ELSEVIER

INTRODUCING FOREIGN DNA INTO TIGER SHRIMP (Penaeus monodon) BY ELECTROPORATION

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ABSTRACT

Electroporation was used to introduce pFLAG-CMV-1-BAP, a DNA fragment that includes a bacterial alkaline phosphatase gene driven by a human cytomegalovirus (CMV) promoter, into Penaeus monodon zygotes. The transgenic tiger shrimp was achieved by using 10kV, 2^8 pulses, 120 μsec pulse time, 10 cycles, and a DNA concentration of 37.5 μg/mL. The hatching rate of electroporated zygotes (46%) was significantly lower than that of zygotes in the untreated group (89%). The survival rate of postlarvae in the electroporated group using a DNA concentration of 37.5 μg/mL decreased from 0.6% for postlarva 45 to 0.4 % for postlarva 120. Based on dot blot analysis, the rate of gene transfer was 37% in mysis-stage, 23% postlarva 15(PL15), 19% postlarva 45(PL45), and 21% 4-month-old (about PL120). Genomic Southern blotting demonstrated that DNA from transgenic tiger shrimp contained fragments of exogenous DNA that were smaller, larger and of the same molecular size as pFLAG-CMV-1-BAP. Transferred DNA fragments were integrated into the genomes of 31% of the transgenic tiger shrimp. The exogenous DNA was mosaically distributed in a wide variety of tissues. Immunohistochemical staining revealed that the FLAG-BAP fused-protein encoded by pFLAG-CMV-1-BAP was present in the ovaries of some transgenic tiger shrimp.

Key words: electroporation, Penaeid shrimp, zygote, gene-transfer and fused-protein

INTRODUCTION

In the past 30 years, the shrimp-culture industry has grown rapidly and so too has the number of pathogens of penaeid shrimp. It has become increasingly important to identify shrimp pathogens. Only six viruses were known to infect shrimp in 1988, but that number climbed to 11 in 1993, 14 in 1996, and more than 20 in 1998 (11). Several species of bacteria also have been associated with shrimp mortality. Lightner et al. (10) found that Vibrio alginolyticus and V. anguillarum were the etiological agents of shrimp diseases. Song et al. (14,15,16) isolated V. damselae, V. harveyi and V. vulnificus from diseased shrimp. Lavilla-Pitogo et al. (9) determined that infection by V. harveyi and V. splendidus occurred via the gastro-intestinal tract. Thus far, at least 15 Vibrio species have been associated with shrimp diseases.

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Genetic transformation has been used to confer disease resistance in insects (2,3), and may provide a fast, feasible method for producing disease-resistant strains of economically important shrimp. Many disease-resistant and immunno-modulation genes have been cloned. There have been few studies of gene transfer in invertebrates, especially shrimp. Powers et al. (12) electroporated zygotes of the red abalone (Haliotis rufescens) with a recombinant plasmid containing a Drosophila β-actin promoter coupled to a β-galactosidase cassette. The vector was integrated in head-to-tail concatamers in the abalone genome. Tsai et al. (18) introduced a DNA fragment into the oocyte of Japanese abalone (H. diversicolor) using electroporated-sperm as a carrier. The reporter gene integrated into the genome of transgenic abalone and was expressed in specific tissues. Gendreau et al. (5) showed that Artemia franciscana (Crustacea) embryos that survived bombardment with microparticles coated with a luciferase reporter gene expressed luciferase. Cabrera et al. (1) microinjected the lacZ reporter gene under the control of SV40 early promoter into Penaeus schmitti zygotes. Thirty percent of the embryos in the injected group developed to the nauplius stage and one nauplius expressed the exogenous gene. Gomez-Chiarri et al. (6) microinjected and electroporated P. schmitti zygotes with an expression vector containing SV40 early promoter coupled to the gene coding for β-galactosidase. Microinjected embryos were not analyzed either by Southern blot or by immunoistochemistry for the transferred gene. From 25 to 30% of microinjected embryos reached the nauplius stage and 10% of the nauplii showed β-galactosidase expression. Of the electroporated zygotes, 16% reached the nauplius stage and 4% of these nauplii exhibited β-galactosidase expression. However, none of these methods of gene-transfer have been able to efficiently transfer exogenous genes to large numbers of zygotes. The efficiency of gene transfer still needs to be improved.

