Isolation and Characterization of Tilapia (*Oreochromis mossambicus*) Insulin-Like Growth Factors Gene and Proximal Promoter Region

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ABSTRACT

To understand the molecular mechanism which controls the transcription of the insulin-like growth factors (*IGFs*) gene, we have cloned and sequenced the cDNA for the proximal promoter region of the tilapia *IGFs* gene and have characterized its activity by chloramphenicol acetyltransferase (CAT) transient transfected expression assays. Tilapia (*Oreochromis mossambicus*) *IGF-I* cDNA (549 bp) was amplified by PCR from single-stranded cDNA of growth hormone (GH)-induced liver RNA using a pair of oligonucleotides specific for fish *IGF-I* as amplification primers. Tilapia *IGF-I* and *IGF-II* 5' termini were analyzed by rapid amplification of cDNA 5' ends (5'RACE). Analysis of the 5'RACE results revealed two transcription start sites in *IGF-I* and one transcription start site in *IGF-II*. Different fragments of the 5' flanking region were transfected into human lung adenocarcinoma cells. In the cell line, maximum promoter activity was located in the distal 657 basepairs of the *IGF-I* 5' flanking region and in the distal 450 basepairs of the *IGF-II* 5' flanking region. The *in vivo* actions of the *IGFs* promoter on developmental stage expression were investigated further in transgenic zebrafish in which an *IGFs* promoter-driven green fluorescent protein (GFP) encoding the cDNA transgene was microinjected into embryos. Morphologic and RT-PCR studies of the transgenic zebrafish indicated that *IGF-I* promoter-driven GFP transcripts appeared for the first time in the 1-K-cell stage and the *IGF-II* promoter-driven GFP transcripts appeared for the first time in the 32-cell stage. Fluorescent (GFP) distribution was apparent within 48 h in *IGF-II*-transgenic zebrafish embryos, especially in eye, muscle, corpuscle, floor plate, horizontal myoseptum, yolk sac extension, and yolk sac. These results indicate that the *IGF-I* and *IGF-II* promoters are active in tissue and in a development-specific manner. Our findings also indicate that the *IGF-II* promoter influences the growth of fish embryos earlier than does *IGF-I*, and *IGF-II* has higher levels of expression than does *IGF-I*. These results suggest that the *IGF-II* promoter plays a growth factor role in teleost embryo development.

INTRODUCTION

MATURE INSULIN-LIKE GROWTH FACTOR-I (IGF-I) is a single-chain polypeptide with 70 amino acid residues. It plays a central role in regulating somatic growth in all vertebrates via the modulation of growth hormone (GH) (Mathews *et al.*, 1986). The IGF-I prepropeptide consists of S, B, C, A, D, and E domains; the S and E domains are cleaved from the others during post-translational processing of the propeptide;

then, the mature IGF-I is secreted into serum. In vertebrates, the effects of IGF-I on somatic growth and cell differentiation are regulated by GH and nutritional conditions (Clemmons and Underwood, 1991; Conover *et al.*, 1993). Growth hormone increases the abundance of IGF-I mRNA by enhancing the transcription of the *IGF-I* gene, as well as affecting the processing of the IGF-I mRNA (Daughaday and Rotwein, 1989).

Piscine cDNA sequences have been cloned and sequenced from several species, including salmon (Cao *et al.*, 1989;

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Duguay *et al.*, 1992), hagfish (Nagamasu *et al.*, 1991), rainbow trout (Shamblott and Chen, 1992), catfish (McRory and Sherwood 1994), *Sparus aurata* (Duguay *et al.*, 1996), carp (Liang *et al.*, 1996), and tilapia (this paper), and show a high degree of homology. Fish IGF-I peptides have been conserved over the evolutionary time scale from hagfish approximately 550 Ma to the present, suggesting that IGF-I function is important in fish. Yet fish IGF-I endocrine function and structural comparisons with mammals and birds are not understood clearly. In order to further understand comparative fish endocrine physiology, we have initiated a study of the growth regulation of IGF-I in the tilapia, *Oreochromis mossambicus*.

Insulin-like growth factors (IGFs) consist of two polypeptides, IGF-I and IGF-II. The peptides have high homology of protein folding structure and play important regulatory roles in growth and differentiation (Daughaday and Rotwein, 1989). Although IGFs have similar protein folding structure and growth-promotion effects, their biologic effects are mediated by different receptors. The IGF-I receptor is a tyrosine kinase receptor; the IGF-II receptor is a mannose-6-phosphate receptor (Czech, 1989). The transduction of signals operates through different mechanisms. Receptors that bind the IGFs are associated with different biologic effects *in vivo* and *in vitro*.

The *IGF-I* gene structure has been analyzed in humans (Rotwein *et al.*, 1986), rats (Shimatsu and Rotwein, 1987; Hall *et al.*, 1992), sheep (Dickson *et al.*, 1991), chickens (Kajimoto and Rotwein, 1991), and salmon (Kavsan *et al.*, 1993, 1994). In general, the canonical fish gene consists of five exons: exon 1 encodes the 5' untranslated region and part of the signal peptide; exon 2 encodes the remainder of the signal peptide and the B domain; exon 3 encodes part of the B domain, the C, A, D domains, and the greater part of the E domain; exon 4, a mini exon, encodes a little of the E domain; while exon 5 encodes a little of the E domain and the 3' untranslated region. The complete mature IGF-I peptide is distributed in exons 2 and 3. Because the complicated gene structure produces multiple E peptides, there are many types of alternative splicing. In fish (salmon) a second nonallelic *IGF-I* gene (designated *IGF-I.2*) has been found (Kavsan *et al.*, 1994); the existence of these two alleles (*IGF-I.1* and *IGF-I.2*) gives rise to the multidiversity of IGF-I mRNA species, even though the salmon *IGF-I* gene is less than 20 kb in length. In salmon, the *IGF-I* promoter region has not produced a consensus TATA box-like element, but potent stimulatory action by hepatocyte nuclear factor 1 has been found (Koval *et al.*, 1994; Kulik *et al.*, 1995) and may play an important regulatory role by stimulating IGF-I expression with transcription factor binding, especially of the liver-specific transcription factor. The rat promoter 1 has been shown to lack TATA and CCAAT boxes in the region 20 to 80 bp upstream of the start sites (Kim *et al.*, 1991; Hall *et al.*, 1992), but TATA and CCAAT-like elements are found in the major exon 2 start site (Adamo *et al.*, 1991). The rat exon 1 and exon 2 transcripts have a tissue-specific distribution according to two promoters under different regulation. In other words, exon 1 transcripts can be detected in all rat tissue, whereas exon 2 transcripts are present in only a few tissues, particularly liver and kidney (Adamo *et al.*, 1989, 1991).

The human *IGF-II* gene consists of 10 exons about 30 kb in length (de Pagter-Holthuisen *et al.*, 1987, 1988; Holthuisen *et al.*, 1990). The sequence encoding the mature, circulating 70-

amino acid polypeptides is contained within exons 8, 9, and 10. Rat and mouse *IGF-II* genes consist of six exons and span about 12 kb of DNA (Rotwein and Hall, 1990; Ikejiri *et al.*, 1990, 1991). The prepropeptide is also contained within exons 4, 5, and 6. The ovine *IGF-II* gene is comprised of nine exons that span approximately 25 kb, and the coding region is contained within exons 8, 9, and 10 (Ohlsen *et al.*, 1994). The fish (tilapia) *IGF-II* gene has a different structure, with four exons encoding prepropeptides (Chen *et al.*, 1997). The human *IGF-II* gene is controlled by four different promoters. Human promoters P2, P3, and P4 are highly activated in fetal tissue and most adult nonhepatic tissues (de Pagter-Holthuisen *et al.*, 1988), but in adult human liver, the promoter P1 is activated, and the other promoters are not. Analysis of the human *IGF-II* P4 promoter reveals that there are three possible Sp1 binding sequences within 100 bp upstream of the transcription start site. Several GC-rich regions and TATA-like sequences are contained in the human *IGF-II* P4 promoter region (Hyun *et al.*, 1993). The six types of human IGF-II mRNA species are expressed by tissue-specific and development-specific pathways. The expression of these mRNA species is regulated by four promoters (de Pagter-Holthuisen *et al.*, 1988) that also represent *IGF-II* promoters provided with tissue-specific and development-specific expression.

Little is known about promoter control of the expression of IGFs during various developmental stages in teleosts; however, in *Sparus aurata*, IGF-I mRNA was detected in unfertilized eggs and in developing embryos (Funkenstein *et al.*, 1996). *Onchorynchus mykiss* mRNA is maternally stored in eggs and was detected from the cleavage stage to the feeding stage (Greene and Chen, 1997). These data indicate that IGFs play an important role in embryonic development. However, up to now, no reports have been published concerning which *IGF-I* or *IGF-II* promoter plays what role in the development of fish.

Recently, we isolated and sequenced the tilapia *IGF-I* and *IGF-II* cDNA, expressed the recombinant polypeptides in an *E. coli* system, and characterized the structure of the *IGF-II* coding region (Chen *et al.*, 1997). Our main objective in this paper is to describe the characterization of the proximal promoter regions of tilapia *IGF-I* and *IGF-II* genes. We have used not only the chloramphenicol acetyltransferase (CAT) system to analyze the proximal promoter activity but also the green fluorescent protein (GFP) system to analyze the proximal promoter features of tissue-specific and development-specific expression. The results indicate that the *IGF-I* and *IGF-II* proximal promoters are important regulators of embryonic growth and development.

