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# ESTABLISHMENT AND CHARACTERIZATION OF 13 CELL LINES FROM A GREEN TURTLE (*CHELONIA MYDAS*) WITH FIBROPAPILLOMAS

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## SUMMARY

Thirteen cell lines were established and characterized from brain, kidney, lung, spleen, heart, liver, gall bladder, urinary bladder, pancreas, testis, skin, and periorbital and tumor tissues of an immature male green turtle (*Chelonia mydas*) with fibropapillomas. Cell lines were optimally maintained at 30° C in RPMI 1640 medium supplemented with 10% fetal bovine serum. Propagation of the turtle cell lines was serum dependent, and plating efficiencies ranged from 13 to 37%. The cell lines, which have been subcultivated more than 20 times, had a doubling time of approximately 30 to 36 h. When tested for their sensitivity to several fish viruses, most of the cell lines were susceptible to a rhabdovirus, spring viremia carp virus, but refractory to channel catfish virus (a herpesvirus), infectious pancreatic necrosis virus (a birnavirus), and two other fish rhabdoviruses, infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus. During in vitro subcultivation, tumor-like cell aggregates appeared in cell lines derived from lungs, testis, and periorbital and tumor tissues, and small, naked intranuclear virus particles were detected by thin-section electron microscopy. These cell lines are currently being used in attempts to isolate the putative etiologic virus of green turtle fibropapilloma.

*Key words:* green turtle fibropapilloma; primary culture; cell line; tumor; turtle virus.

## INTRODUCTION

Fibropapillomas were originally documented as a rare disease of green turtles (*Chelonia mydas*) in Hawaii in 1958 (2). Epidemic spread of this debilitating, frequently fatal tumor now significantly threatens the survival of this marine reptile (2,3,27), which is protected under provisions of the U.S. Endangered Species Act (12). The disease, which disrupts the daily activities of affected animals, is characterized by the formation of lobulated fibrous tumors, frequently around the neck, eye, mouth, flipper and/or oral cavity, and occasionally within visceral organs, such as lung, kidney, and heart.

Recent studies have implicated viruses, notably a herpesvirus and retrovirus, as the potential cause of green turtle fibropapilloma (GTFP) (1,6,13,25). However, to date, no virus has been isolated in cell culture, and the pathogenetic role of herpesviruses or retroviruses has not been established (11). A major obstacle to isolating the causative agent of GTFP has been the lack of convenient and sensitive in vitro cell culture systems. Viruses are obligatory intracellular parasites and their in vitro replication requires permissive cell lines. In addition to specific cell-tropism requirements, some viruses can be propagated only in cell lines derived from the same host species. Although three green turtle cell lines have been established previously, including an epithelial cell line derived from skin (16) and fibroblastic cell lines from cutaneous fibropapillomas (20) and green turtle embryos (24), there has been no documentation of virus isolation in these cell lines. In this report, we describe the successful establishment and characterization of 13 cell lines from various tissues/organs of a green turtle with fibropapillomas in an effort to rescue the possible latent viruses and to facilitate the isolation and identification of the possible etiologic virus(es) of GTFP.

## MATERIALS AND METHODS

*Primary cultures.* A severely debilitated, immature male green turtle (National Marine Fisheries Service, identification no. 04-11-97 straight carapace length, 72.3 cm; weight, 27 kg), found stranded in the Hawaiian Islands and suffering from several fibropapillomas around the mouth and eyes, was euthanized. Various tissues and organs were collected, including tumor tissues as well as normal-appearing kidney, lung, heart, spleen, liver, neck skin, pancreas, gall bladder, urinary bladder, testis, and periorbital tissue. Tissue samples were incubated at room temperature (25 ± 2° C) for 2 h in antibiotic incubation medium (AIM) consisting of 2× RPMI 1640, 1000 μg streptomycin per ml, 1000 IU penicillin per ml, 25 μg amphotericin B per ml, and 250 μg gentamicin per ml. Following two additional washes in AIM (15 min/wash), tissue samples were minced with sterile instruments, and 20–30 tissue fragments were explanted in each 25-cm² Primaria-brand tissue culture flask (Primaria, Falcon). In addition, minced tissue fragments were digested with 0.25% trypsin at 30° C for 1 h. Dissociated cells were then washed once with RPMI 1640 medium, and approximately 1 to 2 × $10^6$ cells were transferred into each of several 25-cm² Primaria-brand tissue culture flasks.

