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Journal of Virological Methods 86 (2000) 25–33

Journal of Virological Methods

www.elsevier.com/locate/jviromet

Identification of a small, naked virus in tumor-like aggregates in cell lines derived from a green turtle, *Chelonia mydas*, with fibropapillomas

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Received 1 September 1999; received in revised form 22 November 1999; accepted 23 November 1999

Abstract

Serial cultivation of cell lines derived from lung, testis, periorbital and tumor tissues of a green turtle (*Chelonia mydas*) with fibropapillomas resulted in the in vitro formation of tumor-like cell aggregates, ranging in size from 0.5 to 2.0 mm in diameter. Successful induction of tumor-like aggregates was achieved in a cell line derived from lung tissue of healthy green turtles, following inoculation with cell-free media from these tumor-bearing cell lines, suggesting the presence of a transmissible agent. Thin-section electron microscopy of the cell aggregates revealed massive collagen deposits and intranuclear naked viral particles, measuring 50 ± 5 nm in diameter. These findings, together with the morphological similarity between these tumor-like cell aggregates and the naturally occurring tumor, suggest a possible association between this novel virus and the disease. Further characterization of this small naked virus will clarify its role in etiology of green turtle fibropapilloma, a life-threatening disease of this endangered marine species.

Keywords: Green turtle fibropapilloma; *Chelonia mydas*; Cell line; Virus

1. Introduction

The incidence of green turtle fibropapilloma, a tumor disease of unknown etiology, has reached epidemic proportion during the past two decades

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0166-0934/00/$ - see front matter
PII: S0166-0934(99)00175-5

(Balazs, 1991; Balazs and Pooley, 1997; Herbst, 1994). These tumors, which range in size from a few millimeters to 30 cm or more in diameter, are frequently observed around the eyes, mouth, neck, flippers and tail, and less frequently in the parenchyma of lung, kidney and other organs (Work and Balazs, 1998). Not just a cosmetic problem, these tumors interfere with normal activities (including feeding and swimming) and threaten further the survival of the green turtle (*Chelonia mydas*), which lives and breeds in the waters surrounding the Hawaiian Archipelago and is protected under provisions of the US Endangered Species Act (Groombridge and Wright, 1982).

The causative agent(s) of green turtle fibropapilloma has not been identified, but considerable circumstantial evidence supports a viral etiology (Herbst, 1995; Aguirre and Spraker, 1996; Herbst et al., 1996; Balazs and Pooley, 1997; Casey et al., 1997; George, 1997; Quackenbush et al., 1998): electron microscopic examination of spontaneous and experimentally induced tumors has revealed herpesvirus-like particles (Herbst, 1995; Aguirre and Spraker, 1996); herpesviral DNA has been detected by the polymerase chain reaction using degenerate oligonucleotide primers designed from conserved regions of herpesviral DNA polymerases (Quackenbush et al., 1998); reverse transcriptase activity has been detected in tumor tissues (Casey et al., 1997); and green turtles, reared in captivity in Florida, have developed fibropapillomas following inoculation with cell-free homogenates of tumor tissues (Herbst et al., 1995). Collectively, these data suggest that green turtle fibropapilloma is caused by a filterable agent (Herbst et al., 1995). However, a virus has not been isolated in cell culture, and no definitive proof exists for the role of herpesviruses and/or retroviruses in the causation of fibropapilloma (Herbst, 1994; George, 1997).

To facilitate the isolation of the causative agent(s) of green turtle fibropapilloma, 13 cell lines, now in their 20th to 40th passage, have been established recently from various tissues of green turtles (Lu et al., 1999). This report describes the in vitro formation of tumor-like cell aggregates in cell lines derived from lungs, testis, periorbital and tumor tissues of an immature green turtle with fibropapillomas, and the identification of a small, naked intranuclear virus in such cell aggregates.

