Introduction of Carp $\alpha$-Globin Gene into Rainbow Trout

Goro Yoshizaki,* Takashi Oshiro,* and Fumio Takashima*
(Received September 21, 1990)

Carp $\alpha$-globin gene (CaG) was microinjected into the cytoplasm of fertilized rainbow trout eggs at 1 cell stage treated with glutathione (reduced) to prevent hardening of chorion. About 67% of microinjected eggs hatched out and grew normally. DNA was extracted from the muscle 130-day-old fish or from adipose fin of 10 to 12-month-old fish and analyzed by Southern blot hybridization to determine the presence of CrG. Forty percent of 130-day-old fish and 39% of 10 to 12-month-old fish tested positive for CaG. In 130-day-old fish, CaG was integrated in the host genome, and formed head-to-tail, head-to-head, and tail-to-tail concatemers. In 10 to 12-month-old fish, most of the positive individuals carried several copies of CaG per cell. About 10% of these individuals, however, carried CaG at less than 1 copy per cell on average. This result suggests that both CaG-integrated and non-integrated cells coexisted mosaically in host fish.

Introduction of cloned foreign genes into animal embryos is a powerful approach for studying gene regulation in vivo. Recently, this technique has been applied to domestic animals. Transgenic techniques can introduce desirable genetic characters in individuals, and foreign genes integrated into host genomes would be transmitted to the offspring through the germ line. Although transgenic techniques have the potential to become useful tools in fish breeding, reports on transgenic fish are rather scarce in the literature. The nucleus of the fertilized egg in fish is often too small for microinjection of the gene. In addition, egg chorion is hard and difficult to penetrate with micropipettes.

Most reports on transgenic fish relate to the introduction of mammalian growth hormone genes for the promotion of growth. Rainbow trout growth hormone cDNA was introduced into carp and successfully promoted growth. Antifreeze protein gene of winter flounder was introduced into Atlantic salmon for the acquisition of the tolerance to low temperature environments. For studying of tissue as well as stage specific gene expression, chicken $\delta$-crystallin gene was introduced into medaka Oryzias latipes and for inserional mutation, hygromycin resistant gene was introduced into zebrafish. Furthermore, expression of introduced $\beta$-galactosidase gene and neomycin resistant gene were detected in salmon and goldfish respectively. Most of the introduced genes mentioned above were from animals other than fish, such as procaryotes and mammals. Genomic clone of fish gene, however, may result in more efficient and accurate expression, and in the production of protein with higher physiological activity in host fish.

In this study, we microinjected the gene encoding carp $\alpha$-globin, a subunit of hemoglobin, into fertilized rainbow trout eggs. Furthermore, the early survival of microinjected eggs and the existence of the foreign gene in the fingerlings and under-yearling fish were determined.

Materials and Methods

Egg Collection

Ripe eggs were stripped from 3 or 4-year-old rainbow trout Oncorhynchus mykiss reared at 10°C in Oizumi Research Station, Tokyo University of Fisheries. After insemination, the eggs were activated and incubated in 1 mM glutathione (reduced) solution (pH 8.0) to prevent hardening of the chorion.

Preparation of DNA

The carp $\alpha$-globin gene was isolated from a carp genomic library, using the carp $\alpha$-globin cDNA as probe and subcloned into pBR 322 (pCaG) (Fig. 1). The microinjected gene was a 2.2 kb BamHI-HindIII fragment (Fig. 2) isolated from pCaG (Fig. 1). The following digestion with two restriction enzymes (Takara Shuzo Co.,
carp $\alpha$-globin gene ($C\alpha G$) was separated from the pBR322 vector by agarose gel electrophoresis, and purified with the GENECLEAN Kit (BIO 101 Inc.). The purified $C\alpha G$ was dissolved in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA at a concentration of 11 ng/$\mu$l.

**Fig. 1.** Structure of recombinant plasmid p$C\alpha G$ containing carp $\alpha$-globin gene. Solid bars represent exon. Open bars represent intron and flanking sequences. The thin line shows the sequence for pBR322.

