

Transgenic Fish and Aquaculture

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Abstract

Transgenic fish species can be routinely produced by transferring foreign DNA into developing embryos via microinjection or electroporation. This technology offers an excellent opportunity for modifying or improving the genetic traits of commercially important fishes, mollusks, and crustaceans for aquaculture. Studies have shown that administration of recombinant fish or mammalian growth hormone (GH) to juvenile fish or oysters resulted in significant growth enhancement. Thus, it is possible to improve the growth rates of marine animals by manipulating GH or its gene. This paper reviews the results of studies to determine the efficacy of recombinant fish GH in improving the growth rates of fishes, mollusks, and crustaceans, and of gene transfer technology in producing fast-growing transgenic animals.

Introduction

The worldwide harvest of fishery products traditionally depends upon the natural populations of fishes, mollusks, and crustaceans from fresh and marine waters. Due to the rapid increase in consumption of fishery products by the general public, as well as uncontrolled fishing and poor management, the total annual harvest has already approached the maximum potential of about 150 million tons as forecast by the US Department of Commerce. Accumulation of chemical pollutants in aquatic environments has further affected the fisheries production. A number of regions have experienced significant declines in the catches of important species such as salmon, striped bass, sturgeons, eels, jacks, mullets, mackerel, abalones, oysters and crabs (FAO 1986). Fishing fleets now travel great distances to exploit more productive areas. They have switched to alternative species and begun to employ a variety of sophisticated technologies. These developments have caused significant increases in fish prices.

In the past decades, many countries have turned to aquaculture to increase fish production. In 1992, the world production from aquaculture exceeded 17 million tons, about 32.2% of the total fisheries production (Csavas 1995). Thus, aquaculture has the potential to substantially meet the world demand for fishery products.

The success of aquaculture depends on: (1) complete control of reproduction and the life cycle, (2) excellent genetic background of the broodstock, (3) efficient detection and effective prevention of diseases, (4) thorough understanding of the optimal physiological, environmental, and nutritional conditions for growth and development, (5) sufficient supply of good quality water; and (6) innovative management techniques. By improving some of these factors, the aquaculture industry has already made impressive progress over the last several years. The application of molecular biology and biotechnology will further speed up the expansion of the industry. These applications include enhancing growth rates, controlling reproductive cycles, improving feed composition, producing new vaccines, and developing disease-resistant and hardier genetic stocks. Over the last several years, our laboratory and others have been searching for innovative strategies to increase fish production by applying the methods of contemporary molecular biology and biotechnology. In this paper, I will summarize results of our studies and those of many others to demonstrate the efficacy of modern biological techniques, including transgenic fish technology, in increasing the production from aquaculture.

Effect of Recombinant Fish Growth Hormone on Somatic Growth

Thanks to the rapid advances in recombinant DNA technology, complementary DNA (cDNA) and the genomic sequence of growth hormone (GH) have been isolated and characterized for several fish species in recent years (Gill et al. 1985, Agellon and Chen 1986, Gonzales-Villaseñor et al. 1986, Momota et al. 1988, Watahiki et al. 1989). Our laboratory has prepared biologically active recombinant GH by expressing rainbow trout (rt) GH1 cDNA in *E. coli* cells (Agellon et al. 1988). Since rainbow trout GH molecule is highly hydrophobic, the resulting polypeptide synthesized in *E. coli* cells forms insoluble inclusion bodies, which are inactive but can be easily recovered by differential centrifugation. The protein is dissolved in a 100 mM Tris buffer pH 9.0 with 8 M urea. Renaturation of the recombinant hormone is carried out by slowly diluting the solution to 4.5 M urea and the protein concentration below 0.5 mg/ml while stirring gently at 40°C for 24 hours. The biological activity of the resulting hormone preparation is assessed by its ability to stimulate the uptake of radioactive sulfate into gill cartilage *in vitro* and the accumulation of insulin-like growth factor (IGF)-I in the liver *in vivo*.

Agellon et al. (1988) showed in a series of studies that application of this recombinant hormone to yearling rainbow trout resulted in a significant growth enhancement. After treatment with the recombinant rtGH for four weeks at a dose of 1 µg/g body weight each week, the weight gain among the hormone-treated rainbow trout was two times greater than among the controls. Significant length gain was also evident in hormone-treated animals. When the same recombinant hormone was administered to small juvenile rainbow trout by immersing them in GH-containing solutions, the same growth-promoting effect was also observed (Table 1; also Leong and Chen, unpublished results). These results are in agreement with those reported by Sekine et al. (1985), Gill et al. (1985), and others (Schulte et al. 1986, Sato et al. 1988a, 1988b, Moriyama et al. 1990). However, it is important to mention that the growth enhancement effect of the biosynthetic hormone was markedly reduced when more than 2 µg/g was applied to the test animals (Agellon et al. 1988). These results suggest that when the total amount of GH exceeds the maximal threshold level, homeostasis is disturbed and growth is affected.

