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[REVIEW]

Recent Progress in the Generation of Transgenic Medaka (*Oryzias latipes*)

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ABSTRACT—Medaka (*Oryzias latipes*) has been recognized as a potential model vertebrate to which modern molecular genetics can be applied. Recent progress in transgenic techniques emphasizes the importance of the use of medaka in a variety of biological fields, particularly in view of the development of transgenic vectors. The first transgenic medaka was produced by microinjection of DNA into the pronucleus of unfertilized oocytes. Several new methods have since been developed for producing transgenic medaka, and an appropriate method can be selected according to the aims of experiments and application. Transgenic medaka have been used in many fields, including aquaculture, toxicology, developmental biology (phenotypic rescue experiments), and for successful characterization of transcriptional regulatory regions. Here, we describe examples of the application of transgenics in these fields. We also summarize the recent progress in transgenic techniques and transgenic vectors, focusing on the elements and the marker genes that enable identification and/or induction of the expression of exogenous genes in transgenics.

INTRODUCTION

The establishment of methods to introduce exogenous genes into organisms, to transmit exogenous genes to the next generations, and to direct proper expression from the exogenous gene is one of the basic and indispensable criteria for an organism to be referred to as a model organism. Medaka (*Oryzias latipes*) is an excellent model organism to which molecular genetics can be applied, and there have been many studies over the past ten years using medaka in a wide variety of biological fields, including physiology, toxicology, genetics and behavior. In addition, the development of pigment-free medaka by successive crossing of different color mutants offers a potentially excellent means for investigating phenomena in living medaka, especially if used in combination with newly developed transgenic techniques for the expression of fluorescent proteins in certain cell types (Wada *et al*., 1998; Tanaka *et al*., 2001). It is expected that the pigment-free transgenic medaka will enable visualization of at cellular levels not only in living embryos but also in living adult medaka. Since such observations would be eventually impossible using other vertebrates, observations using pigment-free transgenic medaka are expected to shed light on new aspects of classical physiological, toxicological and endocrinological fields. Here, we first describe the basic skills required for producing transgenic medaka and then summarize recent examples of the use of transgenic medaka.

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Production of Transgenic Medaka

Transgenic fish were the first produced by microinjection of the chicken delta-crystallin gene into the pronuclei of matured medaka oocytes (Ozato *et al*., 1986; Inoue *et al*., 1989). Surprisingly, the exogenous gene (chick delta-crystallin) showed proper expression in medaka eyes, suggesting that genomic *cis*-elements regulating the transcription may be partially functional beyond the species. However, the microinjection into oocyte pronuclei and the subsequent insemination process were time-consuming techniques. Therefore, several more convenient methods for producing transgenic medaka have been developed. For example, electroporation was used to introduce exogenous DNA into medaka (Inoue *et al*., 1990; Ono *et al*., 1997). A transgenic vector was designed so that trout growth hormone cDNA was flanked by the mouse metallothionein promoter and the SV40 poly(A)$^+$ signal. Although germline-transmitted transgenic medaka have been successfully produced, electroporation has not been widely used because of the difficulties in electric pulse settings and low efficiency in the production of transgenics. Microinjection into a fertilized egg at one or two cell stage is

currently the most widely accepted method.

Transient expression can be seen in 10–80% of microinjected medaka during embryogenesis (Tanaka and Kinoshita, unpublished data). As described in detail below, the gene expressed in muscle is inclined to show particularly high transient expression. This is probably because muscle fiber is multinucleated and one of the nuclei possessing an exogenous gene can provide a gene product in large and long muscle fiber, allowing detection of transient expression in the fiber much more easily than in other single cells. A transient expression assay is used to characterize the promoter and identify *cis*-elements, as is described in detail below. However, before going into the topics, it should first be noted that there are some pitfalls we should consider in a transient expression assay. First, introduced DNA is transcribed as extra chromosomal concatemers during early embryogenesis and integrated into the chromosome as multiple copies in later stages (Winkler *et al*., 1991). The transcription is unlikely to be regulated properly as it occurs in the cells of living medaka. Second, exogenous DNA is not uniformly distributed in all of the cells during embryogenesis. This chimeric distribution of exogenous DNA is characteristics of fish transgenics and is the main reason for the low production of germline-transmitted transgenics. Third, the promoter regions regulating the exogenous gene of interest should be carefully chosen to assay transient expression. In past studies, the regulatory regions derived from viral origins were used. Some viral promoters (RSVLTR, vTK, CMV, MoLV and SV40) showing strong transcriptional activities in mammalian cells transiently drove the downstream reporter genes in F1 generation (Winkler *et al*., 1991; Alestrom *et al*., 1992; Lu *et al*., 1992; Sato *et al*., 1992; Tsai *et al*., 1995). However, promoter activities of viral origins are often not so strong, probably due to incompatibility between virus and host transcriptional mechanisms.

