Transgenic Fish Technology and Its Application in Fish Production

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I. INTRODUCTION

Organisms into which heterologous DNA (transgene) has been artificially introduced and integrated in their genomes are called *transgenics.* Since th,e early 1980s, transgenic plants [1], nematodes [2], fruit flies [3], sea urchins [4,5], frogs [6], laboratory mice [7,8], and farm animals, such as cows, pigs, and sheep [9], have been successfully produced. In plants, transgenes are introduced into cells by infection with *Agrobacterium tumefadens* or by physical means, such as ballistic bombardment. In animals, transgenes are introduced into the pronuclei of fertilized eggs by injection, and the injected embryos are incubated in vitro or implanted into the uterus of a pseudopregnant female for subsequent development. In these studies, multiple copies of transgenes are integrated at random locations in the genome of the transgenic individuals. If the transgenes are linked with functional promoters, expression of transgenes as well as display of change in phenotype is expected in some of the transgenic individuals. Furthermore, the transgenes in many transgenic individuals are also transmitted through the germline to subsequent generations. These transgenic animals play important roles in basic research as well as applied biotechnology. In basic research, transgenic an1mals provide excellent models for studying molecular genetics of early vertebrate development, actions of oncogenes, and the biological functions of hormones at different stages of development. In applied biotechnology, transgenic animals offer unique opportunities for producing animal models for biomedical research, improving the genetic background of broodstock for animal husbandry or aquaculture, and designing bioreactors for producing valuable proteins for pharmaceutical or industrial purposes.

Since 1985, a wide of transgenic fish species have been produced [10-12] by microinjecting or electroporating homologous or heterologous transgenes into newly fertilized or unfertilized eggs. Several important steps are routinely taken to produce a desired transgenic fissh. First, an appropriate fish species must be chosen, depending on the nature of the studies and the availability of the fish-holding facility. Second, a specific gene construct must be prepared. The

gene construct contains the structural gene encoding a gene product of interest and the regulatory elements that regulate the expression of the gene in a temporal,. spatial, and developmental manner. Third, the gene construct has to be introduced into the developing embryos for the transgene to be integrated stably onto the genome of every cell. Fourth, because not all instances of gene transfer are efficient, a screening method must be adopted for identifying transgenic individuals.

Although remarkable progress has been made in producing transgenic fish by gene transfer technology, a critical review of the published results has shown that most of the research effort has been devoted to confirming the phenomenon of foreign gene transfer into various fish species. Very few attempts have been made to explore the application of transgenic fish technology in basic as well as applied research. Recently, we have devoted a substantial amount of our research effort to this problem with promising results.

The worldwide harvest of fishery products traditionally depends on natural populations of finfish, shellfish, and crustaceans in fresh and marine water. In recent years, however, the total annual worldwide harvest of roo products has approached, or even surpassed,. the maximal potential level of about 150 million metric tons (as calculated by the US Department of Commerce and the US National Oceanic and Atmospheric Administration). To cope with the worldwide demand of fish products and the escalating increase in fish price, many countries have turned to aquaculture for increasing production of fish products. In 1985, the world production of finfish, shellfish, and macroalgae by aquaculture reached 10.6 million metric tons, or approximately 12.3% of the worldwide catch generated by international fishery efforts. Although aquaculture clearly has the potential for increasing worldwide fish production, innovative strategies are needed to improve efficiency. What can transgenic technology offer?

Success in aquaculture depends on six factors: (1) complete control of the reproductive cycle of the fish species in culture; (2) excellent genetic background of the broodstock; (3) efficient prevention and detection of disease infection; (4) thorough understanding of the optimal physiological, environmental, and nutritional conditions for growth and development; (5) sufficient supply of excellent quality water; and (6) application of innovative maoagement techniques. By improving these factors, the aquaculture industry has developed to a remarkable extent during the last decade. To sustain this growth, however, newly developed technologies in molecular biology and transgenesis will have to be increasingly applied by the aquaculture industry. These technologies can be employed to enhance growth rates, control reproductive cycles, improve feed compositions, produce new vaccines, and develop disease-resistant and hardier genetic stocks. In the last several years, we have been searching for strategies to increase fish production by manipulating fish growth hormone and growth factor genes. In this chapter, we will review results from our laboratory and those from others to demonstrate this point.

II. METHODS OF TRANSGENIC FISH PRODUCTION

A. Transgene Constructs

A transgene used in producing transgenic fish for basic research or application should be a recombinant gene construct that produces a gene product at an appropriate level in the desired tissue(s) at the desired time(s). Therefore, the prototype of a transgene is usually constructed in a plasmid to contain an appropriate promoter-enhancer element and a structural gene sequence.

Depending on the purpose of gene transfer studies. transgenes are grouped into three main types: (I) *gain-oj-function.* (2) *reporter function.* and (3) *loss-of-function.* The *gain-of-function*

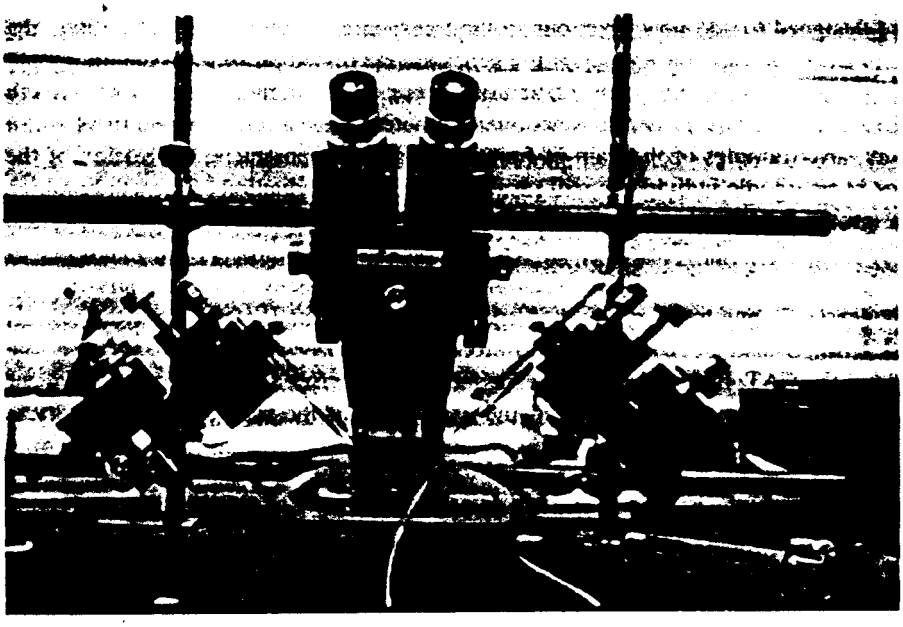
transgenes are designed to add new functions to the transgenic individuals or to facilitate the identification of the transgenic individuals if the genes are expressed properly in the transgenic individuals. Transgenes containing the structural genes of mammalian and fish growth hormones (GH, or their cDNAs) fused to functional promoters, such as chicken and fish β-actin gene promoters, are examples of the gain-of-function transgene constructs. Expression of the GH transgenes in transgenic individuals will result in increased production of growth hormone and ultimate growth enhancement [13–16]. Bacterial chloramphenicol acetyl transferase (CAT), β-galactosidase, or luciferase genes fused to functional promoters are examples of transgenes with *reporter function*. These *reporter function* transgenes are commonly used to identify the success of gene transfer effort. A more important function of a reporter gene is used to identify and measure the strength of a promoter–enhancer element. In this case, the structural gene of the CAT, β-galactosidase, or luciferase gene is fused to a promoter–enhancer element in question. Following gene transfer, the expression of the reporter gene activity is used to determine the transcriptional regulatory sequence of a gene or the strength of a promoter [17].

