Journal of Aquaculture 9 (3) : 293~300 (1996)

Production of Transgenic Homozygous Diploid in Mud Loach (*Misgurnus mizolepis*)
I. Transfer of Luciferase Gene and Evaluation of Mud Loach Expression Vector

Yoon Kwon Nam, Moo-Sang Kim*, Hyung-Ho Lee* and Dong Soo Kim

*Department of Aquaculture and *Department of Biotechnology and Bioengineering, Pukyong National University, Pusan 608-737, Korea*

Validities of several gene transfer methods including microinjection, electroporation and lipofection with luciferase gene (pRSVL), and effectiveness of mud loach expression vector which contains ARS from mud loach on production of transgenic mud loach were evaluated. Microinjection revealed the 0~8% of transgene incidence in 2-week-old fish with significant mosaicism. Electroporation and lipofection of mud loach sperm also successfully introduced the transgene into sperm cells, and transferred the foreign DNA into zygote. Gene transfer by electroporation and lipofection showed a range of 0~28% and 0~48.1% of transgene incidence, respectively in newly hatched larvae, although most DNA introduced were gradually degraded with the development of fish. Microinjections of mud loach expression vector caused a significantly reduced survival rate of mud loach embryos with severe teratogenic effects, and ARS/Luc transgene could not be detected in normally developed fish after microinjection.

Key words : Microinjection, Electroporation, Lipofection, Luciferase, Mud loach expression vector, *Misgurnus mizolepis*

Introduction

Transgenic technology has been considered as a powerful tool in the research fields for developmental biology, medicine, regulations of gene expression, physiology, and immunology of eukaryote. Production of valuable transgenic animals in commercially important species could also give potential economic benefits by providing a quantum leap over traditional selection and breeding method (Hew et al., 1995).

Since 1980s, fishes have been given much attention as one of the best potential model system for transgenic technology because they may give more advantages such as external fertilization, faster embryonic development, amenability for chromosome manipulation, and relatively short generation timeover than other vertebrates. Several successful results have been already reported in connection with desirable changes in phenotypes (Hew and Fletcher, 1992).

However, there were many hurdles in the successful production of transgenic fish. Major problems are (1) limited knowledge on genome and cellular function of fish, (2) restricted scope of methodology for gene transfer, and (3) absence of established useful model system for gene transfer in fish (Pan-

This study was supported by grant of Genetic Engineering Program (No. 174-2, 1995) from Ministry of Education.

dian and Marian, 1994).

Mud loach is an important fish both for food and ceremonial works by Buddhists in Korea (Kim et al., 1994). This species has attracted attention as an useful model material because of relatively small body size, fast growth rate, fast embryonic development, short generation time, year-round spawning under controlled conditions, and good tolerance to low oxygen and disease. On the basis of thease advantages, mud loach has been being subjected for genetic manipulations, including ploidy manipulation (Kim et al., 1994), sex control (Kim et al., 1996), hybridization (Kim et al., 1995), and transgenesis (Kim and Nam, 1994, 1995). Recently, replication origins (autonomously replicating sequences, ARSs) were cloned from mud loach and characterized in order to develop an effective expression vector for transgenesis in mud loach (Lim et al., 1995, 1996).

The objective of this study is to examine the potential use of various gene transfer methods using firefly luciferase gene as a reporter, and to evaluate the effectiveness of mud loach expression vector containing ARS for producing transgenic mud loach.

Materials and Methods

Fish and gamete collection

Mud loach broodstocks used in this study were the strain maintained at Genetic Engineering Laboratory, Department of Aquaculture, Pukyong National University. Eggs and sperm were obtained by IP injection of HCG as described by Kim et al. (1995).

Plasmid

pRSVL (de Wet et al., 1987) containing American common firefly luciferase gene fused to RSV/LTR promoter was kindly provided by Dr. Wolf, University of Wisconsin, USA, and used for gene transfer experiments.

