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RESEARCH ARTICLE

Tilapia chromosomal growth hormone gene expression accelerates growth in transgenic zebrafish (*Danio rerio*)

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Gene transfer is economically important and model fish species has produced a great impact in modern biology and biotechnology. Transgenic zebrafish (*Danio rerio*) were generated through the co-injection of a GFP-expressing plasmid and an "all fish" transgene composed by the carp b-actin promoter and the chromosomal tilapia (*Oreochromis hornorum*) growth hormone gene. The GFP expression was a good indicator of stable transformation and allowed for high efficiency selection of transgenic fish. Transgenic F1 zebrafish grew 20% faster than full sibling non-transgenic controls.

Gene transfer technology has produced a great impact in modern biology and biotechnology (Powers et al. 1998). A number of fish species are in focus for gene transfer experiments and can be divided into two main groups: animals used in aquaculture (Fletcher and Davies, 1991; Hew et al. 1995; Chen and Lu, 1998) and model fish used in basic research (Chen and Lu, 1998). Among the major food fish species are carp (*Cyprinus sp*.), tilapia (*Oreochromis sp.*), salmon (*Salmo sp*., *Oncorhynchus sp.*) and channel catfish (*Ictalurus punctatus*) while zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and goldfish (*Carassius auratus*) are used in basic research.

Zebrafish is an already well-established model organism (Kimmel, 1989; Westerfield, 1995). This fish offers the possibility of combining rapid early development, which is amenable to direct observation and manipulation, large numbers of progeny from a single mating, and a relatively short generation time (2 to 3 months).

Transgenic technology through DNA microinjection into zebrafish embryos has made great gain in the last decade. Stuart et al. (1990) first showed that the DNA injected into the cytoplasm of fertilized zebrafish eggs could integrate into the fish genome and be inherited in the germ line. Culp et al. (1991) demonstrated that the frequency of germline transmission of a microinjected DNA could be as a high as 20% in zebrafish. This technology, however, still has as the major constrains the low efficiency generation of transgenics.

To improve the efficiency of selection of transgenics, genetic markers are co-injected with the transgene to monitor for transformed zygotes. The green fluorescent protein (GFP) from Jellyfish (*Aequorea victoria)* has been used for this purpose in zebrafish (Amsterdam et al. 1995; Peters et al. 1995). Methods to increase transgenic efficiency in zebrafish have been reported through SV40 T antigen nuclear localizing signal (NLS)-mediated gene transfer (Aleström et al. 1998).

The purpose to improve growth performance to create novel strains for aquaculture is one of the most promising applications of gene transfer in fish. Although some fast-growing fish strains created after the transfer of growth hormone (GH) transgenes will be soon commercially available, more knowledge is needed to optimally manipulate this process.

To investigate the effect of an "all fish" transgene containing the carp β-actin promoter (cβp) fused to the tilapia (*Oreochromis hornorum*) GH chromosomal gene (chrtiGH), transgenic zebrafish were generated co-injecting the linear GH transgene (cβp-chrtiGH) with the GFP-expressing plasmid pRSGFP (Clontech, USA) in a 10:1 ratio. The results showed that GFP expression is a good indicator of stable transformation. Transgenic F1 zebrafish grew 20% faster than full sibling non-transgenic controls.