**Fig. 2.** Restriction map of carp $\alpha$-globin gene used for microinjection.

Microinjection
Microinjection was carried out between 3 and 7 hrs after fertilization in isotonic salt solution (NaCl 90.4 g, KCl 2.4 g, CaCl$_2$·2H$_2$O 2.6 g/10 l) (Fig. 3). Eggs were placed on concavities of plastic petri dish and held in place with a wire loop. Two nl of DNA solution containing 10$^7$ copies of $C\alpha G$ was injected into the center of the blastodisc with a micropipette (diameter, 5-8 $\mu$m) mounted on a micromanipulator (Narishige Co., Ltd). These procedures were conducted under constant temperature of 10°C. The eggs were then incubated at 10°C.

**DNA Extraction**
DNA was extracted from muscle of 130-day-old fish or from adipose fin of 10 to 12-month-old fish. The tissue was then digested with 500 $\mu$g/ml proteinase K (Boehringer Manheim GmbH) in lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1.0% SDS) for 5 to 8 hrs at 60°C under gentle agitation. The sample was extracted 3 times with phenol and once with chloroform-isooamyl alcohol (24:1). Then, the aqueous phase was treated with 50 $\mu$g/ml RNaseA for 1 hr at 37°C. The DNA was extracted again with phenol, and dialyzed overnight at 4°C against 10 mM Tris-HCl (pH 8.0)-1 mM EDTA.

**Southern Hybridization**
Ten $\mu$g of genomic DNA was digested with Dra I (Takara Shuzo Co., Ltd) and electrophoresed on a 0.7% agarose gel. The DNA was transferred to a nylon membrane and prehybridized overnight at 42°C in 5X SSPE (10X SSPE=1.5 M NaCl, 0.1 M NaH$_2$PO$_4$·2H$_2$O, 0.01 M EDTA, adjusted to pH 7.4), 5X Denhardt's solution (100X Denhardt's solution=2% Ficoll, 2% polyvinylpyrrolidone, 2% bovine serum albumin), 50% formamide and 10% dextran sulfate. Hybridization was carried out in the same buffer containing the denatured probe $C\alpha G$ labelled with $\alpha$-32P dCTP by random priming. The membrane was washed in 2X SSPE for 15 minutes at room temperature, then in 2X SSPE and 0.5% SDS for 15 minutes at 65°C, and finally in 0.5X SSPE and 0.5% SDS for 15 minutes at 65°C, before autoradiography. In the case of the positive samples of 130-day-old fish, undigested DNA and Pst I (Takara Shuzo CO., LTD)-digested DNA were also analyzed by the same method. Furthermore the carrier 10 to 12-month-old fish were inserted with electronic tags (Identification Devices Inc.) intraperitoneally for individual identification during future analysis.

**Results**

**Survival of Microinjected Eggs**
The survival of 774 microinjected eggs was 98.8% at fertilization, 74.4% at the eyed stage,
66.7% at hatching, and 60.3% at swimming-up (Table 1). This data corresponds with the 99.3%, 80.2%, 74.5%, and 70.5% to those of non-injected eggs respectively. Most of the injected swim-up fry grew normally as judged by external features and behavior.

**Fate of Microinjected Gene**

From 130-day-old fish, DNA was extracted, digested with Dra I, and analyzed by Southern hybridization (Fig. 4, top). After digestion of CaG with Dra I, a 1.2 kb fragment containing all three exons occurred (Fig. 4, bottom). This fragment was detected in fish No. 1, 7, 9, and 10. These signals were equal in molecular weight to those of the positive control, but no signals were detected in the negative control (Fig. 4, top, N). Therefore, it appears that CaG was present in host cells. In relation to the positive control, the intensity of 1.2 kb signals of No. 1 and 10 carried 20 to 30 copies of CaG, No. 7 carried 5 to 10 copies, and No. 9 carried 10 or more copies per cell on the average.

Undigested DNA from positive samples (1, 7, 9, and 10) were analyzed by Southern hybridization (Fig. 5). All signals were of high molecular weight. This finding indicates that CaG was integrated into the host genome.

In addition, the samples were digested with Pst I and analyzed by Southern hybridization.
Fig. 6 left). PstI cleaved the CAG at one site and generated 2 fragments of 1.53 kb and 0.7 kb (Fig. 6 right). However, digestion of DNA from positive samples with PstI generated 2.2 kb, 3.1 kb, and 1.4 kb fragments (Fig. 6), which seemed to be derived from head-to-tail and head-to-head concatenated CAG, respectively, in the host genome. These results suggest that CAG formed the 3 types of concatemers cited above, in the host genome.

