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Genetics

Production of transgenic dwarf surfclams, *Mulinia lateralis*, with pantropic retroviral vectors

(transgene/invertebrate/mollusk/marine bivalve)

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ABSTRACT **A pantropic pseudotyped retroviral vector containing the envelope protein of vesicular stomatitis virus was used as a gene transfer vector in the dwarf surfclam, *Mulinia lateralis*. These pantropic retroviral vectors have an extremely broad host cell range and can infect many non-mammalian species. Newly fertilized dwarf surfclam eggs were electroporated at 700 V in the presence of 1×10^4 colony-forming units of pantropic pseudotyped retroviral particles. Infection was well tolerated and did not affect the survival rate of the embryos. Gametes collected from P_1 presumptive transgenic animals were analyzed for the presence of provirus by PCR, and in different experiments 13–33% of the gamete pools were positive for the transgene. Dot blot hybridization of DNA samples from the F_1 offspring of two different crosses between infected P_1 and wild-type individuals revealed that 28% and 31% of F_1 offspring were transgenic, respectively. Southern blot analysis of DNA isolated from PCR-positive F_1 animals confirmed integration of a single copy of the provirus into the host genome. Thus, the germ lines of these two P_1 transgenic animals were mosaic for the transgene. Expression of β-galactosidase encoded by the provirus was detected in transgenic but not control surfclam embryos. Pantropic pseudotyped retroviral vectors provide a useful method for the stable introduction of foreign genetic information into surfclams and may facilitate the introduction of desirable genetic traits into commercially important shellfish and crustaceans.**

The stable transfer of heterologous genetic information with the creation of transgenic organisms has been used to address a variety of fundamental questions in biology in species ranging from *Drosophila* to mice (see, for review, ref. 1). Marine bivalve mollusks represent a scientifically and economically important class of organisms for which no successful gene transfer has been reported. Natural populations are threatened by disease and environmental stress (2). Although candidate genes for improving disease resistance and hardiness have been identified in commercially important bivalve mollusks, no method currently exists for the genetic manipulation of these species. The absence of transformed cell lines has also hampered gene transfer studies. Attempts in oyster primary cell culture have not resulted in heritable gene expression (3).

The dwarf surfclam (*Mulinia lateralis*, family *Mactridae*) is a useful model species for shellfish genetic studies (4). Its short generation time (2–3 months), ease of laboratory cultivation, small space requirements, reasonable longevity (2 years), high fecundity (0.5–2 million eggs per spawning), and ease of *in vitro* fertilization make it an ideal bivalve model for gene transfer studies. Since males and females are easily distinguished in the mature clam, it is possible to separate them and make specific crosses. Although a large number of eggs are produced, the small size (50 mm) and opacity of the oocyte make the introduction of foreign DNA by microinjection technically difficult. Electroporation of foreign nucleic acid into eukaryotic cells has been used successfully to produce transgenic fish (5, 6). In this study, we used electroporation to facilitate infection with a class of retroviral vectors that contain the vesicular stomatitis virus G glycoprotein (VSV-G).

The broad host range of pantropic vectors containing the VSV-G envelope glycoprotein has been demonstrated through the infection of fish, newt, and *Xenopus* cell lines (7–9). Infection *in vivo* with creation of transgenic zebrafish lines has also been reported (10). We used electroporation to mediate retroviral vector penetration of the vitelline layer of the fertilized clam egg. Transgenic F_1 offspring were produced that contained a single, integrated copy of the provirus. This approach offers new possibilities for the phenotypic alteration of cultivated mollusks with the introduction of genes for desirable traits such as disease resistance and accelerated growth.

METHODS

Preparation of Embryos. *M. lateralis* were obtained from stocks held at the Haskin Shellfish Research Laboratory. Eggs and sperm were prepared from sacrificed ripe males and females (11) and were combined for 30–40 min to allow fertilization and completion of meiosis. The fertilized eggs were collected by brief centrifugation (6400 rpm for 2–3 s) and transferred to an electroporation cuvette.

