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Uniform GFP-expression in transgenic medaka (*Oryzias latipes*) at the F0 generation

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Abstract

A green fluorescent protein (GFP) cDNA flanked by inverted terminal repeats (ITR) of adeno-associated virus was constructed. The construct sharply improved the efficiency and specificity of the transient expression of genes driven by two general promoters (cytomegalovirus and medaka β-actin) and one muscle-specific promoter (zebrafish α-actin) in transgenic medaka. In addition, treatment with ITR sequence-containing constructs resulted in a dramatic increase in the number of embryos showing uniform GFP-expression at F0. Of the GFP-positive embryos, 34.6% (81/234), 10% (10/60), and 18% (38/212) showed homogenous GFP-expression for the derivative constructs of the cytomegalovirus, α-actin, and β-actin promoters, respectively. As a result of uniform GFP-expression, green fluorescence in founders was (a) extended for an entire lifetime without degradation, and (b) transmitted as a genetic trait to F1 and F2 progeny of some transgenic lines via Mendelian inheritance. A Southern blot analysis revealed a random integration of the transgene into the genome of founders and progeny in both head-to-tail and tail-to-tail concatemerization patterns. Interestingly, some transgenic medaka with uniform and strong fluorescence could be visually noticeable to the unaided eye.

Introduction

Due to its transparent chorion, easy injectability, controllable spawning, short generational lifespan, and ability to breed year-round, medaka (*Oryzias latipes*) is one of several vertebrates frequently used for *in vivo* studies of the functions, regulation, and inheritance of transgenes (Ozato et al., 1986). Transgenic fish studies make use of genes that (a) are driven by both heterologous and homologous sources of regulatory elements, and (b) originate from constitutive or tissue-specific expression genes. Control elements include genes from antifreeze protein (Fletcher et al., 1988; Du et al., 1992; Tsai et al., 1995a), mouse metallothionein (Maclean et al., 1987), chicken δ-crystalline (Ozato et al., 1986), carp β-actin (Hew, 1989; Iyengar & Maclean, 1995), salmon histone H3 (Chan & Devlin, 1993; Hanley et al., 1998), carp α-globin (Yoshizaki et al., 1991), *Xenopus* elongation factor (Lin et al., 1994), and a number of viruses (Guyomard et al., 1989; Zhang et al., 1990; Tsai et al., 1995b). However, there are important drawbacks to the use of these DNA elements in transgenic fish, including low expression efficiency (0–20%) (Culp et al., 1991; Higashijima et al., 1997) and the mosaic expression of transgene patterns (Stuart et al., 1990; Ju et al., 1999). At the F0 generation, transferred genes are rarely expressed as a homogeneous pattern; the uniform expression of a reporter gene in transgenic fish normally occurs in generation F1 or F2.

The microinjection into medaka eggs of a *lacZ* reporter gene driven by the medaka β-actin promoter results in the transient expression of the *lacZ* gene, even in the F1 generation, though expression is low and highly mosaic (Takagi et al., 1994). Hamada et al. (1998) reported a similar result in medaka embryos derived from eggs microinjected with green fluorescent protein (GFP) fused with the medaka β-actin promoter. Neither a heterologous nor homologous promoter serves to markedly decrease transgenic

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mosaicism. Another approach to decreasing mosaic expression entails the introduction of foreign DNA fragments (i.e., sperm-mediated gene transfer) into the gametes of embryos prior to their first cleavage (Khoo et al., 1992; Symonds et al., 1994; Tsai et al., 1995a, 1997). However, it has been noted that the transferred DNA fragments are not evenly distributed throughout different tissue types of transgenic samples. Collas and Alestrom (1998) reported that the binding of nuclear localization signal (NLS) peptides to plasmid DNA increases gene transfer efficiency and enhances transient expression. Nevertheless, it is generally accepted that transgene integration mosaicism is inevitable (Liang et al., 2000). Recently Gibbs and Schmale (2000) reported that GFP driven by carp β-actin 5′ sequence, including insulator, promoter and first intron, was expressed from the egg to the adult. But this construct is less versatile in term of studying other promoters. Besides, the uniform phenotype is not stably inherited from the uniform GFP-expression parent. Therefore, the search continues for a simple but effective means of improving the F0 expression of transgene and the stable transmission of phenotype in transgenic fish.

Type 2 adeno-associated virus (AAV) contains an approximately 4.7 kb-long single-strand DNA genome with two inverted terminal repeats (ITR) (Srivastava et al., 1983); each ITR, consisting of 145 nucleotides, is in the form of a palindromic hairpin (Samulski et al., 1989). Fu et al. (1998) reported that the inclusion of an ITR in a DNA plasmid significantly increases the efficiency of transgene expression in *Xenopus* embryos. Accordingly, we set out to learn whether an ITR sequence can also enhance the ubiquitous and tissue-specific expression of transgenes in transgenic fish. If true, it might solve some of the difficulties encountered when using conventional DNA constructs in fish gene transfers. The present report describes the ability of a useful DNA construct, flanked at both ends by ITRs, to increase the efficient expression of transgenic genes in medaka. A uniform transgene expression was achieved in the F0 and the following two generations.

Materials and methods

Plasmids

All plasmids used in this study are presented in Figure 1. The pGREEN-LANTERN (pCMV-EGFP, 5.03 kb) (GIBCO) contained an enhancer and promoter of an immediate early gene of cytomegalovirus (CMV) fused with mutated GFP cDNA (S65T) and SV40 polyA. EGFP cDNA (740 bp) was obtained from pEGFP-1 (Clontech) and digested with *Hin*dIII and *Not*I; ends were filled in. A blunted fragment was ligated to a *Stu*I fragment from vector CS2ITR (5 kb; provided by S.M. Evans) (Fu et al., 1998). The resulting plasmid, pCMV-EGFP-ITR (5.74 kb), contained a CMV promoter fused with EGFP cDNA, followed by SV40 polyA and flanked at both ends by AAV-ITR.

