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Enhanced Expression and Stable Transmission of Transgenes Flanked by Inverted Terminal Repeats From Adeno-Associated Virus in Zebrafish

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ABSTRACT **Mosaic expression of transgenes in the F0 generation severely hinders the study of transient expression in transgenic fish. To avoid mosaicism, enhanced green fluorescent protein (EGFP) gene cassettes were constructed and introduced into one-celled zebrafish embryos. These EGFP gene cassettes were flanked by inverted terminal repeats (ITRs) from adeno-associated virus (AAV) and driven by zebrafish α-actin (pα-actin-EGFP-ITR) or medaka β-actin promoters (pβ-actin-EGFP-ITR). EGFP was expressed specifically and uniformly in the skeletal muscle of 56% ± 8% of the pα-actin-EGFP-ITR-injected survivors and in the entire body of 1.3% ± 0.8% of the pβ-actin-EGFP-ITR-injected survivors. Uniform transient expression never occurred in zebrafish embryos injected with EGFP genes that were not flanked by AAV-ITRs. In the F0 generation, uniformly distributed EGFP could mimic the stable expression in transgenic lines early in development. We established five transgenic lines derived from pα-actin-EGFP-ITR-injected embryos crossed with wild-type fish and 11 transgenic lines derived from pβ-actin-EGFP-ITR-injected embryos crossed with wild-type fish. None of these transgenic lines failed to express the transgene, a result confirmed by polymerase chain reaction analysis. Stable mendelian transmission of the transgenes was achieved in both α-actin and β-actin transgenic lines without changing the patterns of expression and integration. Progeny inheritance test and Southern blot analysis results strongly suggest that transgenes flanked by AAV-ITRs were integrated randomly into the genome at a single locus with a concatamerized multiplier. Thus, incorporating AAV-ITRs into transgenes results in uniform gene expression in the F0 generation and stable transmission of transgenes in zebrafish.**

Key words: actin promoter; adeno-associated virus; green fluorescent protein; germline transmission; inverted terminal repeats; transgenic zebrafish; uniform expression

INTRODUCTION

Zebrafish (*Danio rerio)* have transparent embryos that are easy to inject, easily controlled spawning, a short generation time, and year-round breeding. These traits make them excellent models for studying embryonic development, gene function, and the expression and inheritance of transgenes in vertebrates (Ingham, 1997; Kimmel, 1989; Nüsslein-Volhard, 1994; Vascotto et al., 1997). A large quantity of expressed sequence tags from fish have been identified and sequenced (Gong et al., 1997; Douglas et al., 1999). Mapping the spatial and temporal expression patterns of mRNAs using in situ hybridization facilitates investigation of the gene expression profile (Henrich and Wittbrodt, 2000). It is preferable to conduct functional analysis of the regulatory elements of newly isolated genes in vivo rather than in vitro. In zebrafish, transgenes containing an endogenous promoter and the green fluorescent protein (GFP) gene have been used to map the *cis*-acting elements of the promoter (Chen et al., 2001; Drivenes et al., 2000; Meng et al., 1997, 1999). They also have been used to target gene expression in specific tissues (Higashijima et al., 1997, 2000; Ju et al., 1999; Long et al., 1997). In stable transgenic lines, the dynamic expression patterns of genes are followed easily in real time in living fish, but because cleavage of fish embryos is rapid, uneven distribution and late integration of transgenes are common, and uniform expression of the transgene in the F0 generation is rare (Gibbs and Schmale, 2000). Large numbers of embryos should be analyzed during transient expression assays to account for the expression patterns of endogenous genes. Alternatively, stable transgenic lines must be established, but limiting the number of regulatory sequences that can be tested.

Although many approaches have been tried to overcome these limitations, they were only marginally successful at reducing mosaicism in the F0 generation.

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Transferring the foreign genes into the germinal vesicle (Ozato et al., 1986; Tsai et al., 1995b) or sperm (Tsai et al., 1995a; 1997) before fertilization still resulted in mosaic and variegated patterns of transgene expression. Use of a homologous promoter also did little to reduce mosaicism (Fu et al., 2000; Higashijima et al., 1997; Ju et al., 1999). In zebrafish, mosaic expression of the GATA2-GFP construct was reduced by a bacterial artificial chromosome (BAC) clone harboring a 70–80 kb promoter (Jessen et al., 1998). The BAC construct is suitable for targeting gene expression, but it is not always necessary for mapping promoter activity, because tissue- and stage-dependent *cis*-acting elements may be located in the proximal region of the promoter. Binding nuclear localization signal peptides to plasmid DNA also enhanced transient expression and increased the efficiency of gene transfer and germ-line transmission (Collas and Alestrom, 1998; Liang et al., 2000). Reporter gene expression in the F0 generation was still mosaic, however, and the transgene was not expressed in some transgenic progeny. A strong expression vector, constructed by fusing the carp β-actin promoter, GFP cDNA, ocean pout antifreeze protein terminator, and putative boundary element, was developed to drive uniform GFP expression in the F0 generation in zebrafish (Gibbs and Schmale, 2000). Unfortunately, a low percentage of embryos expressed GFP uniformly, and the transgene was silent in more than half the transgenic lines. Thus, we still need a simple and effective method for enhancing uniform expression of transgenes in the F0 generation and preventing the silencing and unstable transmission of transgenes in subsequent generations of transgenic fish.

Adeno-associated virus (AAV) type 2, a nonpathogenic human virus, has a single-stranded DNA genome of approximately 4.7 kb with two characteristic inverted terminal repeats (ITRs) (Srivastava et al., 1983). Each ITR consists of 145 nucleotides and forms a palindromic hairpin (Samulski et al., 1989). Fu et al. (1998) showed that inclusion of AAV-ITRs in plasmid DNA significantly increased the efficiency and specificity of transgene expression in *Xenopus* embryos. Because *Xenopus* has a long generation time, making germ-line transmission and the pattern of integration in the transgenic lines difficult to follow, we chose to study the effect of AAV-ITR sequences on expression and transmission of transgenes in zebrafish. We found that incorporating AAV-ITRs into DNA constructs effectively reduced mosaic transgene expression and resulted in stable transmission of transgenes.

RESULTS

Effects of AAV-ITRs on Transient Transgene Expression

To verify the effect of AAV-ITRs on transient transgene expression in zebrafish, enhanced green fluorescent protein (EGFP) gene cassettes were constructed (Fig. 1) and introduced into one-celled zebrafish embryos. These EGFP gene cassettes were flanked by AAV-ITRs and driven by a skeletal muscle–specific promoter (zebrafish α-actin) or a ubiquitous promoter (medaka β-actin). Another two EGFP gene cassettes with the same construction but without flanking AAV-ITRs also were performed for comparison. At 5 days post-fertilization (dpf), the EGFP expression rate in embryos injected with pα-actin-EGFP (93%, 67/72) did not differ from that of embryos injected with pα-actin-EGFP-ITR (98%, 268/273). EGFP expression was much stronger, however, in pα-actin-EGFP-ITR-injected embryos than in pα-actin-EGFP-injected embryos. Moreover, more than half (56% ± 8%) of the surviving pα-actin-EGFP-ITR-injected embryos expressed EGFP specifically and uniformly in skeletal muscle. This was designated the uniform (U) expression pattern. In contrast, embryos injected with pα-actin-EGFP never exhibited uniform expression (Fig. 2A). The EGFP expression rates in embryos injected with pβ-actin-EGFP and pβ-actin-EGFP-ITR constructs were about the same (93% and 88%, respectively), but about 1.3% ± 0.8% of surviving pβ-actin-EGFP-ITR-injected embryos expressed EGFP uniformly throughout their bodies at 1 dpf. Embryos injected with pβ-actin-EGFP never exhibited uniform expression (Fig. 2B).

Because injection with pβ-actin-EGFP-ITR alone produced very few U-type survivors, DNA and protamine, at mass ratios ranging from 1:0 to 1:9, were co-injected into zebrafish embryos to test their ability to enhance transient expression of the pβ-actin-EGFP-ITR transgene. The rate of uniform EGFP expression increased as the DNA/protamine mass ratio decreased from 1:0 to 1:3, but the rate decreased dramatically at mass ratios less than 1:3 (data not shown). At the optimal ratio of 1:3, EGFP was expressed uniformly in the entire body of 14% of the pβ-actin-EGFP-ITR-injected survivors (Fig. 2B). Thus, incorporating AAV-ITRs into transgenes greatly enhanced transient expression of the transgenes. This effect was increased by adding precise amounts of protamine to the DNA fragments.