2. Materials and methods

Cell lines, derived from tissues of an immature male green turtle humanely euthanized due to massive tumors around the eyes, mouth and flippers, were maintained at 25–30°C in RPMI-1640 medium, supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT), epidermal growth factor (50 ng/ml), fibroblast growth factor (50 pg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (25 μg/ml) and amphotericin B (2.5 μg/ml), and were subcultured at 5- to 7-day intervals. During subcultivation, tumor-like cell aggregates were observed in cell lines derived from lung, testis, and periorbital and tumor tissues. To investigate the ultrastructural character of these tumor-like aggregates, cells were fixed for 30 min with 2.5% glutaraldehyde, prepared in 0.05 M cacodylate buffer (pH 7.0), and processed for thin-section transmission electron microscopy. Following fixation, cell aggregates were pelleted, rinsed, then post-fixed with 2% osmium tetroxide for 30 min. Ultrathin sections of 700–1000 Å, stained with 2% uranyl acetate for 45 min, followed by lead citrate for 8 min, were examined using a Zeiss 10/A electron microscope at 80 kV.

To detect viral sequences, DNA was extracted from cell lines by the conventional phenol/chloroform method. Cell DNA was ethanol precipitated and resuspended in TE buffer (pH 8.0) to a final concentration of 0.5 μg/μl. Amplification of herpesvirus DNA was conducted using consensus primers derived from herpesviral DNA polymerase gene, according to previously described methods (VanDevanter et al., 1996). DNAs were also tested for the presence of papillomaviral sequence by polymerase chain reaction (PCR), using several oligonucleotide primer pairs, including MY09/11 (Lorincz et al., 1992), L1C1/C2 (Yoshikawa et al., 1991) and JLP15/16 (Agostini et al., 1998) designed from highly conserved regions of the papovaviral genome. Amplification conditions for MY09/11 were 45 cycles, where each cycle consisted of the following steps: denaturation at 94°C, annealing

at 55°C, and extension at 72°C, 1 min per step. Thermocycling conditions for L1C1/C2 and JLP15/16 were 40 cycles at 94°C for 1 min, following by 48°C for 1 min and 72°C for 1 min, and 50 cycles at 63°C for 1 min followed by 94°C for 1 min, respectively. Ten microliters of the amplified PCR product was subjected to electrophoresis on 1–2% agarose gels.

3. Results

3.1. Morphological features of tumor-like aggregates

Green turtle lung-, periorbital-, and tumor-derived cell lines were fibroblastic in appearance (Fig. 1A), while green turtle testis cell line was

