Isolation of growth hormone and in vitro translation of mRNA isolated from pituitaries of the gilthead sea bream Sparus aurata

B. Cavari, Pierre-Yves Le Bail, B. Levavi-Sivan, P. Melamed, H. Kawauchi, B. Funkenstein

GENERAL AND COMPARATIVE ENDOCRINOLOGY 95, 321–329 (1994)

Isolation of Growth Hormone and *in Vitro* Translation of mRNA Isolated from Pituitaries of the Gilthead Sea Bream *Sparus aurata*

B. CAVARI,* P.-Y. LE BAIL,† B. LEVAVI-SIVAN,‡ P. MELAMED,‡ H. KAWAUCHI,§ AND B. FUNKENSTEIN*

*National Institute of Oceanography, Israel Oceanographic & Limnological Research, Tel Shikmona, Haifa 31080, Israel; †Laboratoire de Physiologie des Poissons (INRA), Campus de Beaulieu, 35042 Rennes Cedex, France; ‡Department of Zoology, Tel-Aviv University, Tel-Aviv, Israel; and §Laboratory of Molecular Endocrinology, School of Fisheries Sciences, Kitasato University, Sanriku, Iwate, Japan

Accepted March 22, 1994

Growth hormone (GH) polypeptide was purified from pituitary glands of the gilthead sea bream (*Sparus aurata*) by a two-step procedure involving gel filtration on Sephadex G-100 and reverse-phase high-performance liquid chromatography (rpHPLC). At each stage of purification, fractions were monitored by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and by immunoblotting using anti-bonito GH antiserum. The molecular weight of the sea bream GH was estimated by SDS–PAGE to be 21 kDa when electrophoresed in the absence of β-mercaptoethanol (nonreduced conditions) and 22 kDa when electrophoresed under reduced conditions (in the presence of 1% β-mercaptoethanol). Pituitary RNA was used to direct cell-free translation. When specific immunoisolation from ^{35}S-labeled proteins was conducted, using antisera against *Sparus* or tilapia GH, a larger prehormone was immunoprecipitated. The size of the pre-GH was estimated to be 27–28 kDa under reduced conditions and 26–27 kDa under nonreduced conditions, in agreement with the calculated molecular weight of *Sparus* pre-GH of 26,296 based on the deduced amino acid sequence of *Sparus* GH cDNA. The specificity of the immunoprecipitation reaction was demonstrated by the ability of recombinant tilapia GH to compete with the radioactively labeled translation product. No such competition was found after the addition of BSA. Our results demonstrate that the sea bream GH is similar in its size to other purified fish GHs and provide direct evidence for the synthesis of GH as a prepeptide, thus supporting the conclusions presented earlier by GH cDNA cloning.

Growth hormone (GH) is a single-chain polypeptide of about 22 kDa which is produced in the somatotrophs of the anterior part of the pituitary gland and is secreted into the vascular system. The mature hormone is processed from a precursor by removal of a short signal peptide. GH is essential for normal growth and development of all vertebrates. Considerable information exists regarding the structure of fish GH. GH polypeptides have been purified and characterized from many freshwater and seawater fish species. Among them are the eel (Yamaguchi *et al.*, 1987), chum salmon (Kawauchi and Moriyama, 1988), bonito (Noso *et al.*, 1988), yellow tail (Kawazoe *et al.*, 1988), tuna (Kariya *et al.*, 1989), blue shark (Yamaguchi *et al.*, 1989), Atlantic cod (Rand-Weaver *et al.*, 1989, 1991), tilapia (Yamaguchi *et al.*, 1991), and catfish (Watanabe *et al.*, 1992). The GHs purified from the different fish appear to have a molecular weight of 21–22 kDa, as estimated from SDS–polyacrylamide gel electrophoresis.

Cloning and sequence analysis of cDNAs coding for fish pre-GHs suggested the presence of a putative signal peptide similar to mammalian pre-GH cDNAs (Seeburg *et al.*, 1977; Martial *et al.*, 1979; Miller *et al.*, 1980). The putative signal peptide differs in its size among different fish GHs. Its length

0016-6480/94 $5.00

ranges from 22 amino acids (a.a.) in the cyprinids such as the common carp (Chao *et al.*, 1989; Koren *et al.*, 1989), the grass carp (Ho *et al.*, 1989), and the bighead carp (Chang *et al.*, 1992), the northern pike (Schneider *et al.*, 1992), the salmonids [rainbow trout (Agellon *et al.*, 1988; Rentier-Delrue *et al.*, 1989a), chum salmon (Sekine *et al.*, 1985), coho salmon (Nicoll *et al.*, 1987; Gonzalez-Villasenor *et al.*, 1988), and the Atlantic salmon (Lorens *et al.*, 1989)], to 19 a.a. in the eel (Saito *et al.*, 1988) and 17 a.a. in the tilapia (Rentier-Delrue *et al.*, 1989b), red sea bream (Momota *et al.*, 1988), yellow tail (Watahiki *et al.*, 1988), tuna (Sato *et al.*, 1988), and our own study of the gilthead sea bream (Funkenstein *et al.*, 1991). Interestingly, the length of the putative signal peptide can be correlated with the degree of homology between the different fish GHs. Thus, the gilthead sea bream GH is highly homologous with the red sea bream, tuna, yellowtail, and tilapia GH, and all have a putative signal peptide of 17 a.a. This group of GHs has a much lower homology with the salmonid GH, which exhibits a 22-a.a. signal peptide. For comparison, the rat, human, and bovine GH have a signal peptide of 26 amino acids (Seeburg *et al.*, 1977; Martial *et al.*, 1979; Miller *et al.*, 1980).

