GENERAL AND COMPARATIVE ENDOCRINOLOGY 96, 179–188 (1994)

Cloning, Expression, and Characterization of a Recombinant Gilthead Seabream Growth Hormone

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Accepted July 1, 1994

cDNA clones coding for the gilthead seabream (*Sparus aurata*) growth hormone (sbGH) were isolated from a pituitary expression library using a flounder cDNA probe. The nucleotide sequence of a GH cDNA clone containing an insert of 896 nucleotides was determined. The cDNA encoded a polypeptide of 204 amino acids including a signal peptide of 17 amino acids and contained a 5′ and a 3′ untranslated region of 48 and 233 nucleotides, respectively. The mRNA determined by Northern blot was approximately 1 kb. Amino acid sequence homologies of 97.1% with red seabream GH, 88.9% with the tuna GH, and 67% with the coho salmon GH was found. Transient expression of a sbGH cDNA was done in HeLa cells by induction with a vaccinia virus system, and the expressed GH was detected by immunofluorescence and immunoprecipitation with a specific antibody to the native sbGH. The sbGH cDNA was expressed in *Escherichia coli* by using the pGEX-3X and the pET-3a expression systems. The recombinant sbGH expressed in the pET-3a system was similar, if not identical, to the native hormone when analyzed by homologous radioimmunoassay and receptor binding assay.

Pituitary growth hormone (GH) is a single-chain polypeptide of about 22 kDa, which is essential for normal growth and development of vertebrates (Holly and Wass, 1989). GH has been isolated from pituitaries of several fish species (Farmer *et al.*, 1976; Kawauchi *et al.*, 1986; Rand-Weaver *et al.*, 1989; Sakata *et al.*, 1993) and the mode of action and mechanisms that control its secretion in Teleost species has been addressed (Sakamoto *et al.*, 1993). However, for these studies a major problem is the difficulty in obtaining sufficient quantities of purified hormone.

Progress in recombinant DNA technology provides a means to produce suitable amounts of biologically active GHs. Growth hormone cDNA has been cloned and expressed for chum salmon (Sekine *et al.*, 1985), rainbow trout (Agellon and Chen, 1986), Japanese eel (Saito *et al.*, 1988; Sugimoto *et al.*, 1991); tilapia (Rentier-Delrue *et al.*, 1989), tuna (Kimura, 1991), yellowtail (Watahiki *et al.*, 1992), Japanese flounder (Watahiki *et al.*, 1992), and two Sparidae species, red seabream (Kosugi *et al.*, 1992) and yellowfin porgy (Tsai *et al.*, 1993). Funkenstein *et al.* (1991) cloned and sequenced a GH cDNA for another Sparidae, the gilthead seabream, *Sparus aurata*. No biologically active, recombinant gilthead seabream GH (rsbGH) has been expressed and characterized. Accordingly this study describes the molecular cloning, sequencing, and expression of a cDNA coding for the sbGH and characterizes the resulting recombinant GHs. The structure of the gene and the physiological role of the hormone may now be investigated.

0016-6480/94 $5.00

MATERIALS AND METHODS

Animals were obtained from a local fish-farming research facility (Pemares, Puerto de Santa María, Spain). Restriction enzymes were from Boehringer Mannheim. Tissue culture reagents were from Gibco-BRL and nitrocellulose and nylon membranes were from Millipore. [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{35}S]dATP (600 Ci/mmol) were purchased from Amersham, UK. Plasmid pGEX-3X was from Pharmacia LKB Biotechnology Inc., and pET-3a vector was kindly provided by Dr. F. Martínez Abarca (Centro de Biología Molecular, Madrid). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Boehringer Mannheim.

Preparation of cDNA Library, Screening, and Sequencing

Gilthead seabream pituitary glands were collected from approximately 200 juvenile fish weighing around 150 g. Total RNA was extracted by a guanidine isothiocyanate procedure and mRNA was isolated by chromatography on an oligo (dT)-cellulose column (Maniatis *et al.*, 1982). cDNA libraries were constructed using the cDNA Synthesis Plus and a λ-gt11 cDNA Cloning System (Amersham, UK). The cDNA was ligated into the *Eco*RI site of the λgt11 vector and then packaged following standard protocols. The phage library obtained consisted of 1.5×10^6 recombinants. The library was screened for GH sequences by hybridization with a flounder GH cDNA probe kindly provided by Dr. Hideo Ohgai. Hybridization was carried out by standard procedures at 50° (Maniatis *et al.*, 1982). Filters were washed twice at 50° in 1× SSC, 0.1% SDS for 30 min and once in 0.5× SSC, 0.1% SDS at 55° for 15 min. The cDNA inserts from positive clones were first amplified by PCR using λgt11 primers and subcloned into a Bluescript vector (Stratagene). DNA sequencing was performed by the dideoxynucleotide procedure (Sanger *et al.*, 1977).