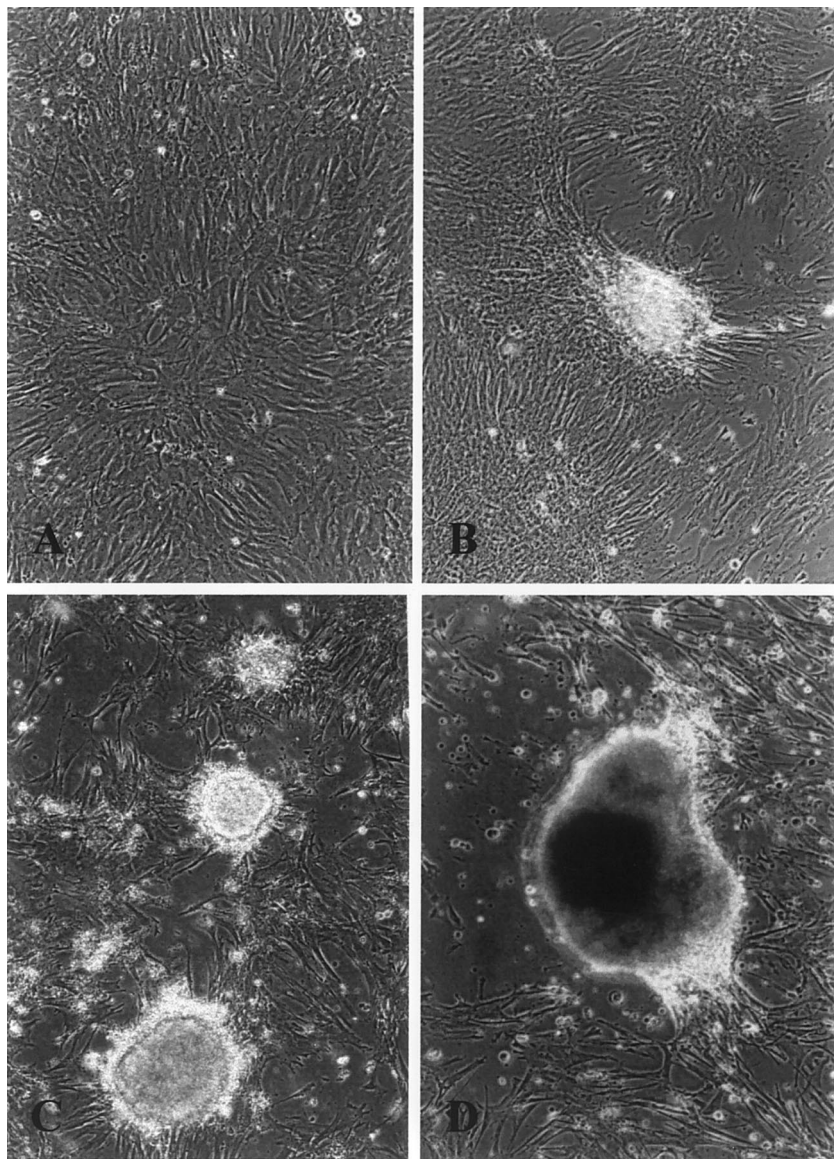

Fig. 1. Photomicrographs of in vitro formation of tumor-like aggregates in a cell line derived from lungs of a green turtle with fibropapilloma at various post-incubation times. (A) Normal fibroblastic lung cells; (B) day 5; (C) day 11 and (D) day 21.

more epithelial-like in character during primary culture. The morphology of testis-derived cells became fibroblastic from the sixth passage, at which time tumor-like cell aggregates were observed within this cell culture, as well as in green turtle tumor-, lung- and periorbital-derived cells. By contrast, this type of cell aggregation did not occur in other cell lines established from the same turtle. Moreover, seven cell lines have been established recently from lungs, heart, flipper skin, spleen, liver, brain and periorbital tissue of newly hatched green turtles, and no tumor-like aggregates developed in these cell lines following serial subcultivation (up to 11–19 passages) under identical growth conditions (data not shown).

The morphological appearance of the tumor-like cell aggregates was the same in all cultures. Formation of the aggregates was first detected along the edges of the culture flask; they then became more extensive over the entire flask surface following serial subcultivation. Initially, cell aggregates were characterized by coalescence of cells to form a dense center, which often resulted in an 'empty' space along or around the center (Fig. 1B). By incubation day 4–5, spindle-shaped cell aggregates containing multiple layers of cells became more distinct, but individual cells within the aggregates could still be differentiated (Fig. 1B). The size and density of the cell aggregates increased rapidly with time and ranged from 0.5 to 1.0 mm in diameter by day 10 (Fig. 1C). With prolonged incubation (15 days or longer), cell aggregates, now measuring approximately 1.0–2.0 mm in diameter, developed dark coloration within the central core and eventually detached from the flask surface (Fig. 1D). Digestion of cell aggregates with 0.25% trypsin-versene solution resulted in complete dissociation or dispersion during the early stage, but only partial dissociation occurred during the later stages. Cell growth and formation of new aggregates were observed when individual cell aggregates at the early developmental stage were isolated and reseeded into another flask.