Primary cell cultures were initially maintained in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories Inc., Logan, UT) and antibiotics (200 U penicillin, 200 μg streptomycin, 50 μg gentamicin, and 5 μg amphotericin B/ml), according to pre-

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TABLE 1

MORPHOLOGICAL CHARACTERISTICS OF CELL LINES ESTABLISHED FROM AN IMMATURE MALE GREEN TURTLE WITH FIBROPAPILLOMAS AND THEIR SUSCEPTIBILITY TO FIVE FISH VIRUSES

| Cell line | Source | Morphology | Passage | Fish virus[a] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | CCV | IPNV | IHNV | SVCV | VHSV |
| GT-UB | Urinary bladder | Fibroblastic | 20 | — | — | — | — | — |
| GT-BR | Brain | Fibroblastic | 22 | — | + | — | + | — |
| GT-PS | Pancreas | Fibroblastic | 24 | — | ND | ND | — | — |
| GT-SK | Neck skin | Fibroblastic | 26 | — | — | — | + | — |
| GT-HT | Heart | Fibroblastic | 28 | — | ND | — | + | — |
| GT-EYE | Periorbital tissue | Fibroblastic | 36 | — | — | ND | + | — |
| GT-TM | Tumor | Fibroblastic | 38 | — | — | — | + | — |
| GT-LG | Lung | Fibroblastic | 40 | — | + | — | + | — |
| GT-LV | Liver | Epithelial | 20 | — | ND | ± | + | — |
| GT-KD | Kidney | Epithelial | 22 | — | + | — | + | — |
| GT-SP | Spleen | Epithelial | 24 | — | + | ± | + | — |
| GT-GB | Gall bladder | Epithelial | 28 | — | — | — | — | — |
| GT-TS | Testis | Epithelial | 32 | — | — | — | + | — |

[a]CCV, channel catfish virus; IHNV, infectious hematopoietic necrosis virus; IPNV, infectious pancreatic necrosis virus; SVCV, spring viremia carp virus; VHSV, viral hemorrhagic septicemia virus; ND, not done; +, cytophathic effect; —, no cytopathic effect.

viously described methods (9,18). Cell cultures were incubated at 30° C, and when monolayers were confluent, cells were subcultivated at a ratio of 1:2 every 5 to 7 d with a 0.25% trypsin-versene solution (Sigma Chemical Co., St. Louis, MO). Concentrations of FBS and antibiotics were reduced by half after the 10th passage.

*Growth studies.* Growth characteristics of the newly established turtle cell lines were evaluated at selected temperatures, as well as with various media and different concentrations of FBS. To determine the incubation temperature for optimal cell growth, 1–2 × $10^5$ turtle cells at the 12th or later passage were seeded in 25-$cm^2$ tissue culture flasks (Corning Labware, Corning, NY), and cells were incubated at 15, 20, 25, 30, and 37° C. On alternate days for a total of 14 d, cells from two or three flasks at each temperature were trypsinized, then counted with a hemocytometer; average cell counts were calculated. In the same manner, the effects of differing concentrations of FBS (nil, 5, 10, and 20%) and of different tissue culture media (RPMI 1640, medium 199, L15 and minimum essential medium) on cell growth was assessed in duplicate 25-$cm^2$ flasks incubated at 30° C (18).

