and the organization of the gene in fish. In order to gain further insights into the basic regulation of desaturase, the full-length cDNA of *desaturase* from grass carp (*Ctenopharyngodon idella*) was cloned, and its expression was further examined in the present study to pave the way to unraveling the regulation of desaturase. Using PCR strategies, we have circumvented the laborious procedure of constructing and screening a cDNA library. The expression of the gene was analyzed, showing that the predominant transcripts are located in the liver, whereas weak expression exists in brain tissues. Furthermore, for the first time, the genomic structure of *desaturase* from a fish is established. The grass carp desaturase gene is composed of six exons and five introns. This organization is similar to those in mammals but more compact by having shorter introns and a shorter 3' untranslated region.

RESULTS and DISCUSSION

Cloning of cDNA encoding grass carp delta-9 desaturase

The full-length cDNA of *desaturase* from grass carp was successfully cloned by using PCR strategies as illustrated in Fig. 1. A fragment was first amplified from liver mRNA after reverse transcription by two degenerate primers (PN and PC). The amplified fragment was subcloned and sequenced. The PCR product is 469 bp. To obtain the full-length cDNA, the 5' region and 3' region were extended by 5'-RACE and 3'-RACE, respectively. The sequence from the decoded RT-PCR product was further employed in the next cloning procedure. Two antisense nested primers specific to grass carp desaturase (P1 and P2) were utilized in concert with the designed anchor primers (PT1 and PT2). The cDNA fragment amplified by 5' RACE is 446 bp. By a similar approach, the 3' end cDNA of 1654 bp was obtained by 3' RACE.

The full-length cDNA shown in Fig. 2 was revealed by compilation of the three overlapping PCR fragments in the above experiments. The full-length cDNA comprises 2420 bp, including 63 bp in the 5' untranslated region, 975 bp in the open reading frame (ORF) which encodes 324 amino acids, and 1382 bp in the 3' untranslated region containing a polyadenylation signal at nucleotide 2387. The presumed start site of translation at nucleotide 64 shares strong similarity to the Kozak translational initiation sequence (Kozak, 1987). The predicted molecular mass of the protein is 37.6 kDa. Comparative analysis of the amino acid sequences from various vertebrates is depicted in Fig. 3. The deduced amino acid sequence from grass carp shares 65% and 86% identity with that of rat and carp *desaturase*, respectively. It was found that the N-terminal residues are highly variable between fish and mammals, suggesting that this region is irrelevant to its enzymatic function. Indeed, the recombinant protein of rat stearoyl-CoA desaturase lacking the 26-amino-terminal residues function normally both in the enzyme activity and the membrane integration (Strittmatter et al., 1988). Moreover, comparison of hydropathy profiles between rat desaturase and grass carp desaturase also directly supports this notion (Fig. 4). In contrast, the carboxyl-terminal region of desaturases terminates in similar residues. The ORF comprises three

histidine-cluster motifs, one HXXXXH (residues 85-90), and two HXXHH (residues 122-126 and 263-267, underlined in Fig. 3), which are well conserved in other desaturases as well as the bacterial membrane-bound enzymes, alkane hydroxylase and xylene monooxygenase (Shanklin, 1994). These motifs are essential for catalytic activity and iron-atom binding (Shanklin, 1994). By hydropathy analysis, two long hydrophobic regions of grass carp desaturase are conserved at the same positions as in rat desaturase (Fig. 4). These regions have been proposed as membrane-spanning domains and are well conserved among the desaturases (Stukey et al., 1990; Shanklin, 1994). According to the hydropathy profile and the presence of histidine motifs, the topology of the desaturases has been modeled as two membrane-spanning regions with hydrophobic residues embedded within the endoplasmic reticulum membranes, while the catalytic histidine motifs are exposed to the cytoplasmic surface of the membrane (Stukey et al., 1990; Shanklin, 1994).

