Nucleotide Sequence and Growth Hormone-Regulated Expression of Salmon Insulin-Like Growth Factor I mRNA

Qiu-Ping Cao, Stephen J. Duguay, Erika Plisetskaya, Donald F. Steiner, and Shu Jin Chan

Department of Biochemistry and Molecular Biology (Q.-C.P., S.J.C., D.F.S.)
and Howard Hughes Medical Institute (S.J.C., D.F.S.)
The University of Chicago
Chicago, Illinois 60637
and School of Fisheries
The University of Washington
Seattle, Washington 98195
(S.J.D., E.P.)

Protein and cDNA sequence analysis have revealed that the insulin-like growth factor (IGF-I) has been highly conserved among several mammalian species. Using the combined techniques of polymerase chain reaction and molecular cloning, we have now obtained the cDNA sequence encoding preproIGF-I from a teleost species, *Oncorhynchus kisutch* (coho salmon). The 2020 nucleotide (nt) cloned cDNA sequence contains a 528 nt open reading frame encoding 176 amino acids in preproIGF-I and 175 nt and 1317 nt of flanking 5′- and 3′-untranslated regions, respectively. The deduced amino acid sequence of salmon IGF-I is highly conserved relative to its mammalian homologues and there are only 14 amino acid differences out of 70 between salmon and human IGF-I. Interestingly, the C-terminal E domain of salmon proIGF-I, which is presumed to be proteolytically cleaved during biosynthesis, also shows striking amino acid sequence homology with its mammalian counterpart, except for an internal 27 residue segment that is unique to salmon proIGF-I. Northern analysis revealed that salmon preproIGF-I mRNA consists predominately of a single 3900 nt sized band although minor bands were also observed after prolonged autoradiographic exposure. The RNA analysis also revealed that the level of preproIGF-I mRNA is increased 6-fold in liver RNA isolated from salmon injected with bovine GH, as compared to untreated controls. These results demonstrate that the primary structure and regulated expression of IGF-I by GH have been conserved in teleosts. (Molecular Endocrinology 3: 2005–2010, 1989)

0888-8809/89/2005–2010$02.00/0
Molecular Endocrinology

INTRODUCTION

The insulin-like growth factors (IGF-I and IGF-II) are mitogenic peptide hormones with important functions in the regulation of development and somatic growth (1, 2). IGF-I is produced predominantly in the liver under the control of GH while IGF-II is expressed during fetal development. Both IGF-I and II share some overlapping biological activities with insulin and, indeed, were first named on this basis (2, 3). The amino acid sequence determination of human IGF-I and II and computerized modeling studies have shown that this similarity extends to their primary and predicted tertiary structures (4–6). Thus the B and A domains of human IGF-I share 50% identity in amino acid sequence with human insulin (4). However, IGF-I is more like proinsulin in that it is a single polypeptide chain which can be divided into B, C, and A domains. Unlike insulin or proinsulin it contains an extended C-terminal peptide, the D domain. Its immediate precursor form, proIGF-I, contains an additional C-terminal peptide, or E domain, which is presumably removed by proteolysis during biosynthesis (7).

The close similarity between insulin and the IGFs has raised important questions concerning the evolutionary origins of these hormones and the basis for their conserved primary structures. Insulin is found in all vertebrates; a cDNA encoding the preproinsulin of the hagfish, a primitive vertebrate which diverged from the main line of vertebrate evolution over 500 million years ago, has been sequenced (8). To date cDNAs encoding preproIGF-I from several mammalian species have been cloned including human (7), rat (8), mouse (10), and pig (11). The amino acid sequences of cow (12) and sheep (13) IGF-I and a partial sequence for chicken IGF-I (14) have been determined. We report here the first nucleotide sequence and predicted amino acid sequence of

preproIGF-I from a teleost, the coho salmon. Our results clearly show that conserved primary structure and regulated of expression of IGF-I by GH extends phylogenetically to and likely below the bony fishes.

RESULTS

In cloning salmon IGF-I cDNA, we obtained a partial coding sequence by using the polymerase chain reaction (PCR) and screened a λgt10-salmon liver cDNA library to obtain the complete coding sequence and flanking 5′- and 3′-untranslated regions. Since GH is known to elevate hepatic IGF-I mRNA levels in mammals, we injected juvenile coho salmon with bovine GH (bGH) for 2 consecutive days and isolated poly(A)-enriched mRNA from the livers. The GH-treated salmon liver mRNA was subjected to PCR using degenerate oligonucleotide primers which corresponded to conserved sequences in the IGF-I A domain as described in *Materials and Methods*. Analysis of the PCR products by polyacrylamide gel electrophoresis revealed a major DNA fragment with the expected target size of 62 base pairs (bp) (Fig. 1). The 62 bp fragment was cloned into a plasmid(pGEM4Z) and sequence analysis demonstrated that it contained a 24 nucleotide (nt) sequence derived from salmon IGF-I cDNA (corresponding to nt no. 451-475 in Fig. 3).