Materials and Methods

Cloning of the chromosomal tilapia (*O. hornorum*) GH gene

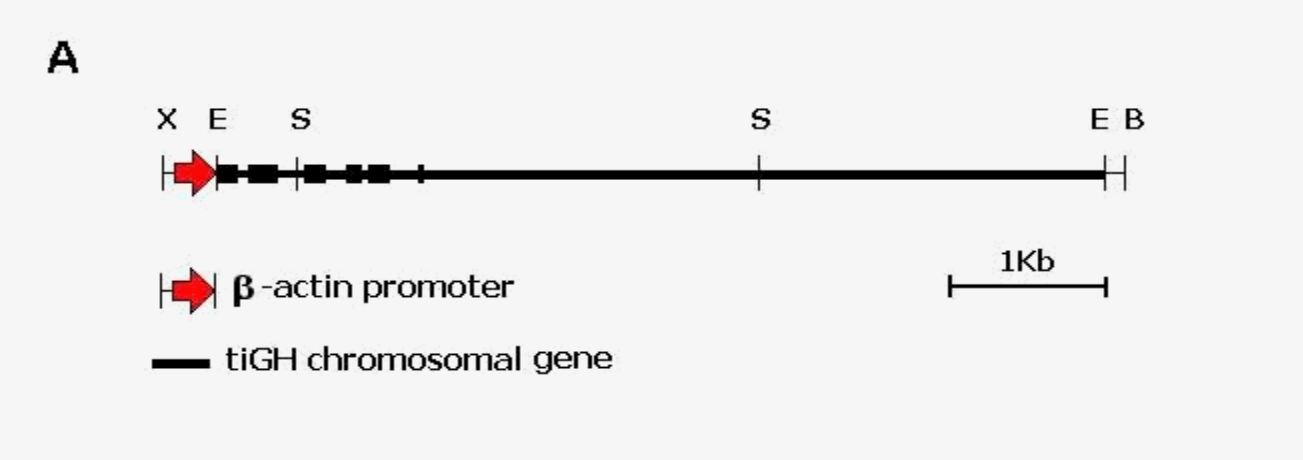
A tilapia genomic DNA library was prepared in the lambda vector EMBL3 (Frischauf et al. 1983). After screening with the tiGH cDNA probe (Guillén et al. 1998), a recombinant phage was isolated containing a 6.3 kb *Eco*R I insert. This fragment containing the chrtiGH gene was subcloned into a Bluescript plasmid for sequencing. (Cat. No.212205, Stratagene, USA).

Cloning of the carp b-actin promoter

For the cloning of the carp (*Cyprinus carpio*) β-actin promoter (Liu et al. 1990), 10 ng chromosomal DNA were used in a polymerase chain reaction (PCR) including 50 pmoles of each oligonucleotide 5´-GATGAAACTCGAGTAGCCCTTGCTCTTC-3´ and 5´-CGTTCGAATTGATATATGCGAGCTG-3´ in 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each dNTP and 9 U *Thermus aquaticus* DNA polymerase (Heber Biotec S.A., Havana, Cuba) in 25 µl final volume. Amplification was obtained after 5 cycles of 1 min at 94ºC, 1 min at 45ºC and 1 min at 72ºC, followed by 30 cycles of 1 min at 94ºC, 1 min at 55ºC and 1 min at 72ºC. The amplified fragment was cloned into the *Eco*R V site of the BlueScript plasmid to generate the plasmid pBC330.

Construction of the cbp-chrtiGH transgene

For the construction of the cβp-chrtiGH transgene, the *Eco*R I tiGH fragment was subcloned into the *Eco*R I site of the plasmid pBC330. For microinjection, the transgene was excised by digesting with *Xho* I and *Bam*H I (Figure 1).



Abbreviations: X: Xba I; E: EcoR I; S: Sac I; B: BamH I

Figure 1. Fragment of DNA (transgene) microinjected into zebrafish embryos. Structure of the cβp-chrtiGH transgene.

Fish maintenance, egg collection and microinjection

Zebrafish (*D. rerio*) were obtained from the local pet store, separated by sex, and maintained in 45 liters aquaria in a 1:1 mixture of tap water and deionized water at 28ºC. Fish were fed 3 times a day (flake food twice and live hatchling artemia once). Eggs for injection usually were obtained by placing 6, 8-10 month old fish, in a female:male ratio of 2:1, in 10-litter tank at least 1 day before eggs were needed. Mating occurred in the morning shortly after the light came on.

Fertilized eggs were collected soon after spawning when most embryos are at the one to two cell stages. Eggs were rinsed in embryo medium (Westerfield, 1995). DNA solution (50 ng/µl) was microinjected into the cytoplasm with the aid of an Eppendorf 5242 (Germany) microinjector. The purified cβp-chrtiGH transgene (6.6 kb *Xho* I - *Bam*H I fragment, Figure 1) and the GFP-expressing plasmid pRSGFP-C1 (Clontech, USA) were co-injected in approximately 350 pl of sterile water containing 0.2% phenol red. Glass needles were prepared using a Narishige PN-3 (Japan) pipette puller. After injection, embryos were incubated in embryo medium until hatching.

DNA analysis

DNA was extracted from fin sections of two to four-month-old zebrafish by means of treatment with proteinase K and phenol, followed by ethanol precipitation.