Increased interest in the potential utilization of GH in fish farming prompted us to clone the GH cDNA from the gilthead sea bream (*Sparus aurata*), which is considered to be an economically important fish species for mariculture. We found that it codes for a polypeptide of 204 amino acids, including a putative signal peptide of 17 amino acids (Funkenstein *et al.*, 1991). Recently, we have expressed *Sparus* GH in *Escherichia coli* (Cavari *et al.*, 1993). In order to compare between the native GH and the recombinant GH, we have purified *Sparus* GH from the pituitary, and the results of the purification are presented in this paper. In addition, we employed specific immunoisolation techniques, using anti-*Sparus* GH antiserum, in order to isolate newly synthesized GH from a cell-free translation system programmed with pituitary RNA. Comparison between the GH purified from *Sparus* pituitaries and the GH translated by pituitary RNA suggests that GH is synthesized in a cell-free system as a larger prehormone, compared to the native hormone isolated from *Sparus* pituitaries.

EXPERIMENTAL PROCEDURES

Protein Purification

Five hundred pituitary glands were removed from *S. aurata* fish cultured in the National Center for Mariculture, Eilat, Israel, during their fast growing season, frozen immediately on dry ice, and kept at −80° until use. The glands were homogenized in a solution of 0.1 *M* ammonium bicarbonate, pH 7.5, containing 5 m*M* EDTA and 1.5 m*M* phenylmethylsulfonyl fluoride (PMSF) using a Polytron homogenizer. The homogenate was centrifuged at 25,000*g* for 30 min. Supernatant was recovered and the pellet was rehomogenized as above. The combined supernatant was subjected to gel filtration on a Sephadex G-100 column (1.9 × 100 cm) equilibrated and eluted with 0.1 *M* bicarbonate buffer, pH 8.2, at a flow rate of 12 ml/hr. Fractions of 3 ml were collected. Fractions were combined into six groups as follows: (1) tubes 27–39, (2) tubes 40–43, (3) tubes 44–54, (4) tubes 55–72, (5) tubes 73–82, (6) tubes 83–101. After lyophilization, each fraction was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot as described below. A putative GH fraction was subjected to a reverse-phase high-performance liquid chromatography (rpHPLC) on a TSK-gel ODS 120T column (0.46 × 25 cm, particle size 5 μm), at a column temperature of 40° and a flow rate of 1 ml/min. Elution was performed with a linear gradient of 20–80% acetonitrile containing 0.1% TFA for 60 min. Proteins were monitored by measuring the absorbance at 220 nm. Fractions were analyzed by SDS–PAGE and Western blot as described below.

Electrophoresis and Western Blot

Fractions from the Sephadex G-100 column and from the rpHPLC were analyzed by SDS–PAGE (12%). Proteins were stained with 0.2% Coomassie blue R-250. The gilthead sea bream GH was identified by Western blot. Proteins were electrotransferred to nitrocellulose membranes (Towbin *et al.*, 1979) and reacted with a 2000 dilution of specific antiserum raised against bonito (*Katsuonus pelamis*) GH (Noso *et al.*, 1988), and then incubated with biotinylated goat

anti-rabbit IgG (Vector Labs, Burlingame, CA). Detection was accomplished by the avidin–biotin reagent (Vectastain ABC kit, Vector Labs) and 3,3-diaminobenzidine in H_2O_2.

RNA Isolation and Cell-Free Translation

Total RNA was isolated from pituitaries of *S. aurata* fish during their fast growing season by the rapid method of Chomczynski and Sacchi (1987). RNA was translated in a commercially available rabbit reticulocyte lysate system (New England Nuclear) in a total volume of 25 μl. Incubations were carried out at 37° for 60 min. The incorporation of [^{35}S]methionine into TCA-precipitable protein was measured in order to assay for translational efficiency.