Based on the 24 bp partial sequence, unique oligonucleotide primers were synthesized and to used to obtain extended cDNA clones using a modification (15) of the RACE protocol developed by Frohman *et al.* (16).

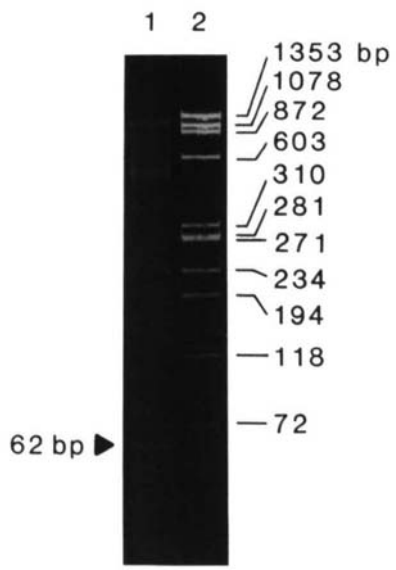

Fig. 1. PCR Amplification of Salmon IGF-I A Domain cDNA Fragment

Complementary DNA reverse transcribed from 50 ng GH-treated salmon liver poly(A$^+$) mRNA was subjected to 30 cycles of PCR using oligonucleotide primers I-1 and I-2 and analyzed on a 8% polyacrylamide gel (lane 1). Molecular size standards correspond to σX174 digested with *Hae*III (lane 2).

Several clones containing 300–400 bp of salmon IGF-I cDNA extended 5′-upstream from the A coding domain were isolated and sequenced. However, attempts to obtain cDNA clones which were extended in the 3′-direction were unsuccessful. This is likely due to a lengthy 3′-untranslated region in the salmon IGF-I mRNA which is difficult to amplify by PCR. To complete the coding sequence for IGF-I, we constructed a λgt10-salmon liver cDNA library and screened it using a partial salmon IGF-I cDNA as a probe. Several positive hybridizing plaques were identified and one, λSIGFI, was purified and further analyzed. Restriction mapping and sequence analysis demonstrated that λSIGFI contained the complete coding sequence for salmon preproIGF-I within a 2.0 kilobase (kb) insert. The restriction map and sequencing strategy used in analyzing the salmon IGF-I cDNA clones is summarized in Fig. 2.

The nucleotide sequence of salmon IGF-I cDNA is presented in Fig. 3. The sequence contains a 175 nt 5′-untranslated region, 528 nt open reading frame encoding 176 amino acids for salmon preproIGF-I, and 1317 nt in the 3′-untranslated region. No poly-(dA) tail or putative polyadenylation signal sequence(AATAAA) was found, indicating that the 3′-untranslated region is incomplete.

As is the case for mammalian preproIGFs, the predicted primary structure for salmon preproIGF-I is divided into six domains: prepeptide, B, C, A, D, and E domains. We have assigned the initiator methionine codon at nt 176–178 and there is an inframe termination codon located 54 nt upstream which precludes a prior start site. This results in a rather long prepeptide of 44 residues. However, both human and rat preproIGF-I mRNAs also encode long prepeptide segments and Rotwein *et al.* (17) have recently shown that the human prepeptide can function to sequester the proIGF-I into the endoplasmic reticulum when the mRNA is translated *in vitro*.

The homology between salmon and mammalian (pre-pro)IGF-I amino acid sequences is compiled in Fig. 4. As shown, sequence identity within the B, C, A, and D domains, which comprise the mature active form of IGF-I, is very high. There are only 14 amino acid changes out of 70 between human and salmon IGF-I and of these, five are located in the C domain and four are in the D domain. The homology between the E domains in salmon and human proIGF-I is also quite high, being 77% identical (27/35 residues), if a unique 27 amino acid segment (residues 87–113) in the salmon sequence is not included in the comparison. We note that the boundaries of this segment contains consensus intron donor/acceptor sequences (GT/AG) and thus may constitute a mini-intron removed by alternative splicing of the mRNA. However, isolation of a correspondingly spliced cDNA will be necessary to prove this. This sequence identity between salmon and mammalian preproIGF-I is lowest in the prepeptide region. However, the salmon prepeptide clearly contains the necessary hydrophobic residues consistent with its pre-

