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Transfer of a foreign gene to Japanese abalone (*Haliotis diversicolor supertexta*) by direct testis-injection

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Abstract

We developed a technique that easily transfers exogenous DNA fragments into the genome of Japanese abalone (*Haliotis diversicolor supertexta*), an important aquaculture shellfish. From 0.1 to 10 μg/μl of the linearized plasmid pOBA–YPGHc, containing the yellowfin porgy (*Acanthopagrus latus*) growth hormone cDNA driven by the medaka β-actin promoter, was directly injected into abalone testis with a microsyringe in a total volume of 10 μl. The sperm activity, fertility, and hatchability of fertilized eggs from testis-injected abalone did not differ ($P<0.05$) from those of abalone in the untreated control group. From extracted genomic DNA from sperm, larvae at the trochophore stage (9 h after fertilization), juveniles (3 weeks after fertilization), and adults (1-year-old) of testis-injected abalone, a product with a molecular weight of 954-bp was amplified using polymerase chain reaction (PCR). Southern blot analysis showed that the amplified PCR product hybridized to the pOBA–YPGHc probe. The gene-transfer efficiency of G_0 in larvae, juveniles, and 1-year-old adults was 90%, 92.5%, and 60%, respectively. Around 20% of G_0 mature abalone contained the transgene in their gonads. Genomic Southern blot analysis of 1-year-old abalone derived from DNA-injected group revealed that the transgene was integrated in the genome of the transgenic abalone. The mean shell lengths and body weights were significantly greater ($P<0.05$) than those of control group. We conclude that direct testis injection is a simple, high-throughput, minimally invasive, non-viral based, and efficient approach for producing transgenic abalone.

Keywords: Abalone; Gene transfer; Testis; Injection

1. Introduction

Transgenic animals provide a powerful system for in vivo study of gene regulation, expression, and function. Transgenics make it possible to generate varieties with special genetic traits encoded by specific genes and to produce mutants with a particular defective gene. Gene delivery to aquatic animals, including commercially important species of finfish and shellfish, has been studied for more than 20 years (reviewed by Gong and Hew, 1995; Sin, 1997). Many approaches have been developed to introduce foreign DNA molecules into gametes or embryos. In finfishes, microinjection is the most common method for transferring the foreign DNA into oocyte nuclei (Ozato et al., 1986; Tsai et al., 1995b), cytoplasm of developing embryos (Chourrout et al., 1986; Dunham et al., 1987) and fertilized eggs (Fletcher et al., 1988;

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Dunham et al., 1992; Lu et al., 1992). Electroporation of exogenous DNA fragments into fertilized eggs (Inoue et al., 1990; Powers et al., 1992; Tsai and Tseng, 1994) is rather simple, convenient, and more efficient than microinjection. In shellfishes, genes have been transferred by electroporation of red abalone (*Haliotis rufescens*) embryos (Powers et al., 1995), retroviral infection for dwarf surf clams (*Mulina lateralis*) (Lu et al., 1996), particle bombardment for Pacific oyster (*Crassostrea gigas*) (Cadoret et al., 1997a), and microinjection, electroporation or chemical-mediated transfection of embryos for Eastern oyster (*C. virginica*) (Cadoret et al., 1997b; Buchanan et al., 2001). In the above studies there was no documentation of transgene integration or inheritance except reported in dwarf surf clams. However, those methods for transferring genes to fertilized eggs are insufficient for quickly treating the tremendously large number of eggs spawned by cultured species of shellfishes.

Aquatic animals produce huge numbers of sperm, which are excellent material for gene transfer because the sperm-mediated method is considered a mass gene transfer technique, sperm can deliver foreign DNA fragments into the nucleus, and sperm can be kept by cryopreservation (reviewed by Tsai, 2000; Li and Tsai, 2000). Such advantages make sperm a potentially useful vector to introduce foreign DNA and produce transgenic finfish and shellfish. Genes have been transferred by sperm-incubation in zebrafish (Khoo et al., 1992), sperm-electroporation in common carp (*Cyprinus carpio*L.) (Muller et al., 1992), catfish (*Clarias gariepinus*) (Muller et al., 1992), tilapia (*Oreochromis niloticus*) (Muller et al., 1992), chinook salmon (*Oncorhynchus tshawytscha*) (Symonds et al., 1994), loach (*Misgurnus anguillicaudatus*) (Tsai et al., 1995a), and Japanese small abalone (*H. diversicolor supertexta*) (Tsai et al., 1997). However, all these methods still have limitations: (1) the facilities required for sperm-mediated gene transfer are expensive; (2) culturing G_0 individuals to reach adulthood is absolutely required before the transgenic founders that enable to transmit the transgene to offspring are identified; and (3) DNA preparation and gamete induction are time-consuming and tedious, especially for abalone, making this method impossible for use in field trials.