To effect mass transfection of foreign DNA fragments into shrimp zygotes, we used the reporter gene (pFLAG-CMV-1-BAP) to determine the optimum parameters for electroporation (Baekonization) of the zygotes of tiger shrimp (P. monodon). Hatching and survival rates of larvae were evaluated at each developmental stage. Dot and Southern blotting were used to detect the presence of transferred DNA fragments in shrimp larvae and adults. Dot blot was used to determine the distribution of the reporter gene (pFLAG-CMV-1-BAP) in different tissues. Expression of the transferred gene also was detected by immunohistochemical staining, using a specific monoclonal antibody as a probe.

MATERIALS AND METHODS

Experimental Animals

Wild tiger shrimp (P. monodon) spawners (25-27 cm long) were captured and exported from Malaysia. In the nursing tank, spawning was induced by spermatophore transplantation and eyestalk ablation. The zygotes were collected 30 min after spawning. The zygotes were washed 2 to 4 times with buffer A (2x: 16g NaCl; 0.4g KCl; 0.18g $NaHCO_3$; 0.14g NaH_2PO_4 with DW dilute 500mL) at room temperature to remove their jelly coat. This was necessary to prevent DNA molecules from sticking in the jelly coat, which would prevent the DNA from entering the zygote during electroporation.

Survival of Two-celled Embryos and Larvae

The number of zygotes that divided into two-celled embryos was counted 60 min after spawning at 30℃ in a photo taken by an anatomic microscope (about 250 zygotes per photo, 6 photos counted). To determine the hatching rate of zygotes, we counted the number of nauplii(I)

in 50 mL (7 to 9 replicates) taken from a fully aerated 200 L hatching pool, 15 hours after spawning at 30℃. The number of surviving zoeae, myses, and postlarvae 15 (PL15) were counted 4, 8 and 27 days after spawning, respectively.

Transferred DNA

A 6.2-kb plasmid, pFLAG-CMV-1-BAP (Kodak Scientific Imaging System), consisting of a bacterial alkaline phosphatase gene driven by a human cytomegalovirus (CMV-1) promoter, was prepared by following the manufacturer's recommendation (QIAGEN endo-free plasmid maxi kit).

Electroporation

We used a non-contact mode Baekon 2000 (Baekon Inc., Fremont, CA, USA) electroporator. The following electroporation parameters were varied: voltages 0, 3, 5, 7, 9, 10 and 10.5 kV; cycles 10; volume 100 μL. The constant parameters were: number of pulses per cycle (Np) 2^8; pulse time (Tp) 120 μsec; burst time (Tb) 1.6 sec and electrode distance 1 mm. We wanted to determine the effect of voltages (5, 10kV), cycles (10, 20) and volume of solution (100 μL, 1mL) on hatching success. Thirty minutes after spawning, 366 zygotes in 100 μL of solution and 10141 zygotes in 1mL of solution were electroporated. Each solution was contained in a 1-mL cuvette. We suspended 9.4, 18.8, 37.5, 75 or 150 μg of pFLAG-CMV-1-BAP and zygotes in 1mL solution. The statistical significance of experimental data was determined by Duncans' test.

Genomic DNA Extraction

During inter-molting periods, genomic DNA was extracted (DNA extraction kit, Acugen Asia) from whole myses, the first or second pleopod of postlarvae (PL45) and the eyestalk, hepatopancreas, intestine, muscle, pereiopod, pleopod, haemolymph and telson of 4-month-old tiger shrimp (about PL120). DNA for gene-transfer was used as a positive control and DNA extracted from spawner or mock-treated tiger shrimp was used as a negative control. Extracted DNA was suspended in sterilized nanopure water at a concentration of 3 ng/ μL.