For Southern blot analysis of founder fish, 10 µg DNA were digested with *Sac* I (Heber Biotec S.A., Cuba), DNA fragments were separated by agarose gel electrophoresis and blotted onto nylon membranes (Hybond N, Amersham, UK) as described by the manufacturer. Blots were hybridized with a ^{32}P-radiolabeled DNA probe (Feinberg and Vogetstein, 1984) comprising the carp β-actin promoter sequence.

PCR was used as a rapid screening method for the analysis of F1 animals and to assess germ line transmission. Twenty nanograms of genomic DNA was used to amplify a fragment of 1 kb extending from the carp β-actin promoter to the chrtiGH, in 50 µl reaction mixture containing 50 pmol of each oligonucleotide primer (5´-CAGCGTCTCAGCCTCACTTTGAG-3´ and 5´-AAGATTCCCGTTTTAAGCTCAG-3´), 50 mM KCl, 10 mM Tris HCl, pH 8.0, 1.5 mM $MgCl_2$, 200 µM each dNTP and 2 u *T. aquaticus* DNA polymerase (Perkin Elmer-Cetus, USA). Samples were subjected to 35 cycles of PCR, each consisting of 1 min of denaturation at 94ºC, 30 s of annealing at 55ºC, and 30 s of polymerization at 72ºC. After amplification, 10 µl of each reaction was analyzed in 1% agarose gels.

Detection of GFP gene expression

Following microinjection, GFP gene expression was followed in 24 h after microinjection embryos using a Zeiss (Germany) epifluorescence microscope (excitation 450-490 nm, barrier filter LP 520 nm). Photographs were taken using Kodak chrome 1200 asa films.

Analysis of growth performance

A random transgenic female zebrafish founder was crossed with a non-transgenic male to produce F1 progeny. Fifty F1 zebrafish of four week-old were randomly selected and grown individually under similar conditions of water temperature (28ºC) and photoperiod (10 h light: 14 h dark). Fish were fed 3 times daily with brine shrimp eggs and brine shrimp flake (Argent Chemical Laboratories, USA). Zebrafish were weighed weekly during 6 weeks to monitor growth performance. In the course of the experiment, fin DNA was extracted and assayed for transgene identification. Weight of transgenic and non-transgenic full siblings was compared employing a Student t-Test.

Table 1. Efficiency in the generation of transgenic zebrafish. Zebrafish embryos were collected and microinjected with cβp-chrtiGH: pRSGFP (10:1 molar ratio). Survival and GFP fluorescence rates were assayed 24 h post-injection. Transgenic fish were screened for the presence of cβp-chrtiGH sequences by PCR analysis of fin DNA.

Microinjected embryos	24 h post-injection survival (%)	GFP positive 24 h post-injection (%)	Transgenic fish (% with respect to)	
			Injected embryos	Fluorescent embryos
252	142 (65)	3 (1.2)	2 (0.8)	2 (67)
214	131 (75)	2 (0.9)	2 (0.9)	2 (100)
281	166 (56)	4 (1.4)	3 (1.1)	3 (75)
293	183 (62)	4 (1.4)	2 (0.7)	2 (50)
233	153 (65)	2 (0.9)	1 (0.4)	1 (50)
225	149 (66)	3 (1.3)	2 (0.9)	2 (67)

Results

Cloning of chrtiGH

The structure of the *O. hornorum* chrtiGH gene was similar to the structure reported for *O. niloticus.* At the nucleotide level, we found in the coding region a change of a guanine instead of an adenine in the position 594 of the *O. niloticus* tiGH cDNA (Ber and Daniel, 1992). However, the deduced aminoacid sequence of the *O. hornorum* tilapia GH was similar to the sequence reported for *O. niloticus* (Ber and Daniel, 1992).

Generation of transgenic zebrafish

Zebrafish embryos were collected and microinjected in several batches with an average of 250 (214-293) embryos per injection batch (Table 1). The survival rate 24 h post-injection averaged 62% (56-75%) (Table 1). Non-injected embryos showed a similar survival rate. GFP was monitored in 24 h post-injection embryos and fluorescence was detected in 1.2% (0.9-1.4%) of the injected embryos (Table 1). After PCR analysis of fin DNA, 0.8% (0.4-1.1%) of injected embryos resulted in transgenic fish (Table 1).

