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Molecular cloning of gilthead seabream (*Sparus aurata*) pituitary transcription factor GHF-1/Pit-1

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Abstract

We report here the complete nucleotide sequence of a cDNA clone encoding *Sparus aurata* GHF-1/Pit-1 isolated from an expression library prepared from gilthead seabream pituitary gland poly(A)$^+$RNA. The cDNA sequence (saGHF-1/Pit-1) encodes a protein of 371 amino acids (aa) containing a POU domain (aa 194–343) and a transactivation, STA domain (aa 1–128). Northern blot hybridization of pituitary RNA detected a single 3.0 kb band and a rat GHF-1/Pit-1 antiserum was found to immunoreact with pituitary protein species of 42 kDa by Western blot analysis. When compared with mammalian GHF-1/Pit-1 aa sequence, the POU and STA domains of saGHF-1/Pit-1 protein show 83% and 48% aa identity, respectively. In spite of the low homology of the transactivation domain, saGHF-1/Pit-1 is able to activate the transcription of the human growth hormone promoter.

Keywords: Tissue-specific gene expression; POU transcription factor; STA domain; Growth hormone; Prolactin

1. Introduction

GHF-1/Pit-1 is a tissue-specific transcription factor that belongs to the family of POU domain proteins (Bodner et al., 1988; Theill et al., 1989; Karin et al., 1990). It is expressed in the anterior pituitary where it plays a critical role in the transcriptional regulation of the *GH*, *PRL*, *TSH*, and *GRFR* genes and the *GHF-1/Pit-1* gene itself (McCormick et al., 1990; Karin et al., 1990; Ruvkun, 1992). *GHF-1/Pit-1* expression is also required for the differentiation, proliferation and survival of the somatotrophic lineage (Dollè et al., 1990; Castrillo et al., 1991; Ruvkun, 1992). *GHF-1/Pit-1* encoding cDNAs have been cloned in several mammalian species including rat, bovine (Bodner et al., 1988), human (Tatsumi et al., 1992) and swine (Tuggle et al., 1993). GHF-1/Pit-1 is characterized by the presence of two conserved regions at the C terminus, designated as POU-specific domain, POU_S (75 aa) and POU homeodomain, POU_{HD} (60 aa), responsible for DNA binding to specific genes. Transcriptional activation is mediated by a less conserved domain at the N terminus that is rich in serine and threonine residues (serine/threonine activation domain, STA) (Theill et al., 1989).

Recently, *GHF-1/Pit-1* cDNAs from two fish species, chum salmon (Ono and Takayama, 1992) and rainbow trout (Yamada et al., 1993) have been isolated. Their encoded proteins are highly homologous to their mammalian counterparts, mainly in the POU_S and the POU_{HD} (83% identity). In fact, both fish and mammalian GHF-1/Pit-1 proteins bind to specific nt sequences that are essentially identical (Yamada et al., 1993). However, the transactivation domain is less conserved (48% identity) and contains two insertions of 26 and 33 aa that are not present in mammalian GHF-1/Pit-1 proteins.

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Abbreviations: aa, amino acid(s); bp, base pair(s); CAT, chloramphenicol acetyltransferase; *CAT*, gene (DNA) encoding CAT; GH, growth hormone; *GH*, gene (DNA) encoding GH; GHF-1, GH transcription factor 1; *GHF-1*, gene (DNA) encoding GHF-1; GRFR, GH releasing hormone receptor; *GRFR*, gene (DNA) encoding GRFR; kb, kilobase(s) or 1000 bp; LTR, long terminal repeat; nt, nucleotide(s); ORF, open reading frame; PCR, polymerase chain reaction; Pit-1, pituitary transcription factor 1; *Pit-1*, gene (DNA) encoding Pit-1; PRL, prolactin; *PRL*, gene (DNA) encoding PRL; RSV, Rous sarcoma virus; *sa*, *Sparus aurata*; TSH, thyrotrop stimutating hormone; *TSH*, gene (DNA) encoding TSH; *UTR*, unstranslated region(s).

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The 26 aa insertion has been found in a GHF-1/Pit-1 variant named GHF-2, which represents an alternative spliced product of the rat and human *GHF-1/Pit-1* genes. GHF-2 exerts different transactivating functions than those proposed for GHF-1/Pit-1 (Theill et al., 1992). As a first step toward understanding the regulation of the *GH* gene family in gilthead seabream, the authors have cloned and characterized a *GHF-1/Pit-1* cDNA from this fish species, that is extensively cultured in the Mediterranean area.