Degrees of Transient Expression and the Frequency of Germ-Line Transmission of Transgenes

Embryos injected with pα-actin-EGFP-ITR and pβ-actin-EGFP-ITR were used to produce transgenic lines. Putative founders were identified based on the presence of transgenes and green fluorescence in their F1 progeny. The germ-line transmission rates estimated by polymerase chain reaction (PCR) and the EGFP phenotype were the same, indicating that EGFP was expressed in all transgenic lines. When pα-actin-EGFP-ITR-injected fish were crossed with wild-type fish, 8% (4/49) and 3% (1/32) of the founders exhibiting uniform (U) and moderate to weak (M/W) expression, respectively, transmitted the transgene to their progeny. If the germ-line transmission rate of null expression (N type) embryos is assumed to be zero, the total germ-line transmission rate of pα-actin-EGFP-ITR-in-

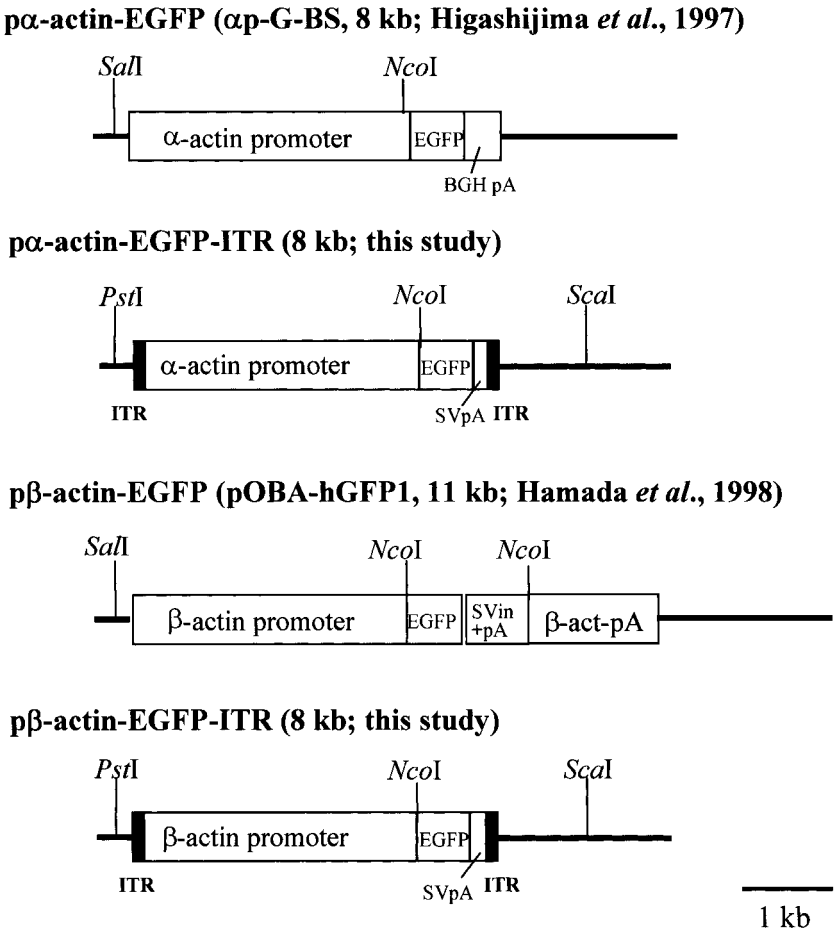

Fig. 1. Plasmid constructs used for transgenesis. The thick lines represent plasmid vectors. α-actin promoter, zebrafish α-actin promoter; β-actin promoter, medaka β-actin promoter; β-act-pA, polyadenylation signal of medaka β-actin gene; BGH pA, polyadenylation signal of bovine growth hormone gene; EGFP, enhanced green fluorescent protein; ITR, inverted terminal repeats of adeno-associated virus; SVin+pA, small t antigen intron and polyadenylation signal of SV40 gene; SVpA, polyadenylation signal of SV40. Scale bar = 1 kb.

jected founders was at least 6% (8% × 0.56 + 3% × 0.43 + 0% × 0.01). Of the founders derived from embryos injected with a mixture of pβ-actin-EGFP-ITR and protamine, 70% (7/10) and 8% (4/48) of those exhibiting U and M/W expression, respectively, transmitted the transgene to their progeny. The total germ-line transmission rate of pβ-actin-EGFP-ITR/protamine-injected founders was at least 16% (70% × 0.14 + 8% × 0.73 + 0% × 0.13). Thus, there was no consistent relationship between the degree of transient expression and the frequency of germ-line transmission of transgenes flanked by AAV-ITRs in zebrafish. The correlation was low in the α-actin transgenic lines, but it was relatively high in the β-actin transgenic lines.

EGFP Expression in the F0 and F1 Generations

After establishing α-actin and β-actin transgenic lines, we determined whether the transient expression pattern of transgenes flanked by AAV-ITRs could mimic the stable expression pattern in transgenic lines. The levels of EGFP expression in F0 and F1 transgenic zebrafish derived from pα-actin-EGFP-ITR- and pβ-actin-EGFP-ITR-injected embryos were compared at several developmental stages.

For pα-actin-EGFP-ITR-injected embryos, EGFP expression was initiated about 8 hr post-fertilization (hpf) in both the F0 and F1 fish. At the 5-somite stage (12 hpf), the EGFP gene was expressed along the body axis and in the head region of both F0 (Fig. 3A-a) and F1 fish (Fig. 3A-b). By the prim-5 stage (24 hpf), EGFP expression gradually had become confined to the somites of F0 (Fig. 3A-c) and F1 fish (Fig. 3A-d). In the swimming-up stage (5 dpf) F0 fish, most skeletal muscle in the trunk expressed EGFP strongly and uniformly, though small portions of skeletal muscle near the eyes and lower jaw were not marked with EGFP (Fig. 3A-e). At early developmental stages, the expression pattern in F0 fish was almost identical to that of F1 fish (Fig. 3A-f). At 30 dpf, the pattern of green fluorescence was mosaic in the skeletal muscle of U-type F0 fish (Fig.3A-g) but uniform in F1 fish (Fig. 3A-h).

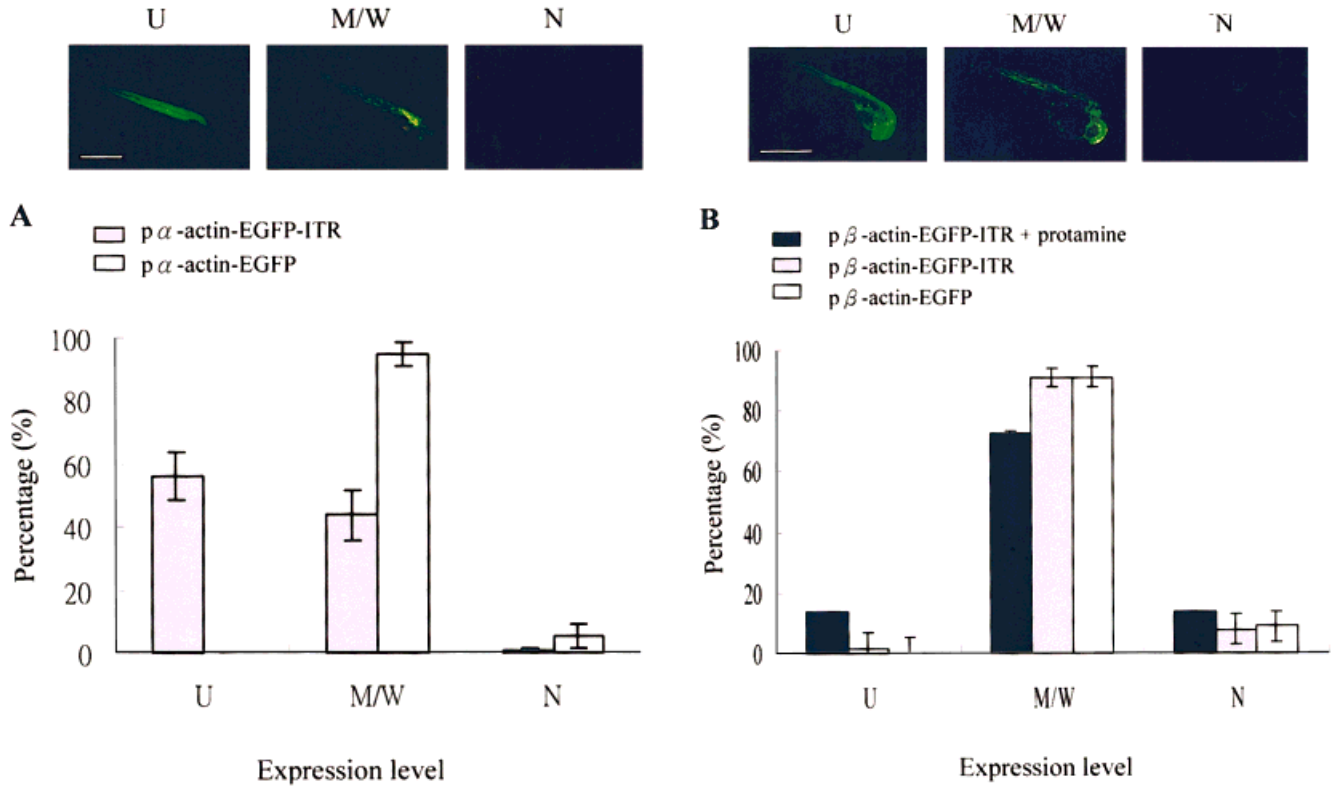

Fig. 2. Effect of AAV-ITRs on transient expression of transgenes in zebrafish embryos. **A:** Transient EGFP expression in pα-actin-EGFP-ITR- and pα-actin-EGFP-injected embryos. **B:** Transient EGFP expression in pβ-actin-EGFP-ITR- (± protamine) and pβ-actin-EGFP-injected embryos. From 30 to 100 injected embryos were used in each experiment. The results from three to five independent experiments on each construct were pooled and analyzed. Embryos were photographed at 5 dpf for pα-actin-EGFP derivatives and at 1 dpf for pβ-actin-EGFP derivatives. EGFP expression in transgenic embryos was classified as uniform (U), moderate to weak (M/W), or null (N). Film exposure times were 16 sec for U embryos and 60 sec for M/W and N embryos. Scale bar = 1 mm.