Gene transfer

1) Microinjection

One-celled embryos were microinjected into the center of the first cell. The attached needle had a diameter of about 5 μm, and constant pressure on the handle of the apparatus kept the DNA solution (50 μg/ml in TE, pH 8.0) running out the tip of the needle. After microinjections, embryos were transferred to a 25℃ incubator until hatch. Hatching success, early survival rate up to yolk sac absorption, and incidence of abnormality were examined.

2) Electroporation

Electroporation of sperm cells was carried out with combinations of electric field strength ranged 0~1,625 V/cm under the given capacitance of 71 μF. Following the electroporation, sperm were artificially fertilized with normal eggs. The effects of electroporation on fertilization, hatching, and the frequencies of gene transfer were evaluated.

3) Lipofection

Liposome suspension prepared from bovine brain phospholipid (Sigma Co., USA) was used for lipofection of mud loach sperm. Liposome suspension (400 μg/ml) was mixed with pRSVL (200 μg/ml), and pre-incubated at 40℃ for 10 min to form liposome-DNA complex. Then liposome-DNA complex were mixed with equal volumes of sperm suspension in PBS, and incubated at 25℃ for 30 or 60 min with gentle agitation. After incubation, sperm cells were collected by centrifugation and then fertilized with normal eggs. The effects of liposome treatments on fertilization, hatching, and gene transfer yields also were evaluated.

4) Electroporation of liposome-treated sperm

Preparation and treatment of liposome were carried out as described in lipofection experiment. After lipofection, sperm cells were electroporated (71 μF, 1,300 V) for 1, 2, or 3 times. Fertilization, hatching, and the incidence of transgene in hatched larvae

were evaluated.

DNA extraction and PCR screening of transgene

DNA was extracted from individual just hatched larvae or fry according to the method described by Kim and Nam (1994). PCR reaction mixture contained 20 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 100 μg/ml gelatin, 20 pmoles each PCR primer, 50 μM of each dNTPs, and 2.5 U Taq DNA polymerase. The reaction was carried out at 94℃ for 1 min, 60℃ for 30 sec, 72℃ for 1 min for 30 cycles with 5-min initial 94℃ denaturation step. Primers were designed as described by Kavumpurath et al. (1993).

Evaluation of mud loach expression vector

To develop the mud loach expression vector, replication origins (autonomously replicating sequence, ARS) were cloned from matrix attachment region (MAR) of mud loach according to the procedure described by Lim et al. (1995). About 2 kb EcoRI fragment of ARS (No. 6) was inserted in pURY 19 which containing the yeast URA3 gene in pUC19. Mud loach expression vector to be evaluated was constructed by adding SV 40 promoter/luciferase gene/poly (A)/SV40 enhancer fragment to pURY19. We named this construct, pURY19N6-luc (Fig. 1).

To evaluate pURY19N6-luc, circular pURY19N6-luc was microinjected to 1-2 cell fertilized eggs, and the development of embryos, hatching, early survival, and efficiency of gene transfer were monitored.

Results

Injected eggs with pRSVL

Survival rate of early embryo and hatching success of microinjected eggs were significantly lower than those of non-injected controls. Also, hatching success of microinjected eggs was different from each microinjection experiment, probably due to the egg quality. Early survival up to yolk sac absorption was not different between microinjected and non-injected groups (Table 1).