The testis of an abalone is easily accessible, because the gonad is exposed when one holds its shell. In this paper, we describe a method of injecting foreign DNA fragments with an all-fish-construct directly into the testis of abalone to achieve faster growth, and demonstrate that this approach is the simplest and most-efficient method for gene transfer for large numbers of abalone.

2. Materials and methods

2.1. Experimental animals

Male and female 2- to 3-year-old, aquacultured Japanese small abalone were cultured in two separate 20 l containers and maintained in 10 l of natural seawater treated with Millipore filtration and ultraviolet light irradiation. The 39 males and 20 females were kept on a natural daylight cycle in a temperature-controlled (25 °C, 30‰) container without feeding during the period of ovulation or spermiation. Males averaged 5.7±0.2 cm in length, 4.6±0.3 cm in width, and 29.3±1.4 g in weight. Females averaged 6.1±0.3 cm in length, 4.9±0.1 cm in width, and 31.6±1.9 g in weight.

2.2. DNA preparation

Expression vector, pOBA–YPGHc, with a molecular weight of 8 kb, consists of a medaka (*Oryzias latipes*) β-actin gene promoter (OBA; Takagi et al., 1994) and a yellowfin porgy (*Acanthopagrus latus*) growth hormone cDNA (YPGHc; Tsai et al., 1993). This plasmid was produced at large scale, as described by Sambrook et al. (1989), extracted by using the VIOGENE V500 kit (VIOGENE, Sunnyvale, CA, U.S.A.), digested by *Nsi*I, and isolated by a gel-recovery kit (Geneaid, Taipei, Taiwan). The purified linear DNA plasmid was resuspended in filtered sterilized seawater at concentrations of 0.1 or 10 μg/μl.

2.3. Testis injection

Foreign DNA, at concentrations of 0.1 or 10 μg/μl in a total volume of 10 μl, was directly injected into the posterior part of the testis of abalone with a microsyringe (Hamilton, Reno, NV, U.S.A.) for testing whether the injection affected milt production. For the mock-treatment control group, filtered (sterilized) seawater was used instead, and two abalone were used for each group. Subsequently, we injected 0.1 and 10 μg/μl foreign DNA fragments (three abalone for each concentration) into the anterior, middle, and posterior part of the testis ($N=18$ for transgene-injections, $N=9$ for mock-treatment overall) to determine whether the successful gene-transfer rate is dependent on the side of injection. After injection, abalone were cultured in a

temperature-controlled (28 °C) container for 3–6 h before they produced milt.

2.4. Gamete collection, sperm mobility and in vitro fertilization

Six, matured male abalone were divided into three groups: one mock-treatment group and two experimental groups (0.1 and 10 μg/μl foreign DNA). Each group was maintained separately in a 10 l container with natural seawater. Twenty mature female abalone were maintained in a 30 l container. The temperature-shift method was used to induce ovulation or milt production (Chen and Yang, 1979). Sperm from two males in each group were collected and kept in a 1 l glass container. The number of gametes obtained was estimated using a hemocytometer slide. Sperm mobility evaluation and in vitro fertilization were described previously (Tsai et al., 1997).

2.5. Fertilization and hatching rates

The fertilization rate was the proportion of eggs that developed into the two-cell or four-cell stage 1 h after fertilization. The hatching rate was the proportion of fertilized eggs that developed into the trochophore stage (9 h after fertilization).

2.6. DNase I treatment and genomic DNA extraction

Sperm or larvae were pooled, collected in a 1.5-ml Eppendorf tube, and preserved with 100% ethanol. Prior to extraction, the samples were centrifuged at 8000 ×*g* for 5 min, the ethanol was removed, seawater was added to wash the pellet three times, and the pellet was resuspended in 500 μl of digestion buffer. To ensure PCR detection of the foreign DNA molecules in the sperm and trochophore larvae and to avoid contamination with non-integrated DNA fragments, DNase I (Sigma, St. Louis, MO, U.S.A.) was added to a final concentration of 100 μg/ml and used to wash samples from each treatment group. The solution was incubated at 37 °C for 1 h. After the reaction, the samples were centrifuged at 8000 ×*g* for 5 min. The supernatant was decanted, and samples were boiled for 10 min to halt DNase activity.