For pβ-actin-EGFP-ITR-injected embryos, EGFP expression was initiated at about 5 hpf in both F0 and F1 fish. At the 90% epiboly stage (9 hpf), EGFP was expressed evenly and strongly in F0 (Fig. 3B-a) and F1 fish (Fig. 3B-b). At the 5-somite stage (12 hpf), EGFP was expressed evenly along the body axis of F0 (Fig. 3B-c) and F1 fish (Fig. 3B-d). At the prim-5 stage (24 hpf), nearly the entire body of F0 (Fig. 3B-e) and F1 fish (Fig.3B-f) was fluorescent green. At 30 dpf, green fluorescence still appeared throughout the body of U-type F0 fish, but it was highly mosaic (Fig. 3B-g). In contrast, F1 progeny (30 dpf) expressed EGFP uniformly throughout the body (Fig. 3B-h). Therefore, in F0 zebrafish derived from embryos transferred by foreign genes flanked by AAV-ITRs, EGFP expression was uniform at the early embryonic stages but not later in development.

Germ-Line Transmission of Transgenes

The transgene inheritance rates of founders derived from pα-actin-EGFP-ITR- and pβ-actin-EGFP-ITR/protamine-injected embryos are summarized in Table 1. Five transgenic lines, four from U-type embryos (α2, α17, α28, α50) and one from M/W-type embryos (mα6), were produced by founders derived from pα-actin-EGFP-ITR-injected embryos. Transgene inheritance rates in F1 α-actin transgenic lines ranged from 7% to 16%. Eleven transgenic lines, seven from U-type embryos (β1, β3, β4, β5, β7, β9, β11) and four from M/W-type embryos (mβ7, mβ34, mβ47, mβ60), were produced by founders derived from pβ-actin-EGFP-ITR/protamine-injected embryos. Transgene inheritance rates in F1 β-actin transgenic lines ranged from 0.5% to 49%. This broad range of inheritance rates indicates that the germ cells of transgenic founders were highly mosaic. Thus, AAV-ITRs cannot facilitate the integration of transgenes into the zebrafish genome soon after microinjection.

To measure the transgene inheritance rate in F2 fish, fish from four transgenic lines (α2, α28, α50, mα6) derived from pα-actin-EGFP-ITR-injected founders and fish from seven transgenic lines (β1, β3, β4, β5, β7, β9, β11) derived from pβ-actin-EGFP-ITR/protamine-injected founders were crossed with wild-type fish (Table 2). From 44% to 59% of the F2 progeny of α-actin and β-actin transgenic F1 males crossed with wild-type females were EGFP positive after the gastrula stage (5 hpf). In contrast, all F2 progeny of β-actin transgenic

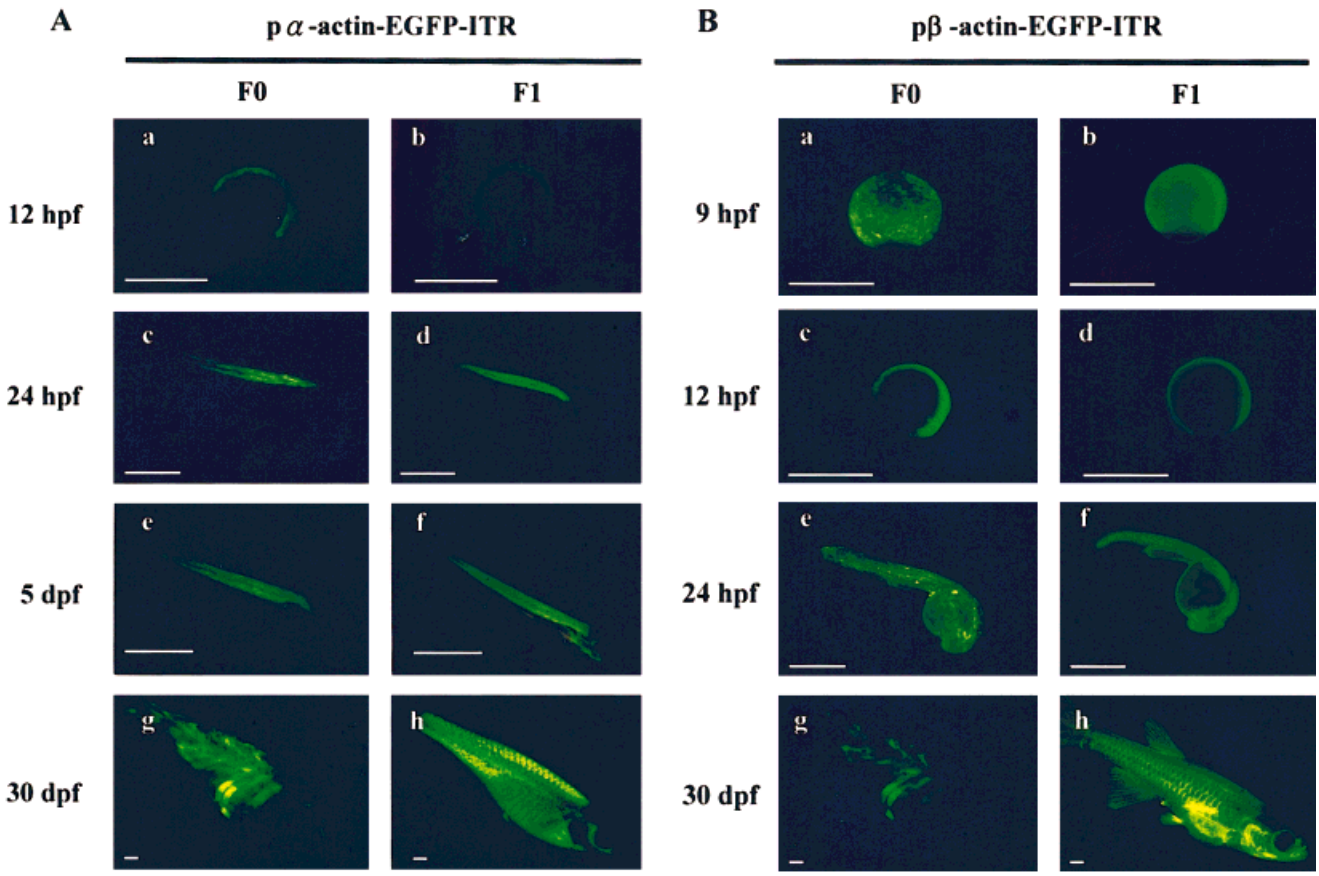

Fig. 3. Comparison of transient and stable EGFP expression patterns in transgenic embryos. **A,B:** EGFP expression patterns in transgenic embryos generated with pα-actin-EGFP-ITR and pβ-actin-EGFP-ITR, respectively. The developmental stage of F0 and F1 embryos is indicated on the left. The U-type transient expression pattern of EGFP in pα-actin-EGFP-ITR-injected embryos (A-a,A-c,A-e) mimicked the stable expression pattern noted in embryos from α-actin transgenic lines (A-b,A-d,A-f) up to 5 dpf. Injected U-type embryos exhibited a mosaic expression pattern in skeletal muscle at 30 dpf (A-g), but expression continued to be uniform in embryos from the stable line (A-h). The U-type transient expression pattern of EGFP in pβ-actin-EGFP-ITR-injected embryos (B-a,B-c,B-e) mimicked the stable expression pattern seen in embryos from β-actin transgenic lines (B-b,B-d,B-f) up to 1 dpf. At 30 dpf, injected U-type embryos exhibited a mosaic expression pattern throughout their bodies (B-g), but expression continued to be uniform in embryos from the stable line (B-h). Film was exposed 180 sec for A-a–A-d; 60 sec for B-g and B-h; 16 sec for A-e,A-f,B-a–B-d. Film was exposed for 4 sec for A-g,A-h,B-e,B-f. Scale bar = 1 mm.

F1 females crossed with wild-type males were EGFP positive at the one-cell stage, but about half progeny faded their EGFP gradually after 14 dpf (data not shown). Sibling crosses of F2 progeny in the seven β-actin transgenic lines (β1, β3, β4, β5, β7, β9, β11) were done to determine the transgene inheritance rate in the F3 generation. In all β-actin transgenic lines tested, the ratio of transgenic F3 progeny to non-transgenic progeny was 3:1 (data not shown). This finding suggests that transgenes flanked by AAV-ITRs were transmitted in a mendelian manner.

Accumulation of High Concentrations of EGFP in the Female Gonads

In the β-actin transgenic lines, the initiation and duration of EGFP expression were dependent on the sex of the founder. Transgenic F1 progeny derived from male founders (lines β7, β9, β11) began expressing EGFP after the gastrula stage. The intensity of EGFP expression did not decline with age. From 1% to 67% of the F1 progeny derived from female founders were EGFP positive in the one-cell stage, but in 44–66% of the EGFP-positive embryos, EGFP fluorescence had faded 2 weeks later. In contrast, F1 progeny derived from male and female α-actin transgenic founders were never positive for EGFP before the gastrula stage.