Immunoprecipitation and Electrophoresis

After the translation reaction was terminated, samples were diluted with 1/5 vol of 50 m*M* methionine in phosphate-buffered saline, vortexed, and then diluted again with an equal volume of 10% SDS. Samples were next heated at 100° for 2 min and diluted 1:10 with phosphate-buffered saline containing 1 m*M* EDTA, 0.5% NP-40, and 10 m*M* methionine. Immunoisolation of GH from translation products was carried out by a modification of the procedure described by Ivarie and Jones (1979) using *Staphylococcus aureus* cell membranes (Pansorbin, Calbiochem). The diluted translation products were first incubated for 1 hr at room temperature with 30 μl of washed Pansorbin (10% suspension). After a 5-min centrifugation at 13,000*g*, the supernatant was removed and incubated with 2 μl nonimmune rabbit serum for 45 min. This was followed by the addition of 30 μl of washed Pansorbin, and incubation was continued for an additional 30 min at room temperature. After centrifugation, the supernatant was incubated overnight at 4° with specific antiserum raised against *S. aurata* GH (Le Bail *et al.*, submitted for publication), or with antiserum raised against recombinant tilapia GH. Thirty microliters of Pansorbin was then added, and the samples were incubated at room temperature for 10 min and then centrifuged as above. At this point the pellet was recovered and suspended in 200 μl of phosphate-buffered saline containing 0.1% SDS, 0.5% NP-40, 10 m*M* methionine, and 1 m*M* EDTA. This suspension was centrifuged through 1 ml of 1.5 *M* sucrose. The pellet was recovered and washed with the above saline solution by suspension and centrifugation 3–4 times. The pellet was suspended in 30 μl of Laemmli electrophoresis sample buffer (Laemmli, 1970) containing 1% β-mercaptoethanol (or alternatively without β-mercaptoethanol) and boiled for 3 min to dissociate the immunoisolated protein from the immune IgG and Pansorbin. Following centrifugation, the supernatant was analyzed by SDS–PAGE utilizing 3 to 15% gradient gels. The gels were dried and subjected to autoradiography. Alignment between the Coomassie-stained gel and the autoradiogram was done by including [^{35}S]methionine dots on the 3 MM paper on which the gel was dried.

Source of Hormones and Antisera

Recombinant tilapia growth hormone (rtGH) was kindly provided by J. Smal (Eurogentec, Liege, Belgium). Antiserum against *Sparus* GH was prepared by P.-Y. LeBail (Rennes, France); antisera against bonito GH was prepared in H. Kawauchi's lab (Noso *et al.*, 1988) and antisera against recombinant tilapia GH was prepared by B. Levavi-Sivan and P. Melamed.

The specificity of the anti-tilapia GH (tiGH) was determined by examining the cross reaction of the radioimmunoassay for tiGH with recombinant tilapia prolactin (tiPRL; Rentier-Delrue, Liege, Belgium) and recombinant common carp GH (cGH; Gertler, Rehovot, Israel). In addition, the ability of the antiserum to bind substances in pituitary extracts of the common carp (*Cyprinus carpio*) and the gilthead sea bream (*S. aurata*) was compared to its affinity for the tilapia pituitary extract (tiPE). The sensitivity of this assay was 0.52 ng/ml.

RESULTS

Isolation of Sparus aurata GH

Sparus aurata GH was isolated from pituitary glands of fast growing fish. Gel filtration on Sephadex G-100 is shown in Fig. 1. Six fractions were pooled, as outlined in the figure. SDS–PAGE of these fractions revealed that fraction 4 contained a protein which reacted positively in a Western blot with anti-bonito GH (Figs. 2A,2B). Fraction 4 was further purified by rpHPLC (Fig. 3). SDS–PAGE analysis of the fractions, illustrated in Fig. 4A, revealed that fraction 16 contained a protein that reacted positively in a Western blot with anti-bonito GH (Fig. 4B). The electrophoretic mobility of the purified protein from fraction 16 was compared to that of recombinant tilapia GH (Fig. 5, lanes 2, 6 and lanes 3, 7, respectively). The molecular weight of *Sparus* GH was estimated to be 21 kDa under nonreduced conditions and 22 kDa in the presence of 1% β-mercaptoethanol (Fig. 5, lanes 2, 6).

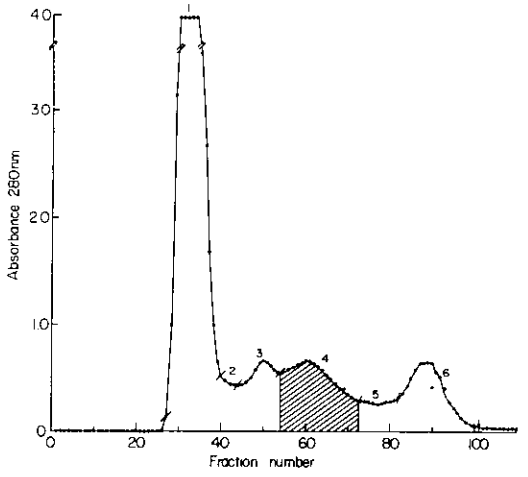

FIG. 1. Gel filtration of *Sparus aurata* pituitary extracts on Sephadex G-100. Elution with 0.1 *M* ammonium bicarbonate buffer, pH 8.2; column size: 1.9 × 100 cm; fraction size: 3 ml/15 min. Fraction G4 was subjected to rpHPLC chromatography.

