Research Article

Improving the production of transgenic fish germlines: *In vivo* evaluation of mosaicism in zebrafish (*Danio rerio*) using a green fluorescent protein (GFP) and growth hormone cDNA transgene co-injection strategy

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Abstract

In fish, microinjection is the method most frequently used for gene transfer. However, due to delayed transgene integration this technique almost invariably produces mosaic individuals and if the gene is not integrated into germ cells its transmission to descendants is difficult or impossible. We evaluated the degree of *in vivo* mosaicism using a strategy where a reporter transgene is co-injected with a transgene of interest so that potential germline founders can be easily identified. Transgenic zebrafish (*Danio rerio*) were produced using two transgenes, both comprised of the carp β-actin promoter driving the expression of either the green fluorescent protein (GFP) reporter gene or the growth hormone cDNA from the marine silverside fish *Odonthestes argentinensis.* The methodology applied allowed a rapid identification of G_0 transgenic fish and also detected which fish were transmitting transgenes to the next generation. This strategy also allowed inferences to be made about genomic transgene integration events in the six lineages produced and allowed the identification of one lineage transmitting both transgenes linked on the same chromosome. These results represent a significant advance in the reduction of the effort invested in producing a stable genetically modified fish lineage.

Key words: transgenesis, genetically modified fish, microinjection, growth hormone cDNA.

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Introduction

Transgenesis involves genomic alteration of an organism through insertion, modification or deletion of a gene with the objective of modifying characteristics of interest (Houdebine, 2002; Carter, 2004). In this manner, new stable and genetically determined characteristics can be incorporated into the genome of the receptor organism and possibly transmitted to the next generations. In the last two decades, this technology has been successfully applied in fish due to the fact that these inferior vertebrates present reproductive and biological characteristics that allow easy manipulation of their genetic and physiological processes in the early stages of ontogenesis (Zhu and Shu, 2000). Studies of gene transfer have been carried out in more than 35 teleost species, most of which are important to aquaculture (Zbikowska, 2003). However, genetically modified fish have also been developed as experimental models for biomedical research, especially in studies involving embryogenesis and organogenesis (Amacher, 1999; Motoike *et al.*, 2000; Goldman *et al.*, 2001; Huang *et al.*, 2001; Takechi *et al.*, 2003) as well as in the study of human diseases (Dodd *et al.*, 2000; Dooley and Zon, 2000; Ward and Lieschke, 2002), xenotransplantation (Wright and Pohajdak, 2001; Leventhal *et al.*, 2004; Pohajdak *et al.*, 2004) and recombinant protein production for producing important therapeutic agents (Anderson and Krummen, 2002).

Several techniques are currently available for transgenic fish production which have been developed to increase the efficiency of transgene integration or to produce a large number of transformed individuals simultaneously. Although these new methods of gene transfer are gaining importance due to the encouraging results reported (Tanaka and Kinoshita, 2001; Lu *et al.*, 2002; Grabher *et al.*, 2003; Hostetler *et al.*, 2003; Kinoshita *et al.*, 2003; Kurita *et al.*, 2004), microinjection in fish is still the most successful technique being used due to its simplicity and trustworthiness (Udvadia and Linney, 2003; Zbikowska, 2003). Maclean *et al.* (2002) stated that microinjection was the

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best technique to use with tilapia (*Oreochromis niloticus*). However, when microinjection is used to produce transgenic fish it almost invariably produces mosaic fish due to delayed transgene integration, which occurs only after a few cycles of embryonic cell division. If the transgene is integrated in only one cell group or tissue but not into germ cells the transmission of the gene to descendants is difficult or impossible (Maclean, 1998).

The use of reporter genes that allow evaluation of the degree of *in vivo* mosaicism in transgenic fish can facilitate identification of probable germline founders and the co-injection of a reporter transgene along with the gene construct of interest represents a considerable reduction in the effort needed for the establishment of transgenic germlines. The gene coding for the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been widely used as a reporter gene because it does not require an exogenous substrate for its activity (Amsterdam *et al.*, 1995) and is stable and non-toxic in receptor organisms (Peters *et al.*, 1995).