The *loss-of-function* transgenes are constructed for interfering with the expression of host genes. These genes might encode an antisense RNA to interfere with the posttranscriptional process or translation of endogenous mRNAs. Alternatively, these genes might encode a catalytic RNA (a ribozyme) that can cleave specific mRNAs and, thereby, cancel the production of the normal gene product [18]. Although these genes have not yet been introduced into a fish model, they could be potentially employed to produce disease-resistant transgenic broodstocks for aquaculture or transgenic model fish defective in a particular gene product for basic research.

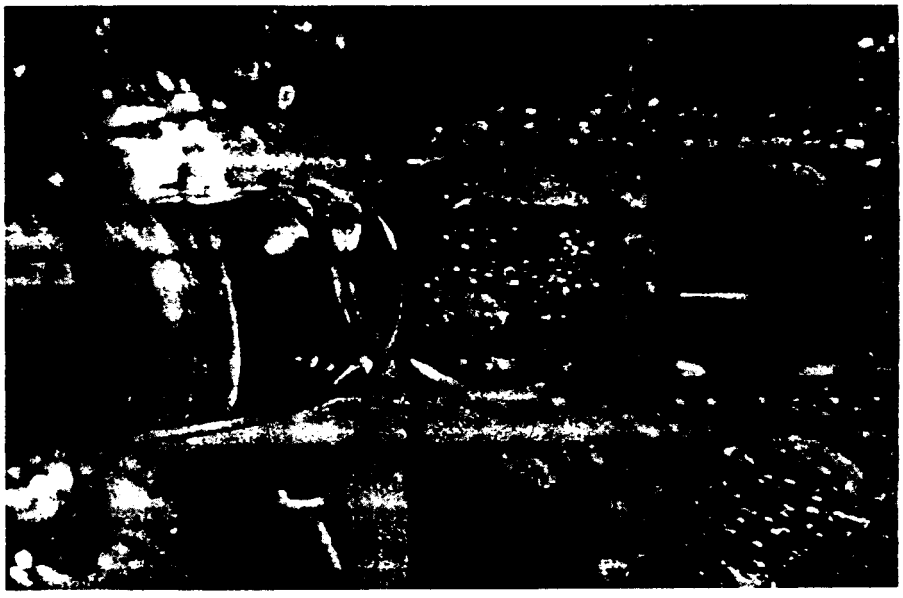
B. Selection of Fish Species

Gene-transfer studies have been conducted in several different fish species, including channel catfish, common carp, goldfish, Japanese medaka, loach, northern pike, rainbow trout, salmon, tilapia, walleye, and zebrafish [for review: 10,12]. Depending on the purpose of the transgenic fish studies, the embryos of some fish species are more suited for gene transfer studies than the others. For example, Japanese medaka (*Oryzias latipes*) and zebrafish (*Brachydanio rerio*) have short life cycles (3 months from hatching to mature adults), produce hundreds of eggs on a regular basis without exhibiting a seasonal breeding cycle, and can be maintained easily in the laboratory for 2–3 years. Eggs from these two fish species are relatively large (diameter, 0.7–1.5 mm) and possess very thin, semitransparent chorions, a feature that permits easy microinjection of DNA into fertilized eggs. Furthermore, inbred lines and various morphological mutants of both fish species are available. Therefore, these fish species are suitable candidates for conducting gene transfer experiments for (1) studying developmental regulation of gene expression and gene action; (2) identifying regulatory elements that regulate the expression of a gene; (3) measuring the activities of promoters; and (4) producing transgenic models for environmental toxicology. However, a major drawback of these two fish species is their small body size that makes them unsuitable for some endocrinological or biochemical analyses.

Channel catfish, common carp, rainbow trout and salmon, are commonly used large–body-sized model fish species in transgenic fish studies. Because the endocrinology, reproductive biology, and basic physiology of these fish species have been well worked out, they are well suited for conducting studies on comparative endocrinology and aquaculture applications. However, the long maturation time of these fish species and a single spawning cycle per year will limit research progress in the field.



(A)

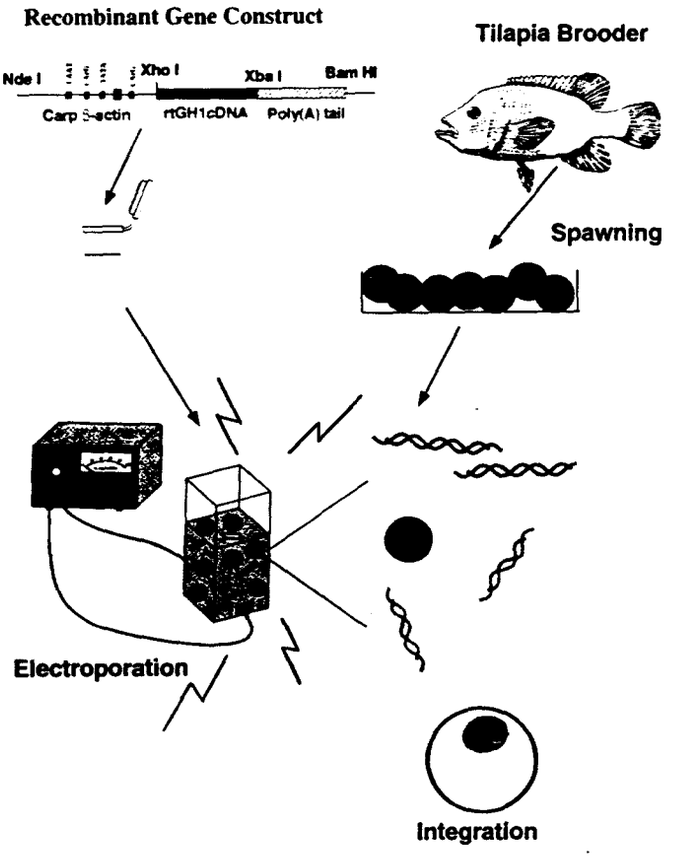


(B)

Fig. 1 Gene transfer apparatus: (A) Microinjection apparatus; (B) a close up view of medaka egg under the microinjection apparatus; (C) scheme of gene transfer by electroporation.