Genomic DNA was extracted from sperm (10^{10} cells); 1, 10, 25, 50 trochophore larvae; a 3-week-old juvenile; and a 1-year-old adult by adding 200 μl of lysis solution (6 M guanidine hydrochloride and 0.1 M sodium acetate, pH 5.5). Samples were crushed quickly and then incubated at room temperature for 3 h on a rotating platform. The genomic DNA was obtained by phenol/chloroform extraction and ethanol precipitation.

2.7. PCR analysis

Two oligonucleotide primers were synthesized for detection of YPGH cDNA by PCR analysis: forward primer (5′-GGTCCCTTTGTGCGCCGTTA-3′) and reverse primer (5′-CTGTCGTTGCTCCTCAGTCAG-3′). The primers for detection of the endogenous β-tubulin gene, which served as an internal control, were forward primer (5′-CCCTTCCCTCGTCTCC-AC-3′) and reverse primer (5′-GCCAGTGTACCA-GTGAAGGGA-3′). Each PCR sample consisted of 20 μl of solution containing 10–20 ng of template, 10 pmol of each primer, 25 μM of each dNTP, and 5 units of ProZyme II (PROtech, Taipei, Taiwan), in a 10× PCR buffer. Amplification was performed with a Perkin-Elmer Cetus DNA Thermal Cycler (Boston, MA, U.S.A.). PCR consisted of 25 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, followed by 10 min extension at 72 °C. Each PCR sample (10 μl) was subjected to electrophoresis on a 3% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME, U.S.A.).

2.8. Probe preparation

The 0.9-kb PCR product containing YPGH cDNA was amplified from plasmid pOBA–YPGHc and purified with a gel-recovery kit. Five micrograms of the 0.9-kb DNA fragments were digoxigenin (DIG)-labeled by the random priming method for 20 h at 37 °C, as recommended by the manufacturer (Roche, Mannheim, Germany).

2.9. Southern blot analysis

PCR products or *Nsi*I-digested genomic DNA were analyzed on an agarose gel and transferred onto a nitrocellulose membrane (Amersham, Little Chalfont, U.K.). After air-drying, the DNA was cross-linked to the membrane by UV irradiation, and then hybridized to the probe of the DIG-labeled YPGHc fragments. Hybridization was carried out overnight, at 42 °C, in a standard buffer solution (Roche, Mannheim, Germany) containing 50% formamide and 10 ng/ml of denatured probe. After the membranes were washed, colorimetric detection was carried out with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate reagents, as

recommended by the manufacturer (Roche, Mannheim, Germany).

2.10. Gene-transfer efficiency

The gene-transfer efficiency was the number of PCR–Southern-positive samples divided by the total number of larvae, 3-week-old juveniles, and 1-year-old adults that were examined.

2.11. Growth performance of abalone after gene transfer

The transferred DNA fragment, pOBA–YPGHc, encodes the growth hormone of a marine fish under the control of the β-actin promoter. To infer whether the exogenous DNA fragments are expressed and functional in the transgenic abalone, we studied their growth performance. After a total volume of 10 μl of the 10 μg/μl DNA fragment was injected into the testes of two parental individuals, we collected sperm and mixed them with eggs that were spawned from six females to carry out in vitro fertilization. After the trochophore stage, all larvae were cultured outdoors in a 1.5×6.0 m^2 hatchery pond. Abalone in the control group were cultured under the same conditions but were kept in a different pond adjacent to that of the experimental group. We measured the densities, shell lengths, and body weights of each group from 100 individuals that were haphazardly collected from 10 different places when they were 1-week, 6-months, and 1-year old.

2.12. Statistical analysis

The fertilization rate, hatching rate, body lengths, and body weights were analyzed statistically with the general linear models procedure (SAS, Inc., Cary, NC, U.S.A.). A one-way analysis of variance was used to compare the mean values between the mock-treatment and the experimental groups. A Duncan's multiple range test was used to separate sample means. A significance level of $P<0.05$ was used in all statistical analyses.

3. Results

3.1. Effect of injection on sperm activity, fertility, and hatchability

Our preliminary results showed that there was no difference ($P<0.05$) in terms of the sperm activity and the number of released sperm (10^6 to 10^7 cells) between the control abalone and the abalone whose testes were injected with sterilized seawater. Then we injected 0 (sterilized seawater) or 0.1 and 10 μg/μl foreign DNA in a total volume of 10 μl into the posterior part of the testis to evaluate the quality of sperm after milt production was induced. Results showed that there were no significant differences ($P<0.05$) in both the activity and the number of sperm (10^6 to 10^7 cells/ml) from the treated and the mock-treated abalone.