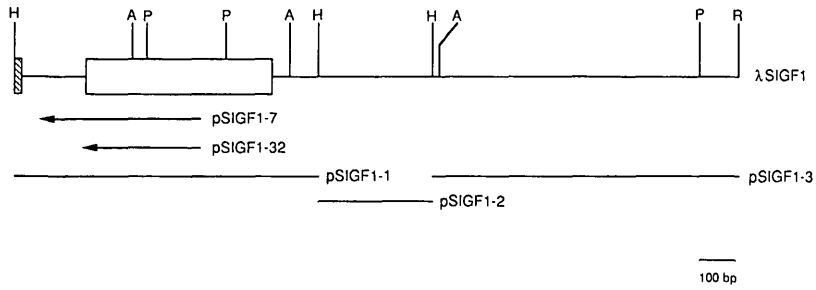

Fig. 2. Molecular Cloning of Salmon preproIGF-I cDNA

The 2.0 kb cDNA insert contained in λSIGF1 is shown and *arrows* indicate cloned cDNA segments (pSIGF1-7, pSIGF1-32) obtained by PCR amplification of the 5′-end using the RACE protocol (16). Restriction fragments of the 2.0 kb insert were subcloned into plasmid form as shown: 1.1 kb *Hind*III (pSIGF1-1), 0.26 kb (pSIGF1-2), and 0.8 kb *Hinb*III-*Eco*RI (pSIGF1-3). The *open box* contains the coding sequence of salmon preproIGF-I; the *stippled box* contains λgt10 sequences which were also subcloned into pSIGF1-1 due to the fact that the *Eco*RI site in the insert was not regenerated during cloning. Restriction sites: A, *Apa*I; H, *Hind*III; P, *Pst*I; R, *Eco*RI.

```
   1 GCTAAATCCGTCTCCAGTTCGCTAAATCTCACTTCTCCAAAACGAGCTGCGCAATGGTTCAAAGTCGGAATATTGAGATGTGACATTGCCTGCATCTTATCCACTTTCTCACTGTTTTT

                                                    -44                 -40                                     -30
                                                    Met Ser Ser Gly His Leu Phe Gln Trp His Leu Cys Asp Val Phe
 119 AATGACTTCAAACAAGTTCATTTTTGCTGGGCTTTGTCGTGCGGAGACCCGTGGGG       ATG TCT AGC GGT CAT TTA TTC CAG TGG CAT TTA TGT GAT GTC TTC

                                  -20                                 -10                                      1
     Lys Ser Ala Met Cys Cys Ile Ser Cys Thr His Thr Leu Ser Leu Leu Leu Cys Val Leu Thr Leu Thr Ser Ala Ala Thr Gly Ala Gly
 221 AAG AGT GCG ATG TGC TGT ATC TCC TGT ACC CAC ACC CTC TCA CTG CTG CTG TGC GTC CTA ACC CTG ACT TCG GCG GCA ACA GGG GCG GGG

                                  10                                  20                                      30
     Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Thr Leu Gln Phe Val Cys Gly Glu Arg Gly Phe Tyr Phe Ser Lys Pro Thr Gly Tyr
 311 CCC GAG ACC CTG TGT GGG GCG GAG CTG GTG GAC ACG CTG CAG TTT GTG TGT GGA GAG AGA GGC TTT TAT TTC AGT AAA CCA ACG GGG TAT

                                  40                                  50                                      60
     Gly Pro Ser Ser Arg Arg Ser His Asn Arg Gly Ile Val Asp Glu Cys Cys Phe Gln Ser Cys Glu Leu Arg Arg Leu Glu Met Tyr Cys
 401 GGC CCC AGT TCA CGG CGG TCA CAT AAC CGT GGT ATT GTG GAC GAG TGC TGC TTC CAG AGT TGC GAG CTG CGG CGG CTC GAA ATG TAC TGT

                                  70                                  80                                      90
     Ala Pro Val Lys Ser Gly Lys Ala Ala Arg Ser Val Arg Ala Gln Arg His Thr Asp Met Pro Arg Thr Pro Lys Val Ser Thr Ala Val
 491 GCC CCT GTC AAG TCT GGC AAG GCA GCT CGC TCT GTG CGC GCA CAG CGC CAC ACA GAC ATG CCA AGA ACA CCC AAG GTT AGT ACT GCA GTG

                                 100                                 110                                     120
     Gln Asn Val Asp Arg Gly Thr Glu Arg Arg Thr Ala Gln His Pro Asp Lys Thr Lys Pro Lys Lys Glu Val His Gln Lys Asn Ser Ser
 581 CAA AAC GTG GAC CGA GGC ACA GAG CGT AGG ACA GCA CAG CAC CCA GAC AAG ACA AAA CCC AAG AAG GAG GTA CAT CAG AAG AAC TCA AGT

                                 130     132
     Arg Gly Asn Thr Gly Gly Arg Asn Tyr Arg Met AM
 671 CGA GGA AAC ACA GGG GGA AGG AAC TAC CGA ATG TAG AAGAAGACAGGAGCTAACGGACAGGCGGACAGGGATAAGAGAGGGCGGGCCCATACCTGGTGCCTCTGTGGA

 779 ATGGTTCACTGTAAAACAACAAAAGGTGGATTATGGTACTAAGATAATGGTAATAAGCTTTGTGTGTAATGTCTTCCCTTAAGGCTGAGAGGTGAAGTGCTGGGTTATATGAGAGGTATT

 899 TATTATTTATACACTGCACCATTTCATATTGGAAGGAATTATATCCATGACAAAACCAATGTACAGACTAGTTAGCTGCTACACATCAAGAGCCTCTCTCTAACTCATGTGTGAGCTGAG

1019 CTATAGTGTCCTGTAGACCTCTGATAGGCTCTGTGCTACATACAGCCAATAGGACGGCAGCCAGCAGACAGTTTTACCTGTCTGTCTATGGGAGCCAATGGTTCATCCTCTTTACCTGAA

1139 CTCTGAAAGAGTTTAAGCTTTACACTGAGAGGTTGGGCCCATGCAAAGCAGTCGGGCTATTGGATAAGTAATCTCCCCCATTCTCATCATACAACCACTGCTTTGATAATATATTAGAAC

1259 CACAAGAGAATGTTATTTCAGCTTCAGTCAGTTCTGACCAAAAGATAATTTCACAGTTGTCACGTGTTCATTAAGGGCTTTATTTACTATTGACTGCTTCAACAGACAATAGCGTATTTA

1379 CATGCAACCATAACATAAGAACCAGGCTTGAGGCACATATGACAGAGACTATCTGATGATACACTCTGACTGCTAGCATCTAGTGAACTGACAGTACTATATTACGTACGTACGAGTCTG

1499 AGGTCATATATTGAGCTCGGTCGCTACTGCTGTAGCTCGCGCCGCTTACTGTAAACAAGAAAGTACTGAAGGAAGATTCAATTTATATTTAATTTTGTAAAGACTCACAGTAGATTCTCT

1619 GGGTCTAATATCTGGTCTGATTAAAAATGGATTCTAATGCAGAGGACAGGACATAGGTGCTCTAAAAATAGCCAGCTACAGGAAGACGATTAATGATTGATTTCACTTGGCCTGCATTCT

1739 TTTATTTTGTTAGCTTAGGGAGGGACCTTTCTTCTTCTGATGAACATGCTGACGATGGATTTTCATTGCAAAAGGCTATAAAATGAACTGCAACACATATAGTTCACTCAAGTGAATGAT

1859 ATCTAGAACTCAAATACTGTTAGAAACACACACCTACAAACAGGGCTGCAGTTTAAAAATGCTGTGCACACACACACACACAGTCCGTTTTGTAGCTCAGCCACTCAGAGCTCTGCTA

1979 CTGTGAGCAAGGTTCACACGCTGGTTCCATCAGACCATGTTC - 2020
```

