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Growth Promotion in Ayu (*Plecoglossus altivelis*) by Gene Transfer of the Rainbow Trout Growth Hormone Gene

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Chao-An Cheng, Kuen-Lin Lu, En-Lieng Lau, Tse-Yeng Yang, Chiou-Yueh Lee, Jen-Leih Wu and Chi-Yao Chang (2002) Growth promotion in Ayu (*Plecoglossus altivelis*) by gene transfer of the rainbow trout growth hormone gene. *Zoological Studies* **41**(3):303-310. A plasmid containing rainbow trout growth hormone cDNA under a carp β-actin promoter was transferred into ayu (*Plecoglossus altivelis*) using a sperm-electroporation method. Hatching and survival rates of ayu into which the gene was transferred were lower than those of the control, and about 10% of ayu were raised from 10^5 treated fertilized eggs. The persistence of transferred DNA was detected using polymerase chain reaction (PCR) with specific primers in genomic DNA from caudal fin tissue after 5 mo. PCR and restriction enzyme digestion analyses showed that the success of gene transfer was as high as 55%. Mosaic expressions of the transgene could be detected in gill, caudal fin, liver, brain, and muscle, but not in the heart. Two-fold growth enhancement in body weight and 1.3-fold in body length were measured. http://www.sinica.edu.tw/zool/zoolstud/41.3/303.pdf

Key words: Gene transfer, Ayu, Sperm-electroporation, Growth hormone.

The 1st transgenic mouse, produced using a rat growth hormone gene under a mouse metallothionein promoter, resulted in a dramatic increase in growth (Palmiter et al. 1982). Series of growth hormone gene transfers into economically important domestic animals have been attempted since then (Du et al. 1992, Devlin et al. 1994, Rahman and Maclean 1999). Several techniques for gene transfer such as microinjection, electroporation, high-velocity microprojectile bombardment, and sperm electroporation have been developed. In fish, microinjection was the earliest technique developed to introduce foreign DNA into fertilized eggs. However, the opaqueness, stickiness, and buoyancy of the embryos, the invisibility of the pronuclei, the toughness of the chorion, and the higher mortality of injected eggs make this a time-consuming technique which requires sophisticated skills, and so it is not suitable for the eggs of many species of fish. At the same time, electroporation and high-velocity microprojectile bombardment methods are able to produce "mass" fish transgenesis, even though lower survival rates and lower efficiencies in gene transfer were reported (Powers et al. 1992). The ease of artificial fertilization enables the use of sperm electroporation, a powerful tool for producing increased transgenic efficiencies (Tsai et al. 1997).

Growth hormone is a member in the hormone family composed of growth hormone, prolactins and somatolactin (Ono et al. 1990, Takayama et al. 1991). It is synthesized and stored in pituitary somatotrophic cells. The secreted growth hormone stimulates the production of insulin-like growth factors (Palmiter et al. 1982, Palmiter et al. 1983), which mediate many of its growth-promot-

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ing effects. Using growth hormone gene transfer, several transgenic fish have been successfully produced (Dunham et al. 1987, Penman et al. 1990, Zhang et al. 1990, Du et al. 1992, Rahman and Maclean 1992 1999, Devlin et al. 1994, Rahman et al. 1998). Growth hormone transgenes of fish origin have universally been shown to be more effective in growth enhancement than are those of non-fish origin (Hew et al. 1992, Devlin et al. 1994).

Ayu, *Plecoglossus altivelis*, the sole member of the Salmoniformes family Plecoglossidae, is an osmerid-like fish found only in streams and coastal waters of regions in Asia. Possessing a special smell and good taste, they are considered a popular and highly valued edible fish in Asia. Moreover, the ayu culture industry is rapidly growing in Taiwan. In this paper, we transferred a plasmid containing rainbow trout growth hormone cDNA under the carp β-actin promoter into ayu, using a sperm-mediated electroporation method, to obtain growth-enhanced transgenic fish.