Expression of the desaturase gene by Northern blot analysis and RT-PCR

In order to confirm the size of the full-length cDNA resolved by RT-PCR and RACE in this study and to determine the distribution of the grass carp *desaturase* transcripts in a variety of tissues, Northern blot analysis of total RNA was performed. Based on the same amount of ribosomal RNA, the relative abundance of a species of RNA can be visualized by blotting with a probe. The results of such an analysis are shown in Fig. 5. Abundant transcripts were detected in the liver (lane 3), but only a very weak band was observed in the brain extract with an equal sample size (lane 2). The size of the detected transcripts is about 2.4 kb, conforming to that of the cDNA (2420 bp) in this study. The weak expression of transcripts in the brain was further confirmed by RT-PCR (Fig. 5, panel C). A DNA fragment with predicted size was acquired from liver and brain tissues. Coherently the expression of this gene is limited only to the liver and brain in grass carp.

Beyond the structural conservation of the molecule in grass carp, the expression pattern of *desaturase* appears different from those in mammals. Northern blot and RT-PCR analyses in grass carp demonstrated that the transcripts of the gene are only located in liver and brain tissues with a high expression in hepatic tissue (Fig. 5). In rodents (mouse and rat), two related desaturase genes, SCD-1 and SCD-2, were expressed in a tissue-specific fashion. SCD-1 is principally expressed in adipose tissue and mildly expressed in the lung, whereas SCD-2 is predominately expressed in the brain and less abundantly in the lung, spleen, and kidney (Ntambi et al., 1988; Kaestner et al., 1989; Mihara, 1990). Starvation and refeeding a high carbohydrate diet induces SCD-1 expression in the liver and increases the transcripts of both genes in the lung and kidney (Ntambi et al., 1988; Kaestner et al., 1989; Mihara, 1990). In sheep, only a single mRNA species was found highly expressed in the liver and mammary gland, whereas a low level of expression was detectable in the brain as well as in a variety of other tissues including muscle, lung, kidney, heart, and adipose tissue (Ward et al., 1998). As to human *desaturase*, two alternative transcripts are generated from a single gene by usage of two tandem polyadenylations. The transcripts are abundant in the brain and liver and also detectable in the lung and heart (Zhang et al., 1999).

In contrast to the expression patterns in mammals, the distribution of the desaturase transcripts in grass carp appears to be more confined: highly detectable in liver and much less so in the brain, and it was not detectable in other tissues by the two methods employed in this study. These differences in the expression profile of *desaturase* between fish and mam-mals imply disparate regulation mechanisms and possibly reflect differences in the physio-logical essentiality and requirements for the enzyme in these animals. Nevertheless, differential expression in different tissues also suggests that

desatruase is regulated through multiple tissue-specific regulators.

Genomic organization of the desaturase gene

To unravel the genomic organization of the desaturase gene in fish, we first established a genomic DNA library from grass carp. Genomic DNA was digested by *Eco*RI, and the library was constructed in a Zap vector from which the phagemid can be directly excised. Thus, the tedious subcloning of lambda DNA was eliminated. By screening about 2×10^6 plaques with probes derived from the 5' and 3' RACE products, four clones of the phagemid were isolated and characterized from the *Eco*RI-digested phage library. Restriction-endonuclease mapping and nucleotide sequencing revealed that three clones, Zap1, Zap2, and Zap3, turned out to be chimeric clones in which a common fragment of 1.9 kb harboring the 5' end of the desaturase gene and some fragments irrelevant to this gene are composed (Fig. 6, the irrelevant fragments are not shown). The Zap 4-clone encompasses the entire region of exons 2-6. Exon-intron boundaries and the size of the introns were determined either by DNA sequencing or measured by agarose-gel electrophoresis of PCR-generated DNA fragments with primers complementary to the flanking region of the exons using phagemid clones and genomic DNA as templates. The analysis reveals that, in all, the gene contains six exons and five introns with the translational start site beginning at the second exon. This organization is almost identical to those of mouse, rat, and human *desaturases*, except the translational start sites in those of mammals begin at the first exon (Ntambi et al., 1988; Mihara 1990; Zhang et al., 1999).

Table 1 summarizes the size of introns and the intron-exon splice-junction sequence. The exon-intron boundaries conform to canonical splice-donor and acceptor consensus sequences beginning with a GT dinucleotide and ending with a AG dinucleotide except that in intron 4 where the splice-donor site is a GC dinucleotide instead. The intron positions related to the cDNA are also marked in Fig. 2.