Retroviral Vector. The pseudotyped pantropic vector LSRNL-(VSV-G), in which the Moloney murine leukemia virus long terminal repeat (L) drives expression of the hepatitis B surface antigen (S) and the Rous sarcoma virus long terminal repeat (R) drives expression of the neomycin-resistance gene (N), was prepared and titered as described (12). Production of pantropic vector Geo4.8 was also as described in Fig. 1 (9).

Electroporation. Electroporation was carried out in a Baekon model 2000 apparatus (Baekon). Conditions for electroporation were standardized using 5000–50,000 eggs (1 h postfertilization) in 250 μl containing either (*i*) equal volumes of UV-irradiated sea water (25 parts per thousand, pH 7.25–8.25) and Dulbecco's minimal essential medium (DMEM) with high glucose, supplemented with 10% fetal calf serum and 1×10^4 colony-forming units of pantropic retrovirus, or (*ii*) sea water/virus medium (2:1, vol/vol). The polycation Polybrene (Sigma; 1 μg/ml final concentration) was included in all infections as described (7). Control electroporations were performed with embryos in sea water/DMEM (1:1, vol/vol) with 10% fetal calf serum. The settings on the Baekon

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Abbreviations: β-gal, β-galactosidase; VSV-G, vesicular stomatitus virus G glycoprotein.

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apparatus were pulse frequency, 2^6 pulses per cycle; burst time, 0.4 s; cycle number, 5; pulse width, 160 ms; and distance of electrode from surface of buffer, 1 mm. Voltage was varied from 500 to 700 V to determine the optimal electroporation amplitude for retroviral penetration of the vitelline membrane. After electroporation, embryos were incubated 0.5–1 h in the virus-containing solution, rinsed in sterile sea water, and transferred to incubation buckets.

Rearing of Clam Embryos. Feeding was initiated 24 h postfertilization (posttrochophore stage) with *Isochrysis galbana* for 4 days. Subsequently, larvae were fed a mixture of *I. galbana* and *Chaetoceros calcitrans* as described (13).

DNA Extraction and PCR Amplification. Pooled gametes (≈8000 oocytes or 40,000 sperm) were collected from adult clams that were electroporated in the presence of retrovirus as embryos and reared to adulthood. The gamete pools were incubated in 20 μl of digestion buffer containing 0.05 mg of proteinase K per ml, 1.7 mM SDS, and 20 μM dithiothreitol for 2 h at 55°C followed by 20 min at 85°C. To amplify a 349-bp DNA fragment of the *neo* gene, 2–4 μl of supernatant was diluted to 40 μl with a PCR buffer (10 mM Tris at pH 8.3, 50 mM KCl, 0.01% gelatin) containing 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, and 0.25 μM of each amplification primer (5′-GCATTGCATCAGCCATGA-3′ and 5′-GATGGATTG-CACGCAGGTTC-3′). Samples were heated to 97°C for 8 min and cooled to 75°C for 15 min, and 1.25 units of *Taq* DNA polymerase (Promega) diluted in 10 μl of PCR buffer was added to each tube. The DNA mixtures were amplified as follows: denaturation for 1.5 min at 94°C, annealing for 2 min at 62°C, and synthesis at 72°C for 40 cycles. The reaction was terminated with a 7-min extension at 72°C. Samples were kept at 4°C until analysis by electrophoresis on a 2% agarose gel and transfer to a nylon membrane for hybridization to ^{32}P-labeled *neo* cDNA.

