Molecular characterization of the insulin-like growth factor-I (IGF-I) gene in channel catfish (Ictalurus punctatus)

LaTonya A. Clay a,b, Shiao Y. Wang b, William R. Wolters a,c, Brian C. Peterson a, Geoffrey C. Waldbieser a,*

a USDA, ARS, Catfish Genetics Research Unit, Thad Cochran National Warmwater Aquaculture Center, 141 Experiment Station Road, Stoneville, MS 38776, USA
b Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MS 38677, USA
c USDA, ARS, National Cold Water Marine Aquaculture Center, University of Maine, Orono, ME 04469, USA

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Abstract

The insulin-like growth factor-I (IGF-I) gene was characterized in channel catfish. Partial cDNA sequence, missing exon 1 and part of exon 2, was obtained in 5' and 3'-RACE experiments. Direct sequencing of two bacterial artificial chromosome clones revealed gene structure and provided sequence from 640 bp upstream of the initiator methionine to 136 bp beyond the polyadenylation site. Genomic sequence contained a putative TATA box 506 bp upstream of the initiator methionine. The 477-bp reading frame within five exons encoded a 159-amino acid (aa) pre-propeptide highly similar to IGF-I in higher vertebrates. The sequence encoding the signal peptide was unique in catfish and contained 70% G+C content with the potential for a stable stem–loop structure. Full-length cDNA was only maintained in recombination-deficient (DH10B) strain E. coli. Levels of IGF-I mRNA were highest in liver, followed by brain and muscle, then heart and kidney (P<0.05). A CT/GA dinucleotide microsatellite in intron 1 was highly polymorphic in commercial channel catfish, and permitted placement of the IGF-I gene on the catfish genetic map. However, specific IGF-I alleles were not correlated with differences in growth rate from 100 to 130 days post-hatch in USDA103 line catfish.

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1. Introduction

Insulin-like growth factor-I (IGF-I) is a single chain peptide hormone that demonstrates structural homology to proinsulin and plays an important role in mammalian growth and development [1]. It belongs to the highly conserved IGF system that is generally composed of the ligands IGF-I and IGF-II, their receptors, and the IGF binding proteins [2,3]. Mature IGF-I peptides share high levels of amino acid sequence similarity between vertebrate species [4].

Along with similarity in IGF-I amino acid sequence, fish and mammals also demonstrate similarities in IGF-I gene expression and peptide function [4]. Teleost IGF-I mRNA is primarily produced in the liver, but is also produced in a variety of extrahepatic tissues [5]. Administration of IGF-I stimulates in vitro sulfation of fish cartilage tissue [6,7]. These data support the conservation of the physiological role of IGF-I between teleosts and mammals.

The channel catfish, Ictalurus punctatus, is a primitive euteleost in the superorder Ostariophysi and native to North America. Commercial production of catfish is the leading industry in United States aquaculture, and 286 million kg were processed in 2004 [8]. Catfish populations exhibit variation in growth rate and demonstrate phenotypic response to selection for growth [9,10]. Because of the central role of IGF-I in vertebrate growth, the present research was designed to characterize the channel catfish IGF-I gene and identify polymorphisms in this gene that could eventually be correlated with variation in growth phenotypes.

* Corresponding author. Tel.: +1 662 686 3593; fax: +1 662 686 3567.
E-mail address: gwaldbieser@ars.usda.gov (G.C. Waldbieser).
2. Materials and methods

2.1. Total RNA isolation

Total RNA was extracted from liver, brain, heart, kidney and muscle tissues of five rapidly growing juvenile (~5-month-old) channel catfish using Trizol reagent (Molecular Research Center, Cincinnati, OH). Total RNA samples used for quantitative PCR analyses were treated with DNase (Ambion Inc., Austin, TX).

2.2. Cloning and sequencing

Degenerate primers (Table 1) were designed from an alignment of ubiquitously expressed broadhead catfish (Clarias macrocephalus), common carp (Cyprinus carpio), chum salmon (Onchorhyncus keta), rainbow trout (Onchorhyncus mykiss), domestic pig (Sus scrofa) and human (Homo sapiens) IGF-I sequences. Total RNA from liver was used in subsequent experiments with 3′- and 5′-RACE (Rapid Amplification of cDNA Ends) Systems (Invitrogen Life Technologies, Carlsbad CA). The first round of RACE PCR included an initial denaturation at 94°C – 2 min; 25 cycles of 94°C – 20 s, 60°C – 1 min, 68°C – 1.5 min; then 68°C – 10 min. Touchdown PCR was used to perform nested amplification on a 100-fold dilution of the first amplification product using a nested IGF-I-specific primer, (IGF1-3′-cen; IGF1-5′c-2 and -1), and an amplification primer provided with the kit. PCR conditions were as follows: 94°C – 3 min; 2 cycles of 94°C – 1 min, 70°C – 2.5 min; 2 cycles of 94°C – 1 min, 68°C – 2.5 min; 6 cycles of 94°C – 1 min, 66°C – 1 min, 68°C – 2 min in which the annealing temperature was lowered 2°C every 2 cycles; then 72°C – 5 min.