There are several examples of exogenous promoters from other vertebrates functioning in medaka (Ozato *et al*., 1986; Inoue *et al*., 1990; Gong *et al*., 1991; Winkler *et al*., 1991; Lu *et al*., 1992; Matsumoto *et al*., 1992; Sato *et al*., 1992). Although it has been reported that fundamental *cis*-elements seem to be functional beyond the species (Nonchev *et al*., 1996), exogenous promoters in F2 generations often do not regulate proper transcription of the downstream gene (Sato *et al*., 1992; Ono *et al*., 1997). Conversely, an approximately 6 kb regulatory region of the medaka *vasa* gene, which is sufficient to show germline expression in medaka, does not direct germline specific expression in the mouse and zebrafish (Tanaka, unpublished data). These results lead to the reasonable conclusions that the promoter functions efficiently in the original organism. Many promoter regions have been isolated from medaka and characterized recently, as described below.

Promoter analysis

Translation elongation factor-1α promoter

In medaka, there are two genes coding translation elongation factor-1α (EF-1α) (Kinoshita *et al*., 1999). They are arrayed in tandem in the medaka chromosome and have been designated EF-1αa and EF-1αb from the 5' end, respectively. To investigate the promoter activity of EF-1α gene, approximately 2.6 kb 5' upstream region from the translation initiation site (ATG) was fused to GFP (Green Fluorescent Protein) gene and introduced into medaka eggs by the microinjection method (Kinoshita *et al*., 2000). Microinjection was performed with 25 ng/μl of circular form DNA in phosphate-buffered saline (PBS) using 1-cell stage embryos. Using the progenies of this transgenic medaka, the promoter activity of EF-1αa gene was investigated during embryonic development and in the tissues of an adult individual. GFP was observed after the early gastrula stage in all cells. As somitogenesis developed, GFP disappeared from the somite. In adult tissues, GFP was observed in all the tissues except for skeletal muscle. These results indicate that EF-1α is a major isoform of medaka EF-1α and suggest that EF-1αa is a muscle-specific isoform.

Tissue-specific muscle actin promoter

Three types of muscle (skeletal, cardiac and smooth) exist in vertebrates, and cells in these muscles express muscle-specific actin genes, respectively. Kusakabe *et al*. (1999) cloned two types of striated muscle actin gene (OlMA1 and OlMA2) from medaka. To investigate the tissue-specific gene expression in skeletal and cardiac muscle, various upstream regions of these gene with truncated, deleted, mutated, and chimeric genomic regions were fused with the GFP gene and introduced into medaka eggs by the microinjection method. Microinjection was performed with the circular form of DNA in PBS using 2-cell stage embryos. GFP fluorescence in living founder embryos was transiently monitored. It was found that two E boxes and unidentified *cis*-regulatory elements cooperatively contribute to the muscle-specific activity of upstream region of OlMA1. The results also suggested that the first intron of OlMA1 has enhancer activity. As for OlMA2, an E box and a CArG box were important for both skeletal and cardiac muscle expression.

Tissue-specific expression of guanylyl cyclase gene family

Guanylyl cyclase (GC) produces cyclic GMP, an intracellular mediator that plays important roles in various biological processes. GC genes constitute one gene family. In medaka eight membrane GCs (OlGC1-7 and OlGC-R2 and two subunits of soluble GC, OlGCS-α_1 and OlGCS-β_1, have been identified (see review by Kusakabe and Suzuki, 2000a). Transgenic medaka embryos that had been microinjected into the 5' upstream region of GC genes followed by a GFP gene revealed tissue-specific expression of GC isoforms. Microinjection was performed with 10 ng/μl of DNA solution using 2-cell stage embryos. OlGC3 and OlGC5 were expressed in retinal photoreceptor cells, and OlGC4 was expressed in the retia, pineal organ, and olfactory pits (Kusakabe and Suzuki, 2000a, b). The soluble GC genes, OlGCS-α_1 and OlGCS-β_2, were arrayed in tandem in this order. The upstream region of OlGCS-α_1 was required for OlGCS-β_1-GFP gene expression, suggesting coordination of the two subunits in gene expression (Mikami *et al*., 1999).

