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Effect of growth hormone on the growth rate of the gilthead seabream (*Sparus aurata*), and use of different constructs for the production of transgenic fish

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ABSTRACT

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 seabream (*Sparus aurata*), and use of different constructs for the production of transgenic fish. *Aquaculture*, 111: 189–197.

When bovine or human growth hormones (GH) were injected into 6 months old (about 10 g) gilthead seabream, the growth rate of the fish, as measured by changes in their weight, increased by only about 15% compared with the saline-injected control. No effect or even slight inhibition of the growth rate was obtained when chicken or porcine GHs were injected. In a preliminary experiment, it was found that injection of the native GH increased the growth rate of the fish by about 20% after treatment for only 2 weeks. An expression vector, using the pRE1 plasmid and transformation into MZ1 cells, produced the gilthead seabream GH, providing a supply for further experiments on the effect of the homologous GH on growth. Two reporter genes, β-galactosidase (lacZ) and melanoma oncogene of *Xiphophorus* (mrk YY), were microinjected into fertilized eggs of *S. aurata*. Expression of these two genes could be demonstrated in 2-day-old embryos, the lacZ gene by staining of its enzymatic product, and the mrk YY gene by its phenotypic expression.

INTRODUCTION

Feed can account for up to about 50% of the operating costs of commercial fish production. Any improvement in the growth rate of fish will significantly reduce expenses in terms of time, labor, feed and space.

In all vertebrates, normal growth is controlled by intricate interactions among several hormonal factors including anabolic steroids, thyroid hor-

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mones, insulin, insulin-like growth factor (IGF) and growth hormone (GH). Of all these hormones, GH plays the most important role.

Growth hormone is a single chain polypeptide of about 22 kD, produced by the somatotrophs of the anterior portion of the pituitary gland. This hormone is required for normal growth and development of pre-adult vertebrates.

Prack et al. (1980) have shown that mammalian growth hormone is effective in increasing growth rate in fish. Agellon et al. (1988) have shown that administration of recombinant trout GH resulted in an acceleration of growth rate of yearling rainbow trout by 2 to 4 times. Higgs et al. (1976) were able to demonstrate weight gain in coho salmon after implanting bovine GH/cholesterol pellets. Cook et al. (1983) showed a very significant (325%) increase in growth rate of goldfish after i.p. injection of bovine or carp GH. Wagner and McKeown (1981) reported an increase in body size and weight of juvenile rainbow trout injected with the purified chum salmon growth hormones, as also found by Weatherley and Gill (1987). In a preliminary experiment, the growth rate of *Sparus aurata* was accelerated when the native GH from the fish was injected twice a week for 2 weeks (unpublished results).

In this report, we have injected GHs from different sources into young *Sparus aurata* fish, to study their effect on growth.

Another way of accelerating growth is by producing transgenic animals with the growth hormone gene. Stable transgenic animals have been produced in *Drosophila* (Rubin and Spradling, 1982), *Xenopus* (Etkin et al., 1984), sea urchins (Flytzanis et al., 1985), mouse (Palmiter and Brinster, 1986), and recently in fish (for review see Chen and Powers, 1990). Since the production of stable transgenic animals is time consuming, transient expression systems can represent alternative test systems for regulatory sequences.

In fish there are several reports showing that foreign DNA microinjected into fertilized eggs can be transiently expressed during development (Chong and Vielkind, 1989; Liu et al., 1990; Winkler et al., 1991).

We report here on microinjection of the lacZ and mrk YY reporter genes into fertilized eggs of *S. aurata*, and followed their expression in 2-day-old embryos.

MATERIALS AND METHODS

Injection of growth hormone

Groups of 45 fish each (5–10 g) were grown in 200 l circular, fiberglass tanks, each being supplied with fresh sea water at ambient temperature (20–22 °C). Recombinant growth hormones from human, bovine, porcine and chicken (prepared by BioTechnology General (Israel) Ltd., Rehovot, Israel) were injected intramuscularly or intraperitoneally biweekly. The concentration of the hormones was 1.0 μg/g of body weight.

