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In Vivo Transfection of Adult Eastern Oysters *Crassostrea virginica*

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Abstract

The eastern oyster *Crassostrea virginica* provides a commercially valuable industry along the eastern and Gulf coasts of the United States. Recently this industry has been damaged by disease problems, creating an interest in the use of gene transfer (transfection) to improve disease resistance. We transfected adult oysters with two genes, red-shifted green fluorescent protein (*rsGFP*), commonly used as a reporter gene, and the lytic peptide cecropin B (*cepB*), known to have antimicrobial properties. Oysters were transfected by injecting DNA mixed with SuperFect™ reagent (Qiagen Inc.) into the adductor muscle sinus. Oysters were assigned to three groups of 15: the first was injected with *rsGFP* complexed with transfecting reagent; the second was injected with *cepB* complexed with transfecting reagent; and the third was injected with saline (control group). Hemolymph was collected at 4 and 10 d after injection. DNA was extracted for analysis by polymerase chain reaction (PCR), and hemocytes were examined by flow cytometry and fluorescence microscopy for detection of green fluorescence due to *rsGFP* expression. The *rsGFP* gene was detected by PCR in hemocytes from 14 of 15 oysters at day 4, and in 15 of 15 oysters at day 10. The *cepB* gene was detected by PCR in 12 of 15 oysters at day 4 and in 14 of 15 oysters at day 10. No oysters from the control group were positive for either gene at days 4 or 10. Green fluorescence was detected by flow cytometry at significantly higher levels ($P < 0.05$) in oysters injected with *rsGFP* than in other oysters at day 4, but not at day 10. This report indicates the ability to introduce DNA into adult eastern oysters with subsequent gene expression. Future work will involve developing these techniques for enhanced disease resistance in oysters.

The eastern oyster *Crassostrea virginica* supports a valuable commercial industry along the Atlantic and Gulf Coasts of the United States. In 1997 Louisiana oysters had a dock-side value in excess of US$51 million (Louisiana Cooperative Extension Service 1998), and in 1992 over 120,000 ha of bottom area in that state were farmed for eastern oyster production (Keithly et al. 1993). Recently, disease problems from protozoan parasites have plagued the industry (Ford and Tripp 1996; Paynter 1996). Another concern to the industry is the transfer of human pathogens (such as *Vibrio vulnificus*) from oysters to human consumers (Jackson et al. 1997). To address these problems, research is underway on selective breeding, ploidy manipulation, and gene transfer, with a long-term goal of producing disease-resistant oysters also capable of eliminating human pathogens.

To evaluate the potential for gene transfer and expression in adult oysters, two genes were tested in this study: red-shifted green fluorescent protein (*rsGFP*) and cecropin B

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(*cepB*). The use of various forms of green fluorescent protein (GFP), originally isolated from the jellyfish *Aequorea victoria,* as reporter molecules is well characterized (Chalfie et al. 1994; Higashijima et al. 1997). A modified form of the original GFP isolate, *rsGFP,* absorbs blue light at a peak of 490 nm and emits green light at a peak of 510 nm (Heim et al. 1995). Because this protein does not require any substrates, cofactors, or enzymes for fluorescence, it can be detected in living cells in real time, making it a useful indicator of successful transfection. Cecropin B is a lytic peptide from the giant silkworm moth *Hyalophora cecropia* (Xanthopoulos et al. 1988). Cecropins are a class of lytic peptides known to have antimicrobial and antiprotozoal capabilities (Hultmark et al. 1982; Gwadz et al. 1989). Previous studies have shown that lytic peptides may be useful as therapeutic agents in aquaculture for fish (Kelly et al. 1993) and shellfish (Morvan et al. 1997).

Research on gene transfer has been commonly conducted on gametes or embryonic stages of organisms, but recently there has been increased research interest in gene delivery to adult organisms. Successful gene delivery and expression in adult organisms through injection of DNA complexed with cationic lipids (Felgner et al. 1987, 1994; Gershon et al. 1993) has become a standard technique, with the majority of research confined to mice (Zhu et al. 1993; Liu et al. 1995). Although transfection has been documented by the injection of DNA only, the injection of DNA complexed to cationic lipids greatly improved transfection efficiency (Liu et al. 1995). There has been little published on the use of such techniques in commercially important aquatic species, in which work with adults has been limited to reports of injection of naked DNA into fish muscle with subsequent gene expression localized to the injection site (Rahman and Maclean 1992; Anderson et al. 1996). Although *in vivo* gene delivery with cationic lipids has become relatively routine, there has been no work done with the use of activated polyamidoamine dendrimers (Tang et al. 1996; Tang and Szoka 1997) to deliver genes to whole organisms. The only similar report has been the use of polyamidoamines to transfect murine cardiac skin grafts (Qin et al. 1998). Activated polyamidoamine dendrimers have been shown *in vitro* to deliver genes at increased efficiency over naked DNA, and to possess advantages over cationic lipids for gene delivery, such as increased transfection efficiency and lower toxicity (Kukowska-Latallo et al. 1996; Tang et al. 1996; Albritton 1997).

Research on gene delivery to aquatic species is needed and is currently underway in fish and shellfish (Mialhe et al. 1995; Sin 1997), however, there are no reports of gene delivery directly to adult shellfish. As part of a project to develop techniques for gene delivery to oysters, the goal of this study was to deliver genes to adult eastern oysters by injection of DNA complexed with activated polyamidoamine dendrimers and to examine transient gene expression. The objectives of this study were to determine the effectiveness of this technique by: 1) detection of a transgene in eastern oyster cells by polymerase chain reaction (PCR); 2) detection of transgene expression in cells by flow cytometry; and 3) detection of transgene expression in cells by microscopic observation. This study is the first documentation of gene delivery and expression to adults of a shellfish species.

Materials and Methods

Oyster Collection

Oysters were collected from Hackberry Bay, Louisiana (29°40′00″N, 90°02′30″W) in July 1998. Oyster meat weights were 3.0 ± 0.9 g (mean ± standard deviation) with shell lengths of 7.0 ± 0.6 cm. Oysters were held at the Louisiana State University Aquaculture Research Station in recirculating systems with artificial seawater (Hawaiian Marine Mix, Hawaiian Marine Imports, Houston, Texas, USA) at 15 ppt without feeding for 3 d before use.

DNA Preparation

Two plasmids were used, pS65T-C1 (Clontech Laboratories Inc., Palo Alto, California, USA) which contains a gene encoding a modified form of green fluorescent protein (*rsGFP*) (Heim et al. 1995) under control of the human cytomegalovirus immediate early promoter (CMV), and pPC-6 (produced in our laboratory), which contains the cecropin B gene (*cepB*) from *Hyalophora cecropia* with its native promoter (Xanthopoulos et al. 1988).