The pα-actin-EGFP (8 kb) (αp-G-BS; Higashijima et al., 1997) used in this study contained a zebrafish α-actin promoter fused with EGFP cDNA and SV40

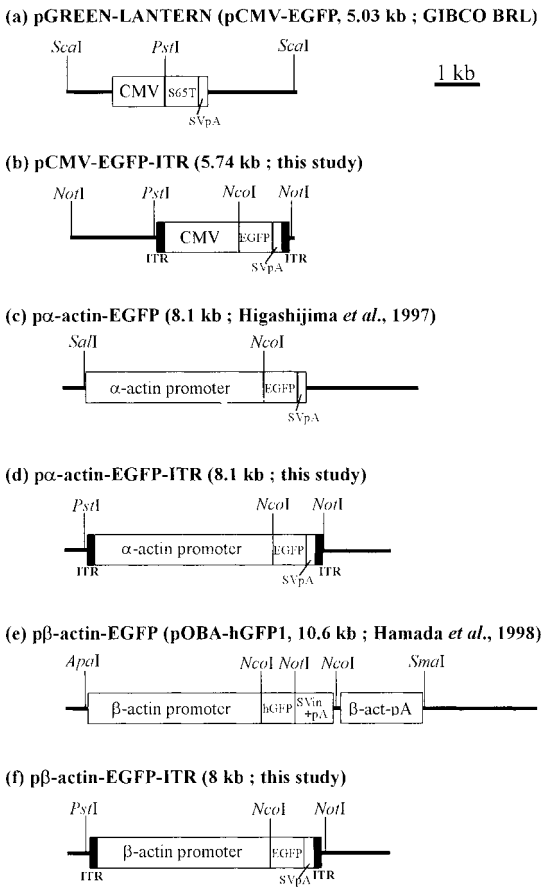

Figure 1. Plasmid constructs used for gene transfer. Thick lines represent plasmid vectors; scale bar indicates 1 kb. EGFP, hGFP, and S65T represent high expression mutations of GFP cDNA. CMV, cytomegalovirus immediate early enhancer/promoter; SvpA: polyadenylation signal of SV40; ITR: inverted terminal repeats of adeno-associated virus; SVin + pA: intron and polyadenylation signal of SV40 small t antigen gene; β-act-pA: polyadenylation signal of medaka β-actin gene.

polyadenylation signal (a gift from S.I. Higashijima). The zebrafish α-actin promoter was obtained from pα-actin-EGFP digested with *Sal*I and *Nco*I. The resulting 3.9 kb fragment was ligated to a 4.2 kb *Sal*I-*Nco*I fragment from pCMV-EGFP-ITR. This in turn resulted in an 8.1 kb pα-actin-EGFP-ITR plasmid, in which EGFP cDNA was driven by the α-actin promoter and flanked at both ends by AAV-ITR.

The pβ-actin-EGFP (10.6 kb) (pOBA-hGFP1; Hamada et al., 1998) used in this study contained a medaka β-actin promoter fused with hGFP1 cDNA, an intron of small t antigen, SV40 polyA, and polyA from the medaka β-actin gene (a gift from K. Ozato). The medaka β-actin promoter was obtained from pOBA-hGFP1 and digested with *Sal*I and *Nco*I. The 3.8 kb fragment end-product was ligated to obtain a 4.2 kb *Sal*I-*Nco*I fragment from pCMV-EGFP-ITR. The final result was an 8 kb pβ-actin-EGFP-ITR plasmid in which EGFP cDNA was driven by the β-actin promoter and flanked at both ends by AAV-ITR.

Preparation of microinjected DNA

All DNA plasmids were prepared via ultra-centrifugation with cesium chloride and ethidium bromide gradient (Radloff et al., 1967). Linearization of pCMV-EGFP and pCMV-EGFP-ITR was performed with *Sca*I and *Not*I, respectively. The molecular masses of the pCMV- EGFP and pCMV-EGFP-ITR fragments were 5.03 and 5.74 kb, respectively; for the pα-actin-EGFP-ITR and pβ-actin-EGFP-ITR DNA fragments, molecular masses were 5.1 and 5.0 kb, also respectively. The plasmids of pα- and pβ-actin-EGFP-ITR were restricted by *Pst*I and *Not*I. pα-actin-EGFP and pβ-actin-EGFP were linearized by *Sal*I and by *Apa*I and *Sma*I, respectively. The molecular mass for the pα-actin-EGFP DNA fragment was 8 kb and for the pβ-actin-EGFP DNA fragment 7.6 kb. All DNA fragments used for microinjection were eluted from agarose gel following electrophoresis.

Cytoplasmic microinjection

The procedures followed for cytoplasmic microinjection are described in detail in Kinoshita and Ozato (1995) and Kinoshita et al. (1996). Briefly, orange-red strain medaka were maintained under artificial conditions of 14 h light and 10 h darkness at 26°C and maintained on a diet of Tetramin (Tetra, Germany). Eggs were collected within 30 min of fertilization and attaching filaments removed. Fertilized eggs were kept at 6°C until microinjected with DNA fragments at a concentration of 10 μg/ml (300 pl) into their cytoplasm prior to the first cleavage. Injected eggs were incubated at 26°C in distilled water.

Fluorescent microscopy and cryosectioning

Embryos were observed under a bright field with a dissecting stereomicroscope (MZAPO, Leica, Germany). Dark field illumination for detecting green fluorescence was performed with a stereomicroscope equipped with a GFP Plus filter (480 nm). Photographs were taken using an MPS60 camera loaded with ISO 400 film and equipped with a controller for film exposure time (Leica, Germany).

In order to examine the distribution of GFP-expression in the tissues of transgenic medaka, 11 d post-fertilization larva which having uniform GFP-expression on appearance were sectioned and observed under fluorescent microscopy. Larva were fixed for 30 min in 4% paraformaldehyde at 4°C, embeded in cryomatrix (Shandon, USA) and frozen at −20°C. Cryostat sections (Cryostat Microtome, HM500 OM, Microm, Germany) with 15 μm thickness were mount on slides and observed the GFP fluorescence immediately.

Genomic DNA extraction

Genomic DNA was extracted using methods described in Chong and Vielkind (1989). Medaka samples were digested with proteinase K (100 μg/ml) solution containing 0.5% SDS for 16 h at 55°C. DNA was then purified using phenol-chloroform extraction and ethanol precipitation. Genomic DNA fragments extracted from embryos, fry and adult of medaka derived from β-actin-EGFP-ITR-injected eggs were digested with *Nco*I and *Not*I. Total restricted DNA was analyzed on a 0.8% agarose gel (FMC, U.S.A).