Dot and Southern Blot Analyses

Genomic DNA (150ng) from myses, PL15 and 4-month-old tiger shrimp was spotted on a nylon membrane (MSI, Westbough, MA, USA). After air-drying, the genomic DNA was cross-linked to the nylon membrane by UV irradiation and then hybridized to a digoxigenin-labeled probe, using a 6.2-kb HindIII-digested plasmid prepared by the random primer method (4). For Southern blot analysis (13), 42 samples of genomic DNA (extracted from the second pleopod of 4-month-old, presumptive transgenic individuals) were digested with HindIII, electrophoresed on 0.8% agarose gel, transferred to a nylon membrane, and then hybridized to an αP^{32}- labeled probe. Prehybridization was carried out at 42℃, overnight, in a solution of 50% formamide, 5x Dehart's solution, 6x SSC, and 0.1% SDS (1x SSC: 0.15 M NaCl and 15mM sodium citrate). Hybridization was carried out at 42℃, overnight, in a prehybridization buffer containing a denatured probe. Prior to autoradiography, membranes were washed twice (30 min each) in a solution containing 4x SSC and 0.1%SDS at room temperature, twice in a solution of 2x SSC and 0.1% SDS at 37℃, and twice in a solution of 1x SSC and 0.1% SDS at 68℃.

Immunohistochemistry

The ovaries of an 11-month-old transgenic tiger shrimp were fixed in Davidson's fixative and embedded in paraffin. Tissue sections (4 μm thick) were cut, deparaffinized and rehydrated. They were incubated for 10 min with 0.02N HCl. Tissue sections were then preincubated for 10 min with 1X blocking solution (Boehringer Mannheim Indianapolis, IN, USA), and incubated for 1 hr at room temperature with mouse monoclonal antibodies specific to FLAG (Kodak, New Haven, CT, USA). The sections then were incubated for 30 min at room temperature with biotinylated goat anti-mouse antibody (Vector, Burlingame, CA, USA). Finally, they were incubated with streptavidin conjugated with alkaline phosphatase complex. The color was developed by adding the substrates, nitroblue tetrazolium and 5'-bromo, 4-chloro, 3-indole phosphate (Boehringer Mannheim) (17), to produce purple and insoluble precipitates. The sections were counterstained with Bismark brown (Sigma, Louis, MO, USA), mounted in a coverslip, and photographed with light microscope.

RESULTS

Effect of Electroporation on Two-celled Embryo Survival and Hatching

Survival of mock-treated, 2-celled embryos declined from 87 to 72% as the voltage increased from 0 to 5 kV. However, survival did not decline further at voltages from 7 to 10.5 kV. In the untreated group, 7% of the early embryos were wrinkled and disrupted and 6% failed to undergo normal development at the first cleavage. In the mock-treated group, 15 to 31% of the embryos were wrinkled and disrupted and the development of 5 to 23% was arrested in the zygote stage (Figure 1).

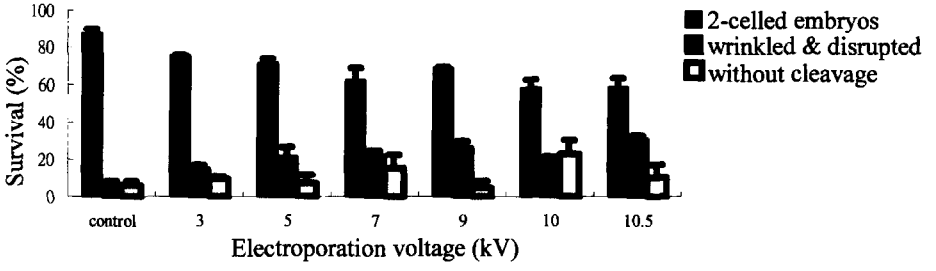

Figure 1.Effect of voltages on the survival of 2-celled Penaeus monodon embryos (n=6) following mock-treatment. Untreated, zygotes were used as a control. Other electroporation parameters were: cycles, 10; Np, 2^8; Tp, 120 μsec; Tb, 1.6 sec; D, 1mm. The bar indicates the standard deviation.