2. Results and discussion

2.1. Isolation and sequencing of cDNAs encoding S. aurata (sa) GHF-1/Pit-1

A PCR-based screen was utilized to isolate a *GHF-1/Pit-1* cDNA from *S. aurata*. Using an oligo(dT)-primed λgt11 gilthead seabream pituitary cDNA library as template with degenerative primers for the most conserved regions among different *GHF-1/Pit-1* nt sequences, a total of six overlapping DNA fragments were isolated. The sizes of these PCR products were 1.2, 1.8, 0.8, 0.4, 2.3 and 1.1 kb (Fig. 1). All these DNA fragments were cloned into the Bluescript vector (Stratagene) and sequenced (Sanger et al., 1977). Fig. 2 shows the *saGHF-1/Pit-1* nt sequence obtained from a series of overlapping clones. The full-length *saGHF-1/Pit-1* cDNA is approx. 2.8 kb in length and contains an ORF of 1116 bp encoding a protein of 371 aa. The *5-UTR* and *3-UTR* regions are 67 bp and 1.6 kb long respectively. A consensus polyadenylation signal AATAAA is present 15 nt upstream from the polyadenylation site.

To confirm the sequence data, two specific *saGHF-1/Pit-1* primers named OL/26 and OL/27 were designed (Fig. 1). These primers hybridized to the 5′ and 3′ end of the *saGHF-1/Pit-1* cDNA and contained the start and stop codon respectively. To facilitate cloning of the PCR products, *Hin*dIII (OL/26) and *Not*I (OL/27) restriction sites were added to the 5′ end of these primers. RT-PCR with total RNA isolated from *S. aurata* pituitaries with OL/26 and OL/27 primers resulted in the amplification of a DNA fragment of the expected size (1.1 kb; see Fig. 1). No additional bands were detected suggesting the absence of an alternative splicing of the *saGHF-1/Pit-1* mRNA (data not shown).

