Transformation of Primary Cultures of Shrimp (*Penaeus stylirostris*) Lymphoid (Oka) Organ with Simian Virus-40 (T) Antigen (43880)

LOURDES M. TAPAY,* YUANAN LU,* JAMES A. BROCK,† ELPIDIO CESAR B. NADALA JR.,‡ AND PHILIP C. LOH*,[1]

Department of Microbiology, University of Hawaii at Manoa, 207 Snyder Hall/2538 The Mall, Honolulu, Hawaii 96822; The Aquaculture Development Program,† Department of Land and Natural Resources, State of Hawaii, Honolulu, Hawaii; and Institute of Molecular and Cell Biology,‡ National University of Singapore, 0410 Singapore*

Abstract. **Primary cultures of lymphoid (Oka) organ from *Penaeus stylirostris* were transformed with naked or Lipofectin-mediated pSV-3 neo, a shuttle vector containing the tumor (T) antigen gene from Simian virus-40. The transformed cells, OKTr-1 and OKTr-23, exhibited the following characteristics: rounded morphology forming grape-like aggregates, loosely adhesive, increased growth rate in Medium-199, resistance to G-418 (a neomycin analog marker in the shuttle vector), cloning efficiencies of 68.7% and 36.7% in soft agarose, respectively, and stability in liquid nitrogen storage. Immunofluorescence staining (IFA) of the transformed cells using a monoclonal antibody against SV-40 tumor antigen showed positive results. In contrast, primary cell cultures exhibited fibroblast-like morphology and formed a tight, adhesive monolayer on the surface of the culture vessel. They were sensitive to G-418, and showed negative results with IFA. To date, OKTr-1 and OKTr-23 have undergone 44 and 18 passages, respectively. Primary cultures of the lymphoid organ have not been successfully passaged beyond the primary stage.** [P.S.E.B.M. 1995, Vol 209]

Progress in the study of shrimp viral diseases and their control has been hampered by the lack of simple technologies, such as *in vitro* cell cultures for the isolation and characterization of the shrimp viruses (1–3). The majority of the eight shrimp viruses which have been described have only been observed through electron microscopy of tissues from infected animals. There is thus a need to establish cell lines from the target organs of shrimp viruses so as to facilitate the study of shrimp viruses and the diseases they cause.

Several attempts have been made to establish a cell culture system for crustaceans. However, these have met with varying degrees of success (1, 3–5), with no continuous cell lines having resulted from these studies. Optimal conditions for the consistent preparation of primary cultures of lymphoid (Oka) organ and nerve cord from *Penaeus stylirostris* and *P. vannamei* were reported recently by our laboratory (6).

Among several approaches extensively used to establish either continuous or stable cell lines from tissues or organs of interest is transformation by the oncogenic simian virus-40 tumor (T) antigen (7). Primary cells of human and rodent origins have been transformed by SV-40 (T) antigen. In contrast, there has been limited application of this technique in transforming cells from notably invertebrate systems.

In this paper, we describe the transfection and transformation of a primary culture of lymphoid (Oka) organ from *P. stylirostris* using pSV-3 neo, a shuttle

[1] To whom requests for reprints should be addressed at Department of Microbiology, University of Hawaii at Manoa, 207 Snyder Hall/2538 The Mall, Honolulu, HI 96822.

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vector containing SV-40 gene sequences and a neomycin resistance marker.

Materials and Methods

Preparation of Primary Culture of Oka Organ. Except for some minor modifications, Oka organs from 50–60 g of *P. stylirostris* were harvested and prepared for cell culture following the procedure of Nadala *et al.* (6). Briefly, 50–60 g of shrimp were soaked in 10% sodium hypochlorite solution for 5 min and then sacrificed for their Oka organs. The Oka organs were pooled and incubated for 1 hr at room temperature (rt) in antibiotic incubation medium (AIM) (2X Leibovitz-15 [L-15], 100 IU/100 μg penicillin/streptomycin per ml, 1% amphotericin B and 0.5% gentamycin) with agitation and then transferred to fresh AIM for a further 0.5-hr incubation. The organ explants were minced to about 1 mm^2 in fresh AIM and transferred to Primaria 6-well plates (Falcon) and growth medium was added. The growth medium, Medium D, consisted of 2X L-15 supplemented with 20% fetal bovine serum, 8% shrimp extract,[2] 20 ng/ml epidermal growth factor (of murine submaxillary origin, CALBIOCHEM, La Jolla, CA), 10 units/ml human recombinant interleukin-2 (CALBIOCHEM), 100 IU/100 μg penicillin/streptomycin per ml and salt solution[3] (to adjust the medium's osmolarity to 750–770 mOsm/kg). The explant cultures were incubated for 18 and 36 hr at rt, after which the tissues were aseptically removed and the cell cultures used in transfection experiments.