The GFP expression pattern in 24 h post-injection zebrafish embryos was patchy and in different regions of the embryo. Under our experimental conditions, GFP expression was a good indicator of embryo transformation as 67% (50-100%) of fluorescent embryos resulted in fish positive for transgene sequences after PCR analysis.

A transgenic female was selected for further characterization and studies. Southern blot analysis of fin DNA indicated that the transgene was present with a size corresponding to the injected fragment (Figure 1). This female was used as P1 founder to obtain F1 descendants after crossing to a non-transgenic male. The transgene was transmitted to 46% of F1 fish.

Characterization of the growth phenotype in transgenic zebrafish

For analysis of growth performance, F1 transgenic and full sibling non-transgenic control fish were grown under similar conditions and weighed weekly during 6 weeks. Transgenic zebrafish grew faster than controls (Figure 2). At the start of the experiment, the weight (mean ± SD) of transgenics (0.12 ± 0.04 g) and controls (0.11 ± 0.04 g) was similar (P = 0.2, Student t-Test). Six weeks later, transgenic fish were 20% heavier than controls (0.36 ± 0.10 g vs. 0.31 ± 0.08 g; P = 0.03, Student t-Test). The increment in weight for the period of study was also statistically significant (P = 0.04, Student t-Test) between transgenic (0.24 ± 0.09 g) and control (0.20 ± 0.07 g) fish.

Discussion

The gene coding for *O. hornorum* tiGH was cloned and its coding sequence compared to the sequence reported for *O. niloticus* tiGH (Ber and Daniel, 1992). Both sequences differed in only one nucleotide. This silent mutation was also present in the cDNA and probably reflects a genetic polymorphism in this locus (Guillén et al. 1998).

The survival rate for injected embryos in our experiments was similar to other reports for zebrafish. However, the fraction of fluorescent embryos 24 h post-injection was much lower than that obtained by Amsterdam et al. (1995). This fact is in accordance with the low efficiency