Southern blot analysis

Following electrophoresis, DNA was transferred onto a Hybond N^+ membrane (Amersham, U.S.A) and cross-linked with UV. Hybridization was carried out using a DIG- and isotope-labeled EGFP cDNA probe (740 bp). EGFP cDNA was prepared via PCR using a forward (5′-atggtgagcaagggcgaggag-3′) and a reverse primer (5′-gatctagagtcgcggccgct-3′). Amplification was performed for 35 cycles in a 50 μl

Table 1. Effects of various DNA constructs on survival and expression rates in transgenic medaka embryos

DNA construct			DNA fragment	Injected DNA size (kb)	No. of injected eggs	No. of surviving embryos (%)	No. of expression embryos (%)	No. of uniformity (%)		
Promoter	GFP	ITR						Stage 21 embryos (%)	Hatched fry (%)	Adult (%)
CMV	+	−	CMV-EGFP	5	226	184 (81.4)	179 (79.2)	0	0	0
CMV	+	+	CMV-EGFP-ITR	5.7	290	238 (82.1)	234 (80.7)	81 (27.9)	0	0
α-actin	+	−	α-actin-EGFP	8	51	46 (90.2)	43 (84.3)	0*	0	0
α-actin	+	+	α-actin-EGFP-ITR	5.1	79	66 (83.5)	60 (75.9)	6 (7.6)*	6 (7.6)	6 (7.6)
β-actin	+	−	β-actin-EGFP	7.6	124	109 (87.9)	109 (87.9)	0	0	0
β-actin	+	+	β-actin-EGFP-ITR	5	251	214 (85.3)	212 (84.5)	38 (15.1)	38 (15.1)	38 (15.1)
–	−	−	Control 1	–	102	94 (92.2)	0	0	0	0
–	−	−	Control 2	–	30	22 (73.3)	0	0	0	0
–	−	−	Control 3	–	56	40 (71.4)	0	0	0	0

Linearized DNA fragments (10 μg/ml) were microinjected into the cytoplasm of fertilized eggs at a volume of 300 pl. Surviving embryos at 36 h post-fertilization (stage 21) were examined to determine GFP expression. Uniformity indicated uniform transgenic GFP expression throughout the entire organism or in muscle tissue only. Controls consisted of parallel groups of non-CMV promoter-treated eggs (Control 1), non-α-actin promoter-treated eggs (Control 2), and non-β-actin promoter-treated eggs (Control 3). The percentages shown in parenthesis were calculated on the basis of the original numbers of injected eggs.
*GFP expressed exclusively in somites.

solution containing 2 pg of template DNA (pEGFP-1), 2.5 mM of each dNTP, 10 μM of each primer, 1X PCR buffer, and 2 units of *Taq* polymerase (GIBCO). For each cycle, denaturation was performed at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min (Perkin Elmer Centus). The resulting PCR product was labeled using a DIG or a radioisotope according to the manufacturer's protocol. Following the hybridization of the DIG-labeled probe, positive signals were visualized 2 h following the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim, Germany). Following the hybridization of the isotope-labeled probe, positive signals were visualized after sequentially washing and exposing to X-ray film at −70°C for several days.

Germ-line transmission

To determine the inheritance of the transferred DNA fragments, uniform GFP-expressions of F0 and F1 founders derived from β-actin-EGFP-ITR-injected embryos were crossed with wild varieties. The F1 and F2 progeny embryos were collected on day 1 and their green fluorescent signals detected at hatching (10 d following fertilization). Southern blot analysis was also performed on genomic DNAs extracted from F1 adults showing uniform and mosaic GFP-expression.

Results

Transgenic expression of GFP in CMV-EGFP- and CMV-EGFP-ITR-injected embryos

Linearized DNA fragments were injected into the cytoplasm of medaka embryos at the single-cell stage. While 79% of the CMV-EGFP-injected embryos tested GFP-positive (Table 1), GFP expression was mosaic and extremely faint (Figures 2A and 2B); none of the GFP-positive embryos showed uniform GFP-expression. In contrast, 80% of the CMV-EGFP-ITR-injected embryos tested GFP-positive, and approximately one-third of these showed GFP expression in almost every tissue at stage 21 (Table 1; Figures 2C and 2D). Neither CMV-EGFP- nor CMV-EGFP-ITR-injected embryos showed green fluorescence after stage 25 (52 h post-fertilization) (Figures 2E-2H).

Transgenic expression of GFP in α-actin-EGFP and α-actin-EGFP-ITR-injected embryos

In the α-actin-EGFP-injected embryos, GFP was primarily expressed in muscle tissue, especially in somites (Figures 3A and 3B). This localization of fluorescence was similar to that reported for the same fragment as expressed in zebrafish by Higashijima et al. (1997). Expression was sporadic at all embryonic stages without degradation in the fry stage. Mosaicism varied among the transgenic individuals. In addition,

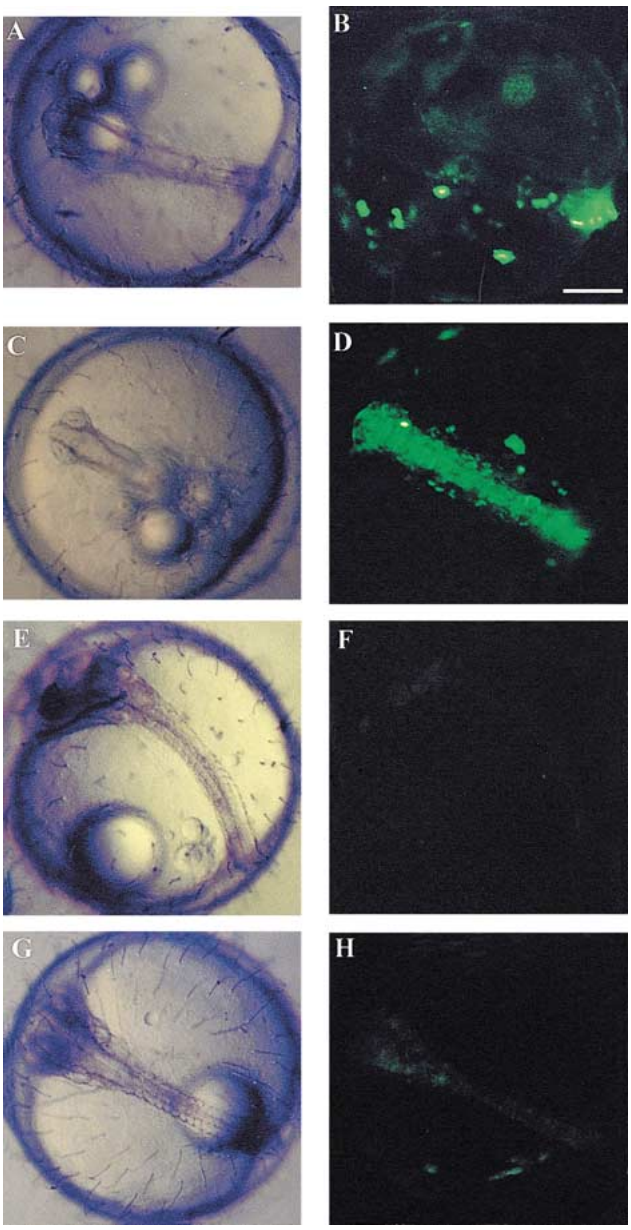

Figure 2. GFP expression in medaka embryos injected with GFP cDNA driven by a CMV promoter. Green fluorescence was observed in CMV-EGFP-injected (A, B, E, F) and CMV-EGFP-ITR-injected medaka embryos (C, D, G, H) at stages 21 (32 h post-fertilization for A, B, C and D) and 25 (52 h post-fertilization for E, F, G and H), respectively. Left panels were exposed under light field illumination; right panels were under dark field illumination. At stage 25, GFP expression decreased dramatically in both CMV-EGFP- and CMV-EGFP-ITR-injected embryos. White scale bar indicates 0.2 mm.