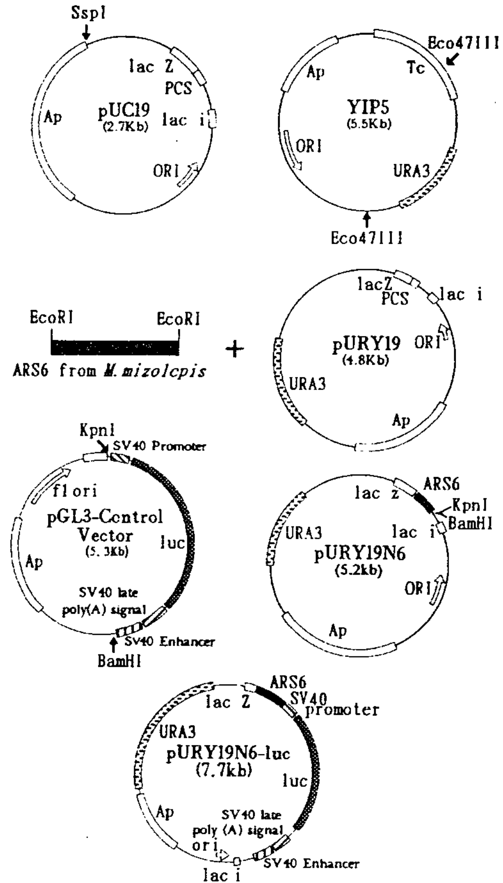

Fig. 1. Schematic flowgram showing the construction of mud loach expression vector.

PCR analysis revealed the incidence of transgene, (0~8%) with 2-week-old fish in their fin DNA (Table 1) (Fig. 2), however, transgenic insert could not be detectable with 5-month-old fish using PCR.

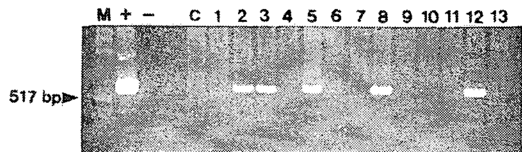

Fig. 2. Et-Br stained agarose gel showing the amplified fragment of transgene by PCR. M, lkb ladder ; +, pRSVL ; −, negative blank, C, non-microinjected fish ; 1~13, microinjected fish.

Table 1. Effects of microinjecting fertilized eggs on hatching success, early survival rate up to yolk sac absorption, and incidence of gene transfer in mud loach

Exp. mating	Hatching success (%)	Early survival rate (%)	Incidence of transgene (%)
Controls	70.5±9.2	81.4±10.6	0.0
1	46.7	78.6	2.1
2	52.5	80.6	0.0
3	60.0	75.9	6.3
4	37.8	89.1	7.2
5	26.8	78.2	8.1
6	50.8	68.5	0.0
7	29.1	58.6	1.8
8	45.5	80.5	3.5
9	37.5	84.2	5.2
10	42.1	86.3	0.0
11	32.3	78.6	2.1
12	14.9	72.0	1.2
13	35.9	70.0	3.8
14	48.6	71.3	4.2
15	57.3	69.8	6.9
16	48.2	81.6	7.0
17	60.0	85.2	5.6
18	65.3	80.0	3.8
19	54.1	78.5	4.1
20	11.8	56.9	0.0
21	19.0	68.2	1.8
22	35.9	78.3	3.7

Table 2. Fate of electroporated DNA (pRSVL) with age of mud loach

Age of fish analyzed	No. of fish analyzed	No. of fish carrying transgene	Incidence of gene transfer (%)
Embryo	46	13	28.26
Just hatching	81	17	20.99
Yolk sac absorption	83	4	4.82
1st feeding	213	2	0.94
1 month	674	1	0.16
3 month	2,029	2	0.09

Electroporation of sperm

Mean fertilization, hatching, and early survival of electroporated groups slightly lower than those of non-electroporated controls, however the differences were not significant (data not shown). Electroporations successfully introduced the pRSVL into mud loach sperm, and introduced pRSVL could also be transferred into oocytes. Overall yields of gene transfer ranged from 0 to 28% in just hatched larvae. There was a trend toward higher gene transfer yield as increasing field strength (Fig. 3). The transgene introduced by electroporation rapidly decreased with development of embryos ; percent of fish carrying transgene was 4.82% at yolk sac absorption ; 0.94% at 1st feeding ; 0.16% at 1 month ; and lower than 0.1% at 3 months old (Table 2).

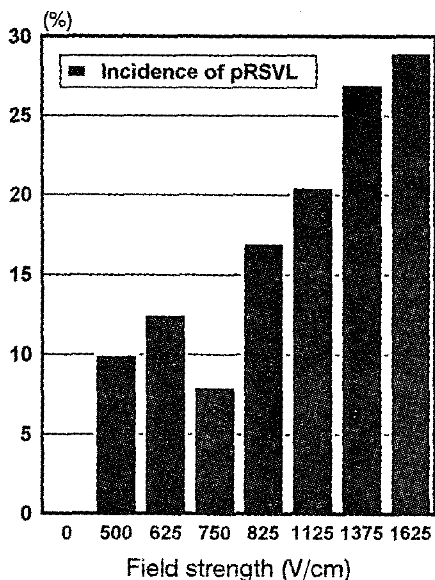

Fig. 3. Effects of field strength on frequency of gene transfer under the given condition of 17 μF capacitance.

