Construction, electroporatic transfer and expression of ZpβypGH and ZpβrtGH in zebrafish†

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Recombinant transformation vectors (ZpβypGH and ZpβrtGH) consisting of fish growth hormone cDNA, and a reporter gene β-galactosidase driven by fish promoter (Zp) were constructed. Freshly fertilized eggs of zebrafish (*Brachydanio rerio*) were electroporated at optimum conditions (0·07 kV voltage; 25 μF capacitance; ∞ ohm resistance and 2 pulses) in the presence of one of these transformation vectors (100 μg circular DNA/ml). In either cases 72% of the electroporated eggs successfully hatched, in comparison to the 85% hatchability of the control eggs. Genomic DNA extracted from fins of randomly chosen F_0 individuals was screened (by Southern blot hybridization); the transgenes were retained in the host genome of all the randomly chosen adult transformants. Fin-positive presumptive founder parents were crossed with control counterparts and the DNA of randomly chosen F_1 progenies was screened for germ-line transformation. Southern analysis of chosen F_1 progenies revealed the persistence of ZpβypGH or ZpβrtGH in 53% of the F_1 progenies. Southern analyses of chosen F_1 progenies and the frequency (53% of F_1 ZpβrtGH and 53% of F_1 ZpβypGH) of transmission revealed the degree of mosaicism in F_0 transformants. Expression was confirmed from the 3–4 times elevated levels of activity of the reporter gene and 30–40% accelerated growth of transgenic F_0 and F_1 progenies.

1. Introduction

Isolation and construction of genes responsible for desirable traits and their transfer into the germ-line of broodstock may produce a quantum leap over traditional breeding and selection methods for the production of fast growing fish (Fletcher and Davies 1991). Many growth hormone (GH) cDNA and full length genes of mammalian, avian and piscine origin have been transferred into fish eggs to accelerate growth (Pandian and Marian 1994). Expression of the transgene depends mainly on the right choice of regulatory elements. So far, a variety of promoters and enhancers derived from mammalian and viral sources have been tested in transgenic fish (Inoue 1992). However, the expression of these heterologous genes was found unsatisfactory for one or other of the following reasons: (i) undesired tissue specific expression (e.g., Moav *et al* 1992a,b) and (ii) side effects leading to impairment of other functions (e.g., Friedenreich and Schartl 1990). Hence, an attempt was made to construct a transformation vector with a unique promoter Zp (Zona pellucida gene) of piscine origin to drive maximum expression of the fish growth promoting genes: yellow porgy growth hormone (ypGHcDNA) and rainbow trout growth hormone (rtGHcDNA).

Transgenic technology also involves an appropriate method of gene transfer (Fletcher and Davies 1991). Popular methods hitherto used for gene transfer in fish are (i) microinjection (Ozato *et al* 1986; Zhang *et al* 1990), (ii) incubation of sperm (Khoo *et al* 1992), and

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(iii) electroporation of sperm (Walker *et al* 1995) or eggs (Powers *et al* 1992b). Gene transfer studies have shown that electroporation is a more promising method for gene transfer (Marian *et al* 1997). It is possible to electroporate unfertilized eggs or just fertilized eggs, but the latter is shown to be ideal, as it results in higher frequency of transgenics (Marian *et al* 1997) and low percentage of deformities (Tsai *et al* 1995a). Besides, electroporation is an alternate method in fishes like the silurids, in which sperm cannot be stripped (Dunham and Smitherman 1987). A second objective of the present study is to subject just fertilized eggs of zebrafish to electroporation with one of the two recombinant transformation vectors: ZpβypGH (6·5 kb) and ZpβrtGH (4·9 kb) and to study (i) the efficiency of electroporation as an alternate gene transfer method, (ii) the efficacy of the recombinant genes ZpβypGH and ZpβrtGH as transformation vectors, and (iii) the integration, expression and transmission of the transgenes in presumptive transgenic founders and their F_1 progenies.

2. Materials and methods

2.1 *Source and description of the recombinant gene*

2.1a *Promoter*: Regulatory sequence of wf♀ gene of the winter flounder, *Pseudopleuconectes americanus* (Lyons *et al* 1993) was used as a promoter and designated as Zp-promoter (gifted by Dr R C Huang, Maryland, USA).

2.1b *Reporter gene*: LacZ of *Escherichia coli* coding for galactosidase enzyme derived from CMVβ was used as the reporter gene (gifted by Dr J D Chen, Keelung, Taiwan).

2.1c *Structural gene*: GHcDNA of the yellow porgy, *Acanthopagrus latus* (ypGH; Tsai *et al* 1991) and GHcDNA of rainbow trout, *Oncorhynchus mykiss* (rtGH; Agnellon and Chen 1986) was used as growth promoting gene (gifted by Dr H J Tsai, National Taiwan University, Taipei and Dr T T Chen, Connecticut, USA).

2.2 *Construction of recombinant plasmids: ZpβypGH and ZpβrtGH*

CMVβ was used as the basic vector for the construction of recombinant plasmids. Zp-promoter of wf♀ gene was excised from pGEM3Z + Zp (8·4 kb length) by double digestion *Eco*RI/*Nco*I. This fragment 1·2 kb in length was inserted into the basic vector CMVβ replacing CMV viral-promoter and designated as pZpβ (figure 1).

2.2a *ZpβypGH*: The ypGHcDNA (915 bp) was inserted between the Zp-promoter and β-galactosidase gene by blunt-end ligation at *Nco*I site. The resultant clone was 6·5 kb in length and was designated as pZpβypGH.

2.2b *ZpβrtGH*: *Hind*III fragment (970 bp) of rtGH cDNA was removed from RSVrtGH and inserted at *Nco*I site of pZpβ by blunt-end ligation. The recombinant clone of 4·9 kb in length was christened as pZpβrtGH.

2.2c *Protocol for electroporation*: Gene Pulser II (Bio-rad, USA) was used to carry out electroporation. Just fertilized eggs of the zebrafish were collected from natural spawn in the laboratory and subjected to electroporation with buffer (FPS). Optimal electroporatic conditions were identified from the following three steps: Initially, varying combinations of voltage (0·05–0·200 kV) and capacitance (1–50 µF in series I and II) were standardized, keeping resistance as a constant factor (figure 2). Subsequently, optimal pulse number (1, 2, 3, 4 or 5) was identified at optimal voltage, capacitance, maintaining resistance at ∞ (table 1). In the above two series, survival was taken as the criteria, based on which optimal voltage, capacitance and pulse number were standardized. In the third series of experiments, the optimal DNA concentration and pulse number were identified, using the presence of the transgene in the host fish (as determined by Southern blot analysis) as the criteria (figure 3). Thus, the DNA uptake by electroporated individuals was standardized by varying combinations of DNA concentration (50, 75, 100 and 200 µg/ml) and pulse number (1, 2, 3 or 4).

