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**Partial sequence and characterization of a growth hormone gene
of the red drum (*Sciaenops ocellatus*)**

Dorworth, Carol Marie, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1991

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

PARTIAL SEQUENCE AND CHARACTERIZATION OF A
GROWTH HORMONE GENE OF THE RED DRUM (*Sciaenops ocellatus*)

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Forestry, Wildlife, and Fisheries

by

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May 1991

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ABSTRACT

A growth hormone (GH) gene was isolated from nuclear DNA of the red drum (Sciaenops ocellatus). Synthetic oligonucleotides were synthesized based on published cDNA sequences of other perciform fishes. Polymerase chain reaction (PCR) methodology was employed to amplify a nucleotide sequence extending from the 5' end of intron I to 15 bases upstream of the 3' end of exon VI of the GH gene. Amplified DNA was cloned into a phagemid sequencing vector. Clones were sequenced. Computer analysis of sequence data was used to determine the internal gene arrangement of exons and introns. Homologies of the nucleotide and deduced amino acid sequences with published GH sequences were determined. The GH nucleotide sequence was examined for the presence of regulatory elements.

The red drum GH gene is comprised of six exons and five introns, an arrangement like that of the salmonid GH genes but unlike the five exon, four intron arrangement of carp (Cyprinus carpio) and mammalian GH genes. Protein coding regions of the red drum GH gene show a high degree of homology with GH cDNA sequences of other perciform fishes. The 3' terminus of exon V of the red drum GH gene shows an 86% similarity with a region of exon V of the human chorionic somatomammotropin gene. A putative glucocorticoid receptor element is present in intron I of the red drum GH gene.

INTRODUCTION

The red drum (*Sciaenops ocellatus*) presents a challenge to the aquaculture industry. The species has been an historically important food fish (Overstreet 1983) but current high market demand cannot be met by natural stocks (Texas Agricultural Extension Service 1986). Pilot red drum culture operations have been inaugurated (Johnson 1990), but production traits of the species and management techniques must be improved before intensive culture will be profitable.

Growth hormone (GH) is a pituitary polypeptide that, in fishes, acts to regulate important production traits: growth, feed conversion ratios, and osmoregulation. There are no reports of studies of red drum GH at the molecular level. Red drum GH DNA sequence data will allow identification of GH gene regulatory elements and prediction of GH polypeptide structure and function. Clones of the red drum GH gene can be used in investigations of gene expression and as probes for library screening, DNA fingerprinting (Gonzalez-Villasenor et al. 1986; Hallerman and Beckman 1987), and restriction fragment length polymorphism analyses (RFLP) (Theilmann et al. 1989). The objectives of the present study were to isolate and clone the gene coding for GH in the red drum, to sequence the gene, and to analyze the sequence data.

LITERATURE REVIEW

Field and laboratory studies have been conducted to extend basic knowledge of important red drum production traits. Lee et al. (1984) studied the effect of temperature on the growth of red drum larvae and Trimble (1981) performed grow-out studies in brackish ponds. Crocker et al. (1981) compared growth and survival of juveniles in fresh and salt water and determined that low salinity tolerance was directly correlated with age. Miranda and Sonski (1985), conducting experiments with calcium chloride treated water, concluded that chloride ion concentration was a factor determining red drum survival rates. Wurts and Stickney (1989), controlling for chloride ion, determined that calcium ion was essential. Osmoregulatory dysfunction has been documented as a factor in handling stress mortality (Caldwell and Tomasso 1985) and correlations between salinity and feed conversion have been reported (Crocker et al. 1981).

Levels of GH in fishes affect production. Serum GH is a single chain protein of about 200 amino acids with a molecular weight about 22 000 (Farmer et al. 1976). Three dimensional structure of the natural protein has not been determined but the structure of a genetically engineered porcine GH is an elongated molecule including four helices (Abdel-Meguid et al. 1987). This structure is, in general, like that predicted for coho salmon (*Oncorhynchus kisutch*) GH (Nicoll et al. 1987). Pituitary preparations of GH are

heterogeneous in size and include fragments of the full length protein (Lewis et al. 1980). These fragments may have some biological activities that have not been identified. Paladini et al. (1979) reviewed the multiple actions of GH in mammals and presented the hypothesis that fragments could have biological significance. A 20 kilodalton variant comprises about 10% of human GH. This variant results from alternative splicing of the primary GH gene transcription product. The alternative splice site represents a site in intron B of the gene (deNoto et al. 1981). The translation product retains full growth promoting activity but lacks normal GH insulin-like activity (Chapman et al. 1981). No analogous variant has been identified in fish GH preparations.

Plasma level of GH is the major factor determining the overall somatic growth rate of fishes (Donaldson et al. 1979). The growth promoting effects of GH apply to skeletal (Raisz and Kream 1981) and soft tissue, especially muscle (Wallis et al. 1985). Endogenous levels of GH in fishes are affected by environmental cues (Jenkin 1970). Bjornsson et al. (1989) found GH levels in Atlantic salmon (*Salmo salar*) to be affected by photoperiod and temperature during smoltification.

Administration of exogenous fish or mammalian GH is effective in promoting fish growth. Injections of naturally produced tuna (*Thunnus thynnus*) GH into snapper (*Pagrus major*) promoted growth in that species (Sato et al. 1988). Administration of natural bovine GH (bGH) accelerated growth of grass pickerel (*Esox americanus*) (Weatherly and Gill 1987), and

recombinant bGH doubled the growth rate of coho salmon (Gill et al. 1985; Down et al. 1988).

Activity of administered exogenous GH may be affected by environmental factors. Danzmann et al. (1990) found a depressive effect of high temperature on growth promoting activity of natural bovine GH or recombinant rainbow trout GH (rtGH) administered to rainbow trout (*Oncorhynchus mykiss*) maintained at 17°C. This temperature would not be expected to adversely affect bGH and the failure of a growth response could have been due to temperature effect on a later step in the growth pathway.

In mammals, the skeletal growth promoting effects of GH appear to be mediated through the actions of a family of peptide hormones, the somatomedins (insulin-like growth factors, IGF-I and IGF-II) (Wallis et al. 1985). Whether an IGF is always necessary for GH action is not clear, but transgenic mice exhibiting high levels of foreign gene expression have high levels of IGF-I and exhibit increased growth (Mathews et al 1988). IGF-I also promotes growth in hypophysectomized rats (Schoenle et al. 1982). There is evidence that somatomedins are present in fishes but it has not been demonstrated that these participate in growth regulation. Lindahl et al. (1985) obtained positive results when they reacted Baltic (Atlantic) salmon serum in a human placental radioreceptor assay for somatomedin; the serum did not cross-react in a radioimmunoassay, however. Nagamatsu et al. (1991) recently cloned a preproinsulin-like growth factor cDNA from the hagfish (*Myxine glutinosa*). The deduced partial amino acid sequence revealed organization

like that of mammalian prepro-IGFs and residues invariant in mammalian IGFs are conserved in the hagfish IGF. Skyrud et al. (1989) found that administration of low dosages of human IGF-I to brook trout (*Salvelinus fontinalis*) had no effect on growth rates and high dosages decreased growth. These results suggest major interspecific differences between teleost and human IGFs or differences in their biological activities.

In mammals, GH acts indirectly, in complex interrelations with other hormones, to promote growth by affecting nutrient metabolism. Administration of GH causes nitrogen retention, possibly due to stimulation of protein synthesis, increased utilization of lipids, and reduced glucose uptake by peripheral tissues (Wallis et al. 1985). Feed utilization efficiency is of particular importance in the culture of those fish species requiring high protein, high cost feeds, and GH has been shown to improve feed conversion. Markert et al (1977) injected yearling coho salmon with bGH and found enhanced growth, improved feed conversion ratios, and accelerated protein synthesis. Foster et al. (1991) studied protein turnover in rainbow trout implanted with ovine GH (oGH) and found oGH significantly improved body growth and tissue protein accretion rates but had little effect on protein degradation rates.

The role of GH in osmoregulation has not been precisely defined. GH is generally considered to be effective in the adaptation of fish to seawater, while the closely related hormone, prolactin (PRL), aids in adaptation of salmonids to fresh water (Prunet et al. 1985) and is essential for the adaptation of

euryhaline species to fresh water (Farmer 1986). Hypophysectomized coho salmon were able to survive in fresh water but not in seawater (Nishioka et al. 1987), suggesting that PRL is not necessary for salmonid survival in fresh water but GH is necessary for survival in seawater. Cortisol may be a controlling agent in the adaptation of euryhaline fishes to seawater (Barron 1986).

Studies of osmoregulatory capacity have been mainly directed toward determining the actions of GH and PRL in the smoltification process of salmonids. Tilapia (*Oreochromus mossambica*) GH facilitated the adaptation of sockeye salmon (*Oncorhynchus nerka*) to seawater (Clarke et al 1977). Komourdjian et al. (1976) found that injections of porcine GH improved growth of Atlantic salmon held in fresh water and improved survival of the fish after transfer to saline water. Hoar (1988) reported that GH improved survival of salmonids in seawater and Young et al. (1989) found elevated levels of GH in coho salmon during periods of seawater adaptation. Prunet et al. (1989) measured plasma GH levels of Atlantic salmon during periods of smoltification and concluded that GH may be a major factor in the development of hypoosmoregulatory activity. Not all studies agree, however. Leatherland and McKeown (1974) found no significant differences in plasma GH in Kokanee sockeye salmon smolts acclimated to either seawater, one-third seawater, or fresh water. The negative results may have been due to the effects of other environmental factors.

There has been less research interest in osmoregulation of non-salmonid fishes and the results of the above studies may not be applicable to non-

salmonid species. However, in a review of endocrine action in smoltification, Barron (1986) notes that all influences on hypoosmoregulatory capacity attributed to GH could be the result of thyroid hormone activity, which increases at the time of smoltification.

Additional effects of GH in fishes have been identified. Regulation of antifreeze protein production in flounder (*Pseudopleuronectes americanus*) by GH has recently been reported (Idler et al. 1989). GH may affect spawning (Reddy and Lam 1991) and the hatching of fish eggs (Stacey et al. 1984), possibly by enhancing the conversion of thyroxine to triiodothyroxine (deLuze and Leloup 1984).

The gene coding for GH is a member of a gene family which, in mammals, includes genes coding for GH, PRL, chorionic somatomammotropin (SMT), and proliferin (PL). These genes are thought to have evolved through duplication of a small ancestral gene (West 1981). Agellon et al. (1988a) did not find confirmatory sequences in the rainbow trout GH gene, however. The human genome contains one GH gene which is fully functional in adult animals, and a second GH gene that is expressed at very low levels and only in placental tissues. The SMT and PL genes are expressed only in placental tissues.

Salmonids appear to have two functional GH genes. Kawauchi et al. (1986) isolated two forms of chum salmon GH. Agellon et al. (1988b) compared two rainbow trout GH mRNA sequences, one sequence was deduced from a genomic GH gene sequence and one from a cloned cDNA sequence. The

investigators found 11 variant residues and concluded that rtGH is transcribed by two gene loci, duplicated during salmonid tetraploidization. Internal arrangement of the mammalian genes is of five exons and four introns (Eberhardt et al. 1988). The GH gene of the common carp (*Cyprinus carpio*) has a mammalian-like arrangement (Chiou et al. 1990) while the rainbow trout GH gene has six exons and five introns (Agellon et al. 1988a). Present classification schemes consider the salmonids a primitive taxon with origins predating the divergence of other teleosts, including the carp (Greenwood et al. 1966). This is not supported by the exon/intron arrangements of the trout, carp, and mammals.