Two genomic clones, CCBL1-36J12 and CCBL1-83F14, were isolated from a channel catfish BAC library with primers IGF1ex3F and IGF1ex3R (Table 1) using established procedures [11]. A 121-bp product was generated from a channel catfish BAC library with primers IGF1ex3F and IGF1ex3R; 38 cycles of 95°C – 1.5 min, 68°C – 2 min. The antisense primer, IGF-QR1, was designed to span an intron/exon boundary to minimize potential amplification of contaminant DNA. A 125-bp fragment of IGF-I cDNA, the antisense primer, IGF-QR1, spanned an intron/exon boundary to minimize potential amplification of contaminant DNA. A 125-bp fragment of IGF-I cDNA was used as a positive control in these experiments as an internal control. The negative controls in the real-time PCR analysis included a no-cDNA control and a DNase-treated liver RNA sample that was not reverse-

### Table 1

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### Quantitative expression

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### BAC identification

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### Genotyping

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<td>[NED]GACACGACGCCATGGTCCA</td>
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SN—sense strand, AS—antisense strand. Position for IGF-I primers correlates with Fig. 2, and position for 18S primers is based on GenBank Accession AF021880. Bases in parentheses were added to promote non-template adenylation. Text in brackets denotes fluorescent label and quencher.
transcribed to ensure that only the cDNA was quantified in each sample. Random hexamer primers were used to synthesize cDNA from 1 μg of total RNA (iScript cDNA synthesis kit, Bio-Rad Laboratories, Hercules, CA). The 25-μl real-time quantification reaction contained 2× IQ Supermix (Bio-Rad Laboratories), 1 ng of template, 300 nM IGF-I primers, 25 nM 18 S primers, and 100 nM each of the IGF-I and 18 S rRNA probes.

The quantitative assay was performed with a thermal profile of 95 °C –45 s; then 45 cycles of 95 °C –15 s, 60 °C –1 min in an iCycler IQ TM Real-Time PCR Detection System (Bio-Rad Laboratories). Fluorescence detection was performed at the end of the 60 °C step and data were quantified using the Relative Quantification method [16]. Serial dilutions of liver cDNA ranging from 10 ng to 100 pg were used to construct the standard curve. Samples from five fish were run in triplicate and IGF-I and 18 S rRNA expression levels were calculated from the standard curve. IGF-I expression levels were normalized against the 18 S rRNA
2.4. Microsatellite genotyping

Genotyping primers (Table 1) were designed flanking a CT/TA repeat in intron 1, and locus heterozygosity was determined using fluorescent genotyping of a general population of outbred commercial catfish. Genotyping reactions and analysis were performed as previously described [12]. Two channel catfish reference families were genotyped to place IGF-I on the catfish genetic linkage map [17]. Fragments were resolved by capillary electrophoresis with POP7 polymer on an ABI PRISM® 3100 Genetic Analyzer, and alleles were determined using GeneMapper software (Applied Biosystems). Linkage was determined using CRI-MAP 2.4 [18] at LOD=3.0. Multipoint linkage analysis was performed using the ALL and FLIPS options to determine the highest likelihood order of linked markers, and the CHROMPIC option to identify unlikely double crossover events.

Full-sib families of the USDA103 strain were placed in a 30-day growth study at 100 days post-hatch [19] and family-wise specific growth rates (SGR) were calculated. Twelve full-siblings from each of the seven families with the highest SGR (range 4.4–4.9) and the eight families with the lowest SGR (range 1.7–2.8) were genotyped as above. Parental genotypes were deduced from Mendelian segregation in full-sib offspring, and allele frequencies were calculated from parental genotypes. A population of 350 USDA103 broodfish, from which the SGR families were derived, were also genotyped. A goodness-of-fit test was used to compare the distribution of alleles in the parent USDA103 population versus the high and low SGR families, and between the high and low SGR families.