Hybridization Studies. Genomic clam DNA (20 μg) was dissolved in 194 μl of TE buffer (10 mM Tris Cl, pH 8.0/1 mM EDTA) and denatured in 0.1 vol of 10 M NaOH at 65°C for 1 h. Samples were neutralized by addition of 0.2 vol of 6 M ammonium acetate and spotted onto a moistened nitrocellulose membrane in a dot blot chamber under vacuum. DNA was fixed to the membrane by exposure to UV light, dried at room temperature, and prehybridized/hybridized as described below. For Southern blot analysis, genomic DNA (25 μg) was digested with *Kpn* I or *Eco*RV, resolved on an 0.8% agarose gel, and transferred to a nylon membrane. Prehybridization was carried out in a 20 mM sodium phosphate buffer (pH 7.5) containing 50% formamide, 5× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 5× Denhart's solution, 0.1% SDS, and denatured yeast RNA (100 μg/ml) at 42°C for at least 3 h. A 3.1-kb ^{32}P-labeled β-galactosidase (β-gal) cDNA probe (0.3–1.0 × 10^9 cpm/μg) was hybridized to the filters with constant shaking for at least 16 h at 42°C. Membranes were washed twice in 2× SSC/0.1% SDS for 10 min each at 42°C, twice in 1× SSC/0.1% SDS for 30 min each at 42°C, and once in 0.5× SSC/0.1% SDS for 15 min at 55°C prior to autoradiography.

β-gal Staining. Experimental and control F_1 embryos (≈5000 embryos per group) were fixed at 2–3 days postfertilization in 1.25% glutaraldehyde in phosphate-buffered saline (PBS, pH 8.2) for 10 min at room temperature. The fixed embryos were incubated in 0.6 mM chloroquine at 30°C for 45 min to inhibit the endogenous lysosomal β-gal activity. After two or three washes in PBS, embryos were stained for 10–12 h at 37°C in a solution containing 1.2 mM 5-bromo-4-chloro-3-indolyl β-D-galactoside 0.1% Triton X-100, 1 mM $MgCl_2$, 0.01% sodium deoxycholate, 6 mM $K_4[Fe(CN)_6]$, and 6 mM $K_3[Fe(CN)_6]$. The stained embryos were then incubated in 1 mM phenylethyl-β-D-thiogalactopyranoside to stop the staining reaction. Embryos were examined for blue color with an Olympus AH2 microscope.

RESULTS

To determine if the pantropic retroviral vector could infect, uncoat, and reverse transcribe in dwarf surfclam embryos, 5000–50,000 fertilized embryos were subjected to electroporation in the presence of 1 × 10^4 colony-forming units of LSRNL-(VSV-G) (Fig. 1). Approximately 3–5% of the embryos in the infected and mock-infected group survived to adulthood. Since survival to adulthood in undisurbed spawns is 10–15% (S.K.A., unpublished results), electroporation was associated with decreased embyro survival. However, no excess mortality was observed in the group exposed to the pantropic vector. Pooled gametes were harvested at the time of sexual maturity (150 days after exposure to the provirus) from 53 randomly selected P_1 individuals: 24 and 29 P_1 adults were exposed to virus-containing medium/sea water in a ratio of 1:1 and 1:2, respectively. DNA was extracted and subjected to PCR amplification for the *neo* proviral-specific sequence. Of the F_1 gamete pools, 8 out of 24 (33.3%) and 7 out of 29 (24.1%) contained the transgene, respectively. Thus, approximately one-third of the electroporated, surviving embryos had retroviral infection of their gametes.

To test for retroviral integration and gene expression, embryos were infected with the pantropic vector Geo4.8 in which the cDNA for β-gal is expressed from the Rous sarcoma long terminal repeat (Fig. 1). Approximately 5000–50,000 embryos were infected at a ratio of sea water to virus-containing medium of 1:1. Gametes were harvested from 22 potentially germ-line transgenic P_1 surfclams, and 3 out of 22 (13.6%) gamete pools were positive by PCR for the *neo* transgene. The 3 P_1 surfclams (1 male, 2 females) from which the positive gamete pools were derived were crossed with wild-type, uninfected surfclams, and the F_1 progeny were screened by DNA dot blot hybridization for the transgene (Fig. 2 *A* and *B*). From the crosses of the 2 female germ-line transgenic clams with wild-type males, 7 out of 20 (35%) and 5 out of 20 (25%) of randomly selected F_1 offspring carried the transgene (Fig. 2*D*). A cross of the germ-line transgenic male with a wild-type female yielded no transgenic F_1 progeny among the 20 offspring screened (data not shown). These results suggest that the germ lines of all 3 P_1 individuals were mosaic for the provirus.