Fig. 3. Nucleotide Sequence and Deduced Amino Acid Sequence of Salmon preproIGF-I cDNA

The 2020 bp sequence was determined from direct sequencing of λSIGF1 and from plasmid subclones pSIGF1-1, pSIGF1-2, and pSIGF1-3 as shown in Fig. 2. The prepeptide is contained in residues numbered −1 to −44; B domain, 1 to 30; C domain, 31 to 41: A domain, 42 to 62; D domain, 63 to 70; E domain, 71 to 132.

sumed function in translocating the prohormone into the endoplasmic reticulum for secretion.

To further characterize salmon preproIGF-I mRNA, Northern blot analysis of salmon liver mRNA was performed. As shown in Fig. 5A, poly(A)-enriched mRNA from GH-treated salmon liver or untreated controls were electrophoresed in a denaturing 1% agarose-formaldehyde gel, transferred onto a nitrocellulose membrane, and hybridized with salmon preproIGF-I cDNA. The autoradiogram revealed a major band 3.9 kb in length; after prolonged exposure several minor bands at 6.0 kb, 2.8 kb, and 1.0 kb were also observed. Figure 5A also demonstrates that hepatic RNA from coho salmon injected with GH contains more preproIGF-I mRNA than RNA from untreated salmon. Based on densitometric scanning of the x-ray film, preproIGF-I mRNA is increased 6-fold in GH-treated liver *vs.* control. In contrast, the same blot, stripped by immersion in boiling water for 5 min and reprobed with labeled mouse actin cDNA, contained the same amount of 1.6 kb actin mRNA in the GH-treated and untreated livers (Fig. 5B).