To investigate EGFP fading in progeny derived from β-actin female founders, we selected transgenic line β4 for further detailed study. At sexual maturity, the green fluorescence in the oocytes of transgenic line β4 was so bright that it could be seen by fluorescence microscopy in living zebrafish (Fig. 4A,B). All unfertilized eggs appeared green. The intensity of green fluorescence in primary-growth-stage oocytes (indicated by the arrowhead in Fig. 4C), which were less than 100 μm in diameter (Selman et al., 1993), was stronger

TABLE 1. Inheritance of Transgenes Flanked by AAV-ITRs by F1 Progeny*

Founders	Sex	Embryonic stage (3 dpf) EGFP(+)/total[a]	Embryonic stage (3 dpf) EGFP(+) (%)	Adult stage (60 dpf) EGFP(+)/total[b]	Adult stage (60 dpf) EGFP(+) (%)	Inheritance rate[c] (%)
α2	f	161/1,360	12	109/109	100	12
α17	f	15/197	8	10/10	100	8
α28	m	34/501	7	16/16	100	7
α50	f	65/406	16	39/39	100	16
mα6	f	62/405	15	10/10	100	15
β1	f	28/883	3[d]	8/18	44	1
β3	f	875/1,302	67[d]	111/201	55	37
β4	f	195/514	38[d]	28/47	60	23
β5	f	1054/1,908	55[d]	71/108	66	36
β7	m	182/511	36	3/3	100	36
β9	m	95/215	44	80/80	100	44
β11	m	21/43	49	11/11	100	49
mβ7	f	153/424	36[d]	23/42	55	20
mβ34	f	5/494	1[d]	2/4	50	0.5
mβ47	f	104/384	27[d]	32/64	50	14
mβ60	f	39/141	28[d]	2/4	50	14

*AAV-ITRs, adeno-associated virus inverted terminal repeats; EGFP, enhanced green fluorescent protein.
[a]Number of EGFP-positive embryos/number of survivors at 3 dpf.
[b]Number of EGFP-positive adult fish/number of survivors that fluoresced green as embryos.
[c]Inheritance rate = [EGFP(+)/total[a] × EGFP(+)/total[b]] × 100%.
[d]EGFP faded after 14 dpf in about half the EGFP-positive embryos derived from female founders generated with pβ-actin-EGFP-ITR.

TABLE 2. Inheritance and Expression of Transgenes Flanked by AAV-ITRs in F2 Progeny*

Lines	Sex[a]	Embryonic stage (3 dpf) EGFP(+)/total[b]	Embryonic stage (3 dpf) EGFP(+)[c] (%)	EGFP content[d] (μg/mg)	Transgene copy number[d]	Normalized EGFP content (μg/mg/copy)
α2	m	101/203	50	50.9	30	1.7
α28	m	241/484	50	18.3	20	0.9
α50	m	125/255	49	90.8	100	0.9
mα6	m	74/148	50	21.6	3	7.2
β1	m	190/392	48	4.1	5	0.8
β3	m	47/79	59	6.7	3	2.2
β4	m	36/69	52	7.2	30	0.2
β5	m	22/43	51	6.3	10	0.6
β7	m	33/75	44	4.0	20	0.2
β9	m	464/956	49	12.4	7	1.8
β11	m	137/277	49	6.5	14	0.5

*AAV-ITRs, adeno-associated virus inverted terminal repeats; EGFP, enhanced green fluorescent protein.
[a]Male F1 progeny were crossed with wild-type females to calculate the inheritance rate of transgenes in F2 progeny.
[b]Number of EGFP-positive survivors/total number of survivors at 3 dpf.
[c]This value is equal to the transgene inheritance rate, because F2 progeny derived from male F1 transgenic fish show no maternal EGFP accumulation.
[d]EGFP content and the number of transgene copies were estimated for the F1 α-actin and F2 β-actin transgenic lines.

than in maturing oocytes (indicated by the arrow in Fig. 4C). By the swimming-up stage (5 dpf), all F2 progeny derived from F1 transgenic females were EGFP positive and expressed EGFP equally (Fig. 4D). At 14 dpf, some F2 fish still expressed EGFP throughout their bodies (Fig. 4E), but others expressed EGFP only in the muscles and eyes (Fig. 4F). Using PCR analysis, EGFP-positive F2 progeny derived from transgenic F1 males proved to have the transgene, whereas those that were EGFP negative lacked the transgene (Fig. 5A). About half the EGFP-positive 3-day-old F2 progeny derived from transgenic F1 females, however, proved to be transgene negative (Fig. 5B). EGFP expression and transgene transmission were not persistent in the progeny of wild-type fish mated with F1 fish in which EGFP expression faded (data not shown). This finding suggests that maternal EGFP in the oocyte, but not the transgene per se, was transmitted into non-transgenic progeny. Over time, the maternal EGFP degraded and EGFP faded in non-transgenic progeny of β-actin transgenic lines.

Levels of EGFP Expression and Transgene Copy Number

To determine whether AAV-ITR sequences were insulators, which allow transgene expression to be independent of its point of insertion, male F1 progeny derived from four α-actin (α2, α28, α50, mα6) transgenic

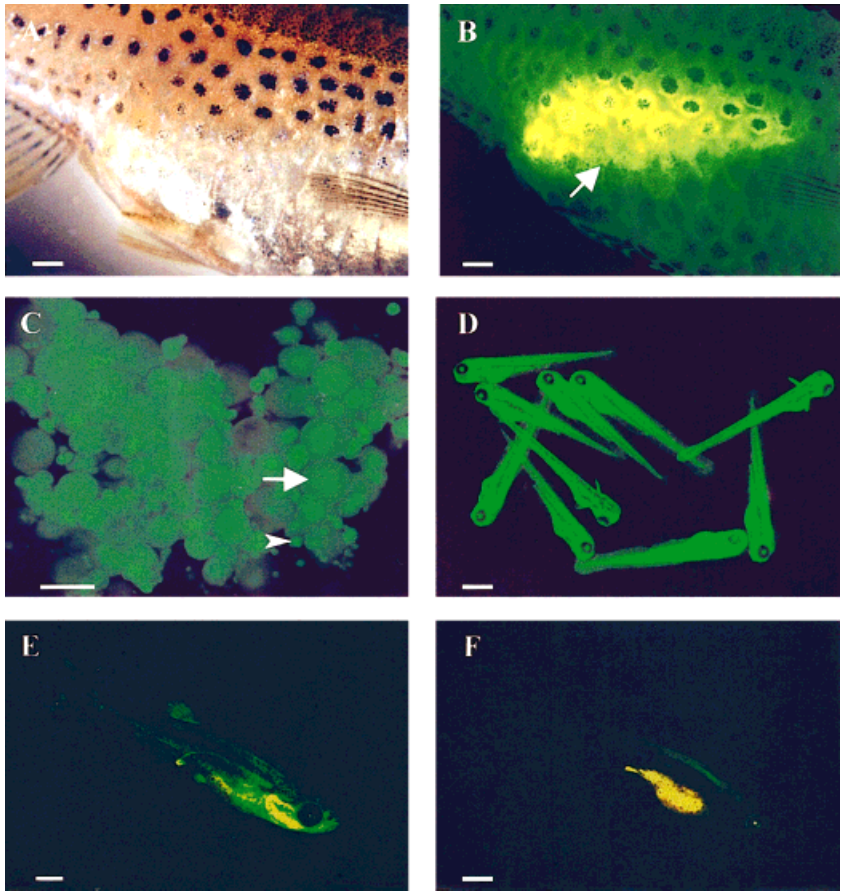

Fig. 4. High concentrations of EGFP were present in female germ cells of β-actin transgenic zebrafish. **A,B:** Lateral views of a 90-day-old female fish from transgenic line β4 in a bright field (A) and dark field (B) through a fluorescence microscope. The arrow indicates the ovary. **C:** Unfertilized eggs dissected from F1 progeny derived from transgenic line β4. All the oocytes were EGFP positive, and EGFP in the primary oocytes (arrowhead) was brighter than in maturing oocytes (arrow). **D:** F2 progeny derived from transgenic line β4 females crossed with wild-type fish. All F2 progeny were EGFP positive and exhibited the same level of EGFP expression at 5 dpf. **E:** EGFP expression showed no signs of fading in transgenic F2 progeny at 14 dpf. **F:** Non-transgenic F2 progeny displayed only trace amounts of EGFP in the muscles and eyes at 14 dpf. Scale bar = 1 mm.

lines and male F2 progeny derived from seven β-actin (β1, β3, β4, β5, β7, β9, β11) transgenic lines were selected for detailed studies. Male transgenic adult fish were selected for measuring EGFP content and transgene copies, to minimize the variations derived from different degrees of EGFP accumulation in developing oocytes of β-actin transgenic females. Exogenous EGFP ranged from 18.3 to 90.8 μg/mg (EGFP/total body protein) in the α-actin transgenic lines and from 4.0 to 12.4 μg/mg (EGFP/total body protein) in the β-actin transgenic lines (Table 2). The number of transgene copies, as determined by dot blot analysis, ranged from three to 100 in the α-actin transgenic lines and from 3 to 30 in the β-actin transgenic lines (Table 2).

Normalizing EGFP content by the number of transgene copies, we found that the level of EGFP expression did not correlate closely with the number of transgene copies. For example, even though transgenic line mα6 contained only three copies of the transgene, the normalized EGFP expression level was extremely high (7.2 μg/mg/copy). Thus, the expression level of transgenes flanked by AAV-ITRs was independent of the number of transgene copies. In both α-actin and β-actin transgenic lines, however, transgenic progeny derived from the same founder always exhibited equal levels and identical patterns of EGFP expression. The only major phenotypic difference between transgenic lines was the relative intensity of EGFP expression. Within any given transgenic line, the pattern and intensity of EGFP expression were predictable and did not change, even after passing through the germ line (data not shown). These results suggest that AAV-ITR sequences are only partial insulators.