Loach, killifish, goldfish, and tilapia are the third group of model fish species suitable for conducting gene transfer studies because their body sizes are large enough for most biochemical and endocrinological studies. Furthermore, shorter maturation times, as compared with catfish, rainbow trout, or salmon, allow easier manipulation of transgenic progeny. Unfortu-

(C)



nately, the lack of a well-defined genetic background and asynchronous reproductive behavior of these fish species render them less amenable to gene transfer studies.

C. Methods of Gene Transfer

Techniques, such as calcium phosphate precipitation, direct microinjection, lipofection, retroviral infection, electroporation, and particle gun bombardment have been used to introduce foreign DNA into animal cells, plant cells, and germ-lines of mammals and other vertebrates. Among these methods, direct microinjection and electroporation of DNA into newly fertilized eggs have proved to be the most reliable methods of gene transfer in fish systems.

1. Microinjection of Eggs or Embryos

Microinjection of foreign DNA into newly fertilized eggs was first developed for the production of transgenic mice in the early 1980s. Since 1985, this technique has been adopted for introducing transgenes into Atlantic salmon, common carp, catfish, goldfish, loach, medaka, rainbow trout, tilapia, and zebrafish [10,11 for review]. The gene constructs that were used in these studies include human or rat growth hormone (GH) gene, rainbow trout or salmon GH cDNA, chicken δ-crystallin protein gene, winter flounder antifreeze protein gene, *Escherichia coli* β-galactosidase gene, and *E. coli* hygromycine-resistance gene [10,11]. In general, transfer

of foreign DNA into fish by direct microinjection is conducted as follows. Eggs and sperm are collected in separate, dry containers. Fertilization is initiated by adding water and sperm to the eggs, with gentle stirring to enhance fertilization. Fertilized eggs are then microinjected within the first few hours after fertilization. The injection apparatus consists of a dissecting stereomicroscope and two micromanipulators, one with a glass microneedle for delivering transgenes and the other with a micropipette for holding fish embryos in place (Fig. 1a). Routinely, about 10^6–10^8 molecules of a linearized transgene (with or without plasmid DNA) in about 20 nL is injected into the egg cytoplasm. Following injection, the embryos are incubated in water until hatching. Because natural spawning in zebrafish or medaka can be induced by adjusting the photoperiod and water temperature, precisely staged newly fertilized eggs can be collected from the aquaria for microinjection. If the medaka eggs are maintained at 4°C immediately after fertilization, the micropyle on the fertilized eggs will remain visible for at least 2 hs. The DNA solution can be easily delivered into the embryos by injection through this opening.

Depending on the fish species, the survival rate of injected fish embryos ranges from 35 to 80% while the rate of DNA integration ranges from 10 to 70% in the survivors (Table 1) [10,11]. The tough chorions of the fertilized eggs in some fish species (e.g., rainbow trout and Atlantic salmon) can frequently make insertion of glass needles difficult. This difficulty can be overcome by any one of the following methods: (1) inserting the injection needles through the micropyle, (2) making an opening on the egg chorions by microsurgery, (3) removing the chorion by mechanical or enzymatic means, (4) reducing chorion hardening by initiating fertilization in a solution containing 1 mM glutathione, or (5) injecting the unfertilized eggs directly.

2. *Electroporation*

Electroporation is a successful method for transferring foreign DNA into bacteria, yeast, and plant and animal cells in culture. This method has become popular for transferring transgenes into fish embryos in the past 3 years [15,63]. Electroporation uses a series of short electrical pulses to permeate cell membranes, thereby permitting the entry of DNA molecules into embryos. The patterns of electrical pulses can be emitted in a single pulse of exponential decay form (i.e., exponential decay generator) or high-frequencies multiple peaks of square waves (i.e., square-wave generator, see Fig. 1b). Studies conducted in our laboratory [15,63] and

Table 1 Transfer of Foreign DNA into Medaka Embryos by Different Gene Transfer Methods

		Electroporation		Pantropic retroviral vector	
Microinjection[a]		I[b]	II[c]	Electroporation[d]	Incubation[e]
Viability (%) (at hatching)	50	70	90	50	70
Integration[f] rate (%)	20	15	25	50	70
Transgene expression	Yes	Yes	Yes	Yes	Yes
Efficiency (eggs per minute)	1–2	200	200	200	200

[a]Injecting is carried out by micropyle before blastodisk formation.

[b]Exponential decay impulse mode.

[c]Square wave impulse mode.

[d]Electroporation with square wave mode at 3.5 Kv.

[e]Fertilized eggs are exposed to a mixture of medaka-hatching enzyme and pancreatin for 2 h. The dechorionated embryos are incubated with the pantropic pseudotyped retrovirus overnight at room temperature.

[f]Integration rate is calculated from the surviving embryos after gene transfer.

Source: Ref. 63.

those of others [19] have shown that the rate of DNA integration in electroporated embryos is one the order of 20% or higher in the survivors (see Table 1). Although the overall rate of DNA integration in transgenic fish produced by electroporation may be equal to or slightly higher than that of microinjection, the actual amount of time required for handling a large number of embryos by electroporation is orders of magnitude less than the time required for microinjection. Recently, several research groups have also reported successful transfer of foreign DNA into fish by electroporating sperm instead of embryos [20,21]. Electroporation, therefore, is considered as an efficient and versatile massive gene transfer technology.

3. *Transfer of Transgenes by Infection with Pantropic Retroviral Vectors*

Although transgenes can be reproducibly introduced into various fish species by microinjection of electroporation, the resulting P_1 transgenic individuals possess mosaic germlines as a result of delayed transgene integration. Furthermore, these two gene transfer methods are not effective or successful in producing transgenics in marine fish and invertebrates. Recently, a new gene transfer vector, a defective pantropic retroviral vector, has been developed [22]. This vector contains the long terminal repeat (LTR) sequence of Moloney murine leukemia virus (MoMLV) and transgenes packaged in a viral envelop with the G protein of vesicular stomatitis virus (VSV). Because the entry of VSV into cells is mediated by interaction of the VSV G protein with a phospholipid component of the cell, this pseudotyped retroviral vector has a very broad host range and is able to transfer transgenes into many different cell types. Using the pantropic pseudotyped defective retrovirus as a gene transfer vector, transgene containing neo^R or β-galactosidase has been introduced into zebrafish [23] and medaka [24] (see Table 1). Recently, the feasibility of using a pantropic pseudotyped retroviral vector for introducing genes into marine invertebrates has been tested in dwarf surf clams and the results have shown that transgenes can be readily transferred into clams at high efficiency [61].