DISCUSSION

Our analysis of the salmon preproIGF-I cDNA has revealed that the highly conserved amino acid sequence

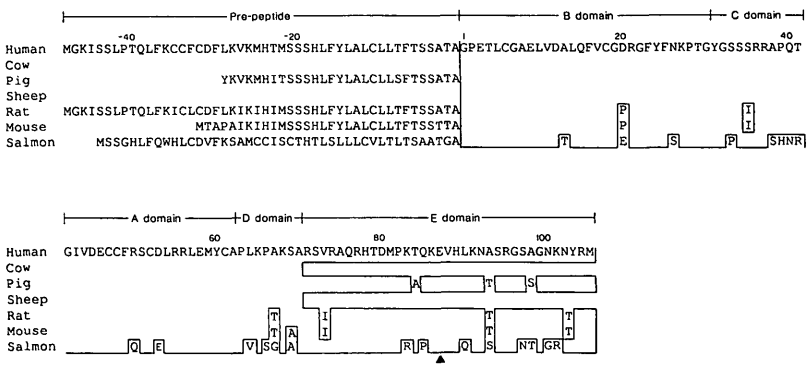

Fig. 4. Comparison of Mammalian and Salmon (prepro)IGF-I Amino Acid Sequences

Human (7), pig (11), rat (9), mouse (10), and salmon preproIGF-I sequences are compared and amino acid residues identical to the human proIGF-I sequence are shown enclosed within the box. The amino acid sequences of cow and sheep IGF-I (12, 13) are also compared. To maximize homology in the E domain, the *triangle* indicates the position where a 27 residue segment predicted by the salmon preproIGF-I cDNA sequence has been omitted.

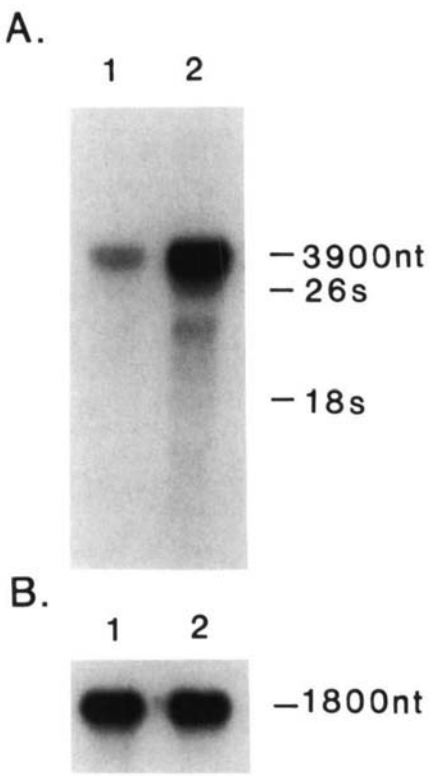

Fig. 5. Northern Blot Analysis of Salmon Liver mRNA

A, Ten micrograms of poly(A$^+$) mRNA from GH-treated (lane 2) or control (lane 1) salmon liver was electrophoresed in a 1% agarose-formaldehyde gel, transferred onto nitrocellulose membrane, and hybridized with 2.0 kb salmon preproIGF-I cDNA labeled by nick translation. The blot was washed in 0.25× SSC at 65 C and autoradiographed for 10 h at −70 C with an intensifying screen. B, The same blot, after immersion in boiling water for 5 min, was reprobed with mouse actin cDNA. After hybridization, the blot was washed in 0.5× SSC, 0.1% SDS at 55 C and autoradiographed for 5 h. Sizes of the mRNAs were determined by comparison with salmon 23s (3700 nt) and 16s (1900 nt) rRNA.

and regulated expression of the IGF-I gene observed in mammalian species can be extended to the bony fishes, which diverged during evolution about 400 million ago. The deduced primary sequence for salmon IGF-I is identical with human IGF-I in 56 out of 70 residues and indeed, the B and A domains contain only 5 out of 50 changes in amino acids. By comparison, the homologous B and A peptide chains in human and salmon insulin differ in 15 out of 51 residues (18).