GFP was expressed from stage 17 (early neurula stage) onwards in α-actin-EGFP-injected embryos.

In the α-actin-EGFP-ITR-injected embryos, GFP was expressed exclusively in muscle tissue. In most of these embryos, over 50% of somites tested GFP-positive after stage 30 (35 somite stage). Some GFP signals were observed at stage 21 (6 somite stage), but widespread observation only occurred after stage 30 (Figure 3C). In total, approximately 10% of the GFP-positive embryos exhibited uniform transgenic GFP expression throughout their somites (Table 1 and Figure 3D). These uniform GFP-expression embryos produced green fluorescence throughout their lives, that was, for at least one year.

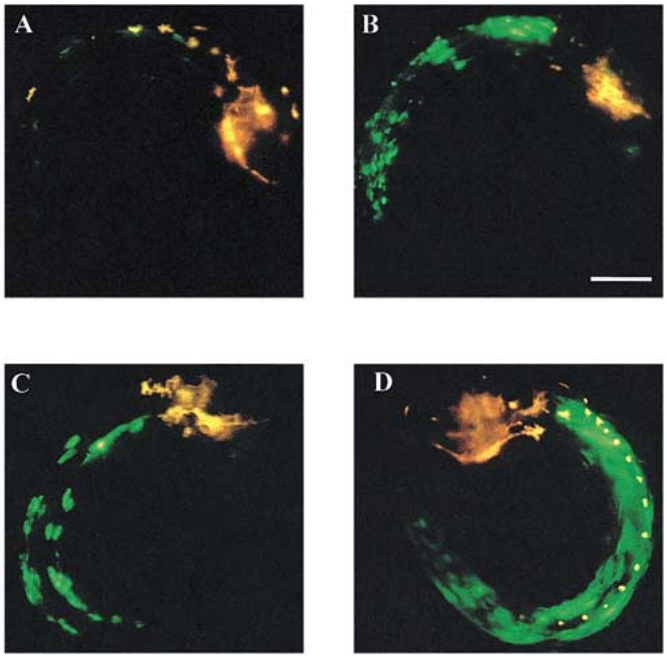

Figure 3. Expression of transgenic GFP driven by the α-actin promoter in medaka embryos at stages 31 (4 d post-fertilization; A, B and C) and 33 (4.5 days post-fertilization; D), respectively. A and B: α-actin-EGFP-injected embryos; GFP was sporadically expressed, primarily in somites. C: α-actin-EGFP-ITR-injected embryos; GFP was tissue-specific and mosaically expressed in muscle. D: α-actin-EGFP-ITR-injected embryos; GFP was uniformly expressed in muscle. White bar indicates 0.2 mm.

Transient expression of GFP in β-actin-EGFP- and β-actin-EGFP-ITR-injected embryos

In the β-actin-EGFP-injected embryos, GFP was primarily expressed in the epidermis, blood vessels, muscle tissue, yolk sac, notochord, finray, and eyes (Figures 4C and 4E); GFP expression also appeared in the hearts and circulating blood cells of some embryos (data not shown). Like the GFP expression in α-actin-EGFP-injected embryos, GFP expression in β-actin-EGFP-injected embryos occurred sporadically at all embryonic stages, and some signals lasted into the fry stage. As with α-actin-EGFP-ITR-injected embryos, β-actin-EGFP-ITR injected embryos exhibited greater GFP expression than those embryos injected with the plasmid construct without ITR (Figure 4D). Over 50% of external body of most of the β-actin-EGFP-ITR-injected embryos appeared GFP-expression. Approximately 18% of the GFP-positive embryos (15% of total injected embryos) showed uniform expression (Table 1; Figures 4A, 4B, and 4F). Green fluorescence appeared uniformly in every tissue of transgenic larvae when cryosections were performed and observed under fluorescent microscopy (Figure 4G). This GFP-expression maintained for two generations, that is, to at least F2 progeny. The green fluorescence in some transgenic embryos was strong enough to be visible to the unaided eye.

Overall, no differences were observed in the frequencies of abnormal transgenic embryos injected with CMV-, α-actin-, or β-actin-derived DNA fragments with or without ITR sequences (Table 1). Moreover, even when CMV-EGFP-, α-actin-EGFP-, or β-actin-EGFP-DNA fragments were injected at ten-fold concentrations (100 μg/ml), transgenic GFP expression did not reach levels where green fluorescence signals were produced by CMV-EGFP-ITR-, α-actin-EGFP-ITR-, or β-actin-EGFP-ITR-injected embryos; higher concentrations instead resulted in greater mortality.

Southern blot analysis of genomic DNA of F0 derived from β-actin-EGFP-ITR-injected embryos

At stages 29 (3 d post-fertilization) and 31 (4 d post-fertilization), genomic DNAs from GFP-positive F0 embryos derived from β-actin-EGFP-ITR-injected eggs were extracted, digested with *Nco*I and *Not*I, and analyzed by Southern blot hybridization using a DIG-labeled probe. Concatemerizations of the transferred DNA fragments that are theoretically possible