Table 1. Effect of recombinant growth hormone (GH) treatment on the growth of young juvenile rainbow trout. Values are mean±SE (n=15). Data from Agellon et al. (1988) with permission.

Treatment	Initial weight (g)	Final weight (g)	%Gain
Saline control	1.33±0.6	3.94±1.8	196
GH 50 µg/l	1.29±0.7	5.51±1.6	327
GH 500 µg/l	1.35±0.7	5.30±1.3	293

Groups of rainbow trout were subjected to osmotic shock in the presence or absence of recombinant GH. Weight was measured before and after 5 weeks of treatment. Final weights were not significantly different between the two GH treatments but both differed from the control.

Several years ago, Morse (1984) reported that bovine insulin and bovine GH enhanced the growth rate of California red abalone. Recently, Paynter and Chen (1991) found that dipping the spats or juveniles of the oyster *Crassostrea virginica* in solutions of recombinant rtGH polypeptide (10-100 nM) also resulted in significant increases in shell size, wet weight, and dry weight (Table 2). Oysters treated with recombinant rtGH, native bovine GH, or bovine insulin consumed more oxygen per unit time than controls. These findings suggest that recombinant fish GH can be used to enhance the growth rate of mollusks under intensive culture conditions. They further suggest that growth in mollusks may also be regulated by hormonal factors similar to mammalian GH and insulin-like growth factors (IGFs).

Table 2. Effect of recombinant rainbow trout growth hormone exogenously applied to oysters for 5 weeks. Values are means±SE. Data from Paynter and Chen (1991), with permission.

GH conc. (nM)	Initial shell height (mm)	Final shell height (mm)	Total weight (mg)	Shell weight (mg)	Dry weight (mg)
0	8.14±0.25	11.68±0.27	206±11	136±8	6.10±0.66
1	8.04±0.27	11.74±0.23	199±9	131±6	6.87±0.66
10	8.72±0.18	12.79±0.27	244±20	171±11	9.42±0.41
100	8.65±0.32	13.00±0.36	252±13	189±13	9.41±0.74

In order to realize the potential of recombinant GH to enhance the somatic growth rate of cultured fish, a series of studies has to be conducted. These studies include: (1) route of hormone delivery, (2) effective dose of hormone, (3) regimen of hormone administration, (4) nutritional requirement, (5) effect of environmental factors, (6) effect of chronic and acute GH treatment, and (7) effect of fish GH on human consumers.

Transgenic Fish Harboring Growth Hormone Gene

Although exogenous application of recombinant GH results in significant growth enhancement in fish, it may not be cost-effective. If new strains of fish producing elevated but optimal levels of GH can be produced, it would bypass many of the problems with exogenous GH treatment. Moreover, once these fish strains have been generated, they would be far more cost-effective than their ordinary counterparts because the fish would produce and deliver the hormone and transmit the enhanced growth characteristics to their offspring.

Gene transfer methodology

Animals into which a segment of foreign DNA has been introduced and stably integrated into the host genome are called 'transgenic'. Since Constantini and Lacy's (1981) transgenic mice, many other transgenic animals including livestock and fishes have been constructed successfully (Palmiter et al. 1982, Gordon and Ruddle 1985, Hammer et al. 1985, Ozato et al. 1986, Dunham et al. 1987, Pursel et al. 1989, Chen and Powers 1990, Chen et al. 1993). These animals play important roles both in basic research as well as in biotechnology application. Various methods have been used to deliver foreign DNA into somatic cells and germlines of mammals and other higher vertebrates. The methods include direct microinjection, retrovirus infection, electroporation, calcium phosphate precipitation, and particle-gun bombardment. Direct microinjection of DNA into the male pronuclei of the fertilized eggs has been the prevalent method.

The microinjection method has been used to deliver foreign genes into several fish species in recent years. These include goldfish (Zhu et al. 1985), medaka (Ozato et al. 1986, Lu et al. 1992), rainbow trout (Chourrout et al. 1986), salmon (Fletcher et al. 1988), tilapia (Brem et al. 1988), zebrafish (Stuart et al. 1988), common carp (Zhang et al. 1990, Chen et al. 1993), and catfish (Dunham et al. 1992, Powers et al. 1991). In general, gene transfer in fish by microinjection is carried out as follows. Eggs and sperm are collected into separate dry containers. Fertilization is initiated by adding water and sperm to eggs and stirring gently. Eggs are water-hardened for various periods of time and then rinsed. Microinjection is done within the first two hours after fertilization. The equipment consists of a dissecting stereomicroscope and two micromanipulators, one with a microneedle for injecting DNA into the embryos and the other with a micropipette for holding the embryos in position during the injection. Since the male pronuclei of the fish embryos studied to date are not visible, the foreign genes are usually injected into the egg cytoplasm and the amount of the DNA injected into each embryo is in the range of one million copies or higher. In zebrafish and medaka, natural spawning can be induced by adjusting photoperiod and water temperature and newly fertilized embryos can be readily collected for microinjection. Within the first two hours after fertilization, the micropyle of the embryo is still visible under the microscope. The DNA solution can be easily delivered into the embryos with a microneedle through the micropyle (Stuart et al. 1988, Lu et al. 1992).