To verify the right combination that could internalize the transgene, genomic DNA extracted from different electroporated groups were subjected to Southern analysis. From each group that was subjected to a particular combination of DNA concentration and pulse number, genomic DNA was extracted from ten randomly chosen 4-week-old fry. After digestion with an appropriate restriction endonuclease, genomic DNA from different groups were subjected to Southern analysis. The group, in which the transgene was internalized into the host, was taken for further analysis. Subsequently, genomic DNA from the tail fin of putative adults (3 months old) was subjected to Southern analysis to trace the persistence of the transgene. Fin positive adult F_0 transformants were crossed with control counterparts to verify germ-line transmission of the transgenes. As in other cases, slot-blot and Southern analyses were carried out in the F_1 progenies also.

2.3 *Extraction of genomic DNA*

DNA was extracted from embryonic stages of the fish, tail fin pieces and different tissues of the adult fish. The samples were homogenized in lysis buffer [50 mM Tris (pH 8·0), 100 mM EDTA (pH 8·0), 100 mM NaCl, 0·1% SDS, 0·5 µg/ml proteinase K] and incubated at 37°C overnight. After incubation, samples were extracted with equal volume of phenol/chloroform (1 : 1) and once with equal volume of chloroform/isoamylalcohol (24 : 1). The resulting aqueous phase was then precipitated with

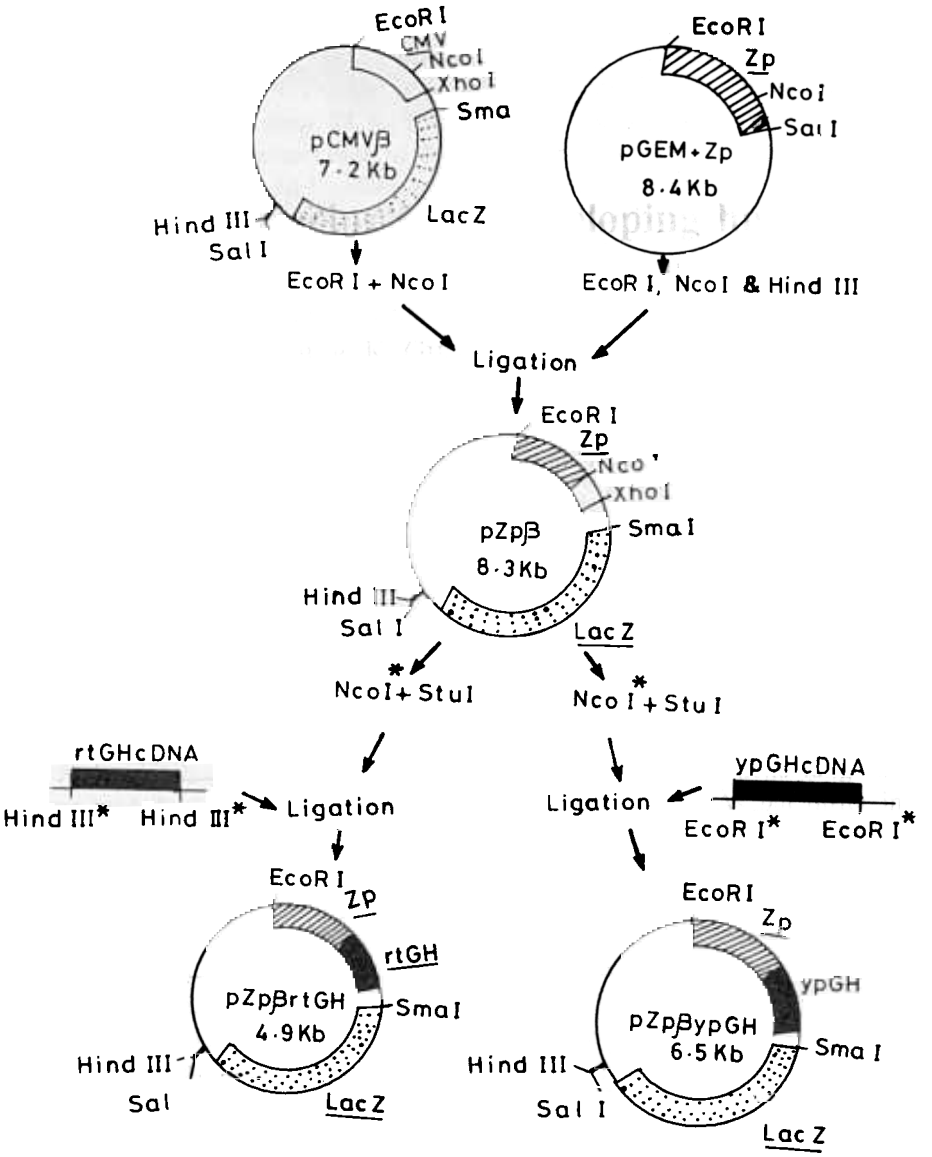

Figure 1. Protocol for construction of Zpβ*y*pGH and Zpβ*r*tGH. *Fill-in reaction.

2·5 volume of ethanol and 1/10th volume of 3 M sodium acetate (pH 5·2). The pelletted DNA was washed with 70% ethanol, air-dried and re-suspended in TE buffer.

2.4 *Slot blot analysis*

Samples containing 2 µg of genomic DNA was diluted in 50 µl of TE buffer denatured by boiling at 100°C for 10 min and chilled immediately in ice for 3 min. Later, they were spotted onto a nylon membrane using the slot blot apparatus of Hybri-BRL (USA). and dried. Subsequently, the membrane was placed on a Whatman filter paper saturated with 0·4 N NaOH for fixing and was given a quick rinse in solution containing 5 × SSC and 5 × SSPE. It was cross-linked on UV light and baked at 80°C for 30 min and stored at 4°C until further analysis.

2.5 *Southern transfer*

Southern transfer was performed following a modified procedure described by Southern (1975). Samples containing 10–20 µg of genomic DNA were electrophoresed on 0·8% agarose gel. After electrophoresis, the gel was de-purinated in 250 mM HCl for 10 min, denatured in 0·5 N NaOH and 1·5 M NaCl for 30 min and neutralized in buffer containing 1 M Tris and 1·5 M NaCl. The DNA was finally transferred onto a nylon membrane by the conventional capillary method and the DNA was fixed to the membrane by UV-cross linking or baking at 80°C for 30 min.