Northern Analysis

Pituitaries from 50 juvenile *S. aurata* were used for RNA purification. Total RNA prepared by the guanidine isothiocyanate–phenol–isopropanol method (Maniatis *et al.*, 1982) was resuspended in diethylpyrocarbonate-treated water. Twenty micrograms of total RNA was loaded onto a 1.5% agarose 2.2 *M* formaldehyde gel and blotted overnight onto a nylon membrane in 20× SSC. The membrane was prehybridized for 2 h at 60° and then hybridized to ^{32}P-labeled GH cDNA. Hybridization was carried out for 16 hr at 60° in 6× SSC (1× = 15 m*M* sodium citrate, 0.15 *M* NaCl, pH 7.6), 2× Denhardt's reagent (1× = 0.02% each ficoll, polyvinylpyrrolidone, and BSA), 0.1% SDS, and 200 μg/ml yeast tRNA. After hybridization, the membrane was washed first in 6× SSC, 0.1% SDS at 60° and then in 1× SSC, 0.1% SDS at 65° and finally exposed to X-ray film.

Infection and Transfection

HeLa cells grown in culture were split 24 hr before infection so that they were at least 75% confluent at the time of the procedure. The cells were washed once with serum-free medium and first infected with vaccinia viruses vT7 (Fuerst *et al.*, 1986) for 1 hr at 37°. The cells were then transfected for 6 hr at 37° with the Bluescript-GH construct driven the T7 promoter using the lipofectin reagent (Gibco-BRL) according to the manufacturer's instructions.

Immunofluorescence and Immunoprecipitation

The immunofluorescence procedure has been described previously (Valdivia *et al.*, 1986). Immunoprecipitation was done on [^{35}S]methionine-labeled HeLa cell extracts by adding a specific anti-native sbGH antibody (Le Bail *et al.*, 1993). The immunocomplex was precipitated with protein A–Sepharose and after several washes with a buffer containing 50 m*M* Tris–HCl, pH 7.0, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 150 m*M* NaCl, and one final wash with 20 m*M* Tris–HCl, pH 7.5, 140 m*M* NaCl, the pellet was analyzed by SDS–PAGE (Laemmli, 1970) and autoradiography.

Expression of sbGH in E. coli

A full-length sbGH cDNA (the whole coding region including the putative signal peptide) was cloned into the *Sma*I site of the pGEX-3X expression vector. DH5α *E. coli* cells were transformed with this vector and grown in L-broth medium containing 50 μg/ml of ampicillin. Protein expression was induced for 2 hr at 37° by adding isopropyl β-D-thiogalactopyranoside (IPTG) up to 0.1 m*M*. The sbGH fusion protein (glutathione transferase-sbGH, GHFP) remained insoluble as inclusion bodies. These were purified from other cellular components by a brief sonication of the bacterial culture and extensive washes with 10 m*M* Tris–HCl, pH 7.1, buffer containing 0.1% Triton X-100. GHFP was solubilized from the inclusion bodies with a phosphate buffered saline (PBS) solution containing 8 *M* urea and 5 m*M* DTT. The solution was centrifuged and the resulting supernatant was extensively dialyzed against PBS containing 3 *M* urea and finally against PBS.

Expression of the sbGH cDNA in the pET-3a system was carried out as follows: a 564-bp amplified

fragment of the sbGH cDNA, corresponding to the full coding area, was generated by PCR using two primers, named DOR-1 and DOR-2, synthesized in a Gene Assembler (Pharmacia Biotech.). The sequences were 5′ CCCGGGCATATGCAGCCGATCACAGACGGCCAG 3′ for DOR-1 and 5′ GGGCCCGGATCCCTACAGGGTGCAGTTGGCCTC 3′ for DOR-2. The synthetic (oligo) DOR-1 contains a *Nde*I site harboring an ATG initiation codon, and the DOR-2 contains a *Bam*HI site and a termination codon TAG. The oligonucleotides were designed so that the signal peptide sequence contained into the sbGH cDNA was deleted after the amplification reactions. The resulting PCR fragment was subcloned into the *Nde*I–*Bam*HI sites of the pET-3a plasmid and used to transform BL21 (DE3) host cells. Growing cells were induced by adding IPTG as described (Studier and Moffatt, 1986). The rsbGH stored as inclusion bodies was isolated after a brief sonication and solubilization in 6 *M* guanidinium–HCl and further dialyzed against 50 m*M* NH_4HCO_3, pH 7.8, as indicated by Paris *et al.* (1990).

Characterization of the Recombinant GH Proteins

To characterize the GH expressed in the pGEX system, the GHFP was cleaved with factor Xa as indicated by the manufacturer. The resulting proteins were purified on an affinity column (Pharmacia LKB Biotechnology Inc.) and analyzed by SDS–PAGE and Western blot (Towbin *et al.*, 1979) using anti-native sbGH antibody. The protein expressed in the pET system (rsbGH) was similarly analyzed.

