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Virology 321 (2004) 101–110
VIROLOGY
www.elsevier.com/locate/yviro

The *Ozobranchus* leech is a candidate mechanical vector for the fibropapilloma-associated turtle herpesvirus found latently infecting skin tumors on Hawaiian green turtles (*Chelonia mydas*)

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Received 14 October 2003; returned to author for revision 9 December 2003; accepted 10 December 2003

Abstract

Fibropapillomatosis (FP) of marine turtles is a neoplastic disease of ecological concern. A fibropapilloma-associated turtle herpesvirus (FPTHV) is consistently present, usually at loads exceeding one virus copy per tumor cell. DNA from an array of parasites of green turtles (*Chelonia mydas*) was examined with quantitative PCR (qPCR) to determine whether any carried viral loads are sufficient to implicate them as vectors for FPTHV. Marine leeches (*Ozobranchus* spp.) were found to carry high viral DNA loads; some samples approached 10 million copies per leech. Isopycnic sucrose density gradient/qPCR analysis confirmed that some of these copies were associated with particles of the density of enveloped viruses. The data implicate the marine leech *Ozobranchus* as a mechanical vector for FPTHV. Quantitative RT-PCR analysis of FPTHV gene expression indicated that most of the FPTHV copies in a fibropapilloma have restricted DNA polymerase expression, suggestive of latent infection.

Keywords: Quantitative PCR; Marine turtle; Leech; Vector; Fibropapillomatosis; Fibropapilloma-associated turtle herpesvirus; Green turtle herpesvirus; Latent

Introduction

Fibropapillomatosis (FP) of marine turtles is an emerging neoplastic disease characterized by the presence of epithelial fibropapillomas. Internal fibromas also develop in approximately 30% of terminal cases (Work and Balazs, 1998). The prevalence of FP exceeds 40% in three monitored residential foraging areas off Florida, Hawaii, and Australia (Aguirre et al., 2000; Balazs and Pooley, 1991; Jacobson et al., 1989). FP has been reported in green (*Chelonia mydas*), loggerhead (*Caretta caretta*), and olive ridley (*Lepidochelys olivacea*) turtles (Herbst, 1994; Quackenbush et al., 2001) and may pose a significant threat to the long-term survival of marine turtles.

FP has a documented association with a turtle herpesvirus (FPTHV) (Lackovich et al., 1999; Lu et al., 2000a; Quackenbush et al., 1998, 2001). FPTHV DNA polymerase (*pol*, U_L30) sequences have been detected by PCR in every tested fibropapilloma and fibroma reported to date (Lackovich et al., 1999; Lu et al., 2000a; Quackenbush et al., 1998, 2001). Moreover, in 79% of fibropapillomas and fibromas examined by quantitative PCR (qPCR), FPTHV *pol* sequences are present at levels exceeding an average of one virus copy per tumor cell (Quackenbush et al., 2001). FP can be transmitted experimentally by injection of a filtered, cell-free tumor homogenate; transmission is abolished by chloroform treatment of the homogenate (Herbst et al., 1995, 1996; Jacobson et al., 1991). On the basis of the order, orientation, and sequence homology of 12 FPTHV open reading frames [Greenblatt et al., the fibropapilloma-associated turtle herpesvirus (FPTHV): alpha-herpesvirus conservation and comparisons across seven geographic areas and three host species, submitted], FPTHV is a member of the *Alphaherpesvirinae* genus. This classifica-

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doi:10.1016/j.virol.2003.12.026

tion is consistent with the apparent tropism of FPTHV for epithelial tissue.