Regulation of GH levels at the level of gene transcription is complex and has been intensively studied in mammals. Cell specificity of transcription is conferred by nuclear factors which bind to the promoter region upstream of the GH gene transcription start site (West et al. 1987; Castrillo et al.; Courtois et al. 1990). GH gene expression is regulated by the hypothalamic growth hormone releasing factor (Barinaga 1985), which also controls GH secretion, and by glucocorticoids (Slater et al. 1985), thyroid hormone (Ye et al. 1988) and retinoic acid (Bedo et al. 1989). Hormone regulation occurs through the binding of ligand-bound hormone receptors to specific DNA sequences, hormone response elements (HREs) located in the 5' or 3' flanking regions or in introns (Slater et al. 1985; Birnbaum and Baxter 1986; Selden et al. 1989). Hormone receptor/ligand elements form homo- or heterodimers before

binding; the complexity of possible interactions and regulatory effects is analyzed by Vedeckis (in press).

Advances in recombinant DNA biotechnology have enabled researchers to genetically engineer fishes by transferring foreign genes into the DNA complement of a host (Maclean 1987). McEvoy et al. (1988) obtained expression of a β -galactosidase gene from a metallothionein promoter after injecting the fusion gene into Atlantic salmon eggs. Dunham et al. (1987) microinjected a fusion product of the mouse metallothionein gene promoter and the genomic human GH gene into channel catfish (*Ictalurus punctatus*) eggs and Brem et al. (1988) injected the same into tilapia (*Oreochromis niloticus*) eggs. In both cases, injected DNA was integrated into the genomic DNA of the host fish. Chen et al. (1990) introduced hGH and rtGH genes into carp spp. and loach (*Misgurnus anguillicaudatus*). The foreign genes were expressed and inherited, and transgenic fish exhibited higher growth rates than sibling controls.

The use of mammalian DNA for gene transfer into fishes has potential drawbacks. The nucleotide and amino acid sequences of the GH gene, its product, and its receptors have diverged with the divergence of species. Fish host cells may not contain the transcription factors necessary for maximal gene production of mammalian GH. There are available genomic GH clones of carp (Chiou et al. 1990) and rainbow trout (Agellon et al. 1988), and perciform fish GH cDNA clones have been produced (Momota et al. 1988; Sato et al. 1988b; Rentier-Delrue et al. 1989), but there is evidence that intronic

sequences are necessary for maximal transcription and translation of inserted foreign genes (Brinster et al. 1988; Ottavio et al. 1990). The presence of a glucocorticoid response element in the first intron of the hGH (Slater et al. 1985) gene and of a thyroid response element in the third intron of the rat growth hormone (rGH) gene (Sap et al. 1990) suggests that intronic regions of fish GH genes may be required for maximal function.

Additionally, the insertion of foreign genes has political implications. To minimize the possibility of objections from the public regarding genetic manipulations of a food or sport fish, the preferred procedure should be to alter a genome with material from the host, or from a closely related species.

MATERIALS AND METHODS

Materials

Laboratory chemicals were obtained from Sigma Aldrich (St. Louis, MO); Amresco (Solon, OH); Boehringer Mannheim (Indianapolis, IN); and BioRad (Richmond, CA). Bacto-tryptone, bacto-yeast extract, and bacto-agar were products of Difco Laboratories (Detroit, MI). Restriction endonucleases were from Bethesda Research Laboratories (BRL) (Bethesda, MD), and United States Biochemical Corporation (USB) (Cleveland, OH). Terminal deoxynucleotidyl transferase, SequenaseTM Version 2.0 DNA polymerase, universal primers, sequencing reagents and buffers were from USB. T4 DNA ligase was from USB and New England Biolabs (Beverly, MA); calf intestinal alkaline phosphatase from Boehringer Mannheim Biochemicals, AmpliTaqTM DNA polymerase from Perkin Elmer Cetus (Norwalk, CT); GeneCleanTM from BIO101 (La Jolla, CA). Poly-PakTM cartridges were from Glen Research (Herndon, VA); Sep-PakTM cartridges from Waters Associates (Milford, MA); and Ultrafree-MCTM NMWL filter units from Millipore Products (Bedford, MA). pBLUESCRIPT KS(+)TM was from Stratagene, (La Jolla, CA). DH5 α cells and DNA size markers were from BRL. [α -³⁵S] dATP was obtained from New England Nuclear/DuPont Co. (Wilmington, DE). Standard buffer solutions, media, and chemical abbreviations are listed in Appendix II.

Isolation of DNA from Red Drum Tissue

Liver tissue was excised from a wild caught, tank maintained, male red drum. Tissue was placed immediately in liquid N₂ and transported to storage at -80°C. DNA isolation essentially followed the protocol of Herrmann and Frischauf (1987). Frozen tissue was broken into pieces and approximately 5 g placed in a 30 ml centrifuge tube with 5.0 ml digestion solution (50 mM TRIS pH 8.0, 100 mM EDTA, 0.5% SDS). 150 µl Proteinase K (10 mg/ml) was added and the mixture was incubated overnight at 55°C. The mixture was extracted sequentially with phenol (pH 7.6), phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform. To the aqueous phase was added 0.5 ml 3M sodium acetate (pH 6.0) and 10 ml ethanol. DNA was spooled out of solution and left to air dry. Before use, samples were dissolved in TE buffer. DNA concentrations were determined spectrophotometrically.

Oligonucleotide Primer Synthesis and Purification

Primer design was based on published fish GH cDNA sequences (Fig. 1). Five sequences were identified that showed little variation among perciform species and no variation in the 3' (primer) terminal three bases. Based on salmon genomic DNA sequence data, the primers were predicted to be located at the 5' and 3' termini and in the second, third and fourth exons. 5' ends of the primers were extended to incorporate restriction enzyme recognition sites and to provide a G + C content of approximately 50%.

		1	
primer 1	5'	GACGGATCCAGACATGGACAGAG	3'
tuna		5' CCAGACATGGACAGAG	3'
yellowtail		CCAGCCATGGACAGAG	
tilapia		CCAGCCATGAACTCAG	
primer 5	5'	GACAGTACTGCTGTCTATCTCCTAT	3'
sea bream		5' CTGCTGTCTATCTCCTAT	3'
tuna		CTGCTGTCGATCTCCTAT	
yellowtail		CTGTTATCGATCTCCTAT	
primer 2		5' GAATTCTGCTCCGAGGAACCA	3'
sea bream		5' TTCTGCTCCGAGGAACCA	3'
tuna		TTCTGCTCCGAGGAACCA	
yellowtail		TTCTGCTCTGAGGAACCA	
primer 3	3'	CCTTAGGTAGAGGACTAGCGCTCAG	5'
sea bream	5'	GGAATCCATCTCCTGATC	3'
tuna		GGAATCCAGCTGCTGATC	
yellowtail		GGAATCCAACCTGCTGATC	
primer 4	3'	TTGACGTGGGACATCGGCTTAAGTCG	5'
sea bream	5'	AACTGCACCCTGTAG	3'
tuna		AACTGCACCCTGTAG	
yellowtail		AACTGCACCCTGTAG	

Figure 1. PCR oligonucleotide primers and published cDNA sequences on which primer design was based. References: sea bream (Momota et al. 1988), tuna (Sato et al. 1988), yellowtail (Watahiki et al. 1988). Primer extensions are shown without matching cDNA sequences.

Oligonucleotide primers were synthesized on an Applied Biosystems 380A DNA Synthesizer by the Louisiana State University College of Basic Sciences Core Facility. Primers were column purified and detritylated on Poly-PakTM cartridges using a modification of the manufacturer's protocol. Columns were flushed with 5 ml 25 mM TEAB. Primer solutions were loaded and forced through the column at a rate of 1 to 2 drops per second. Eluant was reloaded and eluted. The column was flushed 3X with dilute NH_4OH , 2X with 10 ml ddH_2O , 2X with 5 ml TFA, and 2X with 10 ml ddH_2O . Purified, detritylated oligos were flushed off column with 5 ml 30% acetonitrile. Aliquots were removed and scanned spectrophotometrically from 220 to 320 nm. Samples were dried in a SpeedVac (Savant Instrument Co., Farmingdale, N.Y.).

Primer pellets were dissolved in 150 μl loading dye (4 ml deionized formamide, 0.25 ml 10X TBE, 5 mg bromphenol blue, ddH_2O to 5 ml) and purified by electrophoresis on a 20% polyacrylamide gel (19 g acrylamide, 1 g bis-acrylamide, 48 g urea, 5 ml 10X TBE, ddH_2O to 100ml). Primer bands were excised and pulverized, suspended in TEAB, and placed on ClayAdams Nutator overnight at 37°C. Primers were isolated by silica gel reversed phase chromatography on Sep-PakTM C_{18} cartridges, essentially according to manufacturer's recommended protocol. Columns were flushed sequentially with 10 ml acetonitrile; 5 ml 30% acetonitrile in 25 mM TEAB; 5 ml 10% acetonitrile in 25 mM TEAB; 30 ml 25 mM TEAB. The primer containing solution was flushed through two times. Primers were flushed off the column

with 5 ml 30% acetonitrile in 25 ml 25mM TEAB. Samples were dried in a SpeedVac, redissolved in 1 ml ddH₂O and dried in SpeedVac (8X). Samples were dissolved in 100 μ l TE, examined spectrophotometrically and oligonucleotide concentrations were determined.

PCR Amplifications

Amplifications from genomic DNA were performed using a Perkin Elmer DNA Thermal Cycler. Reaction conditions were formulated from protocols recommended by the enzyme supplier's manual, Sambrook et al. (1989), and Innis et al. (1990). Reaction mixtures contained 10 μ l template DNA (0.5 μ g/10 μ l), 10 μ l 10X amplification buffer, 0.5 μ l AmpliTaqTM (2U/ μ l), 5 μ l each of 2 primers (4 μ M), 10 μ l dNTP solution (1mM dATP, 1 mM dTTP, 1 mM dGTP, 1mM dCTP), ddH₂O to 100 μ l. Controls were run substituting 10 μ l ddH₂O for template. Cycling conditions were: cycle 1 - 94°C/4 min; cycles 2 to 35 - 94°C/60 sec, 49°C/60 sec, 72°C/3 min; cycle 36 - 72°C/7 min; cycle 37 - 4°C/until used. Amplifications were performed using primer sets 1 and 4, 1 and 3, 5 and 4, and 2 and 4.

Aliquots of 4 μ l were examined under UV light after electrophoresis on an analytical gel (0.8% agarose gel, 1X TBE buffer, 5 μ l/ml EtBr). Reaction mixtures were run on preparative gels (0.8% LMT agarose, 1X TAE buffer, 5 μ l/ml EtBr) and bands of appropriate size were excised. DNA was isolated from the gel using GeneCleanTM according to the manufacturer's protocol.

DNA was dot quantified by UV visualization, comparing sample with standards of known concentration, each mixed with an equal volume of EtBr solution (2 $\mu\text{g}/\text{ml}$).

Reamplification. Reaction mixtures contained 1 μl amplified DNA (0.01 ng/ μl), 5 μl dNTPs (1 mM each dNTP), 10 μl 10X amplification buffer, 0.5 μl AmpliTaqTM, 5 μl each primer (4 μM), ddH₂O to 100 μl . Cycling conditions were: cycle 1 - 94°C/4 min; cycles 2 to 36 94°C/60 sec, 55°C/60 sec, 72°C/3 min; cycle 37 72°C/7 min; cycle 38 4°C/until used. PCR products were purified using Ultrafree-MC 30 000 NMWL filter units and examined under UV light after electrophoresis on 0.8% agarose analytical gels.

