A Transgene and Its Expression Profile are Stably Transmitted to Offspring in Transgenic Medaka Generated by the Particle Gun Method

Authors: Kinoshita, Masato, Yamauchi, Masatake, Sasanuma, Motoe, Ishikawa, Yuji, Osada, Takashi, et al.

Source: Zoological Science, 20(7) : 869-875

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.20.869

ZOOLOGICAL SCIENCE **20**: 869–875 (2003)

A Transgene and Its Expression Profile are Stably Transmitted to Offspring in Transgenic Medaka Generated by the Particle Gun Method

Masato Kinoshita[1]*, Masatake Yamauchi[2], Motoe Sasanuma[2], Yuji Ishikawa[2], Takashi Osada[3], Koji Inoue[4], Yuko Wakamatsu[3] and Kenjiro Ozato[3]

[1]*Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*
[2]*National Institute of Radiological Sciences, Chiba 263-8555, Japan*
[3]*Laboratory of Freshwater Fish Stocks, Bioscience Center, Nagoya University, Nagoya 464-8601, Japan*
[4]*Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164-8639, Japan*

ABSTRACT—A particle gun is used in a potential method for introducing foreign genes into fish. In this paper, we report on the stable transmission of a transgene and its expression profile of the F4 generation in the transgenic medaka (*Oryzias latipes*). We established four transgenic strains, which contained a green fluorescent protein (GFP) gene controlled by a medaka β–actin promoter, using a particle gun. One more transgenic strain was also generated by microinjection for comparison. In all five strains, the founder was discovered to be mosaic for the transgene. However, from the F1 to F4 generations, transgenes and their expression profiles were stably inherited in the Mendelian manner. The expression profile was common among the five strains regardless of the method for gene transfer: GFP fluorescence became detectable at an early neurula stage. In this stage, the fluorescence was observed ubiquitously in most tissues. As somite developed, GFP fluorescence became intense only in the skeletal muscle and lens but it decreased in other tissues. In adult fish, an intense fluorescence was restricted in the skeletal muscle and lens, while a considerably weak fluorescence was observed in the brain, gill, heart, kidney, spleen, and ovary. From these results, it was concluded that the transgene and its expression profile were stably transmitted to offspring, and thus the particle gun is an effective method for transgenesis in spite of its easiness.

Key words: medaka, transgenic, beta-actin gene, green fluorescence, particle gun

INTRODUCTION

Small fish species, the medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), have been receiving much attention as experimental animals in recent years. These species have many advantageous features for experiment: they are tough and easy to be maintained in small aquariums in a laboratory; they have a relatively short generation time and they frequently spawn eggs; the size of the eggs is large enough for manipulation of the embryos; and the developmental process is visible through the transparent chorion. In addition, especially in medaka, it is possible to observe internal organs without killing fish, even in adult fish, because of a special “see-through” medaka strain lacking pigment through the lifecycle has been generated (Wakamatsu *et al*., 2001).

Recently, the combined use of the green fluorescent protein (GFP) and the transgenic technique is becoming popular in various organisms. Using the GFP gene as a reporter, the expression profiles of regulatory elements of interest can be visualized in live organisms without using additional chemicals and complicated treatments. It is also possible that tissues of interest can be labeled using the GFP gene linked to tissue specific promoters (Higashijima *et al*., 2000; Tanaka *et al*., 2001). The transparency of the chorion, embryos or adult fish of medaka offer a quite advantageous condition for various experiments using GFP.

* Corresponding author: Tel. +81-75-753-6213;
FAX. +81-75-753-6223.
E-mail: kinoshit@kais.kyoto-u.ac.jp

Abbreviations: GFP, green fluorescent protein; NLS, nuclear localization signal; PCR, polymerase chain reaction; SV40, simian virus 40