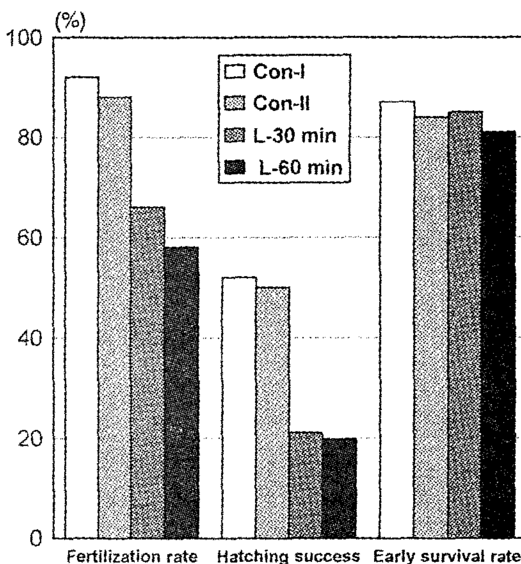

Fig. 4. Effects of liposome treatments on fertilization, hatching and early survival of mud loach. Con-I, non-treated control; Con-II, treated with DNA (100 μg/ml) only; L-30, treated with DNA/liposome complex for 30 min; L-60, treated with DNA/liposome complex for 60 min.

Liposome-mediated gene transfer

Incubation of sperm with liposome-DNA complex significantly lowered the fertilizing ability of sperm and hatching success of fertilized eggs, compared to control groups. However there was no difference between 30 min treated and 60 min treated groups. The early survival rates up to yolk sac absorption of all experimental groups were similar (Fig. 4).

A relatively high incidence of transgene was observed in just hatched embryos. Liposome treatment for 60 min resulted up to 50% of gene transfer which was higher than that of treatment for 30 min (37.0%). This high rate gradually decreased and was only 17.4% in 30 min treated group, and 34.8%

Table 3. Effects of liposome and/or electroporation treatments on the efficiency of gene transfer

Exp. group	Incidence of gene transfer (%)		
	Just hatching	1-week-old	2-week-old
Control	0/20 (0.0)	–	–
L30	10/27 (37.0)	4/23 (17.4)	0/84 (0.0)
L30/E1*	2/29 (6.9)	0/23 (0.0)	–
L30/E2	0/21 (0.0)	–	–
L30/E3	0/18 (0.0)	–	–
L60	13/27 (48.1)	8/23 (34.8)	0/84 (0.0)
L60/E1	2/22 (9.1)	0/23 (0.0)	–
L60/E2	0/21 (0.0)	–	–
L60/E3	0/17 (0.0)	–	–

* Liposome treatment (L) for 30 min followed by 1 time of electroporation (E).

in 60 min treated group by the 1-week-old age with PCR analysis. A further decrease occurred, and after 2 weeks, the transgene could not detected in both 30 min treated and 60 min treated group (Table 3).

Electroporation of liposome treated sperm

Electroporation of liposome treated sperm did not increase the efficiency of gene transfer and cause significant mortality ; in general, fertilization rate and hatching success decreased as both treatment duration of lipofection and time of electroporation increased (data not shown). The incidence of gene transfer in electroporating lipofected sperm was lower than lipofection alone (Table 3).

Evaluation of mud loach expression vector

Microinjection of pURYN6-luc into fertilized eggs revealed the significantly lower hatching success, compared to non-injected control and also to pRSVL injected group. Severe deformation of hatched larvae was also observed in pURYN6-luc (2.5% in non-injected control, 10.8% in pRSVL injected group, and 48.7% in pURYN6-luc injected group). Malformation was noted in vertebra, and most deformed larvae did not survive after yolk sac absorption. No transgene could be detected with normally developed larvae after pURYN6-luc injection.