Isolation and Purification of pSV-3 Neo. Plasmid DNA was isolated from *Escherichia coli* ATCC 37150 (8) and purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients according to the procedure outlined by Maniatis *et al.* (9). The purity of the plasmid preparation was analyzed by agarose gel electrophoresis and by determining the ratio of the absorbance readings at 260 nm/280 nm in a UV-VIS spectrophotometer.

Transfection Experiments. Three DNA concentrations (per milliliter) were used: 2, 3, and 5 μg. The corresponding Lipofectin (Gibco BRL, Grand Island, NY) concentrations used were 5, 10, and 15 μl for each DNA concentration, respectively. Lipofectin, a commercially prepared formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethyl-ammonium chloride (DOTMA) was used to enhance transfection. It has been shown to be effective with over 55 cell lines resistant to transfection by either calcium phosphate or DEAE precipitation methods (10).

Eighteen- and thirty-six-hour-old primary lymphoid cultures in Primaria 6-well plates were used for transfection. The tissue explants were removed and then rinsed with serum-free medium (plain 2X L-15 with osmolarity adjusted to 750–770 mOsm/kg). Duplicate wells of fibroblast-like cells were each treated with different concentrations of DNA or DNA-Lipofectin and incubated for 3 hr at rt. Control wells were inoculated with either naked DNA (5 μg/ml) or Lipofectin (15 μl/ml) or Medium D.

After transfection time, each well was rinsed with serum-free medium as above and replaced with Medium D containing 400 μg/ml Geneticin 418 (Sigma Chemical Co., St. Louis, MO), a neomycin analogue. Microscopic observations were made to monitor any morphological or cultural change.

Sterility tests. One-tenth of a milliliter of the cell suspension was inoculated on Difco Standard Plate Count Agar (supplemented with 10% dextrose) and incubated at 25° and 37°C for 10 days.

Detection of the SV-40 (T) antigen Using Indirect Immunofluorescent Antibody Technique. Twenty-four-hour-old culture of the transformed cells was harvested by centrifugation at 1000 rpm for 5 min at 5°C. The pellet was then resuspended in 0.5 ml of Medium D and a smear of the cell suspension was made on a glass coverslip. The smear was air-dried, fixed in 80% cold acetone for 10 min, and blocked with 5% Carnation skim milk in phosphate-buffered saline (PBS), pH 7.4, for 2 hr. The coverslip was washed two times for 5 min in PBS-Tween 20 and finally once with PBS. The primary antibody (Chemicon MAB 986—a mouse anti-SV-40 (T) antigen monoclonal antibody [Mab], or a mouse monoclonal antibody against SV-40 T/t antigens) was applied at 1:10 and 1:50 dilutions, respectively, for 2 hr at rt. The coverslips were then rinsed as above and treated with a 1:10 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) for 1 hr at rt. The coverslips were rinsed two times for 5 min in PBS, once for 5 min in deionized distilled water, mounted in PBS:glycerol (2:3, pH 7.2) on a glass slide, and viewed under a Nikon inverted microscope equipped with epifluorescence.