Phagemid Vector Isolation and Preparation

DH5 α cells transformed with pBLUESCRIPT (KS+)TM, from laboratory stocks, were streaked on Luria Bertani + ampicillin(50 $\mu\text{g}/\text{ml}$) (LB/Amp) agar plates and incubated overnight at 37°C. A single colony was used to inoculate 5 ml LB/Amp; cultures were grown overnight, 37°C, on roller incubator. The overnight culture (250 μl) was used to inoculate 25 ml LB/Amp. The culture was grown to O.D.₆₀₀=0.6 then used to inoculate 1 L LB/Amp. The 1 L culture was grown to O.D.₆₀₀ = 0.4. Chloramphenicol (5 ml of 34 mg/ml ethanol) was added and incubation continued for 16 h. Cells from 250 ml aliquots were harvested by centrifugation (5000 x g, 10 min), washed with 10 ml STE solution, and recentrifuged. Each cell pellet was resuspended in 6 ml lysis buffer (25 mM TRIS (pH 8.0), 10 mM EDTA, 15% sucrose), and 15 mg

lysozyme added. Tube contents were mixed by inversion and tube placed on ice, 10 min; 12 ml of 0.2N NaOH, 1% SDS solution was added and the tube placed on ice, 10 min. 7.5 ml of 3M sodium acetate (pH 4.5) was added and the tube placed on ice, 20 min. The mixture was centrifuged (10 000 x g, 15 min). The supernatant was transferred to new tube, 10 μ l RNase A (10 mg/ml) added, and tube incubated, 37°C, 30 min. The solution was extracted (2X) with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged (10 000 x g, 10 min). The upper phase was extracted with an equal volume of chloroform, and centrifuged (10 000 x g, 10 min). The upper phase was transferred to new tube. Two volumes ethanol were added, the tube was placed at -20°C, 30 min, then centrifuged (10 000 x g, 30 min). The supernatant was discarded.

The pellet was dissolved in 1.6 ml ddH₂O. 0.4 ml 4M NaCl and 1.0 ml polyethylene glycol (30% solution in ddH₂O) were added and tube placed in ice water for 60 min. DNA was pelleted by centrifugation (10 000 x g, 10 min), the supernatant poured off, the pellet washed with 80% ethanol and air dried. The pellet was dissolved in low TE buffer and concentration determined spectrophotometrically before use.

Single digestions of pBLUESCRIPT KS(+)TM vector were performed with BamH I and with EcoR I. The digested vector was dephosphorylated with calf intestinal alkaline phosphatase according to the supplier's recommended protocol. Double digestions of vector were performed with BamH I and EcoR

I, Sst I and Kpn I, BamH I and Pst I. Digested vector was gel purified, bands of appropriate size excised, and DNA purified from gel with GeneCleanTM. Concentrations were determined by electrophoresis against known standards.

Insert Preparations

Single and double digestions of PCR products were performed using available restriction enzymes which have recognition sites in the pBLUESCRIPT KS(+)TM multiple cloning region. Digestions were performed according to manufacturers' recommendations and using buffers supplied by the manufacturers.

Digestions of PCR product amplified from primer 1 to primer 4 were performed using BamH I, Cla I, EcoR I, EcoR V, Kpn I, Acc I, Hinc II, Xho I, Sst I, Cla I, Not I, Sma I, Pst I, and Xba I. Internal cleavage sites were determined for BamH I, Kpn I, Acc I, HincII, Sst I, and Cla I (Fig. 2). Inserts were prepared by BamH I - EcoR I double digestion and preparative gel purification of digestion products. Appropriate DNA containing bands were excised and used for in-gel ligations. After cloning attempts proved the primer incorporated 5' BamH I cleavage site to be recalcitrant to digestion, the BamH I - BamH I fragment was further manipulated by kination with T4 polynucleotide kinase, concatemerization with T4 ligase, preparative gel purification, cleavage with BamH I, preparative gel purification, and fragment DNA isolation with GeneCleanTM.

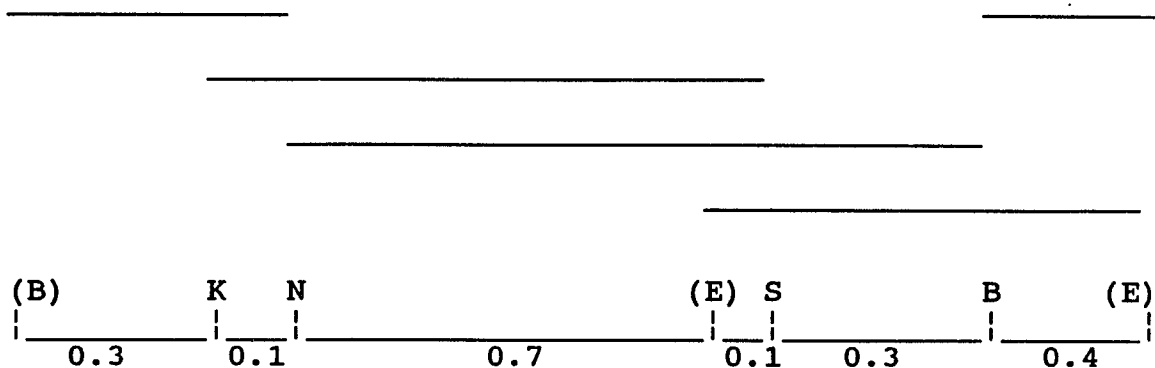


Figure 2. Restriction map of PCR products and cloning inserts. Sites in parentheses are primer incorporated sites. B=BamH I; K=Kpn I; N=Nsi I; E=EcoR I; S=Sst I. Lines above map are cloned inserts used in sequencing strategy. Distances below map are in kilobases.

The PCR product amplified between primer 2 and primer 4 was digested with EcoR I and gel purified. The DNA containing band was excised and used for in-gel ligations.

Cloning and Subcloning

In-gel ligations were performed according to the protocol recommended by the ligase supplier, and after gel slices were melted at 70°C and diluted to the proper DNA concentrations. The vector used for all cloning procedures was pBLUESCRIPT KS(+)TM. Vector:insert molar ratios were 3:1, 2:1 or 1:1, depending upon availability of insert DNA. Ligation products were used directly in cell transformations without purification.

Clones 110503A, 110503H and 100306A. The larger (about 1500 bp) fragment from the BamH I digestion of PCR product from primer 1 to primer 4 amplification was inserted into BamH I digested, dephosphorylated vector. Clones 080705F and 13CC. The small (about 400 bp) fragment from the BamH I, EcoR I double digestion of PCR product from primer 1 to primer 4 amplification was inserted into BamH I, EcoR I doubly digested vector.

Clones 032302A and 012801K. Clones 110503A and 110503H were digested with BamH I and Nsi I. The small (about 400 bp) fragment was inserted into BamH I, Pst I doubly digested vector.

Clones 120501A and 012006A. PCR product was cleaved with BamH I and Kpn I and the large fragment inserted into BamH I, Kpn I vector. The recombinants were cleaved with Sst I, the large fragment gel purified, and religated.

Clones 020201B and 020202B. Clones 110503A and 110503H were digested with BamH I and Nsi I; the large fragment was gel purified and inserted into BamH I, Pst I vector.

Clones 092902B and 092902I. Eco R I digested PCR product from primer 2 to primer 4 amplification was inserted into EcoR I digested, dephosphorylated vector.

Competent Cell Preparation and Cell Transformation

Competent cells used were either subcloning efficiency DH5 α competent cells obtained from BRL or were DH5 α cells cultured in the laboratory and made competent according to the protocol of Hanahan (1985).

Transformations were performed by a protocol modified from the procedure recommended by BRL. Competent cells were removed from -80° storage and aliquoted (50 μ l of commercially obtained cells or 100 μ l of cells made competent in the laboratory) into cold microfuge tubes. Ligation mixtures were melted, the appropriate volume of mixture removed, diluted, and cooled on ice. From 1 to 5 μ l of diluted ligation mixture was pipetted into the competent cells and the tube placed in ice water for 30 min. Cells were heat shocked at 42°C for 90 sec, then placed on ice for 60 min.

LB/Amp agar plates (150 mm) were spread with 40 μ l 2% X-Gal (5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside in N,N dimethylformamide) and 100 μ l 0.1M IPTG (Isopropyl β -D-thiogalactopyranoside in ddH₂O) and allowed to stand for 30 min. Cell preparations, diluted where appropriate were spread on prepared plates and incubated 24 h, 37°C. Recombinant phagemid transformed cells were identified by blue/white selection; colonies appearing to be white colored were picked and streaked on LB/Amp/X-Gal/IPTG plates and incubated overnight, 37°C. Single colonies were picked from white streaks and used to inoculate 5 ml LB/Amp liquid cultures; cells were grown overnight, 37°C.

Phagemid DNA Isolation and Analysis of Recombinants

Phagemid DNA was isolated from overnight cultures according to the alkaline lysis miniprep protocol of Maniatis et al. (1982). Miniprep DNA was digested with appropriate restriction enzymes and digestion patterns and fragment sizes observed after electrophoresis on 0.8% agarose gels.

Preparation of DNA for Sequencing

Phagemid DNA for sequencing was prepared by polyethylene glycol precipitation as described under Phagemid DNA Isolation or by differential ammonium acetate precipitation (Lee and Rasheed 1990). For the latter

procedure, overnight 5 ml cultures of phagemid transformed cells were grown in rich TB broth (Tartof and Hobbs. 1987). Cells from 2 ml culture were spun down and the pellet resuspended in 200 μ l lysis buffer (50 mM glucose, 25mM Tris-Cl (pH8.0), 10 mM EDTA, 5 mg/ml lysozyme). The suspension was let stand for 5 min at room temperature, 400 μ l alkaline solution (0.2 N NaOH, 1% SDS) added, the tube placed on ice 10 min, 300 μ l cold 7.5 M ammonium acetate (pH 7.6) added, and the mixture centrifuged, 12 500 rpm, 5 min. The supernatant was removed to fresh tube, 0.6 vol isopropanol added, placed at room temperature for 10 min, and centrifuged 12 500, 10 min.

The pellet was suspended in 100 μ l of 2 M ammonium acetate (pH 7.4), the tube vortexed placed on ice, 5 min, and centrifuged, 12 500 rpm, 5 min. The supernatant was removed to a fresh tube, 100 μ l isopropanol added, let stand for 10 min, and centrifuged, 12 500, 10 min. Pellet was washed with 80% ethanol, dried, dissolved in 49 μ l TE, 1 μ l RNase A (10 μ g/ml) added and solution incubated, 37°C, 30 min. Final precipitation was by addition of 25 μ l 7.5M ammonium acetate and 75 μ l isopropanol and centrifugation, 12 500 rpm, 10 min. Pellet was dried, dissolved in low TE and DNA examined after agarose gel electrophoresis.

Phagemid template DNA was denatured by adding to approximately 10 μ g DNA in solution, 16 μ l 5X denaturing solution (1M sodium hydroxide, 1mM EDTA) and ddH₂O to 80 μ l. Denaturation was allowed to proceed for 5 min at room temperature. The solution was neutralized by the addition of 8 μ l cold 2M ammonium acetate (pH 4.5). DNA was precipitated by adding 2 volumes ethanol (-20°C), placing the mixture at -80°C for 1 h, and centrifuging 4°C, 30

min, 11 000 rpm. The pellet was washed with 80% ethanol, dried, resuspended in 11 μ l ddH₂O, and placed on ice until used. A 1 μ l aliquot was electrophoresed to estimate DNA concentration and quality.