The present research is merited by the Editor-in-Chief's comments: this paper describ-ed cloning and characterization of a full-length cDNA delta-9 desaturase from grass carp, using RT-PCR and RACE and the characterization of the genomic gene. It is the first time that a desaturase gene is reported for a fish system. The studies report in this manuscript are well designed and carefully executed and the ms is well prepared. In viewing the importance of the finding, this ms should be accepted for publication in the journal of Molecular Reproduction and Development without any revision .

ACKNOWLEDGEMENTS

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LEGENDS

Fig. 1. Strategy of molecular cloning of full-length cDNA of the grass carp desaturase gene. As shown in the schematic drawing, a PCR fragment was amplified after reverse transcription (RT) using degenerate primers (PN and PC). Once the sequence of the region was resolved, some internal sequences were further employed as primers for priming upstream and downstream (P1, P2, Q1, and Q2). In concert with the design anchor primers (PT1 and PT2), the 5' and the 3' regions were amplified through the RACE procedure (rapid amplification of cDNA ends). The full-length cDNA was compiled from three overlapping PCR fragments.

Fig. 2. The nucleotide and deduced amino acid sequences of desaturase cDNA from grass

carp. The amino acid sequence is shown below the nucleotide sequence in a single letter code. It contains 324 deduced amino acids. The translation start and termination codons are marked as M and an asterisk (*), respectively. The polyadenylation signal is underlined. The locations of five intron positions related to cDNA are also marked. Six putative mRNA destabilization signals, the ATTTA motifs, are boxed in the 3' untranslated region.

Fig. 3. Alignment of the amino-acid sequences of delta-9 desaturase from various species. The different amino acid residues among the species are presented with a black background. Residues that diverge between members are aligned by hyphens. Three conserved histidine-cluster motifs are indicated by bars. Amino acid sequences were obtained from the DDBJ/EMBL/GenBank under the accession numbers: grass carp (AJ243835), carp (U31864), mouse (NM009127), rat (J02585), and human (AF097514).

Fig. 4. Hydropathy profiles for rat and grass carp desaturase proteins. The plots were analyzed using a Kyte-Doolittle scale (Kyte-Doolittle, 1982). Two hydrophobic regions are indicated by bold lines. Three histidine-cluster motifs are marked by arrows.

Fig. 5. Northern blot and RT-PCR analyses of the expression of grass carp desaturase in different tissues. The blot shown in panel A was the result of hybridization with an antisense RNA probe corresponding to the region of the 5' RACE product. Total RNA at 10 g from different tissues (lanes 1-6; 1: muscle, 2: brain, 3: liver, 4: gill, 5: kidney, 6: heart) were loaded in each lane, analyzed in 1% agarose gel containing formaldehyde and visualized by ethidium bromide-staining (panel B). An RNA size marker was run in parallel (lane M). The expression of desaturase was also analyzed by RT-PCR with primers specific to grass carp desaturase. The PCR products were analyzed in 1.2% gel with a DNA marker (panel C).

Fig. 6. Schematic representation of the genomic structure of grass carp desaturase. Four genomic DNA clones, Zap 1-Zap 4, have been analyzed, revealing the intron and exon boundaries. There are five introns and six exons in this gene. Exons are presented by boxes: black boxes indicate the coding region; open boxes depict untranslated regions. The boundary between Zap 1 and Zap 4 was confirmed by PCR with primers located at the ends. The translation initiation and termination codons are marked as ATG and TAA, respectively.

Table 1 Exon-intron organization of the grass carp SCD gene

Exon (size)	5'-Splice donor	Intron (size)	3'-Splice acceptor	Exon
Exon 1 (55 bp)	AGTTTGgtgagt	intron 1 (0.153 kb)	tggcagTGATCA	exon 2
Exon 2 (213 bp)	TCTGGAgtaagt	intron 2 (~2.5 kb)	ctgcagCTTTCG	exon 3
Exon 3 (131 bp)	TTCCAGgtacgc	intron 3 (~2.4 kb)	ctgtagAATGAC	exon 4
Exon 4 (206 bp)	GAGGAGgcaagt	intron 4 (0.110 kb)	tctcagGCACTA	exon 5
Exon 5 (233 bp)	CCATAGgtatgt	intron 5 (0.641 kb)	tctcagGTGAAG	exon 6
Exon 6 (1582 bp)				

Exon sequences are marked by capital letters; intron sequences are marked by small letters.