The DNA from two transgenic F_1 clams identified by dot blot analysis was subjected to restriction endonuclease digestion and Southern hybridization to determine if the provirus was integrated into the host genome or maintained as an episomal element. Digestion with *Kpn* I, which releases the full-length 4.5-kb provirus, yielded a single band of the predicted size from each F_1 progeny when hybridized to a 3.1-kb

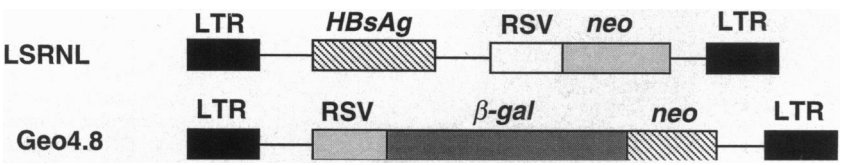

FIG. 1. Genomic organization of pantropic retroviral vectors. LTR, Moloney murine leukemia virus long terminal repeat; HBsAg, hepatitis B surface antigen; RSV, Rous sarcoma virus long terminal repeat; *neo*, neomycin-resistance gene; β-gal, β-gal gene.

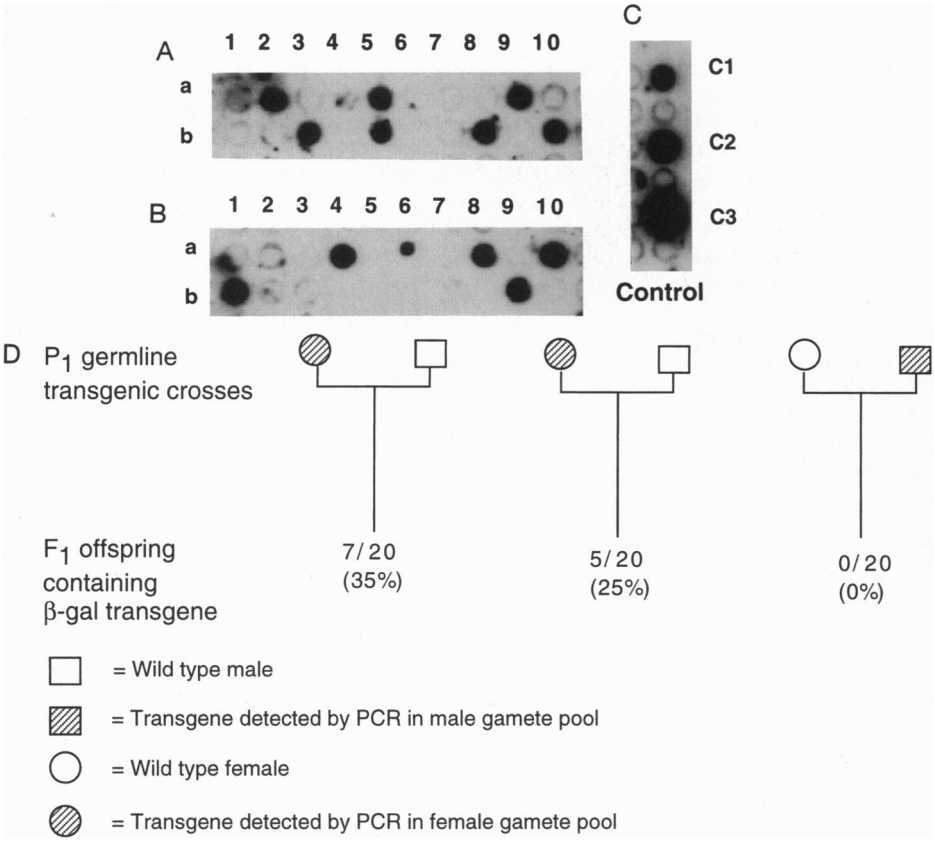

FIG. 2. Dot blot analysis of F_1 progeny. Genomic DNA was isolated from F_1 progeny derived from crosses of two infected, germ line transgenic P_1 female surfclams with wild-type males, spotted on nylon membranes, and hybridized to a ^{32}P-labeled β-gal probe. (*A* and *B*) F_1 progeny derived from the two crosses. Rows a and b are 20 randomly selected F_1 progeny from the same family. (*C*) Hybridization of dilutions of pGeo4 plasmid. C1, 5 ng; C2, 10 ng; C3, 50 ng. (*D*) Schematic of surfclam crosses.