III. CHARACTERIZATION OF TRANSGENIC FISH

A. Identification of Transgenic Fish

The most time-consuming step in producing transgenic fish is the identification of transgenic individuals. Traditionally, the presence of transgene in presumptive transgenic individuals is determined by dot-blot or Southern-blot hybridization of genomic DNA isolated from the test individuals. These methods involve isolation of genomic DNA from tissues of presumptive transgenic individuals, digestion of DNA with restriction enzymes, and dot-blot. Southern-blot hybridization of the digested DNA products. Although this method is expensive, laborious, and insensitive, it offers a definitive answer whether a transgene has been integrated into the host genome. Furthermore, it also reveals the pattern of transgene integration if appropriate restriction enzymes are employed in the Southern-blot analysis. To handle a large number of samples efficiently and economically, a polymerase chain reaction (PCR)-based assay has been adopted [15,16]. The strategy of the assay is outlined in Figure 2. It involves isolation of genomic DNA from a very small piece of fin tissue, PCR amplification of the transgene sequence, and Southern-blot analysis of the amplified products. Although this method does not differentiate whether the transgene is integrated in the host genome or remains as an extrachromosomal unit, it serves as a rapid and sensitive screening method for identifying individuals that contain the transgene at the time of analysis. In our laboratory, we use this method as a preliminary screen for transgenic individuals from thousands of presumptive transgenic fish.

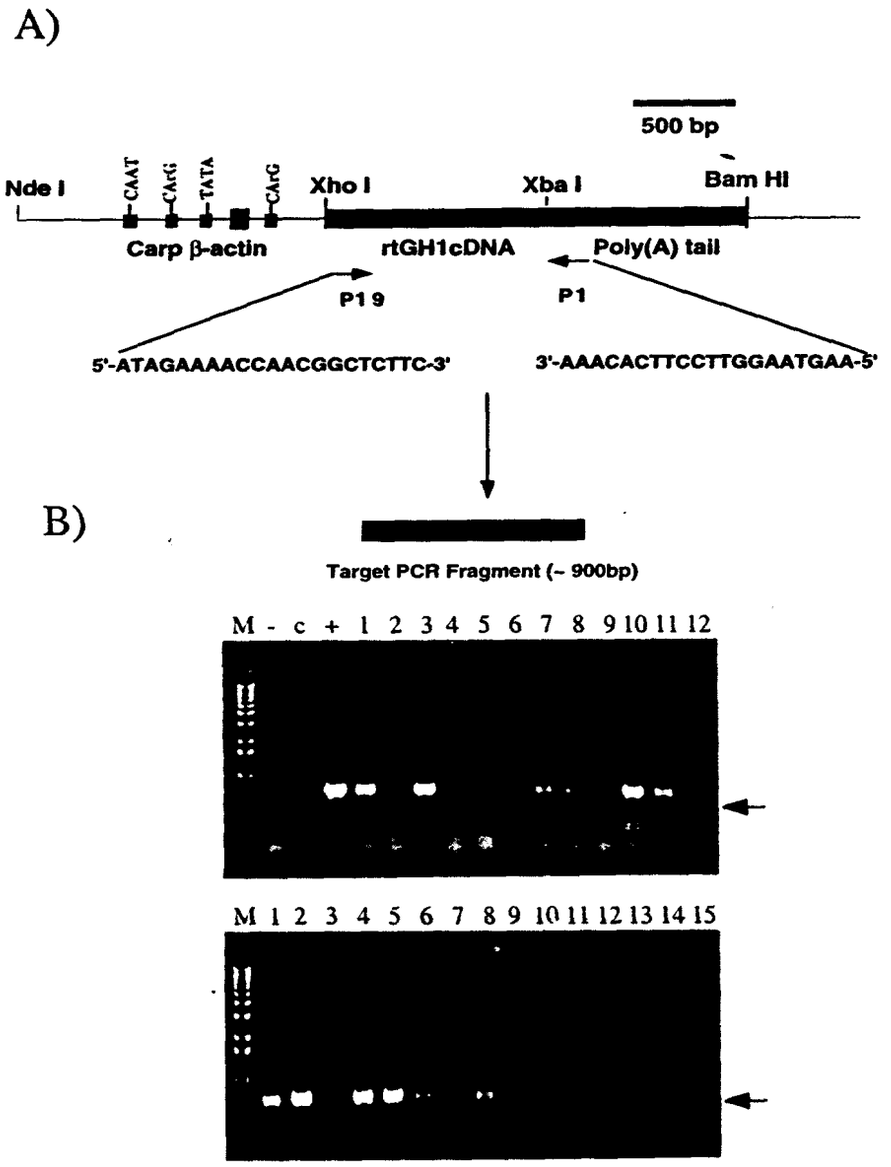


Fig. 2 Strategy for identifying the presence of transgenes in the presumptive transgenic fish by PCR and Southern blot hybridization. DNA samples were isolated from pectoral fin tissues of presumptive transgenic fish and subjected to PCR amplification. The amplified products were analyzed by electrophoresis on agarose gels and Southern blot hybridization. (A) Strategy of PCR amplification; (B) Southern-blot analysis of PCR-amplified products: lane M, molecular weight marker; lane –, PCR reaction without template; lane C, DNA sample from a nontransgenic fish; lane +, transgene construct; lanes 1–12 (upper panel) and 1–15 (lower panel), DNA samples from presumptive transgenic fish. Arrows indicate the size of amplified products.

B. Expression of Transgenes

An important aspect of gene transfer studies is the detection of transgene expression. Depending on the levels of transgene products in the transgenic individuals, the following methods are

commonly employed: (1) RNA Northern or dot-blot hybridization; (2) RNase protection assay; (3) reverse transcription–polymerase chain reaction (RT-PCR); (4) immunoblotting assay; and (5) other biochemical assays for determining the presence of the transgene protein products. Among these assays, RT-PCR is the most sensitive method and only requires a small amount of sample. The strategy of this assay is summarized in Figure 3 [16]. Briefly, it involves the isolation of total RNA from a small piece of tissue, synthesis of single-stranded cDNA by reverse transcription, and PCR amplification of the transgene cDNA by employing a pair of oligonucleotide primers specific to the transgene product. The resulting products are resolved on agarose gels and analyzed by Southern-blot hybridization using a radiolabeled transgene as a hybridization probe. Transgene expression can also be quantified by a quantitative RT-PCR method [25]. Although this method is rapid and sensitive, it can be easily confused by transgene contamination in the reaction unless extra precaution is taken in setting up the reactions.

c. Patterns of Transgene Integration

Studies conducted in many fish species have shown that following injection of linear or circular transgene constructs into fish embryos, the transgenes are maintained as extrachromosomal

