Foreign GH gene expression in GH transgenic salmon

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Growth hormone (GH) gene expression has been examined in control and transgenic coho salmon containing a transgene comprised of the sockeye salmon GH1 gene under the control of the MT-B promoter from the same species. This transgene dramatically enhances the growth of salmonids, and raises serum GH levels some forty-fold. Transcript levels from this transgene were detected by RT-PCR in all tissues examined, and an obvious increase of the transgene expression was obtained in some developing stage. GH mRNA levels were also examined in the pituitary gland, and were found to be significantly lower (p<0.01) in transgenic compared to nontransgenic salmon of the same size. Pituitary glands of transgenic were also smaller than control of the same size. The transgenic fish changed a surface area in the anterior intestine, and the intestine of GH transgenic salmon had more folds and the path tracing the inner circumference around the intestinal folds of a cross section was about 2.7 times longer than in control salmon. Further, the transgenic salmon increased ability to compete for food, and they consumed 2.9 times more pellets that the non-transgenic controls.

KEY WORDS: transgenic salmon, growth hormone, gene expression, metallothionein-B promoter

INTRODUCTION

Since the production of growth-enhanced transgenic mice in the early 1980s, transgenic techniques have been used extensively to study the effects of growth hormone (GH) overexpression in various mammalian systems). This work also stimulated the development of gene transfer methods for several fish species, both for the development of lower vertebrate model systems and to enhance production characteristics for species grown in aquaculture2). The majority of these experiments with fish have involved the transfer of GH gene constructs of both mammalian and piscine origin, and currently have involved over a dozen fish species3).

The phenotypic effects of GH transgenes in mammals and most fish species has ranged from no effect to approximately a doubling of body size4). In contrast, salmonids appear particularly responsive to growth stimulation by somatotropins when administered either by intraperitoneal injection or by transgenesis. For transgenesis, very dramatic growth effects can be observed, typically between 6 and 11-fold but ranging to as much as 37 fold relative to controls5) in several salmonid species. The different responses may arise from the gene constructs utilized: The effects seen in salmonids were obtained with constructs derived from fish DNA, whereas mammalian GH transgenes have not yet produced dramatic phenotypic effects in fish6).

In addition to growth effects, GH overexpression by transgenesis in mammals has produced a range of phenotypic effects, including morphological abnormalities and physiological disorders 7). Similarly, overexpression of GH in salmonids leads to phenotypic effects other than growth, including skin color changes, developmental acceleration of smoltification, precocious sexual maturation, and cranial abnormalities that superficially resemble the acromegaly syndrome seen in mammals with high GH levels. The regulation of endocrine hormones in GH transgenic mammals has been examined, revealing that liver IGF-I mRNA levels and serum IGF-I are increased some two to three fold, whereas pituitary GH mRNA is reduced due to feed-back suppression of GH gene transcription by high levels of circulating GH. In GH-transgenic fish, levels of circulating GH can be approximately 40-fold higher than controls, however, no information has been reported on what effects if any may be occurring to pituitary expression of GH.

The present report examines the expression of the salmon transgene OnMTGH15) (the sockeye salmon metallothionein-B promoter fused to the type-1 growth hormone gene from the same species) in transgenic coho salmon tissues at different developmental stages. Since GH is a multifunctional hormones, feeding behavior and anatomical differences of digestive organs in GH transgenic salmon was also investigated.

MATERIALS AND METHODS

Experimental animals

Transgenic fish were produced by fertilizing wild-type eggs with milt collected from transgenic coho salmon containing the OnMTGH15). Transgenic salmon and wild eggs originate from the Chehalis River hatchery strain from southwestern British Columbia, Canada.
RNA isolation and RT-PCR
Various tissues were homogenized on ice in the presence of guanidine isothiocyanate buffer\(^6\). Total RNA was diluted with RNase-free water, mixed with primer GH6: 5'-CTACAGAGTCGATGGCCT-3' (30 pmol) and 8 μL 1 RNase-free water. The pOnMTGH1 mRNA present in these samples was converted to cDNA in a first strand buffer (50 mM Tris (pH 8.3), 40 mM KCl, 1 mM DTT, 6 mM MgCl\(_2\)) and Superscript reverse transcriptase (200 u; Gibco BRL). The cDNA was amplified by PCR using PCR premix (containing GH33: 5'-TCGATGGAAA ACCAACGGCT-3' (30 pmol) and Taq DNA polymerase (0.05 u/μl)). Reactions were run for 30 cycles with a 55°C annealing cycle (1 min), 72°C extension cycle (2 min), and a 94°C denaturing cycle (1 min). The second PCR was performed using primers specific to the transgene GH gene (sockeye GH1) that would not amplify host (coho) Gill or GH2 sequences (SOCK GH1U: 5'-TGACTCCATCGTGAG CCCAG-3' (10 pmol) and SOCK GH1D: 5'-TCTTGAAGCAAGC ACAAC-3' (10 pmol)).

Southern and northern blot analysis
Southern blot analysis was performed to analyze the PCR products \(^9\). Hybridizations were performed using a cDNA derived from the type-2 growth hormone gene from sockeye salmon \(^10\).