*Cryogenic preservation and stability in liquid nitrogen.* The ability of green turtle cells to be preserved in liquid nitrogen was examined with a previously described technique (9). Briefly, cells growing logarithmically were harvested and prepared at a high cell density suspension of 2–5 × $10^6$ cells/ml in RPMI-1640 culture medium containing 20% FBS and dimethyl sulfoxide (DMSO) at a final concentration of 10%. Aliquots of each cell suspension were dispensed into 1-ml sterile plastic vials (Nalgenunc, Naperville, IL), sealed, and processed for storage in liquid nitrogen. At storage Day 180, the frozen cells were rapidly thawed, and cell viability was assessed by trypan blue exclusion.

*Plating efficiency.* Plating efficiencies of turtle cell lines derived from tumor, lung, and periorbital tissues were ascertained by the method described by Fryer and colleagues (10). Green turtle cells were diluted in RPMI 1640 containing 10% FBS and incubated at 30° C for 14 d. Plating efficiencies were calculated according to the method of Middlebrooks and coworkers (23).

*Chromosomal analysis.* Chromosome counts of green turtle cell lines derived from tumor (GT-TM), testis (GT-TS) and kidney (GT-KD) were performed at Passage 8, as described by Earley (8). A total of 80 or more randomly selected metaphase spreads were counted for each cell line with phase-contrast microscopy.

*Viral susceptibility.* Green turtle cell lines were tested for their susceptibility to five fish viruses: channel catfish virus (CCV, a herpesvirus), infectious hematopoietic necrosis virus (IHNV, a rhabdovirus), infectious pancreatic necrosis virus (IPNV, a birnavirus), spring viremia carp virus (SVCV, a rhabdovirus) and viral hemorrhagic septicemia virus (VHSV, a rhabdovirus). Virus stocks were prepared and virus yields were determined in chinook salmon embryo cells (CHSE-214) for IHNV and IPNV, whereas SVCV and VHSV were titered in epithelioma papulosum cyprini cells (EPC) and CCV was titered in brown bullhead (BB) cells. Infectivity titers were based on cytopathic effect (CPE) observed at 7 d following inoculation. Viral infectivity assays in green turtle cell lines were performed as described previously (17), except that cells were incubated at 30° C until a confluent monolayer was achieved, then transferred to an incubation temperature optimal for virus production following inoculation with 2–5 × $10^4$ $TCID_{50}$ (50% tissue culture infective dose) of each virus. Susceptibility of green turtle cell lines to fish viruses was based on appearance of characteristic, virus-specific CPE.

*Transmission electron microscopy.* Turtle cells exhibiting formation of tumor-like cell aggregates and showing normal-appearing fibroblastic skin (GT-SK) cells were fixed in 2.5% glutaraldehyde prepared in 0.05 *M* cacodylate buffer (pH 7.0), then postfixed with 2% osmium tetroxide, and embedded in Spurrs, as described previously (19). Ultrathin sections of 700–1000 Å were stained with 2% uranyl acetate and lead citrate and examined with a Zeiss 10/A electron microscope at 80 kV.

## RESULTS

*Cell morphology.* Thirteen cell lines were established from tissues of an immature male green turtle with fibropapillomas. These cell lines have been passaged more than 20 times since their initiation in April, 1997. Morphologically, cell lines derived from lung, heart, brain, neck skin, pancreas, urinary bladder, and periorbital and tumor tissues appeared fibroblast-like, whereas those derived from kidney, spleen, gall bladder, liver and testis appeared more epithelial (Table 1, Fig. 1).

*Growth characteristics.* A comparison of the explant technique and enzymatic disaggregation of tissues in initiation of the growth of green turtle cells indicated that both of these methods were equally good for primary cultivation of turtle cells. Following the first passage, cells derived from the same tissue/organ were pooled and subcultured. Initial growth of green turtle cells derived from lung (GT-LG), kidney (GT-KD), testis (GT-TS), pancreas (GT-PS), and periorbital tissue (GT-EYE) was observed 2 to 3 d following seeding of minced tissue explants or dissociated cells. Cell colonies of various sizes formed within 7 d, and confluent cell monolayers were achieved by Days 9 to 12. By contrast, initial growth of cell lines derived from liver (GT-LV), brain (GT-BR), spleen (GT-SP), skin (GT-SK), gall bladder (GT-GB), and urinary bladder (GT-UB) was somewhat slower, becoming evident only after 6 to 7 d of incubation.