Elements of the eel cytochrome P450 1A1 gene responsible for its inducible and constitutive expression

Cytochrome P450 1A1 (CYP1A1) is one of drug-metabolizing enzymes and is induced by certain chemicals, such as 3-methyl cholanthrene. Ogino *et al.* (1999) investigated the *cis*-acting regulatory elements of eel CYP1A1 using transgenic medaka. Deleted and altered 5' upstream sequences of eel CYP1A1 with luciferase gene as a reporter gene were microinjected into the cytoplasm of 1-cell stage medaka embryos, and the transcription efficiency was examined using hatched fry treated with 3-methylcholantholene. Six xenobiotic-responsive elements were found to be responsible for inducible expression but not for constitutive expression. Moreover, it was suggested that some unknown regulatory elements (an estrogen-responsive element being one possible candidate) play important roles in inducible and constitutive expression.

This study showed the successful adaptability of the transgenic medaka system for investigation of transcription elements from exogenous species. However, with the respect of fisheries, it is difficult to carry out transgenic research using commercially valuable fish species. Therefore, medaka is an excellent model for applied sciences as well as for basic sciences.

Basic model for aquaculture

Transgenic techniques can be used to generate profitable fish in the aquaculture industry. For example, growth-enhanced salmon were produced by introducing a growth hormone gene (Devlin *et al.*, 1994). These transgenic salmon showed dramatic increases in weight, 11-fold heavier than non-transgenic controls. In this field, medaka has been used as a basic model for gene therapy. That is, certain intact genes have been introduced into defective fish in attempts to recover lost function caused by lack or mutation of the genes.

Granting L-ascorbic acid biosynthesis activity

L-ascorbic acid (AsA), vitamin C, is one of the essential vitamins for animals. AsA is synthesized from L-gulonic acid, one of the intermediary metabolites of the D-glucronic acid pathway in most animals (Touster, 1962). The last step in which L-gulono-γ-lactone is converted into AsA is catalyzed by L-gulono-γ-lactone oxidase (GLO), an enzyme that is missing enzyme in scurvy-prone animals (Nishikimi *et al.*, 1992; Nishikimi *et al.*, 1994). In case a sufficient amount of AsA is not supplied from food, these animals suffer from scurvy. In some fish, such as trout, salmon, carp and guppy, long-term feeding of an AsA-deficient diet caused scurvy and impaired collagen formation, resulting in anemia, scoliosi and lordosis (Halver, 1972; Ashley, 1972).

Medaka as well as humans lack GLO activity. Medaka have therefore been used as a model to study gene therapy for enzyme deficiencies. A linearized vector that contained GLO cDNA from the rat liver driven by an SV40 early promoter was introduced into the cytoplasm of 1-cell stage medaka embryos by the microinjection method (50 picoliters/egg) in the expectation that AsA biosynthesis activity would recover (Toyohara *et al.*, 1996). AsA synthetic activity of homogenate prepared from the trunk portion of an F1 transgenic progeny was recognized using an electrochemical detector. This result indicates that introduced GLO cDNA revives the AsA biosynthesis pathway and might prevent the occurrence of scurvy in GLO-defective fish species.

This is a good model study showing the possibility to recover lost gene function by a transgenic approach and to generate more beneficial fish by improving genetical defects.

Phenotypic rescue experiment

Transgenic techniques can be applied to complement defect genes. Because of the availability of a wide variety of spontaneous color mutants (Ozato and Wakamatsu, 1994), mutants concerned with body colors provide a useful system for study of the pigmentation mechanism, and epistatic relations have been investigated, especially focusing on albino mutant. *i* or i^3 mutant represents the typical phenotype of albino, showing reduction of melanization. As for melanization, the function of tyrosinase has been investigated with mutants lacking melanophre and/or melanocyte using transgenic techniques. Examples of phenotypic rescue experiments are presented in the next section.

Functional analysis of the tyrosinase gene

Ono *et al.* (1997) introduced a reconstructed mouse tyrosinase gene, which contained the complete cDNA of mouse tyrosinase and the 3' flanking sequence fused with the 5'-flanking genomic non-coding sequence, into orange-colored variant medaka eggs by electroporation. Eight to ten fertilized eggs were placed into an electroporation cuvette containing DNA solution (100 to 200 μg/ml), and the chorion of each egg was scratched with a tungsten hook before pulsation. Square-wave impulse was administered to eggs three times at 4 C° at a voltage of 300 V/cm for 70 to 100 μsec.