Discussion

The production of transgenic fish has been usually attempted by microinjection of DNA into fertilized eggs (Chen and Powers, 19 90 ; Penman et al., 1990, 1991), and this procedure has been considered as a most reliable method. However, microinjection has proved to be difficult and time-consuming due to the hard chorion of fertilized eggs, invisibility of nucleus and restricted available time for microinjection. In this study, several gene transfer methods including microinjection, electroporation, and lipofection were compared to assess their effectiveness for producing transgenic fish.

Microinjection of fertilized eggs lowered the survival of mud loach eggs, and this result was similar to other previous reports where ranged from 5% (Penman et al., 1990) to 80% (Chen and Powers, 1990). Although microinjection was carried out at one cell stage to avoid mosaicism, mosaic fashion of founders was still observed. Transgene mosaicism has been widely reported not only for mammal (Wagner et al., 1981, 1983), but also for many fish species (Pandian and Marian, 1994), and further breeding program should be developed to eliminate the mosaicism.

Electroporation is a simple technique for gene transfer using short electric pulse which results the transient pores on cell membrane. The foreign DNA (pRSVL) was introduced into mud loach sperm. The efficiency of electroporation was 28% in an optimized condition, and it was higher than result reported in chinook salmon (Sin et al., 1993), although most of introduced DNA was rapidly degraded.

Lipofection is also one of the candidate method which has been tried to overcome several problems associated with microinjection. It is the first trial of gene transfer in fish by incubating the sperm with liposome-DNA complex. The high rate of gene transfer (up tp 50%) decreased with increased age of fish. This phenomenon was similar to the result of African catfish (*Clarias gariepinus*) where lipofection had been tried to dechorionated embryos (Szelei et al., 1994).

Electroporation of the liposome-treated sperm did lower the efficiency of gene transfer compared to lipofection alone and also to electroporation alone. One possible explanation is that liposome treatments followed by high voltage of electroporation might cause significant stress to sperm cells, and may have reduced the fertilizing ability of transformed sperm.

To summarize, electroporation and lipofection are potential tools for investigating transient DNA transduction and expression of reporter gene in parallel in fish embryos.

They appear to be promising alternative for microinjection in connection with high efficiency of transfer and relatively simplicity of treatment protocol. However the present problems such as rapid degradation of transferred DNA and lack of integration should be overcome in future study. Further study also needed to determine whether introduced DNA by electroporation or lipofection could express in mud loach embryo.

The expression vector (pURYN6-luc) containing mud loach ARS was injected with the hope to increase the copy number of transgene during cell cycle by function of replication origin (Neri et al., 1992), and also to enhance transcription and recombination (Getzenberg, 1994) by presence of topoisomerase II binding site. Nevertheless, microinjection of pURYN6-luc caused the significant developmental defects and decreased viability. It is not known why such adverse teratogenic effects occurred, and also why all normally developed fish after pURYN6-luc injection did not retain the transgene. More extensive study should be needed to solve these problems associated with pURYN6-luc expression vector. Aspects such as vector sequence and origin, DNA form, use of different ARS clone are being investigated.

References

Chen, T. T. and D. A. Powers, 1990. Transgenic fish. TIB Tech., 8 : 209−215.

de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski and S. Subramani, 1987. Firefly luciferase gene : Structure and expression in mammalian cells. Mol. Cell. Biol., 725−737.

Getwenberg, R. H., 1994. Nuclear matrix and the regulation of gene-expression-tissue-specificity. J. Cell. Biochem. 55 : 22−31.

Hew, C. L. and G. L. Fletcher, 1992. Transgenic Fish : C. L. Hew and G. L. Fletcher, eds. World Scientific Publishing Co., Singapore.

Hew, C. L., G. L. Fletcher and P. L. Davies, 1995. Transgenic salmon : tailoring the genome for food production. J. Fish. Biol., 47 : 1−19.

