Cloning, sequencing and expression of cDNA encoding growth hormone from Indian catfish (*Heteropneustes fossilis*)

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A tissue-specific cDNA library was constructed using polyA$^+$ RNA from pituitary glands of the Indian catfish *Heteropneustes fossilis* (Bloch) and a cDNA clone encoding growth hormone (GH) was isolated. Using polymerase chain reaction (PCR) primers representing the conserved regions of fish GH sequences the 3′ region of catfish GH cDNA (540 bp) was cloned by random amplification of cDNA ends and the clone was used as a probe to isolate recombinant phages carrying the full-length cDNA sequence. The full-length cDNA clone is 1132 bp in length, coding for an open reading frame (ORF) of 603 bp; the reading frame encodes a putative polypeptide of 200 amino acids including the signal sequence of 22 amino acids. The 5′ and 3′ untranslated regions of the cDNA are 58 bp and 456 bp long, respectively. The predicted amino acid sequence of *H. fossils* GH shared 98% homology with other catfishes. Mature GH protein was efficiently expressed in bacterial and zebrafish systems using appropriate expression vectors. The successful expression of the cloned GH cDNA of catfish confirms the functional viability of the clone.

1. Introduction

Growth hormone (GH) is an essential polypeptide required for normal growth and development of vertebrates. Since GH is commercially important in the areas of medicine, animal husbandry, fish farming and animal feed formulations, the gene coding for the hormone has been studied extensively in several mammalian and piscine species (Barat *et al* 1981; DeNoto *et al* 1981; Agellon *et al* 1988; Yamano *et al* 1988; Funkenstein *et al* 1991; Lemaire *et al* 1994; Ayson *et al* 2000; Venugopal T, Anathy V, Pandian T J, Gong G Z and Mathavan S, unpublished results). An analysis of GH cDNA sequences of number of species has shown that the coding regions are highly conserved in mammals, whereas it is less conserved in fishes. However, 3′ ends of the cDNAs are highly conserved in a number of vertebrates including fishes (Nicoll *et al* 1987; Johansen *et al* 1989).

Typical of the Silurids, the Indian catfish *Heteropneustes fossilis*, is a facultative air breather and has the advantage of being cultured in oxygen-deficient waters. However, *H. fossilis* grows relatively slower than the other cultivable Silurids; also the maximum size attainable by *H. fossilis* is just 300 g (Sheela 1999) while it is 30 kg for the riverine *Pangasianodon gigas* (Lemaire *et al* 1994). Therefore, there is a need and scope to increase the growth of Indian catfish. We are attempting to produce transgenic catfish by introducing additional copies of growth hormone cDNA following auto-transgenic technology. Cloning, isolation and characterization of GH cDNA of the target fish is the first step for the construction of "all fish gene transfer vector" and to generate

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Abbreviations used: GH, Growth hormone; IRES, internal ribosome entry site; RACE, random amplification of cDNA ends; RT, reverse transcriptase.

auto-transgeneic fish. Growth hormone cDNAs from the catfishes like *Ictalurus punctatus* (Tang *et al* 1993), *P. gigas* (Lemaire *et al* 1994) and *Pangasius pangasius* (Lemaire and Panyim 1993) have been cloned, sequenced and characterized**.** We have previously isolated and characterized the GH cDNA of the Indian major carp, *Labeo rohita* (Ham.) (Venugopal *et al* 1998; Venugopal T, Anathy V, Pandian T J, Gong G Z and Mathavan S, unpublished results). In the present paper we are reporting the cloning, characterization of GH cDNA from *H. fossilis* and its expression in *Escherichia coli* and zebrafish.

2. Materials and methods

2.1 *RNA extraction and purification*

Pituitary glands were collected from large number of *H. fossilis* and total cellular RNA was extracted following Guanidinium Thiocyanate-Phenol Chloroform method (Chomczynski and Sacchi 1987). The quality of total RNA was checked in denaturing formaldehyde agarose gel electrophoresis and quantity was determined spectrophotometrically. Poly A^+ RNA was purified from non-degraded total cellular RNA by affinity chromatography with oligo dT cellulose using poly A^+ RNA purification kit (Stratagene) following the protocols suggested by the company.

2.2 *Cloning of 3′ end of GH cDNA by random amplification of cDNA ends*

From the total RNA first strand cDNA was synthesized following the standard method. About 2 µg total RNA was incubated with 1 µg of poly dT primer at 65°C for 10 min and quenched on ice. Reverse transcriptase (RT) mix (containing 0·8 µg BSA, 1·4 µg DTT, 0·5 u RNase inhibitor, 800 µM dNTPs, 1× RT buffer and 8 u AMV-RT) was added to the quenched sample. First strand cDNA was synthesized by incubating the above mix at 37°C for 1 h. The mix was boiled at 95°C for 10 min, diluted with sterile distilled water and stored at – 70°C until further use.