Each hormone was injected into a group of 45 fish. One group of 45 fish

was injected with saline, as a control. Fish were weighed and measured at biweekly intervals. They were fed 2–3 times daily, depending upon their size. Daily maintanance included siphoning of the remaining food and feces, removal of any dead fish, and observation of the general health of the fish in the tanks.

Microinjection into fertilized eggs of Sparus aurata

After applying different approaches for microinjection into the fertilized eggs of *S. aurata*, the following protocol was established: During the spawning season, the courting behavior of the fish in the tanks was observed, and when females started to spawn, they were taken from the tank, anesthetized to some extent and eggs collected by gentle stripping. Similarly, sperm was collected from males by squeezing the abdomen. Eggs were fertilized for 90 s in a beaker by mixing the sperm with eggs in sea water diluted to 25 ppt. The fertilized eggs were used for microinjection after washing off the sperm with sea water.

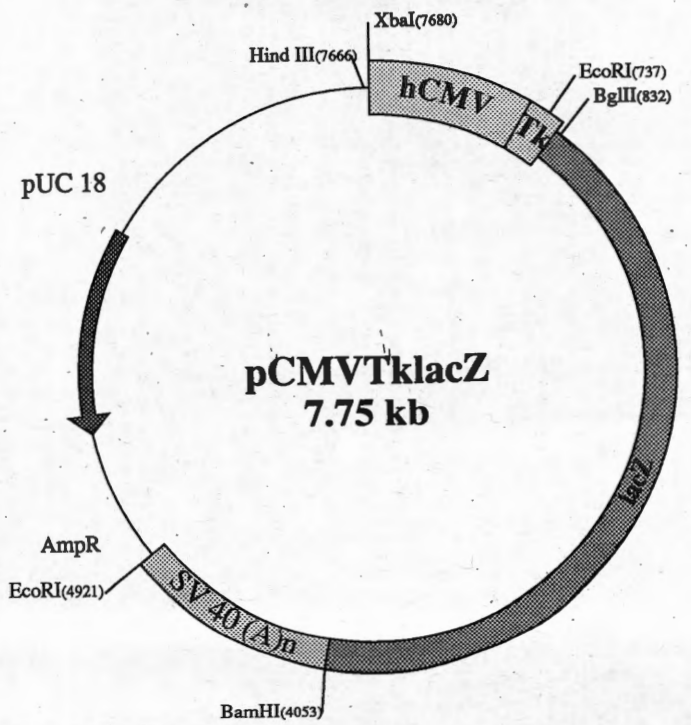

Fig. 1. pCMVTklacZ plasmid.