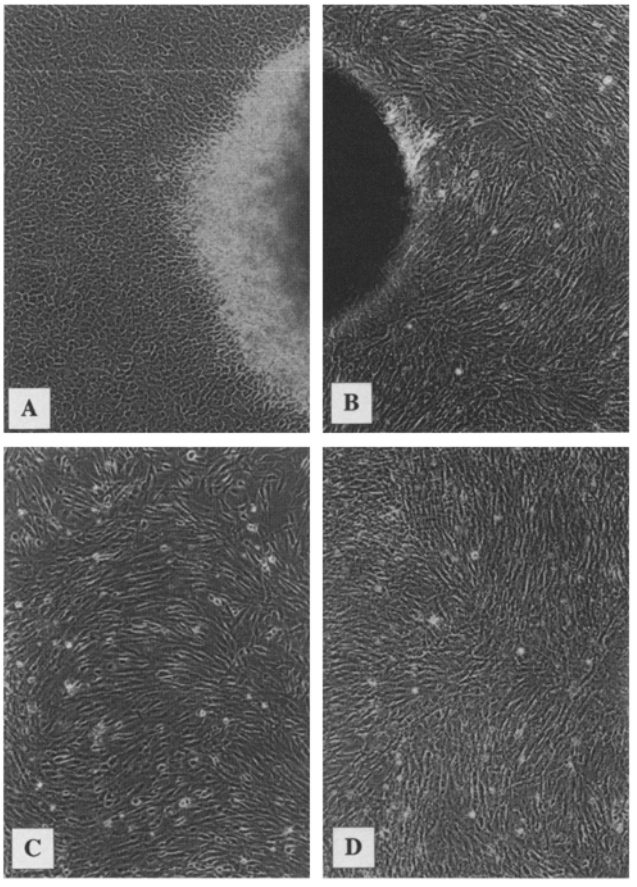


FIG. 1. Photomicrographs of cell lines derived from green turtles. Primary cell cultures derived from explants of (*A*) periorbital and (*B*) tumor tissue; cell monolayers of (*C*) heart and (*D*) lung cells at Passage 8. Magnification, 120×.

TABLE 2

SOME CHARACTERISTICS OF GREEN TURTLE CELL LINES

| | |
|---|---|
| Optimum growth medium | RPMI 1640 |
| Optimum growth temperature | 30°C |
| Plating efficiency (%) | 13–37 |
| FBS requirement | Yes |
| Doubling time[a] | 30–36 h |
| Morphology | Epithelial/fibroblastic |
| Chromomosomal number | 33–88 |

[a]Doubling time was determined while cells were at exponential growth phase.

Tumor-derived cells exhibited a moderate rate of initial growth, but cells replicated more slowly, with the formation of a cell monolayer after 15 d. Despite differences in initial growth rate, all cell lines grew equally well following the first subcultivation and had a doubling time of approximately 30 to 36 h (Table 2). Optimal growth occurred at 30° C with RPMI 1640 supplemented with 10% FBS (Fig. 2). A similar growth pattern was noted for cell lines derived from periorbital and tumor tissues. Although these cells were able to grow at incubation temperatures between 20 and 25° C, replication