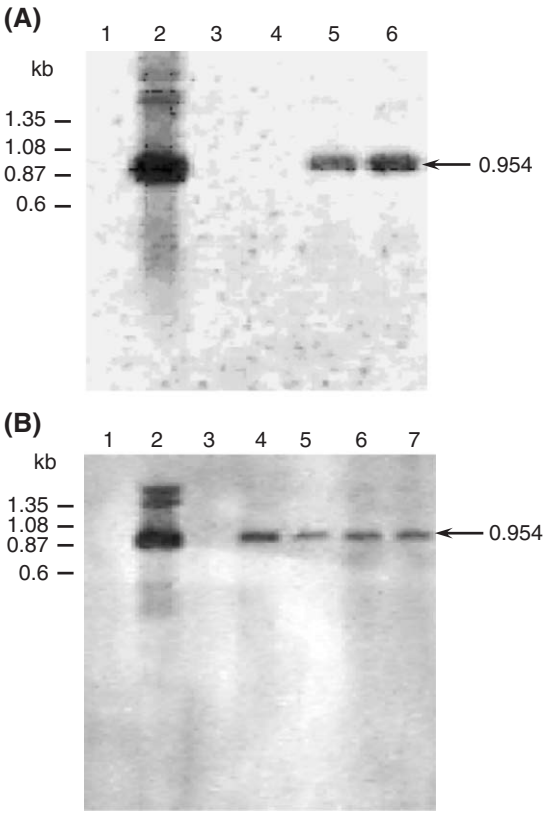

Fig. 1. PCR–Southern blot analysis of genomic DNA isolated from abalone sperm after DNase I treatment (A) and from abalone larvae at the trochophore-stage (B). The purified PCR product of digoxigenin-labeled YPGHc was used as a probe. (A) Lane 1: molecular marker, *Hae*III-digested φX-174-RF DNA; lane 2: 0.1 μg of pOBA–YPGHc as a template (positive control); lane 3: pOBA–YPGHc (10 μg) digested with DNase I (20 μg) for 2 h; lane 4: sperm from abalone injected with seawater (mock-treatment); lanes 5 and 6: sperm from abalone injected with 0.1 and 10 μg of pOBA–YPGHc, respectively, after digestion with DNase I. (B) Lane 1: molecular marker, *Hae*III-digested φX-174-RF DNA; lane 2: 0.1 μg of pOBA–YPGHc as a template (positive control); lane 3: 50 larvae derived from abalone injected with seawater (mock-treatment); lanes 4, 5, 6, and 7: genomic DNA isolated from 50, 25, 10, and 1 larvae, respectively, derived from abalone injected with 10 μg of pOBA–YPGHc. Arrows indicate the PCR product that has the same molecular weight as the control plasmid.

The fertilization rates of sperm from two of six abalone injected with 10 μl of 0.1 or 10 μg/μl foreign DNA were 92±0.6% and 93±0.4%, respectively. The rates were not significantly different ($P<0.05$) from the 95±0.4% fertilization rate for mock-treated abalone sperm. The hatching rates were 91±0.8% and 90±0.6%, similar to that of the mock-treatment control group at 92±0.5%.

3.2. Foreign DNA uptake by sperm

Ejected sperm (10^7 cells) were collected from individuals subjected to injection of the expression vector into the testis. Regardless of whether the foreign DNA fragments were injected into the anterior, middle, or posterior part of the abalone testis, the same 954-bp PCR product was detected and gave a positive signal in Southern blot analysis (data not shown). Gene-transfer efficiency was unaffected by injection location. For subsequent experiments, 0.1 or 10 μg/μl foreign DNA was injected in the middle of the testis. After DNase I digestion, sperm samples were extracted for PCR and Southern blot analyses. A PCR product with a molecular weight of 954-bp was generated. The same product was obtained from the positive control group, for which pOBA–YPGHc was a template (Fig. 1A; lanes 5 and 6).