To introduce gene constructs containing the GFP gene into medaka embryos, microinjection has been used in most of the previous studies (Hamada *et al.*, 1998; Kinoshita *et al.*, 2000; Tanaka and Kinoshita, 2001; Tanaka *et al.*, 2001). The gene transfer efficiency of this method is generally high, and it has been also indicated that introduced genes are transmitted to offspring through the germ-line (Kinoshita *et al.*, 2000, 2001; Tanaka *et al.*, 2001). However, the microinjection operation is performed under a microscope, in which skilled technique is required, and thus a limited number of embryos can be treated at one time. Thus, several other methods such as electroporation (Inoue *et al.*, 1990; Murakami *et al.*, 1994; Ono *et al.*, 1997) and use of a retroviral vector (Lu *et al.*, 1997) have been utilized to achieve foreign gene transfer. Recently, we proposed the particle gun method in high hopes for mass experiments (Yamauchi *et al.*, 2000), in which the successful transfer of a foreign gene construct with high efficiency into medaka embryos and the inheritance of the introduced gene into the next generation were demonstrated. However, no information has been available on the fate of the foreign gene construct introduced by the particle gun and the stability of expression profile in later generations. Here we report on the stable inheritance of the foreign gene construct pβ-Act-GFP-N, containing a GFP gene linked to the medaka β-actin promoter, introduced by the particle gun as well as the common spacio-temporal expression profile between the F1 and F4 generations.

MATERIALS AND METHODS

Fish

The outbred orange-red medaka was maintained under an artificially controlled photoperiod (14 hr of light and 10 hours of darkness) at 26°C.

Plasmid construction

Expression plasmid pβ-Act-GFP-N (Fig. 1), corresponding to pCMX-SAH/Y145F in Yamauchi *et al.* (2000) was prepared by the modification of pCMX-GFP-1 described by Ogawa and Umezono (1998). Namely, a 5'-upstream sequence of medaka beta actin gene reported by Takagi *et al.* (1994) was amplified by a polymerase chain reaction (PCR). The amplified fragment replaced the cytomegalovirus promoter sequence in pCMX-GFP-1. An additional sequence, which contains a SV40 nuclear localization signal (NLS, amino acid sequence of KKKRKV), was ligated to the 3' end of a GFP gene.

Detection of the transgene

DNA was isolated from a three-day-old embryo or tail fin of an adult individual using the conventional proteinase K method (Kinoshita *et al.*, 2000). For the detection of the *GFP* gene, PCR was performed using a GFP-gene-specific primer pair (AFP-FW and SAH-N-RV1), which yielded an 814-bp fragment. The DNA sequences of the primers were as follows: AFP-FW, TACTGGCGCCAAGCTTCTGCAGGTCGACGGATCCGAATTCGGTACCACCATGGTGAGCAAGG; and SAH-N-RV1, CTAAAGCTTCTAGCTAGCCACCTTGCGCTTCTTCTTGGGGCCGCCTGACTTGTACAGCTCG. To confirm successful DNA isolation and PCR, the *EF-1α-A* gene was amplified using an *EF-1α-A* gene-specific primer pair (EF-810F and EF-1160R), which yielded as a 519-bp fragment. The DNA sequences of the primers were as follows: EF-810F, CAGGACGTCTACAAAATCGG; and EF-1160R, AGCTCGTTGAACTTGCAGGCG. The PCR amplification cycles consisted of 96°C for 30 s, 55°C for 30 s and 72°C for 60 s.

Establishment of transgenic medaka strains

In addition to the two transgenic strains reported previously (Yamauchi *et al.*, 2000), two more strains were newly established using the same method. These four F0 individuals that harbored the β-Act-GFP-N gene in germ cells were mated with non-transgenic individuals. One of the F1 transgenics obtained from each F0 individual was mated (back-crossed) with a non-transgenic male to obtain an F2 generation. Brother-sister mating was carried out in the F2 generation, and the F3 individuals homozygous for the β-Act-GFP-N gene were bred as a transgenic medaka strain. The individual, all of whose offspring mated with non-transgenic fish were transgenic, was identified as a homozygote. F4 individuals obtained by brother-sister mating of F3 individuals homozygous for the transgene were used for detailed analysis of the expression profile. An additional strain was also established by the microinjection. The plasmid pβ-Act-GFP-N (25 μg/ml in phosphate buffered saline) was injected into the cytoplasm of a one-cell stage medaka embryo, as described previously (Kinoshita *et al.*, 1996). Out of the ten injected embryos, one individual harbored the transgene in the germ cells. A transgenic F1 individual (female) was mated with a non-transgenic male to obtain an F2 generation and a homozygous F3 and F4 generation were obtained as described above.