MATERIALS AND METHODS

Fish collection and artificial fertilization

Ayu eggs and sperm were collected from a commercial farm in Fushan, a mountain area near Taipei. Artificial fertilization was performed in autumn with natural maturation, which is influenced by water temperature. The mature male and female brood stocks were checked and separated on the day before artificial fertilization. Fertilizable eggs, characterized as having even and fine oil drops, were collected, examined under a stereomicroscope, and mixed with saline-activated sperm. After washing several times with fresh water, the fertilized eggs were stuck onto fibers of a palm leaf in running water until hatching.

Plasmid construction

The recombinant plasmid was constructed as described in Powers et al. (1992). Briefly, the *Nde* I-*Xba* I fragment containing the carp β-actin promoter from pAI 3-6 was inserted into a pRSV2 plasmid (Gorman et al. 1982) after removing the RSV promoter. The resultant plasmid, pAE6, was used for construction of pAE6-rtGH1 as follows. The rtGH1 cDNA fragment was digested using *Hin*d III from pRSVLTR-rtGH1 (Powers et al. 1992) and ligated into the *Hin*d III site of pBS. This plasmid was digested again with *Xho* I and *Xba* I, and the obtained fragment with correct orientation was ligated into the corresponding sites of pAE6.

Sperm electroporation

Fresh sperm of mature ayu, from 500 to 1000 μl per fish, were collected by squeezing the testes. The concentration of pAE6-rtGH1 DNA was adjusted to 100 μg/ml with ISOSALINE solution containing 8.5 mg/ml NaCl, 0.25 mg/ml disodium edetate, and 1.3 mg/ml potassium sorbate (SINTONG, Taiwan). For electroporation, 10 μl of ayu sperm was added to the 500-μl of DNA solution in the receptacle of the electroporation instrument (Baekon 2000, Baekon Saratoga, CA). The electroporation conditions were an electric field strength of 9 kV, a burst time of 0.4 s, 4 reaction cycles, a pulse number of 26, and a pulse-time of 160 ms. After electroporation, treated sperm were immediately aspirated for artificial fertilization.

DNA extraction

Approximately 0.1 g of fresh fish caudal fin tissue was extracted with flame-treated scissors. The fin tissue was first incubated with digestion buffer (100 mM Tris-HCl (pH 8.5), 200 mM NaCl, 5 mM EDTA, 0.2% SDS, and 0.2 mg/ml proteinase K) at 5°C for 5 h with shaking. After phenol/chloroform extraction, the DNA was precipitated, washed with ethanol, and then resuspended in 50 μl of sterile distilled water.

RNA extraction

RNA was isolated by a modified RNAzol™ B method (TEL-TEST, Friendswood, TX). Briefly, a small amount of fresh tissue was homogenized in RNAzol™ B (2 ml/100 mg tissue), transferred to Eppendorf tubes with 1/10 volume of chloroform, and stored at 4°C for 5 min. After 15 min of centrifugation, the aqueous phase was collected and precipitated with 0.4 volumes of isopropanol. The precipitated RNA was washed with 75% ethanol and resuspended in 200 μl of 0.1% diethyl pyrocarbonate (DEPC)-treated water. The dissolved RNA was incubated with 1 unit of RNase-free RQ1 DNase (Promega) at 37°C for 30 min and then extracted with phenol/chloroform. The resulting RNA was again precipitated, washed, and dissolved in 50μl of DEPC-treated water.

Polymerase chain reaction (PCR)

The PCR was used to amplify a 461-bp fragment with primers, β-actin2(+): 5'-TTG TCT GGC ACA TCT GAG-3' designed from the carp β-actin intron 1 region and rtGH2(-): 5'-CGT TCA TCA GGC AAC AGG-3' designed from a rainbow trout growth hormone cDNA sequence. Standard PCR was performed in a final volume of 50 μl containing 250 mM of each dNTP, 2.5 units of *Taq* DNA polymerase, 1 mM of each primer, 1.25 mM $MgCl_2$, and 1 μg of genomic DNA as a template, using a Perkin-Elmer thermocycler 480 programmed for 35 cycles: denaturing at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Following the 1st PCR, 1 μl of the resulting sample was used as template for the 2nd PCR with the same primer set. The re-amplified PCR products were digested with *Xho* I at 37°C for 2 h, and subjected to 2% agarose gel electrophoresis.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