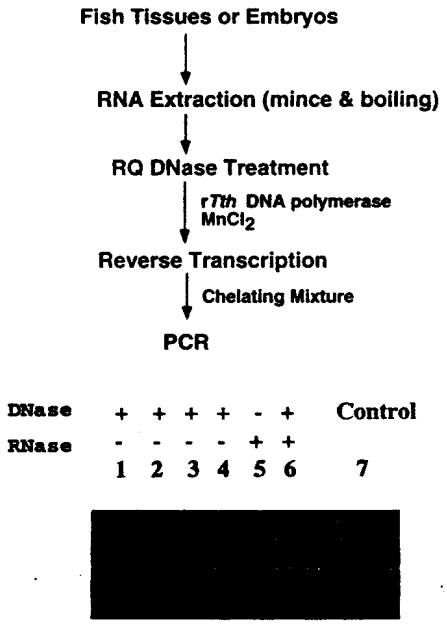


Fig. 3 Strategy of detecting rtGH transgene expression by reverse transcription (RT)–PCR assay: (A) Strategy of RT–PCR; (B) Detection of of rtGH transgene expression in transgenic medaka by RT–PCR. Total RNA was isolated from whole fish of F_1 transgenic and controls fish following the acid guanidinium thiocyanate–phenol–chloroform method. Single-stranded cDNA was prepared by reverse transcription from each total RNA that has been pretreated with RNase-free DNase to remove any contaminating genomic DNA, and used as a template for PCR amplification of rtGH with synthetic oligonucleotides as amplification primers. The resulting products were analyzed by Southern blot analysis, using radio-labeled rtGH cDNA as a hybridization probe. Lanes 1–4, different F_1 transgenic fish; lane 5, PCR of RNA from lane 1 without pretreatment with RNase-free DNase and reverse transcription; lane 6, PCR of RNA from lane 1 with DNase and RNase treatment followed by reverse transcription.

Table 2 Effect of GH Treatment on the Growth of Rainbow Trout Fry

Treatment	Weight (g)		
	Initial	Final	% Gain
Saline control	$1.33 \pm 0.6^{**}$	$3.94 \pm 1.8^{*}$	196
GH (50 μg/L)	$1.29 \pm 0.7^{**}$	$5.51 \pm 1.6^{***}$	327
GH (500 μg/L)	$1.35 \pm 0.7^{**}$	$5.30 \pm 1.3^{***}$	293

Values presented as mean ± SD. Groups of rainbow trout fry ($n = 15$) were subjected to osmotic shock in the presence of absence of GH. Weight was measured before and 5 weeks post-treatment. Differences between mean weights of GH-treated and control groups were evaluated using Student's t-test ($\alpha = 0.01$).

*Significantly different from the GH-treated groups ($P < 0.01$).

**No significant difference between these groups.

***No significant difference between these two treatments.

Source: From Ref. 45.

units through many rounds of DNA replication in the early phase of the embryonic development. At later stages of embryonic development, some of the transgenes are randomly integrated into the host genome, whereas others are degraded, resulting in the production of mosaic transgenic fish [for review, see Ref. 12]. In many fish species studied to date, multiple copies of transgenes were integrated in a head-to-head, head-to-tail, or tail-to-tail form, except in transgenic common carp and channel catfish where single copies of transgenes were integrated at multiple sites on the host chromosomes [13].

D. Inheritance of Transgenes

Stable integration of the transgenes is an absolute requirement for continuous vertical transmission to subsequent generations and establishment of a transgenic fish line. To determine whether the transgene is transmitted to the subsequent generation, P_1 transgenic individuals are mated to non-transgenic individuals and the progeny are assayed for the presence of transgenes by the PCR assay method described earlier [15,16]. Although the transgene may persist into the F_1 generation of transgenic zebrafish as extrachromosomal DNA [26], detailed analysis of the rate of transmission of transgenes to the F_1 and F_2 generations in many transgenic fish species indicates true and stable incorporation of the constructs into the host genome [for review see refs. 10,12]. If the entire germline of the P_1 transgenic fish is transformed with at least one copy of the transgene per haploid genome, at least 50% of the F_1 transgenic progeny will be expected in a backcross involving a P_1 transgenic with a nontransgenic control. In many of such crosses, only about 20% of the progeny are transgenic [13,15,16,26–28]. When the F_1 transgenic is backcrossed with a nontransgenic control, however, at least 50% of the F_2 progeny are transgenics (Table 2). These results clearly suggest that the germlines of the P_1 transgenic fish are mosaic as a result of delayed transgene integration during embryonic development.

IV. APPLICATION OF TRANSGENIC FISH IN BIOTECHNOLOGY

A. Biosynthetic Growth Hormone and Growth Enhancement

In recent years, growth hormone (GH) cDNAs and genomic DNAs have been isolated and characterized for several fish species [for review see Ref. 44]. Expression of rainbow trout or

striped bass GH cDNA in *E. coli* cells results in production of large quantity of recombinant GH polypeptide [45,46]. Because the GH polypeptide is highly hydrophobic and contains four cysteine residues, the newly synthesized recombinant GH polypeptide forms insoluble inclusion bodies in *E. coli* cells, rendering the hormone inactive. In a attempt to regain the biological activity of the recombinant hormone, Cheng et al. [46] developed a procedure for renaturing the protein. It involves dissolving the insoluble recombinant hormone in a buffer containing 8-M urea and renaturing the polypeptide by slowly removing the urea from the protein solution. The biological activity of the renatured protein was then assessed by an in vitro sulfation assay [47].

In a series of in vitro studies, Agellon et al. [45] showed that application of the recombinant trout GH to yearling rainbow trout resulted in a significant growth enhancement. After treatment of yearling rainbow trout with the recombinant GH for 4 weeks at a dose of 1 μg/g body weight per week, the weight gain among the individuals of the hormone-treated group was two times greater than that of the controls (Fig.4). Significant length gain was also evident in hormone-treated animals. When the same recombinant hormone was administered rainbow trout fry (see Table 2) or small juveniles by immersing the fish in a GH-containing solution, the same growth-promoting effect was also observed [45; Leong and Chen, unpublished results]. These results are in agreement with those reported by others [48–52]. However, importantly the growth enhancement effect of the biosynthetic hormone was markedly reduced when more than 2 μg/g body weight of the hormone was applied to the test animals [45]. Recently, Paynter and Chen [53] have observed that administration of recombinant trout GH to spats of juvenile oysters (*Crassostrea virginica*), by the "dipping method", referred to earlier also resulted in significant increases in shell height, shell weight, wet weight, and dry weight (Table 3). Furthermore, they also showed that oysters treated with recombinant trout GH, native bovine GH,