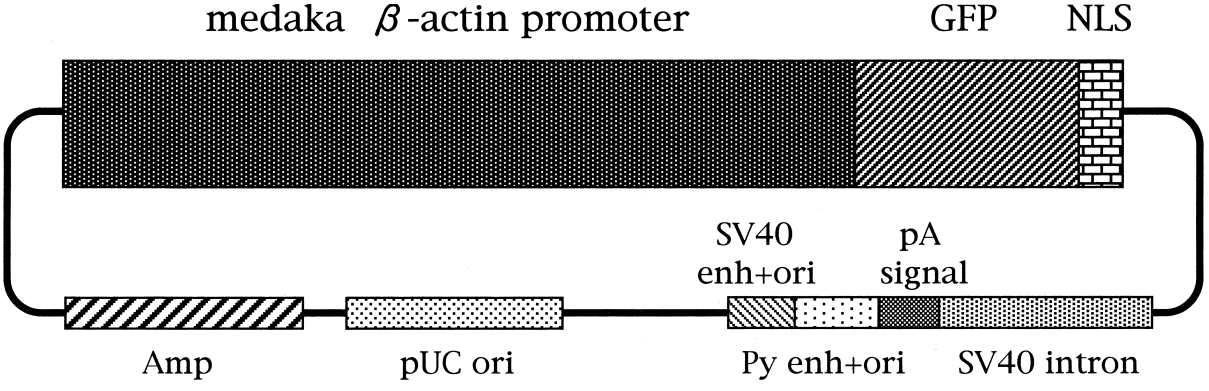

Fig. 1. Structure of pβ-Act-GFP-N. A green fluorescence protein (GFP) gene was followed by a nuclear localization signal (NLS, amino acid sequence of KKKRKV). A PCR-amplified 5'-upstream sequence of medaka beta actin gene reported by Takagi *et al.* (1994) was located at the 5'-end of GFP gene.

Observation of fluorescence

Fluorescence was observed by a stereomicroscope (Leica MZFL III with GFP2 filter set) or a fluorescence microscope (OLYMPUS BX50WI with U-MGFP/XL filter set).

RESULTS

Inheritance of the transgene to offspring

We generated four transgenic strains (strains A, B, C, and D) by the particle gun method in total as follows: 1691 fertilized eggs were treated with the particle gun, 75 were found to have fluorescence derived from the transgene at the hatching stage, 5 of them transmitted the fluorescence to offspring as reported previously (Yamauchi *et al*., 2000),

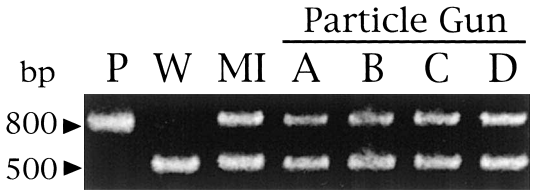

Fig. 2. Detection of inheritance of the GFP gene in the F4 generation. DNA was extracted from the tail fin of each transgenic strain of the F4 generation. For the detection of the *GFP* gene, a GFP gene-specific primer pair, which yielded an 814-bp fragment, was used. A medaka-EF-1α-A-gene-specific primer pair, which yielded a 519-bp fragment, was also used to confirm successful DNA extraction. P, introduced plasmid (pβ-Act-GFP-N); W, non-transgenic medaka; MI, transgenic strain produced by conventional microinjection; A-D, transgenic strain produced by the particle gun method.