β-galactosidase probe (Fig. 3). Digestion of the F_1 clam DNA with *Eco*RV, which cuts once in the β-gal coding sequence, and *Pvu* II, which does not have any restriction sites within the provirus, yielded two fragments of various lengths from each of the clams when hybridized with a full-length probe that should bind to both *Eco*RV fragments. If the provirus was episomally maintained, digestion fragments of ≈2.0 and 2.5 kb would result from both the digestion with *Eco*RV and *Pvu* II as well as the digestion with *Pvu* II alone and the hybridization pattern would be the same for each clam. The observation of much larger fragments that differed between surfclams suggests that host flanking sequence is contained in each of these hybridized fragments and that they are integrated into unique sites in the host genome. In addition, the appearance of only two bands from each clam with *Eco*RV and *Pvu* II digestion and only a single band with *Pvu* II alone suggests integration of a single provirus per clam genome.

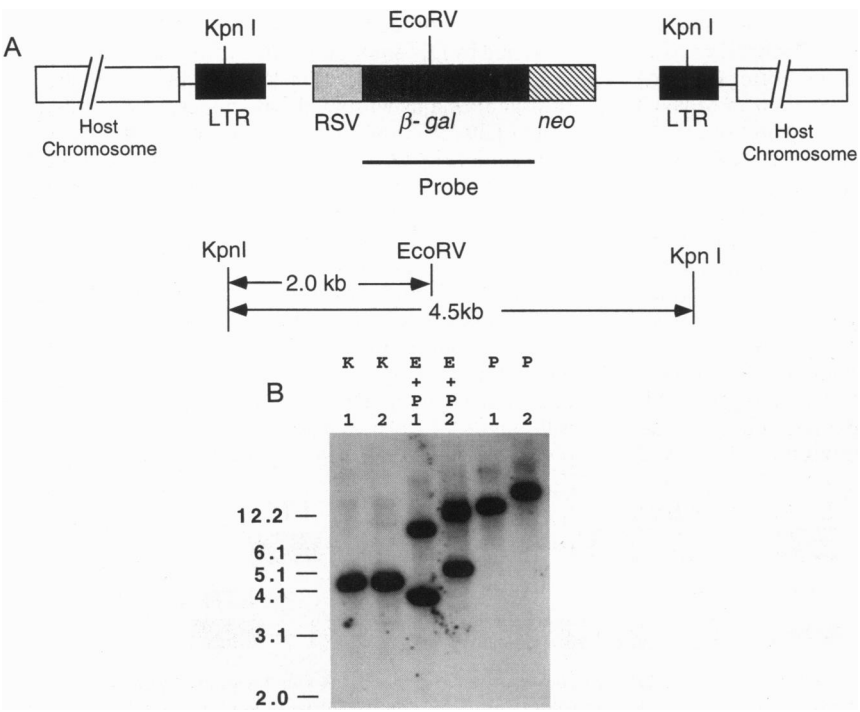

FIG. 3. Southern blot analysis of genomic DNA from two different F_1 transgenic individuals. Genomic DNA was isolated from two F_1 transgenic individuals from two different families (Fig. 2 *A* and *B*), digested with restriction endonucleases as indicated, and resolved on 0.8% agarose gels. The resolved DNA fragments were transferred to nylon membranes and hybridized to ^{32}P-labeled β-gal probe. Lanes 1 and 2, genomic DNA samples of two different F_1 transgenic individuals. K, *Kpn* I; P+E, *Pvu* II and *Eco*RV; P, *Pvu* II. Abbreviations are as in Fig. 1.