Melanization occurred in the transgenic individuals and was restricted to melanophores. No melanin deposition was observed in xanthophores or leucophores. As mentioned above, Ono *et al.* showed that the mouse tyrosinase gene and its regulatory sequence functioned in medaka.

Hyodo-Taguchi *et al.* (1997) revealed the genetic hierarchy concerned with pigmentation. They introduced a mouse tyrosinase minigene consisting of 5.2 kb 5'upstream sequences, exon 1, intron 1 and exon 2-5 into several color mutant strains (i-3bR, ibR, iBR, and HO4C) by the microinjection method. The circular form of DNA (10 ng/μl in Tris-buffered saline, TBS) containing these minigenes was injected into each germinal vesicle of the oocyte. Phenotypic rescue was observed in the i^1 and i^3 mutant strains, suggesting that the i^3 gene acts upstream and that the *b* gene acts downstream of the i^1 gene in pigment cell development.

The genomic nucleotide sequence of the tyrosinase gene and its 5' upstream sequence were cloned from by Inagaki *et al.* (1998). They showed phenotypic rescue of an i^1 mutant with induction of the medaka tyrosinase gene containing 3kb 5' upstream region using microinjection method. They injected digested or undigested plasmids (1 μg/ul in TBS) into 1-cell stage embryos. Fu *et al.* (2000) introduced the medaka tyro-

sinase gene and its regulatory sequence into 1-cell stage medaka embryos. They showed that the transgene was integrated in the chromosome of a tyrosinase-negative i^1 strain, resulting in stable and full rescue of pigmentation through generation. They also suggested the usefulness of the tyrosinase gene as a reporter gene for the detection of transgenic individuals in combination with the tyrosinase-negative strain.

Transgenic medaka for mutation analysis

Recently, water pollution by chemicals such as endocrine-disrupting substances and carcinogens is becoming a serious problem. The use of transgenic fish could contribute to the detection of such chemicals

Winn *et al*. (2000) produced transgenic medaka harboring the cII gene as a mutation target. The cII gene is concerned with the lysogenic cycle of bacteriophage λ in *Escherichia coli*. Mutations in the cII gene can be detected as the appearance of plaques on the bacterial lawn. Winn *et al*. microinjected concatenated DNA into the cytoplasm of fertilized eggs. The frequency of spontaneous and ethylnitrosourea-induced mutagenesis was examined using liver, testis and whole fish of 2–6 month old transgenic medaka. Both spontaneous and induced cII mutational spectra in transgenic fish were similar to those of transgenic rodents. These results, together with those showing that transgenic zebrafish harboring a shuttle vector plasmid can be used for the detection of mutation frequencies and mutagens (Amanuma *et al*., 2000), indicate that transgenic fish is a comparable animal model to a rodent model for in vivo mutagenesis.

Regulatory elements and genes used for transgenic vectors

The most desirable transgenic vector is one that enables exogenous gene of interest to be expressed in any cells at any time. Several elements constituting mammalian transgenic vectors have been characterized in medaka. In the next section, these elements, including reporter genes, are described.

Reporter genes

Several gene expression markers are available for medaka transgenics. In early studies, the lacZ gene was used (Winkler *et al*., 1991; Takagi *et al*., 1994; Tsai *et al*., 1995). The activity of β-galactosidase, which is a product of lacZ gene, can easily be detected using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) as a substrate, enabling detection of the spatial expression pattern of the transgene in early embryos and tissues, while the signal often became confusing because β-galactosidase activity arises in the yolk sac as development proceeds. The chloramphenicol acetyltransferase (CAT) gene has been used to evaluate promoter activity using radiolabeled chloramphenicol as a substrate (Kinoshita *et al*., 1994; Chong *et al*., 1989). These days, the luciferase gene is preferable for this purpose because luciferase activity can be detected without the use of radiolabeled substrate. With the antibody of CAT or luciferase, the spatial expression pattern can also be investigated. In general, these three reporters require the sacrifice of samples. Recently, some fluorescent protein genes have been isolated and altered for the investigation of gene expression. The green fluorescent protein (GFP) gene is the most widely accepted as a marker of gene expression in transgenic medaka. The existence of GFP can be detected as a green fluorescence under the excitation of blue light. This means that gene expression can be observed in the same embryo or individual through the ontogeny without sacrifice. However when using GFP gene, consideration must be given to the time lag between the onset of transcription and emergence of fluorescence because the chromophore is formed post-translationally by a cyclization reaction and oxidation step (Heim *et al*., 1994; see Living Colors User's Manual from Clonthech Laboratories Inc). In cases in which fluorescence is too weak to be detected under a fluorescent microscope, the more sensitve immumochemical method using an anti-GFP antibody can be used. A red fluorescence protein (RFP) is also useful in transgenic study of medaka, while the autofluorescence of gallbladder and gut is sometimes confusing (Fig. 1). The use of a combination of different colored fluorescence proteins enables observation of multiple gene expressions in the same living specimen.