To determine whether an infectious agent was associated with the formation of these cell aggregates, a fibroblast cell line derived from lungs of healthy green turtle hatchlings was inoculated with cell-free medium from green turtle lung, testis, periobital and tumor cell lines. At 6 days post-inoculation, aggregates became visible along the edge of the culture flask, and these aggregates then increased in size with prolonged incubation. By contrast, uninoculated healthy lung cell cultures exhibited no tumor-like aggregate formation during the 21-day incubation period at 25–30°C.

Immunocytochemical staining of paraffin-embedded sections of aggregated green turtle lung and tumor cells revealed the presence of keratin, using a rabbit anti-keratin serum prepared against cow muzzle epidermal keratin (subunits L12824, DAKO) (data not shown). Similar staining of the epidermis from the basal to upper layers was found in sections of tumor tissues.

3.2. Ultrastructural features of tumor-like aggregates

By thin-section transmission electron microscopy, massive deposits of collagen were visualized both intracellularly and extracellularly in the tumor-like aggregates (Fig. 2). The collagen deposits, which varied in number and shape, were either regularly bundled or disorderly tangled, and were confined largely adjacent to more electron-dense cells, in which small naked virus particles were detected.

Viral particles, which were round and naked, with an electron-dense nucleocapsid measuring 50 ± 5 nm in diameter (Fig. 3), were found exclusively within the nuclei of affected cells, either freely scattered (Fig. 3A) or largely aggregated as viral arrays (Fig. 3B). Aggregates of viral particles were seen commonly in affected nuclei. Viral envelopes and virus budding through the nuclear membrane were not observed.

Several types of distinct viral arrays were present usually within any given affected cell. Each of the arrays consisted of viral particles of rather uniform size, ranging from 40 to 55 nm in diameter. However, the number of virions and their relative density varied widely among the affected cells. Intracytoplasmic vacuoles were also observed in cells with intranuclear viral particles (Fig. 4A).

Infected cells were often oval in appearance (Fig. 3A and Fig. 4B) or occasionally more trian-

