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Organization and expression of the chum salmon insulin-like growth factor II gene

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Abstract IGF-II plays an important role in growth and development of vertebrates. In the present study, the characterization of the first fish IGF-II gene, chum salmon IGF-II, is described. The sIGF-II gene consists of four exons, spanning a region of 9 kbp, that encode the 214 aa IGF-II precursor. While the amino acid sequences of fully processed IGF-II of salmon and mammalian species are very similar, the prepro-peptide sequence deviates extensively in the signal- and E-peptide domains. The transcription initiation site of the sIGF-II gene was localized within a 30 nt region employing RT-PCR. Using sIGF-II promoter-luciferase constructs it was demonstrated that the sIGF-II gene has a relatively strong promoter that contains tissue-specific regulatory elements.

Key words: Insulin-like growth factor II; Gene structure; Gene expression; Fish; Salmon; *Oncorhynchus keta*

1. Introduction

The insulin-like growth factors (IGF-I and IGF-II) are mitogenic peptides that play an important role in the regulation of growth and development. The structure of the IGF-II peptide is highly conserved in mammals, birds, and fish (for a review see [1]), [2]. The structures of the human, rat, mouse and ovine IGF-II genes have been studied in detail (for a review see [3]); and all four species show complex patterns of IGF-II expression. Heterogeneity of the IGF-II mRNA population originates from the use of multiple promoters, alternative splicing, and differential use of polyadenylation sites. The extensively studied human IGF-II gene comprises approximately 30 kbp of genomic DNA and contains nine exons. Expression of this gene is regulated in a tissue-specific and developmental stage-dependent manner [4]. Transcription initiation takes place at exons 1, 4, 5, and 6, which are preceded by distinct promoters, P1–P4 [4–6]. Promoter P1 is active exclusively in human adult liver whereas promoters P2–P4 are active in fetal tissues including the liver and in several non-hepatic adult tissues.

Isolation of rainbow trout (*Oncorhynchus mykiss*) IGF-II cDNA [7] was a first step towards the characterization of the fish IGF-II genes. Analysis of the IGF-II mRNA levels in the liver and other tissues of juvenile and adult rainbow trout [8] revealed that IGF-II mRNA levels are two-fold higher in the adult liver than in the juvenile liver. The growth hormone-dependent expression of IGF-II mRNA in the liver and pyloric ceca (pancreatic tissue) suggests an important role for IGF-II in growth hormone-stimulated growth [9]. In the current study we present the nucleotide sequence of the chum salmon (*Oncorhynchus keta*) IGF-II gene as well as the localization of the position of the transcription initiation site and a first characterization of the salmon IGF-II promoter. This information might be helpful to understand the regulation of IGF-II expression and the role of IGF-II 'under water'.

2. Materials and methods

2.1. Materials

Restriction endonucleases, the Klenow fragment of *Escherichia coli* DNA polymerase I, T4 DNA ligase and Taq DNA polymerase were purchased from Fermentas (Vilnius, Lithuania) and Boehringer Mannheim. Ribonuclease inhibitor (RNasin) and calf intestinal phosphatase were from Promega (Madison, WI). Random-primed DNA labelling kits were from Boehringer Mannheim and from Amersham (Amersham, UK), DNA sequencing kits from United States Biochemical Corporation (Cleveland, OH), [α-^{32}P]dATP and [α-^{32}P]dCTP from Isotope (Tashkent, Uzbekistan), and [α-^{35}S]dATP from Amersham (Amersham, UK). Nylon membranes were from Chemifil (Tallinn, Estonia) and GeneScreen membranes from Du Pont de Nemours (Dreieich, Germany). Plasmids pBluescript SK$^+$, and pGEM-3Zf(+), pGEM-5Zf(+) were from Stratagene (La Jolla, CA) and Promega (Madison, WI), respectively. MMLV reverse transcriptase and incubation buffers were from Gibco-BRL (Grand Island, NY).