Escherichia coli strain DH5α transformed with the plasmids of interest were grown overnight in Luria-Bertani (LB) broth at 37 C. Plasmid DNA was extracted and purified using an anion exchange column (QIAFilter Plasmid Maxiprep kit, Qiagen Inc., Valencia, California, USA), and DNA was dissolved in distilled, deionized water (ddH_2O) for use. Concentration and purity of DNA were estimated by spectrophotometry (GeneQuant RNA/DNA Calculator, Pharmacia Biotech, Piscataway, New Jersey, USA) and agarose gel electrophoresis.

Transfection

Eastern oysters were transfected by injection using a sterile 1-mL syringe with a 25-gauge needle. The day before injection, notches were made in the shell along the dorsal margin which allowed access to the adductor muscle sinus and the circulatory system. To deliver DNA to adult oysters, a transfection solution of plasmid DNA complexed with SuperFect™ (Qiagen Inc., Valencia, California, USA) was prepared. SuperFect™ is a commercially available solution of activated polyamidoamine dendrimers (Tang et al. 1996), and the ratio of DNA (μg) to SuperFect™ volume (μL) has been shown to influence transfection efficiency in cell culture (Qiagen SuperFect™ Reagent Handbook). For oysters injected with transfection solution, three ratios of DNA to SuperFect™ (μg to μL) were used: 1 to 2, 1 to 4, and 1 to 8. For each oyster to be transfected, 10 μg of plasmid DNA were diluted in oyster saline solution (0.48 g/L $CaCl_2$, 1.45 g/L $MgSO_4$, 2.18 g/L $MgCl_2 \cdot 6H_2O$, 0.31 KCl g/L, 11.61 g/L NaCl, 0.35 g/L $NaHCO_3$) and mixed with 20, 40 or 80 μL of SuperFect™ for a total volume of 200 μL. The solution was incubated for 10 min at room temperature before injection. A 100-μL volume of hemolymph was removed from the adductor muscle sinus followed by injection of 200 μL of transfection solution into the sinus. Control oysters were injected with 200 μL of oyster saline solution only. Injected oysters were held in air at room temperature for 6 h before they were returned to the recirculating holding system.

Forty-five eastern oysters were used in this experiment, divided into three groups of 15. The first group was injected with *rsGFP* in plasmid pS65T-C1. Within this treatment were three subtreatments of five oysters transfected at the three ratios of DNA (μg) to SuperFect™ (μL). The second group was injected with *cepB* in plasmid pPC-6 using the three DNA to SuperFect™ ratios. Oysters in the third group were injected with oyster saline solution as a control treatment.

Oysters were held in a recirculating system, fed a mixture of the marine algae *Isochrysis galbana* and *Chaetoceros calcitrans,* and sampled at 4 and 10 d after transfection. At each sampling, 300 to 600 μL of hemolymph were withdrawn from the adductor muscle sinus of each oyster with a 25-gauge needle and 1-mL syringe. Hemolymph samples were immediately placed on ice and stored chilled until use (to limit hemocyte clumping). Samples were not washed or pelleted. From each sample, 100 μL was immediately used for flow cytometry, and at least 150 μL was stored frozen at −120 C (in liquid nitrogen vapor) for subsequent DNA extraction. Oyster meats were removed from the shell and weighed after sampling on day 10.

PCR Analysis

DNA was extracted from ~150 μL of each hemolymph sample (QIAmp Blood and Tissue Kit, Qiagen Inc., Valencia, California, USA). From each sample, DNA was eluted into 30 μL of sterile ddH_2O and stored at −20 C. Each PCR reaction received 5 to 10 μL of extracted DNA as template. Primers were designed for PCR using the PC/Gene software package (Intelligenetics, Mountain View, California, USA). Appropriate positive and negative controls were included with all PCR assays.

Polymerase chain reaction amplification of a native oyster gene from the DNA for each sample was used to indicate that DNA extraction was successful (oyster DNA was present in the PCR reaction) and that PCR inhibitors were not evident in that sample. Primers for PCR were designed to target the 28s rRNA gene of the eastern oyster (Littlewood 1994). The primer sequences were GCT-AAA-TAC-TTC-CCG-AGT-CCG-ATA-GC and GCA-CCT-TCC-TCC-AGC-TCT-TCT-GAC (5′ to 3′). Each reaction volume was 100 μL and contained 0.5 μM of each primer, 200 μM of each deoxynucleotide triphosphate (dNTP), 1.0 mM $MgCl_2$, 1 μL of DMSO, 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Inc., Foster City, California, USA), and 1-X AmpliTaq buffer (Perkin Elmer Inc.). After heating to 95 C for 2 min, PCR reaction conditions were 35 cycles at 95 C for 30 sec, 52 C for 30 sec, and 72 C for 1 min. Reaction products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized with an ultraviolet transilluminator. The primers described above were designed to amplify a 286 base pair (bp) DNA fragment from DNA of eastern oysters. The presence of such a band after PCR on a sample (a positive reaction) indicated the presence of oyster DNA in the sample.

Oysters injected with *rsGFP* were assayed for the presence of this gene with primers designed from the pS65T-C1 sequence (Clontech Inc.) for *rsGFP*. Primer sequences were GTC-AGT-GGA-GAG-GGT-GAA-GGT-GAT-GCA-AC and GAA-AGG-GCA-GAT-TGT-GTG-GAC-AGG-TAA-TG (5′ to 3′). Each reaction volume was 100 μL and contained 0.2 μM of each primer, 100 μM of each dNTP, 1.5 U of Vent polymerase (New England Biolabs, Inc., Beverly, Massachusets, USA), and 1-X Thermpol PCR buffer (New England Biolabs). After heating to 95 C for 2 min, PCR reaction conditions were 30 cycles at 95 C for 1 min, 63 C for 1 min, and 72 C for 1 min. Reaction products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. The primers described above were designed to amplify a 539 bp product from *rsGFP*. The presence of such a band after PCR indicated the presence of *rsGFP* in the sample tested.

Oysters injected with *cepB* were assayed for the presence of this gene with primers designed from the *cepB* sequence (Xanthopoulos et al. 1988). The primer sequences were AGA-CTT-GAC-TCC-GCT-GCA-TAA-GTG and TAC-CGT-TTC-TGA-TGT-TGC-GAC-C (5′ to 3′). Each PCR reaction volume was 100 μL and contained 0.5 μM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μL of Biolase DNA Polymerase (ISC Bioexpress, Kaysville, Utah, USA), and 1-X Biolase PCR buffer. After heating to 95 C for 2 min, PCR reaction conditions were 35 cycles at 95 C for 30 sec, 52 C for 30 sec, and 72 C for 1 min. Reaction products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized with a transilluminator. The primers described above were designed to produce and amplify an 863 bp product from the cecropin B gene. The presence of such a band after PCR indicated the presence of the cecropin B gene in the sample tested.