Another type of reporter gene, melanin-concentrating hormone (MCH) gene, is potentially useful in wild type medaka. Mature MCH consists of 17 amino acids and is secreted from the pituitary. The secreted MCH concentrates dispersed melanin granules resulting in a lightened body color (Baker BI, 1991). The advantages of MCH gene as a reporter are 1) gene expression can be detected by simple observation of the body color of living fish without the requirement of special instruments, and 2) because the expressed MCH peptide is secreted and functions in pigment cells of the skin, gene expression can be detected even if it occurs deep inside of body cavity, where excitation of a fluorescent protein can not reach.

Effects of elements derived from bacteria on transgenic medaka

Several bacterial elements have been tested in attempts to develop a useful expression vector for transgenic medaka. The effects of these elements on medaka transgenics are summarized below.

Nuclear localization signal of SV40

The localization of a gene product is critical for its function. Therefore, controlling the location of a transgene product is an important technique in transgenic study. The effect of the nuclear localization signal (NLS) of SV40 has been investigated (Kinoshita, unpublished data). The protein possessing the NLS of SV40 are imported into the nucleus by a heterodimeric receptor that consists of importin- α and inportin-β subunits (Gorlich, 1997). The plasmids pEF-1α-A-GFP-N and pβact-GFP-N were produced by the insertion of the DNA sequence encoding the SV40 NLS peptide into the 3' flanking region of GFP (Fig. 2). The circular forms of these plasmids were introduced into medaka fertilized eggs. Fig. 3 shows the GFP fluorescence in the founder generation. With NLS, fluorescence was accumulated in nuclei in the cells in the yolk sphere (Fig. 3-B) and epidermis (Fig. 3-C). In contrast, fluo-