There are several possible explanations for the high conservation of the primary structure of IGF-I during evolution. One obvious explanation, of course, is that tertiary structural determinants which are required for IGF-I to bind to its receptor and to activate the hormone's biological response, place strong constraints on its primary structure. In addition, unlike insulin, IGF-I does not circulate freely in the blood but is rapidly bound to serum proteins, termed IGF-binding proteins (19). The physiological function of these proteins is not clear but the fact that they bind IGF-I (and IGF-II) with high specificity and high affinity would be expected to place additional structural constraints on the molecule. Recent studies have implicated the B domain of IGF in interactions with these binding proteins (20), particularly the N-terminal tripeptide (21) which is also conserved in salmon IGF-I. Finally, although IGF-I is produced primarily in the liver, significant amounts of IGF-I is synthesized in many other tissues where it acts locally in an autocrine fashion (2). The requirement for biosynthesis of IGF-I in multiple tissues with different internal environments could also inhibit evolutionary change. It is likely that the above factors all contribute to a low mutation acceptance rate for IGF-I during evolution.

In addition to IGF-I, proIGF-I contains a C-terminal peptide extension, the E domain, and the well conserved sequence of this domain among mammalian proIGF-I molecules has led to the proposal that this peptide may have biological activity (9). Our finding that the E domain in salmon proIGF-I is also well conserved provides further support for this hypothesis (Fig. 4). Important caveats here, however, are that in humans,

rats, and mice, differential RNA splicing produces multiple forms of preproIGF-I mRNA which encode E domains with highly variable amino acid sequences (10, 22, 23). Moreover, the sequence identity between salmon and mammalian proIGF-I E domains, shown in Fig. 4, is high only if an internal 27 residue segment, predicted from the salmon cDNA sequence, is deleted. Clearly, further investigation will be necessary to establish the biological function of the E domain.

Northern blot hybridization of liver preproIGF-I mRNA revealed that the regulated expression of the IGF-I gene by GH has been conserved in salmon. As shown in Fig. 5, hepatic RNA from coho salmon injected with bGH showed a 6-fold increase in preproIGF-I mRNA level compared with RNA from untreated salmon liver. Interestingly, the Northern blot also revealed that in salmon, the preproIGF-I mRNA is expressed predominantly as a single 3.9 kb band. In contrast, mammalian species usually contain multiple preproIGF-I mRNAs of different sizes due to alternative RNA processing and/or transcription from different promoters (24, 25). It thus appears possible that the transcription and processing of preproIGF-I mRNA is simpler in salmon.

The amino acid sequence of salmon IGF-I, deduced from the cloned cDNA, should facilitate the development of immunological assays for salmon IGF-I. Previous attempts to measure IGF-I levels in piscine species using antisera prepared against mammalian IGF-I have met with only limited success probably because the antibodies are mostly directed against more variable epitopes located in the C and D domains (26–28). By immunizing with peptides synthesized to the salmon IGF-I C and D domains, more specific antisera should be produced and these can be used to further investigate the biological function and regulated expression of IGF-I in salmon and related fish species.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA modifying enzymes were purchased from Boehringer Mannheim (Indianapolis, IN), BRL (Bethesda, MD), and New England BioLabs (Beverly, MA). Plasmid pGEM4Z was obtained from Promega (Madison, WI); nick-translation kit was from Amersham (Arlington Heights, IL); nitrocellulose disks and membranes were from Schleicher and Schuell (Keene, NH). Radioisotopes were purchased from Dupont NEN (Boston, MA). Oligonucleotides were synthesized on a Applied Biosystems Model 380B DNA Synthesizer and used without further purification. Cloned mouse actin cDNA was a gift from Peter Engler (The University of Chicago). Bovine GH (Lot BGH-B1) was obtained from the USDA Reproduction Laboratory (Beltsville, MD).

Animals

Juvenile coho salmon weighing between 12 and 24 g were maintained in aquatic tanks under natural conditions of photoperiod and water temperature (11 C) and were fed standard rations of Oregon Moist Pellets. Fish were anesthetized with tricaine methanesulfonate and injected ip with 5 μg bGH/g Bw dissolved in sterile 0.15 M NaCl. Control fish received an equivalent volume of 0.15 M NaCl. The injection procedure was repeated after 24 h and after 48 h the fish were killed by a sharp blow to the head. The livers were collected and stored at −70 C until used.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from salmon liver using the guanidine thiocyanate procedure (29); poly(A+) mRNA was prepared by affinity chromatography on oligo dT-cellulose (30). For Northern blots, poly(A^+) mRNA was electrophoresed in 1% agarose gel containing 0.66 M formaldehyde and bound to nitrocellulose membrane by capillary transfer in 10× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na Citrate, pH 7.0). Hybridization was performed in 50% formamide, 5× SSC, 10% dextran sulfate, 2.5× Denhardt's solution (31), 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml calf thymus DNA at 37 C. The blot was washed in 2× SSC, 1× SSC, 0.5× SSC at room temperature, and in 0.25× SSC at 65 C (all wash solutions contained 0.1% SDS), air dried, and exposed to x-ray film at −70 C with an intensifying screen (Dupont Lightning Plus).