The objective of the work described in this paper was to develop an *in vivo* methodology to evaluate the degree of mosaicism and to identify transgenic zebrafish (*Danio rerio*) with the potential to generate germlines for the gene of interest.

Material and Methods

Production of transgenic fish

Adult wild-type zebrafish (*Danio rerio*) obtained from a commercial supplier were kept in a closed water circulation system at 28 °C under a 14 h light/10 h dark photoperiod. Freshly fertilized eggs were collected for microinjection and transgenic zebrafish produced using two transgenes containing the carp (*Cyprinus carpio*) β-actin promoter driving the expression of either the *A. victoria* GFP gene (pcβA/GFP plasmid) or the marine silverside fish (*Odonthestes argentinensis*) growth hormone (msGH) cDNA (pcβA/msGH plasmid). The pcβA/GFP plasmid was kindly provided by Dr. Suzanne Brooks (University of Southampton, UK) and was used to produce the pcβA/msGH plasmid by replacing the GFP gene with the msGH cDNA (Marins *et al.*, 2002). Both plasmids were linearized with the *Spe* I restriction enzyme and co-injected at a 1:1 molar ratio into one-cell embryos using a total DNA concentration of 35 ng μL^{-1}. The linearized transgenes (called cβA/GFP and cβA/msGH) were transferred in an equimolar ratio to provide the same integration and expression probability due to the fact that they had approximately the same length (cβA/GFP = 5.6 kb and cβA/msGH = 5.5 kb) and were both under the action of the same promoter.

The microinjection process followed the general protocol recommended by Vielkind (1992) and used an IM-30 motorized picoinjector (Narishige, Japan) to inject approximately 300 pL of DNA solution representing a final number of 10^6 copies of each transgene per injected embryo. The microinjection needles were produced from Narishige GDC-1 glass capillaries using the Narishige PC-10 micro-electrode puller. A total of 1872 one-cell embryos were injected. Non-injected controls and microinjected embryos were incubated at 28 °C until hatching.

Evaluation of mosaicism in the first transgenic generation (G_0)

One week after fertilization the fish larvae were analyzed by epifluorescence microscope (excitation = 485 nm; emission = 520 nm) and classified according to their GFP expression patterns (Gibbs and Schmale, 2000; Thermes *et al.*, 2002) as follows: weak = few cells expressing GFP; moderate = less than 50% of the body expressing GFP; or strong = more than 50% of the body expressing GFP.

Evaluation of the msGH gene expression by RT-PCR

To confirm G_0 msGH gene expression we used the reverse transcriptase polymerase chain reaction (RT-PCR) to analyze several four-week old fish expressing GFP. Total RNA was extracted by humanely sacrificed 12 G_0 fish and homogenizing their bodies in TRIzol reagent (Invitrogen, Brazil) according to the protocol suggested by the manufacturer. Approximately 3 μg of total RNA from each fish was used as template for the RT-PCR with the AP primer (5'-GGCCACGCGTCGACTAGTAC(T)$_{17}$-3', Invitrogen, Brazil). The complementary DNA (cDNA) synthesis was carried out using the enzyme RT SuperScript III (Invitrogen, Brazil) according to the protocol suggested by the manufacturer. The cDNA obtained was used as template for the msGH gene amplification using the specific primers EXO 293 (5'-GAAAGCTCTCTGCAGACGGA G-3') and GHEX6-RIG (5'-AGAGTGCAGTTTGCCTCT GG-3'), which produce a 467 bp msGH fragment but do not amplify the endogenous zebrafish growth hormone gene. PCR was carried out in a 12.5 μL reaction volume containing 1.25 μL of 10X PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.75 mM of $MgCl_2$, 0.5 unit of Platinum Taq DNA polymerase (Invitrogen, Brazil) and 1 μL of cDNA solution. The reaction was incubated at 94 °C for 2 min followed by 30 cycles of 15 s at 94 °C, 30 s at 65 °C and 30 s at 72 °C, and a final elongation step of 5 min at 72 °C and the PCR products separated on 1% (w/v) agarose gel stained with ethidium bromide (0.5 μg/mL) and visualized by ultraviolet transillumination.