By aligning fish GH cDNA sequence, 3′ conserved sequences were identified and a primer G1 (5′ CGGA ATTCATCGACAARGTSGAGAC 3′) corresponding to the conserved region was synthesized. Using the first strand cDNA as template, the 3′ end of the GH cDNA was amplified by PCR using G1 (forward) and poly dT (reverse) primers (10 pmol each). The reaction mix contained 0·25 u of Taq DNA Polymerase (Perkin Elmer), 1× reaction buffer and 0·25 µm dNTPs. Following conditions were used for amplification: 96°C for 1 min-initial denaturation and 94°C for 40 s denaturation, 55°C for 90 s annealing, 72°C for 3 min-extension for 30 cycles and a final extension at 72°C for 10 min. The resultant PCR product representing the 3′ end of the cDNA was purified and ligated to a T-tailed vector (pMOS blue, Amersham). Recombinant clones were identified by blue-white selection and analysed by restriction digestion. Few of the clones were sequenced and compared with published fish GH cDNA sequences.

2.3 *Construction and screening of cDNA library*

Pituitary gland (tissue) specific cDNA library was constructed using the poly A^+ RNA purified from the pituitary glands of catfish; *l* ZAP cDNA library cloning kit (Stratagene) was used for the construction. Briefly, first strand cDNA was synthesized from the poly A^+ RNA using M-MLV Reverse Transcriptase. After appropriate treatment, the second strand cDNA was synthesized using DNA polymerase. The double stranded cDNA was blunted by enzymatic reaction, ligated to the arms of *l* ZAP vector and packaged using *in vitro* Gigapack II gold packaging extract. The recombinant phages were grown in *E. coli* XLI blue MRF' cells and analysed by plaque hybridization, following the standard protocols (Sambrook *et al* 1989).

The RT-PCR product amplified by random amplification of cDNA ends (RACE) strategy representing the 3′ end of the GH cDNA (last exon of the cDNA and the 3′ untranslated sequences) was used as probe for screening the cDNA library. The probe DNA was labelled with [α^{32}P] dCTP using a random primer labelling kit (Gibco BRL). The plaques (~ 5000 plaques/plate) were transferred onto a nylon membrane by spreading the membrane over the plate for 2 min, and the membrane was dried briefly. The phages on the membrane were lysed and denatured in 0·5 M NaOH solution and neutralized in a solution of 1·5 M NaCl and 0·5 M Tris-HCl. The DNA was cross linked to the membrane by UV irradiation. The membranes were prehybridized and hybridized in a buffer containing 6× SSC, 50% formamide, 0·5% (w/v) SDS, 5× Denhardt's and 100 µg/ml denatured Herring's sperm DNA. The hybridization was carried out at 42°C for 12 h. After hybridization the membranes were washed at high stringency (0·1× SSC and 0·1% SDS at 65°C). Initially a single plaque was isolated and subsequently enriched by secondary and tertiary screening. The strongly hybridizing plaques were isolated and excised as pSK^- plasmid by *in vivo* excision protocol (Stratagene). The isolated plasmid clones (about 30) were screened for the full-length cDNA inserts following restriction analysis and Southern hybridization method.

2.4 *cDNA sequencing and analysis*

The nucleotide sequence of the cloned *H. fossilis* GH cDNA was determined by Sanger's dideoxy chain termination method, using Perkin Elmer bigdye terminator kit in an ABI Prism 377 automated DNA sequencer. All other computational analysis of the GH cDNA was done using GCG-9·1, Wisconsin package.

2.5 *Expression of catfish GH cDNA in* E. coli

2.5a *Cloning of GH-ORF into* E. coli *expression vector*: The ORF coding for the catfish GH was amplified from the full length cDNA by PCR using the forward (cGHF5′AATGCATGCTTCGAGAACCAGCGG3′) and reverse (cGHR 5′AATGTGACCTACGCTTAGATAAG 3′) primers. This PCR fragment (534 bp) representing the mature peptide of the GH was cloned into pSK^+ at the *Eco*RV site and the recombinant clones were identified. The ORF fragment was released from the vector by *Sph*I and *Sal*I digestion and cloned into pQE30 His-tag expression vector in which the ORF was under the control of phage T5 promoter. The 6His residues at the N terminus of the vector facilitated the purification of the expressed GH protein using Immobilized Metal Affinity Chromatography (IMAC). The recombinant vector was transformed into M15 *E. coli* cells and the transformants were selected on LB agar plates containing ampicillin (50 μg/ml) and kanamycin (25 μg/ml). The colonies growing on the ampicillin and kanamycin plates were selected and used for the expression.