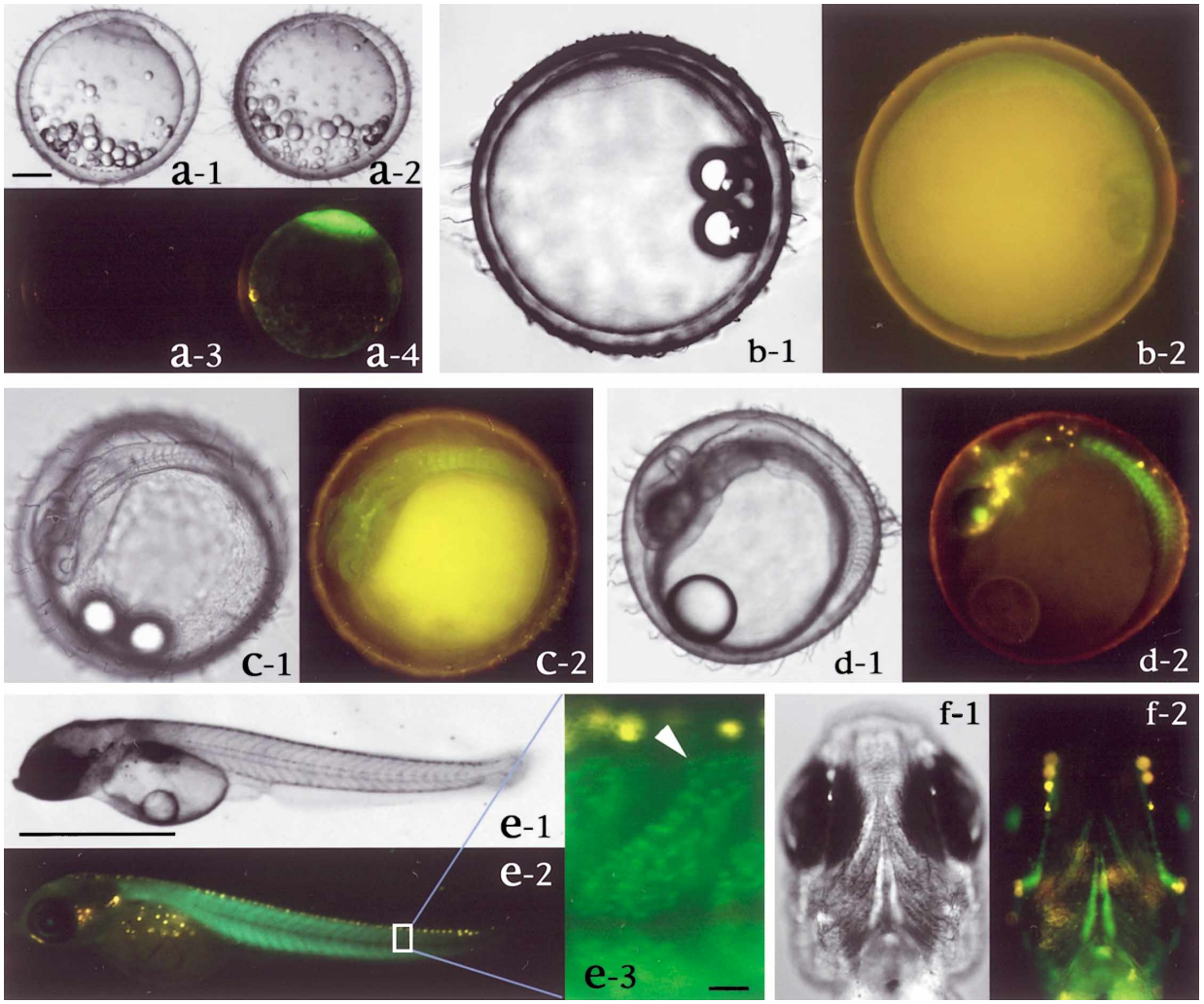

Fig. 3. Expression of the GFP gene in a β-Act-GFP-N transgenic fish produced by the particle gun method during embryonic development. F4 individuals of the transgenic strain A were examined by fluorescence microscopy. In a-2 and a-4, pictures of an embryo of olvas-GFP transgenic fish (Tanaka *et al*., 2001) were shown for comparison. At a one-cell stage, fluorescence of the GFP was not observed in an embryo of β-Act-GFP-N transgenic fish (a-3), while intense fluorescence was observed in olvas-GFP transgenic fish (a-4). The fluorescence was observed ubiquitously at the head forming stage (b-2). The ubiquitous expression of GFP was observed till the 12-somite-stage (c-2). The fluorescence became intense only in the somite and lens (d-2). Intense fluorescence was observed in the skeletal muscle in the trunk at the hatching stage (e-2). An arrowhead in e-3 shows one of the spots revealing intense fluorescence of GFP. Intense fluorescence was also observed in the lens and the muscle in jaw of hatchling (f-2). a-1, a-2, b-1, c-1, d-1, e-1, and f-1 are the bright field images; a-3, a-4, b-2, c-2, d-2, e-2, e-3, and f-2 are the fluorescent images. Yellow spots in the fluorescent images are the auto-fluorescence of pigment cells. Bars in a-1, e-1, and e-3 represent 0.25, 1, and 0.02 mm, respectively.

and 4 stable strains were successfully established. The proportion of transgenics in the F1 generation was 3% for strain A and 5% for strain B, which suggest the mosaic distribution of the introduced transgene. In the F2 generation, the transgene was detected in approximately 50% of all siblings. The crossing of a transgenic male and a female of the F2 generation resulted in the Mendelian inheritance of the transgene to the F3 generation. By crossing transgenic F3 individuals homozygous for the transgene, all the F4 individuals became homozygous transgenic fish.