To test for transgene expression (β-gal) mediated by the Rous sarcoma virus long terminal repeat (Fig. 1), the two P_1 females with mosaic germ lines were bred to wild-type males, and batches of 5000 F_1 progeny embryos were stained. A total of 500 trochophore-stage embryos from each cross was analyzed, and an average of 24.0% and 33.4% of embryos from the two crosses, respectively, showed definite blue staining (Fig. 4). Staining persisted into the late veliger stage 60–72 h postfertilization. No comparable blue staining was seen in 500 control embryos.

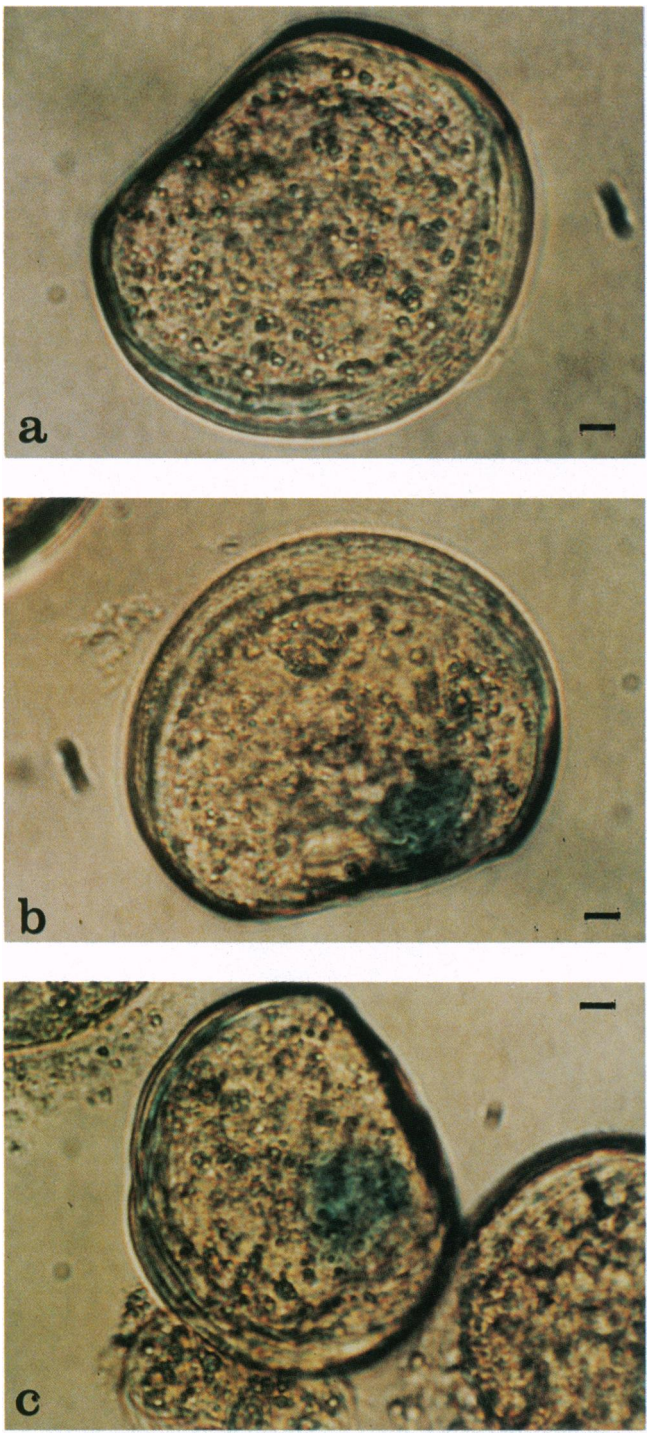

FIG. 4. β-gal activity in F_1 transgenic and control surfclam embryos. Embryos were fixed at the times indicated and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside. (*a*) Control trochophore, 24–36 h postfertilization. (*b*) F_1 transgenic trochophore product of Geo4.8-infected P_1 female with wild-type male, 24–36 h postfertilization. (*c*) Late veliger (60–72 h postfertilization) from cross as in *b*. (Bar = 10 μm.)