2.5b *Expression*: A single colony from the above transformants was inoculated into LB medium (5 ml, starter culture) containing ampicillin and kanamycin antibiotics. This starter culture was grown overnight at 37°C with vigorous shaking and about 500 μl of the above culture was inoculated into 50 ml LB medium containing the antibiotics. The culture was grown at 37°C until the A_{600} reached 0·6. IPTG (0·75 mM) was added to the culture for the induction of GH protein expression and the culture was grown for 2 h. Subsequently, 0·25 mM of IPTG was added again and the culture was allowed to grow for 2–3 h. Control cultures were maintained parallelly. The cells from the control and induced cultures were pelleted, lysed in urea buffer and the protein concentration was quantified by Lowry's method (Lowry *et al* 1951). Proteins expressed in control and induced cultures were analysed in SDS-PAGE. The gel was stained with Coomasie Brilliant blue and destained after 4 h to observe the protein bands.

2.5c *Purification of catfish GH protein*: The over expressed GH protein was purified using NI-NTA matrix (QIAGEN). The crude bacterial lysate (about 4 ml) was mixed with 1 ml of NI-NTA matrix and incubated at room temperature for 1 h with intermittent agitation. The matrix-mix was filled into a column in which bottom outlet was capped with glass wool. The initial flow through was allowed and the matrix was washed sequentially with 20 mM, 50 mM and 75 mM imidazole buffers. After the washes, the protein was eluted with 250 mM imidazole buffer (imidazole buffer contains: 100 mM NaH_2PO_4, 300 mM NaCl, 20/50/75/250 mM imidazole). The fractions collected were subjected to SDS-PAGE to detect the GH protein band.

2.5d *Western blot analysis*: Proteins expressed in the induced and control cultures were separated in SDS-PAGE and transferred to PVDF membrane. The protocols given in the QIA express detection and assay handbook was followed for Western blot analysis for the detection of GH protein using His-tag antibody. The PVDF membrane was incubated with anti penta His monoclonal antibody (1/2000 dilution) solution for 1 h at room temperature and washing steps were carried out as per the standard protocols. Then the membrane was incubated with secondary antibody (1/4000 dilution of antibody stock solution in 3% BSA; conjugated with horse radish peroxidase) for 1 h at room temperature and washed 4 times for 10 min each in TBS-Tween buffer. Subsequently, to detect the expressed protein, the membrane was treated with staining solution [18 mg 4 chloro-1 napthol dissolved in 6 ml of methanol and mixed with 24 ml of 1× Tris-saline containing 60 μl of 30% hydrogen peroxide (H_2O_2)].

2.6 *Expression of catfish GH cDNA in zebrafish* (Danio rerio) *embryos*

2.6a *Construction of pCS2 + cGH-IRES-EGFP vector*: The cGH full length cDNA was cloned into the bi-cistronic expression vector that contained EGFP as the reporter gene. The internal ribosome entry site (IRES) in the bi-cistronic IRES-EGFP expression vector facilitates the expression of the cDNA cloned into the MCS of the vector along with the reporter gene. Catfish GH cDNA was cloned into *Eco*RI–*Sal*I site of the IRES-EGFP vector. The fragment containing the cDNA-IRES-EGFP was released from the vector by *Eco*RI and *Not*I digestion and cloned into the compatible end of the pCS2+ vector. The cloned fragment was placed at the downstream of SP6 promoter-cGH-IRES-EGFP in pCS2+ vector. The fidelity of this vector was confirmed by PCR using EGFP forward primer and vector primer (T7) and also by sequencing.

2.6b *RNA preparation and microinjection*: The vector pCS2+-cGH-IRES-EGFP was linearized with *Not*I and the 5′ capped sense mRNA was synthesized using SP6

MESSAGE MACHINE™ kit (Amion, Austin, USA) following the instructions of the manufacturer. The transcripts obtained contained GH mRNA and also EGFP mRNA in tandem. The RNA was mixed with injection solution (0·1 M Tris-HCl, 0·25% Phenol Red, pH 7·6) to a final concentration of 100 ng/µl. About 5 nl of the transcribed mRNA was injected into one-celled embryos of zebrafish *D. rerio*. After injection, the embryos were kept at 28·5°C and the expression of EGFP was monitored from 4 hpf (hours-post-fertilization) under fluorescent microscope.

3. Results

The sequence of the RACE product (3′ end of the catfish GH cDNA) was compared with GH nucleotide sequences of other Siluride members; the comparison showed 95% homology with the published GH sequences of catfishes (data not shown). This analysis has confirmed that the RACE product represents the partial cDNA of catfish GH containing 84 bp of the ORF (28 aa; last exon) and 456 bp of 3′ UTR. The cDNA library generated for this work had a titer of about 2×10^7 primary plaques/µg of DNA with significant percentage of recombinants. A total of about 5×10^4 plaques were screened by plaque hybridization technique using the 3′ end of the cDNA clone as the probe. On an average 5000 plaques were screened from each plate for the recombinants. The positive plaques from the preliminary screening were subjected to further screening and after tertiary screening almost all the plaques were positive. Full-length GH cDNAs excised as plasmid clones from the recombinant phage following *in vivo* excision method were analysed by restriction digestion. About 30 clones were analysed and all the clones contained the full-length cDNA insert. Few of the clones were subjected to Southern blotting and hybridization using the 3′ end of the cDNA as probe. All the clones hybridized to the probe indicating that the clones had full-length GH cDNA inserts and size of the insert was about 1·13 kbp.