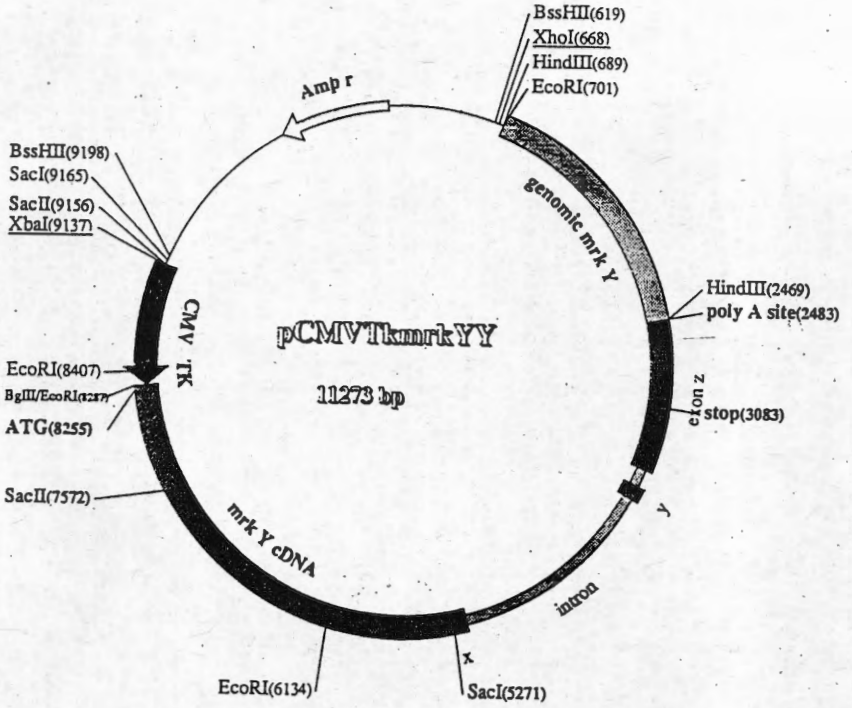

Fig. 2. pCMVTkmrk YY plasmid.

The eggs were put on an agarose plate and microinjected immediately. Injection was done via the germinal disc by using glass capillaries with a diameter of 5–10 μm. DNA solution in a concentration of 0.1–0.2 mg/ml was used, and the amount injected was about 300 pl, which is equivalent to about 10^6 copies of the gene injected. During the first 30 min, the chorion is not hard and penetration with the microneedle was very easy. About 200 embryos could be microinjected during these 30 min. Eggs and sperm from stripped fish were kept at 18°C, and artificial insemination was carried out every hour for 5–6 h after stripping. All injections were completed before the two-cell stage was reached. The injected embryos were washed from the agarose plate into a 25 l v-tank containing filtered (1 μm) sea water at a temperature of 18–19.5°C. Water in the tank was exchanged at a rate of about 6 l/h with continuous aeration. The survival at the hatching stage was about 10%. After hatching, the larvae were transferred to regular 500 l tanks used for growing larvae in the hatchery. Survival rate of the larvae was about 20%, similar to the control larvae developed from non-treated embryos.

Reporter genes used for microinjection

(1) β-galactosidase. pRSVlacZ (Altschmied, Schulz and Renkawitz, unpublished) contains the 600 bp HindIII-NdeI fragment of the Rous sarcoma virus long-terminal repeat (RSVLTR) (Gorman et al., 1982) in front of the lacZ gene and the SV40 polyadenylation site (Fig. 1). For visualizing lacZ enzymatic activity, embryos were fixed in 4% paraformaldehyde and stained for at least 12 h at 37°C. For a detailed description of the histochemical procedures, see Winkler et al. (1991).

(2) mrk YY, melanoma oncogene of *Xiphophorus* (Fig. 2).

RESULTS AND DISCUSSION

Effect of GH on growth

The results shown in Fig. 3 demonstrate a slight increase (about 15%) of fish growth when human or bovine growth hormones were administered. Porcine and chicken growth hormones either had no effect or slightly inhibited growth rate compared with the saline-injected control fish. Since fish exist in an enormous variety of species (close to 18 000) generated in the long process of evolution, it is quite possible that different fishes respond differently to heterologous growth hormones, as also found by Weatherley and Gill (1987). In a preliminary experiment, the growth rate of *Sparus aurata* was accelerated when the native GH from the fish was injected twice a week for 2 weeks (unpublished results). Limited availability of the native GH did not allow further experiments to be carried out with the fish.

Biosynthesis of S. aurata growth hormone

An expression vector was constructed in order to be able to produce the homologous hormone in large quantities. This homologous hormone will be used for studying the effect of GH on the growth rate of the fish. The coding region in the GH cDNA of *Sparus aurata* (Funkenstein et al., 1991) was amplified by using the following primer couple:

primer 1: CGCG CAT ATG CAG CCG ATC ACA GAC
primer 2: GAGT GGA TCC CAC AGA AGA GAG GCG

The product obtained from the PCR amplification was 582 bp. It was treated with Klenow polymerase, phosphorylated with polynucleotide kinase, and then digested with NdeI/Bam HI restriction endonucleases. This insert was ligated to dephosphorylated NdeI/Bam HI cut pRE-1 vector (Reddy et al., 1989). This plasmid was transformed into MZ1 competent cells. Several clones with the expression vector were isolated. The clones with the expression vector were grown at 30°C to OD_{660} of 0.5, and then the temperature was raised to 42°C for 2 h for induction. In one experiment carried out with one of the clones, production of the GH by the expression vector is demonstrated in the