MATERIALS AND METHODS

Oligonucleotides

The PCR primers for cloning *IGF-I* cDNA are located on the S and E domain regions. After comparing several fish *IGF-I* sequences, two universal primers were designed, namely, IGF-I primer 1 and IGF-I primer 2. The 5' end of these primers contains the *EcoRI* restriction site. For *IGF-I* genomic DNA cloning, six primers were designed to amplify the coding region, namely IGF-I exon primers 1 through 6. As for first-strand

TABLE 1. PRIMERS USED

Name	Sequence ^a
IGF-I primer1	5'-CGGAATTCATGGAACCCAGCAAAGATAC
IGF-I primer2	5'-CGGAATTCATTTTGTGACTGACAAAGTTG
IGF-I exon primer1	5'-CGAAGCTTAGATCMAAATTCRAKCCYATTC
IGF-I exon primer2	5'-GTCTCGAGTTGTTTTACAGTGAACCATTCC
IGF-I exon primer3	5'-CGGGATCCGAAATAAAAGCCTCGCTCTCCACAGA
IGF-I exon primer4	5'-CGGGATCCAATAAACCCACAGGCTATGGCCCCAGT
IGF-I exon primer5	5'-CGGAATTCGGCCCTGAGACCCTGTGCGGG
IGF-I exon primer6	5'-CGGAATTCAGAAATCTTGGGAGTCTTGAC
IGF-I GSP1	5'-CGCTCGAGCGCCCCTGTTGCCGTCGGAG
IGF-II GSP1	5'-GGTTGGCCTACTGAAATAAAA
IGF-I P2 primer	5'-CGAAGCTTATTTTTACATTTTCACTC
IGF-I P3 primer	5'-CGAAGCTTCGGTGCCAAATTACGCAC
IGF-I P4 primer	5'-CGCTGCAGGACATTTTCAACAGGAAA
IGF-I P5 primer	5'-CGAAGCTTGCTGGTGGTGGTGGTGGGGCTG
IGF-I P6 primer	5'-CGCTGCAGGACTTTGTTCCATTGCGC
IGF-I P7 primer	5'-CCCTGCAGTTCCAACAAGACAAAGC
IGF-II P3 primer	5'-GGAAGCTTCATGCCTGCATCTAC
IGF-II P4 primer	5'-GGAAGCTTCTGCATCAACCCCG
IGF-II P5 primer	5'-GGAAGCTTAGTGATTCCACTG
IGF-II P6 primer	5'-GGAAGCTTCATTTTTTCTTGGAGTC
IGF-II P7 primer	5'-CGCTGCAGCCTGTGTGTAACATTGTGA
IGF-II P8 primer	5'-GGAAGCTTTGCCCATGGAAGTGTTTC
IGF-II P9 primer	5'-CGCTGCAGTTCAAGCACTTTCATAAAAC
IGF-II P11 primer	5'-GCGTCGACGTCAGATGGCAGTAGTCAAAATG
GFP1	5'-TTCACCGGGGTGGTGGCCATCCTGGTC
GFP2	5'-TTGAAGAAGATGGTGGCTCCTGGACGTAGC

^aW = A or T; Y = C or T; K = G or T; S = C or G; M = A or C; R = A or G.

cDNA synthesis for rapid amplification of 5' cDNA ends, two primers were designed, namely, IGF-I GSP1 and IGF-II GSP1. For IGF-I promoter assay, six primers were designed to amplify the promoter region, namely, IGF-I P2 through IGF-I P7. For IGF-II promoter assay, seven primers were designed to amplify the IGF-II promoter region, namely IGF-II P3 through IGF-II P9 and IGF-II P11. For checking GFP expression, two primers were designed to amplify that coding region, namely GFP 1 and GFP 2. The sequences of all primers are shown in Table 1; they were synthesized by Quality Systems, Inc., Taiwan.

Isolation of total RNA

Tilapia (*Oreochromis mossambicus*) were obtained from the Tainan Fishery Institute and maintained in plastic tanks 2 m in diameter under constant photoperiod (13 h light:11 h dark) at 23°C. In order to obtain high levels of IGF-I RNA, the fish were injected at 8 h intervals with yellowfin porgy (*Acanthopagrus latus*) GH (Tsai *et al.*, 1993) at a dose of 1 µg/g of body weight for three doses. Total liver RNA was prepared by the guanidinium-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987).

Reverse transcription and PCR assay

First-strand cDNA was generated from 30 µg of total RNA in a 37 µl reaction volume. The IGF-I primer 2 was then added,

incubated at 80°C for 10 min, and allowed to cool down slowly to room temperature in order to anneal the IGF-I primers to the corresponding cDNA. This sample was mixed with 5 µl of 10 × first-strand buffer (Stratagene), 2 µl of 100 mM dNTPs (Stratagene), and 2 µl of SuperScript™ II RNase H⁻ reverse transcriptase (200 U/µl; GIBCO BRL). Next, the cDNA synthesis mixture was incubated at 42°C for 60 min and then at 95°C for 5 min. After incubation, the reaction vessel was immediately placed on ice. The PCR reaction was carried out in a final volume of 100 µl. The reaction was constituted by the first-strand cDNA, 10 µl of 10 × PCR buffer (HT Biotechnology), 200 µM of each dNTP, 1 µg of IGF-I primer 1 and primer 2, and 2.5 units of *Taq* DNA polymerase. The reaction mixture was subjected to 35 PCR cycles of 1 min at 94°C (denaturation), 2 min at 42°C (annealing), and 2 min at 72°C (extension); the PCR products were then resolved on 2% agarose gel.

PCR product cloning, sequencing, and analysis

The PCR products were size-separated by gel electrophoresis and digested with *Eco*RI. The digested product of the predicted size (549 bp) was then subcloned into the pUC18 plasmid using 5 U of *T4* DNA ligase (BM). The recombinant plasmid was transformed into the *E. coli* JM109 strain. The nucleotide sequences of each insert were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1992) using forward and reverse primers. The comparison of

the predicted *IGF-I* nucleic acid sequences and predicted amino acid sequences was performed using the Genetics Computer Group Gap program. To compare and align these sequences with those which have been published, we introduced gaps to maximize identity via procedures described by the Gap program (Higgins and Sharp, 1988) and Molecular Evolutionary Genetics Analysis (Kumar *et al.*, 1993).

Isolation and Characterization of *Tilapia* IGFs Genes

Approximately 1 million recombinant bacteriophages from the tilapia genomic DNA library were screened with ³²P-labeled *IGF-I* 549-bp PCR fragments. This DNA library was constructed in a phage charon 40 vector. The hybridization buffer used was 50% formamide containing SDS (7 g/100 ml), 0.5 M EDTA (pH 8.0, 100 μ l/100 ml), 50% PEG8000 (20 ml/100 ml), and formamide (50 ml/100 ml) at 42°C for 16 h; after hybridization, filters were washed in four times with 0.1 $\times$ SSC and 0.1% SDS at 65°C. A total of 18 positive plaques were obtained. Exon primers 1 and 3 were used for PCR to amplify *IGF-I* exons 1 and 2; exon primers 2 and 4 were used to amplify *IGF-I* exons 3 and 4; and exon primers 5 and 6 were used to amplify the *IGF-I* intron between exons 2 and 3. The PCR

was performed with 10- μ l positive phage plaque solutions containing 10 μ l of 10 $\times$ extension buffer, 8 μ l of 2.5 mM dNTP, 1 μ g of each primer, ddH₂O, and 2.5 units of *Taq* polymerase (TakaRa LA Taq™, Japan). The PCR was performed in a thermal cycler (Perkin Elmer-Cetus 2400) for 35 cycles with denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 5 min. Ten microliters of PCR products was analyzed by electrophoresis on 0.9% agarose gels. After ethidium bromide staining, DNA fragments of the predicted size (exon primers 1 and 3 amplified about 1.9 kb, exon primers 2 and 4 about 3.2 kb, and exon primers 5 and 6 about 11 kb) were excised from the agarose gel, and the QIAquick gel extraction kit (QIAGEN GmbH and QIAGEN Inc.) was used to extract the DNA. The PCR products were sequenced by an ABI autosequencer. The nucleic acid sequences were compared with the Genetics Computer Group Gap computer program. In another way of doing this, we isolated one of the *IGF-I* genomic clones, mapped by restriction endonuclease, and subcloned the DNA fragments. These clones contain the 1.3 kb of nucleotides encoding the promoter region.

For *IGF-II* gene structure and promoter region determination, we continued to sequence the recombinant bacteriophage

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      *
1  atgtctagcgctcttttcctttcagtggcatttatgtgatgtcttcaagagtgcgatgtgc
1  M S S A L S F Q W H L C D V F K S A M C

61  tgtatctcctgtagccacaccctctcactactgctgtgcgtcctcaccctgactccgacg
21  C I S C S H T L S L L L C V L T L T P T

121  gcaacagggggcgccctgagaccctgtgcggggcgagctggtcgacacgctgcagttt
41  A T G A G P E T L C G A E L V D T L Q F

181  gtctgtggagagcgaggcttttatattcaataaaccaacaggctatggccccagtgaagg
61  V C G E R G F Y F N K P T G Y G P S A R

241  cggtcacgtggcatcgtggacgagtgctgcttccaaagctgtgagctgcagcgccttgag
81  R S R G I V D E C C F Q S C E L Q R L E

301  atgtactgtgcacctgtcaagactcccaagatttctcgctctgtgcgttcacagcggcac
101  M Y C A P V K T P K I S R S V R S Q R H

361  acagacatgccaaagagcaccgaaggtagtagcagagcgaacaaggggacagagcgtagg
121  T D M P R A P K V S S R A N K G T E R R

421  acagcgcctcagccagacaagacaaaaaacaagaagagaccttcacctggacatagttca
141  T A P Q P D K T K N K K R P S P G H S S

481  tccttcaaggaagtccaccagaaaaaactcaagtcgaggaagttcggggggcagaaactac
161  S F K E V H Q K N S S R G S S G G R N Y

      #
541  agaatgtag
181  R M

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FIG. 1. Nucleotide sequence of *IGF-I* cDNA isolated from *Oreochromis mossambicus*. Predicted amino acid sequence is shown below. Numbers on second line of each row represent amino acid position. Tilapia sequence contains a signal peptide (132 nt; 44 amino acids, 1 to 44), B domain (87 nt; 29 amino acids, 45 to 73), C domain (27 nt; 9 amino acids, 74 to 82), A domain (66 nt; 22 amino acids, 83 to 105), D domain (24 nt; 8 amino acids, 106 to 113), and E domain (210 nt; 70 amino acids, 114 to 183). Tilapia *IGF-I* cDNA insert contains 549 nt, which encode 183 amino acid residues. * = codon; # = stop codon.

clone (Chen *et al.*, 1997), subcloning the DNA fragments and sequencing the upstream ATG (start codon) of approximately 5.7 kb and the downstream TGA (stop codon) of approximately 3.4 kb.