Few of the full-length cDNA clones were sequenced and their nucleotide sequence of is given in figure 1. The full-length sequence was 1132 bp in length (GenBank Accession number: AF 147792). The cDNA had an ORF of 603 bp and untranslated sequences of 58 bp at the 5′ and 456 bp at the 3′ regions. The polyadenylation signal AATAAA was 15 bp upstream of polyadenylation site. Based on the nucleotide sequences the deduced putative peptide contained 200 aa representing 178 aa of matured peptide and 22 aa of signal sequence.

Analysis of proteins expressed in the control and induced *E. coli* cultures in SDS-PAGE showed the over expression of recombinant GH protein in the IPTG induced cultures. Based upon the amino acid composition of the coding region, the predicted size of the GH protein was about 19·5 kDa. From the Coomasie blue stained gel it is clear that over expressed GH protein is about 19·5 kDa and corresponds to the predicted size of the protein (figure 2A). Since the crude extract showed many protein bands, the GH protein was purified using NI-NTA column and purified protein showed a single band upon elution (figure 2A). The Western blot analysis of the purified protein showed a specific binding of the anti-HIS antibody (figure 2B). The specificity of antibody reaction and size of the protein proved beyond doubt that the over expressed protein is indeed the catfish GH protein expressed in fusion with the histidine codons of pQE-30 vector.

The *in vivo* expression of GH-EGFP was monitored in the zebrafish embryos by injecting the *in vitro* transcribed mRNA from the bi-cistronic vector containing cGH-IRES-EGFP. The developing embryos and fish showed green colour indicating the expression of EGFP (figure 3). Since the GH cDNA was cloned into bi-cistronic vector, which has the IRES element, during *in vitro* transcription, the Message Machine kit transcribed the GH mRNA and the EGFP mRNA in tandem. Under this conditions, if EGFP is expressed it implies that GH cDNA is also expressed. The injected mRNA was translated into proteins which could be observed as green colour in the embryo. It is clear from the observation that the GH is fully functional.

4. Discussion

The present paper reports the cloning and expression of growth hormone cDNA of *H. fossilis* and this is the first report for the Indian catfish. The comparison of nucleotide sequences of *H. fossilis* GH cDNA and sequences of other catfish cDNAs (*P. gigas*, *P. pangasius* and *I. punctatus*; data not shown) revealed that the 5′ UTR is almost the same size in the catfishes except in *I. punctatus* which has 10 bp more in this region than the other species. The sequence homology within the ORFs of these catfishes was found to be 98%. *H. fossilis* GH cDNA sequence shared 74% homology with Indian major carp, *Labeo rohita* (Venugopal T, Anathy V, Pandian T J, Gong G Z and Mathavan S, unpublished results). Figure 4 shows the comparison of peptide sequences of catfishes. Invariably, the peptide sequence showed 98% homology among the Siluride members. The predicted protein has a putative signal peptide of 22 aa and a mature peptide of 178 aa. The signal peptide is few amino acids shorter than human/rat GH (26 amino acids) or bovine GH (27 amino acids) (Lewis *et al* 1980; Miller *et al* 1980). In fishes including Silurids, there are two putative N-glycosylation