The hatching rates of untreated group in 100 μL and 1mL of solution were 83% and 81%, respectively. The hatching rates of mock-treated group as zygotes in a volume of 100 μL were 51% (5kV, 10 cycles), 41% (5kV, 20 cycles), 37% (10kV, 10 cycles) and 27% (10kV, 20 cycles). The hatching rates of mock-treated group as zygotes in a volume of 1 mL were 80% (5kV, 10cycles), 66% (5kV, 20cycles), 73% (10kV, 10cycles) and 62% (10kV, 20cycles). The greater the volume that zygotes were suspended in, the higher the hatching rate, indicating that a larger volume protects the zygotes against voltages. However, the hatching rates decreased significantly

as the cycles and voltages increased (Table 1). The hatching rate of groups treated at 10 kV for 10 cycles and at 5kV for 20 cycles in a volume of 100 μL did not differ significantly (P<0.01). To maximize the hatching rates in subsequent experiments, zygotes were electroporated at 10kV for 10 cycles in a volume of 1mL.

Table 1. Effect of voltages and the number of cycles on the hatching success of tiger shrimp that were mock-electroporated as zygotes.

Cycle	Percentage of Hatched Nauplii (mean±SD)					
	Control		5 kV		10 kV	
	100 μL	1 mL	100 μL	1 mL	100 μL	1 mL
0	83.8±6.7[a]	81.02±5.3[a] (n=7)	ND	ND	ND	ND
10	ND	ND	50.85±4.0[b]	80.01±2.2[a] (n=8)	37.3±7.6[c]	73.2±3.0[f] (n=8)
20	ND	ND	41.2±7.2[c]	65.9±4.1[e] (n=9)	26.9±5.7[d]	61.6±4.1[e] (n=9)

Hatched nauplii were counted 15 hours after spawning at 30℃.
The number of replicates is in parentheses.
Electroporation parameters: Tb, 1.6 sec; Np, 2^8; Tp, 120 μsec; D, 1 mm; V, 100 μL or 1mL.
Hatching success was statistically analyzed using Duncan's multiple range tests. Numbers followed by the same superscripts are not significantly different (P<0.01).

Table 2. Effect of DNA concentration and electroporation on hatching.

pFLAG-CMV-1-BAP (μg)	No. of repeated trials	Zygotes	Hatching rate (%)
Untreated	3	10500± 447	80.8± 1.3[a]
Mock-treated	5	10141± 619	40.4± 4.7[b]
9.4	3	9476± 490	38.4± 8.8[b]
18.8	3	9935± 369	29.0± 17.3[bc]
37.5	2	10318± 0	18.3± 1.0[cd]
75	6	10125± 150	7.5± 1.8[d]
150	5	10086± 130	2.8± 0.7[d]

Electroporation parameters: kV, 10; cycles, 10; Np, 2^8; Tb, 1.6sec; Tp, 120 μsec; D, 1 mm; V, 1mL. Hatching success was statistically analyzed using Duncan's multiple range tests. Numbers followed by the same superscripts are not significantly different (P<0.01).

Effect of DNA Concentration on the Hatching

The hatching rate decreased from 40 to 3% as the concentration of DNA was increased from 0 to 150 μg, and was significantly lower (P<0.01) when the concentration of DNA was greater than 37.5 μg (Table 2). Because the foreign gene might inhibit embryogenesis only in shrimp embryos (P. schmitti) that were electroporated and used parameters that yielded lower survivorship (6), we used 37.5 μg DNA in all subsequent experiments. The elder the larvae, the lower their survival following electroporation (Figure. 2). However, the survival rates (12 trials) were 0.6±0.9% in 0. 9%in PL15 being electrporated in the presence of the foreign DNAtr 0.59% in PL45 (718/121692), and 0.44% in PL120 (532/121692). The hatching and survival rates of tiger shrimp that were electroporated and received foreign DNA were similar to those of mock-treated tiger shrimp and much lower than those of untreated tiger shrimp, indicating that electroporation was much more damaging than the toxicity of foreign DNA in the embryo stage.