RNA isolation and rapid amplification of 5' cDNA ends (5'RACE)

Total RNA was isolated from tilapia liver. The RNA extraction method followed the manufacturer's protocols (ULTRASPEC™-II RNA isolation system; Biotecx Laboratories, Inc., U.S.A.). The 5'RACE procedure (Peduel *et al.*, 1994) strictly followed the instruction manual (Life Technologies, U.S.A.) and Frohman (1990, 1993). Briefly, the sample contained about 5 µg of total RNA, and either the IGF-I GSP1 or the IGF-II GSP1 primer. After PCR, the 5'RACE products were analyzed on 1.5% agarose gel and transferred to nylon membranes (BioRad). The membranes were probed separately with [³²P] dCTP-radiolabeled IGF-I P2 → P7 genomic DNA fragments and IGF-II P3 → P11 genomic DNA fragments. The hybridization procedure was the same as that described above for the isolation and characterization of the tilapia IGFs gene. Af-

ter verifying which fragments hybridized with the probe, restriction endonuclease-digested 5'RACE products were cloned into the pBluescript plasmid vector (Stratagene) and sequenced to confirm the genomic sequence.

IGF promoter plasmid constructs, cell culture, and transient transfection assays

An approximately 2 kb *SacI*-*SacI* fragment was obtained from an *Oreochromis mossambicus* IGF-I genomic clone. This fragment includes 1360 bp of the 5' flanking region and the transcription start site sequence. The promoter activity assay of fragments was amplified by PCR (The primer location is referenced below in Fig. 3 and Table 1). The PCR product was digested with *PstI* and *HindIII* restriction endonucleases, and the fragment was ligated into the *HindIII* and *PstI* sites of the pCAT-Basic vector (Promega). The pCAT-Basic vector is a promoterless plasmid containing multiple cloning sites upstream of a CAT cDNA. This resulted in the IGF-I promoter activity assay obtaining seven construct plasmids (see Fig. 6). The clones of IGF-I P2 → P7, IGF-I P3 → P6, IGF-I P3 → P7, IGF-I P5 → P6, and IGF-I P5 → P7 were constructed and con-

Signal peptide

tilapia	MSSALSFQWHLCDVFKSA.MCCISCSHTLSLLL CVLTLTPTATGA
Sparus aurata	MSSALSFQWHLCDVFKSA.MCCISCSHTLSLLL CVLTLTPTATGA
carp	MTSNKFI FAGLLLETQGMSSGHFFQGHCCDVFKCT.MRCLSCHTLSLVLCVLTALPTALEA
rainbow trout	MSSGHFFQWHLCDVFKSA.MCCVSCHTLSLLL CVLTLTSAATGA
salmon	MSSGHLFQWHLCDVFKSA.MCCISCSHTLSLLL CVLTLTSAATGA
hagfish	YIRRVROGSIYSLLVESQQWCKLTLTLLLLLALLTRCTL..
human	MITPTVKMHTMSSSHLFYALCLLTFTSSAT.A
rat	MSSSHLFYALCLLTFTSSAT.A
pig	MHITSSSHLFYALCLLTFTSSAT.A
sheep	MGKISSLPQQLFKCCFCDFLKQVKMPVTSSSHLFYALCLLAFSSAT.A
chicken	MEKINSLSLQVLCDFKFLK.VKMHTVSYIHFFYLGLCLLTSSAA.A
xenopus	METNNLSLQVLCDFKFLK.LKMHKMSCIHLLYLVLCLTLTHSAA.A

	B domain	C domain	A domain	D domain
ancestral fish	GPETLCGAELVDTLQFVCGERGFYFSK???PT	GYGPSSRRSHNR	GIVDECCFQSCELRRLEMYCA	PVK?GKAA
tilapia	GPETLCGAELVDTLQFVCGERGFYFNK...PT	GYGPSARRS...R	GIVDECCFQSCELRRLEMYCA	PVKTPKIS
Sparus aurata	SPETLCGAELVDTLQFVCGERGFYFSK...P	GYGPNARRS...R	GIVDECCFQSCELRRLEMYCA	PAKTSKAA
carp	GPETLCGAELVDTLQFVCGDRGFYFSK...PT	GYGPSSRRSHNR	GIVDECCFQSCELRRLEMYCA	PVKPGKTP
rainbow trout	GPETLCGAELVDTLQFVCGERGFYFSK...PT	GYGPSSRRSHNR	GIVDECCFQSCELRRLEMYCA	PVKSQKAA
salmon	GPETLCGAELVDTLQFVCGERGFYFSK...PT	GYGPSSRRSHNR	GIVDECCFQSCELRRLEMYCA	PVKSQKAA
catfish	GPETLCGAELVDTLQFVCGERRVHFSK...PT	GYGPSSRRSHNR	GIVDECCFQSCELRRLEMYCA	PVKSQKAA
hagfish	GSETLCGSELVDTLQFVCDRGGFFVPOHVPP	RRGAHRRSRARK	GIVECCFKGCSLRLEMYCA	RPSKAE
human	GPETLCGAELVDALQFVCGDRGFYFNK...PT	GYGSSRRAPQT	GIVDECCFRSCDLRRLEMYCA	PLKPAKSA
rat	GPETLCGAELVDALQFVCGPRGFYFNK...PT	GYGSSIRRAPQT	GIVDECCFRSCDLRRLEMYCV	RCKPTKSA
pig	GPETLCGAELVDALQFVCGDRGFYFNK...PT	GYGSSRRAPQT	GIVDECCFRSCDLRRLEMYCA	PLKPAKSA
sheep	GPETLCGAELVDALQFVCGDRGFYFNK...PT	GYGSSRRAPQT	GIVDECCFRSCDLRRLEMYCA	PLKAAKSA
chicken	GPETLCGAELVDALQFVCGDRGFYFSK...PT	GYGSSRRLLHK	GIVDECCFQSCDLRRLEMYCA	PIKPPKSA
xenopus	GPETLCGAELVDTLQFVCGDRGFYFSK...PT	GYGSNNRRSHNR	GIVDECCFQSCDFRREMYCA	PAKPAKSA
ancestral vertebrate	GPETLCGAELVDTLQFVCGDRGFYFSK???PT	GYGSSRRSH?R	GIVDECCFQSCDLRRLEMYCA	PVKP?KSA

FIG. 2. Comparison of amino acid sequences of IGF-I in tilapia, *Sparus aurata*, carp, salmon, rainbow trout, catfish, hagfish, human, rat, pig, sheep, chicken, and Xenopus. Gaps (...) were introduced to maximize potential homologies. Hypothetical ancestral piscine IGF-I sequence is shown above; hypothetical ancestral vertebrate IGF-I sequence is shown below. Mature peptides largely conserved in all fish are underlined. There are four types of amino acid sequences in IGF-I E domain; herein, we do not compare them because of the complexity of this region. Amino acid sequences of F-Y-F are putative receptor binding site in B domain.

tain two transcription start sites. The clones IGF-I P3 → P4 and IGF-I P5 → P4 lack one transcription start site, that connected immediately upstream of the ATG codon.

Fragments of the *IGF-II* gene containing the promoter region were obtained from the *Oreochromis mossambicus* genomic clone. The *IGF-I* and *IGF-II* transcription start site was dependent on the 5'RACE result and assigned at -1 to be near the first nucleotide upstream of the ATG codon, with the definition of the -1 number according to Koval *et al.* (1994). The deletion series of the *IGF-II* promoter was constructed by PCR amplification using oligonucleotides designed to generate ends with *Pst*I-*Sal*I and *Hind*III-*Sal*I restriction sites. This resulted in the *IGF-II* promoter activity assay obtaining eight construct plasmids (see Fig. 6). The PCR products of IGF-II P3 → P11, IGF-II P4 → P11, IGF-II P5 → P11, IGF-II P6 → P11, IGF-II P7 → P11, IGF-II P9 → P11, IGF-II P7 ← P11, and IGF-II P8 ← P11 were then cloned into the pCAT-Basic vector, after which the pCAT-Basic vector was digested with the same enzymes. Next, the QIAGEN plasmid extraction maxi kit was used to extract DNA, and one strand was processed by an ABI autosequencer to identify the orientation and sequence.

Human lung large cell carcinoma cells (ATCC NCI H1299) were grown in DMEM (glucose 4.5 g/l) containing 10% newborn calf serum. Approximately 6×10^5 cells were seeded 12 h before transfection. Calcium phosphate-mediated DNA transfection was as described previously (Tsai *et al.*, 1996). Typically, the transfection lasted 12 h. The CAT activity was measured 48 h after transfection and quantified as described previously (Tsai *et al.*, 1996). Plasmids of pEGFP-1 ligated with a *IGFs* promoter region were transfected into the same cells. The procedure is described below, and the transfection method was the same as that used for the CAT assay.

Reporter gene constructs, preparation of plasmid DNA, fish egg collection, and microinjection

The vector of pEGFP-1 (CLONTECH Laboratories, Inc.; the pEGFP-1 vector sequence can be found in GenBank accession number U55761) was constructed by inserting IGF-I P2 → P7 as a *Hind*III-*Pst*I site from the PCR fragment; the insertion of the IGF-II P5 → P11 PCR fragment was conducted with the

pEGFP-1 vector as a *Hind*III-*Sal*I site. Plasmid DNA was extracted according to the QIAGEN handbook for the plasmid maxi kit (QIAGEN GmbH and QIAGEN Inc.). After purification, the plasmids were precipitated using ethanol and sodium acetate. The DNA pellet was resuspended in ddH₂O and stored at -20°C.