Transgene Integration Pattern

To analyze the integration pattern of transferred genes, Southern blot analyses were used to characterize DNA fragments from the F1 progeny of six β-actin transgenic lines (β1, β3, β4, β5, β9, β11). Genomic DNAs were restricted by *Nco*I and hybridized with an

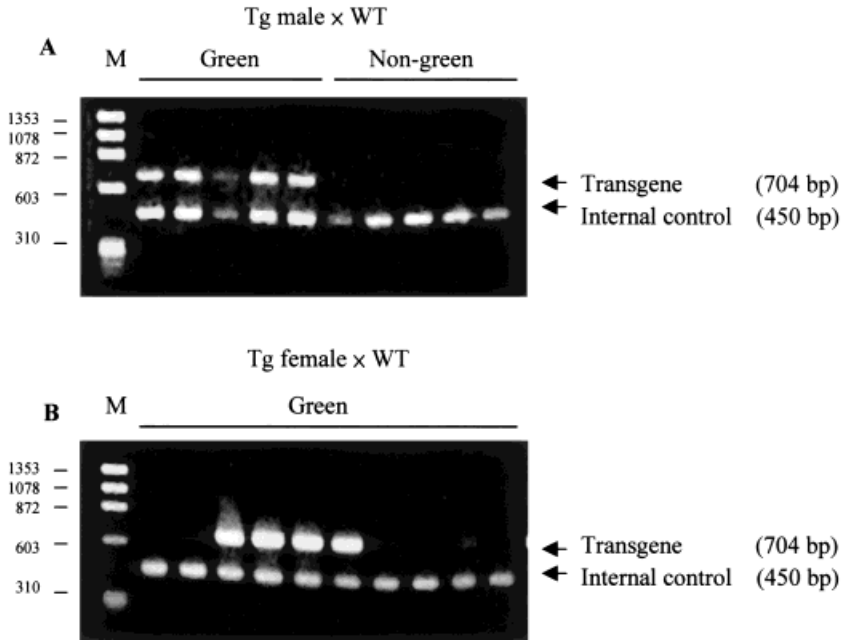

Fig. 5. Polymerase chain reaction (PCR) detection of the transgene in F2 progeny derived from transgenic line β4. **A:** PCR was used to detect the transgene in genomic DNA extracted from EGFP-positive (green) and EGFP-negative (non-green) 3-day-old F2 progeny of transgenic line β4 (Tg) males crossed with wild-type (WT) females. **B:** PCR was used to detect the transgene in genomic DNA extracted from EGFP-positive (green) 3-day-old F2 progeny of transgenic line β4 (Tg) females crossed with wild-type (WT) males. PCR products with molecular masses of 704 bp and 450 bp represent the EGFP transgene and *ZF-21* gene (internal control), respectively. DNA markers (M) are shown on the left; sizes are given in bp.

EGFP-specific radioisotope probe. Concatamerization of transgenes that are theoretically possible is illustrated in Fig. 6A. An 8-kb signal, suggesting a head-to-tail (HtT) or tail-to-tail (TtT) concatamer (indicated by arrows), and some positive bands larger than 4 kb, possibly junction fragments (indicated by asterisks), were found (Fig. 6B). The putative junction fragments in each transgenic line differed in size, and their relative intensity was fainter than that of the putative concatamers. These results clearly indicate that transgenes flanked by AAV-ITRs were integrated randomly into the zebrafish genome in the β-actin transgenic lines. Southern blot analyses of the *Sca*I-digested DNA fragments yielded positive signals for 8-kb (indicated by arrows) and 13-kb (indicated by arrowheads) fragments in transgenic lines β1, β4, β5, β9, and β11. This finding suggests that the transferred DNA fragments were organized into HtT or head-to-head (HtH) concatamers (Fig. 6B). In line β3, only the HtT concatamer was proposed, because the 13-kb HtH concatamer was absent. We also examined the possibility of forming a TtT concatamer by using the full-length plasmid as a hybridization probe. A 3-kb hybridization signal was found in some transgenic lines, indicating that a TtT concatamer may exist (data not shown).

To determine whether there were any modifications in the terminal nucleotides of transgenes within concatamers, genomic DNAs were digested with *Pst*I, a restriction enzyme used to linearize the plasmid for microinjection. A positive 8-kb band (indicated by arrows) was present in all transgenic lines except β1 (Fig. 6B), indicating that, except in line β1, the *Pst*I ends of the transgene within the concatamer had not been modified. Fragments smaller than predicted (indicated by number symbols) also were seen in some transgenic lines. For example, in line β3, cleavage with *Nco*I yielded a 2.3-kb positive fragment, and in lines β3 and β11 cleavage with *Pst*I produced 6.8-kb and 6.5-kb fragments, respectively (Fig. 6B). These fragments may have resulted from rearrangement or partial deletion of the injected plasmid. Some α-actin transgenic lines also yielded fragments that were smaller than expected (data not shown).

Genomic DNAs from transgenic line β4 F1, F2, and F3 progeny were digested with *Sca*I, and the fragments were characterized using Southern blot analysis. The hybridization patterns were the same in each generation (Fig. 6B), indicating that the transgene flanked by AAV-ITRs was integrated stably into the zebrafish genome. Similar results were obtained using different transgenic lines and restriction enzymes. We also evaluated the homogeneity of the transgene integration pattern in the gonads of founders injected with transgenes flanked by AAV-ITRs. The genomic DNA of F1 progeny derived from the same transgenic founder was digested with *Nco*I and characterized using Southern blot analysis. Progeny derived from the same founder always had the same hybridization pattern (data not

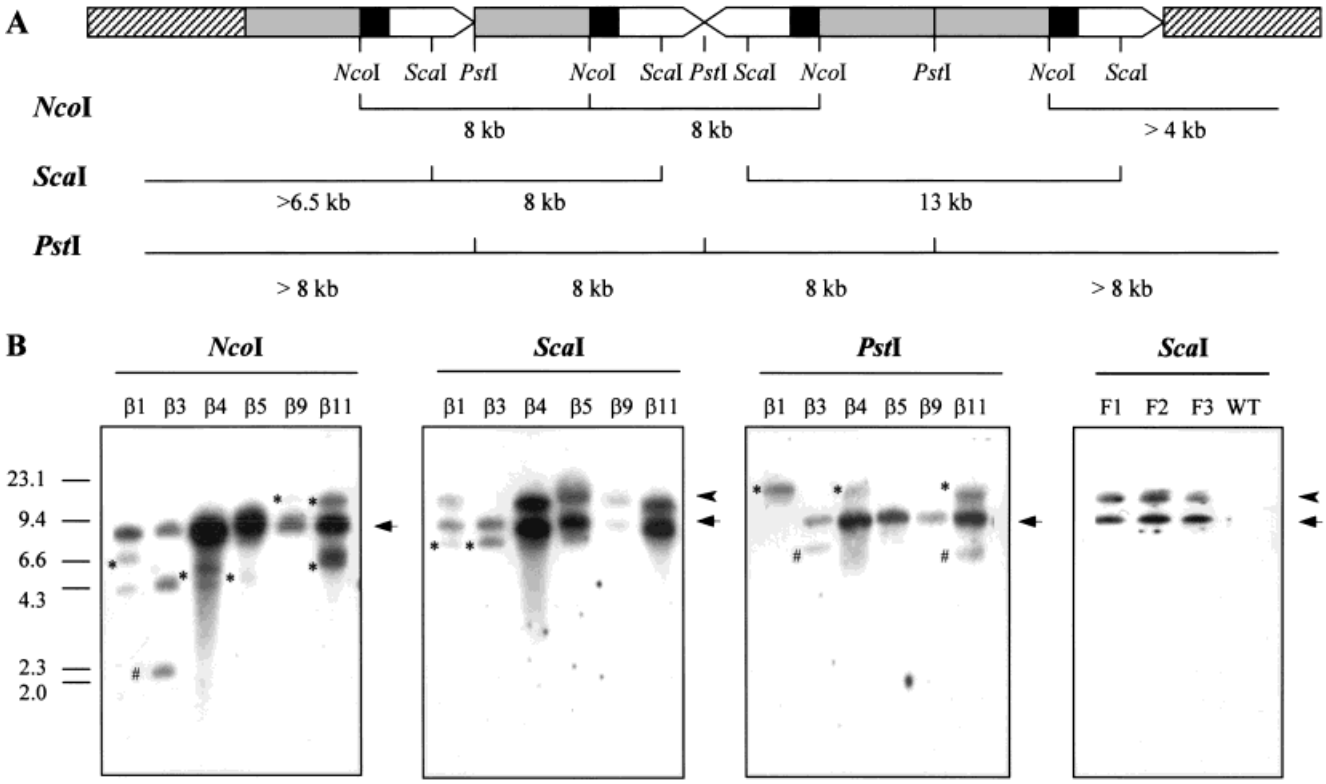

Fig. 6. Southern blot analysis of the integration pattern of transgenes in β-actin transgenic lines. **A:** Diagram shows the expected sizes of DNA fragments produced by digestion of head-to-tail (HtT), tail-to-tail (TtT), and head-to-head (HtH) concatamers with *Pst*I, *Nco*I, or *Sca*I. Hatched box, zebra fish genomic DNA; gray box, medaka β-actin promoter; black box, EGFP cDNA; open box, vector sequences. Arrows indicate the orientation of the gene construct. **B:** Genomic DNAs from six F1 β-actin transgenic lines (β1, β3, β4, β5, β9, and β11) generated with pβ-actin-EGFP-ITR were digested with *Nco*I, *Sca*I, and *Pst*I. Southern blot analysis with an EGFP-specific radioisotope probe was used to identify the resulting DNA fragments. Genomic DNAs from three successive generations (F1, F2, and F3) derived from transgenic line β4 were digested with *Sca*I and analyzed. The arrow indicates DNA fragments from HtT or TtT concatamers; the arrowhead indicates DNA fragments from HtH concatamers. *, putative junction fragments; #, putative fragments from rearrangements or deletions of the transgene. DNA markers are shown on the left; sizes are given in kb. The film was exposed for 9 days.

shown). Thus, although founder germ cells were mosaic for the transgene, in the F1 progeny the integration pattern of transgenes flanked by AAV-ITRs was homogeneous.