Although the microinjection method is successful in transferring foreign DNA into fish embryos, it is very laborious and time-consuming. There is interest in developing convenient mass gene transfer technologies for use in fish transgenesis. Among the mass gene transfer methods are particle-gun bombardment, electroporation, and those mediated by retroviruses, liposomes, or sperm. Electroporation is the most effective means of transferring foreign genes into fish embryos. This method uses a series of short electrical pulses that change the membrane permeability and thereby permit the entry of DNA molecules into embryos. Lu et al. (1992)

showed that the rate of foreign gene integration in transgenic medaka produced by electroporation was 20% or higher. Powers et al. (1992) recently reported a much higher rate of gene transfer in common carp and channel catfish with the same electroporator. The rate of transgene integration in transgenic medaka produced by electroporation was only slightly higher than that of microinjection, but it takes much less time to produce large numbers of transgenic fish by electroporation.

Transgenic fish harboring growth hormone gene

Zhu et al. (1985) reported the first successful transfer into goldfish and loach of human GH gene fused to a mouse metallothionein gene promoter. According to Zhu (personal communication), the F₁ offspring of these transgenic fish grew twice as large as their non-transgenic siblings. Unfortunately, Zhu and his colleagues failed to present compelling evidence for integration and expression of the foreign genes in their transgenic fish. Recently, our laboratory as well as many other laboratories throughout the world have successfully confirmed Zhu's work by demonstrating that human or fish GH and many other genes can be readily transferred into embryos of a number of fish species and integrated into the genome of the host fish (for review, see Chen and Powers 1990). While several groups have demonstrated expression of foreign genes in transgenic fish, only Zhang et al. (1990), Du et al. (1992) and Lu et al. (1992) have documented that a foreign GH gene could be: (a) transferred to the target fish species, (b) integrated into the fish genome, and (c) genetically transmitted to subsequent generations. Furthermore, the expression of the foreign GH gene may result in enhancement of growth rates of both P₁ and F₁ generations of transgenic fish (Zhang et al. 1990, Lu et al. 1992).

In gene transfer studies on common carp and channel catfish, about 10⁶ molecules of a linearized recombinant plasmid containing the long terminal repeat (LTR) sequence of avian Rous sarcoma virus (RSV) and the rainbow trout GH1 or GH2 cDNA were injected into the cytoplasm of one-, two- and four-cell embryos (Zhang et al. 1990, Chen et al. 1990, 1993, Powers et al. 1991, Dunham et al. 1992). Genomic DNA samples extracted from the pectoral fins of presumptive transgenic fish were then analyzed for the presence of RSVLTR-rtGH1-cDNA by PCR amplification, followed by Southern blot hybridization of the amplified DNA samples. About 35% of the injected carp embryos survived at hatching and about 10% of the survivors had integrated the RSVLTR-rtGH1-cDNA sequence (Zhu et al. 1985, Chen and Powers 1990, Chen et al. 1990, Zhang et al. 1990, Du et al. 1992, Lu et al. 1992). Southern-blot analysis of genomic DNA from several transgenic carps revealed that a single copy of the RSVLTR-rtGH1-cDNA sequence was integrated at multiple chromosomal sites (Zhang et al. 1990).

When an RSVLTR-csGH-cDNA construct was injected into catfish embryos, about 10% of the fish also turned out transgenic (Powers et al. 1991, Dunham et al. 1992). In microinjected medaka, 20-30% of the hatched individuals integrated the foreign gene, suggesting that DNA microinjected via the micropyle may have better access to the nucleus situated right beneath it (Lu et al. 1992).

