In vitro biology of fibropapilloma-associated turtle herpesvirus and host cells in Hawaiian green turtles (Chelonia mydas)

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Fibropapillomatosis (FP) of green turtles has a global distribution and causes debilitating tumours of the skin and internal organs in several species of marine turtles. FP is associated with a presently non-cultivable alphaherpesvirus Chelonid fibropapilloma-associated herpesvirus (CFPHV). Our aims were to employ quantitative PCR targeted to pol DNA of CFPHV to determine (i) if DNA sequesters by tumour size and/or cell type, (ii) whether subculturing of cells is a viable strategy for isolating CFPHV and (iii) whether CFPHV can be induced to a lytic growth cycle in vitro using chemical modulators of replication (CMRs), temperature variation or co-cultivation. Additional objectives included determining whether non-tumour and tumour cells behave differently in vitro and confirming the phenotype of cultured cells using cell-type-specific antigens. CFPHV pol DNA was preferentially concentrated in dermal fibroblasts of skin tumours and the amount of viral DNA per cell was independent of tumour size. Copy number of CFPHV pol DNA per cell rapidly decreased with cell doubling of tumour-derived fibroblasts in culture. Attempts to induce viral replication in known CFPHV-DNA-positive cells using temperature or CMR failed. No significant differences were seen in in vitro morphology or growth characteristics of fibroblasts from tumour cells and paired normal skin, nor from CFPHV pol-DNA-positive intestinal tumour cells. Tumour cells were confirmed as fibroblasts or keratinocytes by positive staining with anti-vimentin and anti-pancytokeratin antibodies, respectively. CFPHV continues to be refractory to in vitro cultivation.

INTRODUCTION

Fibropapillomatosis (FP) is a neoplastic disease of marine turtles; it has a global presence and causes significant external (skin) and internal (organ) tumours (Herbst, 1994). FP is a major cause of stranding of green turtles (Chelonia mydas) in Hawaii (Aguirre et al., 1998; Chaloupka et al., 2008; Work et al., 2004) and Florida (Foley et al., 2005), but prevalence of the disease in Hawaii has been decreasing in recent years for unknown reasons. FP is transmissible using cell-free filtrates (Herbst et al., 1995), and molecular studies consistently show alphaherpesviral DNA associated with tumour tissue in green, olive ridley (Lepidochelys olivacea) (Quackenbush et al., 2001) and loggerhead (Caretta caretta) (Lackovich et al., 1999) turtles in the Pacific and Atlantic oceans. Both spontaneous and experimentally induced fibropapillomas have been characterized extensively on the basis of gross and microscopic morphology, revealing that lytic viral production, as evidenced by intranuclear inclusions with viral particles, is rare and limited to epidermal layers of the tumour (Herbst et al., 1999). Greenblatt et al. (2004) found higher levels of Chelonid fibropapilloma-associated herpesvirus (CFPHV) pol mRNA expression in the
superficial portion of some skin tumours; however, whether the virus sequesters in a particular cell layer is unknown. Knowing which cell types contain the highest levels of viral DNA may help target sites of viral replication for in vitro isolation studies.

CFPHV continues to be refractory to in vitro cultivation despite several attempts at virus isolation using green turtle cells and classical cell culture methods (Lu et al., 1999; Moore et al., 1997). This inability to culture the virus makes confirming the role of CFPHV as a cause of tumours difficult. In this context, CFPHV is similar to other tumorigenic herpesviruses such as Marek’s disease virus (MDV) that causes lymphoid tumours in chickens (Venugopal, 2000), Lucke’ frog herpesvirus (LFHV) that causes adenocarcinoma in frogs (Skinner & Mizell, 1972) or Kaposi’s sarcoma virus (KSV) (Ganem, 2006) and Epstein–Barr virus (EBV) (Venugopal, 2000), Lucke’ frog herpesvirus (LFHV) that causes adenocarcinoma in frogs (Skinner & Mizell, 1972) or Kaposi’s sarcoma virus (KSV) (Ganem, 2006) and Epstein–Barr virus (EBV) (Venugopal, 2000), Lucke’ frog herpesvirus (LFHV) that causes adenocarcinoma in frogs (Skinner & Mizell, 1972) or Kaposi’s sarcoma virus (KSV) (Ganem, 2006) and Epstein–Barr virus (EBV) (Venugopal, 2000), Lucke’ frog herpesvirus (LFHV) that causes adenocarcinoma in frogs (Skinner & Mizell, 1972) or Kaposi’s sarcoma virus (KSV) (Ganem, 2006) and Epstein–Barr virus (EBV) (Venugopal, 2000), Lucke’ frog herpesvirus (LFHV) that causes adenocarcinoma in frogs (Skinner & Mizell, 1972) 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10 μM 5-azacytidine for 4 days (Chen et al., 2001) and 1 μM dexamethasone for 24–72 h (Paran et al., 1973). All chemicals were from Sigma, except for dexamethasone and epinephrine, which were from Phoenix Pharmaceutical and MP Biomedicals, respectively. Cells were observed every 1–3 days for presence of cytopathic effects (CPEs) compared with non-treated controls, and were harvested and frozen at 20 days.