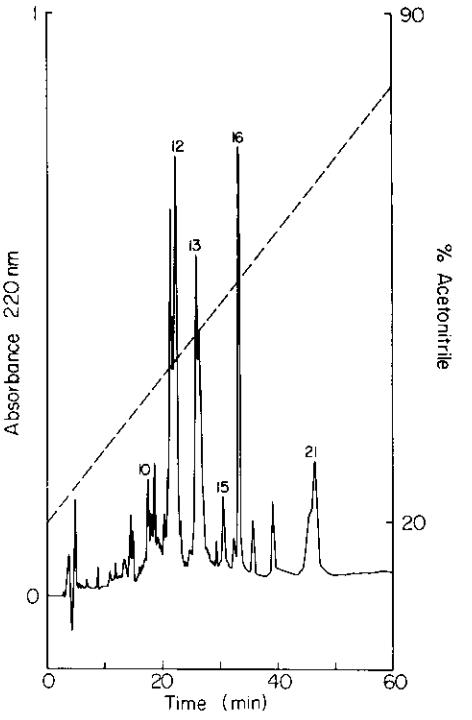

FIG. 3. Reversed-phase high-performance liquid chromatography of G4 on a TSK-gel ODS-120T column with linear gradient of 20–80% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min.

Cell-Free Synthesis of Sparus GH

In a cell-free translation system programmed with pituitary total RNA, GH was synthesized as a larger prehormone, compared to the native hormone isolated from

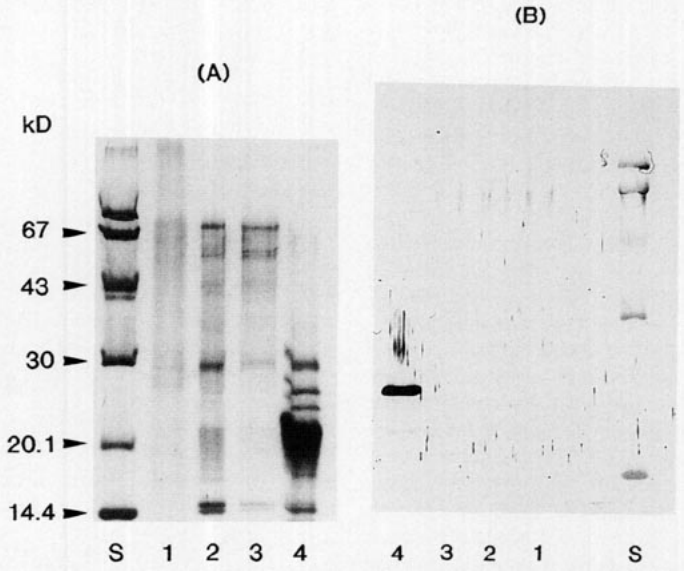

FIG. 2. Analysis of fractions collected from Sephadex G-100 chromatography by SDS–PAGE (A) and Western blot (B). S, standard molecular weight markers. The numbers designate pooled fractions recovered from Sephadex G-100 column.

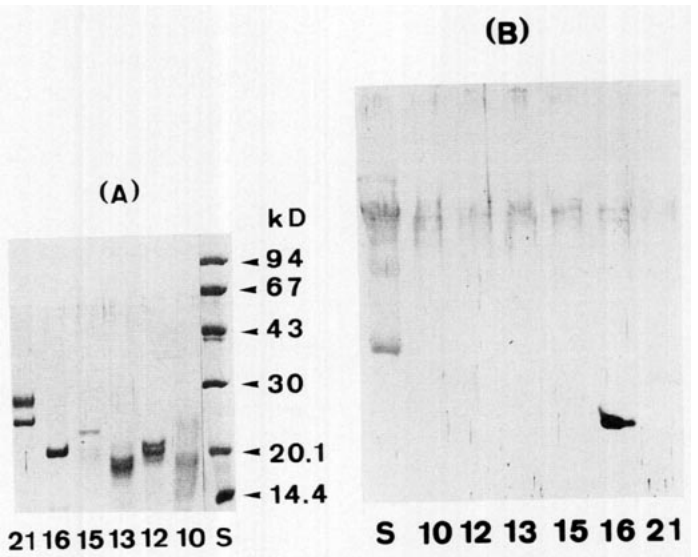

FIG. 4. Analysis of fractions collected from rpHPLC chromatography by SDS–PAGE (A) and Western blot (B). S, standard molecular weight markers. The numbers correspond to the fraction numbers which are shown in Fig. 3.