2.2. Oligonucleotides and plasmids

Based on the rainbow trout IGF-II cDNA sequence [7] two primer sets specific for various regions of the IGF-II coding region were synthesized: (a) 5′-CTGGGAAACTAACTCAACTGCA-3′ and 5′-CCTCTCTGACTTGGCAGGTTT-3′ and (b) 5′-TGCGCTGGCACTTACTCTGTA-3′ and 5′-CAATTGTGGCTGACGTAGTTGT-3′. Employing these primer sets, two coho salmon IGF-II cDNA probes were synthesized by RT-PCR using total coho salmon (*Oncorhynchus kisutch*) liver RNA (generously provided by Dr. S. Duguay, University of Washington, USA) as a template. PCR products were purified and subcloned in pBluescript SK$^+$, generating clones 5′-D IGF-II (425 bp) and Pre-E IGF-II (531 bp), respectively. Promoter fragments were cloned into the promoterless firefly luciferase gene containing vector pFLASH [10]. In all constructs, the *Mun*I site at position −4 relative to the first ATG (methionine start codon) of prepro-IGF-II was used to link the IGF-II promoter fragments to the *Nco*I site of the luciferase gene (both sites were made blunt-ended by the Klenow fragment of DNA polymerase I). Five reporter constructs containing promoter fragments of different lengths were obtained: p2100M, p1500M, p900M, p500M and p300M (Fig. 3). The longest construct (p2100M) was made by cloning a 2.16 kbp long genomic *Bam*HI-

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Abbreviations: nt, nucleotide; bp, base pair; kbp, kilobase pair; aa, amino acid; RT-PCR, coupled reverse transcription-polymerase chain reaction; IGF-II, insulin-like growth factor II; sIGF-II, chum salmon IGF-II; UTR, untranslated region; SDS, sodium dodecyl sulfate; SSC, standard sodium chloride/sodium citrate buffer

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*Mun*I fragment into the *Bam*HI and *Nco*I sites of pFLASH. Subsequent truncations were made using the appropriate restriction enzyme sites at the 5′-end and the blunt-ended *Mun*I and *Nco*I sites. Because of the absence of appropriate restriction sites, the p300M was obtained after treatment with Exonuclease III of a genomic clone and the obtained 299 nt fragment was then cloned into pFLASH and checked by sequencing. The pFLASH vector without the *Bam*HI-*Nco*I polylinker fragment was used as a promoterless control plasmid.

2.3. Screening of the genomic DNA library and DNA sequence analysis

Approximately 4×10^5 bacteriophage plaques from a chum salmon genomic library [11] in λEMBL3 were screened by standard methods [12] using the α^{32}P-labeled inserts of clones 5′-D IGF-II or Pre-E IGF-II as probes. For DNA nucleotide sequence analysis DNA fragments of plaque-purified positive clones were subcloned in pBluescript SK$^+$, pGEM-3Zf(+) and pGEM-5Zf(+) plasmids and were sequenced on both strands employing the dideoxynucleotide method with Sequenase version 2.0 (USB, Cleveland, OH).

2.4. Northern blotting and transcription start site determination

Total RNA was isolated from adult liver of rainbow trout (*Oncorhynchus mykiss*), and chum salmon (*Oncorhynchus keta*) using the single step guanidinium thiocyanate method [13]. RNA (15 μg) was glyoxalated and size-separated on a 1% agarose-10 mM sodium phosphate gel, and transferred to a GeneScreen membrane. The RNA was fixed on the membrane by UV irradiation. Northern blots were hybridized according to GeneScreen protocols with a chum salmon IGF-II cDNA probe. The blot was washed to a final stringency of 0.5×SSC-1% SDS at 65°C and exposed on Fuji RX X-ray film.

In order to localize the transcription start site cDNA was synthesized from 1 μg of adult liver coho salmon total RNA with 200 units of MMLV reverse transcriptase, and subsequent PCRs were performed in the same tube containing 2.5 units of Taq Polymerase in Taq DNA buffer and 0.1 μg of each IGF-II-specific primers as described previously [14].