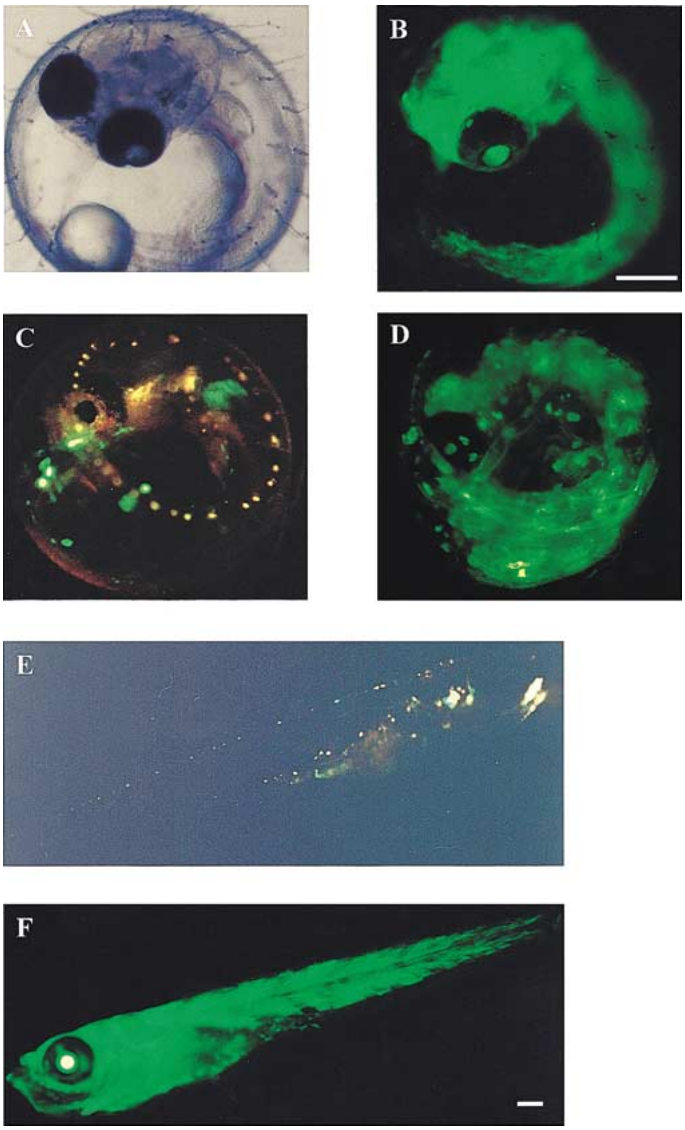

Figure 4. Expression of transgenic GFP driven by the β-actin promoter in medaka embryos. β-actin-EGFP-ITR-injected embryos at stage 36 (6.5 d post-fertilization) were exposed under light (A) and dark (B) field illumination. Other β-actin-EGFP-ITR-injected embryos were exposed under dark field illumination at stage 38 (8 d post-fertilization) (D) and at hatching (12 d post-fertilization) (F). β-actin-EGFP-injected embryos were exposed under dark field illumination at stage 38 (C) and at hatching (E). (G), Cryosection of 11-d larvae derived from β-actin-EGFP-ITR-injected embryo. Scale bar indicates 0.2 mm. Film-exposure time for figures (B) and (F) was 1.0 s, for Figure (D) 4.2 s, for Figure (G) 3 min and 41 s, and for the remaining Figures 8 s.

occurred are illustrated in Figure 5A. Southern blot analyses showed that positive bands with molecular masses of 4.5, 4.7, 5.2, and 4.7 kb in some transgenic medaka whose GFP was expressed mosaically (lanes 1–4 in Figure 5B). Two positive bands (4.5 and 23.1 kb) were also observed in a single medaka showing mosaic GFP expression (lane 8 in Figure 5B), as well as two positive bands (2.2 and 5.2 kb) in transgenic medaka showing uniform GFP expression (lanes 5–7 in Figure 5B). As expected, no positive bands were observed in medaka from the non-transgenic control group (lane N in Figure 5B).

Germ-line transmission of foreign GFP gene

Transgenic medaka derived from embryos microinjected with β-actin-EGFP-ITR fragments and uniformly expressing the foreign GFP gene were crossed with a

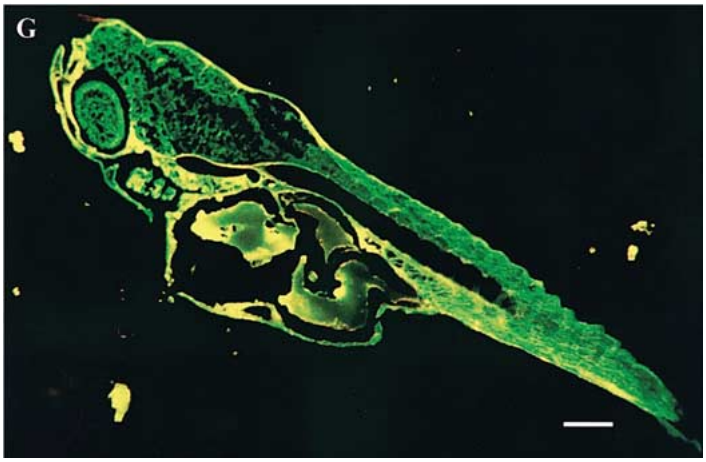

Figure 4. (*continued*)

wild-type medaka strain. Results showed that F1 progeny from three of the nine lines (lines 1, 4, and 6) had the same uniform GFP expression as did their F0 parent. Of the F1 embryos derived from line 4 (male), 46.8% (182/389) showed uniform GFP expression. When three F1 female progeny from line 4 (4F1-1, -2, and -3) were crossed with wild-type medaka individuals, 56.5% (65/115), 51.2% (21/41), and 46.2% (18/39) of the embryos of their F2 progeny showed uniform GFP expression, respectively. These percentages were close to what would be expected from the Mendelian inheritance of green fluorescent signals in F1 and F2 generations. However, one of the nine lines (line 2) showed a mosaic pattern; the remaining lines were sterile.

Genomic DNA from F1 progeny showing uniform GFP expression (lines 1 and 4) and that showing mosaic GFP expression (line 2) were extracted, digested with *Nco*I and *Not*I, and subjected to Southern blot hybridization using a DIG-labeled probe. Results revealed two positive bands with molecular masses of 2.2 and 5.2 kb (data not shown), which were as same as those of their transgenic F0 generation parents. However, when a radioisotope-labeled probe was used, in addition that two prominent bands of 2.2 and 5.2 kb were shown, several extra faint bands with molecular masses of 7.9, 3.8, 1.7 and 1.1 kb were noted for line 1; 9.5 and 3.1 kb were noted for line 2; and 8.9 and 1.1 kb were noted for line 4 (Figure 5C).

In summary, both, what appears to be, head-to-head and tail-to-tail transgene arrangements were detected, particularly in those fish displaying uniform GFP-expression in both F0 and F1 generations.

Discussion

When a DNA fragment consisting of a reporter or target gene of homologous or heterologous origin is transferred, it is very common to find mosaic, transient, or variegated expressions of the gene in transgenic fish. As Liang et al. (2000) point out, mosaic transgene integration in the germ lines of F0 founders is frequently detected even when NLS peptide-DNA complexes are employed. In other words, homogeneous expression of a transferred gene in the F0 generation is a rare occurrence, unlike expression in the F1 generation. Although Gibbs and Schmale (2000) demonstrated that transgenic GFP could be expressed throughout the life, the phenotypic difference occurred between the transgenic lines. In addition, the construct they used was not convenient to be applied in other desired promoter. Here, we believe the results of this study show that ITR technology can be used to overcome these disadvantages.