Sparus pituitaries (compare Fig. 5, lanes 4, 8 to lanes 2, 6). The molecular weight of the precursor form was estimated to be 27–28 kDa when electrophoresed in SDS–PAGE under reduced conditions and 26–27 kDa under nonreduced conditions. To confirm whether the precipitated protein from cell-free translation was indeed GH, immunoisolation was conducted in the presence of excess recombinant tilapia GH or BSA for control. While tilapia GH almost completely displaced the radiolabeled immunoisolate from *in vitro* translation products

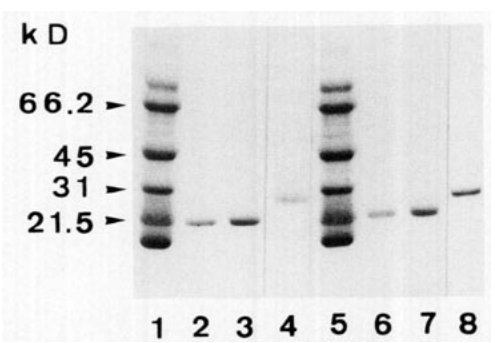

FIG. 5. Electrophoretic comparison between newly synthesized GH immunoisolated from a cell-free system and native GH. Coomassie blue stain of molecular weight standards (lanes 1, 5), purified *Sparus* GH (lanes 2, 6), and recombinant tilapia GH (lanes 3, 7). Lanes 4 and 8, autoradiogram of newly synthesized GH immunoisolated from a cell-free translation system programmed with RNA prepared from pituitary glands using anti-*Sparus* GH. Lanes 1–4 were electrophoresed under nonreduced conditions. Lanes 5–8 were electrophorsed in the presence of 1% β-mercaptoethanol.

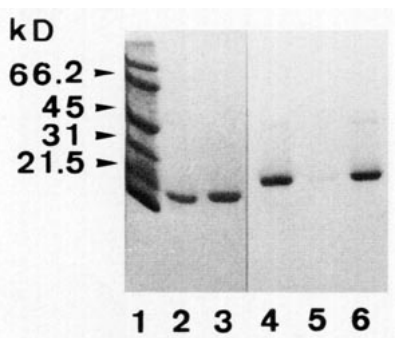

FIG. 6. Specificity of immunoisolation reaction. Coomassie blue stain of molecular weight standards (lane 1), *Sparus* GH (lane 2), or recombinant tilapia GH (lane 3). Lane 4, autoradiogram of newly synthesized GH immunoisolated from a cell-free translation system programmed with pituitary RNA using anti-*Sparus* GH. Lanes 5 and 6, same as lane 4, except that immunoisolation was conducted in the presence of excess tilapia GH or BSA, respectively.

(Fig. 6, lane 5), BSA did not compete for the radiolabeled protein (Fig. 6, lane 6). Due to limitation in the amounts available of purified *Sparus* GH, competition was not performed using *Sparus* GH.

Since recombinant tilapia GH successfully competed with radiolabeled *Sparus* GH in the immunoisolation reaction, the ability of antiserum against tilapia GH to immunoisolate *Sparus* GH was tested. Initially, the specificity of these antibodies was characterized. The displacement curve obtained with the pituitary extract of *S. aurata* was parallel to that of the standard curve of tiGH between 0.002 pit/ml and 0.02 pit/ml, while the pituitary extract of the carp did not (Fig. 7). The cGH was not able to displace the tiGH from its binding to the anti-tiGH, while the tiPRL had succeeded in displacing the tiGH only at a pharmacologic concentration of 10 μg/ml (Fig. 7). Similar results to those obtained with anti-*Sparus* GH were found when anti-recombinant tilapia GH was used in the immunoprecipitation reaction (compare Fig. 8, lanes 3 and 6). A competition experiment showed the ability of recombinant tilapia GH to displace the radiolabeled immunoisolate, while BSA failed (compare Fig. 8, lanes 4 and 5, respectively). The upper faint band detected in all immunoisolations is probably a nonspecific band which is bound and comigrates with the large fragment of IgG. The lower faint band seen in Fig. 8 is probably the result of incomplete reduction of the pre-GH.

DISCUSSION

In the present study, *S. aurata* GH was purified from pituitaries of fast growing fish. Its molecular weight in SDS–PAGE gel electrophoresis under reduced conditions was estimated to be 22 kDa, which is in agreement with the molecular weight of other fish GHs. In the absence of homolo-