2.6 *Hybridization*

The membrane were pre-hybridized in a solution containing 5 × SSPE, 5 × Denharts buffer, 0·1% SDS, 50%

formamide and 500 µg/ml denatured salmon sperm DNA, for 4–6 h at 42°C, and hybridized (12–16 h) in the same solution containing the denatured probe. After hybridization, the membranes were given stringent washing (low or high depending on the need) step by step in decreasing concentration of SSC (2 × SSC + 0·1 SDS; 1 × SSC + 0·1 SDS; 0·1 × SSC + 0·1 SDS at 37°C), wrapped in Saran wrap and exposed to X-ray film (Kodak) for 24 h at –80°C in a autoradiogram cassette. Later the X-ray film was developed, fixed and photographed.

2.7 *Labelling of probe DNA*

Oligolabelling of the DNA was carried out using commercially available kits and as described by the manufacturer (New England Biolabs). Plasmid DNA (10–30 ng) was linearized by digesting with restriction endonuclease and denatured by boiling in a waterbath for 10 min and chilled immediately in ice for 3 min; 5 µl of 10 × labelling mixture, 5 µl of primer, 2 µl of each of dGTP, dATP and dTTP and 50 µCi (5 µl) of [^{32}P]dCTP were added. The total volume was made up to 49 µl with sterile distilled water. The reaction was initiated by adding 1 µl of Klenow enzyme and incubated at 37°C for 1 h. The probe was denatured in boiling water bath and chilled immediately in ice prior to hybridization. For hybridization analyses of Zp*β*ypGH electroporated samples, linearized Zp*β*ypGH plasmid (*Sal*I ~ 6·5 kb) was used as probe DNA; for Zp*β*rtGH electroporated samples, rtGH (970 bp/*Hind*III fragment) was used as probe DNA.

2.8 *Analysis of β-galactosidase activity*

The expression of the lacZ reporter gene was qualitatively and quantitatively assayed for *β*-galactosidase activity.

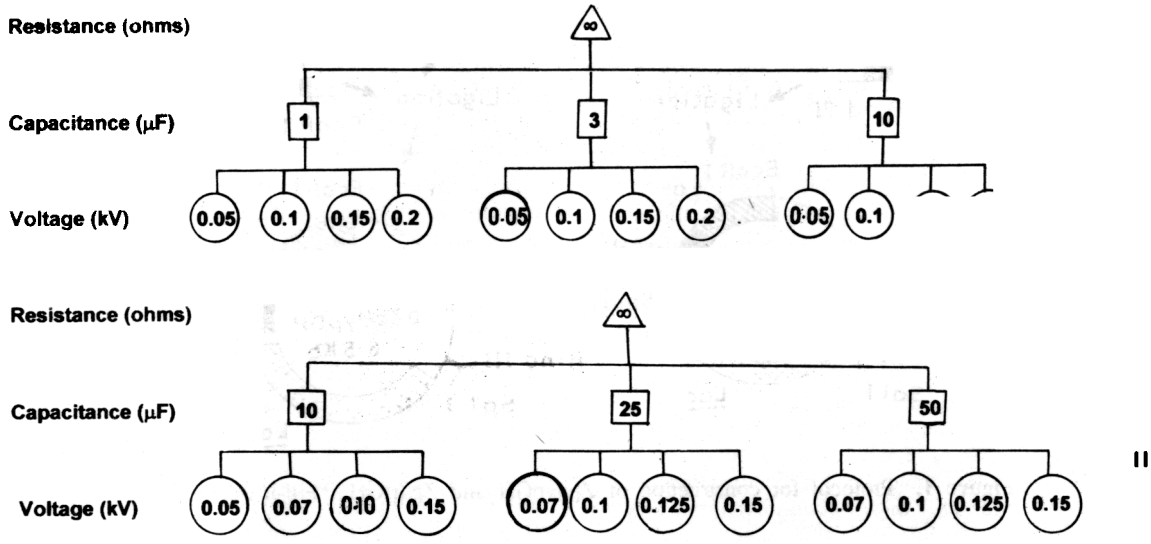

Figure 2. Alternative combinations of voltage and capacitance tested to standardize optimal conditions for electroporation of zebrafish eggs.

Table 1. Combinations of capacitance, voltage and pulse number to select the optima for electroporation of zebrafish eggs soaked in buffer.

Capacitance (µF)	Voltage (kV)	Pulse (No./ms)	Total eggs (No.)	Survival (%) at	
				Hatching	Feeding
25	0·07	1/1·5*	122	81	79
25	0·07	2/1·5, 1· *	97	72	62
25	0·07	3/1·7, 1· 1·6*	111	53	42
25	0·07	4/1·8, 1· 1·6	109	22	12
25	0·07	5/1·6, 1· 1·8, 5, ·7	139	10	0
Control	–	–	251	85	80

*Denotes selected pulse.

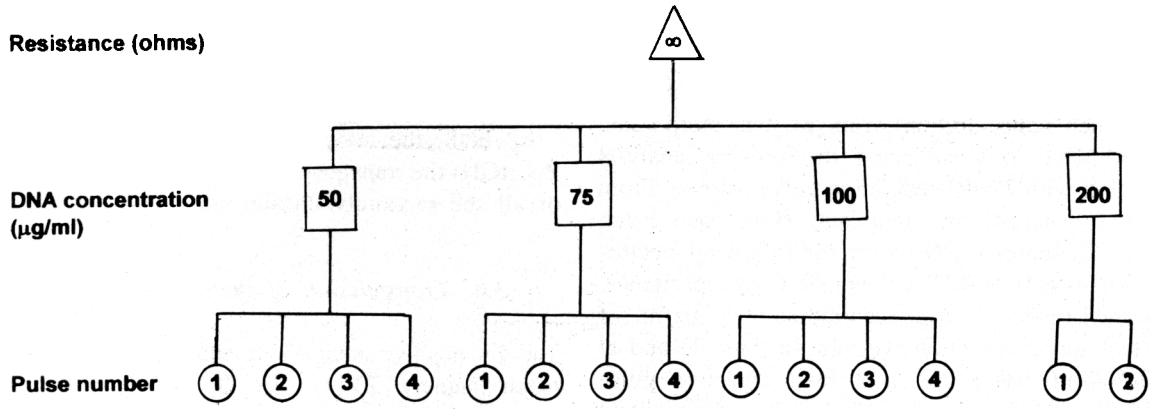

Figure 3. Alternative combinations of DNA concentration and pulse number to standardize optimal conditions.