The GHFP and the rsbGH expressed proteins were immunologically characterized in an homologous radioimmunoassay (RIA) for native sbGH described elsewhere (Le Bail *et al.*, 1993). As a control, proteins expressed in the pGEX system lacking the GH cDNA insert were also tested in the RIA.

These expressed proteins, together with the products of the cleavage of the GHFP with factor Xa, were also tested for their ability to displace iodinated native sbGH in an hepatic radioreceptor assay (Pérez-Sánchez *et al.*, 1993). According to the same reference, the rsbGH was radioiodinated by the chloramine-T method (55 Ci/μg), and tested for specific binding to liver membrane receptors.

RESULTS AND DISCUSSION

Cloning and Nucleotide Sequence Analysis of sbGH

A cDNA library was constructed with 5 μg of mRNA extracted from gilthead seabream pituitaries. A total of 5×10^5 clones were screened by hybridization with a ^{32}P-labeled fragment of a flounder GH cDNA. Three positive clones, namely D1 (896 nt), D2 (865 nt), and D3 (547 nt), were chosen for further characterization. For sequencing purposes the inserts of those clones were amplified by the PCR technique using as primers two oligonucleotides flanking the *Eco*RI site of the λgt11 vector (Saiki *et al.*, 1988). From D1 and D2 clones a single *Cla*I site at nt position 337 was used to originate subclones of the 5′ end and 3′ end in the Bluescript vector. All these DNAs were used for sequence analysis according to Sanger *et al.* (1977). Analysis of the nucleotide sequence shown in Fig 1 reveals an open reading frame of 615 bases encoding a protein of 204 amino acids, with an estimated molecular weight of 23,161 Da. Our sbGH sequence differs in four nucleotides from other previously published (Funkenstein *et al.*, 1991). According to the sbGH sequence deposited with GenBank by those authors (under accession no. S54890), our discrepancies correspond to nt 222 (G by us for C by them), nt 223 (A for G), nt 261 (C for G), and nt 490 (C for G). First, there is a difference between the sequence published by those authors (Funkenstein *et al.*, 1991) and that deposited with GenBank (position 261, TCT for TGT). More significantly, nucleotide sequence in Fig. 2 (of that reference) does not correspond in some of those positions with the amino acid represented in Fig. 3 in that publication (position 223 CCG is proline and not arginine, position 490 AGG is arginine and they assigned serine, and at amino acid 29 they assigned serine to ATC which is actually isoleucine). We conclude that most probably all these changes are because of typing errors, and it may explain the sequences differences found by us.