In 10 to 12-month-old fish, Southern hybridization of Dra I-digested DNA showed that 64 out of 164 carried CAG. Most positive fish carried several copies of CAG per cell, but 7 fish carried less than one copy per cell on the average (Table 2).

**Table 1.** Survival rates (%) of microinjected eggs of the following items

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of eggs used</th>
<th>Fertilization</th>
<th>Eyed stage</th>
<th>Hatch-out</th>
<th>Swim-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microinjected</td>
<td>774</td>
<td>98.8</td>
<td>74.7</td>
<td>66.7</td>
<td>60.3</td>
</tr>
<tr>
<td>Control</td>
<td>2031</td>
<td>99.5</td>
<td>93.1</td>
<td>89.5</td>
<td>85.5</td>
</tr>
</tbody>
</table>

**Discussion**

Microinjection into cytoplasm of fertilized rainbow trout eggs after glutathione treatment would be easier than the 2-step method in which microinjection is carried out through the chorion hole pierced with a metal needle or thick glass pipette.

The hatching rate and swimming-up rate of microinjected eggs were 74.5% and 70.5%, respectively, in relation to those of non-injected control fish. This decrease in survival rate may be due to the effect of the microinjection of DNA, since the survival rate of glutathione (without microinjection) treated eggs was as high as that of non-treated controls.

In this study, 2 nl of DNA solution (11 µg/ml) were injected. Hogan et al. found that survival rate was reduced when more than 10 µg/ml DNA solution was injected into mouse eggs. In zebrafish, as the dose of injected DNA increases from 4.5 pg to 90 pg, the survival rate decreased from 43% to 3%. Penman et al. also reported that increasing the injected DNA concentration from 10⁴ to 10⁶ copies reduced the survival rate of recipient eggs. However, Chourrout et al. reported 77% survival 30 days after 200 pg DNA injection in rainbow trout. In addition, Rokones et al. injected 5 ng DNA into rainbow trout eggs, and obtained 78% survival 28 days after injection. Thus, the survival of microinjected eggs may have been affected not only by concentration and total amount of injected DNA but also by the physical injury of microinjection, the nature of the DNA solvent, and the quality of the eggs.
In this study, 40% of 130-day-old fish and 39% of 10 to 12-month-old fish carried the CaG. On the other hand, 5% of the hatched fry of rainbow trout, 6% of 8-month-old Atlantic salmon, 5% of 90-day-old tilapia, 5% of carp, 5% of 4-month-old zebrafish, and 20% of channel catfish proved to carry foreign genes after microinjection. Therefore, the gene integration rate obtained in the present study is very high by comparison with previous studies. The success rate in this study may be due to the microinjection of large numbers (10⁷) of copies of linear DNA. Chourrout et al. and Rokkones et al. obtained high transformation rate (75%) after DNA injection of 2X10⁹ and 10⁹ copies, respectively, in rainbow trout. Moreover, Chourrout et al. and Penman et al. showed that linear DNA persisted a higher transformation efficiency than circular DNA, when introduced in fish, as well as in mouse and Xenopus.

In 130-day-old fish, CaG were concatenated in the host genome. The same phenomenon was reported in mouse, sea urchin, channel catfish, zebrafish, and rainbow trout. In mouse, most of the concatemers were head-to-tail, but in the present study, head-to-head and tail-to-tail concatemers were also observed. This result may have been caused by injection of enormous copies of DNA into the cytoplasm, so that the injected genes were concatenated at random before integration.

About 10% of the transgenic fish carried less than 1 copy of CaG per cell on average. This result suggests that both CaG-integrated and non-integrated cells coexisted mosaically in the host fish. Similar observations were reported in rainbow trout, zebrafish, carp, and medaka.

In this study, we used genomic clone of carp α-globin gene containing 5' upstream and 3' downstream regions which regulate gene expression, and eliminated the vector pBR322 which may inhibit gene expression in transgenic mouse. In addition, the fish gene used in this study may be more efficient and produce more accurate expression. Further analyses of CaG expression and inheritance are now in progress using fish inserted with electric tags.

Acknowledgement

We would like to thank Dr. Aoki at Miyazaki University for his kind gift of plasmid pCaG, Dr. Reynald Patiño at Texas Tech University for critical reading of the manuscript.

References