Sequencing

All sequencing reactions were performed using denatured double stranded plasmid template DNA and [α -³⁵S] dATP label. Reaction conditions were from the range of conditions suggested in the SequenaseTM manual. All reagents were supplied in SequenaseTM kits.

Sequencing reactions intended for short (2.5 h to 6.5 h) gel runs were performed using dideoxy termination mixes undiluted or diluted 4:1 with extension mix, and five fold dilutions of labeling mix. Reactions intended for long (7h to 15 h) gel runs were performed using termination mixes diluted 4:1 with extension mix, and undiluted labeling mix. Three to five minute labeling reaction times were used for short run reactions; five to fifteen minute labeling times were used for long run labeling reactions. Termination temperatures were either 37°C or 42°C. When available, pyrophosphatase was added to the labeling reactions according to manufacturer's protocol. Where severe stops were encountered, an extra step, modified from the protocol of Fawcett and Bartlett (1990), was included following the termination reactions. For each set of termination reactions, 0.5 μ l terminal deoxynucleotidyl transferase (TdT) was diluted in 4.5 μ l 1X buffer supplied with the enzyme and 1 μ l added to each

termination reaction tube. Incubation of the tubes was continued for 15 min before addition of stop solution.

All sequencing electrophoresis runs were performed using 0.4 mm 6% polyacrylamide (35.6 ml Acryl-40, 37.5 ml 2% bis-acrylamide, 25 ml 10X TBE, 115 g urea, ddH²O to 250 ml) gels. Gels were pre-run for 25 min; samples were heat denatured, 2 min, 90°C before loading. Gels were run at 40 to 45 W. At the completion of each run, gels were soaked in 10% methanol, 10% acetic acid solution, 25 min, transferred to blotting paper, dried at 80°C for 1 h under vacuum, and exposed to Kodak X-OMAT AR film at room temperature.

Data Analysis

Sequence data were examined to determine exon/intron arrangements, homologies, presence of regulatory elements, and presence of repeated elements. Sequence data from protein coding regions were translated and the deduced amino acid sequence examined for homologies with GH of other species, structure elements, and degree of hydrophobicity.

All sequence data analyses were performed using the University of Wisconsin Genetics Computer Group (UWGCG) computer package (Devereux et al. 1984). Sequence data read from sequencing gels were entered into the local database using SEQED, then assembled aligned, and a consensus sequence determined by use of the FRAGMENT ASSEMBLY package. Homologies and alignments were determined using GAP and BESTFIT.

RESULTS

PCR Amplifications

Visualization, after agarose gel electrophoresis, of PCR products generated by amplification of genomic DNA using primers 1 to 4, 2 to 4, and 1 to 3 showed distinct bands at approximately the expected sizes of the fragments. Numerous lighter bands of smaller weight products were present, as were two distinct bands at approximately 300 base pair size. Reamplification of DNA purified from the slowest, most distinct, band produced a single, sharp band of the desired size and two bands of approximately 300 base pair size.

Cloning

Restriction digests of the primer 1 to primer 4 amplification product revealed potential internal cloning sites generated by digestions by Kpn I, BamH I, Cla I, Hinc II, Sst I, and Acc I. Partial sequencing, verified by restriction digest, later identified an Nsi I cleavage site; Nsi I digestion generates ends compatible with Pst I digestion of pBLUESCRIPT KS(+). Judged by cloning results, end digestion of PCR primer 1 to primer 4 fragment with EcoR I was successful; end digestion with BamH I was unsuccessful. After the PCR product was kinased and concatemerized, the 5' end of the PCR product was successfully cleaved by BamH I. Ends generated by Acc I digestion were not clonable into the pBLUESCRIPT KS(+) Acc I site;

subsequent sequencing showed that the insert and vector contained different Acc I recognition sites. Both ends of primer 2 to primer 4 PCR amplification product were cleaved by EcoR I. Products of digestions inserted into appropriately digested pBLUESCRIPT KS(+) are given in Fig. 2.

Red Drum Growth Hormone Gene Nucleotide Sequence

A sequence of 1900 nucleotides of the red drum GH gene was determined between primers 1 and 4 (Fig. 3). This sequence extends from the 5' end of exon I to 12 bp upstream of the stop codon. The protein coding regions of the gene exhibit similarities with the cDNAs of other species in line with their phylogenic relatedness: 9.19% similarity with sea bream (*Pagrus major*) (Momota et al 1988), 89.6 with tuna (Sato et al. 1988b), 59.8% with common carp (Koren et al 1989), and 52.1% with mink (*Mustela vison*) (Shoji et al. 1990). Intron nucleotide sequences were deduced and show no strong similarities with the published sequence of rainbow trout (Agellon et al. 1988b). All splice sites conform to the GT - AT rule (Mount 1982).

The gene has an internal arrangement of 6 exons and 5 introns; exon I is the primer 1 sequence (Fig. 3). Fused nucleotide sequences of exons 5 and 6 of the red drum GH gene match the sequence of the carp GH gene with 77.4% similarity (Fig. 4). Repeated sequences in the red drum GH gene are present flanking the boundary of intron III and exon IV (Fig. 5). An Alu I recognition site is present in the repeated sequence.

ATGGACAGAGgtaagacattcagactgggtttatatgttatgttgatgtgatgaagtcatttagggtaaagggtagactgaacggtcagaccaccaga
 ggaccttagagatctggagcgactcggatcaggactttatttgccggagcaccaaagaacaaacctgaatcagaatcctgaatcggggggcggtagtagc
 tcagtctgtagagacttgtctggacgagtcagtcagtcagcatgaccatagtaggaaggtgggtggtagctggaggggtgccagttcacctcctgg
 kacastgcaagggtacccttgagcaaggcactgaacccccacggggctgccataactccaccatctctctccacatgcatgtctatgagccctgtatg
 tggtcgggggttaaaaatgcatgtaaaataaactgagtgaaaaaagaatttccccgctgaggat taataaagtatctcttctaagaatcagaactcccag
 gctgytggttygttttagacattaggtgctacaaagaactcagtgctgctagtagtactgaggacacatcagcagatgaacctccatgttctctgtctgcct
 gctctgtttatttgtctctcacctgtctatctgtctctacctgrtctctttctcagTG CTC CTC CTG CTG TCG GTG CTG TCT TTG GGT
 V L L L L S V L S L G
 GTG TCC TCT CAG CCA ATC ACA GAC AGC CAG CGT CTG TTC TCC ATC GCT GTG AGC AGA GTT CAA CAC CTC CAC CTG
 V S S Q P I T D S Q R L F S I A V S R V Q H L H L
 CTC GCT CAG AGA CTC TTC TCC GAT TTTgtaagtcctacxgcagcxxxggxcaaaaacagtgataatxaaaaactttgaaactccagatttaa
 L A Q R L F S D F
 ctgaxxatagcaxaaagacgtctaatcctgctgataacaggccgtatggttccctctcctcgtagcccgaggacaaggcgctccatgatgtggactcgtc
 agxcxacaggacagttttcaacctcacctcaatggtggagtttgaactgttgcaattagtgaggatcagagatgaagtggtttgatgcactgccatctgc
 agggcctgaacatcaccgacattgagcagttaaatctggagttttattgtttcacatcagtcgttcttgctacacagtgggcgagacttccgtgtgtga
 taacagtggtatttgtgtgtgtatttctgtgtgtgtttgttagGAG AGC TCT CTG CAG ACA GAG GAA CAG GTC AAG CTC AAC AAA
 E S S L Q T E E Q V K L N K
 ATC TTC CTG CAG GAT TTC TGC AAC TGT GAT TAC ATC ATC AGT CCA ATC GAC AAG CAC GAG ACG CAA CGC AGC TCA
 I F L Q D F C N S D Y I I S P I D K H E T Q R S S
 gtgaggacagcaacacacactacgcaacaacacacactacactggttctaggttctgaagctgctgtctgtctgttgattggttggttctag GTT CTG
 V L
 AAG CTG CTG TCG ATC TCC TAT CGA TTG GTT GAG TCT TGG GAG TTC CCC AGT CGT TCT CTG TCT GGA GGT TCT GCT
 K L L S I S Y R L V E S W E F P S R S L S G G S A
 CCG AGG AAC CAG ATT TCA CCC AAA CTT TCT GAT CTG AAG ACT GGG ATC CTG CTG CTG ATC AGG gtagaagacagacc
 P R N Q I S P K L S D L K T G I L L L I R
 taaatcaaacacactaatctaacctgaccaaccagcctgcctgagtgagtaaccacctgatcctacagGCC AAT CAG GAC GGA GCA GAA ATC
 A N Q D G A E I
 TTT CCT GAT AGC TCC GCC CTC CAG CTG GCT CCA TAT GGG AAC TAT TAT CAA AGT CTG AGC GGC GAC GAG TCG CTG
 F P D S S A L Q L A P Y G N Y Y Q S L S G D E S L
 AGA CGA ACC TAC GAA CTG CTC GCC TGT TTC AAG AAA GAC ATG CAC AAG gtaggaagaggaggaggcaggcgatgatgatgtcac
 R R T Y E L L A C F K K D M H K
 aattatgtaatgatgatgttatcactaagatgtatctgttcacagGTG GAG ACC TAC CTG ACG GTG GCT AAA TGC CGA CTC TCT CCA
 V E T Y L T V A K C R L S P
 GAA GCC AACTGCACCTGTAG
 E A

Figure 3. Nucleotide sequence of the red drum growth hormone gene. PCR primer sequences are underlined. A putative GRE is marked by **. Amino acids are designated by standard one letter abbreviations under the first base of each codon.

A putative glucocorticoid response element is present in intron I (Fig. 3). Computer searches for thyroid response elements (TREs) and retinoic acid response elements (RAREs) (Vedekis in press) were negative. The red drum GH gene shares a region of high homology with the human chorionic somatomammotropin (SMT) gene (Fig. 6).