DISCUSSION

Although Powers *et al.* (14) reported their preliminary success in transferring foreign DNA into California red abalone (*Haliotis rusfescens*) by electroporation, the patterns of transgene integration and inheritance remain to be confirmed by Southern blot analysis of the genomic DNA from presumptive transgenic animals. In this paper, germ-line transformation of marine invertebrates has now been achieved with a modified retroviral vector that can infect a broad range of species. These data demonstrate retroviral infection and stable integration of the provirus into the host genome for at least two generations. In addition, expression of the β-gal transgene in F_1 offspring suggests that the Rous sarcoma virus promoter can mediate foreign protein expression in the surfclam. To our knowledge, this is the first report of protein expression from an integrated transgene in a marine bivalve.

In principle, the techniques applied here can be adapted to infect other marine invertebrates including oysters, abalone, mussels, and shrimp. The method of bringing the virus particle into contact with the cell surface of the dividing embryo may need to be adapted for different species. In these experiments, low-voltage electroporation presumably transiently disrupted the vitelline membrane and allowed direct contact of the virus particle with the embryo surface but did not interrupt the integrity of the embryo. Precise determination of infection efficiency from these data is not possible due to the large variation in estimated numbers of embryos used for each experiment. Because the pantropic retroviral particles can be easily concentrated by ultracentrifugation (7), exposure to greater numbers of infectious particles can be tested as a means to increase the efficiency of embryo infection. Neither the efficiency of embryo infection in the absence of electroporation nor the use of alternate electroporation systems in which the medium is in contact with the electrodes was tested. Therefore, the contribution of electroporation to the successful infection of surfclam embryos cannot be assessed. In fish, direct microinjection of concentrated pantropic retroviral vector into the blastula cavity of the early embryo has resulted in the establishment of transgenic lines (10). This procedure, however, requires great technical precision and is labor intensive. In contrast, the method presented here allows the infection of large numbers of embryos in a single experiment.

Safety issues must always be considered in the creation of new transgenic species. The retroviral vectors used in this study are replication defective and carry no retroviral coding sequences. The particles infect a broad range of cells, including human cells. Thus, care must be exercised in handling the viral stocks. However, once infection of the embryos is completed, no further risk of inadvertent human infection exists. Mutations due to proviral integration have been observed in other species. Insertional mutagenesis may also occur in the dwarf surfclam model and should be monitored in future experiments. Studies of transgenic embryos over subsequent generations will be needed to establish the stability of the integrated provirus over time and the persistence of gene expression. The dwarf surfclam provides an ideal model system for these transgenic studies, since the rearing of these animals can be completely contained within the laboratory, thus allaying concerns about the inadvertent release of transgenic individuals into the environment.

The creation of transgenic mollusks will facilitate studies to improve the growth rate, tolerance to environmental stress, and pathogen resistance. More information is needed on the function of different promoter sequences in bivalves to allow retroviral construction with both endogenous and exogenous regulatory sequences. Because retroviral vectors can accom-

modate between 10 and 13 kb of heterologous sequence, vectors can be constructed that contain coding regions for desirable genes (e.g., growth hormone) in addition to marker genes (e.g., luciferase, β-gal) under the control of different promoters. Thus, pantropic retroviral vectors provide a flexible, efficient, and inexpensive method to introduce coding sequences and to express foreign proteins in surfclams. This technology should be generalizable to other marine bivalves and heralds a new era of transgenic research in these species.

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