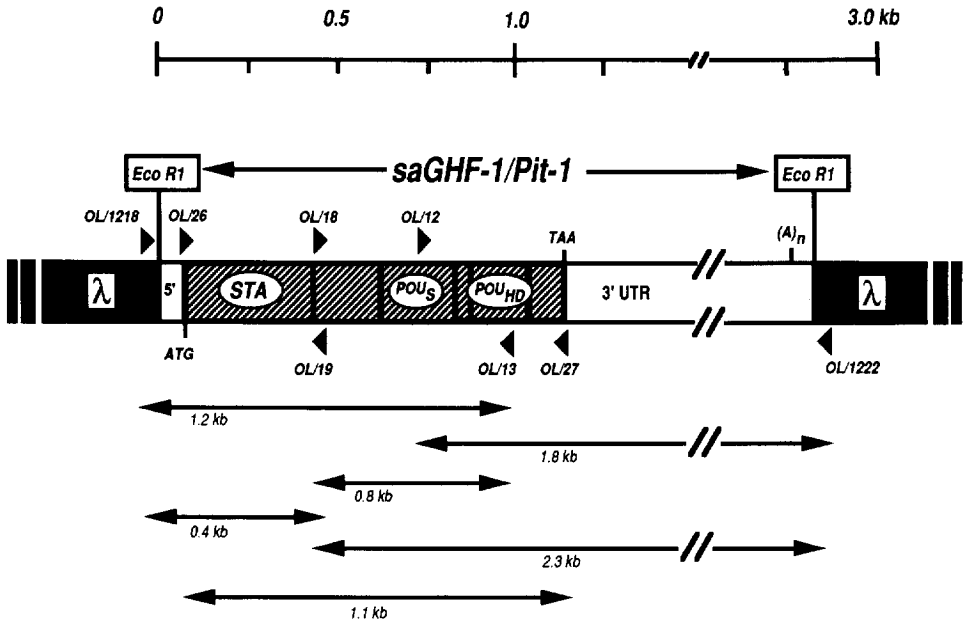
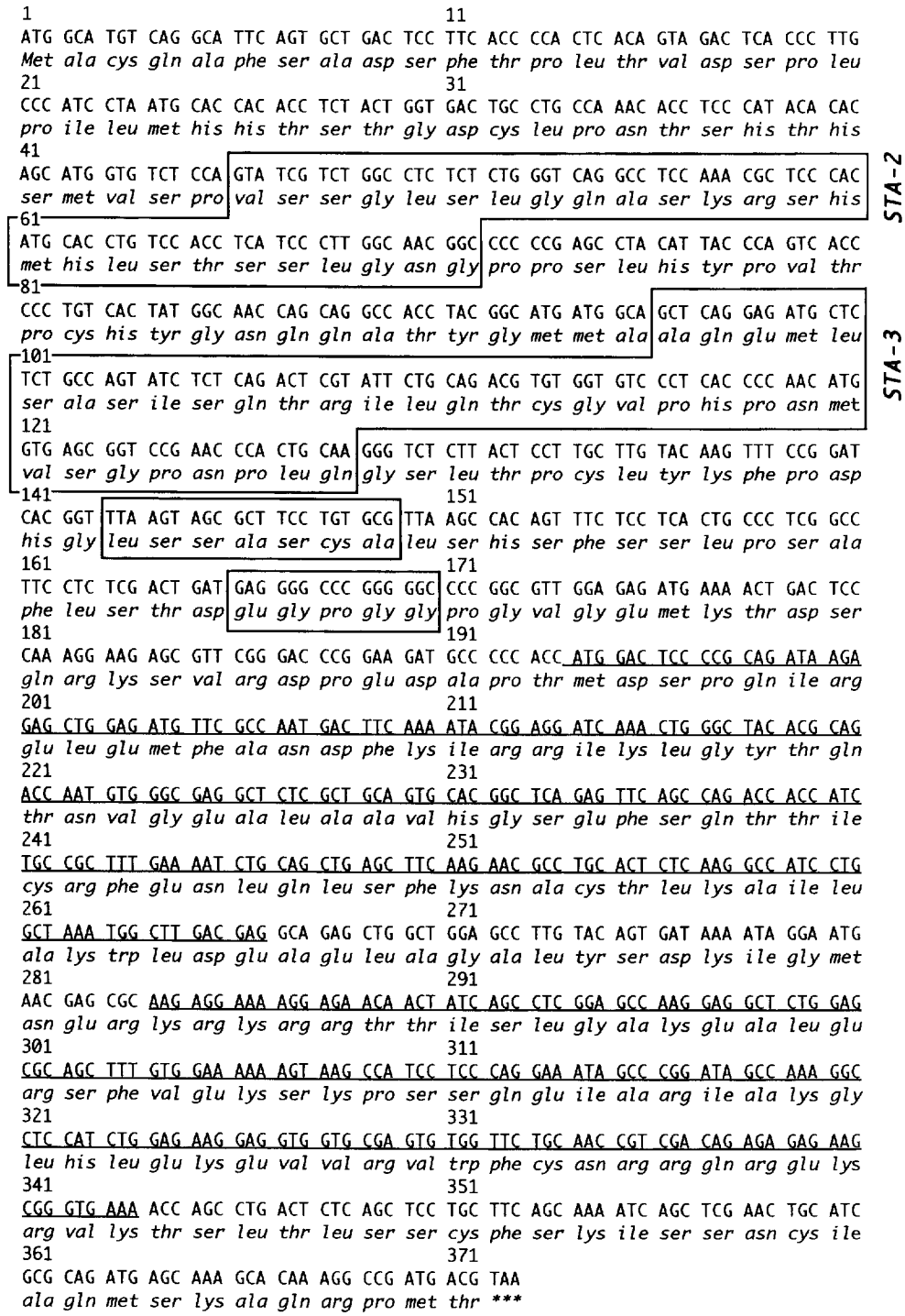


Fig. 1. Schematic representation of the strategy for cloning the gilthead seabream (*S. aurata*) pituitary transcription factor *GHF-1/Pit-1*. The hatched box represents the entire *saGHF-1/Pit-1* coding region containing the *s*erine-*t*hreonine *a*ctivation domain (STA), POU-specific domain (POU_S) and POU homeodomain (POU_{HD}). The white boxes represent the *5′-UTR* and *3′-UTR*. Left and right λ arms are indicated as black boxes at both sides of the full-length *saGHF-1/Pit-1* cDNA. The positions of the primer sequences are indicated (arrowheads). ATG, TAA and $(A)_n$ represent the start and stop codon, and the polyadenylation site, respectively. For cloning, a total of 3×10^6 phages from a λgt11 gilthead seabream pituitary cDNA library were directly added to each PCR reaction as template (Martínez-Barberá et al., 1994). Degenerative primers (OL/12, OL/13, OL/18 and OL/19) for the most conserved regions of the GHF-1/Pit-1 sequences and λ primers (OL/1218 and OL/1222, New England Biolabs) were used to amplify several overlapping *GHF-1/Pit-1* clones. OL/26 and OL/27 hybridize specifically to the *saGHF-1/Pit-1* cDNA at the ATG and TAA regions, respectively. Oligonucleotide sequences are: (OL/12) GCAAAGCTTTCWSCCARACNACCAT; (OL/13) TTCTGTCGACKGTTRCARAACCA; (OL/18) GTGGGATCCTGTCAYTATRGMAACCA; (OL/19) AGTGGATCCTTGGTTKCYATARTGACAGG; (OL/26) AGGATCCAAGCTTATGGCATGTCAGGCATTCAGTGC; (OL/27) TGGATCCGCGGCCGCTTACGTCATCGGCCTTTGTGC. Non-canonical bases: W, A or T; S, G or C; R, A or G; N, G, A, T or C; K, G or T; M, A or C; Y, G or T.