Cryogenic Preservation and Stability in Liquid Nitrogen. Suspensions of transformed cells at a concentration of 10^6 cells/0.5 ml in G-418-supplemented Medium D containing 10% dimethyl sulfoxide (DMSO) were dispensed into sterile glass ampules, sealed, and processed for storage in liquid nitrogen. At various

[2] The shrimp extract was prepared as follows: tissues contained in the cephalothorax (but excluding the hepatopancreas, heart, gonads/ovaries, gut, and gills) of 2–4 g shrimp were homogenized in 5 ml (per shrimp) 2x Leibovitz medium at 15000 rpm for 2 min. The homogenate was centrifuged at 3000*g* for 30 min at 5°C. The supernatant was collected and recentrifuged at 10000 rpm for 1 hr. The supernatant was collected and recentrifuged at 29000 rpm for 3 hr. The final supernatant was filtered through a 0.2 μ membrane, aliquoted to 10 ml volumes and stored at −20°C until further use.

[3] Salt solution consisted (in g) of (per 500 ml 3× distilled water): NaCl, 51.2; KCl, 0.9; $CaCl_2$, 2.55; $MgSO_4$, 5.4; $MgCl_2$, 5.9.

intervals, the frozen cells were rapidly thawed and the viable cells (i.e., those excluding trypan blue) were counted in a hemacytometer.

Growth of Transformed Cells in Various Media. The effect of the following media on the growth of the transformed cells was examined: L-15 Medium Leibovitz (L-15), Medium 199 (M 199), Roswell Park Memorial Institute-1640 (RPMI-1640), Grace's Insect Medium (GIM), and Mitsuhashi and Maramorosch Insect Medium (MMIM) (all were purchased from Sigma Chemical Co., St. Louis, MO). The media were supplemented with the following: 20% FBS, 8% shrimp extract, 100 IU/100 μg per ml penicillin/streptomycin, 20 ng/ml epidermal growth factor, 10 units/ml interleukin-2, 400 μg/ml G-418, and salt solution for adjusting osmolarity to 705–715 mOsm/kg. The pH of the media was adjusted to 7.5 (except for GIM, which was adjusted to a slightly lower pH, 6.5, since heavy precipitation resulted beyond this pH).

Briefly, 25-cm^2 tissue culture flasks (Falcon) containing 5 ml of each medium were seeded with 10^5–10^6 cells of either OKTr-1 or OKTr-23. The seeded flasks were incubated at rt and the number of viable cells were compared at 0 and 5 days postseeding. For viability determinations, the loosely attached transformed cells were removed by gently tapping the culture flask sideways and collected by centrifugation at 1000 rpm for 20 min at 10°C. The cell pellet was resuspended in 0.5 ml of fresh medium, triturated briefly to disperse the aggregates of cells and diluted in trypan blue. Viable cells (unstained) were counted using a hemocytometer. The increase in the number of cells at 5 days postseeding was determined.

Cloning Efficiency. The cloning efficiency of the transformed cells was determined according to Ku and Chen's method (11). Briefly, double-strength growth medium was seeded with either 3×10^2 or 3×10^3 cells and mixed with an equal volume of 1% agarose to yield a final concentration of 0.5% agarose. The seeded soft medium was overlaid onto an agarose base layer medium in a 60-mm culture dish and incubated at rt. Duplicate plates were carried out for each seeding.

Results

Transfection Experiments. In these experiments, 18- and 36-hr-old primary cultures of lymphoid (Oka) cells were transfected with the plasmid (pSV-3 neo), an 8.6-kb construct containing gene sequences from SV-40, pBR 322, and the antibiotic resistance markers G-418 and ampicillin (ATCC 37150). Transfection was accomplished with either naked or liposome-coated DNA.

An 18-hr-old nontransfected fibroblast-like cell culture prepared from shrimp lymphoid (Oka) organ is shown in Figure 1a. Transfected cells which were initially fibroblast-like began to detach from the surface of the culture flask at 5 days post-transfection (pt) and foci of morphologically distinct round cells appeared. By 7 to 9 days pt, these foci of cells gave rise to loosely adhesive grape-like arrangements of aggregated cells (Fig. 1 b, c, d, and e). A similar sequence of events