Northern blot analysis was performed using 50 μg of total RNA electrophoresed in 1.2% agarose-7.0% formaldehyde gels containing MOPS buffer. Hybridization proceeded at 60°C for 16 hr in the same buffer containing the sockeye GH2 probe \(^10\) labeled with \(^32\)P by the method of Feinberg and Vogelstein \(^11\). Chicken b-actin cDNA was used as a control.

GH mRNA quantification by northern dot blot
Digoxigenin-labeled RNA probes were prepared using DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions. The sense and antisense RNA probe was synthesized using linearized GH2 cDNA cloned into pBluescriptII SK+ and transcription reactions were performed using T3 or T7 polymerase in the presence of digoxigenin-11-UTP. Total RNA (50 μg) from various tissues was applied to Hybond-N+ membranes (Amersham). The filters were fixed, baked, pre-hybridized, and hybridized according to the method of the DIG Luminescent detection Kit (Boehringer Mannheim). The expressed GH mRNA was measured by digital densitometry using the Bio Image system (Millipore).

DNA Sequencing
PCR products were purified from 1.8% TAE agarose gels containing ethidium bromide using the Wizard PCR purification system (Promega). Sequencing was performed manually using the Thermo Sequenase kit (Amersham).

In Situ Hybridization
Pituitary glands were fixed with 4% paraformaldehyde in PBS over night at 4°C. The pituitary was washed with PTw (1XPBS+0.1% Tween-20) for 5 min at room temperature, and then dehydrated through a methanol series and stored at -20°C. Linearized GH2 cDNA cloned into pBluescriptII SK+ was used for in vitro transcription in the presence of digoxigenin-11-UTP and the RNA was used as prob. Dehydrated, fixed pituitaries were gradually rehydrated with methanol and PTw. The pituitaries were treated with proteinase K at room temperature. Hybridization was conducted overnight at 60°C. After hybridization, the pituitaries were washed at 55°C in 50% formamide in 2XSSCtw (2XSSC+0.1% Tween-20). RNA hybrids were detected by immunohistochemistry according to the manufacturer's instructions. Pituitaries were stained with NBT (Boehringer Mannheim).

Feeding test
Fish were reared in 5000 L aquaria until 1 week before the start of the experiment, when they were subjected to an initial weighing and then transferred to 250 L aquaria for a 1-week acclimatization period. During this time, fish were fed to satiation twice daily. Twelve size-matched transgenic/ control salmon pairs (about 250g) were created, and each pair was placed in separate tank. The fish were randomly tagged and experiments were conducted indoors in 10°C well water under a simulated natural photoperiod. The experiments repeated twice daily over three consecutive days, each pair was fed a maximum of 30 pellets. Single pellets were provided sequentially, and identified the fish that fed the pellet. Feeding was halted when neither of the fish took three consecutive pellets. The procedure thus yielded six repeated measures for competitive ability and food consumption for each of the 12 pairs.

Intestinal morphology in transgenic salmon
Size-matched transgenic and control salmon were killed with an overdose of anaesthetic and measured. They were injected with buffered formalin, fastened to a wooden tongue depressor with elastics to keep the body straight, then placed in buffered formalin and send to the laboratory in Guelph. All gastrointestinal tract measurements were described in Stevens et al\(^{12}\).

Statistical analysis
Values for mRNA are expressed as means +/- SE. The statistical significance was determined by one-way analysis of variance followed by a posteriori comparisons on
the significant ANOVA results using Fisher PLSD. Statistical significance was set at p<0.05.

RESULTS

OnMTGH1 transgene expression in various tissues

The pattern of OnMTGH1 transgene expression in various tissues was examined by northern hybridization. High-level expression was observed in spleen, pyloric caeca, kidney, and intestine, and smaller amounts were detectable in skin and gill, stomach and liver. GH mRNA was not detected in muscle. The GH mRNA from the transgene was similar in size to the endogenous GH produced in control pituitaries. Primers were designed that could amplify sequences from the sockeye GH1 present in the transgene construct but were not capable of amplifying host (coho) GH1 or GH2 sequences. Analysis of two sizes of transgenic fish by RT-PCR revealed that transgene mRNA could be detected in all tissues examined. As was observed with the northern analysis, a single band was observed from all tissues. The GH bands amplified by RT-PCR were directly sequenced, revealing that they were indeed derived from the transgene.

Quantitation of transgene expression

OnMTGH1 transcripts from different tissues of transgenic fry (3.5-4.0cm in FL, 0.5-1.0g in WT) and from large transgenics (40-47cm in FL, 1.2-1.7kg in WT) were quantitated by RNA dot blot analysis. The highest level of OnMTGH1 transgene mRNA was observed in pyloric caeca, followed in descending order kidney, spleen, intestine, skin, stomach, liver, and muscle. Levels of mRNA in pyloric caeca were significantly (p<0.05) greater than that of the other tissues. Not all tissues could be obtained from transgenic fry, and even in those analyzed (skin, intestine, and muscle), it was difficult to detect GH mRNA suggesting that transcription of the transgene may be lower in very early development, high during the early juvenile growth phase, and then reduced in older larger animals.