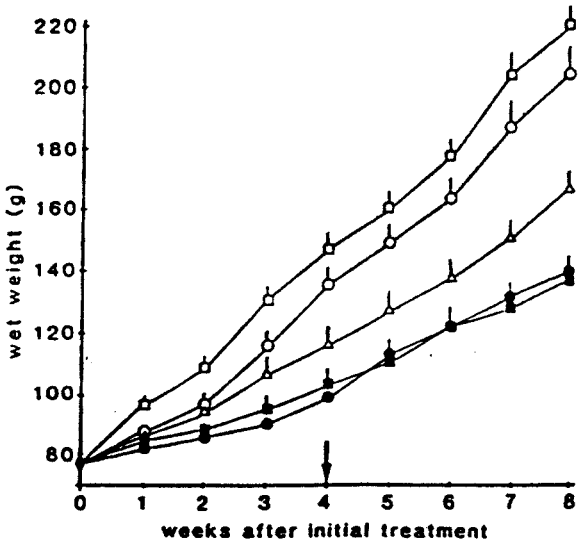


Fig. 4 Effect of recombinant trout GH on growth of yearling rainbow trout: Groups of yearling rainbow trout received intraperitoneal injection of recombinant GH or control extract for 5 weeks. Wet weights of GH-treated and control fish are shown (mean ± SE). Open symbols, GH-treated fish: ○, 0.2 μg/g body weight; □, 1.0 μg/g body weight; △, 2 μg/g body weight. Closed symbols, control fish: ●, mock-treated fish; ■, untreated fish. The arrow indicates the time of the last hormone treatment. (From Ref. 45.)

Table 3 Effect of Exogenously Applied recombinant Rainbow Trout Growth Hormone on Oyster Growth

Treatment	Initial ht (mm)	Final ht (mm)	Total wt (mg)	Shell wt (mg)	Dry wt (mg)
Control	8.14 (0.25)	11.68 (0.27)	206 (11)	136 (8)	6.10 (0.66)
10^{-9} M	8.04 (0.27)	11.74 (0.23)	199 (9)	131 (6)	6.87 (0.66)
10^{-8} M	8.72 (0.18)	12.79 (0.27)[ab]	244 (20)	171 (11)[b]	9.42 (0.41)[ab]
10^{-7} M	8.65 (0.32)	13.00 (0.36)[ab]	252 (13)[b]	189 (13)[ab]	9.41 (0.74)[ab]

[a]Significantly larger than the control group (*t*-test; $P < 0.05$).

[b]Significantly larger than 10^{-9} M treatment group (*t*-test; $P < 0.05$).

Initial ht represents mean size at the beginning of the experiment and final ht, total wt, shell wt, and dry wt are mean values determined after the 5-week treatment cycle was concluded. Height (ht) was measured in millimeters from the umbo to the ventral shell margin; weight (wt) was measured in milligrams. Standard errors of the mean (SEM) are in parentheses.

Source: Ref. 53.

or bovine insulin consumed more oxygen per unit time than controls. The results summarized in the foregoing clearly suggest that exogenous application of recombinant fish growth hormone can enhance the somatic growth of finfish and shellfish.

B. GH and IGF-I Transgenic Fish

Although exogenous application of biosynthetic GH results in a significant growth enhancement in fish, it may not be cost-effective because of the following reasons: (1) high cost in producing large-scale purified biosynthetic GH; (2) treating individual fish with the hormone is labor-intensive; (3) the optimal hormone dosage for each fish species is difficult to determine; and (4) GH uptake into fish from an exogenous source is inefficient. If new strains of fish producing elevated, but optimal, levels of GH can be produce, it would bypass all of the problems associated with exogenous GH treatment. Moreover, once these fish strains have been generated, they would be far more cost-effective than their ordinary counterparts because these fish would have their own means of producing and delivering the hormone, and they could transmit their enhanced growth characteristics to their offspring.

Three aspects of fish growth characteristics that could be improved for aquaculture are (1) initial growth rate so that they reach maturation earlier; (2) enhanced somatic growth rate as adults to provide larger body size for market; and (3) fish with improved feed conversion efficiencies. Among these three, enhanced somatic growth rates by manipulation of GH or insulin-like growth factor gene show considerable promise. Zhu et al. [60] reported the first successful transfer of a human GH gene fused to a mouse metallothionein (MT) gene promoter into goldfish and loach. Although Zhu and his colleagues failed to present compelling evidence for integration and expression of the foreign genes in their transgenic fish studies, recent studies reported by many laboratories have successfully confirmed Zhu's work by demonstrating that human or fish GH gene can be readily transferred into embryos of many fish species and integrated into the host genome. Although a few groups have demonstrated expression of foreign genes in transgenic fish, Zhang et al. [13], Du et al. [14] Lu et al. [15], and Martinez et al. [62] have documented that a foreign GH gene could be (1) transferred to the target fish species; [2] integrated into the fish genome; and [3] genetically transmitted to the subsequent generations. Furthermore, the expression of the foreign GH gene may result in enhancement of growth rates of both P_1 and F_1 generations of transgenic fish [13–15,62].

In gene transfer studies conducted in common carp and channel catfish [13,16,54,55], about 10^6 molecules of a linearized recombinant plasmid containing the long terminal repeat (LTR)

sequence of avian Rous sarcoma virus (RSV) and the rainbow trout GH cDNA were injected into the cytoplasm of one-cell, two-cell, and four-cell embryos. Genomic DNA samples extracted from the pectoral fins of presumptive transgenic fish were analyzed for the presence of RSVLTR–rtGH1–cDNA by PCR amplification and Southern-blot hybridization of the amplified DNA products using radiolabeled LTR of RSV or trout GH1 cDNA as hybridization probes. In the transgenic carp studies [13,16], about 35% of the injected embryos survived at hatching, about 10% of which had stably integrated the RSVLTR–rtGH1–cDNA sequence. A similar percentage of transgenic fish was also obtained when the RSVLTR–csGH–cDNA construct was injected into catfish embryos [55,56]. Southern-blot analysis of genomic DNA samples of several transgenic carp and catfish revealed that a single copy of the RSVLTR–rtGH1–cDNA sequence was integrated at multiple chromosomal sites [13].