DISCUSSION

Flanking AAV-ITRs Enhance Transgene Expression

Mosaic and variegated expression of transgenes in the F0 generation severely hinders the study of transient expression in transgenic fish. Interpretations of transgene transient expression assays must carefully consider ectopic gene expression and the effects of *cis*-acting elements on gene expression. In this study, we clearly showed that in zebrafish the transient expression of transgenes driven by two strong promoters (α-actin and β-actin promoters) was enhanced by AAV-ITRs. Uniform transgene expression was achieved easily in the F0 fish, and it mimicked the stable expression of transgene lines. This finding was consistent with the enhancement by AAV-ITRs of transgene expression in *Xenopus* (Fu et al., 1998). Thus, incorporation of AAV-ITRs into transgenes is an effective method for dissecting promoter activity and targeting uniform gene expression in F0 generation zebrafish. We are investigating whether this strategy will be effective for analyzing weakly expressed and developmentally regulated gene promoters.

Protamine sulfate is a small, arginine-rich peptide (4,000–4,250 d) that quickly folds and compacts DNA. Because naked plasmid DNA has a short half-life (50–90 min) in the cytosol (Lechardeur et al., 1999), protamine sulfate is used widely for gene delivery (Arcasoy et al., 1997; Sorgi et al., 1997). In this study, 14% of the pβ-actin-EGFP-ITR/protamine-injected survivors and only 1% of the pβ-actin-EGFP-ITR-injected survivors exhibited uniform expression as embryos. We think that protamine sulfate prevents degradation of transferred DNA by digestive enzymes and prolongs its half-life in the cytosol. When the protamine-to-DNA ratio exceeded 1:3, however, uniform expression rates declined. This finding suggests that excess protamine abolishes transgene expression by masking the *cis*-acting elements that bind transcription factors.

Because zebrafish have a short generation time, it is easy to follow germ-line transmission and the integration pattern of transgenes flanked by AAV-ITRs in transgenic lines. In transgenic fish, the germ-line transmission rate for plasmid DNAs without flanking AAV-ITRs ranged from 1% to 20% (Higashijima et al., 1997, 2000; Linney et al., 1999; Long et al., 1997; Takagi et al., 1994). In this study, the germ-line transmission rate was at least 6% for pα-actin-EGFP-ITR-injected embryos and 16% for pβ-actin-EGFP-ITR/protamine-injected embryos. These rates were not significantly higher than those for plasmid DNAs that lacked AAV-ITRs. Based on these observations, we believe that the AAV-ITR sequences facilitate even distribution of the transgenes in dividing cells during cleavage but that they do not improve the frequency of genomic integration and germ-line transmission in zebrafish.

Higashijima et al. (1997) tested three DNA constructs and reported that the highest correlation between levels of transient expression and transgene germ-line transmission frequency was 50%. Likewise, in the U-type pα-actin-EGFP-ITR- and pβ-actin-EGFP-ITR/protamine-injected founders, we found no consistent correlation between levels of transient expression and transgene germ-line transmission frequency. The correlation for U-type embryos in the pα-actin-EGFP-ITR injection group was only 8%, but it was 70% for embryos in the pβ-actin-EGFP-ITR/protamine-injected group. Phenotypes that express EGFP uniformly will not necessarily be capable of germ-line transmission. One of the most plausible reasons is that uniform expression of transgenic EGFP results from even distribution of the AAV-ITRs-flanked transgene, not from transgene integration. In addition, because EGFP is water soluble, it might diffuse into surrounding cells that do not express the EGFP gene (Ikawa et al., 1998; Kato et al., 1999). Adding protamine to the DNA fragments used to transfer the transgene greatly increased the percentage of embryos exhibiting U-type EGFP expression. Most U-type embryos are able to transmit the transgene to the next generation. Thus, protamine seems to facilitate the integration of transgenes flanked by AAV-ITRs into the zebrafish genome.

Stable Transmission of Transgenes Flanked by AAV-ITRs

Although many transgenic fish lines have been established, many problems must be overcome, including transgene modification, silencing, and loss during germ-line transmission (Bayer and Campos-Ortega, 1992; Caldovic et al., 1999; Gibbs et al., 1994; Gibbs and Schmale, 2000; Patil et al., 1994; Sato et al., 1992). Cytoplasmic microinjection of transgenes results in highly mosaic founder gonads and low germ-line transmission rates. Thus, many progeny must be screened by dot blot or PCR to find transgenic founders (Stuart et al., 1988; Culp et al., 1991; Liang et al., 2000). A reporter gene helps reduce the screening effort and makes it possible to monitor the expression pattern of the transgene noninvasively. The luciferase gene, driven by an exogenous or viral promoter, sometimes exhibits variegated expression and can be silent in the F1 and F2 generations (Collas and Alestrom, 1998; Gibbs et al., 1994; Patil et al., 1994; Sato et al., 1992). Even with the use of the strong expression vector FRM, which fused the GFP reporter gene with a carp β-actin promoter, some transgenes were silent after passage through the germ line (Gibbs and Schmale, 2000).

In this study, however, none of transgenic lines created with transgenes flanked by AAV-ITRs failed to express the transgene. Mendelian transmission of transgenes flanked by AAV-ITRs, without changes to phenotypic expression and integration patterns in the F1, F2, and F3 generations, showed that the transgenes were stably integrated into the zebrafish genome. They were passed, without modification, through several generations. Sibling F2 fish were crossed to create F3 homozygotes of the α-actin and β-actin transgenic lines. These EGFP-marked homozygotes will greatly aid analyses of cell lineages and cell fates by providing a vital marker for cell transplantation, the creation of chimeric fish, and the screening of expression-based mutagenesis.

In zebrafish, plasmid DNA flanked by border elements could prevent the position effect. The transgene expression level is correlated with the number of integrated copies (Caldovic et al., 1999). We found that the pattern of EGFP expression in different transgenic lines, all of which were derived from the same DNA construct flanked by AAV-ITRs, was predictable and very similar. The level of EGFP expression within transgenic lines was consistent but not closely related to the number of transgene copies. Thus, we believe that AAV-ITRs can serve as incomplete insulators of transgenes in zebrafish.

The α-actin promoter is skeletal muscle specific, and EGFP is not expressed in the oocytes. Thus, the frequency of occurrence of EGFP in embryos from transgenic lines harboring the α-actin promoter and male transgenic lines harboring the β-actin promoter provides an accurate estimate of the germ-line transmission rate. It was difficult to distinguish, however, transgenic and non-transgenic progeny derived from female β-actin transgenic lines based on the appearance of EGFP before 14 dpf. This phenomenon was caused by the large accumulation of EGFP in the oocytes during oogenesis, even after meiotic division, when half the oocytes did not harbor transgenes. Although maternal inheritance of EGFP in oocytes was noted in transgenic zebrafish (Gibbs and Schmale, 2000; Linney et al., 1999), large accumulations of expressed EGFP in the oocytes were reported in our β-actin transgenic zebrafish. It was this store of EGFP that was responsible for the longer persistence of EGFP in non-transgenic progeny. In addition, oocytes with high EGFP intensity were common in our β-actin transgenic lines with different integration sites. Therefore, large

accumulations of EGFP in oocytes were independent of the location on the chromosome where the transgenes were integrated. Based on these observations, we believe that transgenic zebrafish harboring a transgene driven by the medaka β-actin promoter and flanked by AAV-ITRs will be useful bioreactors for producing target gene products in oocytes.

Integration Pattern of Transgenes Flanked by AAV-ITRs

Transgenes flanked by AAV-ITRs were present in extra-chromosomal form in *Xenopus* (Fu et al., 1998). Southern blot, dot blot, and progeny inheritance tests, however, strongly indicated that several copies of transgenes flanked by AAV-ITRs were randomly integrated into the zebrafish genome at a single locus and that this construct was stably transmitted. The integration pattern of transgenes flanked by AAV-ITRs is consistent with the random integration of recombinant AAV in vitro in the absence of Rep and Cap proteins (Ponnazhagan et al., 1997; Miao et al., 1998).

Transgenes flanked by AAV-ITRs tend to form random concatamers, including HtH, HtT, and TtT. Similar complex organization of transgene copies also has been found in *Xenopus* (Kroll and Amaya, 1996) and fish (Chong and Vielkind, 1989; Cretekos and Grunwald, 1999; Iyengar and Maclean, 1995). Concatamerization could result from end-joining of DNA fragments in zebrafish zygotes and early embryos (Hagmann et al., 1998). Rearrangements, partial deletions, and terminal modifications of transgenes were frequent in cell lines after transduction with recombinant AAV vectors (Nakai et al., 1999; Zhang and Fuleihan, 1999). We found similar transgene modifications and think that they may be responsible for the unpredictable expression level of EGFP in transgenic lines with different numbers of transgene copies.