To assess the effects of temperature, CFPHV-DNA-positive cells were incubated at 24 or 30 °C for 20–30 days, observed every 1–3 days for presence of CPE, harvested and frozen. To evaluate the utility of heat shock to induce virus (Moriya et al., 1994), cells incubated at 24 or 30 °C were incubated for 3 h at 37 °C, returned to their original temperature, observed for CPE and harvested 20 days later. To assess whether virus can be reactivated with cryopreservation in DMSO (Reid & Bridgen, 1991), CFPHV-DNA-positive cells (primary culture) were incubated at 30 °C for 30 days, then a portion of those cells were resuspended in FCS containing 10% DMSO and stored in liquid nitrogen for 5 days. The cells were then thawed, subcultured, examined for the presence of CPE, compared with non-treated controls and harvested after the second and seventh subcultures. CFPHV pol DNA levels for cells at day 0 (prior to treatment) and day 20 (after treatment) were measured and compared.

### Effects of virus infection on cell growth and morphology.

To compare growth characteristics and morphology of fibroblasts from tumours versus normal skin, the following cells were established in culture: dermal fibroblasts from normal skin of two immature FP-free captive-bred green turtles that had been raised in captivity, and fibroblasts from paired normal and tumourous skin from two free-range green turtles afflicted with FP. A seventh cell line consisting of chronically infected CFPHV-pol-positive intestinal tumour cells was used for comparison to assess the effects of viral infection on cell growth kinetics. Cells were cultivated for growth characteristics between subcultures 8 and 16. All assays were done in 24-well plates using triplicate wells. We determined seeding density by seeding at the following densities: 2, 8, 40, 200, 1000, 5000 and 25 000 cells cm⁻². Cells were incubated at 30 °C and media was changed every 3 days. After 10 days, adherent cells were detached from culture flask using trypsin/versene buffer and counted with a haemocytometer. To determine plating efficiency, cells were seeded at a density of 50 000 cells cm⁻² and the percentage of non-adhered cells was determined after 30, 60 and 120 min and 24 h. To determine growth response, cells were seeded at 10 000 cells cm⁻², trypsinized at 3, 7, 10, 14, 17, 21, 24 and 28 days, and counted using a haemocytometer. Population doublings were calculated as described previously (Pollard & Walker, 1997). Serum dependency was assessed by seeding cells at 1000 cells cm⁻², incubating with 0, 5 and 10% FCS for 12 days and counting with a haemocytometer.

Cell morphology was assessed using light microscopy, karyotyping and immunohistochemistry. For karyotyping, cells at exponential growth phase at subculture 20 were incubated overnight at 30 °C with 0.5 μg demecolcine ml⁻¹ (Sigma-Aldrich) and chromosomes were prepared as described previously (Pollard & Walker, 1997). Immunohistochemistry was used to confirm that cells in culture were fibroblasts or keratinocytes. Cells were grown at 30 °C to 75–80% confluence, washed three times in PBS, fixed with paraformaldehyde for 15 min at room temperature and then washed again with PBS. Cells were then permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 10 min at room temperature followed by a wash with PBS. Cells were blocked with PBS with 2% milk (PBS-M) for 30 min, followed by incubation with monoclonal mouse IgG2a/κ anti-vimentin (clone Vim 3B4; LabVision) and monoclonal mouse IgG1 anti-pan cytokeratin (clone PCK-26; Sigma) diluted 1:50 and 1:5000, respectively, in PBS-M for 30 min at room temperature. Cells were washed three times in PBS-M and incubated for 30 min with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Sigma-Aldrich) diluted 1:1000 in PBS-M. After three rinses in PBS, diaminobenzidine (DAB) was added to the cells according to the manufacturer’s instructions (Pierce) for colorimetric analysis.