The experimental specimens of *Brachydanio rerio* (zebrafish) were maintained under standard conditions with an artificial photoperiod of 11 h of darkness to 13 h of light to induce and maintain the reproductive cycle. Fertilized eggs were collected, and one of the two cells in the embryo was intracytoplasmically with an appropriate amount of supercoiled plasmid DNA 100 µg/ml. The injection method and GFP assay procedure are described in Winkler *et al.* (1991) and Amsterdam *et al.* (1995, 1996).

Fluorescence microscopy observation and RT-PCR

After injection of supercoiled plasmid DNA (the construction plasmid of pEGFP-1 vector ligated with the *IGF-I* or *IGF-II* promoter region) into the zebrafish embryos, eggs were kept in a 28°C incubator. Embryos were observed and photographed on an Olympus microscope IX70 and Leica MPS 48/52 dissection microscope with a fluorescence filter using a long-wave UV lamp.

The zebrafish embryo RNA extraction method was according to the manufacturer's protocols (ULTRASPEC™-II RNA isolation system; Biotecx). For *IGFs* promoter assay, 10 one-wk-old fish (for *IGF-I* promoter assay) and 10 two-wk-old fish (for *IGF-II* promoter assay) were collected, and their respective extractions were pooled. These embryos and fish were all injected with the construction of pEGFP-1 vector ligated with the *IGF-I* or *IGF-II* promoter region as described above. The concentration of total RNA was measured by an ultraviolet spectrophotometer at an absorbance of 260 nm.

In order to investigate whether the expression of *IGFs* promoters is stage or tissue specific, 1.6 µg of whole embryo or fish total RNA and the GFP 1 and GFP 2 primers were put together in one-tube mixtures and used to amplify internal fragments of GFP first-strand cDNA. The PCR was run continuously to amplify about 317 bp of the GFP coding region according to the manufacturer's instructions (AMPLY RT/PCR

TABLE 2. HOMOLOGY OF INSULIN-LIKE GROWTH FACTOR (IGF) POLYPEPTIDE^a

Insulin-Like Growth Facator	Tilapia IGF-I Mature Peptide Identity (%)	References
Tilapia IGF-II	59.1	Chen <i>et al.</i> , 1997
Catfish IGF-I	83.8	McRory and Sherwood 1994
Hagfish IGF-I	59.7	Nagamasu <i>et al.</i> , 1991
Human IGF-I	79.4	Rindernecht and Humbel, 1978; Klapper <i>et al.</i> , 1983
Mouse IGF-I	79.4	Bell <i>et al.</i> , 1986
Pig IGF-I	79.4	Tavakkol <i>et al.</i> , 1988; Muller and Brem, 1990
Rainbow trout IGF-I	86.8	Shamblott and Chen, 1992
Rat IGF-I	79.4	Roberts <i>et al.</i> , 1987
Salmon IGF-I	89.7	Cao <i>et al.</i> , 1989; Duguay <i>et al.</i> , 1992
Sheep IGF-I	79.4	Wong <i>et al.</i> , 1989

^aComparison of predicted IGF-I amino acid sequence was performed using Genetics Computer Group program.

Tilapia IGF-I Exon 1 and promoter region

-1213 GAGCTCCTGTATCGGAAGCGTGTGTCAGTGTCTGCTGCTGTGACATTTGA
 -1163 TGATGATCGCGTTTTGACCCACTTTACCCAAACAACCTCTAAGTGTGCGT
 -1113 GTCTTAAGTCCCTTTTGAACCAACCTTGTCTGTATGCACACACAAAA
 -1063 CACGGACGGAGCATGCCTGGATACGTGCCGGTGTCTGTTTTATGATTGG
 -1013 GTCACAGCACTGATCTCATGTTTAGTACTGGATCAGTGTGATATCCCA
 -963 TCACGTCTGAGAACAACCTCTTTAAGCCCTTGTCTTCCGTCCTTCTGT
 -913 GACACAGTGGGAACGTGGGGGCCCGACTTCAACAGATGACAGTGGGTG
 -863 GGCCTGCATCAAGAGTCTGTCAAGTCAAAAACAATGGCTCCAGGAATTGAA
 -813 ATTAATGTCTCTCCATCATAAAACCCATGTGTTTTCTTTTTTCTCTC
 -763 CTTTTTTTATTTTTTCCACTCTAGTTTACAGGNGCATCAGATGTCATTAC
 -713 AGCGGAACCCACTTCTATTAAGTGAATAACAGCAAGAAGGAGAGAAAAAC
 -663 CAGAGACATACACAGGNTTATGACAGTGCAGTGTGCAGAAAGGACAGCAA
 -613 ACCCTGCACAGTGGCTCTCAGTTTAGAGGGAACAGGGCTTCACATGTGGC
 -563 TTCTGGCTCAAGTTTGTCTCTTACTGTAAATAAAATGTCACATTTTTAC
 -513 ATTTTCACTCCAGTTCACATTTATTTAGCTTTTCTTGTGAGGTTTTCAG
 P2 Primer
 -463 GTGAGGAGCACTGCGGCTCTAAGTGGGAAAGTGTTTTTAATGATTTATT
 -413 TAATCAAACATTAGTGGAGAGACTTGGTGGTCAGATACACGGTGCCAAA
 -363 TTACGCAACACAGCAAAGGCCATATCACACCGCCAGCTCGTCTTTAAC
 P3 Primer
 -313 TTCTCCTTTATGAGCGTTTCAGGGCAGAAAATAATGCGTTTGTAAAGATG
 -263 TGAGCCGCCACTGCTGCTGCTGGTGGTGGTGGGGCTGATCTGAACTA
 P5 primer
 -213 CTAACCAAGTCTTAGAGAGATCAAAATTAATCCCGTTCGAGAAAAAAC
 -163 AGAAATGACGT TATTTGAATATGTGCCAAAATCCTTAATGAATAACTTA
 HNF 5-like HNF 1-like
 5'RACE
 -113 GGACGAGTAGGAGGCAATAGCTGCCCGAGCTTTTCTCTGTGAAAATGTC
 P4 Primer
 -63 TGTGTAATGTAGATAAATGTGAGGGATTTTCTCTCTAAATCCGCTCTCTG
 5'RACE
 -13 TTCGCTAAATCTC|ACTTCTCCAAAACGAGCCTGCGCAATGGAACAAAGTC
 P6 Primer
 38 GGAATATTGAGATGTGACATTGCCCGCATCTCATCTCTTCTCCCTGTT
 88 TTTAATGACTTTAAACAAGTTCATTTTGTGCGGCTTTGTCTTGTGGAA
 * P7 Primer
 138 AACCGTGGGATGTCTAGCGCTTTTTCCTTTCAGTGGCATTTATGTGATG
 188 TCTTCAAGtaacttacctgatttcctttgacacatacattatcacctt
 238 gattcttcacttgcctactatttgcacagagcatcctcgctactttaaa
 288 aagaacaataaaaggggattcttatcgcttgccttgatttcacagcttt

338 tgaacgcatgcattgggtgaggatttattcacatTTTTTctcttttcatcc
 388 acttatggaacaactctcccgcatgcttggaatctatttttttatt
 438 ttattttccagagataattatattttctacattttgctttaattgattct
 488 tttctcttttttgcaattaaaaaaaatcattgacatggatcgtgcatga
 538 ctttcagcgcaggatgaatttggacattttccgtgcgcccggcgctctt
 588 tttccaccagaaagccctaattgcagctgtgcaactttccaaagtaattac
 638 tgcctgagctgcataatttaagtgtgtaataatctaacctgatgcctctgg
 688 cgggggttgaaagaacgagagcgggagagagagagatttccgaacacttg
 738 cggcgctcgggaacttgcgggttggaagttaataagataacgtcccggtt
 788 t

Tilapia IGF-I Exon 2

1 gttaaagaaactcttttccgatgatgctgtgaaatgttcttgcctc
 51 tccccgttgccagAGTGGCATGTGTGTATCTCCTGTAGCCACACCCCTC
 101 TCACTACTGCTGTGCGTCTCACCTGACTCCGACGGCAACAGGGCGGG
 151 CCCTGAGACCCCTGTGCGGGCGGAGCTGGTCGACACGCTGCAGTTGTCT
 201 GTGAGAGCGAGGCTTTTATTC.....

Tilapia IGF-I Exon 3 and Exon 4

1 AATAAACCAACAGGCTATGGCCCCAGTGCAAGGCGGTCACTGGCATCGT
 51 GGACGAGTGTGCTTCCAAAGCTGTGAGCTGCAGCGCCTTGAGATGTACT
 101 GTGCACCTGTCAAGACTCCCAAGATTTCTCGCTCTGTGCTTCACAGCGG
 151 CACACAGACATGCCAAGAGCACCCAGGTTAGTAGCAGAGCGAACAAGGG
 201 GACAGAGCGTAGGACAGCGCCTCAGCCAGACAAGCAAAAAACAAGAGg
 251 ttgagctaagcatcaaacacagataaactgtaaaaattgttgattggatcg
 301 aatttacatttctgttacatgatcagagcatacgtgcataatatcctgat
 351 taattaaggtagatagcttaataagcagaaactcgtccatccaactaaa
 401 gttacctaatTTTTcttccacagAGACCTTCACTGGACATAGTTTCATCC
 451 TTCAAGgtatgcatgcaaaatgatctcatgttcatgttcttttgcatt
 501 taagctttacacaaacacacaatctctcccatgacataagagtttcaag
 551 caggcgatctttacagaa

Tilapia IGF-I Exon 5

1 tctatatctctgctgtacatctaaaaaaaaaaaaaaaaaaaaaacaatgt
 51 gttttattttgtttgcagGAAGTCCACCAGAAAACTCAAGTCGAGGAAG
 #
 101 TTGGGGGGCAGAACTACAGAAATGTAGGGAAGGAACAAATGGACAAATG
 151 CCCAGCAATTGGGAGGAGAGAAGGAGTGCCTTACCTGGTNACCTGTG
 201 GAATGGTTCAGTGTAAACAA

FIG. 3. Partial nucleotide sequence of *IGF-I* genomic DNA isolated from *Oreochromis mossambicus*. Tilapia *IGF-I* exon sequences and 5' flanking sequences are shown in uppercase letters; intronic sequences are shown in lowercase letters. # = stop codon; * = start codon. Numbered arrows under sequence indicate positions for CAT assay of PCR-constructed primer. Regions of 5'RACE termini are marked by arrows above sequence and labeled 5'RACE.