For synthesizing 1st-strand cDNA, 50 μg of total RNA was treated with methylmercury hydroxide, and subjected to reverse transcription using 75 units of MMLV-RT (Epicentre) and 0.25 μg of oligo-dT primer in a final volume of 70 μl, containing 40 units of RNase block ribonuclease inhibitor (Stratagene), 0.4 mM of each dNTP, and 1x 1st-strand buffer (Epicentre), at 37°C for 1 h. Two microlitres of the resulting solution was then amplified by PCR as described above with primers rtGH3(+): 5'-CTG AGT CAA GGG GCA GCG-3' and rtGH4(-): 5'-TCT GTA TCT GGG AAA CCG-3'.

Southern blot hybridization

Southern blot hybridization was performed as described by Chang et al. (1993). Approximately 1/10 of the RT-PCR products were separated by 2% agarose gel electrophoresis, transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech), and hybridized with a 398-bp internal probe which contained an *Acc* I DNA fragment inside of this RT-PCR product and was eluted from rainbow trout growth hormone cDNA.

RESULTS

Motility of ayu sperm after electroporation

It is essential to find a suitable solution or medium that can maintain the motility of sperm for a sufficiently long time in order to carry out artificial fertilization after electroporation. Hence, as an initial step, we examined sperm motility under a microscope, after mixing the semen with different kinds of solutions including ISOSALINE solution, phosphate-buffered saline (pH 7.4), Tris-EDTA buffer (pH 7.4), and PB-sucrose solution (272 mM sucrose, 7 mM $NaPO_4$, (pH 7.4), and 1 mM $MgCl_2$). The best sperm motility was obtained with ISOSALINE solution, which could maintain the motility for up to 6 min. Therefore, we performed sperm electroporation in ISOSALINE solution in a Baekon 2000 electroporater. Similarly, sperm motility was tested after electroporation, with various electric field strengths and burst times. It was noted that sperm motility decreased with increasing electric field strength or burst time. Finally the electroporation conditions were standardized and performed at an electric field strength of 9 kV, a burst time of 0.4 s, 4 reaction cycles, a pulse number of 26, and a pulse time of 160 ms. Under these conditions, sperm motility could still be maintained for 2 min.

Hatching and survival rates of fertilized eggs after artificial fertilization using electroporated sperm

The pAE6-rtGH1 expression vectors, containing the carp β-actin promoter including its 1st intron to drive rainbow trout growth hormone 1 cDNA and a polyadenylation site for attenuation from the early region of SV40, was used for sperm electroporation. The supercoiled form of the expression vector was purified by 2 passes through a CsCl gradient, after which it was electroporated into ayu sperm before artificial fertilization. Because the quality of eggs determines the efficiency of hatching and survival rates of artificial fertilization, good-quality mature eggs with a round shape and small, even distribution of oil drops were collected and examined under a microscope before sperm electroporation and artificial fertilization. Because of the short life of sperm, the gene-transferred sperms were mixed with eggs in 100-fold volumes of ISOSALINE solution immediately after electroporation. The electric field strength of electroporation on sperm influences the development of fertilized eggs. The high strength of the electric field (9 kV) used resulted in lower hatching and survival rates compared to fertilized eggs

using non-electroporated sperm (data not shown).

Detection of the transgene in fish bred from electroporated sperm

Strategies for transgene detection are illustrated in figure 1A. Total genomic DNA was purified from individual caudal fins, and 2 specific primers, β-actin2(+) and rtGH2(-), were designed from carp β-actin intron 1 and rainbow trout growth hormone 1 cDNA sequence, respectively. Because the quantity of the transgene PCR amplified from total genomic DNA was insufficient for detection by gel electrophoresis, a secondary amplification was performed in a sample with 1/50 of the primary reaction products under the same reaction conditions. The 461-bp DNA fragments subjected to PCR twice (Fig. 1B, lanes 3 and 4) and amplified from the genomic DNA of transgenic ayu co-migrated with those amplified from the pAE6-rtGH1 vector (Fig. 1B, lane 2), while no product was amplified from non-transgenic fish genomic DNA (Fig. 1B, lane 1). For further confirmation, these amplified DNA fragments were digested using *Xho* I to obtain 265- and 196-bp DNA fragments (Fig. 1B, lanes 5 and 6). By this method, we could amplify 44 of 80 samples, revealing that 55% of fish bred from artificial fertilization using electroporated sperm possessed the rtGH transgene.