Constructing plasmids for transferring into medaka embryos, we flanked both ends of the GFP cDNA reporter gene with AAV-ITR. Three promoters from different gene sources were incorporated into the design: an immediate early gene of CMV, the muscle-specific α-actin gene of zebrafish, and the ubiquitous β-actin gene of medaka. The data indicate a remarkable improvement in the transient expression of those promoters in transgenic fish due to the incorporation of the ITR sequence. It is expected that the biological characteristics of this special DNA construct will function in transgenic medaka in a manner comparable to that reported by Fu et al. (1998) in *X. laevis* embryos.

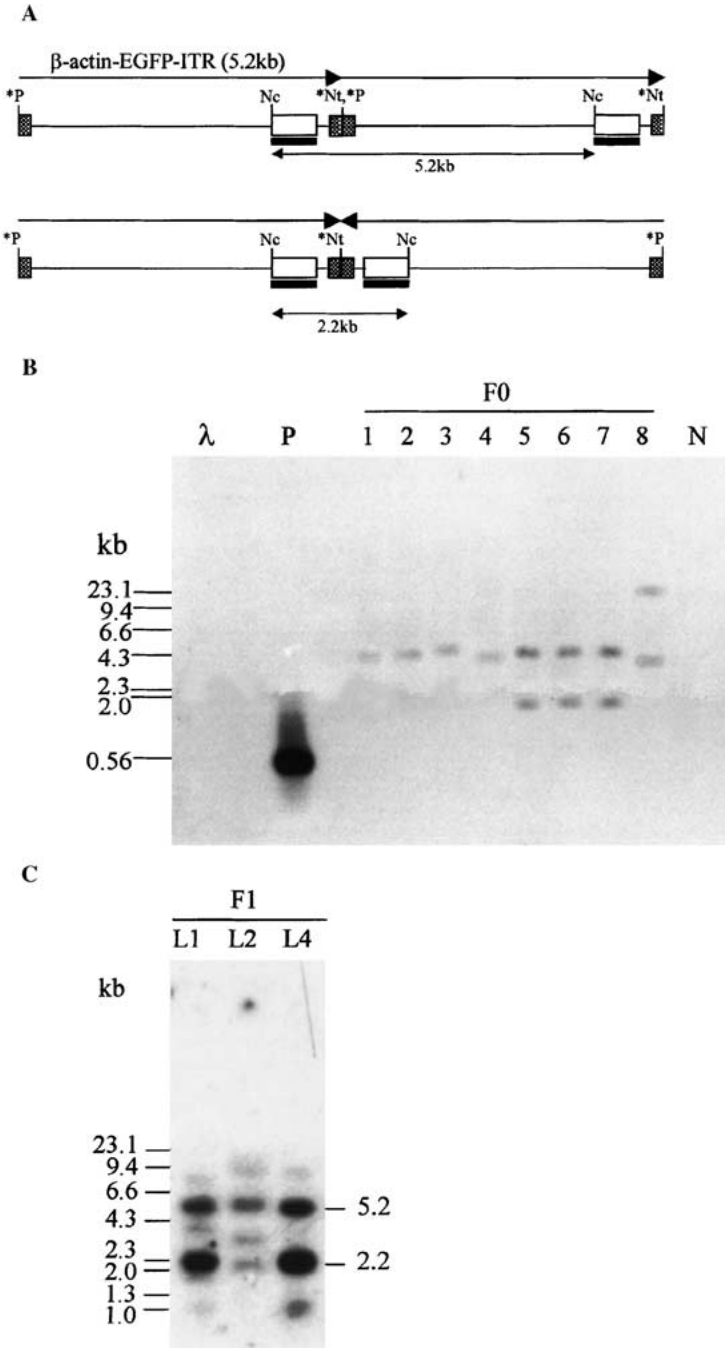

Figure 5. Southern blot analyses of genomic DNAs isolated from embryos, fry and adult medaka derived from β-actin-EGFP-ITR-injected eggs. A: schematic diagram of *Pst*I-*Not*I linearized β-actin-EGFP-ITR construct containing GFP cDNA (open box) and flanked by ITR (hatched box). The products below (5.2 and 2.2 kb) were expected for head-to-tail and tail-to-tail concatemerization, respectively. Probes were indicated by thick blacken lines. Nc: *Nco*I; Nt: *Not*I; P: *Pst*I. *: the restriction site was modified during concatemerization. B: Southern blot analysis of genomic DNA extracted from F0 founders of GFP-positive transgenic medaka and digested with *Nco*I and *Not*I. Lane λ *Hin*dIII-cut λ genome; lane P: positive control (1 ng EGFP cDNA [740-bp]); lanes 1–4: embryos with sporadic GFP-expression at 3 d post-fertilization; lanes 5–7: embryos with uniform GFP-expression at 4 d post-fertilization; lane 8: a single embryo with sporadic GFP-expression at 4 d post-fertilization; lane N: negative control, wild-type embryo at 4 d post-fertilization. C: Southern blot analysis of genomic DNA extracted from F1 progeny of GFP-positive transgenic lines 1 (L1 uniform GFP-expression), 2 (L2 mosaical GFP-expression) and 4 (L4 uniform GFP-expression), and digested with *Nco*I and *Not*I.

Figure 6. Expression of transgenic GFP in F1 progeny whose parent (F0) was derived from embryos microinjected with a β-actin-EGFP-ITR fragment. Hatching fry (12 d post-fertilization) of F1 progeny were exposed under light (A) and dark field illumination (B). (C), adult wild strain (upper) and adult F1 progeny with green fluorescence (lower) photographed under natural lighting. White scale bar in (B) indicates 0.2 mm; black one in (C) indicates 2 mm. Film-exposure time for (B) and (C) were 1.0 and 0.01 s, respectively.

Due to its location in the nuclear membrane (Weitzman et al., 1996), the AAV-ITR sequence makes the embryonic distribution of the transferred DNA fragment more even. As a result, fragment expression is more efficient and tissue-specific.

The observed temporal activation of the ITR-flanked muscle α-actin promoter agrees with the activation timing of the endogenous gene at stage 21 and maximal expression at stage 30, an expression stage similar to that reported in mice (Sassoon et al., 1988). By contrast, the activation of the non-ITR muscle α-actin promoter occurred at stage 17, earlier than expected in normal development. We, therefore, suggest that the addition of AAV-ITR not only enhances tissue-specificity, but also enhances the expression of exogenously introduced genes under the control of a specific promoter.