Kavumpurath, S., O. Andersen, G. Kisen and P. Alestrom, 1993. Gene transfer method and luciferase gene expression in zebrafish, *Brachydanio rerio* (Hamilton). Bamidgeh, 45 : 154−163.

Kim, D. S. and Y. K. Nam, 1994. Transfer of foreign gene into mud loach, *Misgurnus mizolepis*. I. Availability of *lacZ* as a reporter gene for producing transgenic mud loach. J. Aquacult., 7 : 41−54.

Kim, D. S. and Y. K. Nam, 1995. Factors affecting the efficiency of introducing growth hormone gene into mud loach via electroporation. J. Aquacult., 8 : 241−249.

Kim, D. S., Y. K. Nam and I. -S. Park, 1995. Survival and karyological analysis of reciprocal diploid and triploid hybrids between mud loach (*Misgurnus mizolepis*) and cyprinid loach (*M. anguillicaudatus*). Aquaculture 135 : 257−265.

Kim, D. S., J. -Y. Jo and T. -Y. Lee, 1994. Induction of triploid in mud loach (*Misgurnus mizolepis*) and its effect on gonad development and growth. Aquaculture, 120 : 263−270.

Kim, D. S., Y. K. Nam and J. -Y. Jo, 1996. Effect of estradiol-17β immersion treatments on sex reversal in mud loach, *Misgurnus mizolepis*. Fish. Phyiol. Biochem. (in printing).

Lim, H. -S., M. -S. Kim and H. -H. Lee, 1995, Cloning and characterization of replication origins from *Misgurnus mizolepis*. J. Aquacult., 8 : 209−220.

Lim, H. -S., M. -S. Kim, Y. -S. Seok, S. -D. Park and H.H lee, 1996. Characterization and DNA structure analysis of replication origin of *Misgurnus mizolepis*. J. Aquacult., 9 : 93−100.

Maclean, N. and D. Penman, 1990. The application of gene manipulation to aquaculture. Aquaculture, 85 : 1−20.

Neri, L. M., G. Mazzoggi., S. Capitani, N. M. Maraldi, C. Cinti, N. Baldini, R. Rana and A. M. Martelli, 1992. Nuclear matrix-bound replicational sites detected in situ by 5-

bromo-deoxyuridine. Histochemistry 98 : 19 −32.

Pandian, T. J. and L. A. Marian, 1994. Problems and Prospects of transgenic fish production. Curr. Sci., 66 : 635−649.

Penman, D. J., A. Iyengar, A. K. Beeching, A. Rahman, Z. Sulaiman and N. Maclean, 1991. Patterns of transgene inheritance in rainbow trout (*Oncorhynchus mykiss*). Mol. Repr. Dev., 30 : 201−206.

Penman, D. J., A. J. Beeching, S. Penn and N. Maclean, 1990. Factors affecting survival and integration following microinjection of novel DNA into rainbow trout eggs. Aquaculture, 85 : 35−50.

Sin, F. Y. T., A. L. Bartley, S. P. Walker, I. L. Sin, J. E. Symonds, L. Hawke and C. L. Hopkins, 1993. Gene transfer in chinook salmon (*Oncorhynchus tshawytscha*) by electroporating sperm in the presence of pRSV-lacZ DNA. Aquaculture, 117 : 57−69.

Szelei, J., L. Varadi, F. Muller, F. Erdelyi, L. Orban, L. Horvath and E. Duda., 1994. Liposome-mediated gene transfer in fish embryos. Transgen. Res., 3 : 116−119.

Wagner, T. E., P. C. Hoppe, J. P. Jollick, D. R. Scholl, R. L. Hodinka and J. B. Gault, 1981. Microinjection of a rabbit β-globin gene in zygotes and its subsequent expression in adult mice and their offspring. Proc. Natl. Acad. Sci. USA, 78 : 6376−6380.

Wagner, E. F., L. Covarrubias, T. A. Stewart and B. Mintz, 1983. Prenatal lethalities in mice homozygous for human growth hormone gene sequences integrated in the germ line. Cell, 35 : 647−655.