Quantitative assay was carried out according to the modified method of Yano *et al* (1981). Samples from embryonic stages were prepared by homogenizing buffer containing 10 mM sodium phosphate (pH 7·0), 0·1 M NaCl, 1·0 mM $MgCl_2$, 0·1% NaN_3, and 0·1% BSA. To the homogenate, 0·5 ml of substrate solution containing O-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma, USA) was added and incubated at 30°C for 30 min. The reaction was terminated by adding 1·0 ml of stop solution reagent (1·0 M sodium carbonate). The samples were read in a spectrometer at 420 nm and 550 nm (visible light). The β-galactosidase activity was quantified using the formula

$$\beta\text{-gal activity (Muller unit/min)} = \frac{1000\,(420) - (1{\cdot}75 \times 550)}{\text{Time of assay (min)}}$$

2.9 *Detection of β-galactosidase activity by X-gal staining*

β-galactosidase activity was assayed in the F_0 and F_1 individuals to evaluate the expression of the transgenes. Phenotypic expression of the transgenes was also monitored by measuring the live weight and length of the presumptive transgenic fish. Electroporated and control eggs at different developmental stages (cleavage, gastrula, embryonic body formation and hatched) were taken for analysis following the method described by Ueno *et al* (1994). The samples were fixed in 1·25% glutaraldehyde/PBS solution for 6 h and washed in PBS (0·8% NaCl, 0·02% KCl, 0·144% Na_2HPO_4 and 0·024% KH_2PO_4, pH 7·4). Subsequently, the samples were incubated overnight in substrate solution containing 1 mM $MgCl_2$/PBS, 50 mM $K_3[Fe(CN)_6]$, 50 mM $K_4[Fe(CN)_6]$, 10% Triton X-100 and 2% X-galactosidase/dimethylformamide. They were washed in PBS and analysed under microscope and photographed.

3. Results

3.1 *Survival*

Maintaining resistance constant at ∞, 44 alternate combinations of voltage, capacitance, pulse number and DNA concentration were examined for their effect on survival and transgene integration. Survival of the electroporated eggs decreased with increasing voltage, < 0·15 kV. However, within the voltage range of 0·05–0·2 kV, the survival of the electroporated eggs decreased along with the decline in the capacitance i.e., > 10 μF. Subsequently, hatchability of the electroporated eggs (mock-treated) decreased (81–10%) with increase in pulse number at optimal voltage (0·07 kV) and capacitance (25 μF). Based on these preliminary experiments (where hatchability was taken as the deciding factor), the following optimal conditions were chosen for further electroporation: 0·07 kV, 25 μF, 1–3 pulses (one second apart) and ∞ resistance (table 1). Survival of the electroporated eggs (mock treated) at optimal conditions ranged from 53 to 81% against 85% in the untreated control group. The decreases in the hatchability of the electroporated eggs indicate that the mortality was due to electroporation stress. However, no deformed individuals were observed in either series.

3.2 *DNA uptake and transgene integration*

Four different concentrations of the two transgenes were tested (50, 75, 100 and 200 μg/ml), each at four different pulse conditions (1, 2, 3, and 4 pulses one second apart) to identify the optimal combination that could internalize the transgene into the host genome (figure 3).

3.3 *Southern analysis of ZpβypGH and ZpβrtGH electroporated samples*

Genomic DNA extracted from pools of 4-week-old fry (5 fry for each concentration) from each electroporated batch (ZpβypGH) was subjected to Southern analysis after digestion with *Eco*RI restriction endonuclease. From a total of 7 batches, two replicates from each batch were analysed. Genomic DNA from the following electroporated samples at 0·07 (kV) voltage, 25 (μF) capacitance, ∞ (ohms) resistance and a concentration of 75 μg/ml of DNA at two pulse conditions (figure 4a, lane 1) and at 100 μg DNA/ml concentration at one and two pulse conditions (figure 4a, lanes 2, 4, 5) hybridized positively to the probe DNA. *Eco*RI digestion of the genomic DNA was expected to release two fragments of 3·8 and 2·7 kb (figure 4a, lane 6). The sample in lane 5 hybridized at two sites of 6·5 and 5·0 kb, in addition to the expected fragments. This implies a possible integration into the genome of the host. Clearly electroporation at DNA concentration of 100 μg/ml and at 2 pulse conditions (0·07 kV and 25 μF) can internalize the foreign DNA into the genome of the host.

Similarly, Southern analysis of genomic DNA extracted (digested with *Kpn*I) from pools of 4-week-old fry ZpβrtGH electroporated groups (5 fry per group) revealed a positive signal from five groups; a single predominant band at 4·9 kb was observed in groups electroporated at a DNA concentration of 50 μg/ml, at single or double pulse conditions and at a DNA concentration of 75 μg/ml at a single pulse condition. However, a single predominant band at 6·9 kb was observed in lane 1 (figure 4b) indicating integration of the transgene into the genome of the host. Thus electroporation at a DNA concentration of 100 μg/ml and at two pulse conditions, can internalize the foreign DNA into the genome of the host.

3.4 *Retention of transgenes in the adult transformants ZpβypGH*

From 3-month-old ZpβypGH individuals, 14 individuals were chosen randomly to trace the persistence/integration of the transgene. Southern analysis of the tail fin DNA after *Sal*I digestion revealed the presence of the transgene in all the transformants (figure not shown). The probe hybridized at higher level compared to the plasmid size (6·5 kb), indicating the integration of the transgene into the host genome. However, instead of a crisp band, a smear was observed, which might be due to degradation of genomic DNA.

3.5 *ZpβrtGH*

In all the ten randomly chosen adult ZpβrtGH individuals, a predominant band at > 25 kb was observed on Southern analysis after *Kpn*I digestion (figure 5). Nevertheless, bands of different sizes in addition to the 25 kb positive band were also observed in the ninth individual (figure 5, lane 10) i.e., 23 kb, 9 kb and 6·5 kb, implying possible integration of the transgene at two different sites of the host genome.

In both the electroporated groups (ZpβypGH and ZpβrtGH) the transgenes were retained in the host genome of all the randomly chosen adult transformants.