Expression of the transgene in transgenic fish

Strategies for detection of transgene expression are illustrated in figure 2A. Total RNA extracted from gill, heart, caudal fin, liver, brain, and muscle was used for the 1st-strand cDNA synthesis. Using specific primers, a DNA fragment was PCR-amplified from the cDNA region of rainbow trout growth hormone. Further confirmation was made by Southern blot hybridization of the PCR products using an *Acc* I DNA fragment from rainbow trout growth hormone cDNA. Specific rtGH mRNA could be detected from gill, caudal fin, liver, brain, and muscle (Fig. 2B, lanes 1, 3, 4, 5 and 6, respectively), but not from the heart (Fig. 2B, lane 2).

Growth performance of transgenic fish harboring carp β-actin rtGH cDNA

After 5 mo of rearing, about 10^4 sperm-electroporated ayu had grown up. Transgenic and non-transgenic fish were randomly collected and their body weight and length were measured. The average body weight and length of transgenic ayu were 23.44 g and 12.47 cm, compared to 10.95 g and 9.67 cm for non-transgenic ayu (Fig. 3), respectively. The comparison analysis revealed that the growth performance of transgenic fish was approximately 2-fold higher for body weight and 1.3-fold higher for body length than those of non-transgenic fish. Moreover, the transgenic ayu showed a darker body color and had a special scent compared to non-transgenic ones (Fig. 4). This reveals that 5-mo-old transgenic ayu show precocious maturation.

DISCUSSION

In the present study, we show that rainbow trout growth hormone enhances the growth perfor-

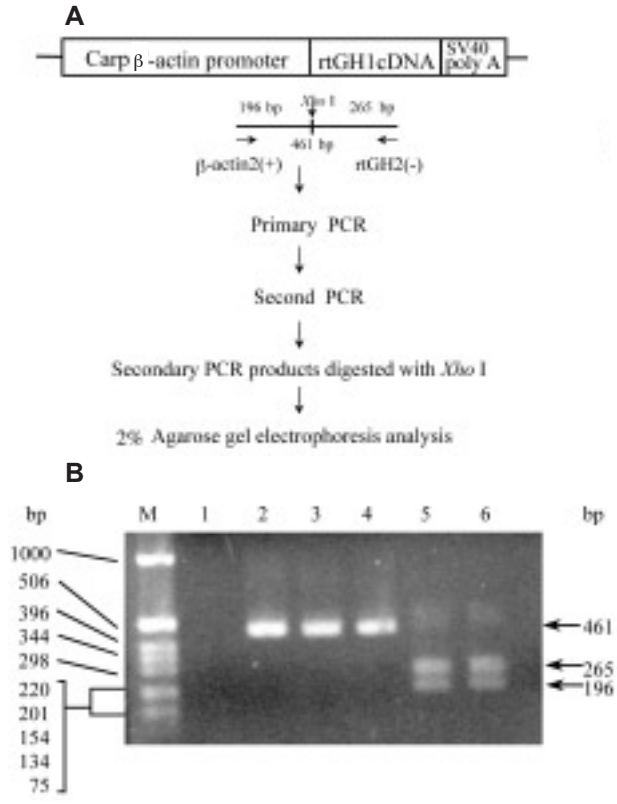

Fig. 1. Detection of transgenes in transgenic ayu. A. Strategy of transgene analysis with genomic DNA extracted from caudal fins by PCR amplification and *Xho* I digestion. B. Gel electrophoresis analysis of PCR products. M: 1-kb DNA ladder marker; lane 1: negative control, non-transgenic ayu genomic DNA as the template; lane 2: positive control, the pAE6-rtGH1 vector as the template; lanes 3 and 4: PCR products amplified from transgenic ayu; lanes 5 and 6: *Xho* I-digested products of lanes 3 and 4.

mance of sperm-electroporated transgenic ayu. Similar results have been reported in mice (Gordon et al. 1980, Wagner 1981, Palmiter et al. 1982), cows, pigs, sheep, and rabbits (Pursel et al. 1987, Pursel et al. 1989). Two-fold growth enhancement by the rainbow trout growth hormone gene was seen in transgenic ayu, which is less than that reported for rainbow trout (Rahman and Maclean 1999), but more than that for carp (Chen et al. 1993).