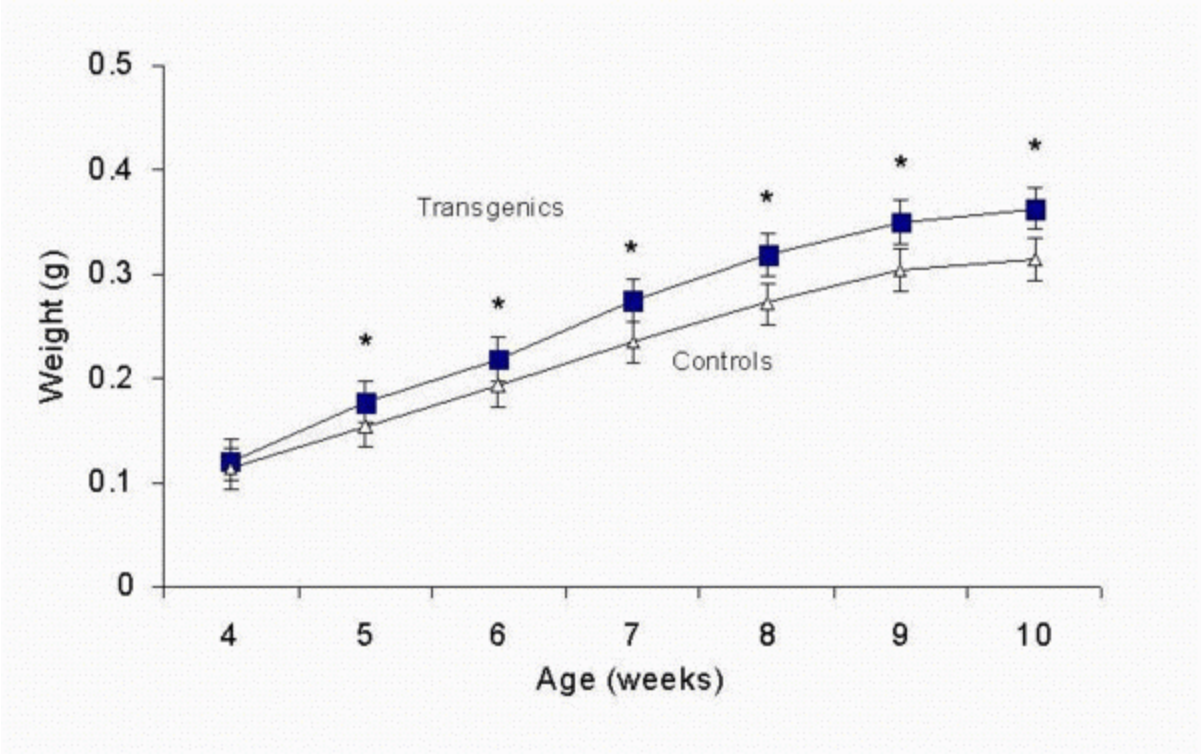


Figure 2. Growth performance in F1 transgenic and full sibling non-transgenic zebrafish. Fifty-four zebrafish of F1 fry were randomly selected and grown individually under similar conditions. At the beginning of the experiment, they were four week old. Zebrafish were weighed weekly during 6 weeks to monitor growth performance. In the course of the experiment, fin DNA was extracted and assayed for transgene identification. Weight of transgenic and non-transgenic full siblings was compared employing a Student t-Test (*, $P < 0.05$).

generation of transgenic zebrafish when compared to published results (Stuart et al. 1990). These results are probably a consequence of having being obtained during the initiation of transgenic zebrafish experiments in our laboratory. As we have shown for transgenic tilapia, the skills of the manipulator among other factors are crucial in the efficiency of generation of transgenics (De la Fuente et al. 1995).

Although it has been reported in zebrafish that approximately only 5% of injected embryos are transgenic (Stuart et al. 1990), this is still low and demands time consuming and tedious work to screen for transgenic fish. To reduce the number of animals to screen, we co-injected with transgene sequences a GFP-expressing plasmid. This permitted us to screen for transgenics only analyzing fish derived from fluorescent embryos, thus reducing the number of potential transgenic founders for analysis.

The expression of GFP 24 h post-injection appeared evenly distributed throughout transgenic embryos. Similar results have been obtained by others (Amsterdam et al. 1995), suggesting that all cell types are capable of expressing GFP, thus extending the possibilities of GFP as a reporter gene for studies of gene expression patterns.

One transgenic female was selected for crossing with a non-transgenic male to analyze in the F1 progeny transgene transmission and growth performance. Southern blot analysis of fin DNA from the transgenic P1 showed the presence of unrearranged transgene sequences. The Mendelian transmission of the transgene to the F1 progeny corroborated that the transgene stably integrated into the germ line of the P1 founder. This result contrasts with the high degree of mosaicism reported for transgenic zebrafish (Stuart et al. 1990).

Growth acceleration in fish has been one of the targets of gene transfer experiments in these species (Powers et al. 1998). Zebrafish are a good model for the rapid study of GH-transgenes, before selecting the construct to use in economically important species.

In this study we assayed an "all fish" transgene, addressing a general concern to as much as possible utilize DNA sequences derived from the same, or closely related species (Du et al. 1992). Furthermore, in our construct we included the chromosomal tiGH gene. In previous experiments we have shown that the expression of the tiGH cDNA in transgenic tilapia results in accelerated growth (Martínez et al. 1996; Martínez et al. 1999) while the inclusion of fish-derived intron sequences in chimeric constructs increases

transgene expression in zebrafish embryos (García del Barco et al. 1994). Both elements are resumed in the chrtiGH gene.

Growth acceleration was demonstrated in F1 transgenic zebrafish. However, the growth acceleration effect was not very pronounced. In transgenic tilapia we have shown that growth acceleration is achieved only at low expression levels of the tiGH-bearing transgene (Hernández et al. 1997; De la Fuente et al. 1998a).

Although we have not measured the tiGH expression levels in transgenic zebrafish, the inclusion of chrtiGH gene could have resulted in high tiGH expression levels, therefore producing a mild effect on growth. Groups working with relatively strong promoters in other species have obtained similar results (Lu et al. 1992; Hernández et al. 1997; Chen and Lu 1998; De la Fuente et al. 1998a; De la Fuente et al. 1998b; Pitkänen et al. 1999).

Two main conclusions can be drawn from our results: (a) the co-injection with the transgene of GFP-expressing plasmids allows for high efficiency selection of transgenic zebrafish and (b) transgenic zebrafish bearing the "all fish" cβp-chrtiGH transgene grow faster than non-transgenic controls.

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