Hemolymph samples from oysters injected with saline only were assayed by PCR for the presence of the 28s rRNA, GFP and

cecropin genes using the above primers and optimized conditions.

Flow Cytometry Analysis

To prepare samples for flow cytometry, 100 μL of fresh hemolymph were suspended in 500 μL of ice-cold modified Alsever's solution (8.82 g/L $C_6H_5Na_3O_7 \cdot 2H_2O$, 3.80 g/L ethylene diamine tetraacetic acid $\cdot 2H_2O$ (EDTA), 20.72 g/L $C_6H_6O_6$, 5.00 g/L NaCl). This solution has been shown to inhibit hemocyte clumping of oysters (Bachere et al. 1988). Analysis was performed using a FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, California, USA) equipped with a 488-nm argon laser, which excites rsGFP to emit green fluorescence. Hemocytes from oysters successfully transfected with and expressing *rsGFP* would be expected to exhibit green fluorescence above background autofluorescence values of cells. Hemocytes from oysters injected with *cepB* or saline would not be expected to exhibit green fluorescence above that of autofluorescence levels. The collection of 10,000 events was attempted for each sample, with the green fluorescence intensity measured for each cell. Data were analyzed with CellQuest™ software (Becton Dickinson). Fluorescence intensity for each cell was reported as a channel number, with increasing brightness of fluorescence yielding increasing channel numbers. Because all cells have some background level of green fluorescence from cell constituents (autofluorescence), it was necessary to count the number of cells with green fluorescence intensities greater than background levels to measure any differences among oysters. Thus, the percentage of cells from each sample with green fluorescence greater than a specific value (channel 100 on a log scale) was used as a conservative measure of *rsGFP* expression for that sample. Cells with green fluorescence intensities in this region were referred to as positive cells. Samples with less than 10,000 events were discarded from analysis. The values for each treatment (rsGFP, cecropin B, oyster saline) were averaged and compared statistically for day 4 and day 10. For data analysis, samples transfected at the different DNA to SuperFect™ ratios (1:2, 1:4, 1:8) were pooled.

Data were analyzed statistically with the General Linear Models procedure (SAS Inc., Cary, North Carolina, USA). A one-way analysis of variance (ANOVA) was used to compare mean values for the percentage of positive cells for each treatment. To better approximate a normal distribution, all data were arcsine square root transformed before ANOVA (Zar 1984). 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.

Analysis by Fluorescent Microscopy

All hemolymph samples were examined with an inverted fluorescent microscope (Zeiss Axiovert 25, Carl Zeiss Inc., Thornwood, New York, USA) for expression of *rsGFP* at 100× or 200× with standard fluorescein isothiocyanate (FITC) filters. Hemocyte samples were examined in blind fashion and were independent of the flow cytometric analysis. As with flow cytometry, hemocytes from oysters expressing *rsGFP* would be expected to fluoresce green. Hemocytes from oysters injected with *cepB* or saline would not be expected to exhibit green fluorescence brighter than background autofluorescence. Between 50 and 100 μL of each hemolymph sample were placed in 24-well tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, New Jersey) for observation.

Results

PCR Analysis

The presence of *rsGFP* was detected in oyster hemocytes at day 4 and at day 10, only in oysters injected with *rsGFP* (Fig. 1). At day 4, 14 of 15 oysters injected with *rsGFP* were positive for the presence of the oyster 28S rRNA gene after PCR (Table 1), indicating successful DNA extraction. Of those 14, all were positive for the presence

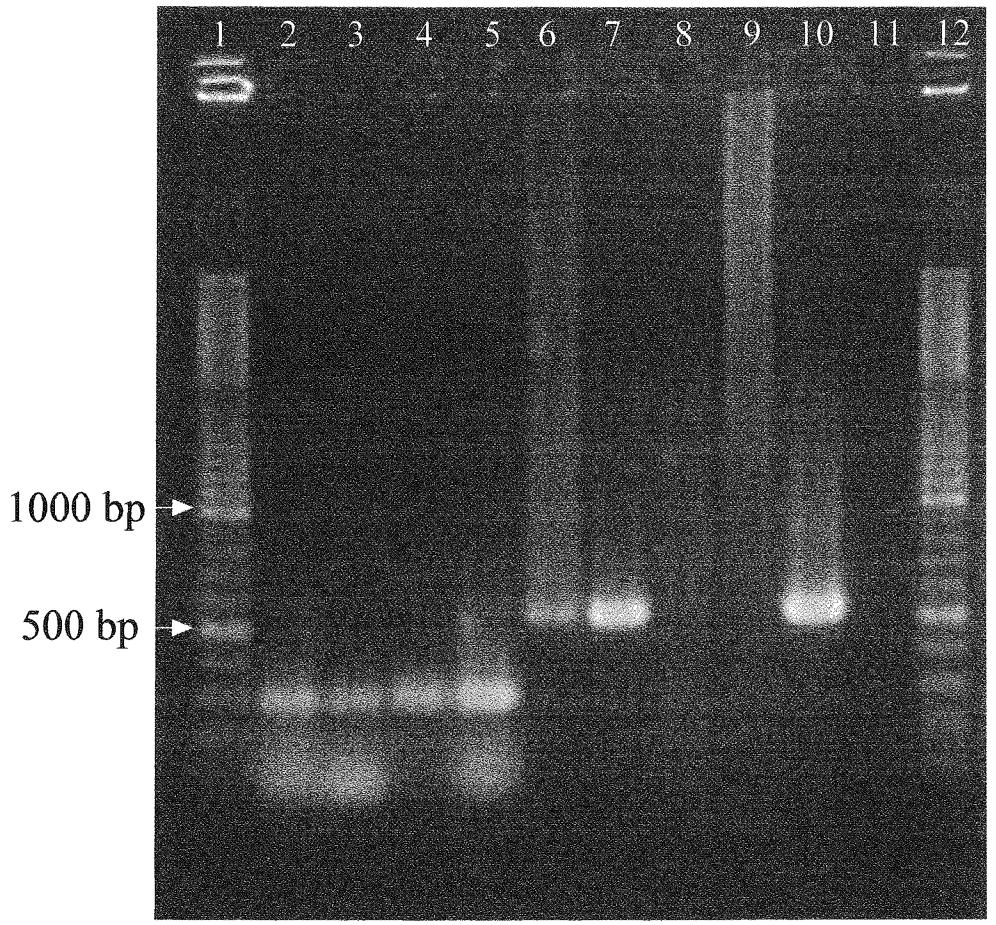

FIGURE 1. *Agarose gel depicting representative results of PCR using DNA extracted from eastern oyster hemocytes 4 d after transfection. Samples in lanes 2–3 and 6–7 were from oysters injected with pS65T-C1* (rsGFP) *complexed with SuperFect™. Samples in lanes 4–5 and 8–9 were from control oysters injected with saline. Lanes 1 and 12 were loaded with a 100-base pair (bp) ladder DNA marker. Lanes 2–5 contained results from PCR to detect the 28s rRNA gene (286 bp product) which served as a control to verify the presence of analyzable oyster DNA. Lanes 6–9 contained results from PCR to detect* rsGFP *(539 bp product). Lane 10 was loaded with a positive control (plasmid pS65T-C1 added to PCR reaction). Lane 11 was loaded with a negative control (no DNA added to PCR reaction). Bands less than 100 bp were PCR artifacts.*