3. Results

3.1. Cloning and sequence analysis of IGF-I

The channel catfish IGF-I cDNA was cloned using degenerate primers designed from conserved regions of this gene in several vertebrate species. A combination of 3′ and 5′ RACE experiments produced cDNA clones with only partial coding sequence beginning in exon 2. The remaining 5′ coding sequence and 640 bp upstream of the initiator methionine were obtained by directly sequencing two BAC clones containing the IGF-I gene. Restriction digestion with NotI polymerase provided sequence 640 bp upstream of the translational initiation site (Figs. 1 and 2). Amplification of cDNA using various sense primers designed from the upstream genomic sequence demonstrated that the 5′ untranslated region extended at least 450 bp upstream of the initiator methionine (Fig. 3) to the site of primer IGF1-450F. A putative TATA sequence was identified 506 bp upstream of the initiator methionine (51 bp upstream of the IGF1-450F primer). This predicted an mRNA of approximately 1100 bp.

The full-length catfish IGF-I cDNA contained an open reading frame encoding a 159 aa peptide. Multiple sequence alignment was performed on available IGF-I peptide sequences from fish of the superorders Ostariophysi, Protacanthopterygii, and Acanthopterygii, chicken, and selected mammals (Fig. 4). The catfish signal peptide demonstrated a low level (12%–48%) of similarity with the other species. The B and A domains were highly conserved (86–100%) between fish, the chicken, and mammals. Sequence similarity in the short C and D domains ranged from 38 to 88%. The E domain sequence of the selected species ranged from 50% to 89% similarity with channel catfish. The Protacanthopterygian and Acanthopterygian fish contained a well-conserved sequence in the E-domain that was not present in the Ostariophysi, chicken, or mammals.

The entire catfish sequence upstream of the initiator methionine was aligned with the zebrafish, common carp, chum salmon, black seabream, tilapia, pufferfish, chicken, and human IGF-I sequences. Similarity from catfish sequence to 640 bp to ~375 bp was 40% with zebrafish and carp but only 24–35% with the other species, and there were no substantial blocks of conserved sequence. However, similarity from ~374 bp to ~52 bp was 70–75% with fish and 65% with chicken and human sequences and there were extensive blocks of conserved sequence (Fig. 5). Similarity in this region between all fish, excluding catfish, ranged from 90 to 99%. The channel catfish 5′ flanking region contained putative binding sites for the CCAAT/enhancer binding protein (C/EBP), hepatocyte nuclear factors-3 beta and 4 (HNF-3β, HNF-4), signal transducer and activator of transcription 5 (STAT5), glucocorticoid receptor (GR), specificity protein 1.
(SP1), and a negative regulatory element in the insulin gene (NRE). Most binding site sequences were well conserved between species. Binding sites that were more specific to catfish were a C/EBP site at \(589\) bp and two SP1 sites at \(342\) bp and \(90\) bp.

### 3.2. IGF-I mRNA expression in channel catfish tissues

Real-time quantitative PCR analysis revealed varying levels of IGF-I mRNA expression in selected juvenile tissues. Levels of IGF-I mRNA in tissues were reported relative to anterior...
kidney, the tissue with lowest expression level. Relative level of IGF-I mRNA expression in channel catfish liver was significantly higher (271-fold, \( P < 0.05 \)) than in muscle, brain, heart, and kidney. Furthermore, IGF-I mRNA in the muscle (72-fold) and brain (56-fold) were significantly higher (\( P < 0.05 \)) than in the heart (5-fold) and kidney (Fig. 6). Expression level of IGF-I in heart tissue was not significantly different from the kidney (\( P < 0.05 \)).

3.3. IGF-I locus polymorphism and mapping

Genomic sequence and genotype analyses revealed a polymorphic CT/GA microsatellite near the end of intron 1 (Fig. 2). Forty-two outbred catfish from a general commercial population contained thirteen alleles at this locus, with an average heterozygosity of 0.850 (Table 2). Analysis of 350 USDA103 strain broodfish revealed only seven IGF-I microsatellite alleles in this population. The five alleles with the highest frequency in the broodfish were also present in the low and high growth rate families that were progeny of these broodfish. A goodness-of-fit test demonstrated no significant difference in allele frequency between the low and high growth families, and no significant difference between these families and the parent population (\( P > 0.05 \)). The only notable difference was due to the presence of one 182/182 homozygous parent in the low growth population. Genetic linkage analysis