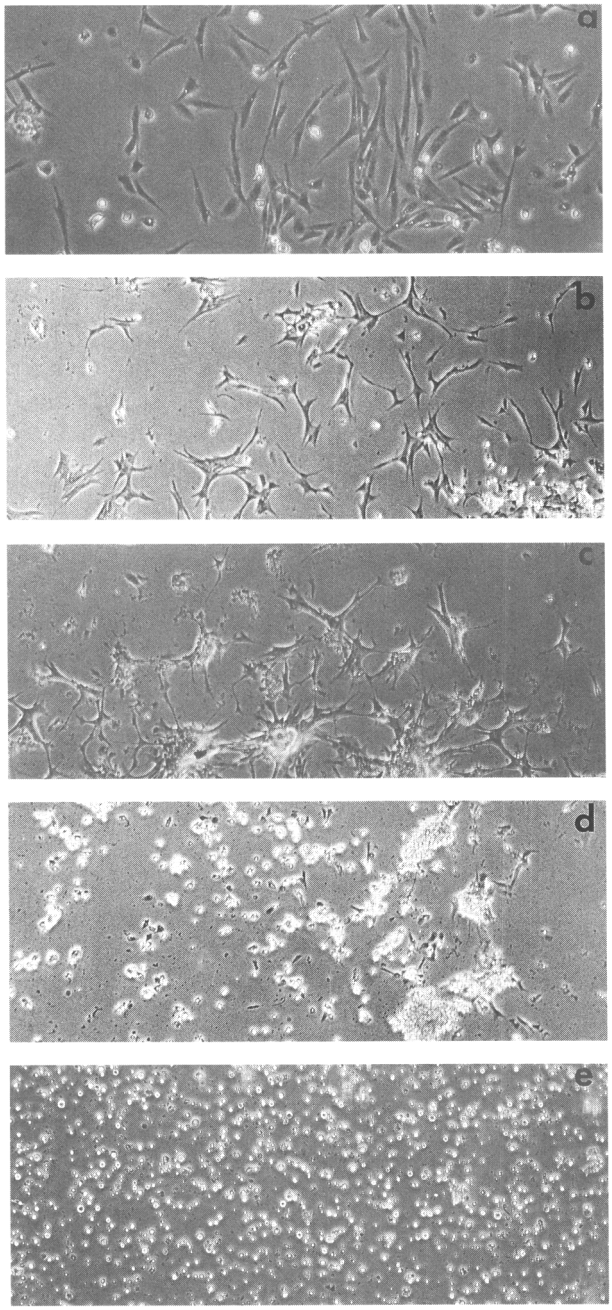

Figure 1. Morphological changes undergone by OKTr-1 after transfection with naked DNA: (a) Eighteen-hour-old primary culture of the shrimp lymphoid organ exhibited fibroblast-like morphology; (b) 2 days pt, the cells still showed normal morphology; 4 days later (c), the cells became constricted and eventually formed foci of round cells. The normal fibroblast-like cells detached from the culture vessel. By Day 9–12, some cells aggregated and formed grape-like clusters of cells (d). Thirty-seven days pt, only the morphologically transformed (round) cells remained in culture (e). Original magnifications: Panel a, ×200; Panel b–e, ×100.

occurred in all three treatments and the transformed cells from these treatments were designated as follows: OKTr-1 (from naked DNA); OKTr-23 (from 3μg DNA-lipofectin); and OKTr-25 (from 5 μg DNA-lipofectin). To date, the transformed cells have undergone 44, 18, and 3 passages, respectively. Passage number in these studies represent the splitting of the transformed cells at a 1:3 ratio weekly. Primary cultures of Oka cells were not successfully passaged beyond the establishment of the explant culture.

When 36-hr-old primary lymphoid (Oka) cultures were transfected similarly as above with the naked plasmid DNA or DNA-Lipofectin, no morphological transformants were observed. All cultures deteriorated after 5 days in G-418-supplemented Medium D.

Sterility tests for bacteria and fungi were negative even after 10 days incubation at 25°C or 37°C.

Revival of Liquid Nitrogen-Frozen Cells. Frozen cells, upon revival after storage for 48 hr in liquid nitrogen, initially showed a one log decrease in viability count but readily recovered after 48 hr of incubation. After 8-months' storage, the cells remained viable upon revival.