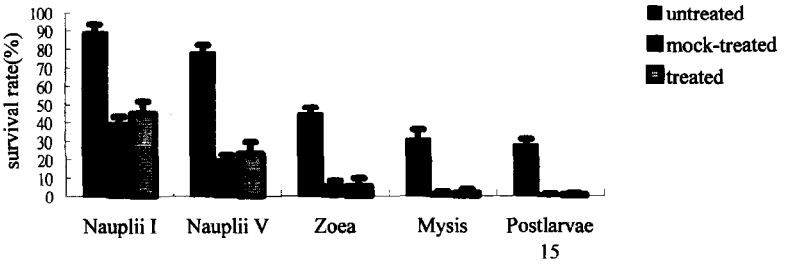

Figure 2. Effect of electroporation with and without the presence of foreign DNA on hatching and larval survival. The zygotes from which nauplii, zoeae, myses and postlarvae 15 developed were untreated, mock-treated or treated with 37.5 μg DNA. Electroporation parameters: kV, 10; cycles, 10; Np, 2^8; Tb, 1.6sec; Tp, 120 μsec; D, 1 mm. The bar indicates the standard deviation.

Analysis of Transgenic Tiger Shrimp by Dot and Southern Blotting

The success rate for gene transfer was 37% in myses (68 positives in 182 DNA samples) (Figure. 3A), 23% in PL15 (27 of 116) (Figure. 3B), 19% in PL 45 (10 of 54 DNA samples extracted from the second pleopods) and 21% in 4-month-old tiger shrimp (10 of 48).

Genomic DNA was extracted from the second pleopods of PL45 and 4-month-old presumptive transgenic individuals. The DNA was digested with HindIII, run on agarose gel, and analyzed by Southern blotting (Figure. 4). Positive reactions were obtained from 41% (17 of 42) of the samples. Of these, three of the 17 transgenic tiger shrimp (18%) exhibited a 6.2-kb positive band of the same size as the transgene (Figure. 4, lanes 15, 22 and 32). However bands of other sizes were observed. These included bands of 23 and 6.2-kb (Figure. 4, lanes 3, 14, 21 and 40), 23, 4.5 and 2.5-kb (Figure. 4, lanes 2 and 20), 23, 5 and 4-kb (Figure. 4, lane 17), 23, 6.2 and 4-kb (Figure. 4, lane 18), 23, 6.2-kb the smear (Figure. 4, lane 26), 6.6 and 5-kb (Figure. 4, lane 35), 6.6-kb (Figure. 4, lanes 36 and 37), 4.5-kb (Figure. 4, lane 25) and the smear (Figure. 4, lane 19) were observed. Based on the positive bands with MW greater than that of the transgene, the transgene was integrated into the genome of 31% (13 of 42) of the transgenic tiger shrimp.

Mosaicism

To determine the distribution of foreign DNA in individual tiger shrimp, genomic DNAs from a variety of tissues from 12, 4-month-old, presumptive transgenic tiger shrimp were subjected to dot blot analysis (Table 3). Foreign DNA was detected in each tiger shrimp in at least one type of tissue, but not all tissues from the same individual tested positive (Figure 3C). Thus, foreign DNA in transgenic tiger shrimp had a mosaic distribution. A tiger shrimp that was transgene-negative in its pleopods could be transgene-positive in other tissues. The real rate of gene transfer in tiger shrimp might be higher than our results indicate because only the pleopods were examined in this study.

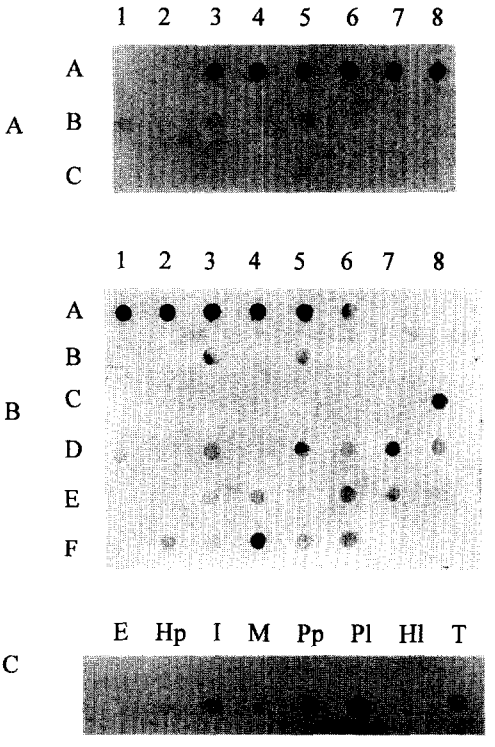

Figure 3. A: Dot blot analyzed genomic DNA extracted from mysis stage shrimp electroporated (B1 through B8). The DNA in A1 & A2 come from myses that developed from the mock-electroporated zygotes and served as negative controls. Wells A3 to A8 are standards containing 0.15, 0.31, 0.62, 1.25, 2.5 and 5ng DNA, respectively. B1, B3, B4, B5, B6, C4 and C5 were positive for transgenic DNA.