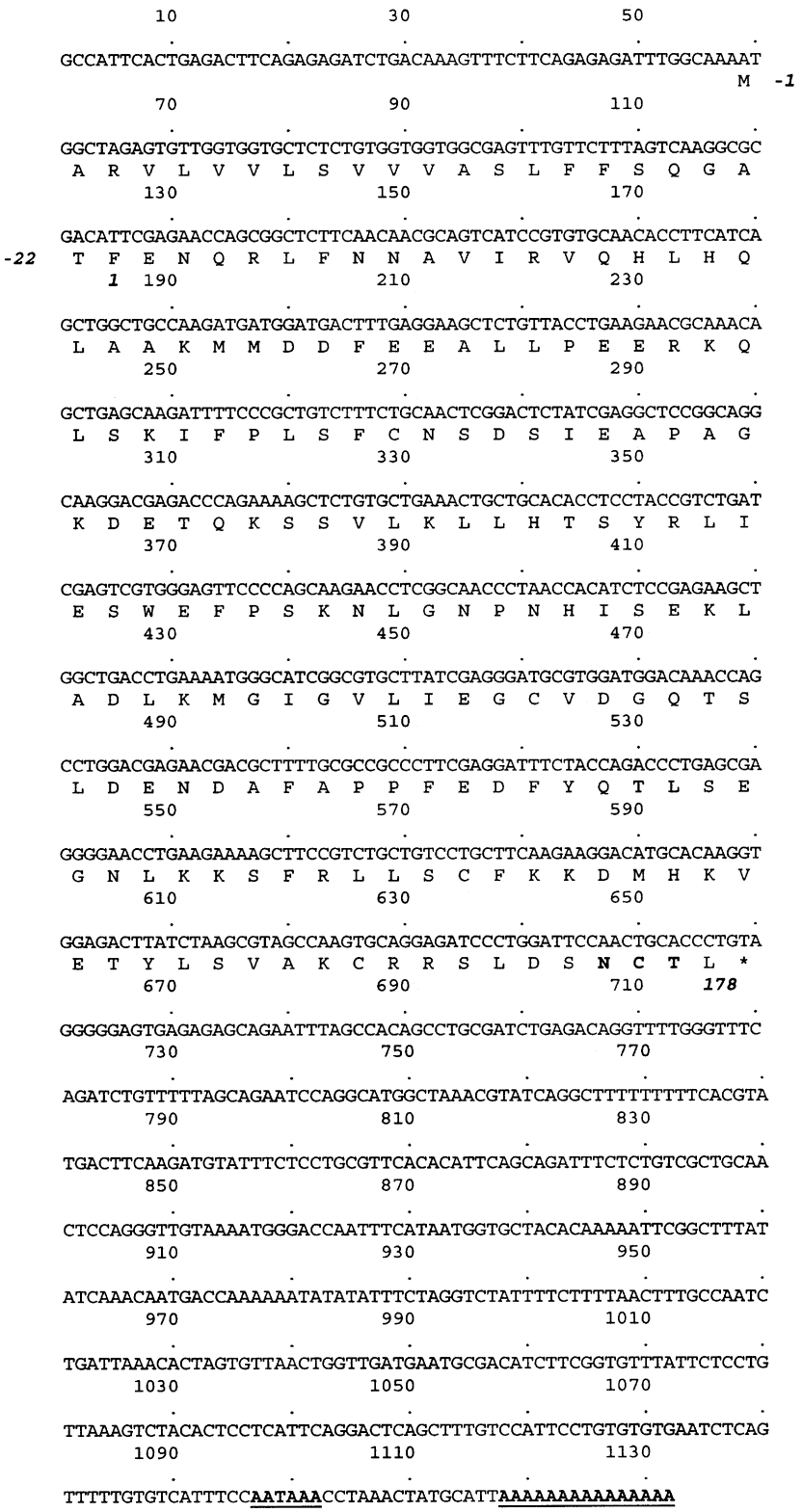

Figure 1. The complete nucleotide sequence of the Indian catfish, *H. fossilis* GH cDNA encoding the prepro GH peptide. The cDNA is 1132 bp long having a signal peptide of 22 residues and the mature peptide of 178 residues. Putative N-glycosylation sites (Asn-Xaa-Thr/Ser) are marked in bold letters. The poly adenylation signal AATAAA and the poly A^+ tails are underlined (GenBank Accession No. AF147792).

sites (Asn-Xaa-Thr or Ser) normally present at 125th and 175th positions of the mature peptide (Lemaire *et al* 1994). However, in *H. fossilis* sequence, there is only one N-glycosylation site at the carboxyl terminus at 175th position of the mature peptide (figure 4). Four Cys residues (49, 113, 151 and 178) are located in almost all the mature GH peptides at an identical positions (Carlacci *et al* 1991); GH protein of *H. fossilis* also have the Cys residues in the identical positions. These Cys residues form 2 disulphide bonds in human and bovine GH molecules (Lewis *et al* 1980; Miller *et al* 1980). Their presence was found to be important for the structural integrity and biological activity of the hormone. Probably these are the regions, from which strong homology could be drawn between vertebrate GH sequences (Schneider *et al* 1992). *H. fossils* GH peptide sequences revealed conserved somatotropin, prolactin and related hormone signatures 1 (71–104) and 2 (173–190). These signatures are also conserved in all the catfishes (figure 4).

There is a need for the over-expression and production of growth hormone in expression systems as this protein has application in medicine, animal husbandry and aquaculture. Substitution of over expressed growth hormone in fish feed has been shown to promote growth and production (Jeh *et al* 1998; Ben-Atia 1999; Chen *et al* 2000). Further, to test the functionality of the cloned gene for its full expression and viability, expression studies are essential. GH genes of few fish have been successfully expressed in *E. coli* (Sekine *et al* 1985; Agellon and Chen 1986; Mahmoud *et al* 1998; Saito *et al* 1988), in yeast (Tsai *et al* 1993; Jin *et al* 1999) and in baculovirus (Ho *et al* 1998). To detect the expression, reporter genes such as βgal (Sheela *et al* 1999; Rahman *et al* 2000) and luciferase (Volckaert *et al* 1995) have been used and these