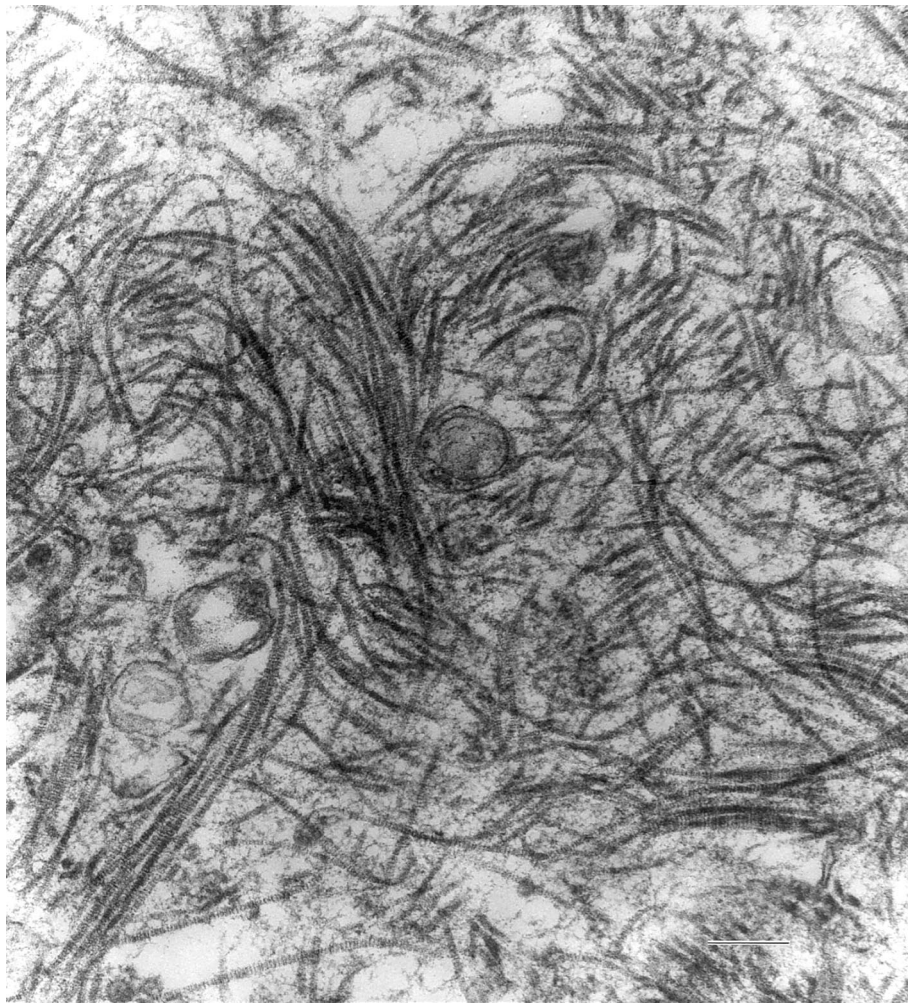

Fig. 2. Thin-section electron micrograph depicting massive collagen deposits appearing as disorganized tangles within the extracellular space of lung cells exhibiting tumor-like aggregates. Bar = 200 nm.

gular in shape (Fig. 3B), and contained very prominent enlarged nuclei (80–90%). The cytoplasm of affected cells was displaced to the perimeter (10–20%), and cellular organelles, such as mitochondria, had all but disappeared. These affected cells were electron dense and could be discriminated easily from unaffected cells. Heavily affected nuclei exhibited loss of heterochromatin. Nuclear degeneration was evident in nuclei containing many scattered virions (Fig. 3A). Many nuclei were hypertrophic, often appearing vacuolated with marginated and rarefied chromatin (Fig. 4). Hypertrophied nuclei containing naked virions were often observed (Figs. 3 and 4).

To detect papovavirus and herpesvirus sequences by polymerase chain reaction amplification, four sets of oligonucleotide primer pairs designed from highly conserved genomic regions of these viruses were used. Amplification of the DNAs prepared from the green turtle cell lines using the degenerate herpesviral primers resulted in no detectable product in any of these cell lines (Table 1). Similarly, no amplification was observed in these cell lines when polymerase chain reaction was carried out using the primers JLP15/16 and L1C1/C2 (data not shown). Amplification of the DNA using primer pair MY09/11 also

failed to generate a 480-bp fragment as expected in the positive control. However, a 235-bp fragment was detected consistently in all cell lines tested, except for cell lines derived from spleen and liver (Table 1 and Fig. 5). Genetic analysis of representative clones indicated sequence identity between different cell lines. Alignment and comparison of the 235-bp fragment showed a low degree of sequence similarity (36–44%) with some known animal and human papillomaviruses (data not shown).