of *rsGFP*. Presence or absence of *rsGFP* was not related to the ratio of DNA to SuperFect™ delivered to the oysters. At day 10, all 15 oysters were positive for both the 28s rRNA gene and *rsGFP* (Table 1).

The presence of *cepB* was detected in oyster hemocytes at day 4 and day 10, and only in oysters injected with *cepB* (Fig. 2). At day 4, 12 of 15 oysters injected with *cepB* were positive for the presence of the oyster 28s rRNA gene after PCR (Table 1). Twelve oysters of this group were also positive for *cepB*; however, two oyster samples positive for the 28s rRNA gene were not positive for *cepB,* and two oyster samples not positive for the 28s rRNA gene were positive for *cepB* (Table 1). Presence or absence of *cepB* was not related to the ratio of DNA to SuperFect™ delivered to the oysters. At day 10, 14 of the 15 oysters were positive for both the 28s rRNA gene and *cepB* (Table 1).

For oysters injected with saline only, at day 4, 12 of the 15 oysters were positive

TABLE 1. *Results of polymerase chain reaction (PCR) at 4 d and 10 d after injection of eastern oysters with genes and SuperFect™ or saline. Ratio indicates the ratio of DNA (μg) to SuperFect™ (μL) injected per oyster. Oysters receiving DNA were injected with 10 μg DNA and a corresponding amount of SuperFect. Genes injected were red-shifted green fluorescent protein* (rsGFP) *and cecropin B* (cepB).

			Positive for 28s rRNA		Positive for GFP		Positive for cecropin	
Treatment	Ratio	Number	4 d	10 d	4 d	10 d	4 d	10 d
RsGFP	1:2	5	5	5	5	5	nt[a]	nt
RsGFP	1:4	5	5	5	5	5	nt	nt
RsGFP	1:8	5	4	5	4	5	nt	nt
Total	—	15	14	15	14	15	nt	nt
CepB	1:2	5	5	5	nt	nt	4	5
CepB	1:4	5	3	5	nt	nt	4	5
CepB	1:8	5	4	4	nt	nt	4	4
Total	—	15	12	14	nt	nt	12	14
Saline only	0:0	15	12	11	nt	nt	0	0

[a] nt, not tested.

for the oyster 28s rRNA gene (Table 1). At day 10, 11 of the 15 oysters were positive for the oyster 28s rRNA gene. No control samples were positive for *rsGFP* or the *cepB* gene (Figs. 1, 2).

Extraction of amplifiable DNA was not successful from every sample (79 of 90 possible for days 4 and 10). However, of the 55 samples from oysters injected with DNA for which DNA extraction was successful, 54 were positive for the presence of a transgene. Sampling at day 4 or day 10 had no effect on transgene detection.

Flow Cytometry Analysis

At day 4, although the total percentage of positive cells was low for all samples (≤5%), analysis of the three treatments revealed significant differences ($P = 0.016$) in green fluorescence (Fig. 3). Oysters injected with *rsGFP* had a significantly higher mean percentage of positive cells (1.0 ± 0.4%) than those injected with the *cepB* (0.3 ± 0.1%) or oyster saline (0.2 ± 0.1%) (Fig. 4). There was variability associated with green fluorescence in the samples, and not all oysters in the *rsGFP* group exhibited levels of green fluorescence noticeably different from oysters in the *cepB* or saline-injected groups. However, no *cepB* or saline-injected samples had greater than 1% positive cells. Four *rsGFP*-injected samples had greater than 1% positive cells, with the highest observed value at 5%.

At day 10, although the mean number of positive cells for *rsGFP* treatment was three times greater than the number of positive cells in *cepB* or saline-injected treatments, there was no statistical difference among the treatments ($P = 0.2027$) (Fig. 4). As with the day 4 samples, there was variability associated with green fluorescence in the samples, and not all oysters in the *rsGFP* group exhibited levels of green fluorescence noticeably different from oysters in the *cepB* or saline-injected groups. Again, no *cepB* or saline-injected samples had greater than 1% positive cells. Four *rsGFP*-injected samples had greater than 1% positive cells, with the highest observed at 7%.

Analysis by Fluorescent Microscopy

Detection of *rsGFP* was possible in oyster hemocytes at day 4 and day 10 (Fig. 5). At day 4, two samples contained more bright green cells than did the other samples upon observation with an inverted fluorescent microscope. Correspondingly, these two oysters (#2 and #13) had the highest percentage of positive cells from flow cytometric analysis. According to our experimental design, these data sets were col-