The size of the cDNA coding for sbGH (896 bp) was in close agreement with the mRNA length determined from the Northern hybridization experiment. Figure 2

```
                                                                    1
   CAGATCTAGTCACCAGAACTTGAACCAAAACCAGAACCTGAACCAGAC ATG GAC AGA
                                                                Met Asp Arg

                                10
GTG GTG CTC ATG CTG TCG GTG ATG TCT CTG GGC GTC TCT TCT CAG CCG
Val Val Leu Met Leu Ser Val Met Ser Leu Gly Val Ser Ser Gln Pro

 20                                                 30
ATC ACA GAC GGC CAG CGT CTG TTC TCC ATC GCT GTC AGC AGA GTT CAA
Ile Thr Asp Gly Gln Arg Leu Phe Ser Ile Ala Val Ser Arg Val Gln

                    40                                          50
CAC CTC CAC CTG CTG GCT CAG AGA CTC TTC TCT GAC TTT GAG AGC TCT
His Leu His Leu Leu Ala Gln Arg Leu Phe Ser Asp Phe Glu Ser Ser

                                    60
CTG CAG ACG GAG GAG CAG CGA CAG CTC AAC AAA ATC TTC CTG CAG GAT
Leu Gln Thr Glu Glu Gln Arg Gln Leu Asn Lys Ile Phe Leu Gln Asp

          70                                            80
TTC TGT AAC TCT GAT TAC ATC ATC AGC CCC ATC GAC AAG CAC GAG ACG
Phe Cys Asn Ser Asp Tyr Ile Ile Ser Pro Ile Asp Lys His Glu Thr

                                90
CAG CGC AGC TCA GTG TTG AAG CTG CTG TCT ATC TCT TAT CGA TTG GTC
Gln Arg Ser Ser Val Leu Lys Leu Leu Ser Ile Ser Tyr Arg Leu Val

100                                                110
GAG TCT TGG GAG TTC CCC AGT CGT TCT CTG TCT GGC GGT TCT GCT CCG
Glu Ser Trp Glu Phe Pro Ser Arg Ser Leu Ser Gly Gly Ser Ala Pro

                    120                                         130
AGG AAC CAG ATT TCA CCC AAA CTG TCT GAG CTG AAG ACG GGC ATC CAT
Arg Asn Gln Ile Ser Pro Lys Leu Ser Glu Leu Lys Thr Gly Ile His

                                    140
CTC CTG ATC AGG GCC AAT GAG GAC GGA GCA GAG ATC TTC CCT GAT AGC
Leu Leu Ile Arg Ala Asn Glu Asp Gly Ala Glu Ile Phe Pro Asp Ser

          150                                           160
TCC GCC CTC CAG CTG GCT CCT TAT GGA AAC TAC TAC CAA AGT CTG GGC
Ser Ala Leu Gln Leu Ala Pro Tyr Gly Asn Tyr Tyr Gln Ser Leu Gly

                                170
ACC GAC GAG TCG CTG AGA CGA ACC TAC GAA CTA CTT GCC TGT TTC AAA
Thr Asp Glu Ser Leu Arg Arg Thr Tyr Glu Leu Leu Ala Cys Phe Lys

180                                                190
AAA GAC ATG CAC AAG GTG GAG ACC TAC CTG ACG GTG GCA AAA TGT AGA
Lys Asp Met His Lys Val Glu Thr Tyr Leu Thr Val Ala Lys Cys Arg

                    200                 204
CTC TCT CCA GAG GCC AAC TGC ACC CTG TAG CCCCGCCTCTCTTCTGTGAAACC
Leu Ser Pro Glu Ala Asn Cys Thr Leu  *   .

ACTGCCCCCGTTGATGATGTAATCGTGTGTGTTCTGTAGCTCCGCCTCCACGCTCTCTGACTC
TGATAAGTGGTGTTAGCACTAGCATTAGCATTAGCTCCTGTTCAGTGGTGGAGGATGTAATTG
AAGGTGGTCTGGTGTCATGATGATGAAAGTTTTGAACAGGAAGTGATGTCATACTGTGAAATA
AAGTCTGTGCTGTTTGCATTCAAAAAAAAAAAAAAAAAAAAAAAAAA
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FIG. 1. Complete nucleotide sequence of full length gilthead seabream GH cDNA and the deduced amino acid sequence. Amino acid numbering is indicated. This sequence has been deposited in the EMBL/GenBank data base (accession No. U01301).

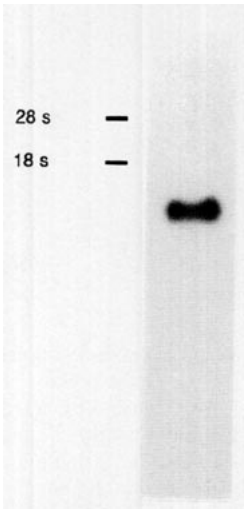

FIG. 2. Northern analysis of the gilthead seabream GH mRNA. Total pituitary RNA was electrophoresed on a 1.5% agarose 2.2 *M* formaldehyde gel and hybridized with ^{32}P-labeled sbGH cDNA. Only one band with an estimated molecular size of 1 kb was detected.

shows that the sbGH clone hybridized with a mRNA species below the 18S RNA marker. The size of this mRNA was estimated to be around 1 kb.

The 5′ noncoding region of the cloned sbGH cDNA contains 48 nucleotides, identical to that described previously (Funkenstein *et al.*, 1991), but three nucleotides shorter. The 3′ noncoding region which starts with the translation stop codon contains 233 bp, upstream from the polyadenylation tail. The sequence AATAAA probably functions as a signal for cleavage and polyadenylation of the pre-mRNA (Momota *et al.*, 1988). Figure 1 shows that sbGH shares structural features also observed in mammals, chicken, and fish GHs. Four Cys residues (Cys-69, Cys-94, Cys-177, and Cys-202) are located in similar positions in all GH polypeptides at the C-terminus; by forming two disulfide bonds, they are assumed to contribute to the tertiary structure of the hormone molecule. The existence of two disulfide bonds has been demonstrated in several GHs, and their presence is essential for the biological activity of the hormone (Lewis *et al.*, 1980).