3.6 *Transmission of the transgenes ZpβypGH*

Tail fin positive adult (3 months old) ZpβypGH transformants namely ZY_1 ♀, ZY_2 ♂, ZY_3 ♀, ZY_4 ♀, ZY_5 ♀,

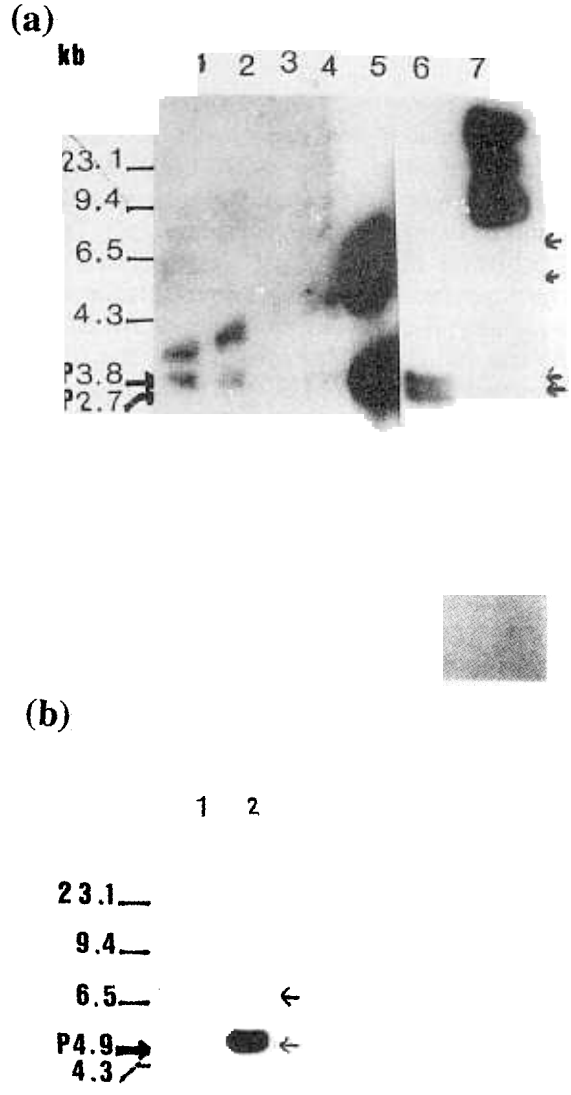

Figure 4. Southern hybridization of genomic DNA from different electroporated groups digested with *Eco*RI (**a**) and *Kpn*I (**b**) prior to electrophoresis. (**a**) Lanes 1–5, test sample; lane 6, positive control ZpβypGH/*Eco*RI (indicating the size of the plasmid DNA); lane 7, uncut ZpβypGH plasmid DNA. (**b**) Lane 1, test samples; lane 2, positive control ZpβrtGH digested with *Kpn*I; P, positive control or plasmid ZpβrtGH; arrow indicates the position of the positive hybridization response.

ZY_6 ♂ and ZY_7 ♂ were crossed with control counterparts. Hatchability of the F_1 progenies of the presumptive ZpβypGH transformants was 81%, against 85% in the control. However, these differences were not significant ($P < 0.05$).

3.6a *Slot-blot analysis*: Genomic extracts from tail fins of randomly chosen F_1 progenies from different crosses were hybridized with the ZpβypGH plasmid. Of 46 samples tested, 14 individuals showed intense hybridization to the probe DNA and 18 samples showed a weak signal (figure 6a, 1A, 1B, 2A, 2B).

3.6b *Southern hybridization of F_1 ZpβypGH transformants*: To confirm persistence/integration of ZpβypGH in F_1 progenies, 18 fry (4-week-old) from ZY_1 ♀ × C ♂ cross were subjected to Southern analysis individually after restriction digestion with *SalI* digestion. Samples in lanes 2 and 18 (figure 6b) hybridized positively to the probe DNA. *SalI* digest of the transgene was expected to release a fragment of 6·5 kb, whereas in the present hybridization, samples showed predominant positive signals at 9·5 kb (lanes 2 and 18). In addition, two bands at 15 and 23 kb for the 18th test sample were also observed in lane 18. This result implies the possible integration of the transgene at a single site of the host genome for the sample of lane 2 and at two independent sites for the sample in lane 18 (figure 6b). Hence of the total of 46 samples analysed in slot blot, 32 showed the persistence of the transgene and of the 18 samples analysed in Southern blot, 2 samples showed the persistence and integration of the transgene.

3.7 *Transmission of the transgene ZpβrtGH*

Tail fin positive adult (3 months old) ZpβrtGH transformants (G_0) were crossed with the control counterparts (namely ZR_1 ♂, ZR_2 ♂, ZR_4 ♀, ZR_5 ♀, ZR_6 ♀, ZR_9 ♂). There was also significant decrease ($P < 0.05$) in the mean hatchability of F_1 progenies (70%) when compared to controls (95%).

3.7a *Slot-blot analyses*: Subsequently, genomic DNA from tail fins of 46 F_1 progenies (4-week-old) were analysed to trace the transmission of the transgene. Of these 11 individuals showed intense hybridization to the probe DNA and 16 showed weak signal on random analysis (figure 7a, 1A and B, 2A and B). Thus 27

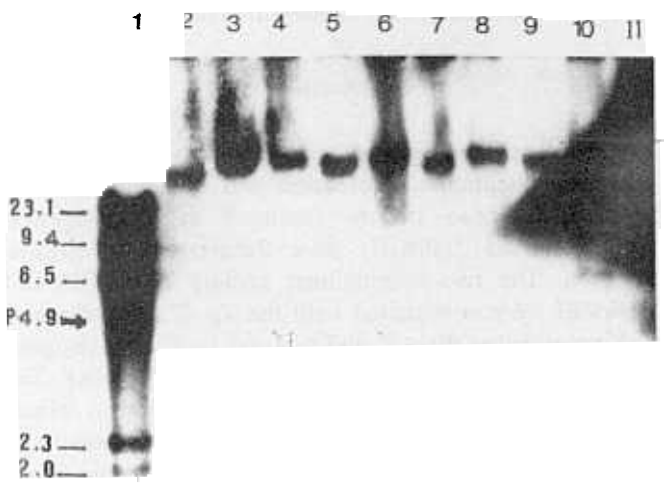

Figure 5. Southern analysis of genomic DNA from F_0 adult prtGH zebrafish transformation digested with *KpnI* prior to electrophoresis. Lane 1, λ marker; lanes 2–11, test individuals; P, positive control (size of plasmid DNA used for electroporation).