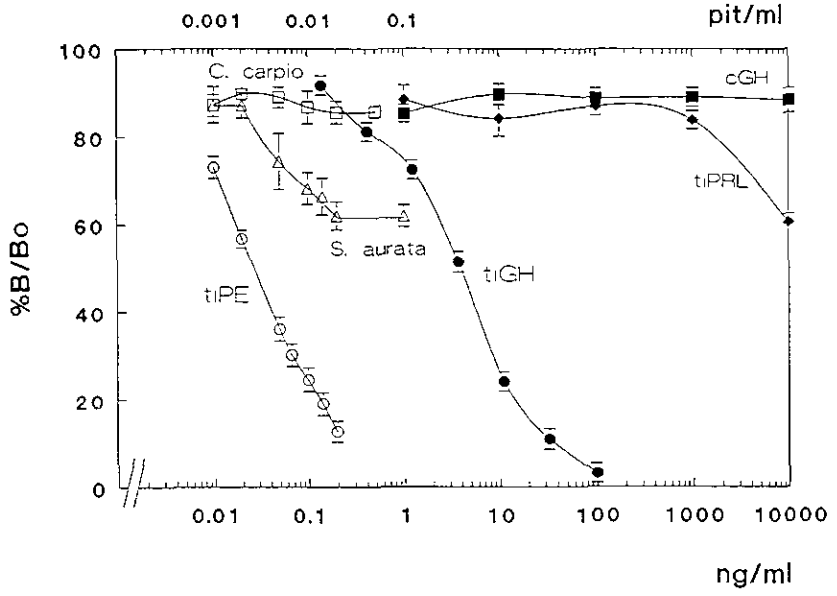

FIG. 7. Cross-reaction of substances in pituitary extracts of tilapia (tiPE; ○), *Sparus aurata* (△), and *Cyprinus carpio* (□), and the recombinant piscine pituitary hormones: carp growth hormone (cGH; ■) and tilapia prolactin (tiPRL; ◆) in the radioimmunoassay for tilapia GH (tiGH; ●). Binding is expressed as % of total binding; the lower *x* axis refers to the concentration (ng/ml) of the pituitary hormones (closed symbols), and the top *x* axis refers to pituitary equivalents per milliliter (open symbols).

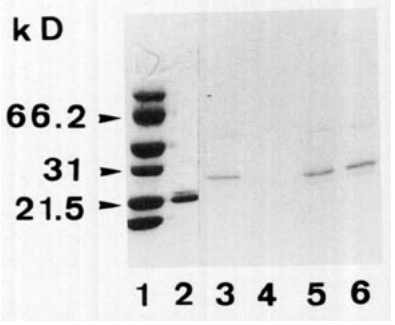

FIG. 8. Comparison between newly synthesized GH immunoisolated from cell-free translation of pituitary RNA using antiserum against *Sparus* GH or tilapia GH. Coomassie blue stain of molecular weight standards (lane 1), recombinant tilapia GH (lane 2). Lanes 3 and 6, autoradiogram of newly synthesized GH immunoisolated from a cell-free translation system programmed with pituitary RNA using anti-tilapia GH (lane 3) or anti-*Sparus* GH (lane 6). Lanes 4 and 5, same as lane 3, except that immunoisolation was conducted in the presence of excess tilapia GH or BSA, respectively.

gous antibodies at the time of purification, the purification steps were monitored by Western blot using anti-bonito GH which was found to cross-react with *Sparus* GH.

The GH synthesized in a cell-free translation system was found to be a larger prehormone. Its molecular weight was estimated to be 27–28 kDa in SDS–PAGE, under reduced conditions, and 26–27 kDa under nonreduced conditions. Based on the deduced amino acid sequence, the molecular weight of *Sparus* pre-GH was calculated to be 26,296. It is well established that peptide hormones, in analogy to other secreted proteins, are synthesized in the rough endoplasmic reticulum as prehormones which undergo cleavage of the signal peptide prior to being secreted (Walter *et al.*, 1984). However, using a specific immunoisolation technique, this is the first direct evidence for the synthesis of fish GH as a prehormone. The reticulocyte system used for cell-free translation is not able to glycosylate the translation product. Thus, the higher molecular weight of the product is not due to sugars which might alter the electrophoretic mobility. The N-terminal portion (signal peptide) of the precursor is thought to be involved in its attachment to the endoplasmic reticulum membranes, a process essential for hormone maturation and secretion. Accordingly, rat and bovine pre-GH were found to be 25 kDa (Seeburg *et al.*, 1977; Miller *et al.*, 1980) when translated *in vitro* and immunoprecipitated by appropriate antisera, about 3 kDa larger than the mature hormone. Similarly, human pre-GH translated by mRNA isolated from GH-producing tumors was found to be a 24-kDa precursor form (Martial *et al.*, 1979). Duck pituitary RNA directed the synthesis of a protein of 25 kDa (Chen *et al.*, 1988).

The characterization of the anti-*Sparus* GH used in this study was described elsewhere (LeBail *et al.*, submitted for publication). It did not cross-react in an RIA with mammalian or recombinant tilapia prolactin. The specificity of the immunoprecipitation reaction was demonstrated in the present study by the ability of excess recombinant tilapia GH to compete with the radioactively labeled translation product. No such reaction was found when BSA was added. In the absence of sufficient amounts of *Sparus* GH, competition experiments were performed using only tilapia GH. Tilapia GH has a homology of 85% with sea bream GH (Yamaguchi *et al.*, 1991). It was found (Le Bail, unpublished results) that purified sea beam GH had a substantial cross reactivity with tilapia GH in an RIA using anti-tilapia GH. Similarly, we demonstrate in this study that extracts of *Sparus* pituitaries are able to displace tilapia GH in RIA using a different preparation of anti-tilapia GH. Taken together, these findings support our present results regarding the ability of recombinant tilapia GH to compete efficiently with sea bream GH in the immunoisolation reaction.