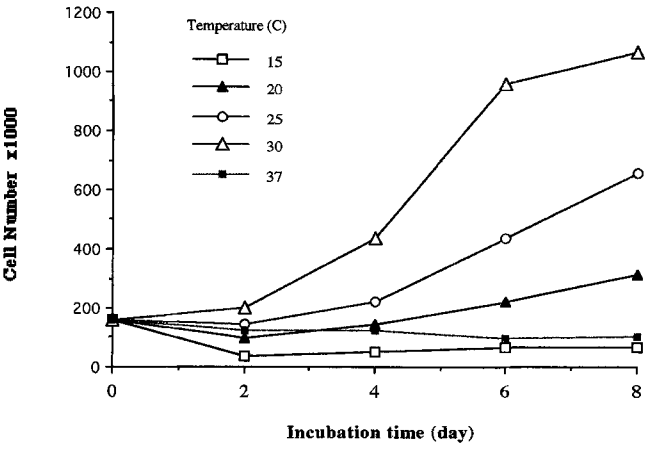


FIG. 2. Comparison of incubation temperatures on growth of green turtle tumor cells (GT-TM, at passage 14 in RPMI 1640 containing 10% fetal bovine serum.

rates were much lower than at 30° C. Cell growth was not observed when cultures were incubated at 37° C or 15° C (Fig. 2).

Comparison of medium 199, L-15, minimum essential medium, and RPMI 1640 to support cell growth indicated that while turtle cells were able to grow well in all media tested (data not shown), cell growth was superior with RPMI 1640. GT-TM and GT-EYE cells grew well in media containing 10 to 20% FBS, with considerably slower growth when 5% FBS was used. No cell growth was observed when FBS was absent in the culture medium. Green turtle cells were stable when stored in liquid nitrogen and analysis of cryopreserved GT-LG, GT-TM, GT-HT, and GT-EYE cell lines showed 90–94% viability after storage in liquid nitrogen for 6 mo. (data not shown).

*Plating efficiency.* Plating efficiencies of GT-TM, GT-EYE, and GT-LG cell lines, as determined at Passages 12 to 14, were relatively low (27%, 19% and 13%, respectively), suggesting that these turtle cell lines, including that derived from tumor tissue, were not transformed.

*Chromosomal analysis.* As judged by chromosomal analysis, turtle cell lines at Passage 8 were heteroploid. From counts of more than 80 metaphase plates in each of three cell lines, the number of chromosomes in GT-TM, GT-TS and GT-KD ranged from 33 to 88, with a vague peak of 45 to 47 for GT-TM and GT-TS and 51 to 53 for GT-KD (data not shown). Turtle chromosome complements were very similar to those identified in avian species. In addition to metacentric, submetacentric, and acrocentric chromosomes, microchromosomes were also frequently identified (30–40%).

*Viral susceptibility.* The 13 turtle cell lines exhibited a similar pattern of sensitivity to the five fish viruses tested. All cell lines except GT-GB, GT-UB, and GT-PS were susceptible to SVCV (Table 3). CPE, which appeared 4 d following inoculation with SVCV, became more extensive with time (data not shown). By contrast, all cell lines were refractory to IHNV, VHSV, and CCV, as evidenced by the absence of CPE. Similarly, except for GT-LG, GT-KD, and GT-SP cells, the other cell lines were not susceptible to IPNV.

*Formation of tumor-like aggregates.* During serial subcultivation, formation of cell aggregates was seen in cell lines derived from testis (GT-TS), lung (GT-LG), periorbital (GT-EYE) tissue, and tumor (GT-TM) (Fig. 3). These aggregates were observed as early as the third passage, initially along the edges of the culture flask, then becoming