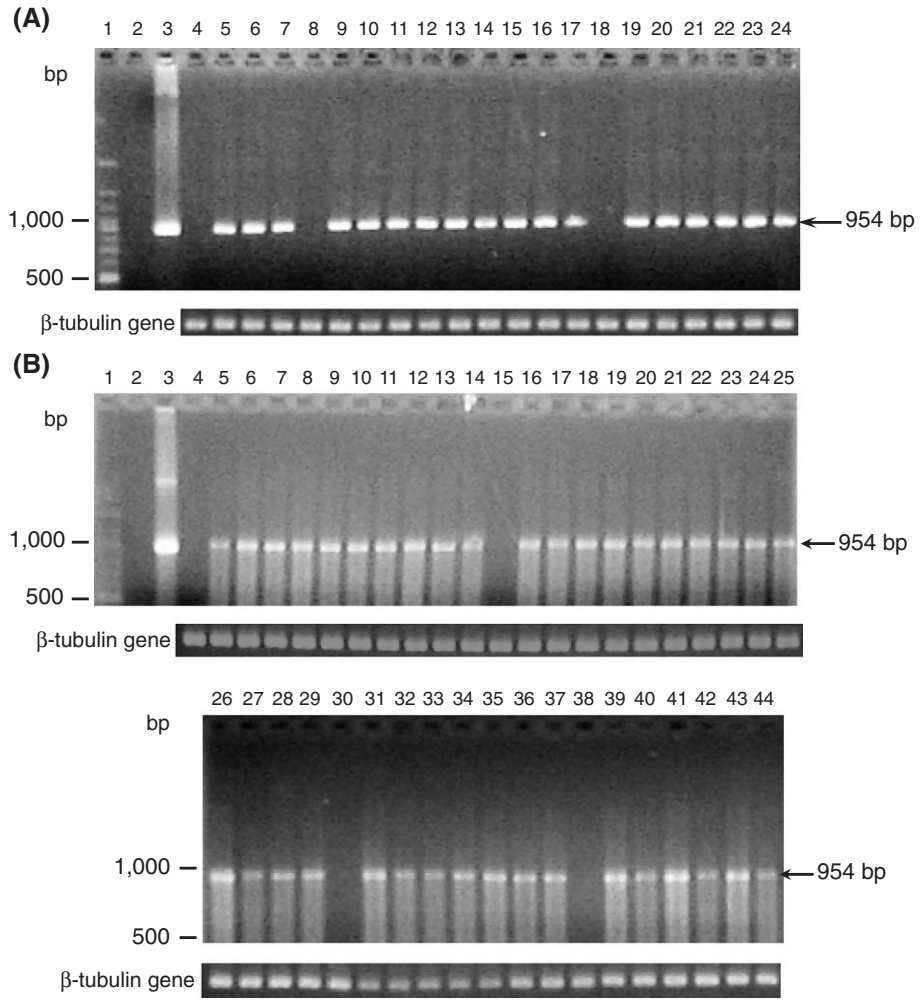

Fig. 2. Use of PCR to detect the transgene and the endogenous β-tubulin gene in the genomic DNA isolated from a trochophore-stage G_0 larva (A) and from a 3-week-old G_0 juvenile (B). Lane 1: 100-bp DNA molecular marker; lane 2: no template added (negative control); lane 3: 0.1 μg of pOBA–YPGHc as a template (positive control); lane 4: larva derived from abalone injected with filtered seawater (mock-treatment group); lanes 5–24 of (A) and lanes 5–44 of (B): derived from founders testis-injected with 10 μg of pOBA–YPGHc. The 954-bp PCR product was amplified from the transgene YPGH cDNA as indicated; whereas the 412-bp PCR product shown on the bottom of each lane was amplified from the endogenous β-tubulin gene (internal control).

The PCR signal for abalone injected with 10 μg/μl foreign DNA was stronger than for abalone injected with 0.1 μg/μl. Ten micrograms per microliter vector DNA in a total volume of 10 μl was used in the subsequent experiments.

3.3. Analysis of transgenic abalone larvae by PCR–Southern blotting

Before we could estimate the gene-transfer rate, we needed to determine how many trochophore larvae were required to have sufficient genomic DNA for analyses. We extracted the samples from 1, 10, 25, and 50 larvae in the 10 μg/μl treatment group and performed PCR–Southern blot analyses (Fig. 1B). We found that DNA extracted from only one trochophore larva was sufficient for analysis. Results of PCR–Southern blot analyses for 1, 10, and 25 larvae did not differ (Fig. 1B; lanes 5, 6 and 7), except that the signal shown in the group of 50 larvae appeared to have a higher intensity (Fig. 1B; lane 4).

We haphazardly collected 20 trochophore larvae from the 10 μg/μl treatment group and performed PCR analysis on their genomes. A single, positive band, with a molecular weight of 954-bp, was observed exclusively in the experimental group (Fig. 2A; lanes 5–24). Based on the appearance of positive signals in the PCR analysis, the rate of gene transfer to abalone larvae was 90% (18 of 20 larvae examined) using this testis-injection method.