PCR and cDNA Cloning

Complementary DNA was synthesized from poly(A^+) mRNA using MLV reverse transcriptase (BRL). PCR was performed as described (15) using two degenerate oligonucleotide primers that corresponded to conserved NH_2-terminal and C-terminal regions in the IGF-I A domains: I-1,

```
5′GGTATCGTGGAGGAGTGCTGC encode the sequence
        A  A  A  A  T  T
        T  C  C
           T  T
         Asp
```

Gly-Ile-Val- Glu-Glu-Cys-Cys; I-2,

```
5′GCGCAGTACATCTCGAG encode the cDNA corresponding
   A  A        T  T
```

to Leu-Glu-Met-Tyr-Cys-Ala.

PCR was performed on a DNA thermal cycler (Perkin Elmer-Cetus) for 30 cycles at 94 C for 1 min, 50 C for 2 min, 65 C for 2 min. Rapid amplification of the IGF-I cDNA ends (RACE) was performed as described (16).

For molecular cloning, salmon liver cDNA was converted into double stranded DNA with *Escherichia coli* DNA polymerase I (Klenow) using a cDNA synthesis kit (BRL). The DNA was treated with *Eco*RI methylase (New England Biolabs) and ligated with *Eco*RI linkers (New England Biolabs) using T4 DNA ligase. After digestion with *Eco*RI, the DNA was precipitated with 2 vol ethanol in 2.5 M ammonia acetate, ligated to λgt10-*Eco*RI vector and packaged *in vitro* (Stratagene). Hybridization screening, plaque purification, and subcloning of the recombinant cDNA insert into pGEM4Z were performed using standard procedures (31). DNA sequence analysis was performed by the dideoxynucleotide chain termination procedure (32) using oligonucleotide primers and Sequenase (U.S. Biochemicals, Cleveland, OH).

Acknowledgments

The authors thank Mrs. Florence Rozenfeld for her skillful assistance in the preparation of this manuscript.

Received August 23, 1989. Revision received September 13, 1989. Accepted September 13, 1989.

Address requests for reprints to: Dr. Shu Jin Chan, Howard Hughes Medical Institute, The University of Chicago, Box 23, 5841 S. Maryland Avenue, Chicago, Illinois 60637.

Work from our laboratories has been supported by NIH Grants DK-13914 and DK-20595 (D.F.S.), NSF Grant DCB861-

5551 (E.P.), and Sea Grant R/A-49 (E.P. and W. W. Dickhoff), and the Howard Hughes Medical Institute (D.F.S. and S.J.C.).