In addition, regardless of promoter type, none of the transgenic embryos injected with non-ITR-containing DNA fragments showed uniform GFP expression at F0; injection with ITR-containing constructs raised the percentage of uniform GFP-expression embryos at F0 to between 10 and 18%. The number of embryos showing uniform and persistent GFP-expression in the β-actin-injected group was higher than that in the α-actin-injected group, a difference that may be attributed to the medaka-origin of the β-actin promoter. Transgenic medaka injected with both α- and β-actin-EGFP-ITR fragments retained their green fluorescence without degradation throughout their lives; this trait of homogeneous GFP expression was inherited by F1 and F2 progeny (Figures 6A and 6B). To our knowledge, this is the first report of a transgenic GFP reporter gene being uni-

formly and strongly expressed in the F0 generation and being possible to identify transgenic medaka with the unaided eye (Figure 6C).

Unlike the persistent GFP expression observed in embryos injected with the α- and β-actin-promoters, embryos injected with the CMV promoter failed to show transient GFP-expression in transgenic medaka after stage 25 (52 h post-fertilization). This finding is similar to Tsai et al.'s (1995b) report of undetected *lac*Z expression in CMV-*lac*Z-injected medaka embryos at 48 h post-fertilization. A probable explanation is that the epigenetic modification of the CMV promoter is due to the endogenous methylation of cytosine in CpG dinucleotides, which results in suppressing transgene expression (Kass et al., 1997; Collas, 1998). On the other hand, embryos injected with the α- and β-actin-promoters, both of fish origin (Higashijima et al., 1997; Hamada et al., 1998), showed prolonged GFP expression in transgenic medaka.

Unexpectedly, some F0 lines failed to transmit uniform GFP expression to their progeny due to their mosaic GFP expression patterns. Xiao et al. (1996) and Wu et al. (1998) both reported a high-level, persistent transgene expression of recombinant AAV (rAAV) in muscle cells, neurons, and non-dividing cells, and Duan et al. (1998) found that the episomal persistence of rAAV was correlated with its long-term transgenic expression. The false-positive result of uniform GFP expression in founders may be explained by the transgenic GFP gene being strongly expressed in skin and muscle tissue, but intermittently expressed in other tissue types, resulting in mosaic germ-line transmission. Another possibility is that the transferred AAV-ITR-flanked gene may be lost, or its expression may be turned off, during gamete formation. This explanation has support from Zhang and Fuleihan (1999), who reported the loss of some rAAV DNA and the silencing of transgenic expression in some cell lines.

We found that female trangenic medaka were capable of transmitting GFP mRNA to such a degree that newly fertilized eggs projected a strong green fluorescence. However, the intensity of the signal decreased during embryonic development, and disappeared entirely at the hatching stage (8–10 d post-fertilization). No positive bands were observed during PCR or Southern blot searches for transgenic GFP fragments in the transiently green eggs (data not shown). Therefore, green fluorescence in hatching eggs was used to study the Mendelian inheritance of the transgene. Approximately 50% of line 4 F1 and F2 progeny tested GFP-positive; this percentage is close to what one would expect from the Mendelian inheritance of a transgene from a uniformly GFP-expressed F0 founder. This uniformly green phenotype was transmitted to F2 progeny to such a strong degree that it was easily noticeable.

Naturally occurring AAV has been reported as showing site-specific integration characteristics at chromosome 19 in the human genome (Samulski et al., 1991; Kotin et al., 1992). On the other hand, rAAV persistence has been attributed to both episomal (Flotte et al., 1994) and random (Kearns et al., 1996; Ponnazhagan et al., 1997) integration in human cell lines. In the present study, Southern blot analysis demonstrated random rAAV integration into the medaka genome at a single locus. The transfer of foreign DNA fragments containing the AAV-ITR sequence strongly enhanced the transient expression of GFP. This effect makes medaka a more practical animal subject for transgene studies. Its transparent physical characteristic makes it easy to note strong GFP expression, which is helpful in screening for the uniform expression of other transgenes.

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References

Chan WK and Devlin RH (1993) Polymerase chain reaction amplification and functional characterization of sockeye salmon histone H3, metallothionein-B and protamine promoters. *Mol Marine Biol Biotechnol* **1**: 97–105.

Chong SSC and Vielkind JR (1989) Expression and fate of CAT reporter gene microinjected into fertilized medaka (*Oryzias latipes*) eggs in the form of plasmid DNA, recombinant phage particles and its DNA. *Theor Appl Genet* **78**: 369–380.

Collas P (1998) Modulation of plasmid DNA methylation and expression in zebrafish embryos. *Nucleic Acids Res* **26**: 4454–4461.

Collas P and Alestrom P (1998) Nuclear localization signals enhance germline transmission of a transgene in zebrafish. *Transgenic Res* **7**: 303–309.

Culp P, Nusslein, Volhard C and Hopkins N (1991) High-efficiency germ-line transmission of plasmid DNA sequences injected into

fertilized zebrafish eggs. *Proc Natl Acad Sci USA* **18**: 7953–7957.

Du SJ, Gong Z, Fletcher GL, Shears MA and Hew CL (1992) Growth hormone gene transfer in Atlantic salmon: Use of fish antifreeze/growth hormone chimeric gene constructs. In: Hew CL and Fletcher GL (eds), *Transgenic Fish* (pp. 176–189) World Scientific Publishing, Singapore.

Duan D, Sharma P, Yang J, Yue Y, Dudus L, Zhang Y, Fisher KJ and Engelhardt JF (1998) Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol* **72**: 8568–8577.

Fletcher GL, Shears MA, King MJ, Davies PL and Hew CL (1988) Evidence of antifreeze protein gene transfer in Atlantic salmon (*Salmo salar*). *Can J Fish Aqua Sci* **45**: 352–357.

Flotte TR, Afione SA and Zeitlin PL (1994) Adeno-associated virus vector gene expression occurs in nondividing cells in the absence of vector DNA integration. *Am J Respir Cell Mol Biol* **11**: 517–521.

Fu Y, Wang Y and Evans SM (1998) Viral sequences enable efficient and tissue-specific expression of transgenes in *Xenopus*. *Nat Biotechnol* **16**: 253–257.

Gibbs PDL and Schmale MC (2000) GFP as a genetic marker scorable throughout the life cycle of transgenic zebrafish. *Marine Biotechnol* **2**: 107–125.

Guyomard R, Chourrout D and Houdebine LM (1989) Production of stable transgenic fish by cytoplasmic injection of purified genes. *Symp Mol Cellular Biol* **87**: 9–18.

Hamada K, Tamaki K, Sasado T, Watai Y, Kani S, Wakamatsu Y, Ozato K, Kinoshita M, Kohno R, Takagi S and Kimura M (1998) Usefulness of the medaka β-actin promoter investigated using a mutant GFP reporter gene in transgenic medaka (*Oryzias latipes*). *Mol Marine Biol Biotechnol* **7**: 173–180.