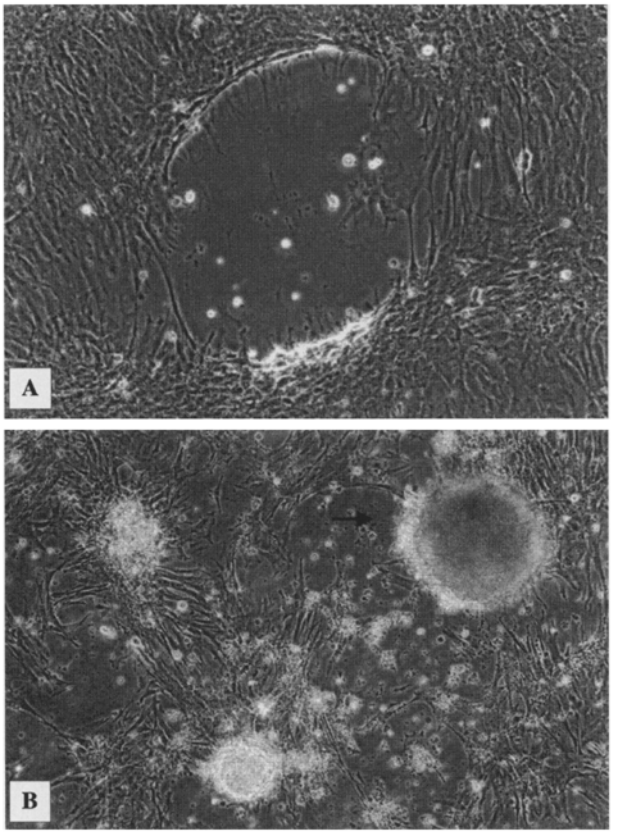


FIG. 3. In vitro formation of tumor-like aggregates in cell line derived from the testis of a green turtle with fibropapillomas. *A*, Initiation of cell aggregation at incubation Day 5, and *B*, formation of tumor-like aggregate at Day 12 (*arrow*). Magnification, 150×.

more extensive over the entire growing surface following subcultivation. These cell aggregates increased rapidly in size and density, attaining diameter measurements of approximately 1 to 2 mm by Day 20 (Fig. 3 *b*). Morphologically identical tumor-like aggregates sharing a similar pattern of growth progression were observed in the GT-LG, GT-TS, GT-EYE, and GT-TM cell lines.

Aggregated GT-LG cells were fixed in formalin and embedded in paraffin. Immunocytochemical staining of cell sections with a rabbit anti-cow muzzle epidermal keratin serum (subunits L12824, DAKO) revealed the deposition of cytokeratin in the peripheral layer of the cell aggregates (data not shown). Similar staining was observed in the epidermis of tumor tissue sections.

Electron microscopic examination of thin sections of tumor-like cell aggregates in the GT-LG, GT-TM and GT-TS cell lines revealed large deposits of collagen both intracellularly and extracellularly. These collagen proliferation centers varied in size and shape (round to oval), and were less electron-dense than adjacent cells. In addition, intranuclear, naked virus particles, measuring 50 ± 5 nm in diameter and resembling papovaviruses, were visualized in the GT-LG, GT-TM, and GT-TS cell lines (Fig. 4). In contrast, examination of normal-appearing GT-SK cells revealed some extracellular collagen deposition but no intracellular viral particles.

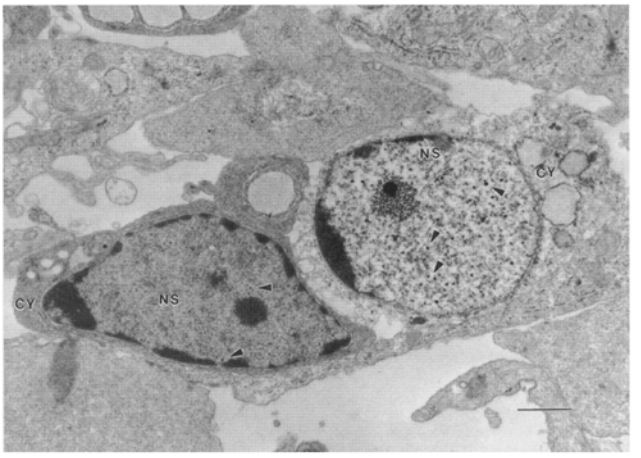


FIG. 4. Photoelectromicrograph of ultrathin section of green sea turtle cell line derived from the testis, showing small, naked virus particles within the nuclei of two cells at different stages of infection. The nucleus of the right cell contains many scattered mature virus particles (*arrowheads*), whereas the nucleus of the left cell contains only a few individual virions (*arrowheads*). NS, nucleus; CY, cytoplasm. Bar = 1 μm.