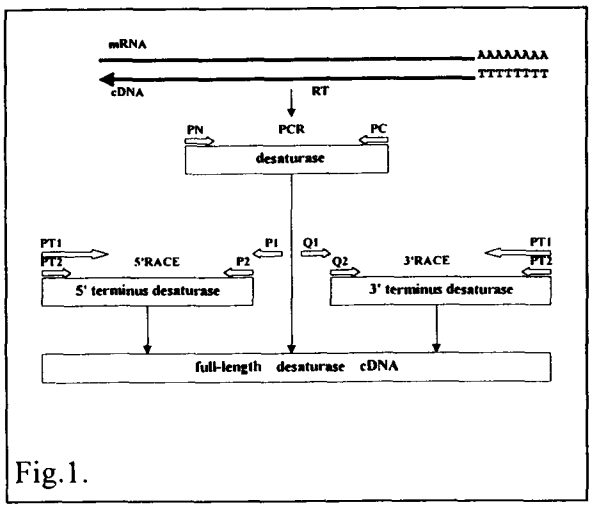

Fig.1.

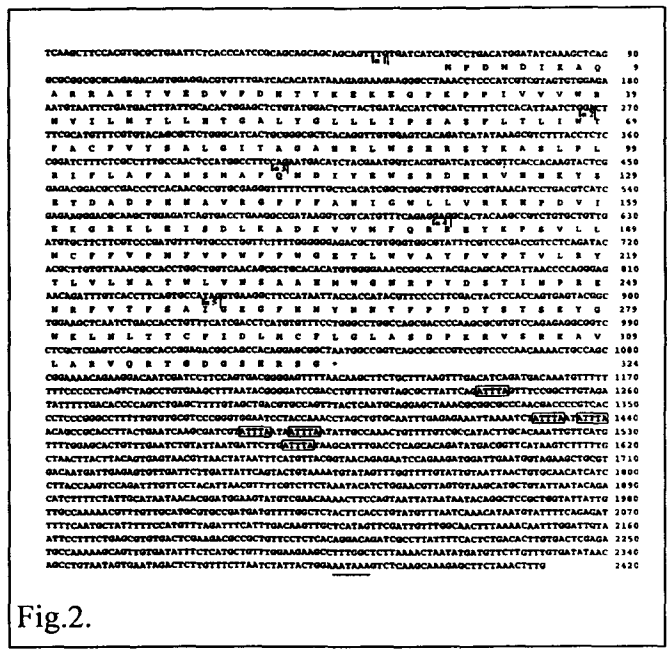

Fig.2.

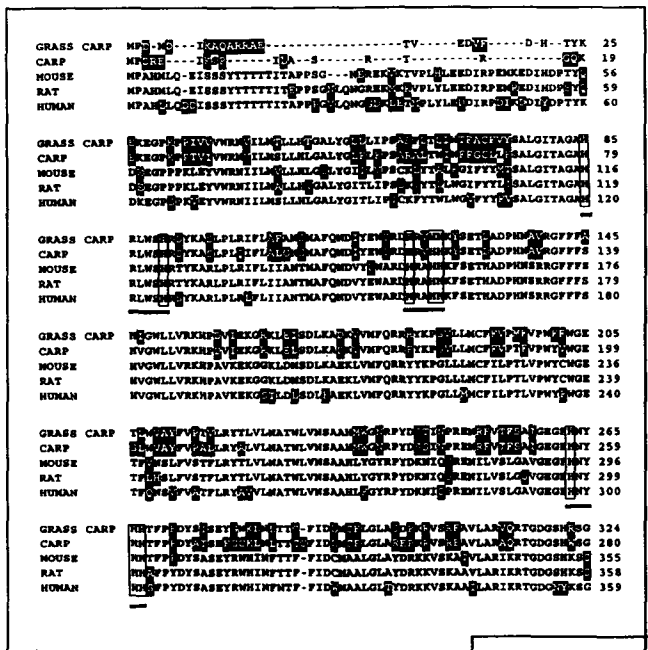

Fig.3.