The patterns of inheritance of RSVLTR–rtGH1 cDNA in transgenic common carp were studied by fertilizing eggs collected from nontransgenic females or p_1 transgenic females with sperm samples collected from several sexually mature P_1 male transgenic fish. DNA samples extracted from the resulting F_1 progeny were assayed for the presence of RSVLTR–rtGH1–cDNA sequence by PCR amplification and dot-blot hybridization [16]. The percentage of the transgenic progeny resulting from nine matings were: 0, 32, 26, 100 (four progeny only), 25, 17, 31, 30 and 23%, respectively (Table 4). If each of the transgenic parents in these nine matings carries at least one copy of the transgene in the gonad cell, about 50–75% transgenic progeny would have been expected in each pairing. Out of these nine matings, two siblots, both control × P_1, transgenic progeny numbers as larger or larger than expected ($P < 0.05$) and the remaining had lower than expected numbers of transgenic progeny. These results indicate that, although most of these P_1 transgenic fish had RSVLTR–rtGH1 cDNA in their germline, they might be mosaics. Similar patterns of mosaicism in the germline of P_1 transgenic fish have been observed in many fish species studied to date [13,15,26,55,57,59].

If the transgene carries a functional promoter, some of the transgenic individuals are expected to express the transgene activity. According to Zhang et al. [13] and Chen et al. [16], many of the P_1 and F_1 transgenic common carp produced rtGH and the levels of rtGH produced by the transgenic individuals varied about tenfold Chen et al. [16] recently confirmed these results by detecting the presence of rtGH mRNA in the F_1 transgenic carp using an assay involving reverse transcription (RT)–PCR amplification. They found that different levels of

Table 4 Percentage of F^1 progeny inheriting PRSVLTR–rtGH1 cDNA

Family	Mating	*N*	Observed % inheritance	Expected % inheritance[a]
1	P_1 × control	17	0	50
2	P_1 × control	96	32	50
3	P_1 × control	26	42[b]	50
4	P_1 × control	4	100[c]	50
5	$P_1 \times P_1$	28	21	75
6	$P_1 \times P_1$	99	21	75
7	$P_1 \times P_1$	312	31	75
8	$P_1 \times P_1$	93	30	75
9	$P_1 \times P_1$	65	23	75

[a]All observed values are less (P 0.05) than except

[b]which is not significantly different ($P > 0.05$) than the expected, and

[c]which is greater ($P < 0.05$) than excepted (X^2).

Source: Ref. 16.

rtGH mRNA were detected in liver, eyes, gonads, intestine, and muscle of the F_1 transgenic individuals.

Because the site of transgene integration differs among individuals in any population of P_1 transgenic fish, they should be considered as totally different transgenic individuals and consequently, inappropriate for direct comparison of the growth performance among these animals. Instead, the growth performance studies should be conducted in F_1 transgenic and nontransgenic siblings derived from the same family. Recently, Chen et al. [16] conducted studies to evaluate the growth performance of F_1 carp in seven families. In these experiments, transgenic and nontransgenic full-siblings were spawned, hatched, and reared communally under the same environment. Results of these studies showed that growth response by families of F_1 transgenic individuals carrying these rtGH1 cDNA varied widely. When compared with nontransgenic full-siblings, the results of four out of seven growth trials showed 20,40,59 and 22% increases in growth, respectively (Table 5 and Fig. 5). The same extent of growth enhancement was also observed in F_2 offspring derived from crossing the fast-growing F_1 transgenics with nontransgenic controls. Similar results were observed when RSVLTR–csGH–cDNA was transferred into channel catfish. Because the response of the transgenic fish to the insertion of the

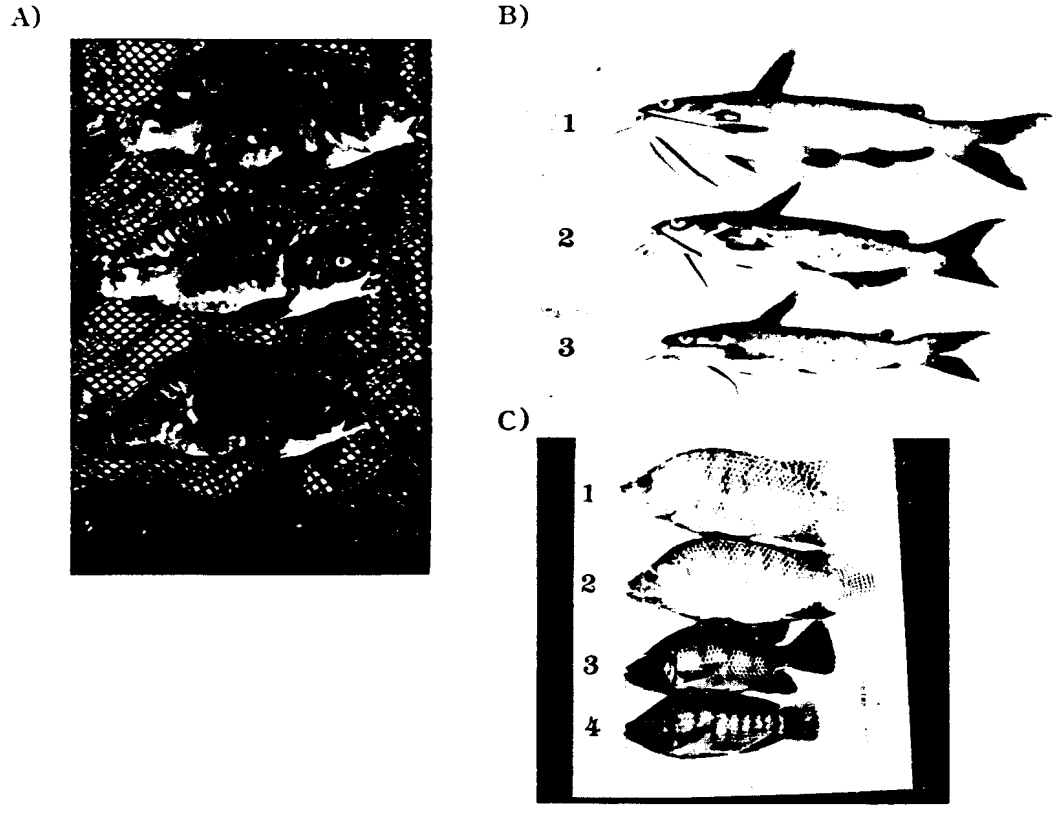


Fig. 5 Transgenic fish carrying rtGH transgene: (A) Transgenic common carp carrying RSV–LTR–rtGH1 cDNA (1, F_1 transgenic fish; 2, P_1 transgenic fish; 3, nontransgenic fish). (B) Transgenic channel catfish carrying RSV–LTR–csGH cDNA (1, F_1 transgenic fish; 2, P_1 transgenic fish; 3, nontransgenic fish). (C) Transgenic tilapia carrying carp β-actin promoter–rtGH1 cDNA (1 and 2, P_1 transgenic fish; 3 and 4, nontransgenic fish).