There is one Asn–X–Thr motif in sbGH amino acid sequence (Asn–Cys–Thr) which is a potential site for N-linked glycosylation (Rentier-Delrue *et al.*, 1989). Similar sequence has also been observed by Sekine *et al.* (1985) for salmon GH. Hydropathy scores for sbGH were also determined. The profile resembles that from other fish species and, to a lesser extent, that from mammals. It reveals a highly hydrophobic

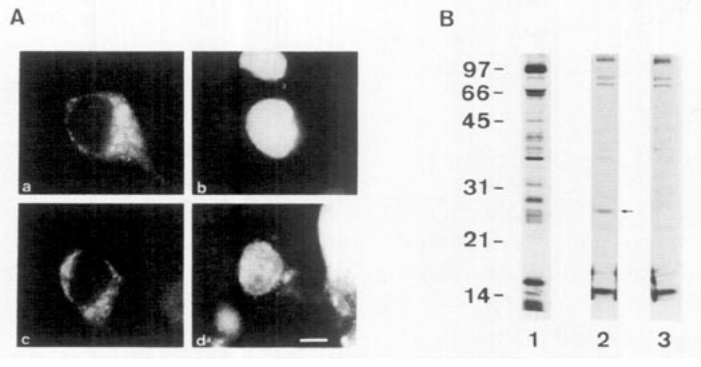

FIG. 3. Transfection of sbGH cDNA in HeLa cells. (A) Immunofluorescence microscopy of cells expressing GH protein. Transient expression of GH cDNA in HeLa cells is indicated by the staining of representative cells at the ER region as shown (a,c). The same cells were stained for DNA with Hoechst 33258 (b,d). Bar, 10 μm. (B) Immunoprecipitation of transfected HeLa cell extracts metabolically labeled for 2 hr with [^{35}S]methionine with anti-native sbGH antibody. Note the specific polypeptide immunoprecipitated from GH cDNA transfected cells (arrow in lane 2). A control in lane 3 represents a HeLa cell extract transfected with the same BS plasmid lacking the sbGH cDNA. Total labeled proteins are shown in lane 1.

amino-terminal polypeptide of 17 amino acids which exhibits all the characteristics of a signal peptide (Rottier *et al.*, 1987) (data not shown). This putative signal peptide is subsequently cleaved off during processing to give a mature protein of 187 amino acids (Watahiki *et al.*, 1989).

Expression of sbGH in HeLa Cells

When a sbGH cDNA was expressed in HeLa cells by the vaccinia virus/T7 system, many cells expressed the exogenous GH after 24 hr of transfection. Immunofluorescence labeling revealed a reticular pattern, indicative of a predominantly endoplasmatic reticulum localization (Figs. 3A, a and c). No other pattern of labeling was detected by extensive observation of positive transfected cells. This expression was verified by immunoprecipitation of labeled HeLa cell extracts. After a 2-hr [^{35}S]methionine pulse, a specific polypeptide was recovered by immunoprecipitation with an antibody against native sbGH (Le Bail *et al.*, 1993) (Fig. 3B, lane 2). This protein migrated in SDS–PAGE with an apparent molecular weight of 22 kDa, which is identical to that of the native sbGH and matched well with the recombinant full-length expressed sbGH cDNA (see below). The appropriate control (Fig. 3B, lane 3) demonstrated the specificity of the immunoprecipitation assay.

Expression of sbGH in E. coli Cells

DH5α *E. coli* cells were transformed with the pGEX-3X expression vector carrying the sbGH cDNA insert. Cell extracts obtained at different times after IPTG induction gave a prominent band of 48 kDa when analyzed on SDS–PAGE (Fig. 4A, lanes 2, 3, and 4). This size fits well with that predicted for the fusion of the glutathione transferase (26 kDa) and the sbGH (22 kDa) polypeptides. Figure 4B (lanes 2, 3, and 4) shows by Western blot analysis that an anti-native sbGH binds to the expressed GHFP.

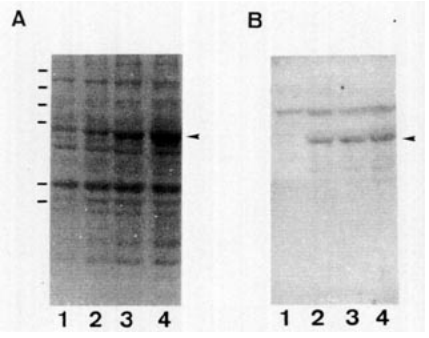

FIG. 4. Expression of sbGH and Western blot analysis. (A) Analysis of sbGH expressed as a fusion protein in the pGEX system by SDS/PAGE and Coomassie brilliant blue staining of uninduced (lane 1) and induced cells by IPTG for 30 min (lane 2), 1 hr (lane 3), and 3 hr (lane 4). (B) Western blotting for sbGH expressed protein with anti-native sbGH serum. The band running at 48 kDa represents the GH fusion protein (arrowheads). Molecular weight standard proteins correspond to 116, 84, 58, 48, 36, and 26 kDa from top to bottom.

The GHFP was cleaved with factor Xa to generate glutathione transferase and recombinant sbGH separately. The cleaved sbGH fragment ran parallel to native sbGH on SDS–PAGE, thus showing that the expressed sbGH cDNA codified for a 22-kDa protein similar to native sbGH (data not shown). However, cleavage with factor Xa was a very inefficient method for yielding the pure rsbGH fragment as also observed by Nagai and Thogersen (1984).