Kit; LTK BioLaboratories, R.O.C.). The amplification program was established for a Perkin-Elmer 2400 thermocycler and was as follows: 1 cycle of 5 min at 60°C, 30 min at 42°C, 35 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C followed by a final extension of 7 min at 72°C then immediate storage at 4°C. Each RT-PCR included control groups: one tube contained only GFP 1 and GFP 2 primers, and another tube

contained only embryos or fish total RNA mixtures. The PCR amplification products (5 µl of 20 µl) were analyzed on 1.5% agarose gel and transferred to nylon membranes (BioRad). The membranes were probed with [³²P] dCTP-radiolabeled GFP cDNA fragments. The hybridization procedure was the same as that used for isolation and characterization of tilapia *IGFs* genes described above.

-377	CCCACTGCCTGCATCTACACAGTCTGAGGAAAGCCGAAACCTTGGCCAC	374	ttttgatttgtatgcagcagagaaaattcaagatgcataaacggcgagga
-327	AGTGATTCCACTGAATGTTAATCTTAATTACAGCAGGCGGAGACATTGA	424	gaagcctttgcggcatttgggatagaaaaagaacaagtaatttttagtcttc
	P5 Primer	474	tcgcgccttggatgggtgtcgtggacacggagcaaacagctgattttttacc
-277	CATTTTCTTGGAGTGAAGGAATTACCCCTATTCTTGTTCAGTCTCGCT	524	ggcagatagggggcgagggtttgagggtcagtagctcattccgggggtg
	P6 Primer	574	agcaaaagggaataatatactgctgagtttgggtgttttcgctagttaactat
-227	CCTGTGTGTAACATTTGTGATTGTAGGTTACAGTGCCTAACGTGGAAAA	624	cgagcagccggccttgcaacttcaggatgggtgtgtaagagtggaagct
	P7 Primer	674	ggtgatactggcgacttgtgttttttccccaataagcacttcttca
-177	TGCCCATGGAAGTGTTCATATTTTGTGACTCTCACCCCTCTTATTCTCCC	724	ggtgcgacagacatgacagcagctgataatagcggcgagggaagtgaaag
	P8 Primer	774	agcgggtctgcacattgtgacttgatttgagatgattttcttttctgaa
-127	TTCAAGCACTTTCTATAAAACGTCTCTGGGCCCTTTTTTTTTTCATGGGCGA	824	tttctactttgtcgaacatttgagcagctacaccgtacacaccgggagcc
	P9 Primer	874	tgtgatgaagactgtaccctcttcgtctgaaaaaaaaaaaaaaaaaactctg
-77	AGAGGAGGAGCAAGGGGTGGGTGGGTGTAAGGGGCGTCTTTAGTATAT	924	gctgatttttgattaaaaaaatggatttaactgtcatlaactgttattttt
		974	gttaacgatttctgtatgccacaactttcgcataatcatggctacatttg
-27	AATACCTCTCCCTGAGAAGTTTTCCTTGTGCTAGTCTTTGGGACAGCT	1024	gtgacccccatgcttcataccgcagGTCAAGAAGATGTCTTCCACGAATC
24	TCTCACTCAACATCTCTATAGTTTAACCAACTGGGAACTAACTCACTG	1074	CCGCGCTGCTCTTTGCACTGGCCCTGACGCTCTACCTAATGGAAATGGCC
74	CAATCACCAACCAAAATATCCCAACATTTTGACTACTGCCACTCTGAC	1124	TCCGCGGAAACCCCTGTTTGGGGGAAAACTGGTGGATGCGCTGCAATTTGT
	*	1174	CTGTGAAGACAGAGAAGCTTTTATTTCAGtaagttcaaaagcattacnagtt
		1224	tcccaaatggctgctgattgtcatttgcctgttgaatctctctgttgt
124	ATGGAAACCCAGCAAGATACGGACATCACTACTTTGCCACACCTGCCG	1274	gcccttgacacatctgtttggagcaaaagtgggaagattaccactacna
174	GAGAACGCAGAACAGCAGATGGAAGtaaccaagaacaagcaaatgtt	1324	atacttcgttactgtactccagtagattttcagtagaatttttggccc
224	ttatactctccgggtctgcgtgctgtaataaagacgtctgacagggt	1374	ctacatttttaacagatatctgtactttctactcc
274	tatagcgggtgtgtcttgggtttataagtaggaaaaccagttcgggttct		
324	gtagatgcagtgctgcacatcagttttgtgcagtatgagccccgggacatc		

FIG. 4. Continued

RESULTS

RT-PCR cloning and characterization of tilapia IGF-I cDNA

To isolate the *IGF-I* coding region from tilapia as quickly as possible, our strategy included injecting seabream GH and comparing multiple piscine *IGF-I* sequences (salmon, rainbow trout, seabream, hagfish, and catfish); after this, two primers were designed. For simplicity, an *EcoRI* restriction enzyme site was designed to clone the *IGF-I* RT-PCR product. A total of 20 *E. coli* clones were selected and sequenced, and three clones were identified as tilapia *IGF-I* cDNA sequences. The nucleotide sequence and predicted amino acid sequences of tilapia *IGF-I* are described in Figure 1. The tilapia *IGF-I* cDNA insert contains 549 nt, which encode 183 amino acid residues. Herein, we identify a tilapia *IGF-I* cDNA sequence containing a signal peptide (132 nt; 44 amino acids), B domain (87 nt; 29 amino acids), C domain (27 nt; 9 amino acids), A domain (66 nt; 22 amino acids), D domain (24 nt; 8 amino acids), and E domain (210 nt; 70 amino acids). The tilapia *IGF-I* nucleotide and amino acid sequences were compared with those of rainbow trout *IGF-I* and possessed 86.6% and 84.9% homology, respectively. The tilapia *IGF-I* nucleotide and amino acid sequences were compared with those of salmon *IGF-I* and possessed 86.8% and 85.4% homology, respectively. Comparison of the amino acid sequences for fish in Figure 2 and Table 2 shows high evolutionary conservation. The homology between IGF-Is indicates that amino acid substitutions are relatively few, and the IGF-I mature peptide sequence is highly conserved (Fig. 2). The mature insulin and IGF peptides are highly conserved among vertebrate species; it can be inferred that IGF-I-like molecules were found in

species with origins from 550 million years ago (LeRoith *et al.*, 1993), which is near the beginning of the Cambrian Era between the Paleozoic and Proterozoic.

Characterization of tilapia *IGF-I* gene structure

In tilapia *IGF-I*, the first exon encodes a partial signal peptide and is highly conserved in the 5' untranslated region. The B, C, A, and D domains of the mature peptide are encoded by exons 2 and 3. The first E domain of 40 amino acids is encoded by exon 3. The second E domain of 11 amino acids is encoded by mini-exon 4. The last E domain and part of the 3' untranslated region are encoded by exon 5.

The partial nucleotide sequence of the *Oreochromis mossambicus IGF-I* gene is shown in Figure 3. The gene contains five exons, and the exon structure is like that of chum salmon. All five exons and part of the introns were sequenced, and the genomic DNA sequence was compared with the *Oreochromis mossambicus IGF-I* cDNA sequence. From exon 2 to exon 3, the junction of the intron could not be amplified by PCR. So, unlike chum salmon, it could not be determined whether the tilapia sequence of exon 2 to exon 3 is divided into about a 10-kb intron. We surmise that the separated intron may have the same structure and nucleotide length as that in the chum salmon gene. In tilapia, exon 1 to exon 2 is about 1.8 kb, and exons 3 to 5 are about 3.1 kb. Exon 1 is separated from exon 2 by a 1.2-kb intron. Exon 3 is separated from exon 4 by a 173-bp intron, a mini-intron. Exon 4 is separated from exon 5 by a 2.5-kb intron. In the chicken, human, rat, and salmon (Kajimoto and Rotwein, 1990; Kavsian *et al.*, 1993), there is a TATA-like consensus sequence of about 300 bp upstream of the first methionine, but no CCAAT-like or GAGA sequence was found.