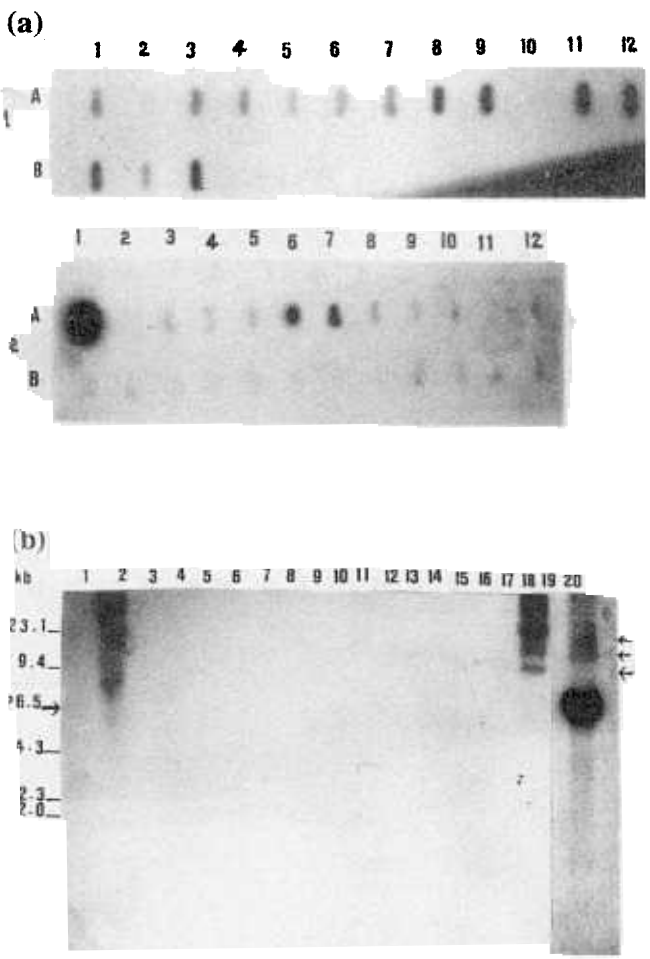

Figure 6. (**a**) Slot-blot Analysis of tail fin tissues from presumptive ZpβypGH transgenic fish 1A1, 2A1-positive control, {1A 2-12, 1B 1-12, 2A 2-12, 2B 1-12}, test samples. (**b**) Southern analyses of genomic DNA from F1 ZpβypGH transformants digested with *SalI* prior to electrophoresis. Lane 1, λ marker; lanes 2–19, test samples; lane 20, positive control ZpβypGH digested with *SalI*; P, positive control.

samples hybridized positively (58%) to the probe DNA from a total of 46 samples analysed.

3.7b *Southern analyses*: 14 F_1 progenies (4-week-old) from the ZR_5 ♀ × C ♂ cross were subjected to Southern analyses individually, after digestion with *Kpn*I, to confirm integration of the transgene (figure 7b). Four individuals responded positively at 25 kb size, i.e., larger than the plasmid (4·9 kb), confirming the integration of the transgene into the host genome. Thus 27 samples from slot-blot analysis and 4 samples from Southern analysis hybridized positively to the probe DNA from a total of 60 samples analysed (31/60, 53%).

It is clear from these random analyses of the F_1 progenies of both ZpβypGH and ZpβrtGH transformants that the transgenes have been transmitted to the respective F_1 progenies. Moreover, Southern analysis confirmed the integration of the transgene into the host genome (germline positive). Exceptionally, ZY_5 ♀ × C ♂ (though fin positive) did not give any fin positive F_1 progenies on random analysis, indicating probable mosaicism in the F_0.

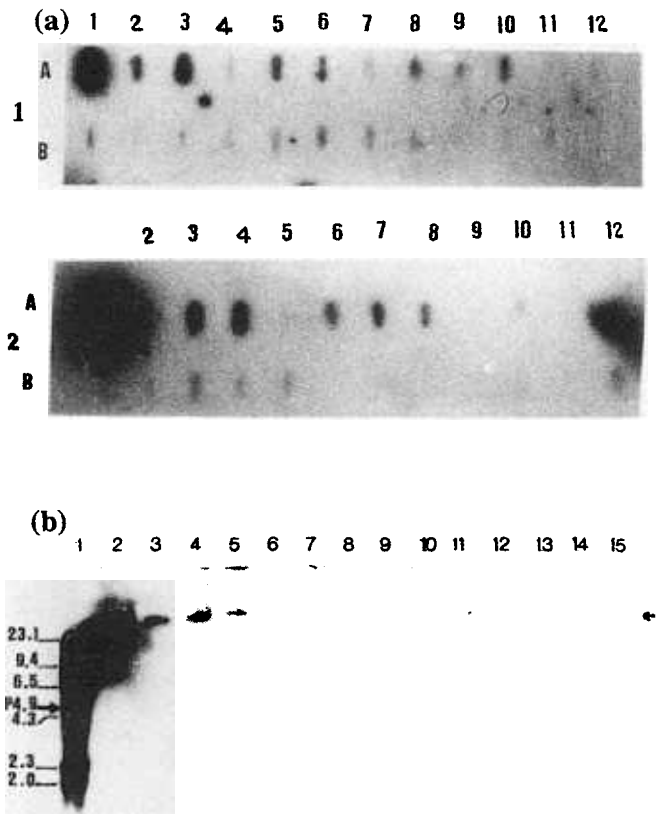

Figure 7. (a) Slot-blot analysis of tail fin tissues from presumptive ZpβrtGH transgenic fish 1A1, 2A1-positive control, {1A 2-12, 1B 1-12, 2A 2-12, 2B 1-12}, test samples. (b) Southern analyses of genomic DNA from F1 ZpβrtGH transformants digested with *Kpn*II prior to electrophoresis. Lane 1, λ marker; lanes 2–15, test samples; P, positive control.

3.8 *Expression*

Analysis of the putative ZpβypGH and ZpβrtGH transformants at translation level gave positive results indicating the functional ability of the integrated lacZ marker gene. Since lacZ and GH genes were in the same reading frame and were driven by the same Zp promoter, β-gal activity was taken as an indirect index of gene expression.

The β-gal activity of endogenous origin could not be detected in the control embryos until the blastula stage (table 2). But in the electroporated individuals, lacZ expression was quantitatively traced in the 32-cell stage prior to the blastula stage confirming the exogenous lacZ expression (table 2). In addition to the regular broad pattern in the yolk sac of the hatched fry, defined blue spots were observed in the tail region (figure 8). In the other embryonic stages, the level of β-gal activity was 2 times higher in the presumptive transformants indicating the presence of measurable quantity of β-gal of exogenous origin (table 2).

Growth of the presumptive transgenic fish was measured at regular intervals as a measure of phenotypic expression of the GH gene. The transformants grew (30–40%) faster than the control counterparts. Early maturity was encountered in the transformants. The observed faster growth of the transformants was maintained throughout the growth phase and even after sexual maturity (figure 9). A constant increase in the wet weight of putative transgenic fish was observed even after maturity when compared to control fish.

4. Discussion

The present investigation represents a comprehensive study on integration, expression and transmission in a fish, into whose freshly fertilized eggs transgenes, ZpβypGH and ZpβrtGH, were transferred by electroporation. The two recombinant vectors ZpβypGH and ZpβrtGH were constructed with the Zp (Zona pellucida) fish promoter at their 5′ end and the lacZ reporter gene at their 3′ end, of the target sequence (GHcDNA). The promoter region of (Zp) wf ♀ gene derived from winter flounder was chosen in the present study for the following reasons. The wf ♀ gene is known to express predominantly in the liver and secondly, it expresses constitutively throughout the year (Lyons *et al* 1993). Moreover the Zp promoter does not require transient ions to be activated unlike the metallothionein promoter (Fletcher and Davies 1991). Since the Zp promoter was linked to growth

Table 2. β-galactosidase activity in the presumptive (G_0) and the control, as function of developmental stages of the zebrafish, whose just-fertilized eggs were subjected to electroporation.