In all transgenic lines, F1 progeny derived from the same founder always exhibited the same integration pattern, indicating that the integration pattern of the transgene flanked by AAV-ITRs was homogeneous in all F1 progeny. This differs from the results of other studies, in which transgenes often had multiple integration patterns in F1 progeny derived from the same founder (Alam et al., 1996; Culp et al., 1991; Liang et al., 2000; Penman et al., 1991). This clonal integration pattern makes it much easier to establish stable homozygotes. Without examining the integration status of the transgenes, important and useful genetic traits can be fixed rapidly in the F2 generation by F1 sibling crosses. Because the generation time of cultured fish is much longer than that of model fish, this strategy will be especially useful for establishing stable transgenic lines of economically valuable, cultured fish. In conclusion, we believe that a transgene flanked by AAV-ITRs can be used effectively to analyze promoter activity and specificity in assays of transient and stable transgene expression.

EXPERIMENTAL PROCEDURES

Plasmid Constructions

pα-actin-EGFP (αp-G-BS, 8 kb; Higashijima et al., 1997) contained a zebrafish α-actin promoter, EGFP cDNA, and the bovine growth hormone polyadenylation signal. A 0.74-kb, blunted *Hin*dIII/*Not*I fragment of EGFP cDNA obtained from pEGFP-1 (Clontech) was inserted into *Stu*I-digested pCS2ITR (5 kb; Fu et al., 1998). The resultant plasmid, pCMV-EGFP-ITR (5.8 kb), contained a cytomegalovirus (CMV) promoter fused with EGFP cDNA followed by an SV40 polyadenylation signal, and the entire gene cassette was flanked by AAV-ITRs. A 3.9-kb *Sal*I/*Nco*I fragment of zebrafish α-actin promoter obtained from pα-actin-EGFP was ligated with a 4.2-kb *Sal*I/*Nco*I fragment obtained from pCMV-EGFP-ITR. The resultant plasmid, pα-actin-EGFP-ITR (8.1 kb), was driven by zebrafish α-actin promoter fused with EGFP cDNA followed by an SV40 polyadenylation signal, and was flanked by AAV-ITRs.

pβ-actin-EGFP (pOBA-hGFP1, 10.6 kb; Hamada et al., 1998) contained medaka β-actin promoter, EGFP cDNA, the intron of t antigen, and the medaka β-actin gene polyadenylation signal. A 3.8-kb *Sal*I/*Nco*I fragment of the medaka β-actin promoter was released from pβ-actin-EGFP and ligated with a 4.2-kb *Sal*I/*Nco*I fragment released from pCMV-EGFP-ITR. The resultant plasmid, pβ-actin-EGFP-ITR (8 kb) was driven by medaka β-actin promoter fused with EGFP cDNA followed by an SV40 polyadenylation signal, and was flanked by AAV-ITRs.

Plasmid Preparation for Microinjection

Plasmids containing AAV-ITR sequences were amplified in *Escherichia coli* STBL2 cells. Plasmids pα-actin-EGFP-ITR and pβ-actin-EGFP-ITR were linearized by *Pst*I, and plasmids pα-actin-EGFP and pβ-actin-EGFP were linearized by *Sal*I. Linearized fragments were separated by 0.8% agarose gel electrophoresis, purified with a gel elution kit (Viogene), and resuspended at a final concentration of 10 μg/mL in 0.25 mol/L KCl with 0.1% (v/v) phenol red. Protamine sulfate from salmon sperm (Sigma) was added only to the injection solution containing the pβ-actin-EGFP-ITR vector. Plasmid and protamine sulfate mixtures were mixed at mass ratios of 0:1, 1:1, 1:3, 1:5, 1:7, and 1:9. The mixtures were kept at 4°C for at least 30 min and then injected into zebrafish embryos.

Transient Expression of EGFP Gene

Zebrafish were maintained as described by Westerfield (1995). The leopard variant (*D. rerio leo*) was used, because it has less pigmentation than the AB strain (McClure, 1999). Fertilized eggs were harvested within 20 min of spawning, and 10^6 copies of linearized DNA were injected into the cytoplasm of one-cell embryos. Injected embryos were cultured in embryonic solution at 28.5°C. The transient expression of each construct

was evaluated four to five times using 30–100 embryos in each test. About half the injected embryos died or exhibited abnormalities before the swimming-up stage. Only survivors with normal morphologic characteristics were scored.

Transient expression was determined at 5 dpf for pα-actin-EGFP- and pα-actin-EGFP-ITR-injected embryos and at 1 dpf for pβ-actin-EGFP- and pβ-actin-EGFP-ITR-injected embryos. The developmental stages of zebrafish were determined using the criteria of Kimmel et al. (1995). Based on the intensity and distribution of green fluorescence, expression was classified as U, M/W, or N. For expression to be considered uniform, 90–100% of the skeletal muscle tissue (pα-actin-EGFP derivatives) or the entire body (pβ-actin-EGFP derivatives) had to be EGFP positive. Expression was M/W if less than 90% of the skeletal muscle tissue (pα-actin-EGFP derivates) or less than 90% of the entire body (pβ-actin-EGFP derivatives) was EGFP positive.

Identification of Germ-Line Transmission

After mating with wild-type fish, founders capable of transmitting the EGFP gene were identified, along with their progeny, by both PCR analysis and green fluorescence detection. Three-day-old embryos derived from each potential founder were pooled in batches of 50–100. Their genomic DNA was extracted as described by Westerfield (1995). PCR analysis was done using forward primer, 5′-ATGGTGAGCAAGGGCGAGGA-3′ (1–20 nucleotides of EGFP cDNA), and reverse primer, 5′-TCCATGCCGAGAGTGATCCC-3′ (685–704 nucleotides of the anti-sense strand of EGFP cDNA). Forward primer, 5′-TGCTATTTTGCAGGCACGTC-3′, and reverse primer, 5′-AGCTTGCTGTCGCATAATCG-3′, designed to amplify the *ZF-21* gene (Njølstad et al., 1988), served as internal controls. PCR was done on 50 μL of solution containing about 100 ng genomic DNA extract, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 0.025 mg gelatin, 0.45% NP 40, 0.45% Triton X-100, 250 μmol/L dNTPs, 0.25 U *Taq* DNA polymerase, and 10 mmol/L Tris-HCl (pH 8.6). PCR was conducted for 35 cycles with an initial denaturation at 94°C for 3 min, followed by incubation at 94°C, 60°C, and 72°C for 30, 30, and 60 sec, respectively. The final extension step was performed at 72°C for 10 min.

To determine the pattern and extent of each embryo's fluorescence, 3-day-old embryos from each potential founder were observed under a dissecting stereo microscope (MZ12; Leica) equipped with a GFP2 filter. Photographs were taken using an MPS60 camera (Leica) and ASA 400 film (Fujicolor).

Quantitation of EGFP in Transgenic Lines

The amount of EGFP produced by F1 and F2 progeny derived from α-actin and β-actin transgenic lines was measured. The entire body of a 60-day-old male transgenic fish was frozen in liquid nitrogen and homogenized in a solution containing 0.5 mol/L Tris-HCl (pH 7.0), 0.5 N NaCl, 5 mmol/L EDTA, and 1 mmol/L phenylmethyl sulfonyl fluoride. After the homogenate was centrifuged at 10,000*g* for 10 min at 4°C, the total soluble protein concentration in the supernatant was measured with a protein assay kit (BioRad) using bovine serum albumin as a standard solution. After the total protein concentration was normalized at 100 μg/mL, the intensity of fluorescence was measured by fluorescence spectrophotometer (F-2000; Hitachi) using 488 nm for excitation and 509 nm for emission. The amount of EGFP in the samples was calculated based on a standard curve using recombinant EGFP (Clontech) dilution series.

Southern Blot Analysis

Caudal fins were removed from 60-day-old F1 and F2 zebrafish derived from the α-actin and β-actin transgenic lines. The fins were digested directly in DNA extraction buffer (25 mmol/L EDTA, 0.5% sodium dodecyl sulfate [SDS], 10 mmol/L NaCl, 200 μg/mL proteinase K, and 10 mmol/L Tris-HCl at pH 8.0) and incubated overnight at 55°C. After phenol-chloroform extraction and ethanol precipitation, 3–5 μg genomic DNA was digested by 50–100 U restriction enzyme. Samples were precipitated with ethanol and resuspended in TE buffer before electrophoresis on 0.8% agarose gel. Following electrophoresis, the DNA was blotted onto a Hybond N+ membrane (Amersham) with a vacuum transfer apparatus. After UV crosslinking, hybridization was carried out overnight at 65°C in the presence of a [^{32}P]dCTP-labeled probe. The probe was a 704-bp, PCR-amplified product from EGFP cDNA. After hybridization, membranes were washed twice in 2× SSC (1× SSC: 150 mmol/L sodium chloride and 15 mmol/L sodium citrate at pH 7.0) and 0.1% SDS (37°C, 30 min each wash), twice in 1× SSC and 0.1% SDS (65°C, 30 min each wash), and once in 0.1× SSC and 0.1% SDS (65°C, overnight). Autoradiographs were developed from X-ray film (BioMS; Kodak) that was exposed for 3–10 days at −70°C.