Successful transfer of genes into aquatic organisms has been demonstrated by electroporation of sperm using exogenous DNA before fertil-

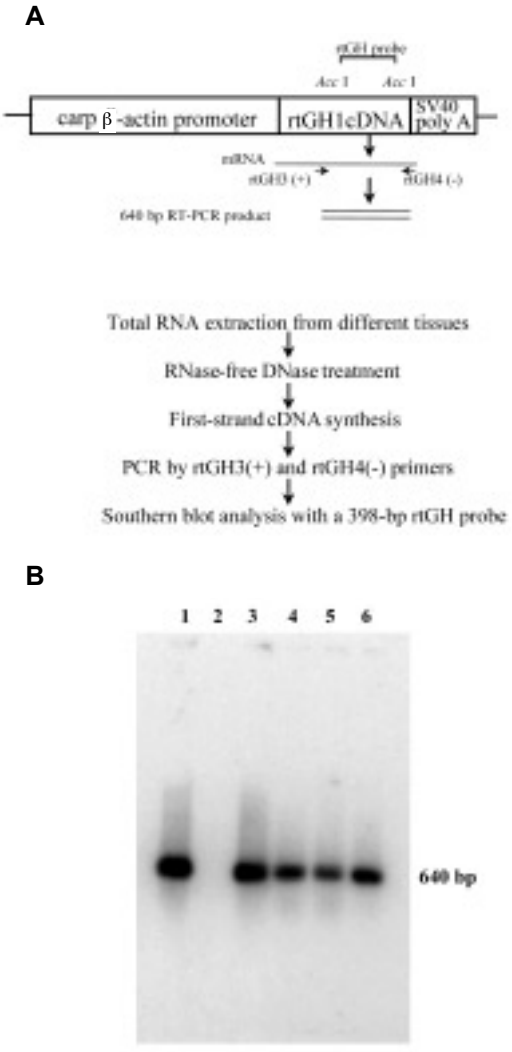

Fig. 2. Detection of transgene expression in transgenic ayu. A. Strategy of determining transgene expression from different tissues detected by RT-PCR amplification and Southern hybridization. B. Southern blot analysis of RT-PCR products. Five microliters of RT-PCR products from different tissues was separated by electrophoresis through a 2% agarose gel, transferred to a Hybond nylon membrane, and hybridized with a rainbow trout growth hormone probe. Lane 1: gill; lane 2: heart; lane 3: caudal fin; lane 4: liver; lane 5: brain; lane 6: muscle.

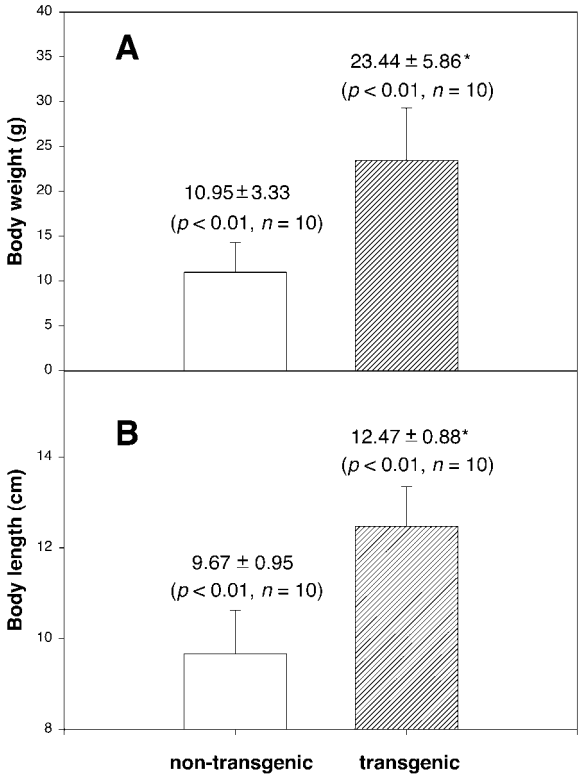

Fig. 3. Comparison of body weight and length of transgenic and non-transgenic ayu. After 5 mo of culture, body weight and length were measured from 10 randomly selected transgenic and non-transgenic ayu. *Indicates that means statistically differ at the 1% significance level, by *t*-test.