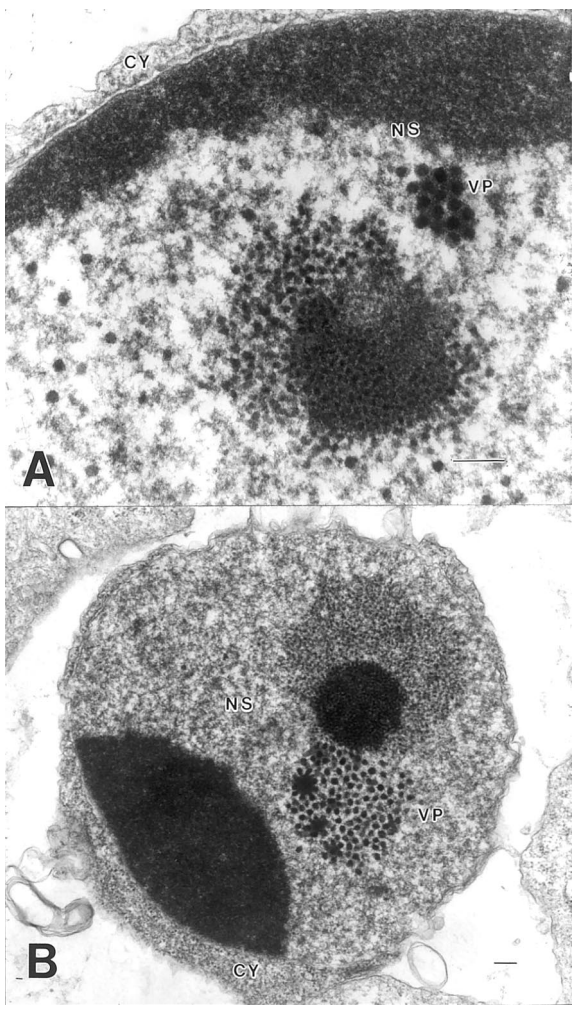

Fig. 3. Transmission electron micrographs of the GST-LG cell line, stained with 2% uranyl acetate and lead citrate. Naked virus particles appearing either (A) scattered or (B) crystallized within the nucleus of affected lung cells. VP, Virus particles; NS, nucleus; CY, cytoplasm. Bar = 200 nm.

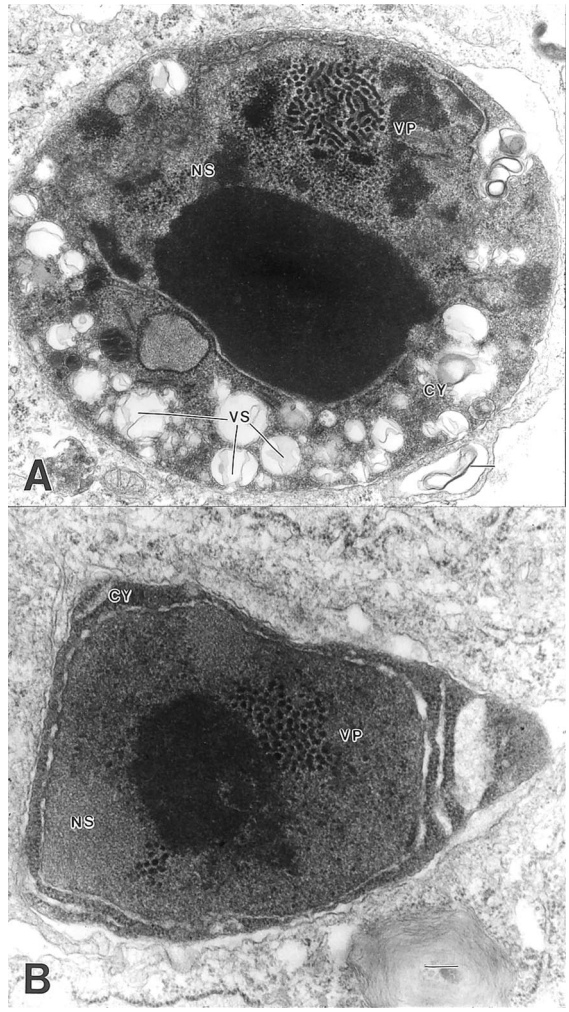

Fig. 4. Thin-section electron micrographs of green turtle lung cell line. (A) Vacuolar formation within cytoplasm and virus particles packaged within hypertrophic nuclei of affected cells; and (B) electron-dense cell with hypertrophic nucleus. VP, Virus particles; VS, vacuoles; NS, nucleus; CY, cytoplasm. Bar = 200 nm.

4. Discussion

Formation of tumor-like cell aggregates in cell lines derived from tissues of a tumored green turtle represents the first evidence, to our knowl-

edge, of in vitro tumor production within turtle cells. These cell aggregates occurred only in cell lines derived from lung, testis, periorbital and tumor tissues, not in cell lines established from other tissues, suggesting that certain cell types or tissues might be more sensitive to tumor induction and that they may serve as better primary targets for the etiologic agent(s) of green turtle fibropapilloma. Moreover, the successful induction of tumor-like aggregates in a cell line derived from healthy turtles inoculated with cell free-medium from tumor-like cell aggregates indicated that the aggregated cell lines contain a transmissible agent. Identification of small, naked viral particles from these tumor cells implies a possible etiologic role of this new viral isolate in the disease. Based on the size, morphology and intranuclear location, this small, naked virus most closely resembles papovavirus. This new virus has not been reported from green turtles and represents the first case of isolation a turtle virus in cell cultures derived from the same species.