Characterization of anti-tilapia GH used in this study revealed no cross-reactivity with carp GH or recombinant tilapia prolactin. As mentioned earlier, extracts from

Sparus pituitaries displaced labeled tilapia GH in a manner parallel to that of tilapia GH itself (see Fig. 7). This antiserum efficiently immunoprecipitated *Sparus* pre-GH from translation products directed by *Sparus* pituitary RNA, indicating substantial cross reactivity between the anti-tilapia GH and *Sparus* GH.

Recently, we have cloned the gilthead sea bream (*S. aurata*) GH cDNA and showed that it encodes for a polypeptide of 204 amino acids, including a putative signal peptide of 17 amino acids (Funkenstein *et al.*, 1991). Using this cDNA as a radioactive probe, we have shown previously that *Sparus* larvae collected 6 days after hatching express high levels of GH mRNA (Funkenstein *et al.*, 1992). Using antiserum against *Sparus* GH, purified in the course of this study, we are currently studying the presence of immunoreactive GH in extracts of whole *Sparus* larvae. This question is of particular importance since we have found recently that IGF-I, which is believed to be a mediator of GH action, is expressed very early during larval development (Cohen *et al.*, unpublished results). The immunoisolation reaction used in this study will be used in future studies to test the ability of poly(A^+)RNA prepared from *S. aurata* larvae to direct the synthesis of GH peptide.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Smal (Eurogentec, Belgium) for the recombinant tilapia GH. We also thank Keren Diskin for skilled editorial assistance. This work was supported in part by the United States–Israel Binational Agricultural Research and Development Fund (BARD, Project IS-1973-91).

REFERENCES

Agellon, L. B., Davies, S. L., Lin, C. M., Chen, T. T., and Powers, D. A. (1988). Rainbow trout has two genes for growth hormone. *Mol. Reprod. Dev.* **1**, 11–17.

Cavari, B., Funkenstein, B., Chen, T. T., Gonzalez-Villasenor, L. I., and Schartl, M. (1993). Effect of growth hormone on the growth rate of the gilthead sea bream (*Sparus aurata*), and use of different constructs for production of transgenic fish. *Aquaculture* **111**, 189–197.

Chang, Y. S., Liu, C. S., Huang, F. L., and Lo, T. B. (1992). The primary structures of growth hormones of three cyprinid species: Big head carp, silver carp and grass carp. *Gen. Comp. Endocrinol.* **87**, 385–393.

Chao, S.-C., Pan, F.-M., and Chang, W.-C. (1989). Purification of carp growth hormone and cloning of the complementary DNA. *Biochem. Biophys. Acta* **1007**, 233–236.

Chen, H. T., Pan, F. M., and Chang, W. C. (1988). Purification of duck growth hormone and cloning of the complementary DNA. *Bioch. Biophys. Acta* **949**, 247–251.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

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

Funkenstein, B., Tandler, A., and Cavari, B. (1992). Developmental expression of the growth hormone gene in the gilthead sea bream *Sparus aurata*. *Mol. Cell. Endocrinol.* **87**, R7–R9.

Gonzalez-Villasenor, L. I., Zhang, P., Chen, T. T., and Powers, D. A. (1988). Molecular cloning and sequencing of coho salmon growth hormone cDNA. *Gene* **65**, 239–246.

Ho, W. K. K., Tsang, W. H., and Dias, N. P. (1989). Cloning of the grass carp growth hormone cDNA. *Biochem. Biophys. Res. Commun.* **161**, 1230–1243.

Ivarie, R. D., and Jones, P. P. (1979). A rapid sensitive assay for specific protein synthesis in cells and in cell-free translations: Use of *Staphylococcus aureus* as an absorbent for immune complexes. *Anal. Biochem.* **97**, 24–35.

Kariya, Y., Sato, N., Kawazoe, I., Kimura, S., Miyazaki, N., Nonaka, M., and Kawauchi, H. (1989). Isolation and characterization of growth hormone from a marine fish, tuna (*Thunnus albacares*). *Agric. Biol. Chem.* **53**, 1679–1687.

Kawauchi, H., and Moriyama, S. (1988). Chum salmon growth hormone: Isolation and effects on growth of juvenile rainbow trout. *In* "New and Innovative Advances in Biology/Engineering with Potential for Use in Aquaculture" (Sparks, A. K., Ed.), NOAA Technical Report NMFS 70, National Marine Fisheries Service, Seattle, WA.

Kawazoe, I., Noso, T., Kuriyama, S., Akasaka, A., and Kawauchi, H. (1988). Isolation and characterization of growth hormone from yellowtail *Seriola quinequeradiata*. *Nippon Swisan Gakkaishi* **54**, 393–399.