To confirm the nature of binding of monoclonal mouse IgG2a/κ anti-vimentin to turtle vimentin, fibroblasts or keratinocytes, 2×10⁵ cells ml⁻¹ were homogenized in PBS, homogenates were mixed 1:2 in Laemmli buffer and proteins were separated on a 10% polyacrylamide gel with a 4% stacking gel using a Mini-Protein II electrophoresis system (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane using a mini transblot electrotransfer cell (Bio-Rad). The membrane was blocked with PBS-M and treated with mouse anti-vimentin and rabbit anti-mouse conjugate as described above for immunohistochemistry. Positive control antigen for anti-vimentin and anti-pan-cytokeratin antibodies included vimentin Ab-2 and cytokeratin 20 (both LabVision), respectively. Negative control antigens for anti-vimentin and anti-pan-cytokeratin antibodies were keratinocyte and fibroblast homogenates, respectively.

### RESULTS

There was no significant relationship between tumour size and levels of CFPHV pol DNA (Fig. 1a). CFPHV pol DNA copy number in the dermis of tumours were significantly higher (t=7.07, P<0.0001) than those in the epidermis (Fig. 1b). For both external and internal tumours, viral DNA decreased rapidly to less than 10% of original tissue

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**Fig. 1.** (a) Scatterplot of CFPHV pol DNA load in dermis versus tumour size (n=28). Note, two tumours at what appears to be 0 actually measured 0.2 cm². (b) Box plot of CFPHV pol DNA load in dermis and epidermis from paired samples (n=28) of tumours from green turtles afflicted with FP. Whiskers indicate SD, upper and lower bounds of the boxes indicate the 5 and 95 percentiles, and horizontal lines in the middle of the boxes indicate the means.
burdens by subculture 1 and continued decreasing to essentially undetectable levels by subculture 10 (Fig. 2). No CPE was seen in cells at any time during the experiment. One cell line (intestine tumour) remained consistently CFPHV-pol-DNA-positive through to subculture 46, whereupon viral DNA disappeared with subsequent subcultures after the cells were frozen and thawed.

Induction of known infected cells with CMR, at various temperatures, by cryopreservation with DMSO or keratinocytes–fibroblast co-cultivations uniformly failed to induce viral replication. No CPE was seen in any cultures for any treatment and DNA levels in known CFPHV- induced viral replication. No CPE was seen in any cultures.

Cells grew from primary explants in 4–10 days and formed confluent monolayers in 20–29 days in the case of normal skin, 30–32 days for paired tumour-affected skin from FP-affected turtles and 43 days for skin from FP-free turtles. Optimal plating density for all cell types ranged from 40 to 1000 cells cm$^{-2}$ and, based on this, 1000 cells cm$^{-2}$ were used for all subsequent studies except plating efficiency. Fibroblasts from skin tumours or intestinal tumour cells that were CFPHV-pol-DNA-positive for 46 subcultures generally grew more slowly than those from paired normal skin or normal skin from FP-free turtles; however, these differences were not significant for serum dependency, growth kinetics, seeding density or plating efficiency (Fig. 3).

Modal karyotype for all six cell lines was 55–56. Morphologically, fibroblasts had a fibroblast-like appearance (Fig. 4a), whereas keratinocytes had a distinct cobblestone pattern (Fig. 4b). Fibroblasts stained with vimentin whereas keratinocytes did not (Fig. 4c), and keratinocytes stained with pancytokeratin whereas fibroblasts did not (Fig. 4d). Under phase-contrast or when stained with Wright's Giemsa, cells from normal and tumour-affected skin were morphologically indistinguishable. Mouse anti-vimentin antibodies recognized several proteins in fibroblast homogenates in the 50–60 kDa range, but failed to stain any bands for keratinocyte homogenates. Mouse anti-pancytokeratin antibodies recognized proteins in keratinocyte homogenates in the 45–60 kDa range but failed to stain any bands for fibroblast homogenates (Fig. 5).