On the other hand, lysates of IPTG induced BL21(DE3) *E. coli* cells harboring the pET-3a-sbGH expression plasmid were similarly analyzed. A prominent 22-kDa band identified by binding to anti-native sbGH antibody was obtained. This band was absent in the controls (data not shown).

Analysis of Recombinant sbGHs

An homologous radioimmunoassay for native sbGH was used to analyze the recombinant proteins. Both the fusion protein expressed in the pGEX-3X system (GHFP), and the recombinant GH ex-

pressed in the pET-3a system (rsbGH), competitively displaced radioiodinated native sbGH (Fig. 5). An appropriate control of expressed glutathione transferase alone showed no displacement of the labeled native sbGH (data not shown). The ED_{50}, calculated as the amount of unlabeled hormone that causes a 50% displacement of the labeled hormone, was 1.7 ng/ml for native sbGH. For the GHFP, ED_{50} was approximately 220 ng/ml, equivalent to 100 ng/ml of the GH moiety. Thus, the specificity of the GHFP in this assay was very low, as a 70-fold excess is needed to displace similar amount of native sbGH. In contrast, the ED_{50} for rsbGH was ca. 5 ng/ml, or a threefold excess over the native sbGH. The recombinant proteins were further analyzed in a homologous radioreceptor assay for native sbGH. Displacement curves for both native sbGH and rsbGH overlapped (Fig. 6A); thus the rsbGH showed the same receptor binding activity as the native hormone. Neither the GHFP nor its factor-Xa cleavage products showed significant receptor binding activity (data not shown). When radiolabeled rsbGH was incubated with increasing amounts of liver membrane preparations, a maximum specific binding close to 30% was found (Fig. 6B), approaching that for native sbGH (Pérez-Sánchez *et al.*, 1993).

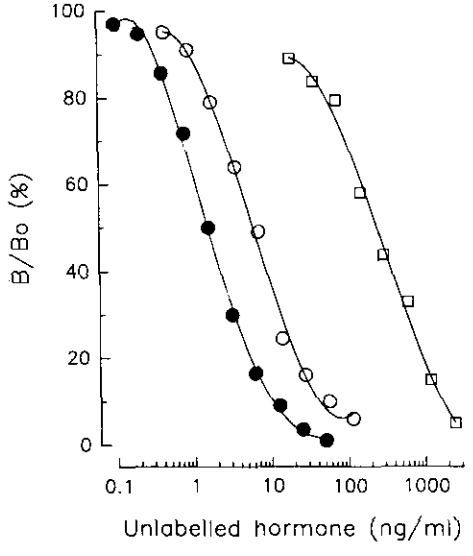

FIG. 5. Dose displacement curves of native gilthead seabream GH (sbGH, ○), recombinant gilthead seabream GH (rsbGH, ●), and recombinant fusion protein (GHFP, □) in a homologous radioimmunoassay for native sbGH. Bo and B represent, respectively, the radioactivity specifically bound in the absence and in the presence of varying amounts of the unlabeled hormone.

Some proteins expressed as fusion proteins fully conserve their activity (for an example, see Nasrim *et al.*, 1991; Maeda *et al.*, 1992). It is also known that after the cleavage of proteins expressed in the pGEX-3X system with factor Xa, full biological activity can be recovered (for a reference, see Baksh *et al.*, 1992). In this study, although the denatured fusion protein (GHFP) was recognized by a specific anti-native sbGH serum in Western blots, it reacted only minimally with the same antibody under radioimmunoassay conditions and was totally ineffective in the radioreceptor assay. The GHFP contains a glutathione transferase moiety, and a putative signal peptide of 17 amino acids, highly hydrophobic. This may contribute to a defective folding of the GH moiety during the renaturation process, and/or to an alteration of the steric conformation, all impairing the binding to the receptor. If the steric impediment for the binding to liver receptors comes from the presence of a glutathione transferase moiety, it could possibly be removed after the cleavage of the GHFP by factor Xa. However, the GH fragment so produced did not bind to liver receptor neither, although it was electrophoretically identical to the native sbGH and was also recognized by the anti-native antibody in Western blots. So, if such steric alteration existed, it seems to be irreversible. Although other experimental conditions could be further investigated, this fusion system does not seem to be the method of choice to express a biologically active rsbGH.

All these restrictions are circumvented

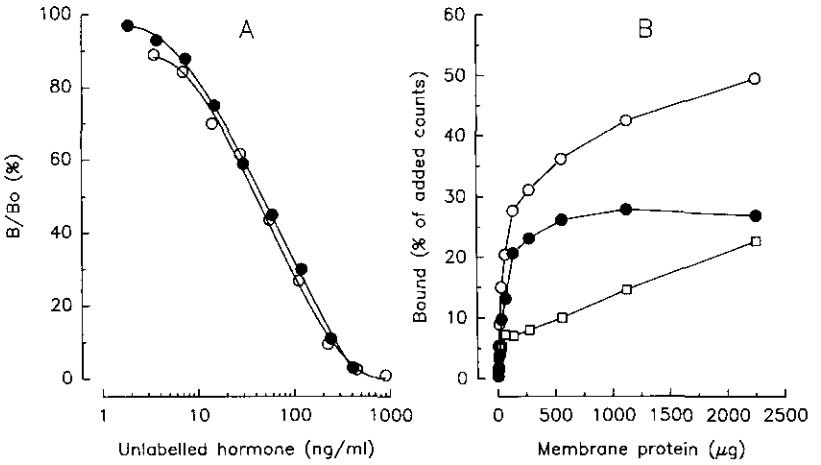

FIG. 6. (A) Dose displacement curves for native gilthead seabream GH (sbGH, ○) and recombinant gilthead seabream GH (rsbGH, ●) in a homologous radioreceptor assay for native sbGH. Bo and B are as described in the legend to Fig. 5. (B) Binding of rsbGH to increasing amounts of a liver membrane preparation; ○, total binding; □, nonspecific binding; ●, specific binding (total binding–nonspecific binding).