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1                                                  11
ATG GCA TGT CAG GCA TTC AGT GCT GAC TCC TTC ACC CCA CTC ACA GTA GAC TCA CCC TTG
Met ala cys gln ala phe ser ala asp ser phe thr pro leu thr val asp ser pro leu
21                                                 31
CCC ATC CTA ATG CAC CAC ACC TCT ACT GGT GAC TGC CTG CCA AAC ACC TCC CAT ACA CAC
pro ile leu met his his thr ser thr gly asp cys leu pro asn thr ser his thr his
41
AGC ATG GTG TCT CCA GTA TCG TCT GGC CTC TCT CTG GGT CAG GCC TCC AAA CGC TCC CAC
ser met val ser pro val ser ser gly leu ser leu gly gln ala ser lys arg ser his
61
ATG CAC CTG TCC ACC TCA TCC CTT GGC AAC GGC CCC CCG AGC CTA CAT TAC CCA GTC ACC
met his leu ser thr ser ser leu gly asn gly pro pro ser leu his tyr pro val thr
81
CCC TGT CAC TAT GGC AAC CAG CAG GCC ACC TAC GGC ATG ATG GCA GCT CAG GAG ATG CTC
pro cys his tyr gly asn gln gln ala thr tyr gly met met ala ala gln glu met leu
101
TCT GCC AGT ATC TCT CAG ACT CGT ATT CTG CAG ACG TGT GGT GTC CCT CAC CCC AAC ATG
ser ala ser ile ser gln thr arg ile leu gln thr cys gly val pro his pro asn met
121
GTG AGC GGT CCG AAC CCA CTG CAA GGG TCT CTT ACT CCT TGC TTG TAC AAG TTT CCG GAT
val ser gly pro asn pro leu gln gly ser leu thr pro cys leu tyr lys phe pro asp
141                                                151
CAC GGT TTA AGT AGC GCT TCC TGT GCG TTA AGC CAC AGT TTC TCC TCA CTG CCC TCG GCC
his gly leu ser ser ala ser cys ala leu ser his ser phe ser ser leu pro ser ala
161                                                171
TTC CTC TCG ACT GAT GAG GGG CCC GGG GGC CCC GGC GTT GGA GAG ATG AAA ACT GAC TCC
phe leu ser thr asp glu gly pro gly gly pro gly val gly glu met lys thr asp ser
181                                                191
CAA AGG AAG AGC GTT CGG GAC CCG GAA GAT GCC CCC ACC ATG GAC TCC CCG CAG ATA AGA
gln arg lys ser val arg asp pro glu asp ala pro thr met asp ser pro gln ile arg
201                                                211
GAG CTG GAG ATG TTC GCC AAT GAC TTC AAA ATA CGG AGG ATC AAA CTG GGC TAC ACG CAG
glu leu glu met phe ala asn asp phe lys ile arg arg ile lys leu gly tyr thr gln
221                                                231
ACC AAT GTG GGC GAG GCT CTC GCT GCA GTG CAC GGC TCA GAG TTC AGC CAG ACC ACC ATC
thr asn val gly glu ala leu ala ala val his gly ser glu phe ser gln thr thr ile
241                                                251
TGC CGC TTT GAA AAT CTG CAG CTG AGC TTC AAG AAC GCC TGC ACT CTC AAG GCC ATC CTG
cys arg phe glu asn leu gln leu ser phe lys asn ala cys thr leu lys ala ile leu
261                                                271
GCT AAA TGG CTT GAC GAG GCA GAG CTG GCT GGA GCC TTG TAC AGT GAT AAA ATA GGA ATG
ala lys trp leu asp glu ala glu leu ala gly ala leu tyr ser asp lys ile gly met
281                                                291
AAC GAG CGC AAG AGG AAA AGG AGA ACA ACT ATC AGC CTC GGA GCC AAG GAG GCT CTG GAG
asn glu arg lys arg lys arg arg thr thr ile ser leu gly ala lys glu ala leu glu
301                                                311
CGC AGC TTT GTG GAA AAA AGT AAG CCA TCC TCC CAG GAA ATA GCC CGG ATA GCC AAA GGC
arg ser phe val glu lys ser lys pro ser ser gln glu ile ala arg ile ala lys gly
321                                                331
CTC CAT CTG GAG AAG GAG GTG GTG CGA GTG TGG TTC TGC AAC CGT CGA CAG AGA GAG AAG
leu his leu glu lys glu val val arg val trp phe cys asn arg arg gln arg glu lys
341                                                351
CGG GTG AAA ACC AGC CTG ACT CTC AGC TCC TGC TTC AGC AAA ATC AGC TCG AAC TGC ATC
arg val lys thr ser leu thr leu ser ser cys phe ser lys ile ser ser asn cys ile
361                                                371
GCG CAG ATG AGC AAA GCA CAA AGG CCG ATG ACG TAA
ala gln met ser lys ala gln arg pro met thr ***
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Fig. 2. Nucleotide sequence of the *saGHF-1/Pit-1* cDNA and deduced aa sequence. Composite nt sequence is derived from different overlapping *GHF-1/Pit-1* fragments obtained by PCR. The POU_S domain (Met^{194}-Glu^{266}) and the POU_{HD} (Lys^{284}-Lys^{343}) are underlined. Amino acid insertions of 26 and 33 aa in the N-terminal region are boxed (STA-2 and STA-3, respectively). Two smaller insertions of 7 and 5 aa are also boxed. The GenBank accession number is X81646.