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1   attgaacaatatnttataacntaatgaatgatccttcttttcccttttt
51  cttctatttttccgccacgccacaatagGAAGTTCAGAAGAAGCAACAT
101 GTGACCGTGAAGATTTCCAAATACGAGGTGTGGCAGAGGAAGGCGGCCA
151 GCGGCTCCGAGGGGTGTCCCCGCCATTCTGAGGCCAGAAAGTATAAGA
201 GGCACGCGGAGAAGATTAAAGCCAAGGAGCAGGCTATCTTCCACAGGCC
251 CTGATCAGCCTTCTAGCAAGCTGCCCTCCCGTGTGCTCACCACGGACAA
    #
301 CTTTGTCACTCACAAATGAGCCCGCTGCCAGCCCTTTGCACAGACAAGAG
351 TTTTGAAGGTGAAAAAAGACTAGGGGATTATAGCTTTTGGTCTTCTGACG
401 TCATTTCTGTGGCAGTCTCTTTGACCTCCCTGCCCTGTCCGAGCTCAC
451 CAATCCCTCCCTTGCACATATCCACTACGTCTTGAACCCCTGGCCCTTT
501 TCTAATGACCCNNTTAAACCCGAACCTCCCTCCCAACCAACCCACCT
551 CCTCTGGCACACAGACATGCCCTTCACATTCTTCTGTCTGAACCTTTCT
601 CTCCCACTCTCTTTCACTCACTGATACAAAAGGCACAAACAAAAACGTC
651 GAACAAAAAGTTAACAATTTGGCTGAATGCGGTTCAAGTGGATCCTTAAG
701 CAAAAGACAAAAGAGAAGGAAAAAGAGATGAAAGAGATCTGTGTTT
751 GCAAGTGTCAAGAGGACACCTAGCGGAATGTTTTTGTCTTGTGGAAGA
801 CAAGTGAAGTGAAGAGCTGTGCTGAAAGAAATCCATCCACCTCATT
851 TTCTGAGGCAAAAGAAATCTCTGTAGTCTTTAGTTTGCACCTCTAC
901 CTGTAATGGGACTTCCACACTGTAAGGAATTTTGTAAAATTAGATTCT
951 CTGTTCCAGCACCTTTTGATCACAACAAAAAGCAGAAAAGAGTCTGCAA
1001 AATTGCACATTGCCACGATTACGTCTTTGTAAGAAAAAATGGGCACTA
1051 TTTTTTTTGAACAATGAACGTGTAGCTTAAAAAATGTCATGGTGTAG
1101 CTTTGGGAATGGACTCAAAGAAGAGGTGAAAAAGCAGTTTTTTTTCTT
1151 TGAATTAATAATTAAGCTTTCCGTTTTTAAGGAAAGTGTGACTTTTTAAA
1201 AAAAGGAAAAATTTGGATATGGGGAGCTCAGCCGGGACCAGGCCCGGC
1251 AGCGNCAGGGGGGGGNCACCGGGGAAAAACCGGGCGCGATGGCACC
1301 TAGGCTCATGGTGGGAGCTAGCGGCTGCTATTTACTAGCTTGCCAGCATA
1351 AGCAGCAAGGGAGGCCCCAGGCGCTAGTCCCTGCTCCTCCTGCCCTCTG
1401 AGGCTGCTGGACACATGGAGCACTATGGGACACATACGGGACACCATGG
1451 ACCACCTGGATTGGGACAGTACTATAGTTCCGGGACAGTACTTCTGTTT
1501 GCCATGGCTTTGCGGACTGTTCTGGCAGGAAGTAACATGGCATGGACTAA
1551 GAACGAGTGGGATAATGATGATTATATTTATTTTTTGTCTTTTTTCATA
1601 TCAATGTTTCAATGAAAAAAGGAAAAACCTGTCAAGTTTCTGGTACCG
1651 TGACATTCTGTTTTTCCAAAGTTAGGCTTTTTTTTATGTTATGATGTTA
1701 TTTTACAGTTATAAAGAAAGGAAAAAACAGCAATAATGTCAACTAACAT
1751 TTTATTTTTATGTATCTCAGTAGTATACACATGCTTGGCGCGAACACA
1801 CATACGTGAATATATATATATATATATATATATATATATACACATG
1851 TACACATGTATATATATATATATATATATATATATATATATACACATG
1901 TACACATGTATATATATATATACACATATATATGTGCGCATATATATAGA
1951 TATATATATAGACTCATATATATATATATATATATATATATATATATAT
2001 TATATATATATATACACATATATATATATATATATATATATATATATATA
2051 TAGACATCCCCCATATATATATATATATATATATATATATATATATATAT
2101 ATATATACATATATATATATATATATATATATATATATATATATAGATTTC
2151 ATATATATATATATATATATATATATATATATATATATATATATATATAGAT
2201 ATACTATTAGTTTCCAGGACATTTGTGTTATTTTGTACTAGTCTGAGCT
2251 GTACCTTGCCTAATGAGCCGCAAGCTTTTGTACCTTGCCTAATGAGCCG
2301 CCAAGCTTTTTTGTTCGTTTGTGAAATACAAATAAGGGCACTGTATAAA
2351 GGCATTATTTATTTTGTATAAAAATATATTTGAAAAAATGGTCCAAATAA
2401 TATACGTAGCACTGATGCTCACTGCTAGATTTATTTTGTATGGTCTCTG
2451 TTTTCCAATTGGAGTTGTAAGGCTTTTTTTTGTAGATGCTTTGTACCAAA
2501 AATGTTGTTTTTTTCTGTTTCATTTGCCATAGTCTTTAAATTAATATT
2551 TTAACAGCAGCATAGCCTGGATTGCTTAGGCATGTTATGTCACAAAAATA
2601 GGCACATACTCCTAATCTGTGCGTCTGCATGGATTTCAGAGACCTGTCA
2651 GGGTGTCTGAGAAAATGAAAGCATCAATGAGTAACTTTTTCATTGCTGA
2701 AAAAAATGAGAAATAACTGTTTCAAGCATGGATATGCTCAAGCCCGCTA
2751 TGTTTTTTTAAGCCACTTTAGTATTGATGGCAGGGAGGGGAATGACGA
2801 GTCCTTCATTAAGTGTGCTTTTATAAGTGTGCATGGTGTCTATGATCT
2851 TCTAAGGAACATCTAATGCAGGTGAGTCCCACTGATACTCTTGGTGGGT
2901 CATGAGGCCATAAATGTCCTCACTGAGTGTGGAGTTGTGGTTTGCATTAC
2951 ACATGACCTAACCAGTCATCCTATTTCATGCCGACGTTGGTGTGATGCTA
3001 GCAAACATCAAGGTCATTTGAAAGTCACTGTCTGTCAAGAGAGACTGA
3051 CGATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCT
3101 ATCTATCTAAGTCTCTTTTGGTATAGCAGTGAAGTCTGTGTCATCATCA
3151 GCATATCTTGAGCTTGTGCATCATCAATATGCATCTGTATTTGCATTGGG
3201 TTTGGGGTTCGGCATTCATCCCTCGGAATCTTCCACTAATGCATCCATTA
3251 TGCATTTAATGAAGGGCTATCATGCTGAAGCATTTCTGAAGCGCTCAGGA
3301 AAAAAAATGTGCTCTCTAAGGAGAGTGAAGTCTGCTGAGTTTAGG
3351 AGATAATCTTGTGTGGAAGGTGAGCTGAGGTTGTTACCCATACCTGGCC
3401 ACAATAATATAGTTCTCTGTAAACAAACCACTGAAGCCCATGTGAGAAGCA
3451 AGGTCTATCTGTGCCACATGTGCAGTTTCTCCTCTGTTGTGTGTCATGC
3501 ATCACCCCATGAAACGGTGACTCAATGGATAGTTGCCATATTGTGTGTGTA
3551 GAGTCGAGAGAACACTTTGCTTTTTTCTGTGAGAGATCCGGGCCCGG
3601 CGCCC

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FIG. 5. Partial nucleotide sequence of last exon of tilapia *IGF-II* gene. There are putative polyadenylation signals at positions 1153–1158, 1160–1165, and 2541–2546, which are underlined. # = stop codon. PreproIGF-II stop codon (TGA) is underlined and occurs at position 317–319. A poly(AT) stretch is present between positions 1801 and 2201. Exon sequences and 3' flanking sequences are shown in uppercase letters; intronic sequences are shown in lowercase letters.

Examination of the 5' flanking region of tilapia showed several potential transcriptional factor-binding sequences, a TATA-like consensus sequence of about 300 bp upstream of the first methionine, one possible HNF-5-binding sequence, one possible HNF-1-binding sequence, and one possible AP-1-binding sequence.

Characterization of tilapia 5' and 3' flanking regions of *IGF-II* gene

Using data published before (Chen *et al.*, 1997), we continued to sequence the subclone of *IGF-II* genomic DNA. The entire DNA sequences are shown in Figures 4 and 5. The partial

nucleotide sequence of the *Oreochromis mossambicus IGF-II* gene is shown in Figure 4, which depicts the sequence of about 5.7 kb 5' upstream of the first methionine sequence containing the tilapia *IGF-II* promoter, as determined by the research reported in this paper. As in the human *IGF-II* P4 promoter sequence (Hyun *et al.*, 1993), there are a TATA-like sequence, several G+C-rich regions, three possible Sp1-binding sequences, one AP-1-binding sequence, and a C/EBP-binding sequence. In tilapia, there is an undefined TATA-like sequence of 25 bp in front of the 5'RACE end. There is one possible Sp1-binding sequence. Between –5477 bp and –4477 bp, there are one possible DBP-binding sequence, one possible GHF-1-binding sequence, one possible AR-binding sequence, and one possible

FIG. 6. Identification of promoter activity in 5' flanking region of tilapia *IGF-I* gene (A) and *IGF-II* gene (B). The IGF-CAT fusion plasmids containing fragments of 5' flanking region were transfected into human lung large cell carcinoma, and CAT activity was measured 48 h later as described previously (Tsai *et al.*, 1996). All transfections were performed in triplicate. Values (mean ± SEM) represent CAT activity relative to that in cells transfected with promoterless plasmid pCAT-basic vector, defined as 1.0.

HNF-5-binding sequence. Although we did not characterize this region, we surmise that it contains another promoter. In the 3' flanking regions of the tilapia *IGF-II* gene, we found that the last exon contains an A + T-rich sequence, which proved to include the complete sequence of a 3.6-kb genomic region downstream of the 5' boundary of the last coding exon (Fig. 5). In the rat, a 3' terminal exon can be found, and two polyadenylation signals (5'-AATAAA-3') can be distinguished (de Pagter-Holthuisen *et al.*, 1988), but in the tilapia 3' last coding exon sequence, we

could not find a legitimate polyadenylation signal; we found only three less common variants of the AATAAA signal.