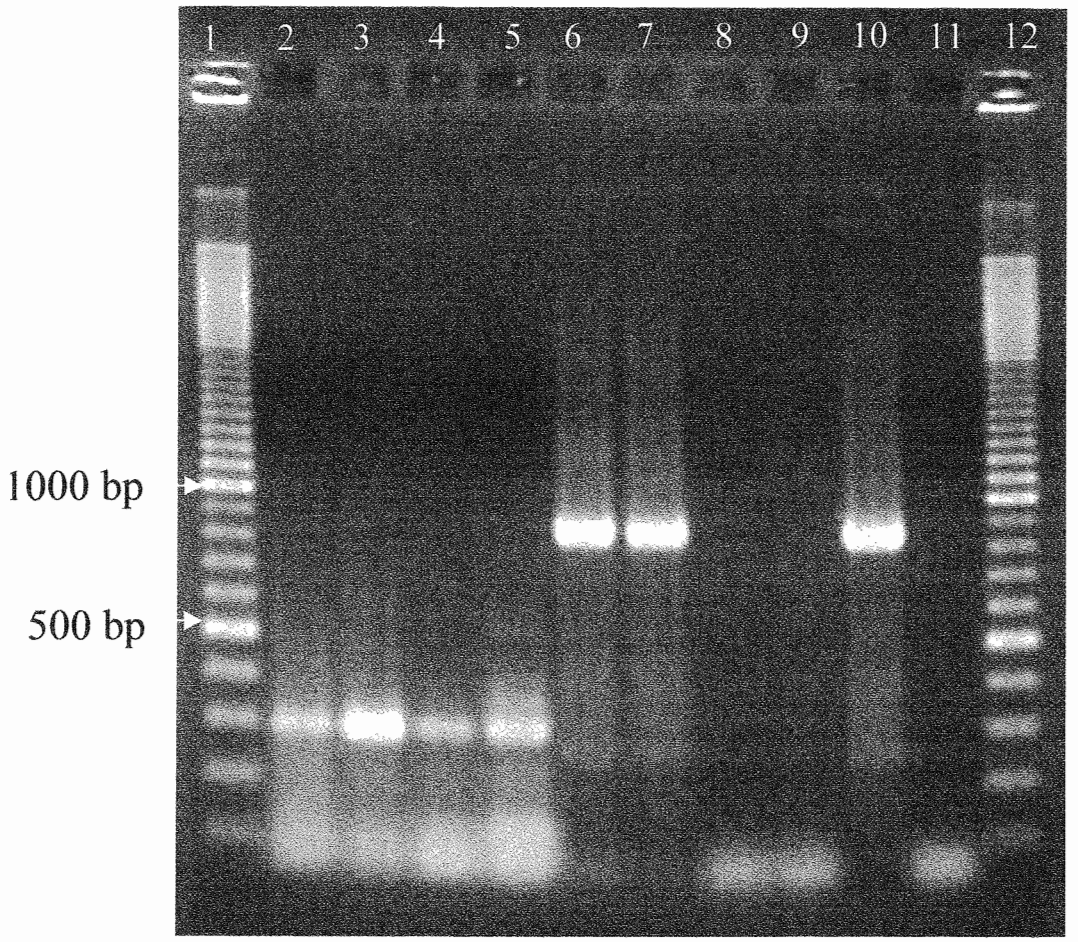

FIGURE 2. *Same as Figure 1 except hemocytes were collected 10 d after transfection and treated oysters were injected with pPC-6 (cecropin) and lanes 6-9 contained results from PCR to detect the cecropin B gene (863 base pair product).*

lected independently and comparison of results was not made until after data collection. Cells expressing *rsGFP* were detected rarely in other *rsGFP*-injected samples, but none were detected in cecropin B or saline-injected samples.

Results were similar at day 10, although only hemocytes from one oyster (#13) were obviously brighter than all other samples with observation using an inverted fluorescent microscope. Correspondingly, this oyster had the highest percentage of green fluorescent cells from flow cytometric analysis (these data were also collected in blind fashion). Cells expressing *rsGFP* were detected rarely in other *rsGFP*-injected samples, but none were detected in cecropin B or saline-injected samples.

Discussion

In most reports, techniques for transfection involve gene delivery to gametes or embryos; however, there are potential benefits to transfection of adult organisms. The ability to efficiently transfect large numbers of somatic cells in adults would provide a rapid and reproducible source of animals for study of the expression of transferred genes *in vivo*. Additionally, determination of gene expression in adult organisms

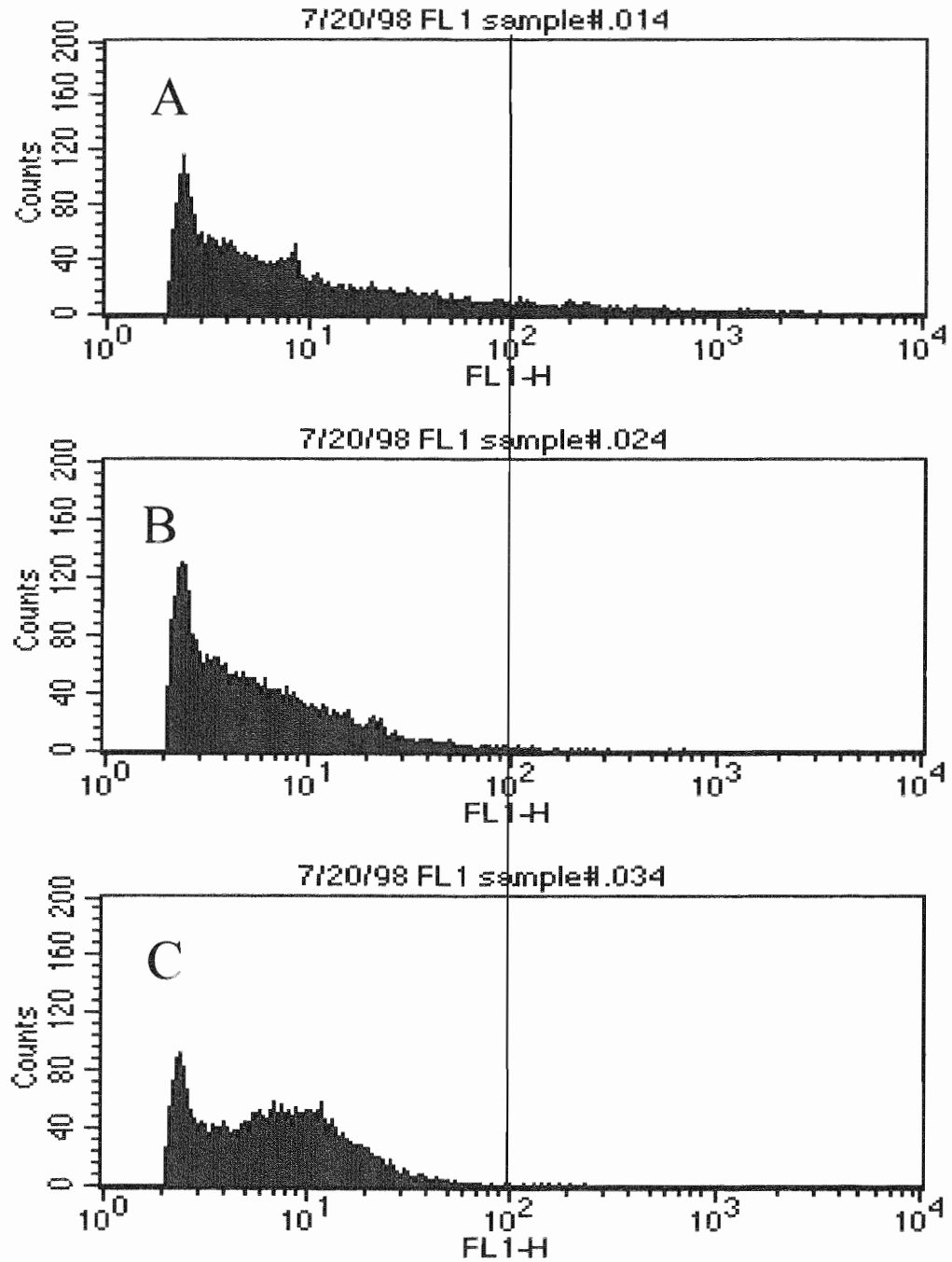

FIGURE 3. *Flow cytometry histograms generated from analysis of 10,000 hemocytes. Panel A was from a sample collected from an eastern oyster injected with* rsGFP *complexed with SuperFect®; panel B was from a sample collected from an eastern oyster injected with* cepB *complexed with SuperFect®; and panel C was from a sample collected from an eastern oyster injected with saline. Cells with fluorescence intensity beyond the threshold indicated by the vertical line were considered positive.*