Partial sequencing of this DNA product showed it to be identical to that obtained from the overlapping clones. Moreover, the same 1.1 kb DNA fragment was also amplified from the λgt11 gilthead seabream pituitary cDNA library, thus confirming the suitability of our cloning strategy.

2.2. Structural features of saGHF-1/Pit-1 protein

We have compared the aa sequence of *S. aurata* GHF-1/Pit-1 with GHF-1/Pit-1 sequences from different species. saGHF-1/Pit-1 protein is similar to the rainbow trout, chum salmon and rat GHF-1/Pit-1 at 80%, 78%

and 48% aa identity, respectively. The low homology between fish and mammalian GHF-1/Pit-1 proteins is mainly in the N terminus, whereas the C terminus is the most conserved region of the protein. Fig. 3 shows a schematic comparison between gilthead seabream and rat GHF-1/Pit-1 proteins. Gilthead seabream GHF-1/Pit-1 (371 aa) is larger than its rat counterpart (291 aa). This difference in size is mainly due to two insertions of 26 and 33 aa in the N-terminal half (STA-2 and STA-3 respectively). These two additional aa sequences have also been found in other fish and avian GHF-1/Pit-1 proteins, but are not present in mammalian GHF-1/Pit-1 proteins (Wong et al., 1992; Yamada et al., 1993; Vila et al., 1995). However, a novel protein named GHF-2 has been cloned and characterized from rat and human pituitaries (Theill et al., 1992; Delhase et al., 1995). GHF-2 is an alternative splicing product between exons 1 and 2 of the *GHF-1/Pit-1* gene. The aa sequence of GHF-2 is identical to that of GHF-1, except for an insertion of 26 aa located in the STA domain, at the same position as one of the insertions in the fish GHF-1/Pit-1 proteins. GHF-2 retains the binding activity of GHF-1/Pit-1 and can activate the GH promoter, but has lost the ability to activate the PRL and GHF-1/Pit-1 promoters. The presence of additional aa sequences in fish GHF-1/Pit-1 proteins suggests that they may carry out novel functions from those common to mammalian GHF-1/Pit-1, such as differentiation of somatolactin (SL)-producing cells and/or pituitary-specific expression of the *SL* gene. It has been demonstrated that chum salmon GHF-1/Pit-1 binds the SL promoter and activates the transcription of a fusion reporter gene (Ono et al., 1994).