In the transgenic strain generated by microinjection, the proportion of embryos revealed that the fluorescence at the hatching stage was 40% (4 of 10 injected embryos) and one of them inherited the transgene to the F1 generation. The proportion of transgenics in F1 individuals was 23% (24 of 103 tested embryos), which also suggests the mosaic incorporation of the transgene. In this strain, the transgene was also transmitted from F1 to F2 by the back-crossing with nontransgenic fish, and F3 and F4 generations were obtained as in the four strains described above.

Fig. 2 shows the representative results of the PCR analysis for the existence of the transgene in the F4 generation. The GFP gene was clearly detected in F4 individuals derived from all four transgenic strains produced with the particle gun method, as well as those from a transgenic strain produced by the conventional microinjection method.

GFP expression during embryogenesis

The pattern of expression in developing embryos and hatchlings was common among four transgenic strains generated by the particle gun method and one strain generated by microinjection. The expression pattern was also common between heterozygous F1 and homozygous F4 generations in all five strains. In transgenic embryos at a one-cell stage (stage 2b of Iwamatsu, 1994), fluorescence was not detected (Fig. 3 a-1 and a-3), although intense fluorescence of GFP was observed in some transgenic medaka strains; for example, the olvas-GFP medaka strain (Fig. 3 a-2 and a-4) (Tanaka *et al.*, 2001). The fluorescence began to be observed ubiquitously in the embryonic body at the head forming stage (stage 17) (Fig. 3 b-1 and b-2). The ubiquitous expression of GFP was observed until the 12-somite stage (stage 23, Fig. 3 c-1 and c-2). After this stage, the fluorescence became intense only in the somite and lens. The fluorescence in other tissues, on the other hand, decreased gradually and became hardly detectable (Fig. 3 d-1 and d-2).

In hatchlings, intense fluorescence was observed in the skeletal muscle in the trunk (Fig. 3 e-1 and e-2), so that each somite was recognized clearly in the fluorescent image. The magnified image of the skeletal muscle revealed the spots (about 10 μm in length) of a more intense fluorescence of GFP (one of these spots was indicated by arrowhead in Fig. 3, e-3). Intense fluorescence was also observed in the lens (Fig. 3 e-2, f-2) and the muscle in the jaw (Fig. 3 f-1 and f-2).