Koren, Y., Sarid, S., Ber, R., and Daniel, V. (1989). Carp growth hormone: Molecular cloning and sequencing of cDNA. *Gene* **77**, 309–315.

Laemmli, U. K. (1970). Cleavage of structural protein during the assembly of the bacteriophage T4. *Nature* **227**, 680–685.

Lorens, J., Nerland, A. H., Male, R., Lossius, I., Telle, W., and Totland, G. (1989). The nucleotide sequence of Atlantic salmon growth hormone cDNA. *Nucleic Acids Res.* **17**, 2352.

Martial, J. A., Hallewell, R. A., Baxter, J. D., and Goodman, H. M. (1979). Human growth hormone: Complementary DNA cloning and expression in bacteria. *Science* **205**, 602–607.

Miller, W. L., Martial, J. A., and Baxter, J. D. (1980). Molecular cloning of DNA complementary to bovine growth hormone mRNA. *J. Biol. Chem.* **255**, 7521–7524.

Momota, H., Kosugi, R., Hiramatsu, H., Ohgai, H., Hara, A., and Ishioka, H. (1988). Nucleotide sequence of cDNA encoding the pregrowth hormone of red seabream (*Pagrus major*). *Nucleic Acids Res.* **16**, 3107.

Nicoll, C. S., Steiny, S. S., King, D. S., Nishioka, R. S., Mayer, G. L., Eberhardt, N. L., Baxter, J. D., Yamanaka, M. K., Miller, J. A., Sckilkamer, J. J., Schilling, J. W., and Johnson, L. K. (1987). The primary structure of coho salmon growth hormone and its cDNA. *Gen. Comp. Endocrinol.* **68**, 387–399.

Noso, T., Yasuda, A., Kawazoe, I., Takehara, H., Takahasi, A., Sakai, K., and Kawauchi, H. (1988). Isolation and characterization of growth hormone from a marine fish, bonito (*Katsuwonus pelamis*). *Int. J. Peptide Protein Res.* **32**, 579–589.

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

Rand-Weaver, M., Noso, T., and Kawauchi, H. (1991). The complete amino acid sequences of two variants of growth hormone from Atlantic cod (*Gadus morhua*). *Gen. Comp. Endocrinol.* **81**, 39–50.

Rentier-Delrue, F., Swennen, D., Mercier, L., Lion, M., Benrubi, O., and Martial, J. A. (1989a). Molecular cloning and characterization of two forms of trout growth hormone cDNA: Expression and secretion of tGHII by *E. coli*. *DNA* **8**, 109–117.

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

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

Sato, N., Watanabe, K., Murata, K., Sakaguchi, M., Kariya, Y., Kimura, S., Nonaka, M., and Kimura, A. (1988). Molecular cloning and nucleotide sequence of tuna growth hormone cDNA. *Biochem. Biophys. Acta* **949**, 35–42.

Schneider, J. F., Myster, S. H., Hackett, P. B., Guise, K. S., and Faras, A. J. (1992). Molecular cloning and sequence analysis of the cDNA for northern pike (*Esox lucius*) growth hormone. *Mol. Mar. Biol. Biotech.* **1**, 106–112.

Seeburg, P. H., Shine, J., Martial, J. A., Baxter, J. D., and Goodman, H. M. (1977). Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone. *Nature* **270**, 486–494.

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.

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.

Walter, L., Gilmore, R., and Blobel, G. (1984). Protein translocation across the endoplasmic reticulum. *Cell* **38**, 5–8.

Watahiki, M., Tanaka, M., Masuda, N., Yamakawa, M., Yoneda, Y., and Nakashima, K. (1988). cDNA cloning and primary structure of yellow tail (*Seriola quinquerodiata*) pregrowth hormone. *Gen. Comp. Endocrinol.* **70**, 401–406.

Watanabe, K., Igarashi, A., Noso, T., Chen, T. T., Dunham, R. A., and Kawauchi, H. (1992). Chemical identification of catfish growth hormone and prolactin. *Mol. Mar. Biol. Biotech.* **1**, 239–249.

Yamaguchi, K., Yasuda, A., Kishida, M., Hirano, T., Sano, H., and Kawauchi, H. (1987). Primary structure of eel (*Anguilla japonica*) growth hormone. *Gen. Comp. Endocrinol.* **66**, 447–453.

Yamaguchi, K., Yasuda, A., Lewis, U. J., Yokoo, Y., and Kawauchi, H. (1989). The complete amino acid sequence of growth hormone of an elasmobranch, the blue shark (*Prionace glauca*). *Gen. Comp. Endocrinol.* **73**, 252–259.

Yamaguchi, K., King, D. S., Specker, J. L., Nishioka, R. S., Hirano, T., and Bern, H. A. (1991). Amino acid sequence of growth hormone isolated from medium of incubated pituitary glands of tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.* **81**, 323–331.