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**Fig. 5.** Multiple sequence alignment of the IGF-I proximal promoter and 5’ untranslated region between channel catfish (Ipu: Ictalurus punctatus, DQ088970), zebrafish (Dre: Danio rerio, BX510924), common carp (Cca: Cyprinus carpio, AF465830), chum salmon (Oke: Oncorhynchus keta, AF063216), black seabream (Asc: Acanthropagrus schlegeli, AF030573), gilthead seabream (Sau: Sparus aurata, [20]), tilapia (Omo: Oreochromis mossambicus, AF033797), pufferfish (Fru: Fugu rubripes, Fugu genome v.3.0-http://genome.jgi-psf.org/fugu6/fugu6.home.html), chicken (Gga: Gallus gallus, M74176), and human (Hsad: Homo sapiens, genome build 35.1, http://www.ncbi.nlm.nih.gov/mapview). Consensus sequence similarity is indicated with a black background. Numbers represent position upstream of the translation initiation site in the channel catfish IGF-I gene. The initiator methionine codon is the common 3’ end. Putative transcription factor binding sites in the promoter and 5’ untranslated sequence are indicated by a double line above the sequence and bases shared in adjacent recognition sequences are indicated by an asterisk.
revealed Mendelian inheritance of the IGF-I microsatellite marker and allowed placement of the IGF-I gene on linkage group U19 [17]. The IGF-I gene was positioned between a terminally-located gene for 40S ribosomal protein S16 (20.4 cM, recombination fraction = 0.16, LOD = 15.31) and marker IpCG0011 (19.9 cM, r.f. = 0.21, LOD = 5.91) based on 176 informative meioses.

4. Discussion

4.1. Cloning and sequence analysis of IGF-I

Reverse transcriptase PCR experiments suggested the 5′-untranslated region of the catfish IGF-I gene extended at least 450 bp upstream of the translation initiation site, compared with 400–410 bp for the gilthead seabream [20] and 114 bp for the common carp [21] IGF-I genes. While the approximate position of the catfish initiation site was identified by reverse transcriptase PCR and not primer extension, the RT-PCR primers spanned a 1-kb intron and the amplification product was consistent with accurate intron splicing. The proximal 374 bp upstream of the initiation site contained high levels of sequence conservation between catfish, other teleosts, chicken, and human. This region contained conserved binding sites for transcriptional factors such as the HNF’s, SP1, and STAT5 which are known to modify IGF-I expression [22–24]. The region also contained C/EBP binding sites which modify expression of other genes in liver [25], and a negative regulator of expression for the insulin gene [26]. The high degree of conservation of the 5′ untranslated region points to its importance in IGF-I transcriptional regulation. It remains
unknown whether differences in transcriptional regulatory recognition sequences in catfish, such as the C/EBP and SP1 sites, lead to differences in IGF-I gene regulation compared with the other species.

The IGF-I coding region in vertebrates is characterized by sequence encoding domains that are characterized by post-translational processing of the pre-propeptide. These include the signal peptide, and the B-, C-, A-, D-, and E-domains. Multiple sequence alignment showed the catfish IGF-I signal peptide was quite different from the selected fish, avian, and mammalian species. When the full-length catfish IGF-I cDNA was ligated to a plasmid and transfected into recombination-competent DH5α strain *E. coli*, large segments of exon 2 (encoding the signal peptide) were always excised in resulting clones. However, plasmids transfected into recombination-deficient DH10B strain *E. coli* always contained full-length cDNA inserts. Free energies of secondary structure in the signal peptide-encoding region (calculated at temperatures optimal for species growth) also predicted the formation of structure more stable in the other fish species \((\Delta G = -34.1 \text{ to } -48.4 \text{ kcal/mole})\) than in chicken \((\Delta G = -15.24 \text{ kcal/mole})\) or mammals \((\Delta G = -15.6 \text{ to } -25.8 \text{ kcal})\). Thus, secondary structure in the mRNA may have caused the cloning instability observed in repeated experiments. Secondary structure has been implicated in the translational control of several genes [27] and this structure could play a role in the regulation of IGF-I production in teleosts.

The mature IGF-I molecule consists of the B-, C-, A-, and D-domains [28], and multiple sequence alignment revealed a high level of peptide sequence conservation at the B-, C- and A- domains among vertebrates, but marked differences between species at the other domains. In general, peptide sequence identity was highest within each teleost superfamily, and the chicken was more similar to the mammalian species than to the fish. Multiple sequence alignment demonstrated that the selected Protacanthopterygians and Acanthopterygians contained an insertion in the E domain that did not exist in the Ostariophysians, chicken, or mammals (Fig. 4). This insertion occurred at the junction of exons 3 and 4 in the Ostariophysian genes, and could indicate the existence of alternate splicing mechanisms in the more differentiated teleosts. Four E-domain variants have been reported in rainbow trout [29], and the Ostariophysian genomic sequence encoding the E-domain most resembles trout variant Ea-2. Recent research has demonstrated potential biological activity of rainbow trout E domain peptides transfected into cultured cells [30]. It is evident that the more differentiated teleosts contain a unique E-domain compared with the more primitive teleosts, but it is speculative to state that differences in IGF-I peptide sequence lead to alternate IGF-I transcriptional processing or functional differences between E-domain peptides.