REFERENCES

1. Van Wyk JJ 1984 In: Li CH (ed) Hormonal Proteins and Peptides: Growth Factors. Academic Press, New York, vol 12:81–125
2. Froesch ER, Schmid C, Schwander J, Zapf J 1985 Actions of insulin-like growth factors. Annu Rev Physiol 47:443–467
3. Froesch ER, Bürgi H, Ramseier EB, Bally P, Labhart A 1963 Antibody-suppressible and non suppressible insulin-like activities in human serum and their physiologic significance. J Clin Invest 42:1816–1834
4. Rinderknecht E, Humbel RE 1978 The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 253:2769–2776
5. Rinderknecht E, Humbel RE 1978 Primary structure of human insulin-like growth factor II. FEBS Lett 89:283–286
6. Blundell TL, Bedarkar S, Rinderknecht E, Humbel RE 1978 Insulin-like growth factor: A model for tertiary structure accounting for immunoreactivity and receptor binding. Proc Natl Acad Sci USA 75:180–184
7. Jansen M, Van Schaik FMA, Ricker AT, Bullock B, Woods DE, Gabby KH, Nussbaum AL, Sussenback JS, Van Den Grande JL 1983 Sequence of a cDNA encoding human insulin-like growth factor I precursor. Nature 306:609–611
8. Chan SJ, Emdin SO, Kwok SCM, Kramer JM, Falkmer S, Steiner DF 1981 Messenger RNA sequence and primary structure of preproinsulin in a primitive vertebrate, the Atlantic hagfish. J Biol Chem 256:7595–7602
9. Shimatsu A, Rotwein P 1987 Mosaic evolution of the insulin-like growth factors. J Biol Chem 262:7894–7900
10. Bell GI, Stempien MM, Fong NM, Rall LB 1986 Sequences of liver cDNAs encoding two different mouse insulin-like growth factor I precursors. Nucleic Acids Res 14:7873–7882
11. Tavakkol A, Simmen FA, Simmen RCM 1988 Porcine insulin-like growth factor-I (pIGF-I): complementary deoxyribonucleic acid cloning and uterine expression of messenger ribonucleic acid encoding evolutionary conserved IGF-I peptides. Mol Endocrinol 2:674–681
12. Honegger A, Humbel RE 1986 Insulin-like growth factors I and II in fetal and adult bovine serum. J Biol Chem 261:569–575
13. Francis GL, McNeil KA, Wallace JC, Ballard FJ, Owens PC 1989 Sheep insulin-like growth factors I and II: sequences, activities and assays. Endocrinology 124:1173–1183
14. Dawe SR, Francis GL, McNamara PJ, Wallace JC, Ballard FJ 1988 Purification, partial sequences and properties of chicken insulin-like growth factors. J Endocrinol 177:173–181
15. Nishi M, Chan SJ, Nagamatsu S, Bell GI, Steiner DF 1989 Conservation of the sequence of islet amyloid polypeptide in five mammals is consistent with its putative role as an islet hormone. Proc Natl Acad Sci USA, in press
16. Frohman MA, Dush MK, Martin GR 1988 Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85:8998–9002
17. Rotwein P, Folz RJ, Gordon JI 1987 Biosynthesis of human insulin-like growth factor I (IGF-I). J Biol Chem 262:11807–11812
18. Plisetskaya EM, Pollock HG, Rouse JB, Hamilton JW, Kimmel JR, Gorbman A 1985 Characterization of coho salmon (*Oncorhynchus kisutch*) insulin. Regul Pept 22:105–116
19. Hintz RL 1984 Plasma forms of somatomedin and the binding protein phenomenon. Clin Endocrinol Metab 13:31–42
20. Joshi S, Burke T, Katsoyannis PG 1985 Synthesis of an insulin-like compound consisting of the A chain of insulin and a B chain corresponding to the B domain of human insulin-like growth factor I. Biochemistry 24:4208–4214
21. Szabo L, Mottershead DG, Ballard FJ, Wallace JC 1988 The bovine insulin-like growth factor (IGF) binding protein purified from conditioned medium requires the N-terminal tripeptide in IGF-I for binding. Biochem Biophys Res Comm 151:207–214
22. Roberts Jr CT, Lasky SR, Lowe Jr WL, Seaman WT, LeRoith D 1987 Molecular cloning of rat insulin-like growth factor I complementary deoxyribonucleic acids: differential messenger ribonucleic acid processing and regulation by growth hormone in extrahepatic tissues. Mol Endocrinol 1:244–248
23. Rotwein P 1986 Two insulin-like growth factor I messenger RNAs are expressed in human liver. Proc Natl Acad Sci USA 83:77–81
24. Rotwein P, Pollock KM, Didier DK, Frivi GG 1986 Organization and sequence of the human insulin-like growth factor I gene. J Biol Chem 261:4828–4832
25. Lowe Jr WL, Roberts Jr CT, Lasky SR, LeRoith D 1987 Differential expression of alternative 5′ untranslated regions in mRNAs encoding rat insulin-like growth factor I. Proc Natl Acad Sci USA 84:8946–8950
26. Wilson DM, Hintz RL 1982 Inter-species comparison of somatomedin structure using immunological probes. J Endocrinol 95:59–64
27. Daughaday WH, Kapadia M, Yanow CE, Fabrick K, Mariz IK 1985 Insulin-like growth factors I and II of nonmammalian sera. Gen Comp Endocrinol 59:316–325
28. Funkenstein B, Sibergeld A, Cavari B, Laron Z 1989 Growth hormone increases plasma levels of insulin-like growth factor (IGF-I) in a teleost, the gilthread seabream (*Sparus aurata*). J Endocrinol 120:R19–R21
29. Chirgwin JM, Przbyla AE, McDonald RJ, Rutter WJ 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299
30. Aviv H, Leder P 1972 Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA 69:1408–1412
31. Maniatis T, Fritsch EF, Sambrook J 1982 In: Molecular Cloning—A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Sanger FS, Nicklen S, Coulson AR 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 75:5463–5467