Three weeks after hatching, we haphazardly collected 40 juvenile abalone from the experimental group treated with 10 μg/μl foreign DNA. We extracted genomic DNA from each juvenile and performed PCR analysis. A single positive band, as the positive control, with a molecular weight of 954-bp was observed (Fig. 2B; lanes 5–44). In the PCR analysis, positive signals were detected for 37 of 40 juveniles, yielding a gene-transfer rate of 92.5%.

After 1 year of culture, we haphazardly collected 10 adults from the 10 μg/μl foreign DNA-injection group. Genomic DNA was extracted from three different tissues (muscle, gonad, and others), and PCR analysis was performed. Results showed that positive signal was detected for 8 of 30 samples examined that originated from six individuals. Thus, the gene-transfer rate should be around 60% (6 of 10 abalone). Furthermore, when we detected the transgene in the gonad specifically, gonad cells were isolated directly from the gonad of the 2-year-old G_0 abalone and analyzed by PCR. Results showed that 31% (4 of 13 males) and 33% (4 of 12 females) of the examined abalone were positive (Fig. 3), suggesting the transgene was carried by the gametes of G_0 abalone. After genomic DNA from these eight PCR-positive samples was digested with *Nsi*I (data not shown) and Southern blot analysis was performed, we found positive band(s) with a molecular mass higher than that of the transferred DNA, 8 kb (Fig. 4; lanes 4–7, 9, and 10), indicating that the transgene was integrated in the host genome. But we noticed that some positive bands

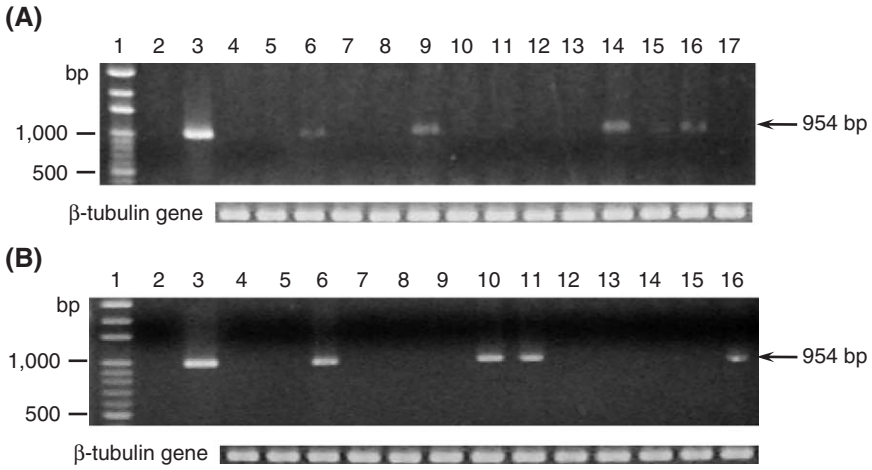

Fig. 3. Use of PCR to detect the transgene DNA fragment in gonad cells of G_0 mature abalone. Gonad cells from testis (A) and from ovary (B) were isolated by microsyringe from the gonad of G_0 2-year-old abalone derived from founder testis-injected with 10 μg of pOBA–YPGHc. Lane 1: 100-bp DNA molecular marker; lane 2; no template added (negative control); lane 3: 0.1 μg of pOBA–YPGHc as a template (positive control); lane 4: abalone from mock-treated founder; lanes 5–17 of (A), and lanes 5–16 of (B): individuals from DNA injected founders. The 954-bp PCR product was amplified from the transgene YPGH cDNA as indicated, whereas the 412-bp shown on the bottom was amplified from the endogenous β-tubulin gene (internal control).

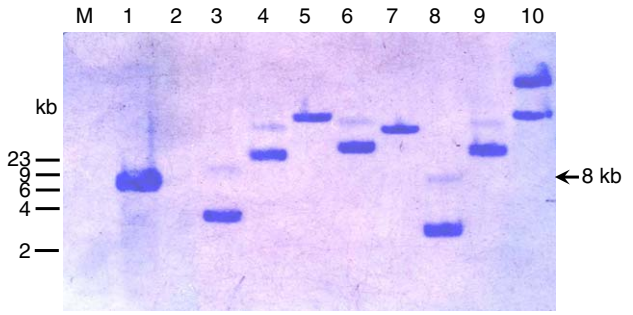

Fig. 4. Southern blot analysis of genomic DNA extracted from 1-year-old abalone derived from founders that were injected with pOBA–YPGHc into the testis. Southern blot analysis was performed by using a digoxigenin-labeled YPGHc as a probe for hybridization. Lane 1: plasmid pOBA–YPGHc used for gene transfer (positive control); lane 2: untreated abalone (negative control); lanes 3–6: muscle from PCR-positive abalone; lanes 7–9: gill and digestive tissues from PCR-positive abalone; lane 10: gonad of PCR-positive abalone. The arrow indicates the molecular size of the linearized transgene (8.0 kb) fragment.

appeared smaller than 8 kb (Fig. 4; lanes 3 and 8), suggesting these DNA fragments may be deleted partially.