Inheritance and Expression of Foreign Growth Hormone Gene in Transgenic Fish

The patterns of inheritance of RSVLTR-rtGH1 cDNA in transgenic common carp were studied by fertilizing eggs collected from non-transgenic females or P₁ transgenic females with sperm samples of several sexually mature P₁ male transgenic fish (Chen et al. 1993). The DNA samples extracted from the resulting F₁ progeny were assayed for the presence of RSVLTR-rtGH1 - cDNA sequence by PCR amplification and dot-blot hybridization. The percentage of transgenic progeny resulting from nine matings were 0, 32, 26, 100 (4 progeny only), 25, 17, 31, 30, and 23. If each of the transgenic parents in these nine matings carried at least one copy of the transgene in the gamete cell, about 50-75% transgenic progeny would have been expected in each pairing. Of these nine matings, two sib lots, both control x P₁, gave transgenic progeny as many as or more than expected. The other matings gave less than expected numbers of transgenic progeny. These results indicate that although most of these P₁ transgenic fish had RSVLTR-rtGH1 cDNA in their germline, they might be mosaics. Similar patterns of mosaicism in the germline of P₁ transgenic fish have been observed in many fish species studied to date (Ozato et al. 1986, Dunham et al. 1987, 1992, Stuart et al. 1988, Zhang et al. 1990, Lu et al. 1992).

If the transgene carries a functional promoter, some of the transgenic individuals are expected to express the transgene activity. Many of the P₁ and F₁ transgenic common carp produced rtGH at levels that varied about 10-fold (Zhang et al. 1990, Chen et al. 1993). Chen et al. (1993) recently detected rtGH mRNA in the F₁ transgenic carp with an assay involving reverse transcription, PCR amplification, and RNA dot-blot hybridization. Different levels of rtGH mRNA were detected in liver, eyes, gonads, intestine and muscle of the F₁ transgenic individuals.

Growth Performance of Fish Transgenic for Growth Hormone

Since the site of transgene integration differs among the individuals in any population of P₁ transgenic fish, they should be considered as totally different transgenic individuals and cannot be directly compared for growth performance among themselves. Instead, the growth studies should be conducted in F₁ transgenic and non-transgenic siblings derived from the same family. Chen et al. (1993) evaluated the growth of F₁ transgenic carp in seven families. In these experiments, transgenic and non-transgenic full siblings were spawned, hatched, and reared communally under the same environment. Results showed that growth of F₁ transgenic individuals in response to rtGH1 cDNA varied widely. The seven trials showed that transgenics grew 20, 40, 59, and 22% faster, or 27, 15 and 2% slower than non-transgenic full siblings.

In three of the four families where F₁ transgenics grew faster than their non-transgenic full siblings, the highest and lowest body weights of the transgenics were larger than those of the non-transgenics. In the fourth family, the minimum but not the maximum body weight of the transgenics was larger than that of the non-transgenics. In two of the three transgenic families in which the transgenic siblings grew slower than the non-transgenics, the lowest and highest body weights of the transgenics were less than those of the non-transgenics. In the third family, however, one of the F₁ transgenics was the largest fish in the family. Since the response of the transgenic fish to the insertion of the RSVLTR-rtGH1 cDNA appears to be variable, as a result of random integration of the transgene, the fastest growing genotype will likely be developed by utilizing a combination of family selection and mass selection of transgenic individuals after the

insertion of the foreign gene. Among transgenic medaka carrying chicken b-actin gene promoter human GH gene construct, the F₁ transgenics grew significantly faster than the non-transgenic siblings (Lu et al. 1992).

In an effort to study the biological effect of elevated levels of IGF-I on somatic growth, transgenic medaka harboring trout IGF cDNA driven by carp β -actin gene promoter have been produced in our laboratory. Both P₁ and F₁ IGF-I transgenic medaka hatched two days earlier than their non-transgenic siblings. The P₁ transgenics also grew faster than the non-transgenics.

General Conclusion and Prospective

Transgenic fish technology has great potential in the aquaculture industry. By introducing desirable genetic traits into fishes, mollusks, and crustaceans, superior transgenic strains can be produced for aquaculture. These traits include faster growth rates, improved food conversion efficiency, resistance to some known diseases, tolerance to low oxygen concentrations, and tolerance to extreme temperatures. Our laboratory and those of others have shown that transfer, expression and inheritance of fish growth hormone transgenes can be achieved in several fish species and that the resulting transgenics grow substantially faster than their non-transgenic siblings. This is a vivid example of the potential application of the gene transfer technology to aquaculture.

However, to realize the full potential of the transgenic fish technology in aquaculture or other biotechnological applications, several important scientific breakthroughs are required. These include: (1) more efficient technologies for mass gene transfer, (2) targeted gene transfer technologies such as embryonic stem cell gene transfer or ribozyme gene inactivation, (3) suitable promoters to direct the expression of transgenes at optimal levels during the desired developmental stages, (4) identified genes of desirable traits for aquaculture and other applications, (5) information on the physiological, nutritional, immunological and environmental factors that maximize the performance of the transgenics, and (6) safety and environmental impacts of transgenic fish. Once these problems are resolved, the commercial application of the transgenic fish technology will be readily attained.

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