2.5. Transient transfection experiments

The human hepatoma cell line Hep 3B [15] was cultured in α-modified minimum essential medium (αMEM) and the adenovirus-transformed kidney cell line 293 [16] was cultured in Dulbecco's modified Eagle's medium (DMEM). The media were supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml glutamine. Cells from both cell lines were transfected at 60% confluence in 25 cm^2 flasks using the calcium phosphate coprecipitation method [17]. Each flask was transfected with 4.5 μg of a promoter-luciferase construct and 0.5 μg of RSV-LacZ which served as an internal control for transfection efficiency. Four hours after the addition of the precipitate, cells were shocked with 10% dimethylsulfoxide in serum-free αMEM or DMEM. Fresh medium was added and the cells were harvested after 24 h. Preparation of cell extracts, luciferase assays and β-galactosidase assays were performed as described [12]. Luciferase levels were recorded on a Lumac/3M Biocounter M2010A. Luciferin was purchased from Boehringer Mannheim.

3. Results

Since previous analysis of members of the Pacific salmon family showed a very high degree of homology for the IGF-I sequence [18,19], we surmised that the coding sequences of IGF-II genes from members of this family would also be very similar. Based on this assumption, primers derived from the published rainbow trout (*Oncorhynchus mykiss*) IGF-II cDNA sequence [7] were used to synthesize coho salmon (*Oncorhynchus kisutch*) IGF-II cDNA from adult liver mRNA by means of RT-PCR. Two different cDNAs were constructed, 5′-D IGF-II and Pre-E IGF-II, which together cover the entire coding region of IGF-II. Subsequently, the cDNA clone representing the region between the 5′-UTR and the D-domain of coho salmon IGF-II was used as a probe to screen a chum salmon (*Oncorhynchus keta*) genomic library consisting of approximately 4×10^5 recombinant bacteriophages. Two recombinant clones were isolated and characterized by restriction endonuclease mapping and Southern blot hybridization. One of the positive clones (λsIGFII 1) contained 17 kbp of chum salmon genomic DNA, while the other (λsIGFII 2) contained 13 kbp of DNA from the same locus. Using the two IGF-II cDNA clones as probes, the salmon IGF-II locus was analyzed as shown in Fig. 1A. Four different exon-containing regions were identified, designated exons 1–4 (Fig. 1A). All exon-intron boundaries contained canonical donor and acceptor splice sequences.

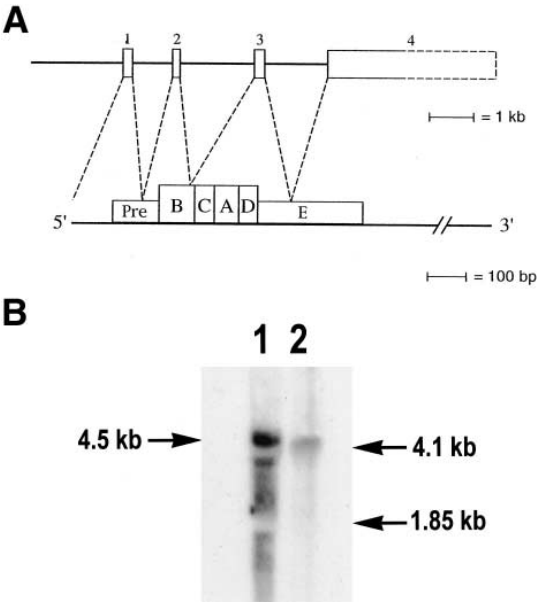


Fig. 1. Structure and expression of the chum salmon IGF-II gene. A: Schematic representation of 17 kbp of contiguous chromosomal DNA from the chum salmon IGF-II locus. Exons are depicted by boxes, and introns and flanking regions by thin lines. The structure of the corresponding mRNA and the domain structure of the salmon prepro-IGF-II peptide are displayed below the gene map. The nucleotide sequence data are in GenBank under accession number X97225. B: Northern blot analysis of total RNA from adult rainbow trout liver (lane 1) and from adult chum salmon liver (lane 2). 15 μg of total RNA was hybridized with a sIGF-II cDNA probe containing the coding sequence. The size of the major IGF-II transcript is indicated. Stained ribosomal RNA bands served as size markers; for fish 4.1 kbp and 1.85 kbp [23].

The chum salmon IGF-II gene locus was subcloned in plasmid vectors and the IGF-II gene nucleotide sequence was determined. First, the coding sequence of the chum salmon gene was compared with that of the isolated coho salmon cDNAs. As expected, the coding sequences were completely identical with the exception of a silent single nucleotide change in the D-domain region. Second, the chum salmon gene sequence was compared with the published rainbow trout cDNA sequence [7]. Comparison of the 87 nt region of the 5′-UTR revealed 3 nt differences, while three nucleotide changes were found in the signal peptide sequence, one of which led to an amino acid substitution. The IGF-II mature peptide encoding sequences of chum salmon and rainbow trout contained one silent nucleotide change. In the first 400 nt of the 3′-UTR four deletion/insertions were found and one point mutation.