Assessment of the biological effect of msGH in the G_0 generation

The objective of this experiment was to verify whether the msGH transgene produced significant biological effects in the growth performance of the transgenic indi-

viduals. Groups of non-transgenic and G_0 transgenic fish classified as strongly expressing GFP were reared until one-year old for comparison of their final average weight. Fish were fed *ad libitum* twice a day with a commercial fish food (ColorBits, Tetra) containing 47.5% protein. The average weight data were statistically analyzed using a t-test for heterogeneous variances contained in the Statistica program v. 6.0 (Statsoft, USA).

Transmission of transgenes to the second (G_1) and third (G_2) transgenic generations

A group of larvae showing strong GFP expression were selected and reared until sexual maturity as being probable germline founders. For the transgene transmission study, eight G_0 mosaic fish classified as strong for GFP expression were separated in individual aquariums and crossed with non-transgenic fish. Two of these transgenic fish did not developed reproductive behavior. Six sexually mature transgenic fish were mated with wild-type fish to produce G_1 offspring which were assessed by epifluorescence microscopy as described above to verify the presence of the uniform GFP expression expected after genomic integration. To confirm the presence of the msGH transgene in the G_1 fish genome, GFP positive larvae were cultivated until adulthood and a small fin clip was taken from each fish and the genomic DNA extracted (Sambrook *et al.*, 1989). The msGH gene was amplified using the specific primers EXO-293 and GHEX6-RIG and the PCR conditions described above. Only GFP positive G_1 fish were tested for msGH because the objective was to identify G_0 fish which were transmitting both transgenes to the descendants.

Twelve G_1 fish, six from each of two G_1 parents carrying both transgenes, were crossed with wild-type fish to produce G_2 progeny, which were assessed using epifluorescence microscopy as described above. To verify whether or not the transgenes had integrated on the same chromosome, sub-samples of GFP positive and negative fish (N = 12) were randomly selected from each lineage and their DNA extracted and subjected to PCR, using the conditions described above, to detect the presence of msGH.

Results

Transgenic zebrafish were produced by the co-injection of 1872 one-cell embryos, using the transgenes cβA/GFP and cβA/msGH in an equimolar ratio (1:1). At the time of assessment by epifluorescence microscopy, one week after fertilization, the survival rate for untreated fish embryos was 1414 out of 1872 (75.5%) while the survival rate of the microinjected embryos was 877 out of 1872 (46.8%), of which 275 out of 877 (31.4%) were classified as GFP negative (no expression), 315 out of 877 (35.9%) as weakly GFP positive, 198 out of 877 (22.6%) as moderately GFP positive and 89 out of 877 (10.1%) as strongly

GFP positive. The sum of the three GFP expression classes was 602 out of 877 fish (68.6%).

All of the 12 one-month old G_0 GFP positive fish assessed for msGH expression by RT-PCR were msGH transgene positive. The analysis of the average weight after 12 months demonstrated a significant increase ($p < 0.01$) in the transgenic group ($1.79 \text{ g} \pm 0.37$) in relation to wild-type fish of the same age ($0.68 \text{ g} \pm 0.13$). This represents an increase in growth of 2.6 times for the transgenic group as compared with the non-transgenic fish, and shows the biological effect of expression of the cβA/msGH transgene in zebrafish (Figure 1).