Determination of tilapia IGFs 5' cDNA terminus

To ascertain the tilapia *IGFs* 5' cDNA terminus, the 5'RACE method was applied. The results identified two 5' cDNA ends in the tilapia *IGF-I* gene and one in the tilapia *IGF-II* gene. The 5' cDNA ends of the tilapia *IGF-I* gene compared with the

salmon *IGF-I* gene show a different location for the transcription start site. The first 5' cDNA end is the same as the salmon 5'UTR, which is 450 to 550 nt long. The second 5' cDNA end is the same as in the salmon 5'UTR, which is 200 to 250 nt long (Koval *et al.*, 1994). In tilapia *IGF-II*, the 5' cDNA end is different from the human P4 promoter transcription start site. In humans, there are two different transcription start sites (Hyun *et al.*, 1993), but in tilapia, we found only one 5' cDNA end. The data are shown in Figures 3 and 4.

Demonstration of promoter activity in tilapia IGFs gene

To determine which regions of the tilapia *IGFs* promoter are involved in its regulation, we constructed sequential 5' deletions of the assumed promoter region, including the flanking transcription start sites described above (Figs. 3, 4, and 6), ligated with the CAT coding sequence. The constructs were then transfected into human lung large cell carcinoma cells in triplicate, and, after transient transfection expression, CAT activity was analyzed (Fig. 6). The fragment of IGF-I P2 → P7 (46.4 ± 7.8) contained enzymatic activity with a maximum value comparable to that of the other *IGF-I* promoter-region constructs and the promoterless plasmid, pCAT-basic. Deletion of the fragment from IGF-I P2 → P7 to IGF-I P5 → P7 decreased the CAT activity. In contrast, minimal CAT activity was generated with the fragments IGF-I P3 → P6 and IGF-I P5 → P6. Comparison of the activities of fragments IGF-I P3 → P4 (32.2 ± 4.5), IGF-I P3 → P6 (1.0 ± 0), IGF-I P3 → P7 (27.8 ± 4.4), and IGF-I P5 → P4 (13.6 ± 2.2), IGF-I P5 → P6 (1.3 ± 0.1), IGF-I P5 → P7 (28.2 ± 5.0) showed that the se-

quence from P4 to P6 contains a positive regulatory element. Surprisingly, the fragment narrows to a 184-bp length (IGF-I P5 → P4); the CAT activity represents only basal transcription in this fragment, although the fragment contains all possible transcription factor-binding sequences (Figs. 3 and 6A). In the rat, the minimal exon 2 promoter is contained within the 73 bp upstream and 44 bp downstream of the transcription start site cluster. These minimal promoters contain specific elements for basal transcription (Wang *et al.*, 1997). However, our data show that the region downstream of the transcription start site and the sequence of about 120 bp from P6 to P7 may contain a negative regulatory element, which gives rise to decreased CAT activity.

Figure 6B reveals that the fragment of IGF-II P5 → P11 (82.6 ± 11.2) has enzymatic activity with a maximum value comparable to that of the other *IGF-II* promoter region constructs and the promoterless plasmid pCAT-basic. Deletion of the fragment from IGF-II P5 → P11 to IGF-II P6 → P11 rapidly decreased CAT activity, and deletion of the fragment from IGF-II P6 → P11 (38.0 ± 7.1) to IGF-II P7 → P11 (79.4 ± 9.2) and IGF-II P9 → P11 (71.5 ± 12.1) sharply increased CAT activity. Inverted orientation of the fragments (IGF-II P7 ← P11

FIG. 7. Human lung large-cell carcinoma cells transiently transfected with *IGF* promoter ligated to pEGFP-1 vector and expressing GFP.

FIG. 8. Expression of *IGF-I* promoter-GFP vector microinjected into zebrafish 2-cell-stage fertilized eggs. After 24 h of development in freshwater on culture dishes, GFP was first visibly expressed in whole trunk and head. Other embryo at the same stage injected with *IGF-I* promoter-GFP vector shows no GFP. Brightfield photograph and fluorescence image (above) of same 24-h injected embryo. All photographs were taken with 45× lens on Olympus microscope IX70.

FIG. 9. The *IGF-II* promoter-GFP vector microinjected into zebrafish 2-cell-stage fertilized eggs. Fluorescence (bottom) and brightfield (top) photographs. A. The GFP was first visibly expressed at 8 h (75% epiboly, gastrula period). Photograph was taken with 100 $\times$ lens on Olympus microscope IX70. The *IGF-II* promoter-GFP vector was highly expressed from embryo to embryo (B) and from 1 stage to the next (panel C is 24 h after injection, and panel D is 48 h after injection). Panel D shows two fish of which the upper was not injected. Green fluorescence accumulated in yolk sac, yolk sac extension, and notochord (D). The GFP is visible in horizontal myoseptum (E), eye (F), and floor plate (G) 48 h after microinjection.

[19.2 ± 2.3] and IGF-II P8 ← P11 [24.4 ± 2.9]) produced lower promoter activity than was seen with the fragment of IGF-II P7 → P11 and the other fragment. These results show that the sequence from IGF-II P5 → P6 contains a negative regulatory element, and the sequence from IGF-II P6 → P7 contains a positive regulatory element. The sequence from IGF-II P5 → P6 contains a possible Sp1-binding regulatory element. This putative Sp1 element may play an important role in regulating *IGF-II* promoter function in tilapia.

Expression of IGFs promoter-GFP in human cell line and zebrafish embryos

To understand the *IGFs* promoter regulation in fish, we constructed the fragment IGF-I P2 → P7 and the fragment IGF-II P5 → P11 ligated with the pEGFP-1 vector. The promoter activity of these regions produces enzymatic activity to a maximum value identified by transient transfection expression in human lung large cell carcinoma cells, as described above. A plasmid containing the *IGF* promoter driving the *GFP* cDNA was transfected into lung carcinoma cells, and the fluorescence was observed (Fig. 7). The data show that the *IGF-II* promoter is stronger than the *IGF-I* promoter, and the fish promoter can take advantage of the GFP system in the human cell line to be expressed. About 24 h after microinjection of the plasmid of pEGFP-1 and *IGF-I* promoter region construct, patches of fluorescent cells could be observed in a zebrafish embryo injected with the IGF-I P2 → P7 plasmid construct (Fig. 8). The low fluorescence in the whole embryo implies that the *IGF-I* promoter has little expression during the zebrafish straightening

period. Otherwise, 8 h (75% epiboly, gastrula period; Fig. 9A) after injection of the plasmid of pEGFP-1 and *IGF-II* promoter construct, patches of fluorescent cells could be observed in 42 of the 60 embryos. Strongly fluorescent cells were visible from embryo to embryo (Fig. 9B) and from one stage to the next (Fig. 9C, D). The fluorescence was visible for up to 1 wk; in 24 h, it was visible in the horizontal myoseptum and the eye (Fig. 9E, F); in 48 h, the fluorescence appeared in the yolk sac (Fig. 9D) and floor plate (Fig. 9G). The fluorescence from the segmentation period to the straightening period also appeared in the heart and blood cells (data not shown). The expression of Xex-GFP in zebrafish embryos appears in a pattern different from what was seen in our studies (Amsterdam *et al.*, 1995).

After injection of the fragment IGF-I P2 → P7 and the fragment IGF-II P5 → P11 ligated with pEGFP-1 vector into zebrafish embryos, RT-PCR was run using primers GFP1 and GFP2 (see Table 1), followed by Southern blotting by hybridization assay. The data show that the tilapia *IGF-I* promoter drives the *GFP* cDNA the first time the transcripts rise in the 1-K-cell period; after 1 wk, RT-PCR cannot detect the tilapia *IGF-I* promoter-driven transcript products (Fig. 10). In *IGF-II*, the promoter drives the *GFP* cDNA the first time the transcripts rise in the 32-cell period; after 2 wk, the RT-PCR can continuously detect the *IGF-II* promoter-driven transcript products, but expression is weak (Fig. 10).

DISCUSSION

The tilapia *IGF-I* cDNA insert contains 549 nt, which encode 183 amino acid residues. In the tilapia E domain, there are 11 amino acid insertions. The *IGF-I* has four size forms, and the size differences are attributable to insertions or deletions of E-domain amino acids (Chen *et al.*, 1994). According to the insertions or deletions, the *IGF-I* forms are designated Ea-1, Ea-2, Ea-3, or Ea-4 (Duguay *et al.*, 1992; Holthuisen *et al.*, 1991; Shambloott and Chen, 1993). Tilapia *IGF-I* RT-PCR products belong to Ea-4. The *IGF-I* Ea-4 form is also found in coho salmon (Duguay *et al.*, 1992), rainbow trout (Shambloott and Chen, 1993), and chinook salmon (Wallis and Devlin, 1993). The quantity of Ea-4 mRNA was not changed after GH treatment, and the Ea-4 transcript was expressed only in most nonhepatic tissues (Duguay *et al.*, 1994). In our experiment, the first step was to inject GH to induce high expression of *IGF-I*. Growth hormone can increase immunoreactive *IGF-I* levels in fish plasma and mRNA levels of *IGF-I* in salmon liver. *Gillichthys mirabilis* was injected with tilapia GH, and it has been suggested that *IGF-I* is sensitive to the GH status of the animal (Gray and Kelley 1991). Plasma *IGF-I* levels increased significantly after 48 h in *Oncorhynchus mykiss* injected with a higher dose of GH (Moriyama *et al.*, 1995).