Mature chum salmon IGF-II is encoded by two exons (exons 2 and 3) as in other species (man, mouse, and sheep). The leader sequence is located in exons 1 and 2 together

encoding a 47 aa long signal peptide with high homology to the rainbow trout sequence, but it deviates completely from all other known IGF-II leader sequences. The E-domain sequences are located in exons 3 and 4 and encode a 97 aa long E-peptide, which is conserved between chum salmon and rainbow trout but shows only partial homology to the E-peptides of IGF-II from other species.

Since the IGF-II cDNA sequences lack a poly(A) tail, the size of the last exon and the precise location of the polyadenylation site are unknown. Therefore, assuming that the 5′-end of the known cDNA [7] was located close to the transcription start site region, we performed Northern blot analysis to establish the approximate size of the salmon IGF-II transcript. Total RNA from adult chum salmon liver and adult rainbow trout liver were hybridized with the coho salmon cDNA probe (Fig. 1B). A single IGF-II specific mRNA band was detected with an approximate size of 4.5 kbp.

To determine the size of exon 1 and the transcription start site of the salmon IGF-II gene, RT-PCR analysis was performed on total RNA from adult liver coho salmon. A single reverse primer (D1) complementary to sequences of exon 2 and a set of contiguous primers (P1–P5) representing different sequences in the putative 5′-flanking/5′-untranslated region were used for analysis (Fig. 2A). PCR products of the expected size were synthesized with the primer pairs D1/P5 and D1/P4 (Fig. 2B). No products were obtained with the primer pairs D1/P3, D1/P2, and D1/P1. These results pinpoint the sIGF-II transcription start site within a region of approximately 30 nt situated 110–140 nt upstream of first methionine codon of sIGF-II (Fig. 2A).

Finally, to localize the salmon IGF-II promoter and to analyze its activity we constructed a set of five plasmids containing the putative promoter sequence linked to the luciferase reporter gene. The longest sIGF-II promoter-luciferase expression construct, p2100M, contained an upstream region of 2.16 kbp relative to the first methionine codon. Subsequent promoter truncation clones were made of which the smallest promoter construct, p300M, still contained 160–190 nucleotides upstream of the transcription start site (Fig. 3). Since previous studies revealed that the expression of the salmon IGF-II gene is regulated in a tissue-specific and developmental stage-specific fashion [8], we employed a liver-specific and a non-liver-derived cell line (Hep 3B and 293 cells, respectively) for transient expression studies of the five salmon IGF-II promoter-luciferase expression constructs. All salmon IGF-II promoter constructs possess promoter activity. In 293 cells the maximal activity was observed with the p2100M and the p1500M constructs, and a two-fold decrease was observed when the promoter region was truncated from −500 to −300. In Hep 3B cells p500M reveals maximal promoter activity while additional upstream regions have a negative effect on the promoter activity. Truncation of the region from −500 to −300 results in a 2.4-fold reduction of the promoter activity in Hep 3B cells. In fact, the promoter is quite strong in both cell lines, since the activity of p500M in Hep 3B cells was 17% of the activity of the human thymidine kinase promoter cloned in the pFLASH vector (pTK-Luc) and in 293 cells the activity of p1500M is 60% of pTK-Luc (results not shown). The activity of the negative control, a promoterless reporter-luciferase construct, was less than 1% of the activity of the salmon promoter constructs.

Cell type-specific differences in expression of the salmon IGF-II promoter constructs in the two cell lines employed could be mapped in the region upstream of the p500M construct. The proximal promoter region that is necessary for basal transcription is situated within 300 bp upstream of the first methionine codon and activating factors might be located in the region between −500 and −300. Further analysis of these regions is necessary to identify the specific factors that may be involved in promoter activation.