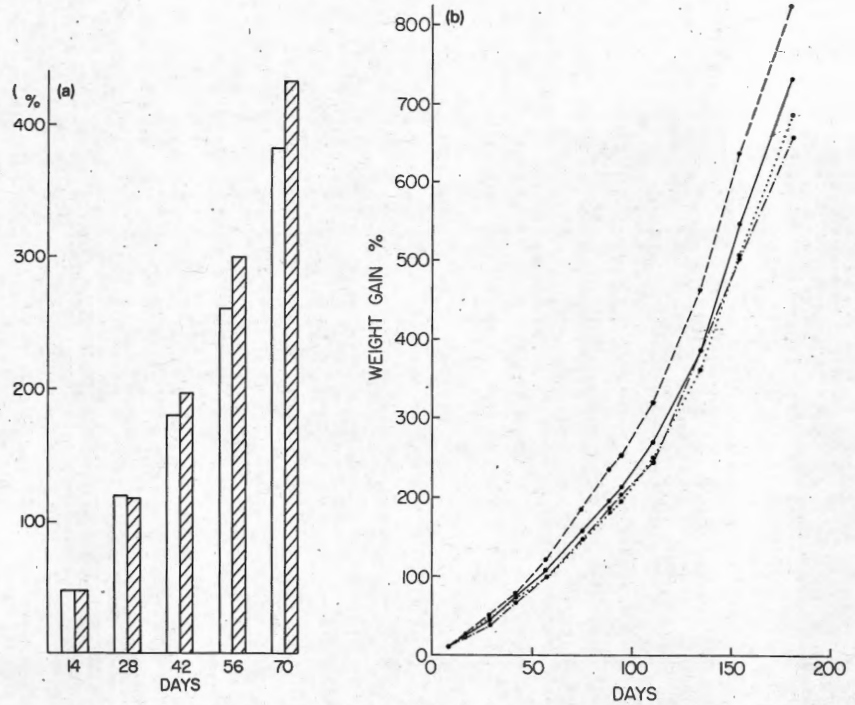

Fig. 3. Effect of the GH on growth of *Sparus aurata*. (a) Fish received intramuscular injections of human GH. Open bars represent control injected with saline. Dashed bar — injected with GH. (b) Fish received intraperitoneal injections of bovine, porcine or chicken GH. —— control, – – – – bovine GH, ···· chicken GH, –·–·–·– porcine GH.

Western analysis using a universal antibody for the detection of animal growth hormones [Gonzalez-Villasenor, L.I., 1992. Uni-GH-IgG™ (Trade Mark of Biotrax, Inc.) U.S. Patent pending]. GH produced was about 5% of total protein (Fig. 4).

Transient expression of reporter genes

The two constructs, pRSVlacZ and mrk YY, were microinjected into fertilized eggs of *S. aurata*. Expression of these two genes was observed in 2-day-old embryos (Fig. 5 and Fig. 6), either by staining of lacZ enzymatic activity or by the phenotypic effect of the mrk oncogene (embryonal tumor).

The pattern observed is explained as lacZ expressing cell clones derived from a common precursor. The mosaic expression pattern was also observed in medaka (Friedenreich and Schartl, 1990). The low proportion of stained

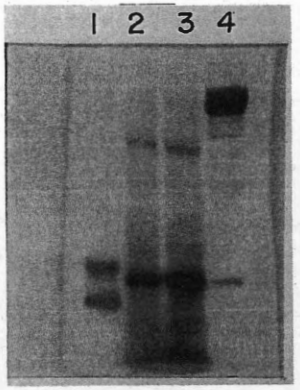

Fig. 4. Western blot of the protein extract of the expression vector. Lane 1, bovine GH; lane 2, unpurified inclusion bodies — 10 μl; lane 3, unpurified inclusion bodies — 20 μl; and lane 4, total protein extract — 10 μl.