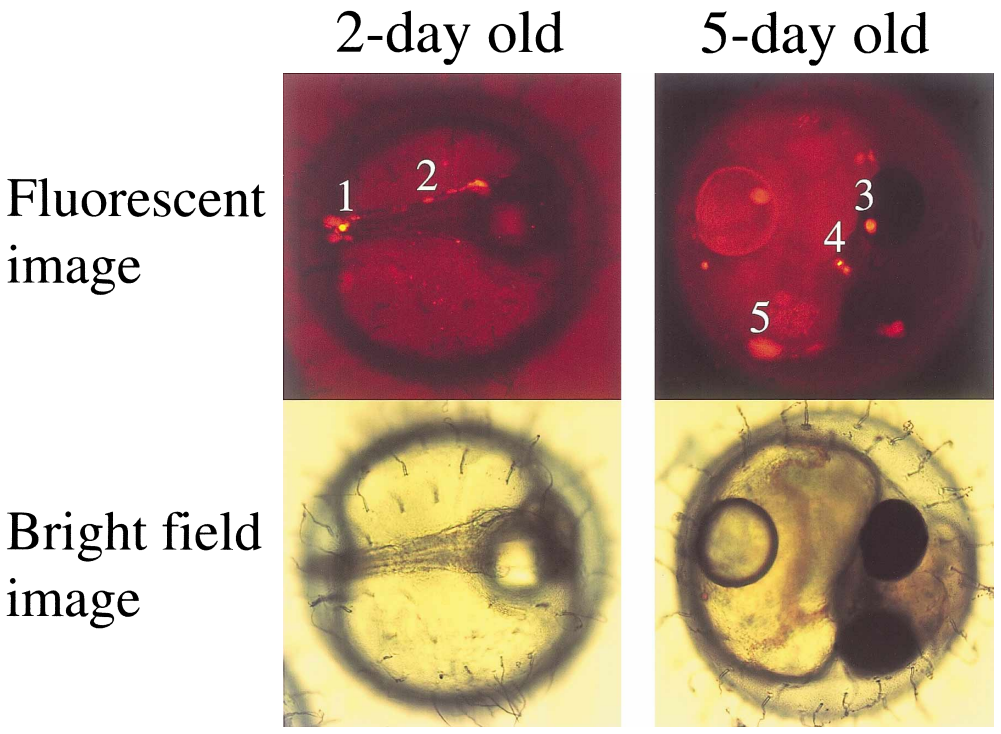

Fig. 1. Expression of red fluorescent protein (RFP) in medaka embryo. A linearized pβacti-RFP (20 ng/μl) was purified by agarose gel electrophoresis after the digestion of pβacti-RFP with *Nde* I. The vector was introduced into cytoplasm of fertilized medaka egg by microinjection method Transient expression of RFP was observed in somites (1), epidermis (2), eye (3), heart (4) and yolk sphere (5) of 2- or 5-day old embryos. Information on the plasmids is given in the legend to Fig. 2.

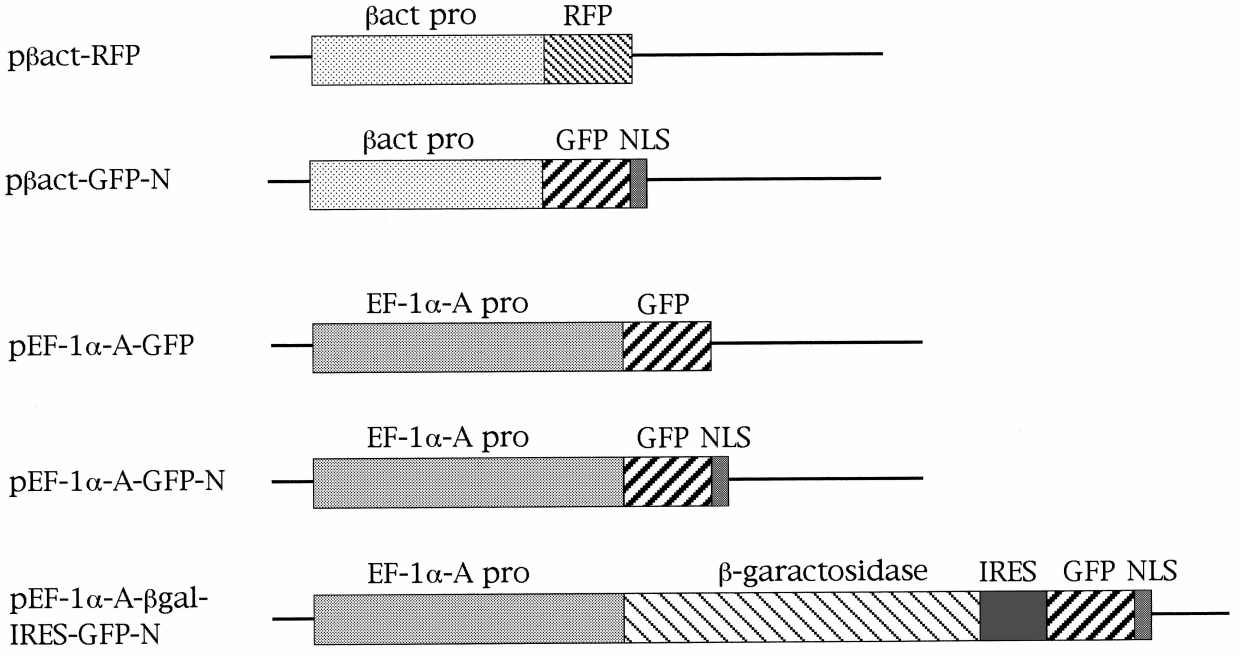

Fig. 2. Structures of fluorescent protein expression vectors. βact pro is the PCR-amplified fragment corresponding to the 5' upstream sequence (ca. 2.1 kb) from the translation initiation site (ATG) of the medaka βactin gene. EF-1α-A pro is the PCR-amplified fragment corresponding to 5' upstream sequence (ca 2.8 kb) from translation initiation site (ATG) of medaka EF-1α -A gene (Kinoshita *et al.*, 2000). The RFP gene is derived from pDsRed1-N1 (Clontech Laboratories Inc., Palo Alto USA). The GFP gene is derived from pCMX-hGR-GFP (Ogawa and Umezono, 1998). NLS represents a typical nuclear localization signal (KKKRKV) of simian virus 40. The internal ribosome entry site (IRES) is derived from encephalomyocarditis virus (Sugimoto *et al.*, 1994). The drawing is not to scale.

rescence was observed uniformly in the cells without NLS (Fig. 3-A). In muscle cells, fluorescence was not accumulated in nuclei (Fig. 3-E). These results indicate that the effect of NLS derived from SV40 depends on cell types in medaka.