by subcloning the sbGH cDNA lacking the peptide signal nucleotides into the pET-3a plasmid. The recombinant protein so expressed is electrophoretically identical to the native hormone, is recognized by an anti-native sbGH antibody in an immunoblotting assay, and closely resembles the native hormone in a homologous radioimmunoassay. Furthermore, this recombinant hormone binds to hepatic GH receptors apparently with the same affinity as the native hormone in a radioreceptor assay.

In conclusion, a sbGH cDNA has been cloned and sequenced and the sequence differs only slightly from others previously published. By expression in HeLa cells, and by subclonage and expression in two different vectors, pGEX-3X and pET-3a, this cDNA has been shown to codify for a recombinant protein resembling the native hormone, corroborating the sequence data. In addition a recombinant hormone (rsbGH) which is similar, if not identical, to the native hormone when analyzed by radioimmunoassay and by receptor binding assay has been obtained.

Both the cloned cDNA, and the rsbGH, should provide useful tools for studies on the functional roles of GH in fish, as well as for the development of potential applications to fish farming.

ACKNOWLEDGMENTS

We are grateful to Dr. Hideo Ohgai (Cellular Technology Institute, Otsuka Pharmaceutical Co. Ltd., Ako, Japan) for kindly providing the flounder GH cDNA plasmid and to PEMARES (Puerto de Santa María, Spain), especially to Mr. Salvador Cárdenas, for providing the animals used in this project. We thank Concepción Iglesias for her technical assistance with tissue culture experiments. We also thank Dr. E. *Domingo and Dr. C. Escarmis for their collaboration* with the sequencing part of this work and Dr. M. Medina for his help with the transfection experiments. This research was financially supported by D.G.I.C.Y.T. (Ministerio de Educación y Ciencia, Spain, Ref. MAR91 1020), by Plan Nacional de Investigación Científica y Desarrollo Tecnológico, P.E.T.R.I. (Ref. PTR90-0097), and Plan Andaluz de Investigación (grupo 3127) to M.M.V.

REFERENCES

Agellon, L. B., and Chen, T. T. (1986). Rainbow trout growth hormone: Molecular cloning of cDNA and expression in *Escherichia coli*. *DNA* 5, 463–471.

Baksh, S., Burns, K., Busaan, J., and Michalak, M. (1992). Expression and purification of a recombinant and native calreticulin: Protein Expression and Purification. 3, 322–331.

Farmer, S. W., Papkoff, H., Hayashida, T., Bewley, T. A., Bern, H. A., and Li, C. H. (1976). Purification and properties of teleost growth hormone. *Gen. Comp. Endocrinol.* **30**, 91–100.

Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**, 8122–8126.

Funkenstein, B., Chen, T. T., Powers, D. D., and Cavari, B. (1991). Cloning and sequencing of the gilthead seabream (*Sparus aurata*) growth hormone encoding cDNA. *Gene* **103**, 243–247.

Holly, J. M. P., and Wass, J. A. H. (1989). Insulin-like growth factors; autocrine, paracrine or endocrine? New perspectives of the somatomedin hypothesis in the light of recent developments. *J. Endocrinol.* **122**, 611–618.

Kawauchi, H., Moriyama, S., Yasuda, A., Yamaguchi, K., Shirahata, K., Kubota, J., and Hirano, T. (1986). Isolation and characterization of chum salmon growth hormone. *Arch. Biochem. Biophys.* **244**, 542–552.

Kimura, A. (1991). Microbial production of fish (*Tuna*) growth hormone. *Crit. Rev. Biotech.* **11**, 113–127.

Kosugi, R., Momota, H., Kawai, S., Ohgai, H., Ishioka, H., and Hara, A. (1992). Production of recombinant red seabream growth hormone using a secretion plasmid of *Escherichia coli* and its binding activity to the membranes of red seabream liver. *Nippon Suisan Gakkaishi* **58**, 1115–1122.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277, 680–685.

Le Bail, P. Y., Mouront, B., Zohar, Y., and Pérez-Sánchez, J. (1993). Application of a sensitive radioimmunoassay for the measurement of growth hormone in gilthead seabream, *Sparus aurata*, and other sparid fish. *Can. J. Zool.* **71**, 1500–1505.