**DISCUSSION**

Most herpesviruses undergo intermittent lytic replication followed by latency (Young & Rickinson, 2004). In herpesviral diseases that induce tumours, latent viral proteins are thought to be responsible for tumorigenesis (Young & Rickinson, 2004). Alternatively, the virus infects cells and then disappears after inducing irreversible genetic damage that leads to cell transformation and generation of tumours (the hit-and-run hypothesis) (Ambinder, 2000). In turtles, larger tumours are presumably older, so if a hit-and-run mechanism is responsible for tumour formation, larger tumours should have little to no viral DNA. The lack of a relationship between tumour size and levels of CFPHV viral DNA suggests otherwise and indicates that CFPHV somehow plays a role in tumour formation in vivo.

Skin tumours in green turtles affected by FP are characterized by the presence of a sessile to arborizing collagen matrix mixed with pleomorphic haphazardly arranged fibroblasts overlaid by markedly acanthotic epidermis (Herbst et al., 1999). On occasion, rare clumps of herpesvirus-like intranuclear inclusions are present within the keratinized epidermis (Jacobson et al., 1989) but not fibroblasts. The presence of higher levels of CFPHV pol DNA in dermis versus epidermis suggests that while vegetative viral production occurs in keratinocytes, a majority of the CFPHV viral genomes resides in fibroblasts integrated either into host DNA or as episomes (Ganem, 2006). Given that less than 2% of skin tumours have intranuclear inclusions visible on histology (Herbst et al., 1999), the lack of viral DNA in epidermal cells is not surprising. However, other investigators have occasionally found higher levels of CFPHV pol DNA in superficial tumour tissues versus deeper tissues (Greenblatt et al., 2004), seemingly contradicting our findings. In the Greenblatt study, superficial tumour tissue included biopsies of both epidermis and dermis. In contrast, in the present study, we analysed epidermal and dermal cells separately, thereby providing a more accurate picture of the distribution of viral DNA in turtle tumours. Two potential explanations for the inconsistently higher levels of viral DNA in superficial tumours found by Greenblatt et al. (2004) exist: (i) superficial fibroblasts contain more viral DNA than deep fibroblasts or (ii) some superficial tumour biopsies may have contained actively replicating virus within the epidermis.

Treatment with CMR has been successfully used to induce replication of retroviruses (Paran et al., 1973) and herpesviruses (Countryman et al., 2008); however, the mechanisms by which they do this are unclear. For herpesviruses, the ability to induce replication depends...
Table 1. Effects of various treatments on copies of CFPHV pol DNA in cultured cells from green turtles afflicted with FP

FB indicates the number of attempts on fibroblasts only, while FBKC indicates the number of attempts on co-cultures of fibroblasts and keratinocytes. Mean (±SD) number of copies of CFPHV pol DNA per cell at the start (DNA-Start) and end (DNA-End) of the experiment along with percentage decrease of CFPHV pol DNA are shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FB</th>
<th>FBKC</th>
<th>DNA-Start</th>
<th>DNA-End</th>
<th>% Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Azagctidine</td>
<td>3</td>
<td>2</td>
<td>101 (69)</td>
<td>20 (30)</td>
<td>80</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>3</td>
<td>2</td>
<td>101 (69)</td>
<td>19 (33)</td>
<td>80</td>
</tr>
<tr>
<td>DMSO</td>
<td>2</td>
<td>2</td>
<td>135 (20)</td>
<td>11 (16)</td>
<td>91</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>2</td>
<td>2</td>
<td>105 (44)</td>
<td>34 (57)</td>
<td>67</td>
</tr>
<tr>
<td>Forskolin</td>
<td>2</td>
<td>2</td>
<td>105 (44)</td>
<td>77 (149)</td>
<td>26</td>
</tr>
<tr>
<td>Hexamethylenebisacetamide</td>
<td>3</td>
<td>2</td>
<td>101 (69)</td>
<td>15 (28)</td>
<td>85</td>
</tr>
<tr>
<td>Isobutylmetaxanthine</td>
<td>1</td>
<td>2</td>
<td>105 (44)</td>
<td>42 (81)</td>
<td>60</td>
</tr>
<tr>
<td>IUDR</td>
<td>3</td>
<td>2</td>
<td>101 (69)</td>
<td>21 (35)</td>
<td>78</td>
</tr>
<tr>
<td>IUDR + dexamethasone</td>
<td>2</td>
<td>2</td>
<td>115 (28)</td>
<td>16 (25)</td>
<td>86</td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>3</td>
<td>0</td>
<td>1825 (283)</td>
<td>1239 (338)</td>
<td>32</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1</td>
<td>2</td>
<td>105 (44)</td>
<td>32 (58)</td>
<td>68</td>
</tr>
<tr>
<td>TNF-α</td>
<td>3</td>
<td>2</td>
<td>101 (69)</td>
<td>11 (18)</td>
<td>88</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate</td>
<td>3</td>
<td>0</td>
<td>1825 (283)</td>
<td>1432 (238)</td>
<td>21</td>
</tr>
<tr>
<td>Trichostatin A</td>
<td>2</td>
<td>2</td>
<td>105 (44)</td>
<td>50 (90)</td>
<td>52</td>
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<tr>
<td>Valproic acid</td>
<td>1</td>
<td>2</td>
<td>129 (22)</td>
<td>49 (83)</td>
<td>62</td>
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<tr>
<td>Heat shock (37 °C, 3 h)</td>
<td>3</td>
<td>2</td>
<td>28 (4)</td>
<td>3 (3)</td>
<td>88</td>
</tr>
<tr>
<td>DMSO and freezing (30 °C)</td>
<td>1</td>
<td>0</td>
<td>15.1</td>
<td>3.4</td>
<td>77</td>
</tr>
<tr>
<td>DMSO and freezing (24 °C)</td>
<td>1</td>
<td>0</td>
<td>15.1</td>
<td>0.08</td>
<td>99</td>
</tr>
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</table>