Evidence has been presented by our laboratory and others implicating FPTHV as a major agent of marine turtle fibropapillomatosis (Herbst et al., 1995, 1996; Lackovich et al., 1999; Quackenbush et al., 1998, 2001). These reports raise questions about the mechanism of viral transmission. One possibility is that the virus is transferred among turtles by a vector organism. In this study, quantitative PCR (qPCR) was used to examine FPTHV loads in common ecto- and endoparasites of Hawaiian green turtles. The parasites examined included marine leeches (*Ozobranchus* spp.), blood flukes of the genera *Carretacola*, *Hapalotrema*, and *Laeredius*, a pool of *Pyelosomum longicaecum* bladder parasites, barnacles (*Platylepas* spp.), and amphipods of the skin and oral cavity (order Talitroidea). Strikingly high FPTHV DNA loads were found in the *Ozobranchus* leeches, suggesting the leech as a candidate vector for FPTHV. Quantitative RT-PCR was then used to examine the transcriptional state of the virus in fibropapillomas, particularly in the superficial tumor layer that is parasitized by the leech. We hypothesized that most FPTHV copies in tumor tissue would be maintained in the latent phase of infection, like human herpesvirus 8 in Kaposi's sarcoma in humans or gallid herpesvirus 2 (Marek's disease virus) in the tumors associated with Marek's disease of chickens.

Results

qPCR of parasites

Fig. 1 displays the FPTHV *pol* copy numbers normalized to whole parasite individuals or pools. Because of the wide range of these values (0–1.6 × 10^7 copies), the *Y* axis is tripartate: the lower scale is 0–1000 copies, the middle scale is 6000 to 4.6 × 10^4 copies, and the upper scale is 5 × 10^4 to 1.6 × 10^7 copies. The different species analyzed are color-coded as shown.