GFP expression in adult tissues

The expression sites in adult fish of the F1 and F4 gen-

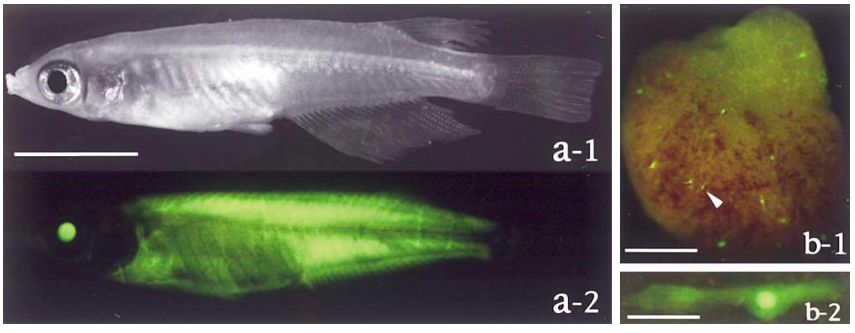

Fig. 4. Expression of the GFP gene in a β-Act-GFP-N transgenic fish produced by the particle gun method. F4 individuals (strain A) were examined by fluorescence microscopy. a, external appearance of adult individual; b-1, heart; b-2, magnified image of a GFP expressing cell indicated in b-1 by arrowhead. a-1 is a bright field image of an adult male fish. a-2, b-1, and b-2 are fluorescent images. Bars in a-1, b-1, and b-2 represent 5 mm, 300 mm, and 10 μm, respectively.

Table 1. Expression of GFP in adult fish.

Tissue	brain	gill	heart	liver	spleen	kidney	gut	ovary	testis
Intensity	+	++	+	–	++	++	–	++	–

Anatomical observation on a F4 individual produced by the particle gun method (line A) was performed under a stereomicroscope. In this individual, the intensity of green fluorescence in tissues (except the muscle and the lens) was considerably weak; the fluoescence was observed in a small portion of cells of each tissue (see Fig. 4b-1). Fluorescence intensity is expressed in three categories: weak (++), fairly weak (+) and undetectable (–).

erations were also common among the four strains generated by the particle gun method and the one by microinjection. The external view of an adult transgenic individual of a strain generated by the particle gun was shown in Fig. 4 a-1 and a-2. Intense fluorescence was observed in the body of the trunk and lens. The intense fluorescence enables us to identify transgenic individuals more easily under the fluorescent microscope. The results of anatomical studies on adult fish were summarized in Table 1. The fluorescence was observed in the brain, gill, heart (Fig. 4b), spleen, kidney, and ovary. The intensity of fluorescence in these tissues was considerably weaker than that of the skeletal muscle. In these tissues, however, all cells did not show green fluorescence. For example, only a small portion of the cells showed the fluorescence in the heart (Fig. 4b-1). The magnified image of the cells showing fluorescence in the heart (Fig. 4b-2) represented that the GFP was concentrated in the nucleus. We could not detect green fluorescence in the liver, gut, and testis using a stereomicroscope.

DISCUSSION

In this study, we successfully established four transgenic strains bearing the transgene pβ-Act-GFP-N containing the GFP gene controlled by a 5'-upstream sequence of the medaka β-actin gene. We demonstrated the Mendelian transmission of the transgene to the F4 generation. The expression patterns in embryos, hatchlings and adult fish were faithfully reproduced in each generation, which was the same as the patterns in the strain generated by microinjection. Thus, we conclude that the transgene introduced by the new method, the particle gun, is stably transmitted to offspring as that introduced by the traditional microinjection technique. Considering that the procedure is much easier than microinjection, the particle gun method is one of the most promising methods for mass transfer experiments.