3.4. Growth performance of abalone after gene transfer

As shown in Table 1, there was not much difference between the control group and the DNA-injected group in term of density when they were cultured outdoors in the hatchery ponds for 1 week, 6 months, and 1 year. We randomly collected 100 individuals from each group and found that shell lengths and body weights of abalone from the DNA-injected group were significantly greater than those of abalone from the untreated group at the same stage (Table 1): the DNA-injected group showed an increase ($P<0.05$) in weight approximately 72.2% and 81.8% relative to the control group at 6 months and 1 year, respectively.

4. Discussion

Genes have been transferred into abalone by electroporating fertilized eggs (Powers et al., 1995) and sperm (Tsai et al., 1997). However, electroporation requires an expensive electroporator. Processing abalone gametes and performing in vitro fertilization are laborious and time-consuming. In this study, we developed a direct testis-injection system, which is a one-shot, minimally invasive, and high-throughput technique for transferring DNA fragments into the testis of abalone. Neither the instruments of gene transfer nor the preparations of gametes or fertilized eggs are required. The greatest advantage of using direct testis injection is that the gonads of abalone are exposed when one holds the shell. Unlike bivalve mollusks, with abalone, we do not have to force the shells open mechanically. In addition, we found that the fertilization and hatching rates of the experimental and mock-treatment control groups were not significantly different ($P<0.05$). Therefore, direct injection of DNA fragments in abalone is easy and minimally invasive.

Gene-transfer efficiency is the percentage of embryos, larvae, juveniles, or adults that possess the DNA fragment among the samples examined after treatment. Gene-transfer rates vary, depending on

Table 1
Comparison of abalone growth between the gene-transfer (injected) group and the control (untreated) group

		Age		
		1 week	6 months	1 year
Density (individuals/m^2)	Untreated	$3.9\pm0.8\times10^{5,a}$	$2.7\pm0.5\times10^{2,a}$	$0.8\pm0.2\times10^{2,a}$
	Injected	$4.0\pm1.1\times10^{5,a}$	$2.8\pm0.7\times10^{2,a}$	$0.9\pm0.3\times10^{2,a}$
Length (cm)	Untreated	0.24 ± 0.01^{b}	1.5 ± 0.13^{b}	3.3 ± 0.21^{b}
	Injected	0.23 ± 0.01^{b}	2.2 ± 0.22^{c}	4.2 ± 0.34^{c}
Weight (g)	Untreated	–	2.2 ± 0.32^{d}	4.3 ± 0.47^{d}
	Injected	–	3.8 ± 0.23^{e}	7.8 ± 0.72^{e}

The presented data are means±SD, which in the same column sharing a common superscript letter are not significantly different ($P<0.05$). Dashes indicate that the animal was too small to weigh.

animal species and transfer method. In shellfishes, gene-transfer rates were 72% using electroporation in red abalone embryos (Powers et al., 1995), 2% to 5% using retroviral infection in dwarf surf clams (Lu et al., 1996), 40% with particle bombardment of the Pacific oyster (Cadoret et al., 1997a), 65% with electroporation of abalone sperm (Tsai et al., 1997), 48% with by microinjection and electroporation, and 20% using chemical-mediated transfection in Eastern oyster embryos, respectively (Cadoret et al., 1997b; Buchanan et al., 2001). In this study, we used PCR–Southern analysis of the genomic DNA extracted from sperm after incubation with DNase I, which enabled the digestion of DNA that remained outside the sperm without entering. A 954-bp PCR–Southern-positive finding was the basis determining the gene-transfer efficiency. The gene-transfer efficiency was not affected by what part of the testis received the injection of foreign DNA. However, a higher gene-transfer rate was obtained using a higher concentration of DNA fragments. Interestingly, the gene-transfer efficiencies we found in the 1-week trochophore larvae, 3-week-old juveniles, and 1-year-old adults were 90%, 92.5%, and 60%, respectively. Our findings are consistent with those from studies of testis injection in other animal species, including DNA injection into the vas deferens (Huguet and Esponda, 1998) and testis (Sato et al., 2002) of mouse, the testis of silkworm (Shamila and Mathavan, 1998), and the spermatophore of giant freshwater prawn (Li and Tsai, 2000). Moreover, we find that the transgene is detected in the gonad of around 20% (60% × 31–33% = 18–19%) of surviving mature abalone of G_0 derived from the DNA-injection group, suggesting that the gene transmission is easier to generate, compared to the conventional gene transfer. In addition, it is not necessary to culture the treated G_0 individuals to adulthood before the transgenic founders able to transmit the transgene to offspring are identified. Therefore, we believe the direct testis-injection technique reported in this study is the highest efficiency and the most convenient of gene-transfer techniques to date, although the germ-line transmission is needed for further confirmation.