By contrast, the C-terminal half of the GHF-1/Pit-1 protein responsible for the DNA binding activity shows a high homology between fish and mammals. Gilthead seabream and rat GHF-1/Pit-1 proteins are 91% and 82% identical within the POU_S domain and the

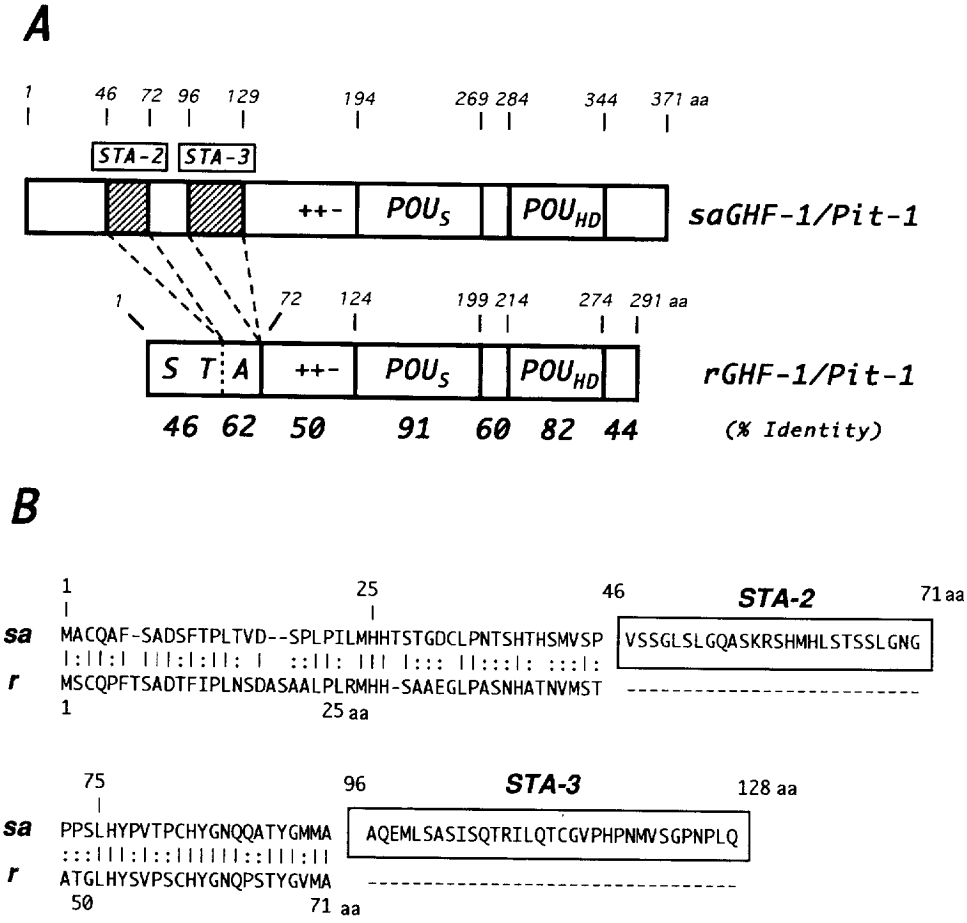

Fig. 3. Schematic comparison of the structure of the gilthead seabream and rat GHF-1/Pit-1 proteins. (**A**) saGHF-1/Pit-1 protein contains a 60 aa POU homeodomain (POU_{HD}) near its C terminus that is preceded by a 75 aa POU-specific domain (POU_S). The transactivation domain at the N terminus contains two peptide insertions, STA-2 (26 aa) and STA-3 (33 aa), that are not present in the rat counterpart. Bold numbers below the box indicate the % identity between different segments of the gilthead seabream and rat GHF-1/Pit-1 proteins. Amino acid position is indicated. (**B**) Amino acid sequence comparison (one-letter code) of the entire serine-threonine activation domain for gilthead seabream and rat GHF-1/Pit-1 protein. Identical aa are indicated by vertical lines (|), conserved substitutions by two dots (:) and aa insertions by dashes (–). Peptide insertions in the saGHF-1/Pit-1 protein are boxed (STA-2 and STA-3).

POU_{HD} respectively (Fig. 3A). These data are in agreement with those previously published for other fish species (Ono and Takayama, 1992; Yamada et al., 1993) and support the idea that GHF-1/Pit-1 binding sequences have been conserved throughout evolution.