### 4.2. IGF-I mRNA expression in channel catfish tissues

In order to compare relative IGF-I gene expression between published studies in fish, expression in liver was calculated related to expression in kidney. The liver/kidney (L/K) ratio was 271 in channel catfish, 40 in adult tilapia [31], 20 in juvenile common carp [32], and 12 in juvenile coho salmon [33]. This suggested catfish liver produced substantially more IGF-I than the other species and/or less IGF-I in kidney tissue. However, the life stage and/or nutritional status of the fish could affect relative IGF-I mRNA levels. Restriction of feeding lowered IGF-I mRNA expression in livers of coho salmon and the snapper, *Pagrus auratus* [34,35] and in rainbow trout muscle [36], while IGF-I mRNA levels increased after restoration of feeding. Nonetheless, liver always exhibited the highest levels of IGF-I gene expression in these species, and supported other data showing liver is the main site of endocrine IGF-I production in teleosts [4].

Table 2
IGF-I microsatellite alleles (number observed and frequency) in randomly selected commercial catfish, USDA 103 broodstock, and parents of USDA103 families with low or high specific growth rate

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<th>USDA103 broodfish 350 fish</th>
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* SGR-specific growth rate (low=1.7– 2.8; high =4.4 – 4.9). Parental genotypes were deduced from Mendelian inheritance of 12 offspring that were genotyped from each family (180 offspring total). Allele frequencies were calculated based on the parental alleles.

b Measured in basepairs.

c Number of observed alleles.

d Allele frequency.
It is unclear whether the high levels of IGF-I gene expression in channel catfish brain and muscle reflect a species difference, or a difference in nutrition or environment during sampling. Levels of IGF-I mRNA from extrahepatic tissues in coho salmon, common carp, and tilapia generally did not differ significantly from kidney [31–33]. An exception was the tilapia brain, which contained 6-fold higher IGF-I mRNA levels than kidney. Detection of IGF-I mRNA in extrahepatic tissues suggests organ-specific paracrine and autocrine roles of IGF-I in channel catfish as in mammals [37]. Coupled with research revealing growth hormone production in the catfish pituitary [38], the present data suggest channel catfish have a growth hormone–IGF-I axis similar to other teleosts and mammals.

4.3. IGF-I locus polymorphism and mapping

We have previously observed, in outbred catfish populations, an average of eight alleles per locus and heterozygosity near 0.70 at type II (non-coding) microsatellite loci [17]. The IGF-I locus displayed a high level of polymorphism in commercial catfish, and this likely reflected the lack of sustained genetic selection in these populations. The USDA103 strain contained fewer alleles than the commercial population, although the missing alleles were at a low frequency in the commercial fish. Within the USDA103 strain, there was no apparent difference in IGF-I microsatellite allele frequency between the families with low and high growth rates, but these families also demonstrated no difference in levels of IGF-I gene expression in liver and muscle [19]. Although it appears molecular variation at the IGF-I locus is not linked to phenotypic variation within the USDA103 population, this relationship may exist in other catfish populations. Also, the present research was performed on a growth rate trial from 100 to 130 days post-hatch, so IGF-I expression could be correlated with growth at other life stages.

Genetic linkage of IGF-I with ribosomal protein S16 (RPS16) in catfish is not conserved in other species. In zebrafish, IGF-I and RPS16 are located on chromosomes 4 and 11, respectively (build Zv5, http://www.ensembl.org/Danio_rerio/index.html). The IGF-I and RPS16 genes are located on human chromosomes 12 and 19, respectively, and on mouse chromosomes 10 and 7, respectively (human build 35.1 and mouse build 34.1, http://www.ncbi.nlm.nih.gov/mapview/). The catfish RPS16 gene was mapped using a microsatellite marker located within the third intron [39], thus it was not a retrotransposed pseudogene. However, it is possible RPS16 is duplicated in the catfish genome. Analysis of IGF-I-RPS16 synteny in less differentiated teleosts would help clarify whether the catfish has retained an ancestral synteny at this genomic locus.

Acknowledgements

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dation or endorsement by the U. S. Department of Agriculture. GenBank accessions for channel catfish IGF-I are AY353852 (cDNA), DQ088970–DQ088972 (gene), and AAQ56592 (protein).

References