All four known mammalian IGF-II genes have been found to form a conserved linkage group with the insulin gene. The

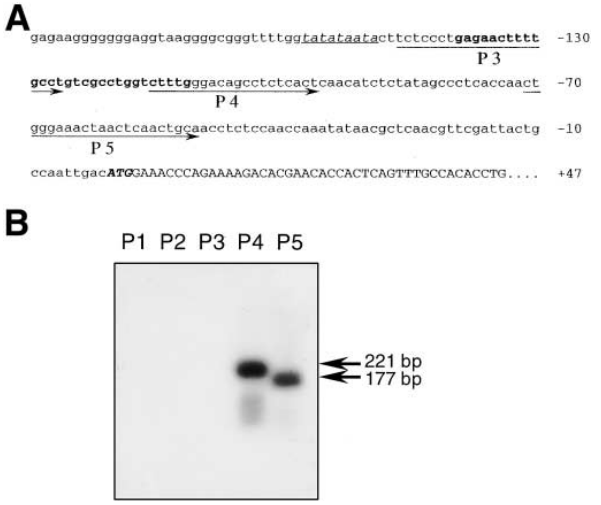
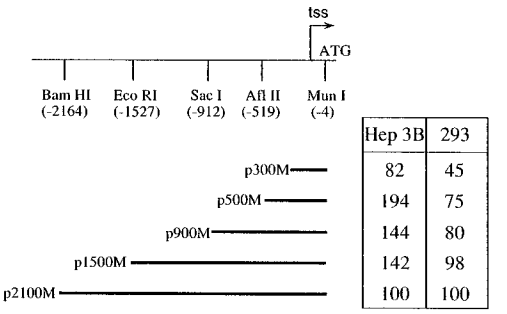


Fig. 2. Mapping of the sIGF-II transcription start site using RT-PCR. A: Genomic nucleotide sequence of exon 1 of the chum salmon IGF-II gene. Underlined sequences with numbered arrows represent the positions of primers P3–P5 used in PCR; primer D1 (+906 to +926) in exon 2 served as the reverse primer. Primers P1 and P2 were located from −670 to −650 and −293 to −273, respectively. The TATA box-like sequence is shown in underlined italics. The identified region for the transcription start site location is shown in bold. The translation initiation codon is shown in bold italics and the A-residue is numbered +1. B: Southern blot analysis of the RT-PCR reaction products obtained with forward primers P1–P5 and reverse primer D1. The blot was hybridized with the 5′-D IGF-II cDNA probe.



	Hep 3B	293
p300M	82	45
p500M	194	75
p900M	144	80
p1500M	142	98
p2100M	100	100

Fig. 3. Determination of salmon IGF-II promoter activity. Luciferase activities of the salmon IGF-II gene promoter constructs in Hep 3B and 293 cell lines. The various sIGF-II promoter-luciferase constructs used for the transfections are depicted. Positions are numbered relative to the A nucleotide of the first methionine codon of sIGF-II (Fig. 2A). The luciferase activity obtained upon transfection with the longest p2100M construct was arbitrarily set at 100. Each value represents the average of at least three independent, triplicate transfection experiments. The standard deviation was within 10% of the indicated value. The activity of the promoterless vector alone resulted in values lower than 1% of p2100M.

IGF-II genes are located downstream of the insulin genes in the same transcriptional polarity. In this study we could not find linkage of the salmon insulin and IGF-II genes, at least, there were no overlapping sequences in 10 kbp of genomic sequence 3′ to the insulin gene and 5 kbp of genomic sequence 5′ to the IGF-II gene (results not shown).

4. Discussion

In this paper we report the isolation and characterization of the first fish IGF-II gene. The organization and nucleotide sequence of the chum salmon IGF-II gene were determined and its promoter was identified. Comparison of IGF-II genes from several species which belong to different classes of animals shows that the overall organization of the IGF-II genes is similar. The mature IGF-II protein is encoded by two exons (exons 2 and 3 in fish; exons 4 and 5 in rodents; exons 8 and 9 in sheep; and exons 7 and 8 in man (review [3])) and the amino acid sequence of the fully processed IGF-II peptide is well conserved. However, as shown in this paper, the sequence of the leader exon of the salmon IGF-II gene deviates strongly from the known leader exons of the mammalian IGF-II genes. While the IGF-II signal peptide in mammals is 24 aa long, the salmon signal peptide has a length of 47 aa and shows no homology with that of the mammalian species. The salmon IGF-II gene is expressed in adult liver tissue yielding a single mRNA species of 4.5 kbp as was shown by Northern blotting. In addition, it was possible to detect the presence of sIGF-II mRNA from the early stages of embryonic development until the adult stage using primers for exons 1 and 3 by RT-PCR assay (data not shown). This implies that mRNA derived from the first exon is present in early embryonic as well as adult stages of development and that the corresponding promoter is active in these stages. Since the size of salmon IGF-II mRNA is approximately 4.5 kbp, it implies that the salmon IGF-II gene, similar to the mammalian IGF-II genes, has a very long 3′-UTR.