Fig. 4. Transgenic (right) and non-transgenic (left) ayu siblings at the age of 5 mo showing size difference and precocious coloration.

ization (Powers et al. 1992, Sin et al. 1993, Tsai et al. 1995). The efficiency of DNA transfer into ayu eggs by the sperm-mediated electroporation method in this study was 55%, better than that of 50% in loach (Tsai et al. 1995), but less than that of 80% in zebrafish (Powers et al. 1992). Compared to conventional microinjection and electroporation for aquacultured animals, sperm-mediated gene transfer is a relatively simple and efficient method, especially for ayu, because several hundred thousand eggs can be processed at the same time by using artificial fertilization. Therefore, we remain cautiously optimistic about sperm-mediated electroporation as a means for the mass treatment of fish gametes to produce transgenic fish.

The sperm motility time of ayu is rather short under in vitro conditions. Motility times in NaCl (25-175 mM) and KCl (25 -75 mM) solutions were found to be 30-50 and 50-100 s, respectively (Utsugi 1993). However, with electroporation as well as artificial fertilization, sperm must be motile for long time. Hence, several solutions were assessed for long-term sperm motility and ISOSALINE solution was ultimately selected; motility could be maintained in it for up to 6 min. Moreover, the solution influences the success of electroporation to a great extent. It has been reported that natural seawater and MIPS are suitable solutions for abalone sperm electroporation (Tsai et al. 1997). In the present study, fortunately, the ISOSALINE solution itself was also found to be suitable for electroporation. Unlike that of vertebrates, invertebrate sperm is more resistant to high-voltage treatment. Tsai et al. (1997) revealed that abalone sperm was resistant to high voltages (10 kV). Here, the mortality of ayu sperm decreased to 50% after 120 s when 9 kV was used. Symonds et al. (1994) also demonstrated that the activity of salmon sperm decreased from 82% to 2% as sperm were electroporated at voltages increasing from 625 to 1000 V/cm.

The 5-mo-old transgenic ayu showed precocious maturation with a darkened body coloration. Similar precocious maturation has also been reported in transgenic tilapia (Rahman and Maclean 1999) and transgenic salmon (Devlin et al. 1994), which implies an elevated expression level of growth hormone from the transgenes. The early change of body coloration in transgenic ayu suggests that the increasing growth hormone may be involved in the regulation of sexual maturation. Lobie et al. (1990) observed the widespread distribution of the growth hormone receptor/binding protein in the reproductive system of the rat in which growth hormone may exert a direct action on reproductive functions. Through unique receptors, growth hormone may stimulate ovarian follicles and testicular Leydig cells, by functioning alone or synergistically with luteinizing hormone and follicle-stimulating hormone (Childs 2000).

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轉殖虹鱒生長激素基因促進香魚生長之研究

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我們利用精子電穿孔轉殖法將含鯉魚β-肌動蛋白啓動子的虹鱒生長激素cDNA質體轉殖到香魚受精卵中。基因轉殖香魚之孵化率及存活率都較控制組低，且在十萬個處理過的受精卵中約有10%的育成率。經過5個月飼養後，萃取尾鰭基因體DNA，利用特定引子進行聚合酶鏈鎖反應來檢測轉殖基因。聚合酶鏈鎖反應和限制酶切割分析證實基因轉殖成功率約為55%。以反轉錄酶-聚合酶鏈鎖反應來分析轉殖基因之表現，發現其表現型為鑲嵌式，可在鰓、尾鰭、肝臟、腦和肌肉中表現，而不在心臟表現。此基因轉殖香魚促進生長之效率，以體重而言為非基因轉殖組的2倍，體長則為非基因轉殖組的1.3倍。

關鍵詞：基因轉殖，香魚，精子電穿孔法，生長激素。

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