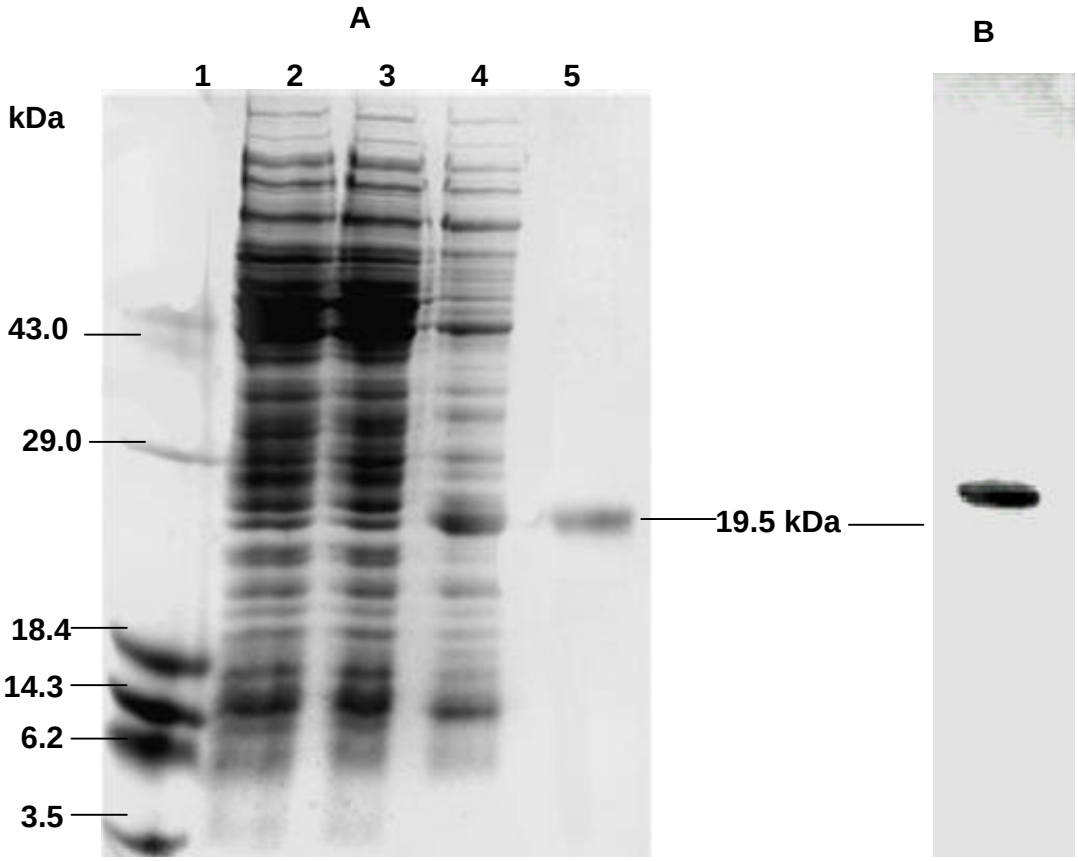

Figure 2. (**A**) SDS-PAGE analysis of the crude and purified GH protein of the *H. fossilis* over expressed in *E. coli* cultures. The coding region of the GH cDNA was cloned into pQE30 expression vector and transformed using M15 *E. coli* cells. GH was over expressed using the transformed cells in culture induced by IPTG. Over expressed protein was purified NI-NAT matrix and analysed in SDS-PAGE. Lanes: 1. Marker, 2. Crude lysate of the *E. coli* harbouring pQE30 vector with out the GH ORF (induced with IPTG), 3. Crude lysate of the *E. coli* harbouring pQE30 vector with the GH ORF (un-induced), 4. Crude lysate of the *E. coli* harbouring pQE30 vector with the GH ORF (induced with IPTG) 5. Purified GH. (**B**) Western blot analysis: Purified GH protein was immobilized to PVDF membrane and detected using anti-HIS antibodies. Specific binding of the antibody to the purified GH protein indicate the proper expression and processing of the protein with out degradation.

genes are mainly expressed as fusion proteins. In the present study we have expressed the catfish GH cDNA in *E. coli* (*in vitro*) and also in zebrafish (*in vivo*) using system specific vectors.