Figure 2 shows that the mature tilapia *IGF-I* peptide has nine amino acids different from the conjectured ancestral piscine peptide. They are located at B26(Asn), C6(Cys), A14(Gln), D4(Thr), D5(Pro), D7(Ile), and D8(Ser). The peptide changes include SerB26(Asn → Ser) and AlaD8(Ser → Ala), but the other five amino acids could not be distinguished from the conjectured ancestral peptide. Compared with the ancestral piscine *IGF-I*, the tilapia *IGF-I* B26 was changed to a polar amino acid (Asn), but ArgA14 was changed to a charged GlnA14, and hy-

FIG. 10. Detection of *IGF-I* (lane 1 to lane 5) and *IGF-II* (lane 6 to lane 9) promoters driving GFP cDNA production of mRNA in each stage of embryos and fish (*Brachydanio rerio*). Whole-embryo total RNA and juvenile tilapia total RNA were extracted, and RT-PCR and Southern blot analyses were conducted as described in the text. Upper picture shows ethidium bromide staining of RT-PCR products using GFP-specific primers. Lower picture shows Southern blot analysis of the gel using ³²P-labeled GFP full-length cDNA as a probe. Lane M is ϕ X174 *Hae*III marker. Lanes 1 and 6: 32-cell stage; lanes 2 and 7: 1-K-cell stage; lanes 3 and 8: 30% epiboly stage; lane 4: gastrula stage; lane 5: 1-wk-old fish; lane 9: 2-wk-old fish.

drophobic AlaD8 was changed to a SerD8. The implications for the protein structure and stereospecific form of the two changes here are still unknown. The human native IGF-I molecule contains three disulfide bonds linking six cysteine residues. They are located at CysB6-CysA7, CysA6-CysA11, and CysB18-CysA20 (Iwai *et al.*, 1989); in tilapia, the linking disulfide bonds are located at the same sites. In the C domain, HisC10 and AsnC11 exist in rainbow trout, salmon, hagfish, and catfish but have been deleted in tilapia. In the E domain, the actual mechanism is at present unknown. The N-linked glycosylation site (Asn-X-Ser/Thr) exists at the amino acid position E22-24 in humans, while the rat and mouse also have a glycosylation site at E30-E32 (Howard and Charles, 1991). In contrast, the tilapia sequences contain a putative glycosylation site at residues E57-E59 (see Fig. 1). In the E peptide processing site there is a Lys-Lys-Lys motif. Chum salmon IGF-I.1 and IGF-I.2 sequences have Pro and Thr instead of Lys (Kavsan *et al.*, 1994), but in tilapia, the possible motif is an Asn-Lys-Lys sequence. The actual physiological role of this sequence in tilapia is unknown. Comparing this with the known sequences in other fish species shows that fish IGF-Is are highly conserved.

We have sequenced and identified *IGF-I* and *IGF-II* proximal promoter regions in the tilapia *IGFs* gene. In the chicken, the *IGF-I* gene is about 50 kb in length (Kajimoto and Rotwein, 1991), and those of rat and human genes are from 80 to 100 kb (Rotwein *et al.*, 1986; Shimatsu and Rotwein, 1987). If the tilapia *IGF-I* gene is the same length as the chum salmon gene, then, compared with avian and mammalian *IGF-I* genes, the fish gene is very small. In tilapia, in contrast to the salmon *IGF-I* gene (Koval *et al.*, 1994), there is a TATA-like consensus sequence upstream of the first methionine to about 300 bp. The vicinity of the tilapia *IGF-I* proximal promoter sequence contains a potential hepatocyte nuclear factor 1 (HNF-1) and hepatocyte nuclear factor 5 (HNF-5) binding site; however the HNF-1-binding site sequence is identical in chum salmon (Kulik *et al.*, 1995) and is also found in the *Sparus aurata* *IGF-I* genomic sequence (Funkenstein *et al.*, 1996).

Here, we confirm that the tilapia *IGF-I* gene contains five exons. Tilapia exon 4 is the same as the mammalian exon 5 (Holthuisen *et al.*, 1991). Tilapia exon 4 is a "cassette" exon; the cassette is retained in some *IGF-I* mRNAs but not in others. There are "cassette" exons retained in mRNA termed Eb *IGF-I* mRNAs, otherwise termed Ea *IGF-I* mRNAs. The two types of *IGF-I* prepropeptides (*IGF-I* Ea and Eb) give rise to the alternative splicings. In rats, the two types, *IGF-I* Ea and Eb, depend on whether the differential splicing contains the 52-nt mini-exon (Adamo *et al.*, 1991), and in rats, the mini-exon is suggested to cause a frameshift and the introduction of a stop codon. In chinook salmon, four distinct *IGF-I* genes were found by Southern blotting, which suggests that there are at least two *IGF-I* loci in salmon (Wallis and Devlin, 1993). In tilapia, this remains unknown, although we have performed Southern blotting in this species. In chum salmon, there are two nonallelic *IGF-I* genes (Kavsan *et al.*, 1994), but in tilapia, we have not yet obtained this sequence.

Analysis of the tilapia *IGF-II* proximal promoter region sequence has shown possible transcription factor-binding elements such as Sp1. The human *IGF-II* P4 promoter shows several G + C-rich regions and three possible Sp1-binding

sequences (Hyun *et al.*, 1993). In the rat, the results suggest that there are four proximal upstream GC boxes binding the general transcription factor Sp1 (Soares *et al.*, 1986; Evans *et al.*, 1988). Comparison of the tilapia *IGF-II* proximal promoter region upstream of the first methionine DNA sequence with those of the human and rat indicates that homology is low among the three species. The potential transcription factor-binding sequence represents only an Sp1 site but does not include an Egr-1 site in the tilapia *IGF-II* proximal promoter region. The tilapia last exon shows a poly(AT) stretch different from the human poly(AC) stretch (de Pagter-Holthuisen *et al.*, 1988). Additional *IGF-II* transcripts of between 1.2 and 3.0 kb have been detected in fetal rat tissue and transformed cells, but their origins are unclear. A stable 1.8-kb RNA is formed that is derived from the 3' untranslated region of exon 9; this RNA is formed through site-specific endonucleolytic cleavage of *IGF-II* mRNAs (Meinsma *et al.*, 1992; Nielsen and Christiansen, 1992). Unusually, cleavages of the *IGF-II* mRNA trailer will occur at a guanosine-rich region and a putative hairpin (Meinsma *et al.*, 1992; Nielsen and Christiansen, 1992). The poly(AT) stretch of the tilapia last exon was shown to play an important role in gene regulation of general mechanisms of mRNA degradation; alternatively, specific mRNAs may be selectively targeted for degradation (Ratnasabapathy *et al.*, 1990).

The CAT analyses presented here suggest that the tilapia *IGF-I* promoter's more distal 5' flanking region has greater activity (*IGF-I* P2 → P7). In rat *IGF-I*, exon 2 transcripts are expressed at high levels in the OVCAR-3 cell line (Tobin *et al.*, 1990; Yee *et al.*, 1991). The construction containing 1500 bp of 5' flanking sequence and 44 bp of exon 2 stimulates luciferase activity 13- to 23-fold, whereas the fragment containing only 36 bp of the 5' flanking sequence is inactive (Wang *et al.*, 1997). In tilapia, if the fragment is shortened to 132 bp (*IGF-I* P5 → P4), the activity is weak (13.6 ± 2.2). When the tilapia *IGF-II* proximal promoter region is shortened to 450 bp (*IGF-II* P5 → P11), it shows maximum activity. The fragment *IGF-II* P5 → P6 shows a negative regulatory effect, and the fragment *IGF-II* P6 → P7 shows a positive regulatory effect. The fragment *IGF-II* P5 → P6 shows a putative Sp1 transcription factor-binding element. Here, Sp1 may play a negative regulatory role, but in humans, Sp1 binds to the P4 promoter and is involved in regulation of the transcription of the *IGF-II* gene (Hyun *et al.*, 1993).

Salmon *IGF-I* genes have negative regulatory effects that may be due to the tissue-specific and developmental regulation of *IGF-I* gene expression (Koval *et al.*, 1993). Human *IGF-II* mRNAs show transcription can be initiated at three distinct promoter sites, suggesting that these promoters may be subject to tissue-specific and development-specific regulation (de Pagter-Holthuisen *et al.*, 1987, 1988). To integrate these reports, we can say that the *IGFs* promoter is regulated by a complicated pathway. To understand *IGFs* promoter function, we used the GFP system as a reporter gene (Amsterdam *et al.*, 1995; Ogawa *et al.*, 1995; Chiu *et al.*, 1996; Corbo *et al.*, 1997). In the analyses presented here, GFP mRNA was detected using primers designed to amplify GFP cDNA specifically, and the result was confirmed by Southern blot analysis. In rainbow trout, the early detection in embryos of *IGFs* mRNA expression is similar to that in bovine embryos (Telford *et al.*, 1990; Schultz and Heyner, 1992; Schultz *et al.*, 1993; Greene and Chen, 1997).

However, those reports could not confirm the presence of mature IGF peptides in embryos. In our GFP system, the *IGFs* proximal promoter drove the GFP cDNA, which then produced the GFP protein, and fluorescence could be observed. In other words, the construction of GFP cDNA is replaced by the *IGF* coding region of genomic DNA sequences. When the promoter starts to drive, the mature IGF peptide may be present in the natural state of the fish. From our GFP analysis data, we found that the *IGF-II* proximal promoter was expressed more strongly than was the *IGF-I* proximal promoter in the embryo stage and began expression earlier than did *IGF-I*. Although we used RT-PCR to check GFP transcripts, we found that *IGF-I* proximal promoter-driven GFP transcripts appeared for the first time at the 1-K cell stage, and the *IGF-II* proximal promoter-driven GFP transcripts appeared for the first time at the 32-cell stage. These data will need further checking in the next generation of *IGF* promoter-GFP transgenic fish. The *IGFs* proximal promoter may play an important role in teleost embryos for controlling IGF expression.

In summary, we have located two 5' cDNA termini in tilapia *IGF-I* and one in tilapia *IGF-II* using the 5'RACE method. We have determined the proximal promoter region in the tilapia *IGFs* gene using CAT analysis and have presented GFP and GFP/RT-PCR data suggesting that the fish *IGFs* first proximal promoter region plays a very important role in controlling IGF expression in embryo development. Future studies will focus on characterization of the tilapia second promoter and on finding interactions between IGFs and specific transcription factors controlling gene expression in fish.

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