Effect of the internal ribosome entry site on transgenic medaka

The internal ribosome entry site (IRES) has been isolated from several viruses (Wimmer *et al*, 1993; Brown *et al*., 1994) and permits two genes to be translated independently from a single mRNA controlled by a single upstream promoter. The gene located in the 5' or 3' region of IRES is translated in a cap-dependent or IRES-dependent manner, respectively. The effects of IRES derived from encephalomyocarditis virus (ECMV) on transgenesis in mammalian cells have already been revealed (Morgan *et al*., 1992; Sugimoto *et al*., 1994). However, it is also known that the activity of IRES depends on the host species. To evaluate the activity of IRES derived from ECMV in medaka transgenesis, pEF-1α-A-βgal-IRES-GFP-N, which contained the EF-1α-A promoter, β galactosidase gene, IRES, and GFP gene with NLS coding sequence at 3' end in this order, was constructed and microinjected into fertilized medaka eggs in circular form. If IRES functions in medaka, both β galactosidase activity and GFP fluorescence will be observed in the same cells. Some of the embryos that had been injected with pEF-1α-A-GFP-N as controls exhib-

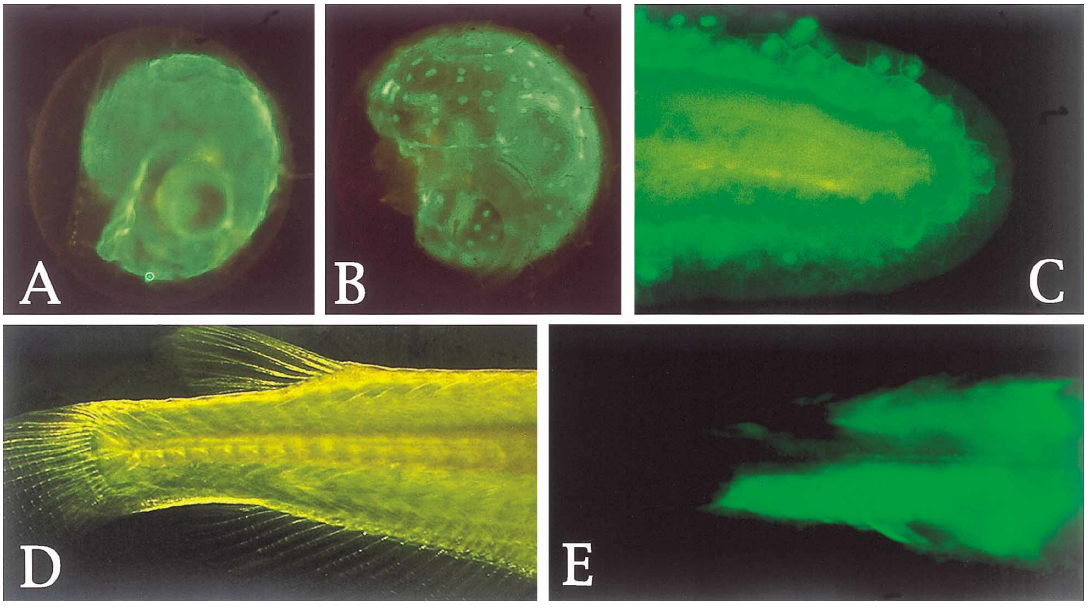

Fig. 3. Effect of the nuclear localization signal (NLS) of simian virus 40 (SV40) on transgenic medaka. The circular forms of plasmids with or without SV40 NLS were introduced into the cytoplasm of fertilized medaka eggs before the first cleavage by the microinjection method. (A) A 2-day old embryo injected with pEF-1α -A-GFP. Fluorescence is observed uniformly in the yolk sphere. (B) A 2-day old embryo injected with pEF-1α-A-GFP-N. (C) The tail portion of a 5-day old embryo injected with pEF-1α-A-GFP-N. Fluorescence is accumulated in nuclei in an embryo that had been injected with NLS fused GFP (B and C). A bright field image (D) and a fluorescent image (E) of the tail portion of juvenile fish injected with pβact-GFP-N. In the developed muscle, fluorescence is not accumulated in nuclei. The information on plasmids is given in the legend to Fig. 2.