	β-galactosidase activity at the embryonic stages					
Plasmid	2-cell	4-cell	32-cell	Blastula	Gastrula	Hatching
Control	–, 0	–, 0	–, 0	–, 0	+, 5·0	+, 15·0
ZpβypGH	+, 4·0	+, 5·0	+, 5·8	+, 6·5	++, 12·0	++, 38·0
ZpβrtGH	+, 3·8	+, 4·7	+, 5·5	+, 7·0	++, 10·0	++, 32·0

–, Denotes total absence; + and ++, presence and intensity of the β-gal activity respectively; values indicate β-gal activity as Muller unit/min.

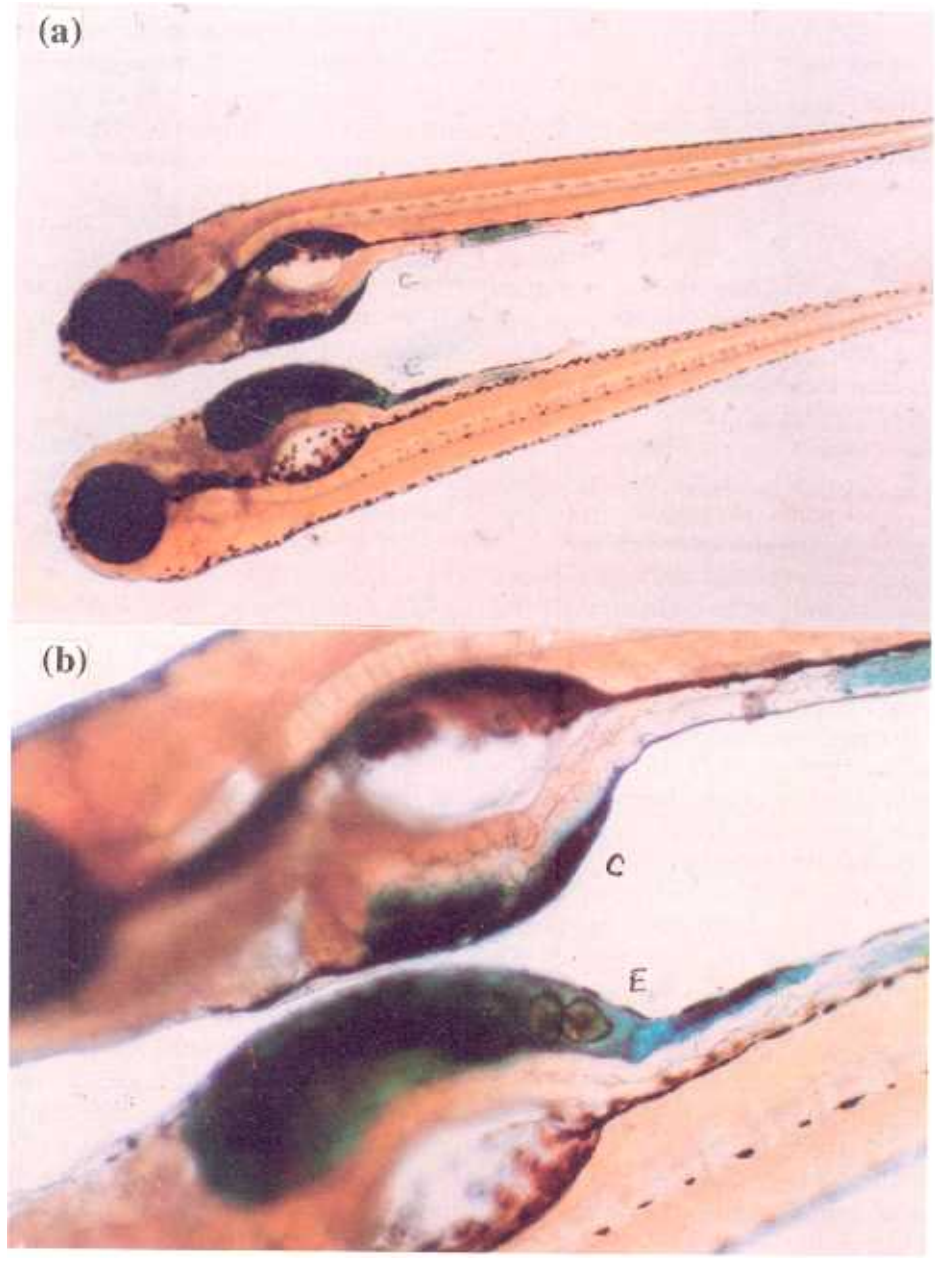

Figure 8. β-galactosidase activity in (**a**) control (C) and ZpβypGH electroporated transformant (E). (**b**) Control (C) and ZpβypGH electroporated transformant (E) enlarged view.

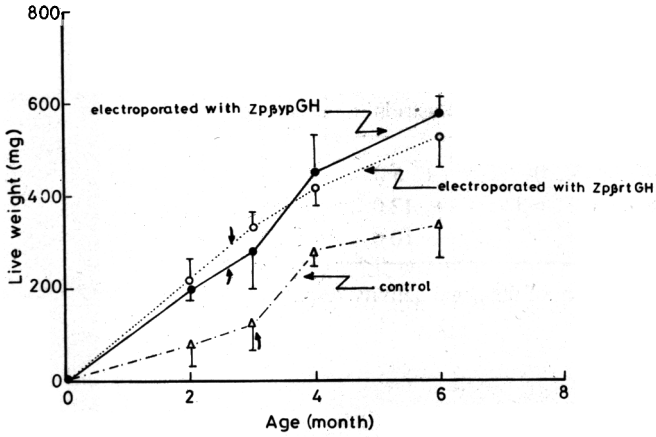

Figure 9. Growth difference between the control and electroporated transformants (arrows indicate the onset of sexual maturity).

promoting genes, its specific ability to express predominantly in the liver tissue may facilitate effective interaction between GH and the GH receptors on the liver cell membranes. However, the transgenes could not induce dramatic growth, i.e., such as those of 10 or 30 times larger transgenic fish (Du *et al* 1992b; Devlin *et al* 1994a). It is not clear whether tissue- or species-specificity of the Zp promoter is responsible for this low expression, resulting in lower acceleration of growth. Hence the utility of the Zp promoter remains questionable.