Dot Blot Analysis

The number of transgene copies in the genomic DNA of 60-day-old F1 and F2 zebrafish derived from α-actin and β-actin transgenic lines were determined by dot blot analysis. Genomic DNAs were purified as described earlier herein for Southern blot analysis. Three micrograms of genomic DNA was denatured under alkaline conditions (Kafatos et al., 1979) and blotted onto a Hybond N+ membrane (Amersham) using a Schleicher and Schuell minifold II apparatus. The blot was hybridized with EGFP-specific probe and washed as described for Southern blot analysis. Positive signals were quantitated by comparing their intensities with those of signals from known amounts of plasmid DNA. A haploid zebra fish genome is estimated to have 1.7×10^9 bp (Hinegardner and Rosen, 1972). We calculated that a single copy of the transgene in a haploid genome would be equivalent to 12 pg of transgene in 3 μg of genomic DNA.

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REFERENCES

Alam MS, Popplewell A, Maclean N. 1996. Germ line transmission and expression of a *lacZ* containing transgene in tilapia (*Oreochromis niloticus*). Transgenic Res 5:87–95.

Arcasoy SM, Latoche JD, Gondor M, Pitt BR, Pilewski JM.1997. Polycations increase the efficiency of adenovirus-mediated gene transfer to epithelial and endothelial cells in vitro. Gene Ther 4:32–38.

Bayer TA, Campos-Ortega JA. 1992. A transgene containing *lacZ* is expressed in primary sensory neurons in zebrafish. Development 115:421–426.

Caldovic L, Agalliu D, Hackett PB. 1999. Position-independent expression of transgenes in zebrafish. Transgenic Res 8:321–334.

Chen YH, Lee WC, Liu CF, Tsai HJ. 2001. Molecular structure, dynamic expression and promoter analysis of zebrafish (*Danio rerio*) *myf-5* gene. Genesis 29:22–35.

Chong SSC, 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, Alestrom P. 1998. Nuclear localization signals enhance germline transmission of a transgene in zebrafish. Transgenic Res 7:303–309.

Cretekos CJ, Grunwald DJ. 1999. *Alyron*, an insertional mutation affecting early neural crest development in zebrafish. Dev Biol 210:322–338.

Culp P, Nüsslein-Volhard C, Hopkins N. 1991. High frequency germline transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. Proc Natl Acad Sci U S A 88:7953–7957.

Douglas SE, Gallant JW, Bullerwell CE, Wolff C, Munholland J, Reith ME. 1999. Winter flounder expressed sequence tags: establishment of an EST database and identification of novel fish genes. Mar Biotechnol 1:458–464.

Drivenes O, Seo HC, Fjose A. 2000. Characterisation of the promoter region of the zebrafish six gene. Biochim Biophys Acta 1491:240–247.

Fu L, Mambrini M, Perrot E, Chourrout D. 2000. Stable and full rescue of the pigmentation in a medaka albino mutant by transfer of a 17 kb genomic clone containing the medaka tyrosinase gene. Gene 241:205–211.

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

Gibbs PDL, Schmale MC. 2000. GFP as a genetic marker scorable throughout the life cycle of transgenic zebrafish. Mar Biotechnol 2:107–125.

Gibbs PDL, Peek A, Thorgaard G. 1994. An *in vivo* screen for the luciferase transgene in zebrafish. Mol Mar Biol Biotechnol 3:307–316.

Gong Z, Yan T, Liao J, Lee SE, He J, Hew CL. 1997. Rapid identification and isolation of zebrafish cDNA clones. Gene 201:87–98.

Hagmann M, Bruggmann R, Xue L, Georgiev O, Schaffner W, Rungger D, Spaniol P, Gerster T. 1998. Homologous recombination and DNA-end joining reactions in zygotes and early embryos of zebrafish (*Danio rerio*) and *Drosophila melanogaster*. Biol Chem 379: 673–681.

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

Henrich T, Wittbrodt J. 2000. An *in situ* hybridization screen for the rapid isolation of differentially expressed genes. Dev Genes Evol 210:28–33.

Higashijima SI, Hotta Y, Okamoto H. 2000. Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the *islet-1* promoter/enhancer. J Neurosci 20:206–218.

Higashijima SI, Okamoto H, Ueno N, Hotta Y, 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.

Hinegardner R, Rosen DE. 1972. Cellular DNA content and the evolution of teleostean fishes. Am Nat 106:311–319.

Ikawa M, Yamada S, Nakanishi T, Okabe M. 1998. Green mice and their potential usage in biological research. FEBS Lett 430:83–87.

Ingham PW. 1997. Zebrafish genetics and its implications for understanding vertebrate development. Hum Mol Genet 6:1755–1760.

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

Jessen JR, Meng A, McFarlane RJ, Paw BH, Zon LI, Smith GR, Lin S. 1998. Modification of bacterial artificial chromosomes through Chi-stimulated homologous recombination and its application in zebrafish transgenesis. Proc Natl Acad Sci U S A 95:5121–5126.

Ju B, Xu Y, He J, Liao J, Yan T, Hew CL, Lam TJ, 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.

Kafatos FC, Jones CW, Efstratiadis A. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res 7:1541–1552.

Kato M, Yamanouchi K, Ikawa M, Okabe M, Naito K, Tojo H. 1999. Efficient selection of transgenic mouse embryos using EGFP as a marker gene. Mol Reprod Dev 54:43–48.

Kimmel CB. 1989. Genetics and early development of zebrafish. Trends Genet 5:283–288.

Kimmel CB, Ballard WW, Kimmel, SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310.

Kroll KL, Amaya E. 1996. Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122:3173–3183.

Lechardeur D, Sohn KJ, Haardt M, Joshi PB, Monck M, Graham RW, Beatty B, Squire J, O'Brodovich H, Lukacs GL. 1999. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. Gene Ther 6:482–497.

Liang MR, Alestrom P, 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.

Linney E, Hardison NL, Lonze BE, Lyons S, DiNapoli L. 1999. Transgene expression in zebrafish: a comparison of retroviral-vector and DNA-injection approaches. Dev Biol 213:207–216.

Long G, Meng A, Wang H, Jessen JR, Farrell MJ, Lin S. 1997. GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. Development 124:4105–4111.

McClure M. 1999. Development and evolution of melanophore patterns in fishes of the genus *Danio* (Teleostei: Cyprinidae). J Morphol 241:83–105.

Meng A, Tang H, Ong BA, Farrell MJ, Lin S. 1997. Promoter analysis in living zebrafish embryos identifies a cis-acting motif required for neuronal expression of GATA-2. Proc Natl Acad Sci U S A 94:6267–6272.

Meng A, Tang H, Yuan B, Ong BA, Long Q, Lin S. 1999. Positive and negative cis-acting elements are required for hematopoietic expression of zebrafish GATA-1. Blood 93:500–508.

Miao CH, Snyder RO, Schowalter DB, Patijn GA, Donahue B, Winther B, Kay MA. 1998. The kinetics of rAAV integration in the liver. Nat Genet 19:13–15.

Nakai H, Iwaki Y, Kay MA, Couto LB. 1999. Isolation of recombinant adeno-associated virus vector-cellular DNA junctions from mouse liver. J Virol 73:5438–5447.

Njølstad PR, Molven A, Fjose A. 1988. A zebrafish homologue of the murine *Hox 2.1* gene. FEBS Lett 230:25–30.

Nüsslein-Volhard C. 1994. Of flies and fishes. Science 266:572–574.

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

Patil JG, Wong V, Khoo HW. 1994. Assessment of pMTL construct for detection in vivo of luciferase expression and fate of the transgene in the zebrafish, *Brachydanio rerio*. Zool Sci 11:63–68.

Penman DJ, Iyengar A, Beeching AJ, Rahman MA, Sulaiman Z, Maclean N. 1991. Pattern of transgene inheritance in rainbow trout (*Oncorhynchus mykiss*). Mol Reprod Dev 30:201–206.

Ponnazhagan S, Erikson D, Kearns W, Zhou SZ, Nahreini P, Wang XS, 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.

Samulski RJ, Chang LS, Shenk T 1989. Helper-free stocks of adeno-associated viruses: normal integration does not require viral gene expression. J Virol 63:3822–3828.

Sato A, Komura JI, Masahito P, Matsukuma S, Aoki K, Ishikawa T. 1992. Firefly luciferase gene transmission and expression in transgenic medaka (*Oryzias latipes*). Mol Mar Biol Biotechnol 1:318–325.

Selman K, Wallace RA, Sarka A, Qi X. 1993. Stages of oocyte development in the zebrafish, *Brachydanio rerio*. J Morphol 218:203–224.

Sorgi FL, Bhattacharya S, Huang L. 1997. Protamine sulfate enhances lipid-mediated gene transfer. Gene Ther 4:961–968.

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

Stuart GW, McMurray JV, Westerfield M. 1988. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development 103:403–412.

Takagi S, Sasado T, Tamiya G, Ozato K, Wakamatsu Y, Takashita A, Kimura M. 1994. An efficient expression vector for transgenic medaka construction. Mol Mar Biol Biotechnol 3:192–199.

Tsai HJ, Tseng FS, Liao IC. 1995a. Electroporation of sperm to introduce foreign DNA into the genome of loach (*Misgurnus anguillicaudatus*). Can J Fish Aquat Sci 52:776–787.

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

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

Vascotto SG, Beckham Y, Kelly GM. 1997. The zebrafish's swim to fame as an experimental model in biology. Biochem Cell Biol 75: 479–485.

Westerfield M. 1995. The zebrafish book. Eugene: University of Oregon Press.

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