Red Drum Growth Hormone

The amino acid sequence deduced from translation of the protein coding regions of the red drum GH gene shares a high degree of homology with other fish GH amino acid sequences (Fig. 7). A hydropathy plot of the hormone (Fig. 8) shows a hydrophobic region at the NH₂ terminus, an internal region of alternating hydrophobic and hydrophilic segments, and an amphipathic segment upstream of the COOH terminus. The predicted structure (Table 1) contains four α -helices, comprising 30% of the residues. A three dimensional computer representation of the predicted structure is shown in Figs. 9. Codon usage is given in Table 2; G + C = 61.7%.

```

[-----2-----][-----3-----
rGH QPITDSQRLFSIAVSRVQHLHLLAQRLFSDFESSLQTEEQRLNKIFLQ-
tGH .....VK.....-
yGH .....H.....I.N.....N...T....D.....-
sGH ---IEN....N.....KN.N..DGT.LPD.R.....L-
eGH ---ISLYN..TS..N.A....T..AEIYKE..R.IPP.AH...S.TSPL-
hGH F.TIPLS...DN.SL.AHR..Q..FDTYQE..EAYIPK..KYSFLQNP.T

```

```

-----][-----4-----
rGH DFCNSDYIISPIDKHETQRSSVLKLLSISYRLVESWEFSPRSLS--GGSA
tGH .....
yGH .....S..F.--....
sGH .....S.V..V.....K.....H..F..I....Y..QT.--IISNS
eGH AG.Y..S.PT.TG.D...EK.DGY..R..SA.IQ..VY.LKT..DAFSNS
hGH SL.F.ES.PT.SNRE...QK.N.E..R..LL.IQ..LE.VQF.RSVFANS

```

```

-----][-----5-----
rGH P--R--NQIS-PKLSELKTGIHLLIRANQDGDENFADSSALQLAPYGNYY
tGH .--.--.....D.....L.....A.I.P.....
yGH L--.--.....R.....TQ...T....A...S.V.....F.
sGH LMV.NA....-E...D..V..N...TGS...VLSLD.NDSQ..P.....
eGH LMFGTSDGIF-D..ED.NK..NE.NKVG...--GIYIEDVRN.RYENFDV
hGH LVYGASDSNVYDL.KD.EE..QT.NGRLE...--SPRTGQIFKQTYSKFDT

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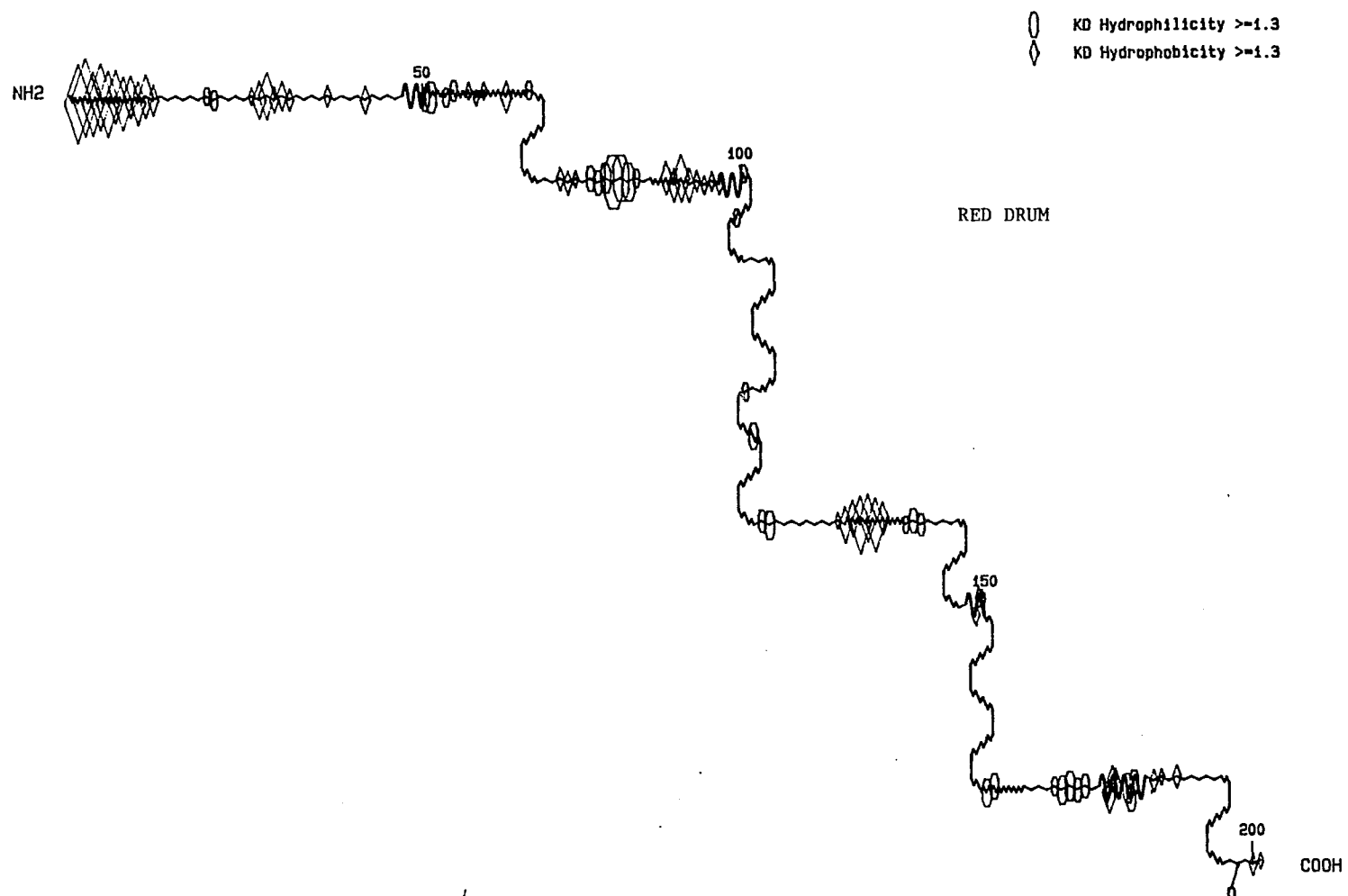
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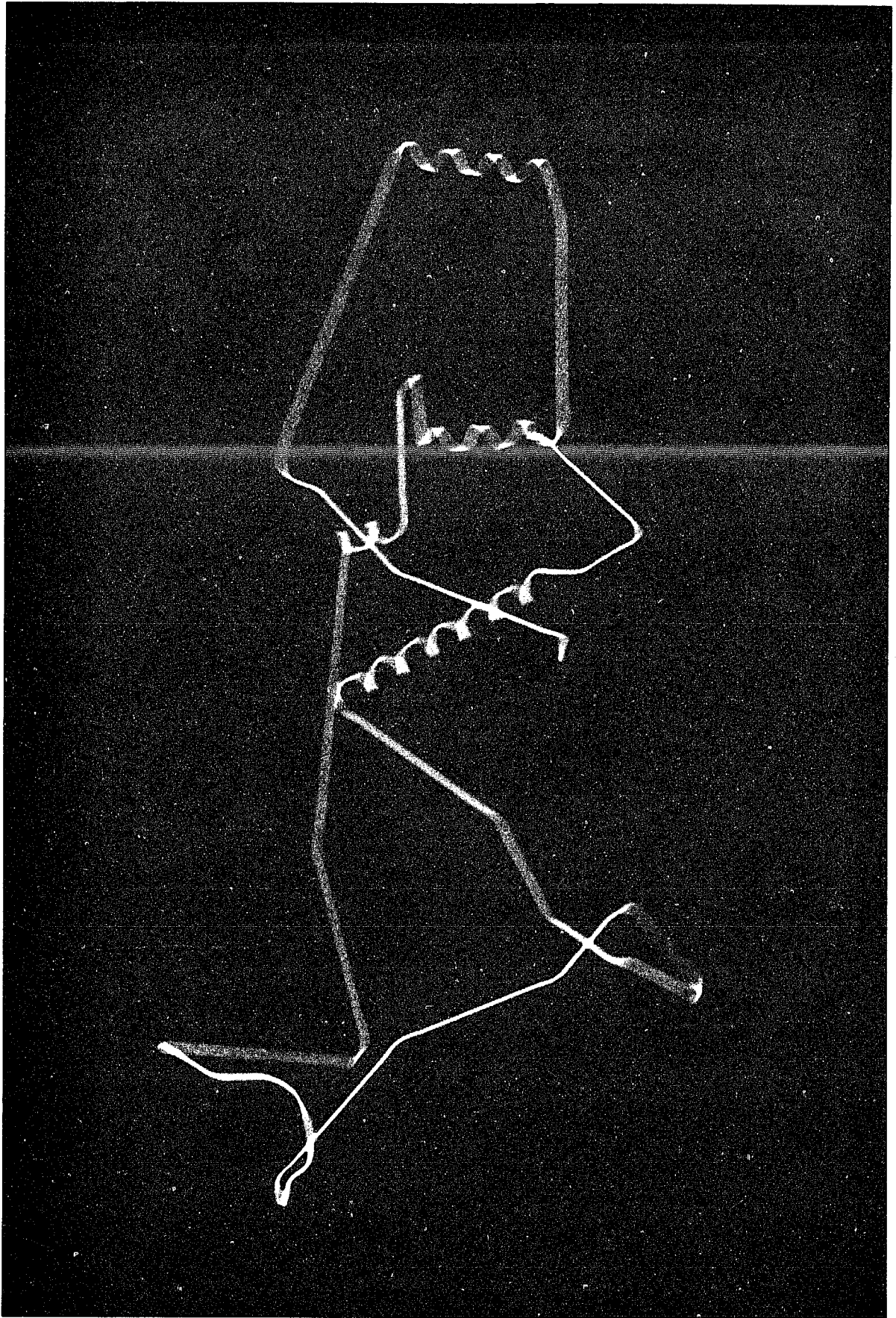
-----][-----6-----]
rGH QSLGADESLRRSYELLACFKKDMHKVETYLTVAKCRLSPEANCTL
tGH ...SG.....T.....
yGH ....GE.L...N.....
sGH .N..G.GNV..N.....K.L.....
eGH HLRNDAGLNK-N.G.....K.T...RFV.S....
hGH N.HND.AL.K-N.G..Y..R...D....F.RIVO..S-V.GS.GF

```

Figure 7. Alignment of deduced amino acid sequence of red drum GH. t=tuna; y=yellowtail; s=salmon; e=eel; h=human. Dots represent perfect matches with red drum GH; hyphens represent gaps. Exons boundaries are marked on top line.

Figure 8. Hydropathy plot of red drum growth hormone.





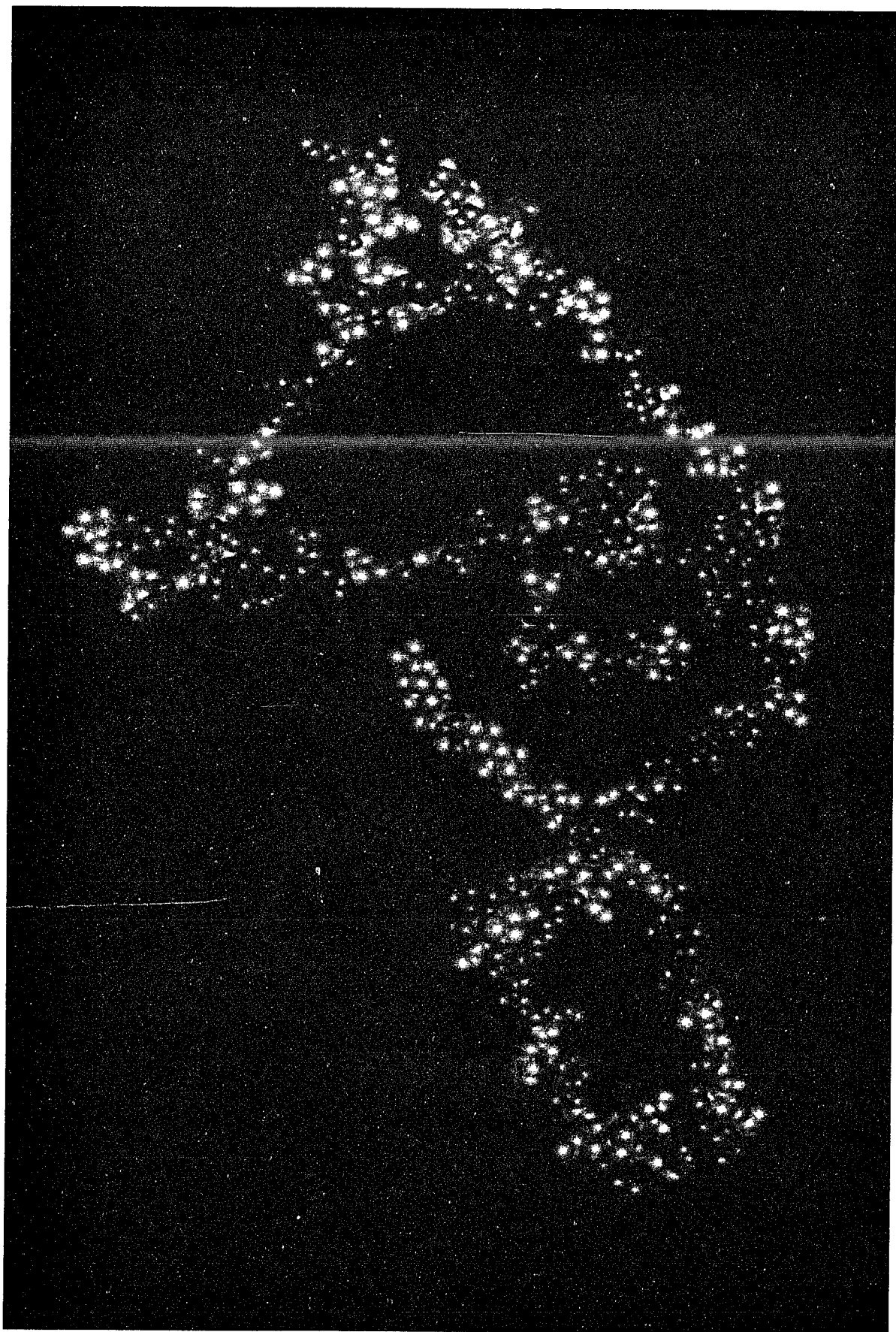


Figure 9. Three-dimensional structure of red drum growth hormone as predicted by INFORMATION THEORY and generated by the SYBL molecular modeling system. (a and b). Ribbon model. c. Space- filling model.