The amino acid sequence alignment of IGF-II from mammals, birds, and fish reveals high homology in the B-, A-, and D-domains as well as in the first six residues of the E-peptide. The C-domain of salmon IGF-II is 3 aa longer than that of the mammalian and avian IGF-II. The Lys-X-X-Arg motif at the D-domain/E-peptide junction was identified as required for processing of proIGF-I [20]. The same motif has been conserved in the D-domain/E-peptide junction of all IGF-II sequences except for shark IGF-II [1], suggesting that the mechanism for IGF-II processing may be similar to that of IGF-I. When comparing the IGF-II E-peptides of different higher vertebrates it is interesting to note that the salmon IGF-II E-peptide contains 97 aa (that is 9 aa longer than that of sheep and 8 aa longer than that of other known mammalian IGF-II E-peptides). Although the overall sequence diverges extensively, several regions of strong homology are present in the IGF-II E-peptides. The first region of homology is situated at the amino-terminus of the E-peptide which is important for the cleavage of proIGF-II to mature IGF-II. Pulse-chase experiments have shown that the E-peptide of pro-IGF-II is cleaved at several sites during transport through the cell and most steps appear to occur very late in the secretory pathway and may also occur extracellularly [21]. Conservation of distinct regions in the E-peptide may imply a regulated conversion of pro-IGF-II that could be a potential mechanism by which its biological activity can be modulated.

A TATA box-like sequence (TATATAATA) was found 157 bp upstream of the ATG codon encoding the first methionine of the signal peptide and 69 bp upstream of the 5′-end of the known rainbow trout IGF-II cDNA. Subsequent RT-PCR experiments on total salmon liver RNA located the major transcription initiation site within a 28 bp long region that is situated 111–139 bp upstream of the first methionine codon and 10–38 nucleotides downstream of the putative TATA box.

The salmon IGF-II promoter is a relatively strong promoter with tissue-specific regulation of expression. Using up to 2 kbp long sIGF-II promoter-luciferase expression constructs it was shown that the basal promoter is contained within the first 380–410 nucleotides upstream of the transcription start site, whereas tissue-specific factors may be located up to 2 kbp upstream of the transcription start site. Further analysis of the specific factors involved in regulation of expression is required to locate such transcription factors.

Studies of IGF-II mRNA expression in eight different tissues of both juvenile and adult rainbow trout revealed interesting differences in fish and mammalian IGF-II expression [8]. In rainbow trout, IGF-II mRNA is present in both developmental stages and in all tissues examined. This deviates from the mouse and rat expression patterns where IGF-II is not expressed after birth, since no adult liver-specific promoter is present [3]. Interestingly, the human IGF-II gene does contain a separate promoter that is exclusively activated in adult liver tissue [5]. In rainbow trout the level of IGF-II mRNA is much higher in adult liver than in juvenile liver. This observation could be explained by the fact that the regulation of IGF-II expression in rainbow trout is growth hormone-dependent in the liver and pyloric ceca [9]. In contrast, in the human liver the synthesis of IGF-II is not regulated by growth hormone [22]. The high levels of IGF-II in adult liver of rainbow trout suggest that salmon, like humans but unlike rodents, still express high levels of IGF-II in the adult stage. Since only one promoter and a single mRNA species for IGF-II have been identified in the chum salmon gene, the regulation of expression of IGF-II in adult liver is most likely regulated by a different mechanism in fish than in human adult liver. The growth hormone-dependent activation of salmon adult liver IGF-II mRNA might be regulated by such a developmental stage-specific factor.

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