In the transgene transmission study, eight G_0 mosaic fish classified as strongly expressing GFP were crossed with non-transgenic fish. Two of the transgenic fish did not developed reproductive behavior but two males (M0104 and M0204) and four females (F0104, F0204, F0304 and F0404) reproduced, four of which (M0104, F0104, F0204 and F0304) transmitted the GFP gene to the G_1 in percentages that varied from 2.2% to 42% (Table 1). The GFP expression pattern observed in the G_1 offspring showed fish expressing the transferred gene in all body cells. However, the msGH gene was detected only in G_1 descents obtained from the M0104 and F0104 parent fish. Only half the GFP-positive offspring of the M0104 parent were also carrying the msGH gene but for the F0104 parent all the GFP-positive offspring were also positive for the exoge-
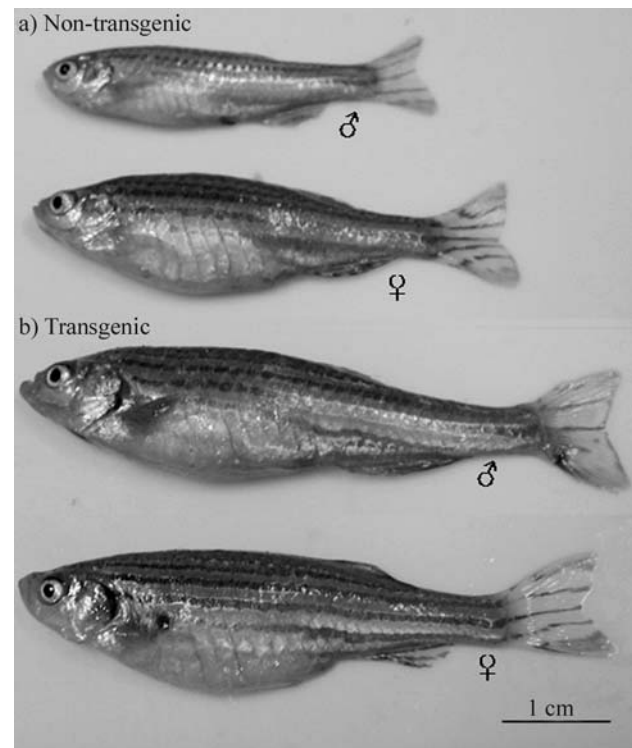
Figure 1 - Zebrafish (*Danio rerio*): (a) one-year old non-transgenic fish (average weight = 0.68 ± 0.13) and (b) one-year old G_0 transgenic fish (average weight = $1.79 \text{ g} \pm 0.37$).

Table 1 - Germline transmission and expression of a green florescent protein (GFP) reporter transgene (cβA/GFP) and growth hormone transgene (cβA/msGH) in transgenic zebrafish (*Danio rerio*).

Transgenic lines	Number of G_1 embryos expressing GFP	G_1 GFP+ embryos carrying the msGH gene	Percentage of cβA/GFP and cβA/msGH segregation in G_2 embryos (%)			
(G_0)*	(% transmission to G_1)	(%)	GFP+	GH+	GFP+/GH+	GFP-/GH-
M0104	50 out of 757 (6.6)	3.3	25	25	25	25
F0104	18 out of 812 (2.2)	2.2	0	0	50	50
F0204	119 out of 283 (42)	0	-	-	-	-
F0304	51 out of 167 (30.5)	0	-	-	-	-
M0204	0 out of 115 (0)	-	-	-	-	-
F0404	0 out of 107 (0)	-	-	-	-	-

*M = male; F = female.

nous GH gene (Table 1). No GFP gene expression was detected in the offspring of the M0204 and F0404 parents.

The G_1 offspring of the M0104 and F0104 G_0 mosaic parents which were positive for both transgenes were reared until sexual maturation and six G_1 fish from each parent were mated with wild-type fish to produce G_2 offspring. A total of 466 G_2 offspring resulted from the M0104 matings while 588 G_2 offspring were obtained from the F0104 matings. These results are summarized in Table 1 and indicate two different transmission patterns for each lineage. For the male M0104 parent the G_2 offspring consisted of 25% negative for both transgenes, 25% GFP-positive only, 25% msGH-positive only and 25% GFP/msGH-positive, while for the female F0104 parent the G_2 offspring consisted of 50% negative for both transgenes and positive for both transgenes.

Discussion

Although some methodologies have presented promising results in increasing first generation (G_0) transgenic fish production, there is still the problem of rearing large numbers of G_0 fish and the subsequent identification of those with the potential to generate stable germlines carrying the active transgene. This process is even more difficult for large fish such as carp, salmon, tilapia or trout which need very complex culture facilities. The methodology applied in our study not only allowed the production of transgenic fish carrying the active transgene of interest (cβA/msGH) but also the reporter transgene (cβA/GFP) which allowed the evaluation of mosaicism in all the G_0 transgenic fish generated. The analysis of the GFP expression patterns permitted the selection of possible germline founders from the fairly low number of fish in the G_0 generation.