Almost all transgenes present in extra-chromosomal form in transgenic animals should exhibit mosaicism and cannot generate germ-line transmission. Many attempts are reported to improve the genomic integration of the transgene in the founder individuals, such as protamine sulfate (Lechardeur et al., 1999), DNA mixed with nuclear transport peptide (Sebestyen et al., 1998; Liang et al., 2000), and DNA flanked with inverted terminal repeats from adeno-associated viruses (Fu et al., 1998; Hsiao et al., 2001). These methods do improve the transgene integration and expression in the transgenic fish. However, tedious work is needed to prepare either chemical reagents or gene constructs. Surprisingly, in this study, we find that even naked DNA fragments delivered by a direct-testis injection still have a great chance to integrate into the genome of abalone, which was demonstrated by Southern blot analysis of genomic DNA (Fig. 4). Moreover, we can find positive hybridization bands with molecular weights that are higher than that of the transferred gene in the gonad of founder individuals. Non-homologous re-combination end-joining of the transgene, such as rearrangements, partial deletions, and terminal modifications, was frequent after gene transfer both in cell lines (Nakai et al., 1999) and in transgenic animals (Hsiao et al., 2001; Chou et al., 2001). In this study, our results suggested similar modifications of the transgene in the transgenic abalone.

We randomly collected 100 individuals from the DNA-injected group and found that both shell lengths and body weights of abalone were significantly higher than those of abalone from the untreated group at the same stage (Table 1). Although the mechanism of how fish growth hormone affects the growth of abalone is unclear, this evidence suggests that the transgene might be expressed and functioned, resulting in growth enhancement of transgenic abalone. Using RT-PCR to detect the YPGH mRNA or using Western blot to detect the YPGH protein in the transgenic abalone remains to further study.

Most sperm-mediated gene transfers have been performed in mammals, including mice (Spadafora, 1998; Perry et al., 1999), pigs (Farre et al., 1999), bovids (Gagne et al., 1991), and humans (Spadafora, 1998); and other vertebrates, including amphibians (Takac et al., 1998) and zebrafish (Khoo et al., 1992). Also, sperm-mediated gene transfer has been studied in invertebrates, including insects (Atkinson et al., 1991; Shamila and Mathavan, 1998), abalone (Tsai et al., 1997), and mussel (Kuznetsov et al., 2001). The interaction between the living spermatozoa and naked DNA fragments has been reviewed by Spadafora (1998), Gandofi (1998), and Sato et al. (2002). It is thought that the interaction between sperm cells and foreign DNA from a variety of species is regulated by specific protein factors (Lavitrano et al., 1997; Perry et al., 1999). DNA binding proteins on the membrane of spermatozoa reportedly direct the internalization of naked DNA (Zani et al., 1995; Lavitrano et al., 1997; Shamila and Mathavan, 2000). The molecular mass of the binding protein is 30 to 35 kDa in mammals (Lavitrano et al., 1992; Zani et al., 1995), and 26 to 50 kDa in silkworm (Shamila and

Mathavan, 2000). The ability of animal spermatozoa to easily incorporate foreign DNA fragments may be attributed to the absence of a normal nuclear envelope (Baccetti and Afzelius, 1976) or to the character of the nuclear sperm constituents, which are different from those of the typical eukaryotic cell (Balhorn, 1982; Bedford and Hoskins, 1990). For shellfishes, the molecular mechanisms modulating the interaction between the sperm cells and foreign DNA and the internalization of the DNA are not yet understood. Nevertheless, the reason that testis injection in this study causes a high occurrence of gene transfer in abalone is the most interesting issue to be addressed. We speculate that the incubation for 3–6 h after injection greatly facilitates the entry of foreign DNA into abalone sperm. Sato et al. (2002) demonstrated that the longer the foreign plasmids remain in the spermatophore and testis, the greater the probability they will enter sperm. Further research is needed on factors affecting gene-transfer rate, including any special characteristics of abalone testis, the biology of abalone sperm, and their associated proteins.

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