Growth of Transformed Cells in Different Media. Various media were examined to find a suitable medium for the cultivation of OKTr-1 and OKTr-23 cells. Although Medium 199 proved to be a good medium for both transformants as shown by the 13.9- and 8.5-fold increase in OKTr-1 and OKTr-23 cell numbers, respectively (Table I), L-15 was found to show better buffering capacity than Medium 199 and was the medium used for the maintenance of the cells and for future experimental studies. No increase in cell number was observed in Mitsuhashi and Maramorosch Insect Medium (MMIM). With Grace's Insect Medium (GIM), a 0.6-fold decrease in cell number of OKTr-1 transformants occurred (Table I). OKTr-23 exhibited increase in cell number in all the media examined. All the growth media contained the antibiotic Geneticin 418.

Table I. Effect of Various Media on Growth of the Transformed Cells at rt

Cell Culture/ Medium	0 Days ($\times 10^6$)	5 Days ($\times 10^6$)	Fold Increase (Decrease)
OKTr-1			
L-15	1.8	4.2 ± 0.8	2.3
M 199	1.0	13.9 ± 1.4	13.9
RPMI-1640	1.8	10.5 ± 1.5	5.8
GIM	1.8	1.0 ± 0.1	(0.6)
MMIM	1.8	1.8 ± 0.2	0
OKTr-23			
L-15	0.8	2.4 ± 1.1	3.0
M 199	1.1	9.4 ± 0.2	8.5
RPMI-1640	0.8	2.2 ± 0.4	2.8
GIM	0.8	5.0 ± 1.5	6.2
MMIM	0.8	8.5 ± 1.5	10.6

Note. All media were purchased from Sigma Chemicals Co., St. Louis, MO. Each medium was supplemented with the following: 20% FBS, 8% shrimp extract, 100 IU/100 μg per ml penicillin/streptomycin, 20 ng/ml epidermal growth factor, 10 units/ml interleukin-2, salt solution for adjusting osmolarity to 705–715 mOsm/kg plus G-418. The pH of the media was adjusted to 7.5 (except for GIM, which was adjusted to 6.5). L-15 = Leibovitz L-15 Medium; M 199 = Medium 199; RPMI-1640 = Roswell Park Memorial Institute-1640; GIM = Grace's Insect Medium; MMIM = Mitsuhashi and Maramorosch Insect Medium.

Growth in 0.5% Agarose. When cloned in 0.5% agarose, single cells of both OKTr-1 and OKTr-23 transformants formed colonies after 14 days of incubation at 28°C. The OKTr-1 cultures showed a higher cloning efficiency (68.7%) than OKTr-23 (36.7%).

Indirect Immunofluorescence Staining for SV-40 (T) Antigen. Employing the indirect immunofluorescence antibody technique, the majority (>90%) of the transformed cells both at the early passages and at the later passages (24th for OKTr-1 and 13th for OKTr-23) were found to stain positively for SV-40 (T) antigen (Fig. 2A). In contrast, controls consisting of both transformed cells stained only with FITC-labeled secondary antibody (Fig. 2B), and untransformed pri-