A week after microinjection 68.6% of the fish embryos expressed GFP, which represents a high efficiency of transgenic fish production. However, part of this observed expression can be attributed to transitory expression due to the transcription of unintegrated transgenes (Chong and

Vielkind, 1989). Our results are significant when compared to the 1.95% transgenic zebrafish obtained by Morales *et al.* (2001) and the 10% transgenic tilapia obtained by Rahman *et al.* (1997) using the same reporter transgene co-injection strategy. We found that 10% of the fish analyzed by us presented strong GFP reporter gene expression, significantly more than the 5% with strong GFP expression reported by Gibbs and Schmale (2000) for G_0 transgenic zebrafish and the 3% with strong GFP expression reported by Thermes *et al.* (2002) for G_0 transgenic medaka. The conditions used by these authors were similar to ours and they also used linearized transgenes in which the GFP gene was controlled by ubiquitous promoters (α and β-actin).

Although our RT-PCR analyses showed that 100% of the G_0 GFP positive fish were expressing the msGH gene not all these fish were carrying the msGH transgene in their germ cells and could transmit the msGH transgene to the next generation, this being evident when the G_0 and wild-type fish were crossed. The growth experiment data supported our RT-PCR results and indicated that the transgenic group increased in weight 2.6 times more than the non-transgenic group. These results demonstrate that the cβA/msGH transgene was producing an active hormone. The higher weight of the transgenic fish was probably related to increased circulating msGH which could not be controlled by the negative feedback mechanism which regulates endogenous GH gene expression (Peter and Marchant, 1995).

The main negative consequence of mosaicism in transgenic fish germline production is the fact that the germ cells of G_0 fish can receive few or no copies of the transgene, making transgene transmission to the next generation difficult or impossible (Maclean, 1998). However, this problem can be minimized by evaluating mosaicism using a reporter transgene co-injection strategy. This is supported in our study by the presence of strongly GFP-positive fish (2 out of 6, or 33.3%) transmitting both transgenes to the G_1 generation, indicating a considerable increase in the possibility of identification of germline founder fish. According to Maclean (1998), the rate of transgene transmission to the

G_1 is low, with only approximately 5% of G_0 transgenic fish having the capacity to transmit transgenes to the next generation.

The percentage of GFP positive G_1 fish produced in our study indicated the degree of mosaicism in the germ cells of the G_0 fish. We found that two fish did not transmit any transgene to their descents, indicating that the transgenes were not integrated into the germ cells, while four fish transmitted the cβA/GFP transgene to produce 2.2% to 42% of GFP-positive descents. Therefore, despite the fact that some of our fish strongly expressed GFP, transgene integration into germ cells was extremely variable. In theory, if all the germ cells of a transgenic fish contained the transgene (*i.e.* no germ cell mosaicism) and this fish was mated with a wild-type fish 50% of the offspring would express the transgene. Maclean (1998) pointed out that generally only a small percentage of the offspring from G_1 mosaics are transgenic, which makes identification difficult when an easy detectable marker is absent. In our study, the identification of G_1 transgenic fish was greatly facilitated by the presence of the GFP gene reporter and simple evaluation using epifluorescence microscopy allowed rapid identification of the transgenic fish. Additionally, G_1 fish originating from the F0104 female and the M0104 male (which were carrying both transgenes) were crossed with wild-type fish to verify how the transgenes integrated in the G_2 fish genome. In the G_2 produced from the M0104 lineage a number of GFP-positive fish did not carry the exogenous GH transgene while some GFP-negative fish were carrying it. This indicates that the cβA/GFP and cβA/msGH transgenes segregated in the G_2, since the observed genotypic ratio (Table 1) is in accordance with genes situated on different chromosomes. However, for the F0104 lineage G_2 descents all the GFP-positive individuals were carrying the cβA/msGH transgene as well as the cβA/GFP transgene, indicating that both transgenes had been integrated on the same chromosome.

The methodology described in this paper allowed the rapid identification of G_0 transgenic fish and also identified which fish were transmitting transgenes to the next generation. This strategy also allowed inferences to be made regarding genomic transgene integration events, and permitted the identification of the one lineage (out of the six produced) which contained and transmitted both transgenes linked on the same chromosome. These results represent a significant advance in the reduction of the effort involved in the production of genetically stable modified fish lineages.

Acknowledgments

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