Lewis, U. J., Singh, R. N. P., Tutwiller, G. F., Sigel, M. B., Vanderlaan, E. F., and Vanderlaan, W. (1980). Human growth hormone: A complex of protein. *Rec. Prog. Hormone Res.* **36**, 477–509.

Maeda, Y., Hisatake, K., Kondo, T., Hanada, K.-I., Song, C.-Z., Nishimura, T., and Muramatsu, M. (1992). Mouse rRNA gene transcription factor mUBF requires both HMG-box1 and an acidic tail for nucleolus accumulation: Molecular analysis of the nucleolar targeting mechanism. *EMBO J.* 11, 3695–3704.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor. NY.

Momota, H., Kosugi, R., Ohgai, H., Hara, A., and Ishioka, H. (1988). Amino acid sequence of flounder growth hormone deduced from a cDNA sequence. *Nucleic Acids Res.* **16**, 10362–10370.

Nagai, K., and Thogersen, H. C. (1984). Generation of β-globin by sequence-specific proteolysis of a hybrid protein produced in *Escherichia coli*. *Nature* **309**, 810–312.

Nasrim, N., Buggs, C., Kong, X. F., Carnazza, J., Goebl, M., and Alexander-Bridges, M. (1991). DNA-binding properties of the product of the testis-determining gene and related protein. *Nature* 354, 317–320.

Paris, N., Rentier-Delrue, F., Denfontaine, A., Goffin, V., Lebrun, J. J., Mercier, L., and Martial, J. A. (1990). Bacterial production and purification of recombinant human prolactin. *Biotech. Appl. Biochem.* **12**, 436–449.

Pérez-Sánchez, J., Martí-Palanca, H., and Le Bail, P. Y. (1993). Homologous growth hormone (GH) binding in the gilthead seabream, *Sparus aurata:* Effect of fasting and refeeding on hepatic GH and plasma somatomedin-like immunoreactivity. *J. Fish Biol.*, in press.

Rand-Weaver, M., Walther, B. T., and Kawauchi, H. (1989). Isolation and characterization of growth hormone from Atlantic cod (*Gadus morhua*). *Gen. Comp. Endocrinol.* **73**, 260–269.

Rentier-Delrue, F., Swennen, D., Philippart, J. C., L'Hoir, C., Lion, M., Benrubi, O., and Martial, J. A. (1989). Tilapia growth hormone: Molecular cloning of cDNA and expression in *Escherichia coli*. *DNA* **8**, 271–278.

Rottier, P. J. M., Florkiewicz, R. Z., Shaw, A. S., and Rose, J. K. (1987). An internalized amino-terminal signal sequence retains full activity *in vivo* but not *in vitro*. *J. Biol. Chem.* **262**, 8889–8895.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.

Saito, N., Sekine, S., Komatu, Y., Sato, M., Hirano, T., and Ito, S. (1988). Molecular cloning of eel growth hormone cDNA and its expression in *Escherichia coli*. *Gene* **73**, 545–551.

Sakamoto, T., McCormick, S. D., and Hirano, T. (1993). Osmoregulatory actions of growth hormone and its mode of action in salmonids: A review. *Fish Physiol. Biochem.* **11**, 155–164.

Sakata, S., Noso, T., Moriyama, S., Hirano, T., and Kawauchi, H. (1993). Flounder growth hormone: Isolation, characterization, and effects on growth of juvenile rainbow trout. *Gen. Comp. Endocrinol.* **89**, 396–404.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Sekine, S., Mizukami, T., Nishi, T., Kuwana, Y., Saito, A., Sato, M., Itoh, S., and Kawauchi, H. (1985). Cloning and expression of cDNA for salmon growth hormone in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **82**, 4306–4310.

Sugimoto, S., Yokoo, Y., Inui, Y., and Hirano, T. (1991). Large-scale purification and refolding of recombinant eel growth hormone. *Agric. Biol. Chem.* **55**, 1635–1637.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.

Tsai, H.-J., Lin, K.-L., and Chen, T. (1993). Molecular cloning and expression of yellowfin porgy (*Acanthopagrus latus houttuyn*) growth hormone cDNA. *Comp. Biochem. Physiol. B* **104**, 803–310.

Valdivia, M. M., Tousson, A., and Brinkley, B. R. (1986). Human antibodies and their use for the study of chromosome organization. *In* "Methods and Achievements in Experimental Pathology" (G. Jasmin and R. Simard, Eds.), Vol. 12, pp. 200–223. Karger, Basel.

Watahiki, M., Yamamoto, M., Yamakawa, M., Tanaka, M., and Nakashima, K. (1989). Conserved and unique amino acid residues in the domains of the growth hormones. *J. Biol. Chem.* **264**, 312–316.

Watahiki, M., Ohara, H., Tsuda, M., Shouji, K., Masuji, A., Tanaka, M., Yamakawa, M., Ushiro, H., Yoneda, Y., and Nakashima, K. (1992). Synthesis of recombinant yellow tail and flounder growth hormone in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **56**, 1012–1016.