At present, on the other hand, there are some points needed to be improved. The frequency of germ-line-transmitting founders is one of the most important factors to select a gene delivery method for the production of a transgenic strain. In the case of the microinjection method, the frequency reported so far is 2% (3 germ-line-transmitting founders / 150 injected eggs, Kinoshita *et al*., 2000), 3% (13/410, Tanaka *et al*., 2001), and 10% (1/10, this study). On the other hand, the frequency using the particle gun method was 0.4% (2/564 at the optimum condition, Yamauchi *et al*., 2000), which was quite low compared with those of the microinjection method. Chimerism of germ cells in F0 founders is also a critical parameter for the selection of the gene delivery method. The chimerism observed in the particle gun method was relatively low (3% in strain A and 5% in strain B) compared with those observed in the microinjection method (23%, in this study and 5~67%, Tanaka *et al*., 2001). The disadvantages of the particle gun method may be due to the following reasons: In the case of microinjection, DNA is perfectly delivered into the cytoplasm of a 1-cell-stage embryo. On the contrary, in the case of the particle gun method, some particles coated with DNA reach the cytoplasm and some stay in the chorion, perivitelline space, and/or the yolk sac. In other words, the total volume of DNA delivered into cytoplasm is not enough to elevate the frequency of germ-line-transmitting founders and chimerism up to those of the microinjection method. Another unknown feature of DNA may also reduce the frequency of the particle gun method. In the case of microinjection, the feature of DNA that is aqueous fluid makes it easy to disperse into cytoplasm. As a result, this dispersion elevates the number of cells in which the integration of the transgene may occur. In the case of the particle gun method, DNA is attached to the gold particle. Therefore, DNA must be dissolved into cytoplasmic fluid before dispersion, while cleavage is already ongoing. This delay may reduce the chance of integration of the transgene. The low frequency of germ-line-transmitting founders and chimerism requires laborious work to search a great number of individuals or eggs for the identification of germ-line-transmitting founders or transgenics. We think that the combination of a gene, which we want to introduce into medaka, with a reliable selective marker gene, for example olvas-GFP as a germ-cell-transmitter marker (Tanaka *et al*., 2001), helps us to identify transgenics easily and cancels the disadvantages of the particle gun method.

Since the expression pattern was common among the four strains and the one strain generated by microinjection, it is evident that the expression pattern reflects the activity of regulatory elements contained in the plasmid pβ-Act-GFP-N. In the developmental process, the expression of pβ-Act-GFP-N became detectable at the head forming stage in this study. This result is in contrast with the case of the transgenic medaka bearing the GFP gene driven by a ubiquitous promoter EF-1α, in which the GFP expression was already detectable at the early gastrula stage (Kinoshita *et al*., 2000). It has been shown that the zygotic gene expression generally starts at the midblastula stage in developing medaka (Chong and Vielkind, 1989; Tsai *et al*., 1995). Taking these facts into consideration, the regulatory elements contained in pβ-Act-GFP-N becomes active with a time lag after the onset of the zygotic gene expression. It has been also reported that GFP fluorescence is already detectable before the midblastula stage in some transgenic medaka and zebrafish strains. For example, the transgenic medaka with the olvas-GFP gene (Tanaka *et al*., 2001), which is shown for comparison in Fig. 3 a-2 and a-4, a strong fluorescence is detected in the blastdisc of the one-cell stage. The fluorescence in a one-cell stage is, however, obviously derived from the maternal expression. In the case of pβ-Act-GFP-N used in this study, the lack of expression in the cleavage stages is consistent with the very low-level of expression in the gonad of the adult fish.

Next, we added NLS of SV40 to the end of the GFP-coding region. As a result, a more intense green fluorescence was observed at the nucleus than in the cytoplasm

(Fig. 3 e-3 and Fig. 4 b-2). It is evident that the NLS concentrated GFP in the nucleus and the low-level of fluorescence in the cytoplasm is supposed to be the result of the overflow from the nucleus. These results suggested that the intra-cellular localization of the transgene product could be controlled in the medaka using NLS of SV40. This element may be helpful for the transgenic studies, for example, the low level of expression may become detectable by concentrating the GFP to the nucleus.