Some members of the *Papovaviridae*, such as BK and JC polyomaviruses, can be grown in in vitro cell culture and produce cytopathic effects (Mahy and Kangro, 1996). However, papillomaviruses, the other genus, are highly species and tissue specific, and are generally refractory to propagation in vitro. There is no tissue culture system for the in vitro cultivation of papillomaviruses. Although the transmission electron microscopic findings suggest the presence of a papova-like virus, these data are preliminary and the taxonomic classification of this small, naked virus is unknown. Polymerase chain reaction amplification of DNAs prepared from these cell lines using primer pair MY09/11 designed from the conserved L1 gene of papillomaviruses produced no product of the expected size (480 bp), but a product of 235 bp. Sequence analysis showed a sequence similarity of 36–44% to some papillomaviruses of animals and human. Because of the smaller size and the low sequence similarity, it is unclear if the 235-bp product is the result of specific amplification of a reptilian papillomavirus. It should be noted that little is known presently for the reptile papovavirus and reptile papillomaviral sequence, and their genetic localization among other papovaviruses. Future approaches will be aimed toward obtaining more sequence and to explore the phylogenetic distance of this small naked virus to other members of *Papovaviridae*.

Table 1
Polymerase chain reaction detection of viral sequences in green turtle cell lines

Cell line	Source	Papillomavirus[a]	Herpesvirus[b]
GT-SK	Skin	+	–
GT-EYE	Periorbital tissue	+	–
GT-LG	Lung	+	–
GT-KD	Kidney	+	–
GT-HT	Heart	+	–
GT-SP	Spleen	–	–
GT-LV	Liver	–	–
GT-GB	Gall bladder	+	–
GT-BR	Brain	+	–
GT-TS	Testis	+	–
GT-TM	Tumor	+	–
GT-PS	Pancreas	ND	ND
GT-UB	Urinary bladder	+	–

[a] Polymerase chain reaction was performed using oligonucleotide primer pair MY09/11. ND, Not determined.

[b] Polymerase chain reaction was performed using degenerate herpesviral primers, as described by VanDevanter et al. (1996).

Recent studies have suggested herpesviruses and retroviruses as etiologic agents of green turtle fibropapilloma (Herbst, 1995; Aguirre and Spraker, 1996; Casey et al., 1997; Quackenbush et al., 1998). However, such viruses have not been isolated either in cell cultures or purified from tissues of affected animals. Therefore, the causative agent of green turtle fibropapilloma remains unknown and Koch's postulate has not been fulfilled (Herbst, 1994; George, 1997). Findings of intranuclear virions resembling papovavirus within cell lines derived from a tumored turtle and their association with tumor-like aggregate formation suggest that such particles could represent the causative agent of fibropapilloma. Further characterization of this virus will clarify its role in the pathogenesis of green turtle fibropapilloma. Members of the *Papovaviridae*, such as papillomaviruses, have been implicated in the