Fig. 3. Cultured fibroblasts from paired normal (▲) and tumourous (△) skin, skin from tumour-free turtles (●) and CFPHV-positive intestinal tumour cells (■). Except for intestinal tumour cells, each symbol represents the mean of duplicates (two turtles each). (a) Seeding density versus population doublings, (b) growth kinetics, (c) serum dependency and (d) plating efficiency.
on not only the type of CMR but also the type of cell line being induced (Gradoville et al., 2002). The lack of persistently infected cell lines does not help attempts to isolate CFPHV, so we tried to get around this problem by using early subculture cells known to contain CFPHV pol DNA. CMRs work through various mechanisms that inhibit or facilitate DNA replication. Butyrate, trichostatin A and valproic acid all inhibit histone deacetylase, phorbol esters are protein kinase activators and 5 azacytidine is a DNA methyltransferase inhibitor; all of these in various combinations have been successfully used to induce replication of KSV and EBV from persistently infected cell lines (Countryman et al., 2008). Forskolin (an adenylate cyclase activator) and isobutylxanthine (a phosphodiesterase inhibitor) have been used successfully to reactivate herpes simplex virus type 1 (HSV-1) from cell cultures (Smith et al., 1992). Granulocyte precursors latently infected with human cytomegalovirus can be induced to lytic replication by TNF-α because these compounds induce cellular differentiation (Streblow & Nelson, 2003). Cells latently infected with MDV have been induced to lytic replication by administering the DNA replication inhibitor 5 IUDR (Nazerian, 1975). KSV has been induced to replicate from latently infected primary effusion lymphomas by epinephrine and norepinephrine that act through b1-adrenoreceptors (Chang et al., 2005). Finally, dexamethasone alone has successfully been used to increase lytic replication of cytomegalovirus from fibroblasts (Thiele & Woods, 1988) and dexamethasone plus IUDR has induced viral replication of murine leukaemia virus C (Paran et al., 1973). None of these mechanisms appear to be viable strategies for inducing lytic replication of CFPHV from infected turtle cells in vitro.