2.3. Expression of GHF-1/Pit-1 in the pituitary gland from gilthead seabream

The expression of the pituitary *saGHF-1/Pit-1* gene was examined by Northern hybridization. A 1.1 kb cDNA fragment encompassing the entire coding region of the *saGHF-1/Pit-1* was found to hybridize with a single mRNA species of an estimated size of 3.0 kb (Fig. 4, lane B). This mRNA size correlates well with that described for rainbow trout GHF-1/Pit-1 but not with that for chum salmon, where two GHF-1/Pit-1 mRNA species emerge from an alternative polyadenylation site (Ono and Takayama, 1992; Yamada et al., 1993). As shown in Fig. 4, no signal was detected in liver (lane A) and spleen (lane C) from gilthead seabream.

GHF-1/Pit-1 protein expression in the pituitary gland and the liver of gilthead seabream was analyzed by Western blot (Fig. 5). A specific rat *GHF-1/Pit-1* antiserum immunoreacted with protein species of 42 kDa from a gilthead seabream pituitary extract (Fig. 5, lane B). The molecular weight of this polypeptide correlates well with that expected for the *GHF-1/Pit-1* protein deduced from the *saGHF-1/Pit-1* cDNA (40 457 Da), and is markedly higher than that for rat GHF-1/Pit-1 (Fig. 5, lanes A and D). As previously mentioned, saGHF-1/Pit-1 protein contains two aa insertions of 26 an 33 aa in the N-terminal end which are not present in rat GHF-1/Pit-1. These two insertions are likely to be responsible for the higher electrophoretic mobility of the saGHF-1/Pit-1 protein. As shown in Fig. 5, lane C, no immunoreacted bands were observed in protein extracts from gilthead seabream liver.

2.4. Activation of GH gene promoter by saGHF-1/Pit-1

The gene activation functions of saGHF-1/Pit-1 were assayed by DNA-mediated gene transfer experiments in HeLa cells. Rat and gilthead seabream *GHF-1/Pit-1* cDNAs were subcloned into a plasmid containing the RSV-LTR promoter. These effector plasmids were used in cotransfection experiments with different reporter

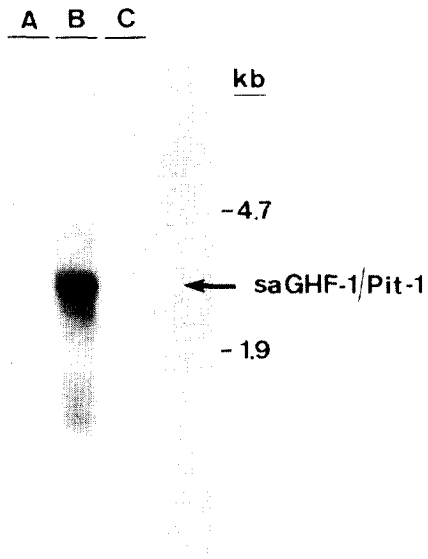

Fig. 4. Northern blot analysis of *saGHF-1/Pit-1* gene expression in gilthead seabream tissues. Total RNA (20 μg) isolated from gilthead seabream liver (lane A), pituitary glands (lane B) and spleen (lane C) was hybridized with a *saGHF-1/Pit-1* probe. A single 3.0 kb mRNA species was detected only in pituitary extracts. No signal was observed in liver and spleen RNA extracts. Total RNA was isolated with the method of Chomczynski and Sacchi (1987). Northern blots of total RNA were prepared according to standard methods and hybridized to ^{32}P-labeled *saGHF-1/Pit-1* cDNA at 45°C for 12–16 h in a solution of 6 × SSC (0.9 M NaCl, 0.9 M Na_3·citrate), 5 × Denhardt's mixture (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 20 mM sodium phosphate, pH 7.0, 1 mM EDTA, pH 8.0, 100 μg/ml sperm salmon DNA, 100 μg/ml yeast tRNA and 1% SDS. Membranes were washed in a solution of 0.1% SSC, 0.1% SDS and exposed at −80°C for 24 h. The used probe was the 1.1 kb cDNA fragment containing the complete *saGHF-1/Pit-1* coding region and labeled by random priming as described (Martínez-Barberá et al., 1994). The size of the GHF-1/Pit-1 transcripts was determined by comparison with the 28S and 18S ribosomal RNA.