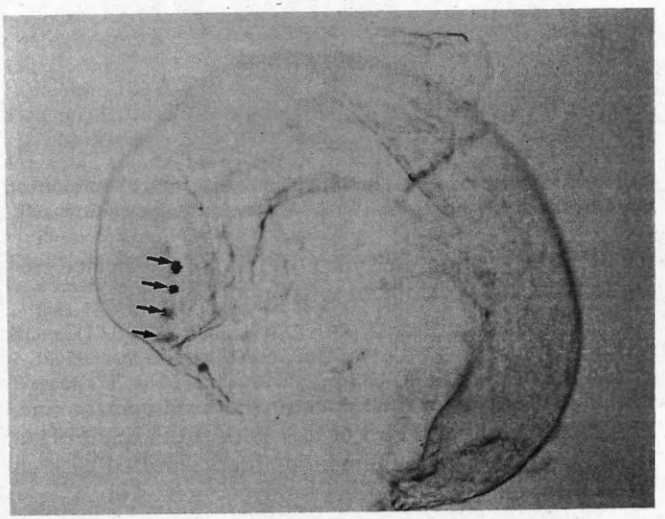

Fig. 5. Larva (1.5 days) of *Sparus aurata* injected with the pCMVTk lacZ construct at the one cell stage. Expression of lacZ is apparent at four distinct regions in the head (arrows).

cells is probably due to the low sensitivity of the histochemical staining procedure.

In the prehatching larvae of *S. aurata* that were microinjected with pmrk YY construct, at the one cell stage, a tumor (arrow in Fig. 6) develops from

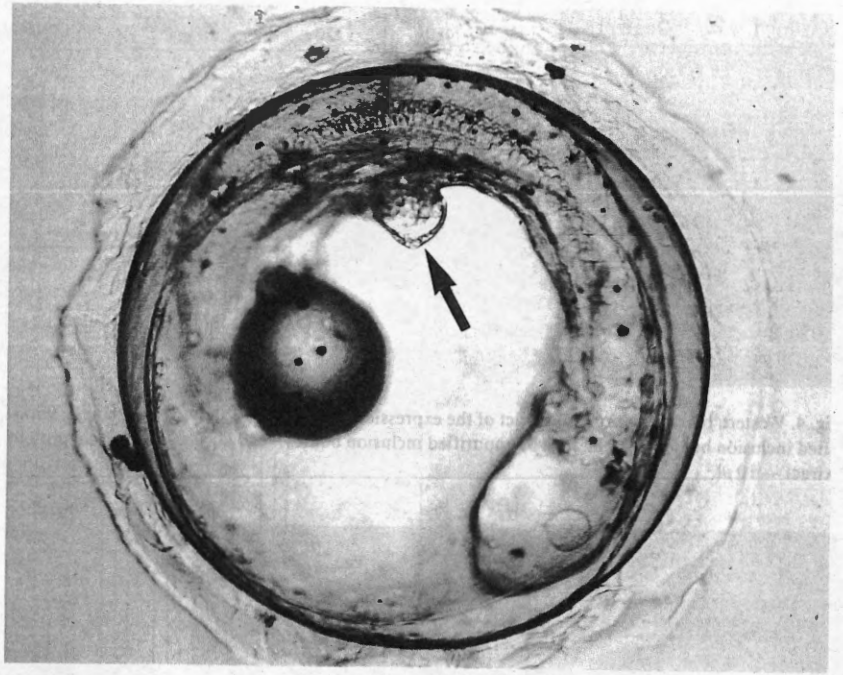

Fig. 6. Prehatching larva (2 days) of *Sparus aurata* that has been injected with pmrk YY construct at the one cell stage. A tumor (arrow) is developing from the ventral abdomen of the larva into the yolk.

the ventral abdomen of the larvae into the yolk. Of a total of 46 injected embryos that developed to this stage, 3 exhibited such tumor outgrowth.

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