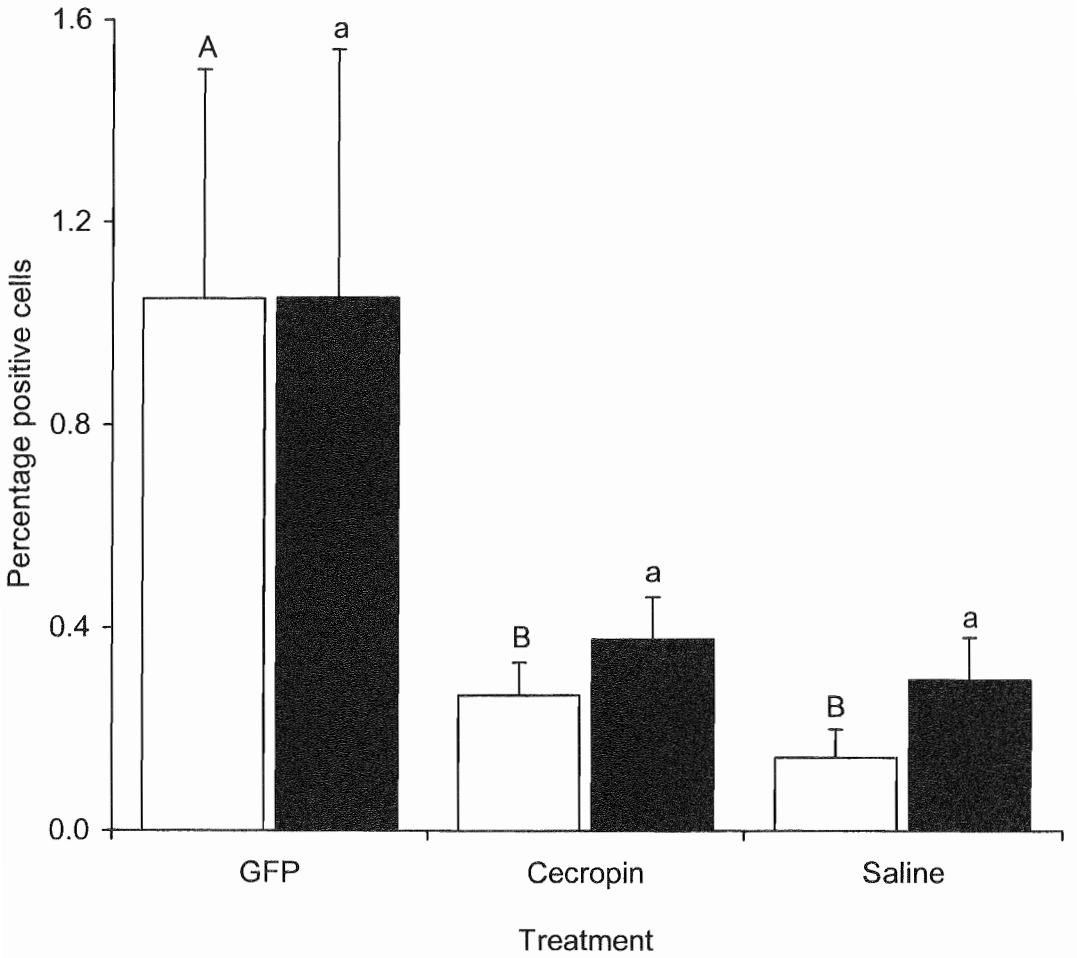

FIGURE 4. *Percentage (mean ± standard error) of eastern oyster hemocytes detected by flow cytometry expressing green fluorescence greater than background levels and indicative of the production of green fluorescent protein (GFP) following transfection of the* rsGFP *gene. Open bars = day 4, closed bars = day 10 after injection. Fifteen eastern oysters each were injected with* rsGFP, *cecropin B gene, or saline solution. All injected DNA was complexed with SuperFect®. Data from day 4 and 10 were analyzed separately as indicated by uppercase and lowercase letters. Bars of each type sharing letters were not significantly different (P > 0.05).*

would not involve introduction of DNA into gametes or embryos and waiting for growth to adults. Concerns with containment and prevention of the accidental release of larval or juvenile stages would be diminished. In organisms, such as oysters, where early life stages are difficult to maintain in a closed artificial environment, transfection of adults would facilitate research. Questions of promoter function, gene expression, and gene effectiveness could be analyzed with transient gene expression in adults and less effort than production of generations of stably expressing transgenic lines. Such techniques may also be useful in the production of transgenic progeny.

Results from PCR indicated the successful delivery of *rsGFP* and *cepB* to oyster hemocytes in most (>90%) treated oysters. At this time, it is unknown whether the genes were delivered to other tissues as well. It is unlikely that extracellular DNA