DISCUSSION

PCR Methodology

The results of this study demonstrate the potential utility of using PCR techniques to obtain genomic DNA sequences of highly conserved genes, or of genes having some segments conserved, where sequence data are available from related species. Limitations are that sequence data at the primer site are lost when the amplification process replicates primer design, and further research is required to obtain flanking sequence data.

The basic methodology, literature and database search followed by primer synthesis and DNA amplification, has considerable potential for use in fishery research, especially population genetics. Polymorphisms in GH and related genes have been detected in restriction fragment length polymorphism (RFLP) analysis of bovine genomic DNA (Cowan et al. 1989; Theilmann et al. 1989), and might be expected to be present in other genomic sequences. Amplified genomic sequences could be an efficient substitute for mitochondrial DNA in RFLP analysis. Disadvantages of mitochondrial DNA analyses are mainly concerned with the labor and expense involved in mitochondrial DNA isolation and with the limited number of polymorphisms to be expected. Genomic DNA isolation is simple, large numbers of samples can be amplified at one time with a DNA thermal cycler, restriction digest patterns are easily interpretable, and the potential number of useful polymorphic sequences large.

GH DNA clones produced in the present study, or sequences cloned using these methods, provide perfectly homologous probes for use in library screening intended to isolate the complete gene with regulatory flanking sequences. Cloned fragments might also, if polymorphisms are found, be used in combination with other probes, to increase the specificity of DNA fingerprinting.

Major problems encountered in the present study concerned the cloning of PCR products. A BamH I site incorporated into synthetic primer 1 could not be digested with the enzyme, and there was complete failure in attempts to blunt end clone PCR products. The former problem might be addressed by extending the primers further beyond the restriction site or by selecting more reliable enzymes than were chosen for this study. Commercial interests have solved the latter problem. Invitrogen Corporation (San Diego, CA) recently made public the information that company researchers had determined the cause of failure in blunt end cloning of PCR products; the polymerization procedure produces a single base overhang at the 3' end of each strand synthesized. The corporation now has made commercially available a vector with compatible ends and cloning is a one day procedure. Sequence data obtained with these methods could also allow the use of inverse PCR techniques (Innis et al. 1989) to isolate flanking sequences.

Advances in PCR single stranded amplification (Innis et al. 1989) are being made. As single stranded sequencing is simpler and provides more easily

interpreted results than double stranded sequencing, it will probably become the method of choice for sequencing projects.

Internal Arrangement of the Red Drum GH Gene

The red drum GH gene has an internal arrangement of five introns and six exons; this arrangement is like that of the salmonids (Agellon et al. 1988b), but unlike the four intron, five exon structure of the common carp (Chiou et al. 1990) and of mammalian species (Moore et al. 1982). Intron V of the red drum GH gene appears to be an insertion into a primordial exon V. If intron V is deleted, the fused boundary nucleotide sequences of red drum exons V and VI share 77% homology with the comparable segment of exon V of the common carp GH gene (Fig. 4). The occurrence of a fifth intron in the GH gene of salmonid and perciform fishes suggests that the five exon/four intron arrangement of the mammalian GH gene is the primordial arrangement. Insertion of the fifth intron occurred in the phylogenetic tree leading to the Perciformes (including the red drum) only after divergence of the superorder Ostariophysi (including the carp). Fish classification schemes have considered the salmonoid fishes a primitive taxon. The classic phylogeny of Greenwood et al. (1966) places this group at the base of an evolutionary tree leading to the teleosts and the Ostariophysi (Fig. 10a). Placement of the Ostariophysi is given as questionable. It is this phylogeny upon which the American Fisheries Society List of Common and Scientific Names of Fishes (Robins et al. 1980) is

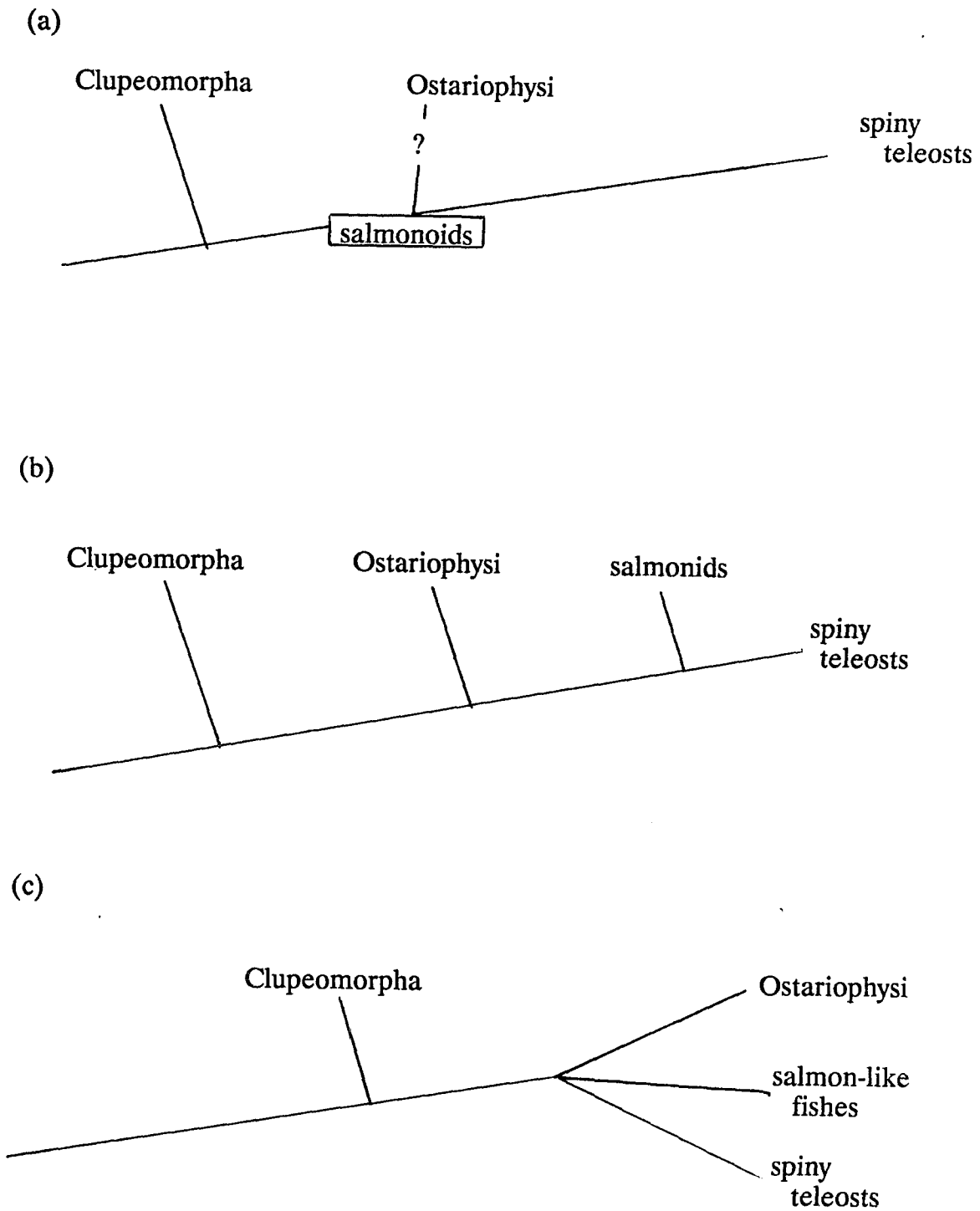


Figure 10. Phylogenetic relationships of Clupeomorpha, Ostariophysi, salmonoid fishes, and spiny fishes. Based on (a) Greenwood et al. (1966); (b) Lauder and Liem (1983); and (c) Carroll (1988).

based. Lauder and Liem (1983) consider the Ostariophysi to be a monophyletic taxon that diverged prior to the emergence of the salmonids. In this scheme, modern spiny teleosts, including the red drum, evolved from the salmonid fishes (Fig. 10b). Carroll (1988) follows the classic phylogeny to the appearance of the Clupeomorpha (herrings, anchovies, sardines) (Fig. 10c). This scheme then divides all more advanced fishes into three groups: salmon-like fishes, the Ostariophysi, and all others. He notes that the relationships between these groups are undetermined.

The presence of a fifth intron in the GH genes of the salmonids and the red drum, and the absence in the carp GH gene, supports the classification of Lauder and Liem. Sequence data tracking the appearance of intron V of GH genes could clarify the relationships between major taxa of modern fishes.

The insertion of intron V was not accompanied by significant changes in flanking protein coding regions. The amino acid sequences of protein regions coded for by exons V and VI of the red drum and salmonid GH genes share a high degree of homology with these regions of other fish GHs (Fig. 7). If intron V has biological significance, its role must be performed in the stages prior to translation. One possible role would be to mediate GH levels by regulating the transport of mRNA. mRNA must enter the cytoplasm before translation into protein. Spliceosome complexes bound to introns prior to splicing events prevent mRNAs from leaving the nucleus (Steitz 1988).

Insertion of an intron into a gene sequence could provide an additional level of control of translation, thus of hormone levels.

Response Elements

Glucocorticoids, thyroid hormone, and retinoic acid stimulate GH synthesis in mammalian cells (Martial et al. 1977; Dobner et al. 1981; Brent et al. 1988; Bedo et al. 1989). Response elements are located in flanking regions and, for glucocorticoids and thyroid hormone, in introns, of the mammalian GH genes (Slater et al. 1985; Sap et al. 1990). The 16 bp putative glucocorticoid response element (GRE) of the red drum GH gene is located in intron I, a location analogous to that of a GRE in mammalian genes, contains the 3' end hexanucleotide TGT(T/C)CT considered essential for response activity, and matches at 11 of 16 bases the GRE present in the 3' flanking region of the rat GH gene (Slater et al 1985). The control mechanism of REs has not been exactly determined, but the formation of homo- or heterodimers of ligand bound receptors in the control process is involved (Vedekis in press). This allows very precise control of GH activities at the transcriptional level as the absolute and relative concentrations of different hormones fluctuate in cells.