B: Dot blot analysis of genomic DNA isolated from the first pleopod of postlarvae 15 shrimp. Wells A1 to A6 are standards containing 5, 2.5, 1.25, 0.62, 0.31 and 0.15 ng DNA, respectively. A7 and A8 contain DNA from spawners and served as negative controls. B1 to F8 contain DNA from presumptive transgenic individuals.

C: Dot blot analysis of DNA isolated from different tissues of 4-month old shrimp, E: eyestalk, Hp: hepatopancreas, I: intestine, M: muscle, Pp: pereiopod, Pl: pleopod, Hl: haemolymph, T: telson.

Table 3. Dot blot analysis of genomic DNA extracted from a variety of tissues from 4-month-old tiger shrimp

Tissue	Individual												Ratio of positive individuals
	1	2	3	4	5	6	7	8	9	10	11	12	
Pleopod	+	+	+	+	+	+	-	+	+	-	-	+	9/12
Pereiopod	+	+	+	-	+	-	-	-	+	+	+	+	8/12
Intestine	+	+	+	+	+	-	+	-	+	-	-	-	7/12
Hepatopancreas	+	+	+	-	-	-	+	+	-	+	-	-	6/12
Muscle	+	-	+	+	-	-	-	-	+	+	-	-	5/12
Telson	+	+	-	+	-	-	+	-	-	-	-	+	5/12
Hemolymph	-	-	+	-	-	+	-	-	-	-	+	+	4/12
Eyestalk	-	-	-	+	-	-	-	-	-	-	-	-	1/12

Genomic DNA was prepared from tiger shrimp in which the first pleopod DNA was dot using DIG-labeled 6.2-kb probe, a positive dot shown +, no dot shown -.