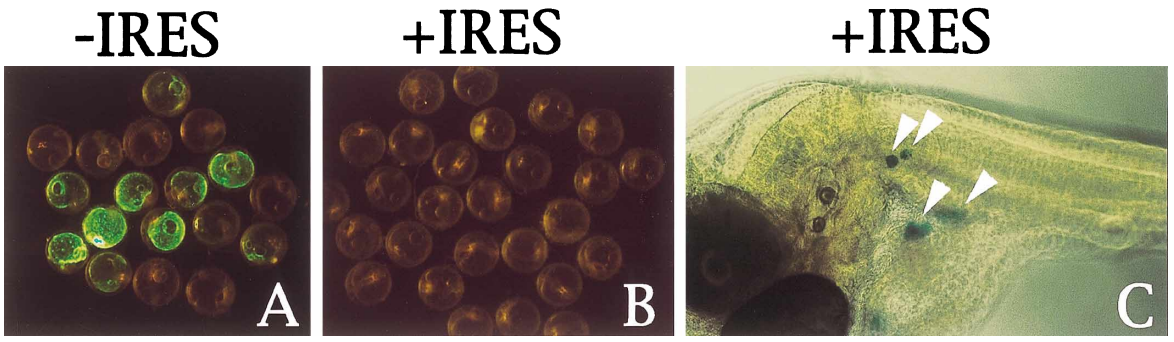

Fig. 4. Effect of the internal ribosome entry site (IRES) on transgenic medaka. The circular form of plasmids with (pEF-1α-βgal-IRES-A-GFP-N) or without (pEF-1α-A-GFP-N) IRES from encephalomyocarditis virus was introduced into fertilized medaka eggs by the microinjection method. (A) The fluorescence image of founder embryos injected with pEF-1α-A-GFP-N. Some of them show fluorescence. (B) A fluorescence image of founder embryos injected with pEF-1α-βgal-IRES-A-GFP-N. None of them show fluorescence. (C) Detection of β-galactosidase activity in the embryo injected with pEF-1α-βgal-IRES-A-GFP-N. β-galactosidase activity was observed in some areas (arrowheads). The yolk sac was removed because of the endogenous β-galactosidase activity. The information on the plasmids is given in the legend to Fig. 2.

ited fluorescence (Fig. 4-A). On the other hand, the fluorescence was not observed in any of embryos that had been injected with pEF-1α-A-βgal-IRES-GFP-N (Fig. 4-B), but β galactosidase activity was recognized in some of them (Fig. 4-C). In other words, in medaka, IRES-dependent translation did not occur, but cap-dependent translation did. Thus, IRES does not function effectively in medaka cells at present. Types of IRES other than ECMV must be evaluated.

Perspective of medaka transgenics

As mentioned in the introduction, one of the most ideal vectors for transgenics is one that enables any DNA to be expressed in any cells at any time. As for the inducible vectors, HSP (heat shock promoter) may be one of the most promising elements for a vector. In zebrafish, the gene of interest is linked downstream of the HSP region and has been microinjected (Halloran *et al*., 2000). Laser illumination was used to induce the gene through the activity of HSP. If the cells can be marked by a fluorescent protein in combination with transparent medaka, the laser can target a single cell of interest and analyze the lineage of the cells and the effect of the gene in the cells.

Further study is needed to establish an efficient method for producing germline-transmitted transgenics. Recently, it has been reported that the parameters of a particle gun were optimized to obtain maximum production of transgenics (Yamauchi *et al*., 2000). The rate is still low compared with microinjection. However the particle gun has the advantage over any other methods in enabling a number of eggs to be simultaneously treated with gold particles.

The gene expressing in germ cells can be a good marker allowing to select germline-transmitted transgenics efficiently. medaka *vasa* gene (*olvas*) is expressed in germline and olvas promoter-GFP transgenic medaka have been established recently (Tanaka *et al*., 2001). It is clearly shown that F0 medaka selected by GFP fluorescence in germ cells produce germline-transmitted F1 offsprings with a high efficiency. Therefore, this promoter can be a good marker for transgenic vectors.

The technique for introducing exogenous DNA into eggs by electric pulses has also been improved by modification of pulse shapes and a cell chamber. There have recently been reports of successful introduction of DNA into chick cells with high efficiency using this new method of electroporation (e.g., Momose *et al*., 1999). This new electroporation technique would be worth trying in medaka.

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