Hanley S, Smith TJ, Muller F, Maclean N, Uzbekova S, Prunet P and Breton B (1998) Isolation and functional analysis of the histone H3 promoter from Atlantic salmon (*Salmo salar* L.). *Mol Marine Biol Biotechnol* **7**: 165–172.

Hew CL (1989) Transgenic fish: Present status and future directions. *Fish Physiol Biochem* **7**: 409–413.

Higashijima SI, Okamoto H, Ueno N, Hotta Y and Eguchi G (1997) High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Dev Biol* **192**: 289–299.

Iyengar A and Maclean N (1995) Transgene concatemerisation and expression in rainbow trout (*Oncorhynchus mykiss*). *Mol Marine Biol Biotechnol* **4**: 248–254.

Ju B, Xu Y, He J, Liao J, Yan T, Hew CL, Lam TJ and Gong Z (1999) Faithful expression of green fluorescent protein (GFP) in transgenic zebrafish embryos under control of zebrafish gene promoters. *Dev Genet* **25**: 158–167.

Kass S, Pruss D and Wolffe A (1997) How does DNA methylation repress transcription? *Trends Genet* **13**: 444–449.

Kearns WG, Afione SA, Fulmer SB, Pang MC, Erikson D, Egan M, Landrum MJ, Flotte, TR and Cutting GR (1996) Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. *Gene Ther* **3**: 748–755.

Khoo HW, Ang LH and Wong KY (1992) Sperm cells as vectors for introducing foreign DNA into zebrafish. *Aquaculture* **107**: 1–19.

Kinoshita M and Ozato K (1995) Cytoplasmic microinjection of DNA into fertilized medaka (*Oryzias latipes*) eggs. *Fish Biol J MEDAKA* **7**: 59–64.

Kinoshita M, Toyohara H, Sakaguchi M, Inoue K, Yamashita S, Satake M, Wakamatsu Y and Ozato K (1996) A stable line of transgenic medaka (*Oryzias latipes*) carrying the CAT gene. *Aquaculture* **143**: 267–276.

Kotin RM, Linden RM and Berns KI (1992) Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *EMBO J* **11**: 5071–5078.

Liang MR, Alestrom P and Collas P (2000) Glowing zebrafish: Integration, transmission, and expression of a single luciferase transgene promoted by noncovalent DNA-nuclear transport peptide complexes. *Mol Reprod Dev* **55**: 8–13.

Lin S, Yang S and Hopkins N (1994) *LacZ* expression in germ-line transgenic zebrafish can be detected in living embryos. *Dev Biol* **161**: 77–83.

Maclean N, Penman D and Zhu Z (1987) Introduction of novel genes into fish. *Biotechnology* **5**: 257–261.

Ozato K, Kondoh H, Inohara H, Iwamatsu T, Wakamatsu Y and Okada TS (1986) Production of transgenic fish: Introduction and expression of chicken δ-crystallin gene in medaka embryos. *Cell Differ* **19**: 237–244.

Ponnazhagan S, Erikson D, Kearns WG, Zhou SZ, Nahreini P, Wang XS and Srivastava A (1997) Lack of site-specific integration of the recombinant adeno-associated virus 2 genomes in human cells. *Hum Gene Ther* **8**: 275–284.

Radloff R, Bauer W and Vinograd J (1967) A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: The closed circular DNA in HeLa cells. *Proc Natl Acad Sci USA* **57**: 1514–1521.

Samulski RJ, Chang L-S and Shenk T (1989) Helper-free stocks of adeno-associated viruses: Normal integration does not require viral gene expression. *J Virol* **63**: 3822–3828.

Samulski RJ, Zhu X, Xiao X, Brook JD, Housman DE, Epstein N and Hunter LA (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J* **10**: 3941–3950.

Sassoon DA, Garner I and Buckingham M (1988) Transcripts of alpha-cardiac and alpha-skeletal actins are early markers for myogenesis in the mouse embryo. *Development* **104**: 155–164.

Srivastava A, Lusby EW and Berns KI (1983) Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J Virol* **45**: 555–562.

Stuart GW, Vielkind JR, McMurray JV and Westerfield M (1990) Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. *Development* **109**: 577–584.

Symonds JE, Walker SP, Sin FYT and Sin I (1994) Development of a mass gene transfer method in chinook salmon: Optimization of gene transfer by electroporated sperm. *Mol Marine Biol Biotechnol* **3**: 104–111.

Takagi A, Sasado T, Tamiya G, Ozato K, Wakamatsu Y, Takeshita A and Kimura M (1994) An efficient expression vector for transgenic medaka construction. *Mol Marine Biol Biotechnol* **3**: 192–199.

Tsai HJ, Tseng FS and Liao I (1995a) Electroporation of sperm to introduce foreign DNA into the genome of loach (*Misgurnus anguillicaudatus*). *Can J Fish Aquat Sci* **52**: 776–778.

Tsai HJ, Wang SH, Inoue K, Takagi S, Kimura M, Wakamatsu Y and Ozato K (1995b) Initiation of the transgenic *lacZ* gene expression in medaka (*Oryzias latipes*) embryos. *Mol Marine Biol Biotechnol* **4**: 1–9.

Tsai HJ, Lai CH and Yang HS (1997) Sperm as a carrier to introduce an exogenous DNA fragment into the oocytes of Japanese abalone (*Haliotis divorsicolor suportexta*). *Transgenic Res* **6**: 85–95.

Weitzman M.D, Fisher KJ and Wilson JM (1996) Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication center. *J Virol* **70**: 1845–1854.

Wu P, Phillips MI, Bui J and Terwilliger EF (1998) Adeno-associated virus vector-mediated transgene integration into neurons and other nondividing cell targets. *J Virol* **72**: 5919–5926.

Xiao X, Li J and Samulski RJ (1996) Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* **70**: 8098–8108.

Yoshizaki G, Oshiro T and Takashima F (1991) Introduction of carp α-globin into rainbow trout. *Nippon Suisan Gakkaishi* **57**: 819–824.

Zhang P, Hayat M, Joyce C, Gonzalez-Villasenor LI, Lin CM, Dunham RA, Chen TT and Powers DA (1990) Gene transfer, expression and inheritance of pRSV-rainbow trout-GH cDNA in the common carp, *Cyprinus carpio*. *Mol Reprod Dev* **25**: 13–25.

Zhang PX and Fuleihan RL (1999) Transfer of activation-dependent gene expression into T-cell lines by recombinant adeno-associated virus. *Gene Ther* **6**: 182–189.