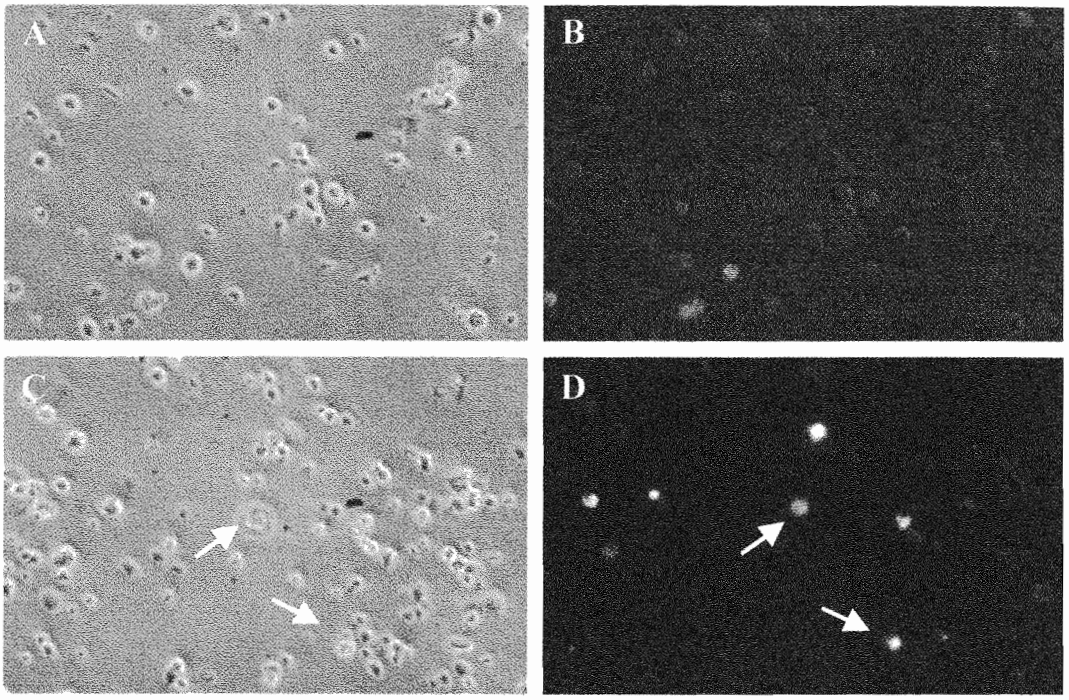

FIGURE 5. *Expression of the gene for red-shifted green fluorescent protein (*rsGFP*) in eastern oyster hemocytes. These cells were removed from oysters 4 d after injection of plasmid DNA containing* rsGFP *complexed with SuperFect™. Panels A and C, brightfield micrographs; panels B and D, fluorescent micrographs. Hemocytes in panels A and B were collected from control oysters. Hemocytes in panels C and D were from eastern oysters transfected with* rsGFP. *Some cells expressing* rsGFP *are indicated by arrows. Presented here in grayscale, cells expressing GFP emitted noticeably brighter green light compared to controls.*

molecules remaining in the oyster circulatory system resulted in positive PCR given the potential for degradation after 4 or 10 d.

Detection of *rsGFP* expression is further evidence that gene transfer was successful. It should be pointed out that the gene expression detected in this study was probably transient and is not proof of integration of foreign DNA into the oyster genome. The expression of *rsGFP* was detected with flow cytometry and corroborated by independent analysis with fluorescent microscopy. However, though the majority of oysters were positive for the presence of *rsGFP* with PCR, there were variable levels of green fluorescence observed in these hemocyte samples. Not all oysters injected with *rsGFP* exhibited levels of green fluorescence noticeably different from controls. Background autofluorescence was a problem as well, and was probably related to variability in hemolymph constituents, hemocyte cell number, and the ratios of different hemocyte cell types that can occur in oysters (Ford et al. 1994; Cheng 1996). In spite of these problems, flow cytometric analysis detected cells with green fluorescence in samples injected with *rsGFP* as brighter than background levels. Detection of *cepB* expression was not attempted in this study. Cecropin B transfection served as a control for possible effects of the injection of DNA and SuperFect™ on background levels of green autofluorescence, and none were detected.

Variability in gene expression after transfection is to be expected, and GFP expression at low levels could be below the threshold of detection. Variability in GFP expression has been reported for embryos

of zebrafish *Danio rerio* after injection of fertilized eggs with DNA containing a GFP gene (Higashijima et al. 1997). In another study, only one third of mice treated with complexes of DNA and liposomes exhibited transgene expression (Alexander and Akhurst 1995). In cell culture, even when DNA was known to be delivered to cells at constant amounts, levels of gene expression varied greatly, especially among different cell types (Tseng et al. 1997; Pollard et al. 1998). It must be noted that we sampled only a small fraction of the circulating hemocytes in these oysters.

In addition to variability in gene expression, the promoter used in this study (pCMV) may be expressed at low levels in oyster hemocytes. Mammalian viral promoters have been shown to drive gene expression in a wide variety of organisms, including mammals, fish, insects, and molluscs. In bivalves, the Rous sarcoma virus long terminal repeat (RSV LTR) functioned in dwarf surf clams *Mulinia lateralis* (Lu et al. 1996). In the Pacific oyster *C. gigas*, CMV and SV40 promoters have been shown to drive gene expression (Boulo et al. 1996; Cadoret et al. 1997). However, in work with *C. gigas*, the CMV promoter was shown to be inefficient compared to the *Drosophila hsp70* promoter (Boulo et al. 1996; Cadoret et al. 1997). Molluscan promoters have been identified recently (Yoshino et al. 1998; Cadoret et al. 1999), and should be evaluated in future work.

This work is the first report of gene transfer to an adult mollusc. Data from PCR, flow cytometry, and fluorescent microscopy indicated that gene delivery and expression were successful in adult oysters. Future work should address increasing transfection efficiency and levels of gene expression. Of course, the application of further advances of this technology in the oyster industry is uncertain and is likely years away. Application will require assessment of ecologic risk, economic risk, and documented safety of modified oysters for human consumption.

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Literature Cited

Albritton, L. M. 1997. Efficient transfection of fibroblast and epithelial cells using an activated-dendrimer reagent. NIH Research 9:52.

Alexander, M. Y. and R. J. Akhurst. 1995. Liposome-mediated gene transfer and expression via the skin. Human Molecular Genetics 4:2279–2285.

Anderson, E. D., D. V. Mourich, and J. C. Leong. 1996. Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. Molecular Marine Biology and Biotechnology 5:105–113.

Bachere, E., D. Chagot, and H. Grizel. 1988. Cell separation by centrifugal elutriation. Pages 169–177 *in* W. S. Fisher, editor. Disease processes in marine bivalve molluscs, volume 18. American Fisheries Society, Bethesda, Maryland, USA.

Boulo, V., J. P. Cadoret, F. Le Marrec, G. Dorange, and E. Mialhe. 1996. Transient expression of luciferase reporter gene after lipofection in oyster (*Crassostrea gigas*) primary cell cultures. Molecular Marine Biology and Biotechnology 5:167–174.

Cadoret, J. P., V. Boulo, S. Gendreau, and E. Mialhe. 1997. Promoters from *Drosophila* heat shock protein and cytomegalovirus drive transient expression of luciferase introduced by particle bombardment into embryos of the oyster *Crassostrea gigas*. Journal of Biotechnology 56:183–189.

Cadoret, J. P., R. Debon, L. Cornudella, V. Lardans, A. Morvan, P. Roch, and V. Boulo. 1999. Transient expression assays with the proximal promoter of a newly characterized actin gene from the oyster *Crassostrea virginica*. FEBS Letters 460:81–85.

Chalfie, M., Y. Tu, G. Eukirchen, W. Ward, and D. Prasher. 1994. Green fluorescent protein as a marker for gene expression. Science 263:802–805.