The expression of catfish GH in *E. coli* adds to the list of the fish GHs expressed in prokaryotic systems and this over-expression may help to formulate fish feeds containing GH to augment catfish growth. The GH protein expressed in *E. coli* system in the present study was not degraded at any stage of expression. There was a single protein band in the purified fraction of the protein; even in the Western blot analysis, the immunoreaction was

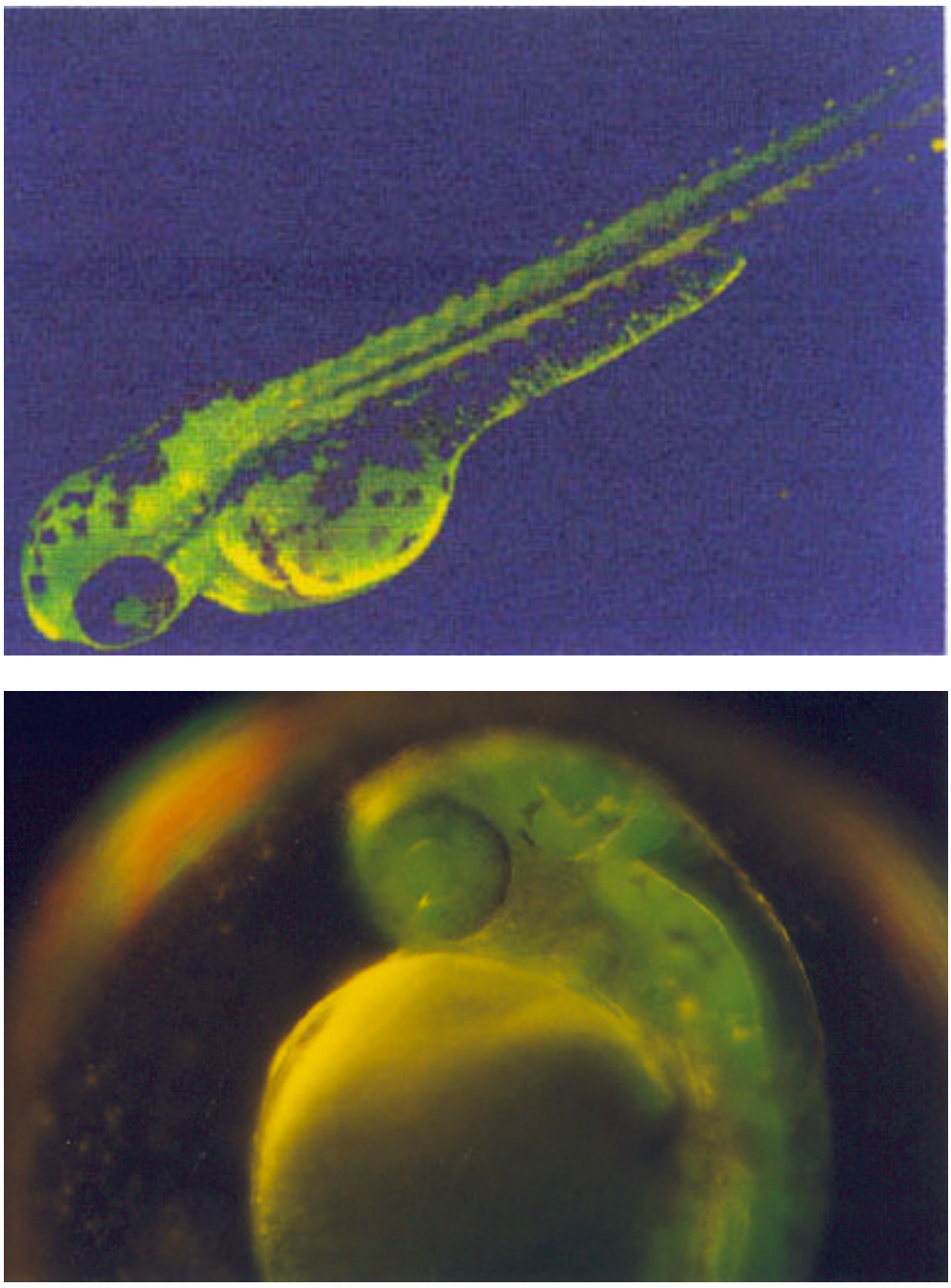

Figure 3. *In vivo* expression of GH-EGFP in zebrafish embryo. The *in vitro* transcribed cGH-IRES-EGFP mRNA was injected into one cell embryo and the expression was monitored continuously. The expression shown here is in developing embryo and freshly hatched fish. The intensity of green colour indicate the strong expression of EGFP in all the tissues of the embryo/fry. The expression of EGPF indicates the co-expression of catfish GH cDNA and the functional viability of the clone.

observed at the expected size of 19·5 kDa and no smaller fragments could be detected indicating the degradation of the protein. These observations suggest the proper processing of the protein in the expression system and confirm that the catfish GH cDNA is intact. The functional viability of the clone was tested following *in vivo* expression in zebrafish.