## DISCUSSION

Recent demonstration of tumor induction in healthy young turtles inoculated intradermally with cell-free homogenates of fibropapilloma strongly suggests a viral etiology for this slow-growing tumor (13). However, no infectious virus has been isolated in cell culture and the causative agent of GTFP has not been identified (11). Establishment of 13 cell lines derived from multiple tissues of a green turtle with fibropapillomas will greatly enhance attempts to isolate and characterize the presumed virus of GTFP. The 13 cell lines replicated well under the described condition, were easy to maintain, and had high viability following storage in liquid nitrogen. As such, the availability of these turtle cell lines from various tissues/organs will provide a useful in vitro cell culture system for exploring the existence and disease potential of other viruses of green turtles.

In vitro propagation of selected green turtle cell lines indicated that growth was maximal at 30° C, which was the temperature selected for the establishment of these turtle cell lines. It should be noted that this optimal growth temperature was not due to the initial adaptation of green turtle cells cultivated at 30° C since the initial growth of green turtle cells progressed much slower when an alternative temperature (25° C) was selected (data not shown). This optimum growth temperature was identical to that reported for three cell lines previously established from green turtles (16,20,24). Interestingly, the incubation temperature for maximal growth was slightly higher than the preferred temperature (21–28° C) of green turtles in nature (15). Wolf (26) suggested culturing fish cells at temperatures slightly above that preferred by the intact animal, and cell lines established from other cold-blooded marine animals similarly show a differential temperature optimum for the host (17,21,22,26). It is currently unclear whether the optimal growth temperature of turtle cells is also optimal for the replication and expression of latent viruses.

All 13 cell lines showed no growth when incubated at either 15° C or 37° C. This result is consistent with that of Moore and coworkers (24) but conflicts with findings by Koment and Haines who demon-

strated rapid replication of turtle skin cells at 37° C and minor growth at 16° C (16). Despite their sensitivity to low temperature, these newly established green turtle cell lines remained viable over 3 mo. at 15° C after plating and attachment at 30° C. This characteristic suggests the possibility of using these cell lines to isolate cold-adapted or temperature-sensitive strains of turtle and other reptilian viruses.

Previously, an epithelial skin cell line derived from a green turtle has been shown to support the replication of several mammalian viruses (16). We did not test our cell lines for susceptibility to mammalian viruses. Instead, susceptibility of the green turtle cell lines to some fish viruses was evaluated because of the suggestion that other aquatic animals could serve as vectors of GTFP. In our study, SVCV, a carp rhabdovirus, replicated in 10 of the 13 green turtle cell lines. Members of the Rhabdoviridae family exhibit a wide host range, including the fruit fly, *Drosophila melanogaster* (5), and their ability to replicate in an unnatural or foreign host system or crossing species barriers has been well documented (4,7,14).

Currently, virtually nothing is known about the source(s) and the principal modes of transmission of the etiologic agent of GTFP. Our primary rationale for developing cell lines from a tumor-bearing turtle was to rescue and amplify potential virus(es) associated with GTFP by in vitro cultivation. The in vitro formation of tumor-like cell aggregates in cell lines derived from tumor, periorbital tissue, testis, and lung was an unanticipated finding. Eyes and lungs are reportedly the most common external and internal sites affected by fibropapillomas (27). Tumor-like aggregate formation has not occurred in other turtle cell lines established from the same animal under the same growth conditions. Our findings suggest preferential targeting or selection of tissue/organ by the infectious agent. These aggregates were successfully passaged by subcultivation and in healthy turtle cell lines inoculated with cell-free medium of aggregated cultures. Although we have not fully characterized the small, naked intranuclear virus particles in these tumor-like cell aggregates or determined its role in the etiology and pathogenesis of GTFP, these cell lines have added potential as an in vitro system in which to investigate tumorigenesis in green turtles.

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