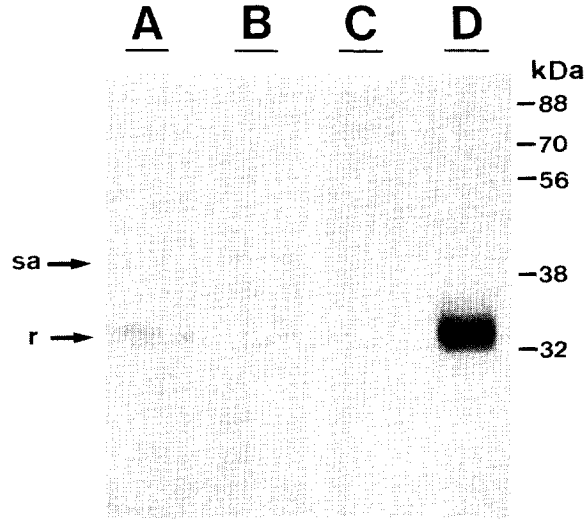

Fig. 5. Western blot analysis of the pituitary saGHF-1/Pit-1 protein expression. Protein extracts from rat and gilthead seabream pituitary glands (lane A and B, respectively) and gilthead seabream liver (lane C) were mixed in sample buffer, separated on a sodium dodecyl sulfate (SDS) – 12.5% polyacrylamide gel and electroblotted onto nitrocellulose membranes. Western blots were performed essentially as described by Towbin et al. (1979) using a specific rat GHF-1/Pit-1 antiserum. Positions of the gilthead seabream (sa) and rat (r) GHF-1/Pit-1 proteins are indicated. In lane D, recombinant GHF-1/Pit-1 protein from rat was loaded as a positive control. Numbers on the right indicate the sizes (kDa) of the prestained protein markers (BioRad).

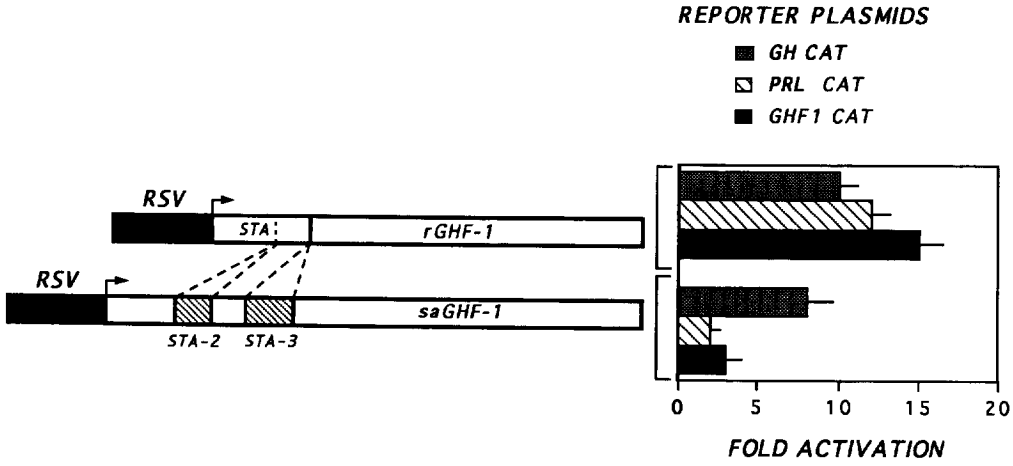

Fig. 6. saGHF-1/Pit-1 activates the human GH promoter. HeLa cells were cotransfected with 10 μg of the effector and reporter plasmids at 37°C for 1 h using lipofectin reagent (Gibco BRL) and recultured for 48 h before analysis. Cells were then harvested and protein extracts were tested for CAT activity by incubation with 1 μCi of ^{14}C-labeled chloramphenicol (Amersham). Acetylated products were separated by thin layer chromatography and then silica plates were exposed to X-ray films. For comparison, CAT conversion is expressed as a factor of the activity seen in protein extracts from HeLa cells only transfected with the reporter plasmid.

plasmids carrying the *CAT* gene under the control of the mammalian GH, PRL and GHF-1/Pit-1 promoters (Theill et al., 1992). As can be seen in Fig. 6, HeLa cells cotransfected with the human GH*CAT* and the RSV*saGHF-1/Pit-1* plasmids showed an increased CAT activity of 8 times with respect to a control transfected only with the reporter plasmid. However, no significant activation was observed when rat PRL and human GHF-1/Pit-1 promoters were used. These data support the idea that the cloned *saGHF-1/Pit-1* encodes a transcription factor with GHF-2-like activity (Theill et al., 1992). Furthermore, these transfection experiments show that a fish GHF-1/Pit-1 is able to activate a mammalian GH promoter in HeLa cells, thus supporting the premise that basic transcriptional mechanisms have been conserved between fish and mammals (see Argenton et al., 1993).

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