A fish whose certain tissues are labeled, is a useful model to study the development of these tissues. For example, a transgenic zebrafish strain with *Islet-1* promoter/enhancer sequence expresses GFP in the cranial motor neurons (Higashijima *et al*., 2000). The medaka strain with olvas-GFP expresses GFP exclusively in germ cells (Tanaka *et al*., 2001). In this study, transgenic strains harboring pβ-Act-GFP-N revealed a specific expression in the lens and the muscle. Since the expression pattern was uniform among the five separate strains, it is a specific characteristic of the regulatory elements contained in β-Act-GFP-N. Some other transgenic medaka and zebrafish strains containing the GFP gene linked to the regulatory region of the β-actin gene have been reported previously. As for the medaka, Takagi *et al*. (1994) , Hamada *et al*. (1998), and Chou *et al*. (2001) reported that the promoter of β-actin exhibits a ubiquitous expression in various tissues. Higashijima *et al*. (1997) produced two transgenic zebrafish strains; one harboring the GFP gene driven by a muscle-specific actin (alpha-actin) promoter specifically expressed the GFP throughout the muscle cells and the other harboring the GFP gene driven by the cytoskeletal β-actin gene promoter expressed the GFP throughout the body. The results in this study, in which strong expression is limited to the muscle and the lens, are different from the ubiquitous expression pattern in previous studies and are very similar to the expression pattern of the skeletal muscle actin. The reason of the difference in the expression patterns is unknown at present, however, it may be due to the difference of the length of the upstream region of the β-actin gene or differences in the construction of the downstream GFP gene. Nevertheless, the intense fluorescence in muscle cells of our transgenic strains may be helpful in the transplantation experiments to identify transplanted cells or nuclei because of its intense fluorescence.

ACKNOWLEGMENT

This work was supported in part by the Grants-in-Aid for Scientific Research on Priority Area (11236208 to M.K.)

REFERENCES

Chou CY, Horng LS, Tsai HJ (2001) Uniform GFP-expression in transgenic medaka (*Oryzias latipes*) at the F0 generation. Transgenic Res 10: 303–315

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

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 Biotech 7: 173–180

Higashijima S, 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

Higashijima S, 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

Inoue K, Yamashita S, Hata J, Kabeno S, Asada S, Nagahisa E, Fujita, T (1990) Electroporation as a new technique for producing transgenic fish. Cell Differ Dev 29: 123–128

Iwamatsu T (1994) Stages of normal development in the medaka *Oryzias latipes*. Zool Sci 11: 825–839

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

Kinoshita M, Kani S, Ozato K, Wakamatsu Y (2000) Activity of the medaka translation elongation factor 1α-A promoter examined using the GFP gene as a reporter. Dev Growth Differ 42: 469–478

Kinoshita M, Morita T, Toyohara H, Hirata T, Sakaguchi M, Ono M, Inoue K, Wakamatsu Y, Ozato K (2001) Transgenic medaka overexpressing a melanin-concentrating hormone exhibit lightened body color but no remarkable abnormality. Mar Biotechnol 3: 536–543

Lu JK, Burns JC, Chen TT (1997) Pantropic retroviral vector integration, expression and germline transmission in medaka fish *(Oryzias latipes*). Mol Mar Biol Biotech 6: 289–295

Murakami Y, Motohashi K, Yano K, Ikebukuro K, Yokoyama K, Tamiya E, Karube I (1994) Micromachined electroporation system for transgenic fish. J Biotechnol 34: 35–42

Ogawa H, Umezono K (1998) Intracellular localization and transcriptional activation by the human glucocorticoid receptor-green fluorescent protein (GFP) fusion proteins. Acta Histochem Cytochem 31: 303–308

Ono H, Hirose E, Miyazaki K, Yamamoto H, Matsumoto J (1997) Transgenic medaka fish bearing the mouse tyrosinase gene: expression and transmission of the transgene following electroporation of the orange-colored variant. Pigment Cell Res 10: 168–175

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

Tanaka M, Kinoshita M (2001) Recent progress in the generation of transgenic medaka (*Oryzias latipes*). Zool Sci 18: 615–622

Tanaka M, Kinoshita M, Kobayashi D, Nagahama Y (2001) Establishment of medaka (*Oryzias latipes*) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: A useful model to monitor germ cells in a live vertebrate. Proc Natl Acad Sci USA 98: 2544–2549

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

Wakamatsu Y, Pristyazhnyuk S, Kinoshita M, Tanaka M, Ozato K (2001) The see-through medaka: A fish model that is transparent throughout life. Proc Natl Acad Sci USA 98: 10046–10050

Yamauchi M, Kinoshita M, Sasanuma M, Tsuji S, Terada M,

Morimyo M, Ishikawa Y (2000) Introduction of a foreign gene into medakafish using the particle gun method. J Exp Zool 287: 285–293

(Received January 8, 2003 / Accepted March 31, 2003)