Pituitary gland structure and GH gene expression

Pituitary glands in transgenic animals increased in size with age, but they were dramatically reduced relative to pituitaries from size-matched control fish. When expressed as a proportion of body weight, all transgenic animals had pituitary glands smaller than controls, and this reduction was negatively correlated with body size. To examine the effect of ectopic GH transgene expression on pituitary GH mRNA levels, northern blots of total pituitary RNA from control and transgenic fish were examined. The amount of GH mRNA was reduced 3.9-fold in transgenic pituitaries relative to control fish of the same size.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was utilized to examine the pattern of GH gene expression in transgenic and control pituitary glands. In control glands hybridized with antisense GH probe, the highest signal was obtained in the proximal pars distalis, with some glands showing a weak signal in the rostral pars distalis, and none in the pars intermedia. In pituitaries from larger animals, GH mRNA was detected in approximately the same position and proportion of cells in control and transgenic pituitaries, but with a reduced intensity in the latter.

Feeding contest between transgenic and size-matched control salmon

For each fish of the 12 pairs, food consumption during the repeated feeding trials was first summarized for each of the 30 sequentially presented pellets. As there were six feeding trials, a fish in a pair could theoretically consume a maximum of six pellets for any of the sequential pellets given. Analysis of the food consumption at the initiation of feeding showed that the transgenic fish took these significantly more often than the control fish. The initial consumption of the transgenic fish was 2.5 times that of the control fish. While the consumption declined gradually in a near-linear fashion for both the transgenic and the control fish during continued feeding. Thus, the transgenic fish consumed 50.3 pellets on average, or about 2.9 times more food than the control fish.

Intestinal morphology in transgenic salmon

Length of the anterior and posterior intestine, and total intestine length did not differ between transgenic and size-matched control. The anterior intestine of GH transgenic salmon had more folds and the path tracing the inner circumference around the intestinal folds of a cross section was about 2.7 times longer than in control salmon. The product of the inner circumference and the length of the anterior intestine were used to estimate the total anterior intestinal surface area. This was 2.4 times greater in GH transgenic salmon than in control salmon. The inner circumference and surface area of the posterior intestine while greater in GH transgenic salmon were not statistically different. The total surface area (anterior plus posterior) was 2.2 times larger in GH transgenic salmon than in control salmon.

DISCUSSION

Mammalian MT promoters have also been used to drive GH gene expression in transgenic fish, and effects on growth have been noted. Further, the mammalian MT promoter has been shown to be active in transient assays in fish cells and microinjected fish embryos.
In the current study, we have examined endogenous and ectopic expression of GH from a salmonid MT promoter that has been previously shown to elevate serum GH levels and dramatically enhance growth in transgenic salmon. As is observed in mammalian systems, the salmon MT promoter was found to be active in a variety of cell types by both northern blot and RT-PCR analysis, resulting in GH mRNA in tissues in addition to the pituitary gland. However, in contrast to mammals, the salmon MT promoter was only weakly expressed in liver relative to other tissues.

Mice containing GH transgenes have elevated plasma GH, and grow to almost twice the size of their normal litter mates. In such mice, endogenous GH synthesis is inhibited due to negative feedback regulation by high serum GH and IGF-I levels 13), and abnormal pituitary development ensues, with a dramatic reduction of acidophilic or GH producing cells detected by immuno cytochemistry. Interestingly, GH gene constructs containing different promoters affect somatotrophs differently in transgenic mice; for example, pituitary GH mRNA levels were reduced approximately 38% and 98% in MT-bGH and PEPCK-bGH transgenic mice, respectively 14). Stefaneanu et al 15) demonstrated significantly decreased pituitary mass and GH mRNA levels in GH transgenic mice pituitary relative to controls, indicating that negative feedback may be occurring. We have observed similar effects on GHmRNA and pituitary size in transgenic fish containing the OnMTGH1 transgene, indicating that such regulatory mechanisms controlling GH expression are also operating in lower vertebrates. (and perhaps also GH1 at even lower levels), and whether functional protein is being produced, secreted, and acting in a paracrine or endocrine fashion.

The elevated competitive food consumption and growth rate of GH transgenic salmon measured in the present study is consistent with the known physiological functions of GH in salmonids. The data are also consistent with the hypothesis that GH increases feeding motivation and appetite in salmonids. As the strain of GH transgenic fish used in this study has been shown to have extraordinarily high plasma GH levels. It can be assumed that the observed differences between the transgenic and non-transgenic fish are GH mediated, and that GH transgenesis and GH treatment thus appear to induce similar changes in the feeding behavior of salmonids. While, the strong appetite of the transgenic fish containing the OnMTGH1 transgene, indicating that such regulatory mechanisms controlling GH expression are also operating in lower vertebrates. (and perhaps also GH1 at even lower levels), and whether functional protein is being produced, secreted, and acting in a paracrine or endocrine fashion.

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REFERENCES