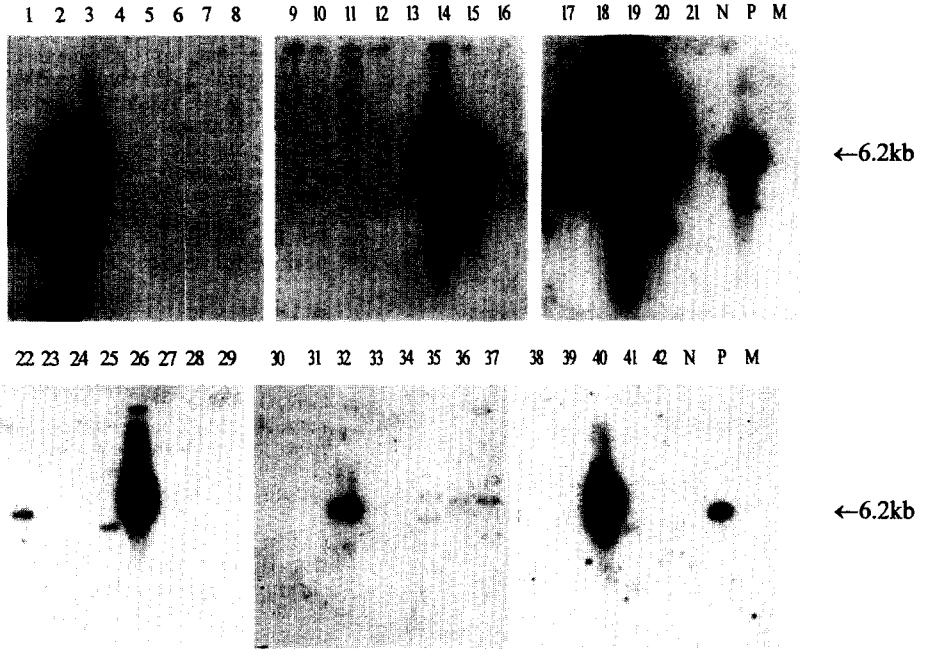

Figure 4. Southern blot analysis of genomic DNA isolated from the second pleopod of 4-month-old, presumptive transgenic individuals. DNA was digested with HindIII at 37℃ for 2.5 hours. DNA from the second pleopod of presumptive transgenic individuals is in lanes 1 to 42. N: DNA from the mock-treated tiger shrimp served as a negative control; P: DNA used for gene transfer was the positive control; M: molecular marker λ HindIII. Arrowhead: the molecular size of the transgene (6.2-kb)

Figure 5. Immunohistochemical staining to detect the protein expressed by the transferred gene. A DNA fragment encoding a FLAG-BAP fusion protein was transferred into tiger shrimp. The ovaries of one 11-month-old mock-treated tiger shrimp (left) and one, 11-month old transgenic tiger shrimp (right) were detected by a specific FLAG monoclonal antibody. Positive signals are blue-purple in color. A, toward the anterior region of the tiger shrimp, D, toward the dorsal region of the tiger shrimp; Cnf, connective fibrous tissue; Ooc, oocytes; Zop, zone of proliferation. Scale bar: 50 μ m.

Expression of the Transferred Gene in Tiger Shrimp

We examined the expression of the FLAG-BAP fusion protein encoded by pFLAG-CMV-1-BAP in the ovaries of an 11-month-old transgenic tiger shrimp. Positive signals with blue-purple precipitates were present in the nucleus of oocytes and follicle cells (Figure 5). The FLAG-BAP fusion protein was predominately expressed in follicle cells surrounding oocytes. However, these relatively light signals also were detected in muscle, neurosecretory cells, granular cytoplasm and partial oocyte yolk of the other transgenic tiger shrimp (data not shown). Some, but not all, germ cells of this positive animal expressed the transgene.

DISCUSSION

Among the techniques used for artificial reproduction of prawn and shrimp, the one best developed and most widely applied is the artificial transplantation of spermatophores into the thelycum of newly molted females. However, it is not the case for penaeid shrimp because spawners reject spermatophores. In addition, penaeid shrimp zygotes that were artificially fertilized in vitro had the low hatching rates. Thus, electroporation for zygotes provides advantages in gene-transfer for penaeid shrimp due to its simplicity and convenience. Electroporation technology has been reported that it is an efficient method for introducing foreign molecules into the zygotes of sea urchins (8) and abalone (12), into the sperm cells of loach (19) and small abalone (18).

In this study, the higher the voltage used for electroporation, the lower the survival rate of two-celled embryos. The survival rate of the two-celled embryos of the mock-treated group was lower than the untreated group. In the mock-treated group, the survival rate of the two-celled embryos did not decline further at voltages from 7kV to 10.5kV. Compared to fish oocytes, shrimp zygotes and small abalone sperm, which were resistant at 10kV, are tolerant of higher voltage (18). Whether this tolerance is related to some structural characteristic of tiger shrimp zygotes remains to be studied. It is well known that high voltages physically disrupt cell and organelle membranes, leading to low hatching and survival rates. It was affected similarly that the electroporation was done between the survival rate of the shrimp zygotes and cultured cell (20).

Hatching rates of shrimp embryos electroporated in the volume of 100 μ L were significantly ($p<0.01$) lower than that of the untreated group, and hatching rates decreased as the voltages and cycles were increased. Interestingly, the hatching rates were close between untreated group and the mock-treated (5 kV, 10 cycles) if they were treated in the volume of 1mL. However, the hatching rates were significantly different between embryos treated in the volume of 100 μ L and 1mL, suggested that the hatching rate of the electroporated zygotes was affected by the volume. It was observed that the electroporation in 100 μ L was more detrimental to zygotes than in 1mL. The greater the volume, the higher the tolerance of high voltages.