GREs were not found in the GH nucleotide sequences of rainbow trout by Agellon et al. (1988b). A computer search of the published carp GH nucleotide sequence (Chiou et al. 1990) for the definitive GRE hexanucleotide produced negative results. Few TRE and RARE sequences have been

determined; failure to identify these in the red drum GH sequence may be due to a lack of available search sequences.

Response activity of the putative GRE in the red drum sequence remains to be demonstrated, but the presence of an internal regulatory element has implications for gene transfer technology. Introns have been found to increase transcriptional efficiency of inserted GH genes in transgenic mice (Brinster et al. 1988). It is possible that, for maximum acceleration of normal growth patterns via introduced GH genes, introns and response elements must be present and be compatible with the physiology of the host fish. Interspecific differences in the functions of growth hormone have been discussed by several authors (Bern 1983; Nicoll et al. 1986) in cautioning researchers to make no assumptions regarding the roles of growth hormone in a species. This should be considered in planning genetic engineering projects for the improvement of fishes.

Repeats

Repeated sequences which incorporate an Alu I recognition site are a common feature of mammalian genes (Darnell et al. 1986). The function of these repeats has not been recognized, but a possible role as mobile gene elements in gene rearrangement has been proposed. The repeated segment found in the red drum GH gene (Fig. 5) is unlike the mammalian Alu family of repeats (Jelinek 1982) in several ways. It crosses an exon/intron boundary,

lacks repeats flanking at both termini, and is shorter than the approximately 300 base pair length associated with the Alu family. The repeated sequence in the exon shares homology with the same exon region in other fish GH genes (Fig. 7), but is not found in the flanking intronic region of the carp or salmon GH genes. The repeat may identify a location responsive to mutational events. Analysis of sequence data from GH genes of related fish species could suggest whether or not the repeated sequence has functional significance.

Other Primary Sequence Features

Two variants of human GH have been isolated (Chapman et al. 1981). A 20 kd variant comprises about 10% of human GH and is the result of alternative splicing of GH mRNA with a shifted splice site at the boundary of intron II and exon III (de Noto et al. 1981). Alternative splicing of GH transcript has been reported only for human GH mRNA. Two GH variants have been reported for rainbow trout (Agellon et al. 1988a) and *Xenopus* (Martens et al. 1989) but both groups of researchers concluded that the variants were coded for by two different genes. The intron II/exonIII region of the red drum GH sequence was examined for alternative splice sites (Mount 1982) and none found.

Homology of exon V of the red drum GH gene with exon V of the human SMT gene provides an opportunity to search a red drum genomic library for new genes of the growth hormone family. Isolation and purification of the

protein products of the gene family is made difficult because of their amino acid similarities. Products produced in small quantities, or in cell types not usually used in protein purification schemes might not be identified. Clones incorporating the conserved 3' end half of the red drum GH gene were produced in the course of this study. These clones, labeled and used to probe a red drum genomic library, should identify all members of the GH gene family present in the red drum genome. GH clones could be identified and eliminated after reprobing with a labeled fragment derived from intron I and intron II regions. These variable sequences would not hybridize to other members of the gene family under stringent reaction conditions.

Future Prospects

Clones produced in the present study may serve to advance work in the study of red drum GH gene regulation, genetic engineering, and gene isolation. Each of the clones would serve as a perfectly homologous probe to isolate the GH gene, with its flanking regulatory sequences, from a genomic library. Alternatively, sequence data obtained in the present study would allow the use of inverse PCR methodology to isolate and amplify the flanking regions. An understanding of the regulatory processes that determine the levels of GH could aid in the design of projects to provide the most beneficial conditions for successful aquaculture of the species. Comparative studies of the GH gene regulatory elements from individuals having desirable characteristics with the

GH gene sequence of less desirable individuals might identify significant nucleotide regions to be examined when choosing breeding stock.

RFLP analysis of mitochondrial DNA for the identification of fish populations is too cumbersome for routine work by most public agency laboratories, and is not an ideal technique for research institutions analyzing large samples. It is highly probable that useful polymorphic loci are present in total genomic DNA and the PCR methods used in the present study would be a practical means for identifying such loci. A database search would provide additional data from other genes for primer design. An advantage of RFLP analysis is that, for applied work, it not necessary that a sequence be identified, only that results be reproducible. Preliminary PCR amplifications would identify potentially useful primer pair sequences that consistently produce single size amplification products. Applying the methods of PCR and restriction endonuclease digestion to sample populations would identify polymorphisms. A useable database would consist of primer sequence data and the sizes of fragments to be expected after digestion with each tested enzyme. The level of discrimination to be expected, individual, subpopulation, population, must be determined empirically; currently available data are not adequate for projections. Primer design used in the present study could be used as the basis for research directed toward tabulating GH gene polymorphisms in most spiny rayed fishes.

The full length PCR product of amplification between primers 1 and 4 could be used in the production of transgenic fish. After fusion with a suitable promoter element the sequence would code for a complete protein. Integration of this full genomic sequence into the red drum genome could provide the most efficient means of accelerating growth in this species. The gene sequence, with introns and associated any regulatory elements, may also be the most useful available sequence for the production of other transgenic euryhaline species.

Experiments to isolate other GH gene family members should be attempted. The roles of PL and SMT in mammals are related to fetal growth. PRL is associated with the reproductive strategy of mammals. It is possible that GH gene family members exist in the lower vertebrates and are active in the reproductive process. Alternatively, the PL and SMT genes may have been adapted to the unique mammalian reproductive strategy. Functions of ancestral genes in the lower vertebrates could have been related to physiological processes no longer needed by terrestrial homeotherms. Roles in temperature adaptation, osmoregulation, and response to photoperiod are possible. The role of introns in regulating cellular levels of gene products could be examined using deletion mutants of the red drum GH gene and of a salmonid GH gene. The fifth introns of these genes share no significant homologies of size or sequence. Comparative studies of mRNA levels before and after deletion of intron V could indicate mechanisms of control. The

extreme interspecific differences in intron V sequences would serve to separate effects due to sequence from effects due to the presence of an intron.

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J l uJ nhI r Rsr n a
I I II IIV I IuF l n
/ / III I I

GGTGGGCTGGTAGCTGGAGGGGTGCCAGTTCACCTCCTGGKACASTGCAAGG
-----+-----+-----+-----+-----+ 312
CCACCCGACCATCGACCTCCCCACGGTCAAGTGGAGGACCMTGTSACGTTCC

N B F
lRK B sD Cn
asp b as vu
Ian v Ja JH
VII I II II
/ /

TACCCTTGAGCAAGGCACTGAACCCCCACGGGGCTGCCCATAACTCCACCAT
-----+-----+-----+-----+-----+ 364
ATGGGAACTCGTTCCGTGACTTGGGGGTGCCCCGACGGGTATTGAGGTGGTA

B
s
N p
l C Bl
aN v a2 M
Is i n8 s
Ip J I6 e
II I II I

CTCTCTCCACATTGCATGTCTATGAGCCCTTGTATGTGTTTCGGGGTTAAAAA
-----+-----+-----+-----+-----+ 416
GAGAGAGGTGTAACGTACAGATACTCGGGAACATACACAAGCCCCAATTTTT

N					B	N		
l					p	s		M
N aN		D			s	M u	SpD	F AM b
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I II		I			I	I I	III	I II I

TGCATGTAAAATAAACTGAGTGAAAAAGAATTTCCCCGCTGAGGATTAATA

-----+-----+-----+-----+-----+-----+-----+----- 468

ACGTACATTTTATTTGACTCACTTTTTTCTTAAAGGGGCGACTCCTAATTAT

		H		B	C	C
DE	iT	M	As	v	v	
da	nf	n	va	i	i	
er	fi	l	aJ	J	J	
II	II	I	II	I	I	

AAGTATCTCTTCTAAGAATCAGAACTCCCGAGGCTGTTGGCTTGTTTTAGAC

-----+-----+-----+-----+-----+-----+-----+----- 520

TTCATAGAGAAGATTCTTAGTCTTGAGGGCTCCGACAACCGAACAAAATCTG

				F		E
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				u		O
B	D		MM	RSD		P
b	d		4 an	scd	F	l
v	e		H el	aae	o	5
I	I		I II	III	I	I

ATTAGGTGCTACAAAGAACTCAGTGCTGCTAGTACTGAGGACACATCAGCAG

-----+-----+-----+-----+-----+-----+-----+----- 572

TAATCCACGATGTTTCTTGAGTCACGACGATCATGACTCCTGTGTAGTCGTC

	N					
D	l					B
r	a				H	s
d	I				p	m
I	I				h	A
I	I				I	I

ATGAACCATCCATGTTCTCTCGTCTGCCTGTCTGTTTATTTGTCTCTCACCT

-----+-----+-----+-----+-----+-----+-----+----- 624

TACTTGGTAGGTACAAGAGAGCAGACGGACAGACAAATAAACAGAGAGTGGA

			B			
			s			T
			p			a
	B		lH			q
H	s	D	2g	M	M	MI
p	m	d	8i	w	n	wI
h	A	e	6A	o	l	o-
I	I	I	II	I	I	I2

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GTCTATCTGTCTCTCACCTGTCTGTTTCTCAGTGCTCCTCCTGCTGTCGGTG

-----+-----+-----+-----+-----+----- 676

CAGATAGACAGAGAGTGGACAGACAAAGAGTCACGAGGAGGACGACAGCCAC

	C		C		A
D	v	M	H	v	l
d	i	n	g	i	w
e	J	l	a	J	N
I	I	I	I	I	I

CTGTCTTTGGGTGTGTCCTCTCAGCCAATCACAGACAGCCAGCGTCTGTTCT

---+-----+-----+-----+-----+----- 728

GACAGAAACCCACACAGGAGAGTCGGTTAGTGTCTGTCGGTCGCAGACAAGA

S		S
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y		M s sDbPy i
L		n m pdoll n
T		l A MeIeT f
I		I I IIIII I

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CCATCGCTGTGAGCAGAGTTCAACACCTCCACCTGCTCGCTCAGAGACTCTT

-+-----+-----+-----+-----+-----+----- 780

GGTAGCGACACTCGTCTCAAGTTGTGGAGGTGGACGAGCGAGTCTCTGAGAA

		F		
		n		
EM	A	u	B	G
al	c	4	b	s
ry	c	H	v	u
II	I	I	I	I

CTCCGATTTTGTAAGTCTACXGCAGCXXXGGXC AAAACAGTGATAATXXAA

-----+-----+-----+-----+-----+----- 832

GAGGCTAAAACATTCAGATGXCGTCGXXXCCXGTTTTTGTCACTATTAXXTT

		AM A	B
	M	ha a	c
	s	ae t	e
	e	II I	f
	I	II I	I

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AAACTTTGAAACTCCAGATTTAACTGAXXATAGCAXAAAGACGTCTAATCCT

-----+-----+-----+-----+-----+----- 884

TTTGAAACTTTGAGGTCTAAATTGACTXXTATCGTXXTTTCTGCAGATTAGGA

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ve	l	M v	MHMNC
iI	a	n	i ngscr
JI	I	l	J lapiF
II	V	I	I IIIII
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GCTGATAACAGGCCGTATGGTTCCTCTCCTCGTTAGCCCGGAGGACAAGGCG
-----+-----+-----+-----+-----+-----+-----
CGACTATTGTCCGGCATAACCAAGGAGAGGAGCAATCGGGCCTCCTGTTCCGC