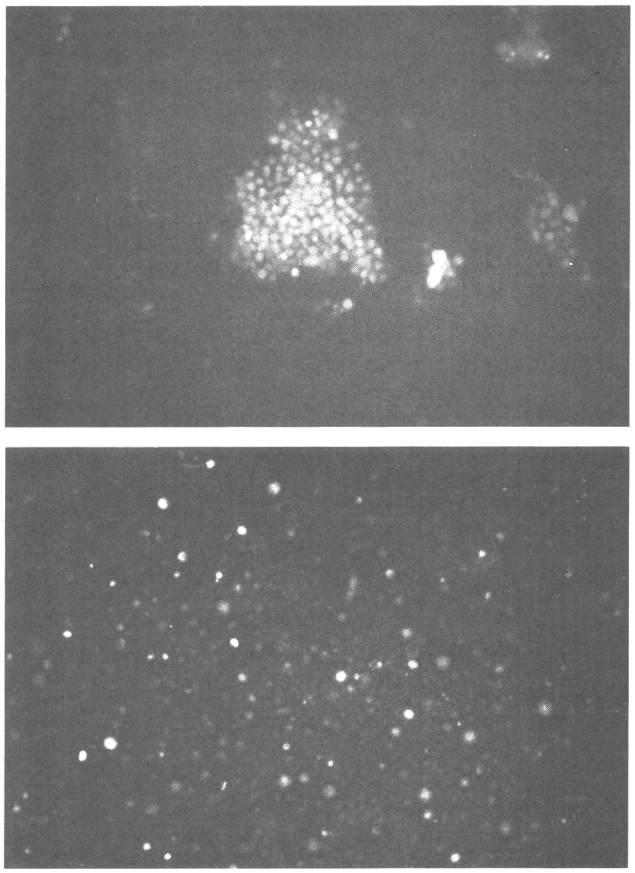

Figure 2. (A) Stained smear of OKTr-1 (24th passage) treated with mouse anti-SV-40 (T) antigen (primary antibody) and FITC-labeled goat anti-mouse antibody. (Original magnification: ×200.) (B) OKTr-1 (24th passage) stained only with FITC-labeled secondary antibody. (Original magnification: ×200.)

mary cells treated with primary and labeled secondary antibodies did not stain for the (T) antigen.

Discussion

The object of the present study to extend the culture life span of penaeid shrimp cells, conditionally identified as lymphoid in origin, was successfully achieved by transfection of primary cells with pSV-3 neo containing SV-40 gene sequences. To the best of our knowledge, this represents the first report of a stable transformation event induced by genes of an oncogenic mammalian virus in cells derived from a marine invertebrate animal. All previous attempts to develop a continuous cell line from penaeid shrimp have not been successful (1, 3–6). The transformation event led to enhanced cell proliferation and the extension of the life span of the cells. In addition, the growth and morphology of the cells were altered. Spindle-shaped fibroblast-like primary explant lymphoid cells which were adhesive to the solid surface of the culture container were converted to less adhesive round cells. These cells grew more rapidly assuming grape-like arrangements reminiscent of EBV-transformed lymphoblast cells. Furthermore, single cells formed colonies in semisolid medium, a characteristic typical of transformed cells. The progenitor of the transformed cells could not be ascertained since the lymphoid organ may consist of more than one cell type. In the hematopoietic nodules of the ridgeback prawn, a close relative of the penaeid shrimp, at least seven cell types have been reported (12). Of these cell types, four are hemocytes, which are involved in phagocytosis (13), wound repair (14), and other cellular functions.

The present results suggest that the transfection phenomenon is relatively sensitive to the DNA concentration used since transformation occurred only in cell cultures transfected with either 3 or 5 μg of DNA. Furthermore, the age of the cell culture plays an important role in the transfection process since no transformation was observed in 36-hr-old primary cultures. In contrast, the younger 18-hr-old cultures, which may contain a greater proportion of actively dividing cells, were more susceptible to transfection. It is also interesting to note that transfection was achieved whether Lipofectin was present or not. The reason for this remains to be elucidated.

The structure and function of SV-40 large (T) antigen have been comprehensively reviewed in a recent article (16). In the present study, expression of the SV-40 antigen(s) was correlated with transformation and successful transfection was also associated with the expression of the antibiotic resistance marker G-418. The exact mechanism of transformation in this case is not known. In mammalian systems, particularly the human mammary epithelial cells transformed with SV-40 (T) antigen, immortalization is thought to occur due to the bypass and inactivation of two mortality stages, M1 and M2, respectively, which are controlled by several genes (15). According to the M1/M2 model, the SV-40 (T) antigen neutralizes or inactivates the M1 genes (probably including the tumor suppressors retinoblastoma, or RB, and p53 genes) and allows the cells to proliferate beyond 20–30 times until a second antiproliferative mechanism, the M2 stage, is reached. When M2 gene(s) are inactivated by a second mutational event, immortalization ensues. Whether such a similar mechanism exists in the current transformation event of shrimp cells is yet to be resolved.

Chromosomal analyses of the transformed cells are currently being undertaken. Presently, we are also testing the susceptibility of the transformed cells against Rhabdovirus of penaeid shrimp (RPS) and other poikilothermic viruses in our laboratory. Further characterization of the transformed cells (particularly at the molecular level) is currently being pursued.

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