Cheng, T. C. 1996. Hemocytes: Forms and functions. Pages 299–333 *in* V. S. Kennedy, R. I. E. Newell, and A. F. Eble, editors. The eastern oyster. *Crassostrea virginica*. Maryland Sea Grant College, College Park, Maryland, USA.

Felgner, J., R. Kumar, C. Sridhar, C. Wheeler, Y. Tsai, R. Border, P. Ramsey, M. Martin, and P. Felgner. 1994. Enhanced gene delivery and

mechanism studies with a novel series of cationic lipid formulations. Journal of Biological Chemistry 269:2550–2561.

Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. 1987. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. Proceedings of the National Academy of Sciences of the USA 84:7413–7417.

Ford, S. E. and M. R. Tripp. 1996. Diseases and disease processes. Pages 581–660 *in* V. S. Kennedy, R. I. E. Newell, and A. F. Eble, editors. The eastern oyster, *Crassostrea virginica.* Maryland Sea Grant College, College Park, Maryland, USA.

Ford, S. E., K. A. Ashton-Alcox, and S. A. Kanaley. 1994. Comparative and microscopic analyses of oyster hemocytes. Journal of Invertebrate Pathology 64:114–122.

Gershon, H., R. Ghirlando, S. B. Guttman, and A. Minsky. 1993. Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. Biochemistry 32:7143–7151.

Gwadz, R. W., D. Kaslow, J. Lee, W. L. Maloy, M. Zasloff, and L. H. Miller. 1989. Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. Infection and Immunity 57:2628–2633.

Heim, R., A. B. Cubitt, and R. Y. Tsien. 1995. Improved green fluorescence. Nature 373:663–664.

Higashijima, S., H. Okamoto, N. Ueno, Y. Hotta, and G. Eguchi. 1997. High frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body using promoters of zebrafish origin. Developmental Biology 192:289–299.

Hultmark, D., A. Engstrom, H. Bennich, R. Kapur, and H. G. Boman. 1982. Insect immunity: isolation and structure of cecropin D and four minor antibacterial components from *cecropia* pupae. European Journal of Biochemistry 127:207–217.

Jackson, J. K., R. L. Murphree, and M. L. Tamplin. 1997. Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous populations in shellfish. Journal of Clinical Microbiology 35:2098–2101.

Keithly, W. R., K. J. Roberts, and R. Dugas. 1993. Dynamics in Louisiana's oyster industry as portrayed through state auctions, 1987–1992. Journal of Shellfish Research 12:9–13.

Kelly, D. G., W. R. Wolters, J. M. Jaynes, and J. C. Newton. 1993. Enhanced disease resistance to enteric septicemia in channel catfish, *Icatlurus punctatus,* administered lytic peptide. Journal of Applied Aquaculture 3:25–34.

Kukowska-Latallo, J. F., A. U. Bielinska, J. Johnson, R. Spindler, D. A. Tomalia, and J. R. Baker. 1996. Efficient transfer of genetic material into mammalian cells using starburst polyamidoamine dendrimers. Proceedings of the National Academy of Sciences USA 93:4897–4902.

Littlewood, D. T. J. 1994. Molecular phylogenetics of cupped oysters based on partial 28S rRNA gene sequences. Molecular Phylogenetics and Evolution 3:221–229.

Liu, Y., D. Liggit, W. Zhong, T. Guanhuan, K. Gaensler, and R. Debs. 1995. Cationic liposome-mediated intravenous gene delivery. The Journal of Biological Chemistry 270:24864–24870.

Louisiana Cooperative Extension Service. 1998. Louisiana summary: agricultural and natural resources 1997. Publication 2382. Louisiana State University Agricultural Center, Baton Rouge, Louisiana, USA.

Lu, J., T. C. Chen, S. K. Allen, T. Matsubara, and J. C. Burns. 1996. Production of transgenic dwarf surfclams, *Mulinia lateralis,* with pantropic retroviral vectors. Proceedings of the National Academy of Sciences of the USA 93:3482–3486.

Mialhe, E., E. Bachere, V. Boulo, and J. P. Cadoret. 1995. Strategy for research and international cooperation in marine invertebrate pathology, immunology, and genetics. Aquaculture 132:33–41.

Morvan, A., S. Iwanaga, M. Comps, and E. Bachere. 1997. *In vitro* activity of the *Limulus* antimicrobial peptide tachyplesin I on marine bivalve pathogens. Journal of Invertebrate Pathology 69: 177–182.

Paynter, K. T. 1996. Introduction. Pages ix–xxxi *in* The oyster, 1996 edition. The Johns Hopkins University Press, Baltimore, Maryland, USA.

Pollard, H., J. S. Remy, G. Loussouarn, S. Demolombe, J. P. Behr, and D. Escande. 1998. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. Journal of Biological Chemistry 273:7507–7511.

Qin, L., D. R. Pahud, Y. Ding, A. U. Bielinska, J. F. Kukowska Latallo, J. R. Baker Jr., and J. S. Bromberg. 1998. Efficient transfer of genes into murine cardiac grafts by starburst polyamidoamine dendrimers. Human Gene Therapy 9:553–560.

Rahman, A. and N. Maclean. 1992. Fish transgene expression by direct injection into fish muscle. Molecular Marine Biology and Biotechnology 1: 286–289.

Sin, F. Y. 1997. Transgenic fish. Reviews in Fish Biology and Fisheries 7:417–441.

Tang, M. X. and F. C. Szoka. 1997. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Therapy 4:823–832.

Tang, M. X., C. T. Redemann, and F. C. Szoka. 1996. *In vitro* gene delivery by degraded polyamidoamine dendrimers. Bioconjugate Chemistry 7: 703–714.

Tseng, W., F. R. Haselton, and T. D. Giorgio. 1997.

Transfection by cationic liposomes using simultaneous single cell measurements of plasmid delivery and transgene expression. Journal of Biological Chemistry 272:25641–25647.

Xanthopoulos, K. G., J. Y. Lee, R. Gan, K. Kockum, L. Faye, and H. G. Boman. 1988. The structure of the gene for cecropin B, an antibacterial immune protein from *Hyalophora cecropia*. European Journal of Biochemistry 172:371–376.

Yoshino, T. P., X. Wu, and H. Liu. 1998. Transfection and heat-inducible expression of molluscan promoter-luciferase reporter gene constructs in the *Biomphalaria glabrata* embryonic snail cell line. American Journal of Tropical Medicine and Hygiene 59:414–420.

Zar, J. H. 1984. Biostatistical analysis. Prentice-Hall, Englewood Cliffs, New Jersey, USA.

Zhu, N., D. Liggitt, Y. Liu, and R. Debs. 1993. Systemic gene delivery after intravenous DNA delivery to adult mice. Science 261:209–211.