936

N				
l P	H			
aPf	i	M	H	M
Ill	n	l	p	n
IeM	f	y	h	l
III	I	I	I	I
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TCCATGATGTGGACTCGTCAGXCXACAGGACAGTTTTCAACCTCACCTCAAT
 ---+-----+-----+-----+-----+-----+-----
 AGGTACTACACCTGAGCAGTCXGXTGTCCTGTCAAAAGTTGGAGTGGAGTTA

988

				T	
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l	p	n	L	111	a
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GGTGGAGTTTGAAC TGTTGCAATTAGTGGAGTCAGAGATGAAGTGTGTTTGA
-+-+-----+-----+-----+-----+-----+-----+
CCACCTCAAAC T TGACAACGTTAATCACCTCAGTCTCTACTTCACACAAACT

1040

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      E
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      OaCAa
S    PluvleH
f    s09iwIp
e    t96JNiH
I    IIIIIII
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TGCACTGCCATCTGCAGGGCCTGAACATCACCGACATTGAGCAGTTAAATCT
-----+-----+-----+-----+-----+--
ACGTGACGGTAGACGTCCCGGACTTGTAGTGGCTGTAACCTCGTCAATTTAGA

1092

G	A	HT
s	c	hh
u	c	aa
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GGAGTTTTATTGTTTCACATCAGTCTGTTCTTGTCTACACAGTGGCGCGACT
 -----+-----+-----+-----+-----+----- 1144
 CCTCAAAATAACAAAGTGTAGTCAGACAAGAACAGATGTGTCCACGCGCTGA

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TCCTGTGTGTATAACAGTGTGTATTGTTGTGTGTATTTCTGTGTGTGTTTGT
 -----+-----+-----+-----+-----+----- 1196
 AGGACACACATATTGTCACACATAACAACACACATAAAGACACACACAAACA

B		
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CB1H	t	C M
Ava2gSMS P	y	Av b
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TAGGAGAGCTCTCTGCAGACAGAGGAACAGGTCAAGCTCAACAAAATCTTCC
 ---+-----+-----+-----+-----+----- 1248
 ATCCTCTCGAGAGACGTCTGTCTCCTTGTCCAGTTCGAGTTGTTTTAGAAGG

		B
P	T	sB
s	a	ms
t	q	AI
I	I	II

TGCAGGATTTCTGCAACTCTGATTACATCATCAGTCCAATCGACAAGCACGA
 -+-----+-----+-----+-----+-----+----- 1300
 ACGTCCTAAAGACGTTGAGACTAATGTAGTAGTCAGGTTAGCTGTTTCGTGCT

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 h F
 E l n C
 M s l uHAvDM B
 w p l 4glidn b
 o 3 I HauJel v
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 GACGCAACGCAGCTCAGTGAGGACAGCAACACACACTACGCAACAACACACA
 -----+-----+-----+-----+-----+----- 1352
 CTGCGTTGCGTCGAGTCACTCCTGTCGTTGTGTGTGATGCGTTGTTGTGTGT

F E
 Cn c
 BBM Avu o B
 sba li4 5 b
 rve uJH 7 v
 III III I I
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 CTACACTGGTTCTAGGTTCTGAAGCTGCTGTCTGTCTGTTGATTGGTTGGTT
 -----+-----+-----+-----+-----+----- 1404
 GATGTGACCAAGATCCAAGACTTCGACGACAGACAGACAACCTAACCAACCAA

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 oF S E
 CRn a c H
 M Avlu Tu D o CT i PM
 a li24 a3 p 5 la n ll
 e uJ4H qA n 7 aq f ey
 I IIII II I I II I II
 / / /
 CTAGGTTCTGAAGCTGCTGTGATCTCCTATCGATTGGTTGAGTCTTGGGAG
 -----+-----+-----+-----+-----+----- 1456
 GATCCAAGACTTCGACGACAGCTAGAGGATAGCTAACCAACTCAGAACCCTC

B ND
 s n M s lrGH
 r l l J adsp
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 VIII
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 TTCCCCAGTCGTTCTCTGTCTGGAGGTTCTGCTCCGAGGAACCAGATTTCAC
 -----+-----+-----+-----+-----+----- 1508
 AAGGGGTCAGCAAGAGACAGACCTCCAAGACGAGGCTCCTTGGTCTAAAGTG

E	T		
CH	t		
op	h		
Ph	l	C	D
II	l	v	d
/	l	i	e
	I	J	I
	I	I	

M		S		H			
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e	A	u	D	vHe		X	G
I	l	3	p	iaI		m	s
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I	I	I	I	III		I	I
				//			

			N	
C			CsPC N	
Av	ES		AvpvvMl	N
li	cp		liBuina	d
uJ	ii		uJIIJlI	e
II	II		IIIIIIIV	I
/	/		/// //	

TGATAGCTCCGCCCTCCAGCTGGCTCCATATGGGAACTATTATCAAAGTCTG
 -----+-----+-----+-----+-----+----- 1716
 ACTATCGAGGCGGGAGGTGACCGAGGTATACCCTTGATAATAGTTTCAGAC

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F
n      H B      E
u      i s      D PM      s
4      n m      d ll      p
H      f A      e ey      3
I      I I      I II      I
AGCGGCGACGAGTCGCTGAGACGAACCTACGAACTGCTCGCCTGTTTCAAGA
---+-----+-----+-----+-----+-----+-----+----- 1768
TCGCCGCTGCTCAGCGACTCTGCTTGGATGCTTGACGAGCGGACAAAGTTCT

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N	D				M	T	
l	r			M	a	s	T
aMN	aEM	M	H	b	e	p	s
Ins	Ian	n	p	o	I	4	p
Ilp	Irl	l	h	I	I	5	E
III	III	I	I	I	I	I	I
/	/						

AAGACATGCACAAGGTGAGGAAGAGGAGGGCAGGCGATGATGATGTCACAAT
 -+-----+-----+-----+-----+-----+-----+ 1820
 TTCTGTACGTGTTCCACTCCTTCTCCTCCCGTCCGCTACTACTACAGTGTTA

		B	E
	D	Bs	C
	d	sm	O
	e	aA	P
	I	II	I
		/	
TATGTAATGATGATGTTATCACTAAGATGTATCTGTTACAGGTGGAGACCT			
-----+-----+-----+-----+-----+-----+-----			
ATACATTACTACTACAATAGTGATTCTACATAGACAAGTGTCCACCTCTGGA			

1872

C		H		C	
vG	P	i		M	S
is	l	n		l	f
Ju	e	f		Y	e
II	I	I		I	I

ACCTGACGGTGGCTAAATGCCGACTCTCTCCAGAAGCCAACTGCACCCTGTAG
 -----+-----+-----+-----+-----+-----1925
 TGGACTGCCACCGATTACGGCTGAGAGAGGTCTTCGGTTGACGTGGGACATC

Enzymes that do cut:

AatII	AccI	AhaII	AluI	AlwI	AlwNI	AseI
AvaI	AvaII	BamHI	BanI	BanII	BbvI	BbvII
BceI	BclI	BglII	Bpu10I	BsaI	BsaJI	BsiI
BsmAI	Bsp1286I	BspCI	BspMI	BsrI	BstYI	Clai
CviJI	DdeI	DpnI	DraIII	DrdII	DsaI	EarI
EciI	Eco57I	EcoO109I	EcoPI	EcoP15I	EcoRII	EcoR124I
Esp3I	FauI	Fnu4HI	FokI	GsuI	HaeI	HaeIII
HgaI	HgiAI	HhaI	HinfI	HphI	KpnI	MaeI
MaeII	MaeIII	MboII	MlyI	MnlI	MseI	MspI
MwoI	NciI	NdeI	NlaIII	NlaIV	NsiI	NspI
NspBII	PflMI	PleI	PpuMI	PstI	PvuII	RsaI
Sau96I	Sau3AI	ScaI	ScrFI	SfaNI	SfeI	SpiI
SstI	StyLTI	TaqI	TaqII-2	TfiI	ThaI	Tsp45I
TspEI	Tth111II	Uba1105I	XmnI			

Enzymes that do not cut:

AflIII	AflIII	AgeI	ApaI	ApaLI	AvrII	BalI
BcgI	BglI	BsaAI	BsaBI	BsmI	BspGI	BspHI
BspMII	BssHII	BstBI	BstEII	BstXI	Bsu36I	Cfr10I
CfrAI	DraI	DrdI	EaeI	Eco47III	EcoAI	EcoBI
EcoDI	EcoDXXI	EcoEI	EcoKI	EcoNI	EcoRI	EcoR124/3I
EcoRV	EspI	FinI	FseI	FspI	GdiII	HaeII
HgiEII	HincII	HindIII	HinfIII	HpaI	McrI	MfeI
MluI	MmeI	NaeI	NarI	NcoI	NheI	NotI
NruI	PmlI	PshAI	PvuI	RleAI	RsrII	SacII
SalI	SfiI	SgrAI	SmaI	SnaI	SnaBI	SpeI
SphI	SplI	SspI	StuI	StyI	StySBI	StySJI
StySPI	StySQI	TaqII-1	Tth111I	Uba1108I	XbaI	XcmI
XhoI	XmaIII					

Appendix II

Reagent Abbreviations, Solutions and Reagents

Reagent Abbreviations

EDTA	Ethylenediaminetetraacetic acid
IPTG	Isopropyl- β -D-thiogalactopyranoside
TEAB	Tetraethylammonium borohydride
TRIS	Tris(hydroxymethyl)Aminomethane
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
EtBr	Ethidium bromide

Bacterial Media

LB (Luria-Bertani)	per liter	Bacto-tryptone	10 g
		Bacto-yeast	5 g
		NaCl	10 g
	Adjust pH to 7.5		
	<hr/>		
	for plates	Bacto-agar	15 g
TB (Terrific Broth)	Solution I		
	per 450 ml	Bacto-tryptone	6 g
		Bacto-yeast	12 g
		Glycerol	2 ml
	Adjust pH to 7.5		
	Solution II		
	per 50 ml	KH ₂ PO ₄	1.6 g
		K ₂ HPO ₄	8.2 g

Autoclave Solutions I and II separately. Combine.

DNA Buffers

TE	10 mM TRIS 1 mM EDTA	
Low TE	10 mM TRIS 0.1 mM EDTA	
TBE	per liter 10X stock solution TRIS base Boric acid EDTA Adjust pH to 8.0	121.1 g 55 g 7.4 g
TAE	per liter 50X stock solution TRIS base Glacial acetic acid 0.5 M EDTA Adjust pH to 8.0	242.2 g 57.1 ml 100 ml
STE	Stock solution 10X 100 mM TRIS (pH 8.0) 1 M NaCl 10 mM EDTA (pH 8.0)	

VITA

Carol Marie Dorworth was born in Philadelphia, Pennsylvania to Charles E. and Bertha M. Dorworth. She earned her B.S. in Biology from The Pennsylvania State University, May 1980, and her M.S. in Natural Resources from Cornell University, May 1984. Currently she is a candidate for the degree of Doctor of Philosophy in the School of Forestry, Wildlife, and Fisheries at Louisiana State University, Baton Rouge, Louisiana.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Carol Marie Dorworth

Major Field: Wildlife and Fisheries Science

Title of Dissertation: Partial Sequence and Characterization of a Growth Hormone Gene of the Red Drum (Sciaenops ocellatus).

Approved:

William R. Walters

Major Professor and Chairman

Heim

Dean of the Graduate School

EXAMINING COMMITTEE:

Robert F. Romaine

Robert C. Righ

Robert W. Chabreck

Albert H. Meier

Steven H. Chang

Date of Examination:

29 April 1991