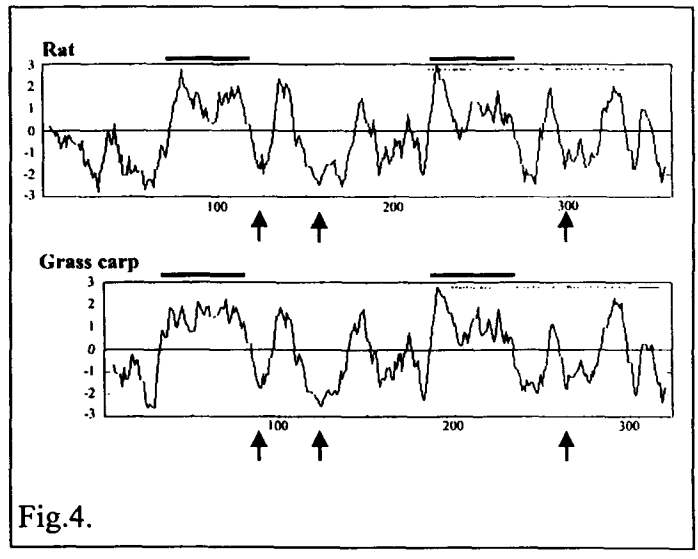

Fig.4.

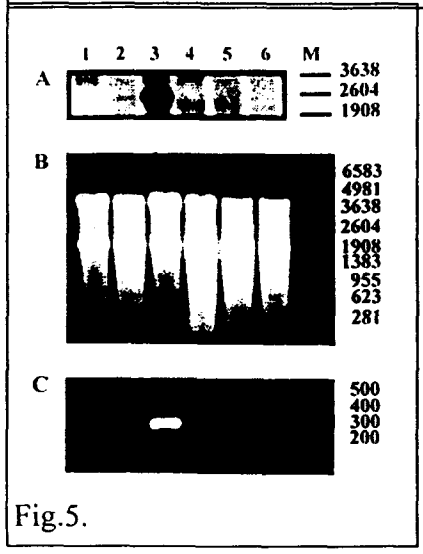

Fig.5.

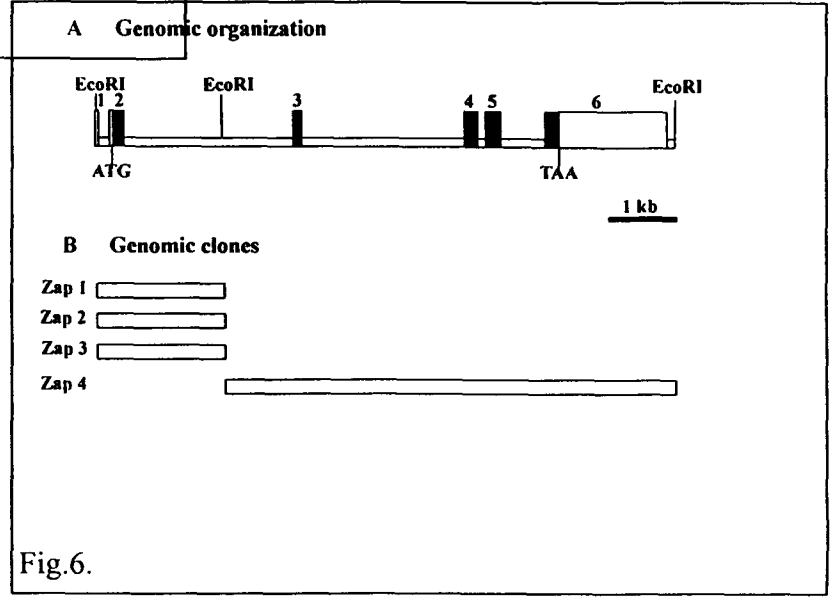

Fig.6.