Other strategies to successfully induce replication of latent viruses have included incubation at varying temperatures, submitting cells to cryopreservation with DMSO and co-cultivation of cells. LFHV grows optimally at approximately 11 °C (Skinner & Mizell, 1972). Our rationale for incubating tissues at 24 and 30 °C was based on seasonal mean ocean temperatures in Hawaii which seldom deviate beyond the range of 23–28 °C. While 30 °C may seem on the high side, sea turtles will haul out on shore and bask, whereupon their core temperature can easily exceed 30 °C (Rice et al., 2000). Cultures of known infected turtle cells at both 24 and 30 °C failed to induce viral replication under the conditions of this study. Likewise, heat shock, which has been used successfully to induce replication of HSV-1 from neuronal cultures (Halford et al., 1996) also failed to induce viral replication. Co-cultivation of duck embryo fibroblasts with lymphoid cells infected with MDV has been used to rescue virus from latently infected cells (Churchill & Biggs, 1967). Although lytic replication of CFPHV appears to be limited to keratinocytes, co-cultivation of this cell type with known infected fibroblasts failed to induce viral replication. In this context, CFPHV may be more akin to papillomaviruses, in which maturation of keratinocytes is needed to induce viral replication (Wang et al., 2009).

Tumour cells in vitro lose herpesviral DNA with subculturing. A similar phenomenon occurs in Kaposi’s sarcoma, a human tumour associated with a herpesvirus. In that system, sarcoma cells lose episomal viral genomic DNA over time in vitro because the pressures that lead to

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**Fig. 4.** Cells from skin tumours of Hawaiian green turtles with FP. (a) Fibroblasts. Note the stellate appearance. Bar, 60 μm. (b) Keratinocytes. Note the cobblestone appearance. Bar, 60 μm. (c) Fibroblasts stained with anti-vimentin antibodies. Bar, 20 μm. (d) Keratinocytes stained with anti-pancytokeratin antibodies. Bar, 30 μm.

**Fig. 5.** Western blot containing green turtle fibroblast (FB) or keratinocyte (KC) homogenates. Lanes: 1, FB + anti-pancytokeratin antibody (APCKab); 2, FB + anti-vimentin antibody (AVab); 3, Vimentin+AVab; 4, KC+AVab; 5, KC+APCKab; 6, cytokeratin 20+APCKab.
maintenance of viral DNA in vivo are absent (Ganem, 2006). Thus, there are two possible reasons why CFPHV DNA in culture is lost over time in cultured green turtle cells: (i) loss of episomal DNA due to a lack of pressures needed to maintain viral DNA or (ii) competitive survival advantage of virus-free cells in culture.

Neoplastic cells typically have lower adherence and serum dependency for growth (Eagle et al., 1970). In this study, we saw no differences in plating efficiency and serum dependency between primary cultures derived from tumourous and normal skin. The lack of differences in morphology and in vitro behaviour of fibroblasts derived from tumourous and normal skin suggests that cells from tumours cultured in vitro lose neoplastic characteristics found in vivo. Comparisons between cell types were done between subcultures 8 to 14, long after viral genomes disappeared from cells; however, the growth characteristics of an infected intestinal tumour cell line also did not differ from those of normal cells. Others have reported that a comparison of in vitro characteristics of tumour and normal cells yields highly variable results (Eagle et al., 1970).

The skin fibroblasts used in this study behaved similarly to turtle skin fibroblasts used by other investigators (Lu et al., 1999; Moore et al., 1997); explants grew out of tissues within 10 days and optimal growth was at 30 °C. A karyotype of 55–56 matched that found by Moore et al. (1997) for green turtles but was less variable than that seen by Lu et al. (1999). The plating efficiencies seen in our study were higher than those reported by either Lu et al. (1999) or Moore et al. (1997), and this may be attributable to media; the aforementioned studies used RPMI 1640 or M199 with CO2 incubation, whereas we used we used L-15 with no CO2.

Green turtle fibroblasts in cultures were stained for vimentin with commercially available anti-vimentin antibodies, and keratinocytes in mixed fibroblast–keratinocyte cultures were stained with commercially available anti-pancytokeratin antibodies. Vimentin is a 57 kDa intermediate filament protein considered as diagnostic for mesenchymal cells such as fibroblasts (Fuchs & Weber, 1994). The presence of vimentin and cytokeratin in green turtle fibroblasts and keratinocytes, respectively, was confirmed by Western blotting, in which mouse monoclonal anti-vimentin antibodies bound protein bands at the appropriate molecular mass range for these proteins. In addition to morphology, positive staining of cultured cells with anti-vimentin antibodies and anti-pancytokeratin antibodies confirmed the identity of cultured cells. Future strategies to isolate CFPHV might consider establishment of organotypic raft cultures (Wang et al., 2009). However, successful implementation of such a strategy awaits cultures of fibroblast-free turtle keratinocytes.

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REFERENCES