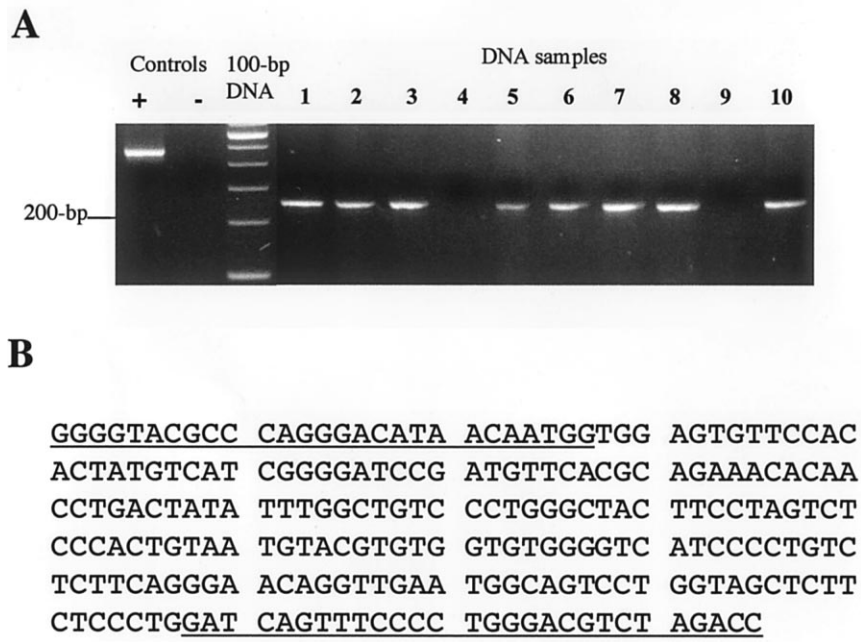

Fig. 5. (A) PCR amplification of a 235-bp sequence using oligonucleotide primer pair MY09/11 designed from the highly conserved L1 gene of papillomavirus. DNA extracted from green turtle cell lines was amplified and then subjected to 2% NuSieve gel electrophoresis. DNA samples represent GT-SK (lane 1), GT-HT (lane 2), GT-KD (lane 3), GT-SP (lane 4), GT-TS (lane 5), GT-LG (lane 6), GT-EYE (lane 7), GT-GB (lane 8) GT-LV (lane 9) and GT-TM (lane 10). (B) Nucleotide sequence of the 235-bp fraqment amplified using oligonucleotide primers MY09/11. Underlined portions represent positions of the PCR primers.

pathogenesis of various types of proliferative and neoplastic lesions in many animal species, including the Bolivian side-necked turtle (*Platemys platycephala*), green lizard (*Lacerta viridis*) and other reptiles (Cooper et al., 1982; Jacobson et al., 1982; Sundberg, 1987; Syrjanen, 1987; Cuzick, 1992; Levy et al., 1994). As evidenced by the localized epithelial hyperplasia with a defined boundary and intact basement membrane, the cutaneous fibropapillomas in green turtles resemble morphologically those induced by papillomavirus in other vertebrate species (Sundberg, 1987). Furthermore, the histological findings of perivascular lymphocytic infiltration, vacuole formation and epithelial degeneration also suggest papillomavirus infection (Dalto and Haguenau, 1973; George, 1997). These preliminary findings, as well as data published previously (Herbst, 1995; Herbst et al., 1996), suggest that the isolation of these candidate viruses will be important and essential to understand their etiologic role in green turtle fibropapilloma.

Green turtle fibropapilloma is a lethal disease that threatens the survival of *Chelonia mydas* in the waters around the Hawaiian Islands, Florida and elsewhere. Successful isolation and characterization of the etiologic agent(s) of the fibropapilloma are paramount for the development of long-term prevention and control strategies for this disease. Moreover, in-depth studies of this naturally occurring model of virus-induced tumorigenesis may provide important insights into the pathogenesis of certain human malignancies.

Acknowledgements

We thank Drs. George Antinelis and Frederick A. Murphy for helpful suggestions, Tina M. Weatherby for technical assistance in TEM, and Dr Takuya Iwasaki for immunocytochemical staining. This work was supported in part by the National Marine Fisheries Service Southwest

Fisheries Science Center, Honolulu Laboratory, and by grants from the Hawaii Community Foundation (435073), the University of Hawaii Research Council (3-89877) and the US Public Health Service (G12RR/AI-03061 from the Research Centers in Minority Institutions Program, National Institutes of Health).

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