The hatching rate of electroporated zygotes using a DNA concentration of 37.5 μ g was lower than that of the mock-treated zygotes, which was significantly different. The hatching rate of electroporated zygotes using a DNA concentration of 37.5 μ g was significantly lower than that of the 18.8 μ g, although it was not significantly different due to limited number of observations. Therefore, we statistically analyzed the hatching rate of electroporated zygotes

using 9.4, 18.8, 37.5, 75 or 150 μ g of DNA, showed that the hatching rate decreased as the DNA concentration increased. Too high DNA concentrations might lead to abnormal transgenic larvae and cause low survivorship. Gomez-Chiarri et al. (6) reported that expression was observed only in embryos that were electroporated under conditions that caused low survivorship. However, the higher voltages increased gene transfer in the presence of low DNA concentrations. The gene-transfer rate also could be improved by using a low voltage and a high concentration of foreign DNA. There was no significant difference in the hatching rate of electroporated zygotes exposed to 9.4 or 18.8 μ g DNA, or to 75 or 150 μ g DNA. But the hatching rate of electroporated zygotes using 9.4 or 18.8 μ g DNA was significantly higher than that of electroporated zygotes using 75 or 150 μ g DNA. Although the hatching rate of electroporated zygotes using 37.5 μ g DNA was lower than the electroporated zygotes using 18.8 μ g DNA, we preferred to use 37.5 μ g DNA for gene transfer because it was acceptable in terms of facilitating gene transfer success. The survival rate calculated as number of the PL45, or PL120 per total number of the zygotes born was even lower. Theoretically, one spawner could yield about 3000 transgenic larvae (PL15) (1.4 million nauplii produced per spawner × 1% survival till PL15 stage × 23% gene transfer rate at PL15 stage).

After hatching, penaeid shrimp go through four molting-stages, the nauplius, zoea, mysis and postlarva, on the way to becoming tiger shrimp. Nauplii and zoeae were too small for us to extract DNA from them individually. Nevertheless genomic DNA could be extracted from a whole mysis, and from the pleopod or pereiopod of older tiger shrimp. The gene-transfer rate varied for each developmental stage. However, we think that 37% of the myses could be transgenic because genomic DNA was extracted from the whole body of an individual mysis and assayed separately. Variation in the gene-transfer rate may have resulted, in part, from the failure of some transgenic larvae to grow and metamorphose successfully. In addition, the mosaic distribution of introduced DNA probably results in false negatives when genomic DNA from only a single tissue type is assayed. Genomic DNA from the pleopod and pereiopod proved the most suitable for assays because it yielded the lowest number of false negatives. More transformants need to be analyzed to determine whether introduced DNA is correlated with integration into the P. monodon genome. We have yet to develop an easy and reliable method for screening living transgenics. We have tried to fuse a target gene with a visible reporter gene so that presumptive transgenics can be screened without sacrificing them.

Genomic DNA was extracted from the second pleopod of a presumptive transgenic tiger shrimp, digested by Hind III, and hybridized with the linear form of pFLAG-CMV-1-BAP. We observed bands of different sizes, including the 6.2-kb transgene and larger combinations of the transgene with genomic DNA. The positive bands larger than 6.2-kb suggest that the transferred gene might be integrated into the tiger tiger shrimp chromosomes. Inoue et al. (7) reported that positive bands the same size and larger than the transferred DNA occurred in transgenic medaka.

Immunohistochemical staining demonstrated that FLAG-BAP fused protein, encoded by transferred pFLAG-CMV-1-BAP, was present in some tissues, such as muscle and the ovaries. In the ovary, the FLAG-BAP fused protein was present in some, but not all, oocytes and follicle cells. Thus, this gene exhibits mosaic expression in the ovary. Dot blot analysis of a 4-month-old transgenic tiger shrimp demonstrated that transferred pFLAG-CMV-1-BAP, which was present in some tissues, had a mosaic distribution. The CMV promoter may be working in some of the tissues examined. Because the FLAG-BAP fused-protein is present in the ovary, it is possible that the foreign DNA fragment could be transmitted in the germ-line

Electroporation greatly facilitated mass transfection of tiger shrimp (P. monodon) zygotes. These developed into larvae, with relatively high rates of survival, and transformed adult tiger shrimp. This technique can be used for the genetic transformation of penaeid shrimp and makes possible the creation of varieties with disease resistance, or other desirable traits, for use in penaeid shrimp aquaculture.

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