Table 5 Mean, Standard Deviation, Coefficient of Variation, and Percentage Difference in Body Weight of Transgenic Common Carp *Cyprinus carpio*, and their Nontransgenic Full-Siblings

Family	Mating	Genotype	*N*	Mean body weight (SD)	Coefficient of variation	% Difference	Range in body weight (g)
1	$P_1 \times$ control	T	31	120.6 (17.4)	14.4	20.8	95–173
		NT	65	99.3 (14.7)	14.8		65–129
2	$P_1 \times$ control	T	11	206.0 (45.2)	21.9	40.1	115–283
		NT	15	147.0 (48)	32.6		67–228
3	$P_1 \times P_1$	T	7	5.8 (3.4)	58.6	−26.6	1.8–11.3
		NT	21	7.9 (3.1)	39.2		3.3–17.9
4	$P_1 \times P_1$	T	28	66.1 (36.9)	55.8	58.5	18.5–338
		NT	65	41.7 (27.8)	66.6		8.3–141
5	$P_1 \times P_1$	T	17	14.7 (6.8)	46.3	21.5	6.5–30.4
		NT	82	12.1 (8.4)	69.4		3.9–56.1
6	$P_1 \times P_1$	T	97	114.2 (81.6)	71.5	−14.5	18.3–565.1
		NT	215	133.6 (83.6)	62.5		20.9–416.2
7	$P_1 \times P_1$	T	15	72.2 (58.0)	80.3	−1.5	7.1–214.4
		NT	48	73.3 (47.6)	64.5		8.7–203.3

T, transgenic; NT, nontransgenic; *N*, number of fish; SD, standard deviation.

Table 6 Growth Performance of Transgenic GH, IGF-I Fish and Control

Transgene	Regression line ($y = a + bx$)	Absolute growth rate (mg/d)	Feed conversion rate	K_n value
cBA–hGH	$y = 4.27 + 1.52\times$	1.52	0.353	$6.19 \pm 0.43^{a*}$
ccBA–rtGH1	$y = 1.20 + 1.49\times$	1.49	0.327	5.98 ± 0.25^{a}
ccBA–rtIGF1	$y = 0.37 + 1.32\times$	1.32	0.316	5.88 ± 0.39^{a}
Control	$y = 0.06 + 1.04\times$	1.04	0.297	7.21 ± 0.56^{b}

*ANPOVA $P < 0.05$

$$\text{Absolute growth rate} = \frac{\text{Final wt (mg)} - \text{initial wt(mg)}}{T - t}$$

$$\text{Condition factor } (K_n \text{ value}) = \frac{\text{Weight(mg)}}{\text{length}^{3.22}\text{ (cm)}} \times 1000$$

$$\text{Feed conversion rate} = \frac{\text{Wet weight (mg)}}{\text{dry food weight (mg)}}$$

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 using a combination of family selection and mass selection of transgenic individuals following the insertion of the foreign gene.

More dramatic growth enhancement in transgenic fist has been obtained by introducing Chinook salmon GH cDNA drive by the promoter of ocean pout antifreeze protein gene into Atlantic salmon embryos [14]. Some of these transgenic animals grew several times faster than their controls. A series of recent studies conducted by Lu et al. [manuscript in preparation] showed that both P_1 and F_1 transgenic medaka carrying a chicken β-actin gene promoter–human GH gene construct [15] or tilapia carrying carp β-actin promoter–rtGH1 cDNA exhibited a significant growth enhancement when compared with the nontransgenic siblings (see Fig. 5; Table 6). Some of the P_1 transgenic tilapia grow several times faster than their controls.

The effect of IGF-I transgene on somatic growth has also been tested in medaka and tilapia. IGF-I transgenic medaka and tilapia not only developed faster during embryonic development, they also exhibited a significant degree of growth enhancement (see Table 6).

V. GENERAL CONCLUSION AND FUTURE PROSPECTIVE

Transgenic fish technology has a great potential in revolutionizing the aquaculture industry. By introducing desirable genetic traits into finfish or shellfish, superior transgenic fish strains can be produced for aquaculture. These traits may include elevated growth enhancement, improved food conversion efficiency, resistance to some known diseases, tolerance to low oxygen concentrations, and tolerance to subzero temperatures. Recent progress in our laboratory and those of others has shown that transfer, expression, and inheritance of fish growth hormone and IGF-1 transgenes can be achieved in several finfish species, and the resulting animals grow substantially faster than their control 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 are (1) identifying genes of desirable traits for aquaculture and other application; (2) developing targeted gene transfer technologies, such as embryonic stem cell gene transfer method or ribozyme gene inactivation methods; (3) identifying suitable promoters to direct the expression of transgenes at optimal levels during

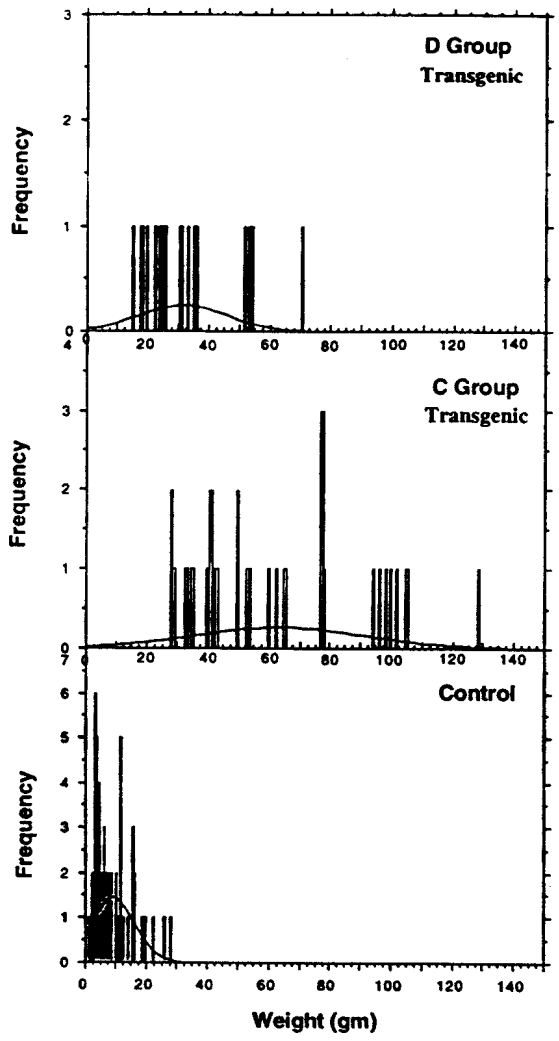


Fig. 6 Body weight distribution of P_1 transgenic and nontransgenic tilapia. D group, embryos electroporated about 2 h after fertilization; C group, embryos electroporated 30 min after fertilization. Frequency, number of animals in each weight group.

the desired developmental stages; (4) determining physiological, nutritional, immunological, and environmental factors that will maximize the performance of the transgenic individuals; and (5) assessing safety and environmental effects of transgenic fish. Once these problems are resolved, the commercial application of the transgenic fish technology in aquaculture will be readily attained

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