A bi-cistronic vector is ideal for the independent expression of two genes (candidate and reporter gene); the IRES element in the vector facilitates the expression the candidate and reporter gene together and hence we have used the bi-cistronic vector with EGFP as reporter gene. Further, EGFP reporter gene is useful because the expression could be detected in live fish. The bi-cistronic vector has been shown to be functional in mammalian expression systems (Mountford and Smith 1995) and also in fish (Xukun *et al* 2000; Venugopal T, Anathy V, Pandian T J, Gong G Z and Mathavan S, unpublished results). Following observations can be made from the results obtained on the expression of catfish GH and EGFP in zebrafish: (i) As explained earlier, the *in vitro* transcribed mRNA contained the GH and EGFP transcripts in tandem and the expression of EGFP amounts to the expression of growth hormone. (ii) The reasons for the appearance of green colour all over the embryo/fry is that the mRNA injected at one/two cell stage of the embryos expressed in these cells and the expressed product has percolated to all the cell lineages giving the colour to all tissues. (iii) The intensity of green colour is quite strong indicating that the expression is intensive. (iv) The expression was observed even after 48 h of injection indicating that the expression is fairly stable. In mRNA injection studies, the expression is normally transient. However, if the resultant product is stable the expression could persist for an extended period. In this study the EGFP appears to be stable and hence the expression has been observed for longer time at the same intensity. These observations of the *in situ* expression of the GH-EGFP indicate that the catfish GH cDNA is intact and functionally viable. The cloning and characterization of *H. fossilis* GH cDNA should enable us to construct an all fish gene transfer vector and for the production of auto-transgenic catfish.

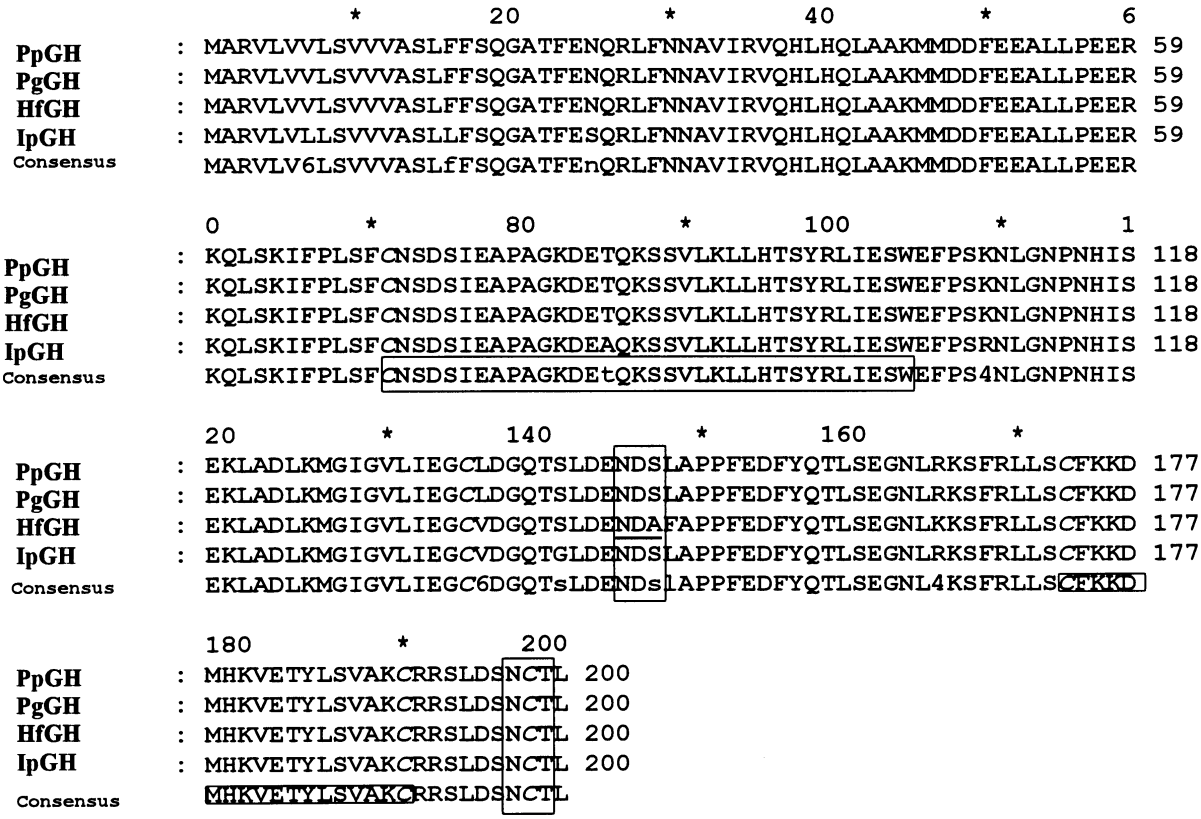

Figure 4. Comparison of GH peptide sequence of catfishes. HfGH; *H. fossilis*; IpGH, *I. punctatus*; PgGH, *P. gigas*; PpGH, *P. pangasius*. Note the presence of Ala instead of Thr/Ser at the 147th position (underlined) of the GH peptide eliminating one potential N-glycosylation site in HfGH (boxes indicates the potential N-glycosylation sites). The conserved Cystiene (C) residues are indicated in italics. The conserved Somatotropin 1 (70–104) and 2 (173–190) signatures are indicated in horizontal box.

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