Low survival of the electroporated embryos (average 66%) compared to the control (85%) indicates the stress suffered by eggs subjected to electroporation. A direct correlation between hatchability and electroporatic conditions such as field strength or pulse number was apparent. Similar findings have been reported by Lu *et al* (1992) in medaka, Powers *et al* (1992b) and Kavumpurath *et al* (1993) in zebrafish. Despite the fact that the survival and hatchability of the electroporated embryos are species-specific characters (Powers *et al* 1992b), a similar declining trend in the hatchability of the treated embryos with increasing field strength or pulse length was observed in all the tested species (Sin *et al* 1997). However, the present study reports the lowest field strength (0·07 kV) at which electroporation has successfully transferred the desired transgene into the host system.

Electroporation of the zebrafish embryos at different concentrations (50, 75, 100 and 200 µg/ml) and pulse number (1, 2, 3, 4) resulted in greater mortality as DNA concentration or pulse number increased. The zebrafish embryos electroporated at 200 µg DNA/ml concentration suffered < 80% mortality. Incidentally, Marian (1995) reported that 100 µg DNA/ml concentration was the critical dose for *Heteropneutes fossilis* that was subjected to electroporation. A comparison between zebrafish and medaka, whose eggs were subjected to identical microinjection procedures indicated that zebrafish are more sensitive to injected DNA, with a dose of 50 µg/embryo being lethal to 84% of the embryos (Stuart *et al* 1988a; Vielkind *et al* 1990; Fletcher and Davies 1991). Hence, the amount of exogenous DNA that can be tolerated by early embryos appears to differ considerably between species (Stuart *et al* 1988b, 1990; Fletcher and Davies 1991; Pandian and Marian 1994).

Pulse number or pulse length (length of each pulse) are important decisive factors in electroporatic experiments, because they control the period during which the cells become permeable to exogenous DNA. In the present study, the 2 pulse condition at 100 µg DNA concentration was found to be optimum based on both hatchability and transgene integration. In the loach and crucian carp, the gene transfer and copy number of the transgene per genome were found to increase proportionally with increasing pulse duration (0·48–4·8 ms) and voltage (Xie *et al* 1993).

In most of the electroporation experiments, detection of the transgene was carried out using DNA extracted from fry of different ages. Powers *et al* (1992b, 1995) observed a decrease in the putative transgenic zebrafish and red abalone with advancing age. Similarly, Marian (1995) reported a decline in transgenics from juvenile (27%) to adult stage (12%) of *H. fossilis*. Thus the transgenic founders detected range from 4% in medaka (Inoue *et al* 1990b), 12% in *H. fossilis* (Marian 1995) to 35–75% in zebrafish (Powers *et al* 1992b). Surprisingly, a random analysis of adult zebrafish transformants in the present study (for both the transgenes) showed integration of the transgene in all the 10 (Zpβ ypGH) or 14 (Zpβ rtGH) surviving individuals. A possible reason for such high percentage of transformants may be that the homologous promoter Zp facilitated stable integration of the transgene into the genome of the fish (Friedenreich and Schartl 1990; Alam *et al* 1996).

Stability of the integration in the zebrafish was established by transmission studies. The level of integration of the transgene into the germ-line of transgenics is perhaps determined by the number of germ-line precursors present at the time of germ cell differentiation. Approximately 53% of F_1 progenies from Zpβ ypGH and Zpβ rtGH F_0 transformants inherited the transgene. The percentage of F_1 transformants observed in the present study is clearly 2 to 3 times higher than that reported for Indian catfish (15%) by Marian (1995) and zebrafish (20%) by Stuart *et al* (1988b). However, it is lower than that reported by Inoue *et al* (1990b) in medaka (100% in F_1). A reason suggested by Stuart *et al* (1988b) for the low frequency of F_1 transformants (20%) among F_1 progenies is that the founder parents (though fin positive)

were perhaps mosaics, especially in their germ cells. Contrary to these observations, Inoue *et al* (1990b) reported 100% transmission to the F_1 progenies. This may be due to the fact that the transgene was inserted into both the chromosomes. In the present study, frequency of transmission to F_1 progenies of zebrafish therefore suggests that the transgene is possibly inserted into a single chromosome.

The reporter gene lacZ or β-galactosidase was used as an indirect measure of GH activity as both of these genes were driven by the same promoter. The present study is the first one to report qualitative and quantitative expression of the lacZ gene in the putative transformants, whose freshly fertilized eggs were electroporated. Fertilizing salmon eggs with electroporated sperm (RSV lacZ), Xie *et al* (1993) failed to observe the expression of β-gal. However, Tsai *et al* (1995c) and Ueno *et al* (1994) reported transient expression of the lacZ gene in microinjected batches of medaka and crucian carp respectively. Hence, expression of the transgene can be possibly altered by the adjacent gene sequence, because of the position of the transgene in the genome (position effect), or by deletion of the transgene, or by reverse orientation of the transgene while integrating into the genome (Iyengar *et al* 1996).

In zebrafish, Bayer and Campos-Ortega (1992) observed specific expression of the lacZ gene in the primary neuron, when RSV lacZ was injected. Similarly, lacZ expression was encountered only in the muscle tissues of the presumptive transgenic zebrafish (Muller *et al* 1997). Thus, these evidences suggest that expression of the lacZ gene may depend on the tissue-specificity of the promoter.

Phenotypic growth has been regarded as a measure of expression of the transgene and has been measured in transformants, F_0 (Devlin *et al* 1994a,b), F_1 (Du *et al* 1992a,b,c), and F_2 (Pandian *et al* 1991). Devlin *et al* (1994a,b) reported a major breakthrough in transgenic biology by producing salmon fish 33 times larger than their control siblings. This may be because he inserted an all-salmon gene construct. Du *et al* (1992c) reported the production of fish 10 times larger than the controls, which he accounted for by the use of an all-fish sequence. However, it is not clear whether their claim of super-fast growth of the transgenic fish was sustained in subsequent progenies. Many other scientists claimed only a modest growth increment in the respective transgenic fish. In the present study also a modest increase of 30–40% growth was noted, but it must be indicated that such modest growth was maintained for a period of 6 months (figure 9) and such a faster growth was also punctuated by early onset of maturity indicating that the overall growth acceleration of the transgenic is faster than the control. Another remarkable feature of these modestly fast growing fish was the absence of wide individual variations in the wet weight of the transformants. Individual variations in the growth performance of transgenics were so high that previous workers had to group them under three categories, gigantism, dwarfism and normal (Palmitter *et al* 1982; Devlin *et al* 1994a; Marian 1995). The absence of size hierarchy and sustained sustenance 30–40% faster growth in the transgenic zebrafish indicate perhaps that faster growth is regulated and a kind of homeostasis has been reached in the transgenics.

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