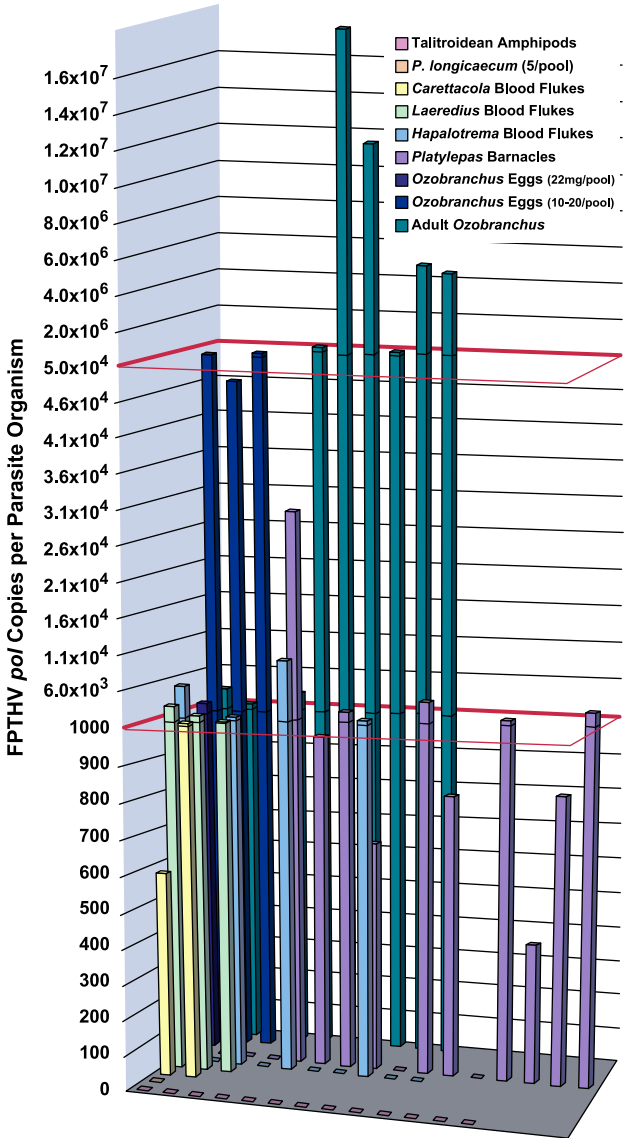

Fig. 1. Copies of FPTHV *pol* DNA assayed in turtle parasite individuals or pools. Note that the *Y* axis is tripartate: the lower scale is 0–1000 copies, the middle scale is 6000 to 4.6 × 10^4 copies, the upper scale is 5 × 10^4 to 1.6 × 10^7 copies. Colors front to back: pink = individual talitrodean amphipods, peach = *P. longicaeum* pool (five individuals), yellow = individual *Carettacola* flukes, light green = individual *Laeredius* flukes, light blue = individual *Hapalotrema* flukes, light purple = individual *Playlepas* barnacles, dark purple = pool of *Ozobranchus* eggs (22 mg), navy = pools of *Ozobranchus* larvae (10–20 individuals per pool), teal = individual adult *Ozobranchus*. Average parasite masses: amphipods 20 mg, barnacles 300 mg, *P. longicaeum* pool 100 mg, flukes 30 mg, leech egg pools 100 mg, leech larvae pools 100 mg, adult leeches 300 mg.

Amphipods (Talitroidea)

Thirteen individuals removed from the skin and oral cavities of 13 free-ranging FP-affected and -unaffected turtles were examined. None carried any copies of FPTHV *pol*.

Bladder parasites (P. longicaecum)

A pool of approximately five parasites removed from one stranded FP(+) turtle was examined. No FPTHV *pol* copies were detected.

Barnacles (Platylepas spp.)

Sixteen individuals removed from the skin of two free-ranging turtles, one FP(+) and one FP(−), were examined. Six of the sixteen (38%) carried no detectable viral copies. The remaining 10 contained 19–180 copies, yielding an overall average of 41 FPTHV copies. Of the 10 positive barnacles, two were from the FP(−) turtle.

Blood flukes of three genera were examined

Carretacola: Two individuals removed from one stranded FP(−) turtle were examined. Their copy numbers were 64 and 55 FPTHV *pol* copies, respectively.

Table 1
Distribution of FPTHV *pol* copies during the preparation of an isopycnic sucrose density gradient from *Ozobranchus* leeches parasitizing an FP(+) Hawaiian green turtle

Leech tissue homogenate	2.1×10^6
Pellet from initial centrifugation (clearing)	2.0×10^6
Supernatant from first ultracentrifugation (pelleting of putative viral material)	2.6×10^3
Pellet from first ultracentrifugation	7.6×10^4
Sum of copies in all sucrose gradient fractions	5.8×10^4
Sum of three sucrose gradient fractions surrounding 1.17g/ml	5.6×10^4

Ninety-five percent of the copies in the tissue homogenate partitioned into the initial pellet during sample clearing, but 96% of the copies loaded on the gradient banded at 1.17g/ml, the density of enveloped virus. Copy numbers given are extrapolated for whole gradient fractions from qPCR performed on aliquots.

Hapalotrema: Ten individuals from one stranded FP(+) turtle were examined. Of these, six (60%) contained no FPTHV *pol* copies. The remaining four carried 22–500 copies, yielding an overall mean of 63 copies.
Laeredius: Three individuals from two stranded turtles, one FP(+) and one FP(−), were examined. Their copy numbers were 100, 210, and 57, respectively.

Marine leeches (Ozobranchus spp.) were examined at three stages of development

Adults: DNA was prepared from 16 individuals removed from 9 turtles: 6 free-ranging FP(+), 1 moribund FP(+), and 2 free-ranging FP(−). Although two of these leeches (13%) carried no *pol* copies [one from a free-ranging FP(+) turtle and one from a free-ranging FP(−)], six others (38%) contained loads in excess of 10^4 copies [the hosts of these high-load leeches were 3 free-ranging FP(+) turtles, one moribund FP(+) turtle, and one free-ranging FP(−) turtle]. These viral loads were two orders of magnitude greater than those observed in any of the other parasites, and are comparable with loads observed in fibropapilloma and fibroma tissue (mean 3.9×10^5 copies per 50 ng Hawaiian fibropapilloma or fibroma DNA in Quackenbush et al., 2001). The remaining eight adult leeches [from three free-ranging FP(+) turtles, one moribund FP(+) turtle, and one free-ranging FP(−) turtle] carried loads ranging from 14 to 9300 copies, yielding an overall mean of 2.2×10^4.
Larvae: Four pools of leech larvae (leeches with less than 5% adult body mass) removed from one moribund FP(+)

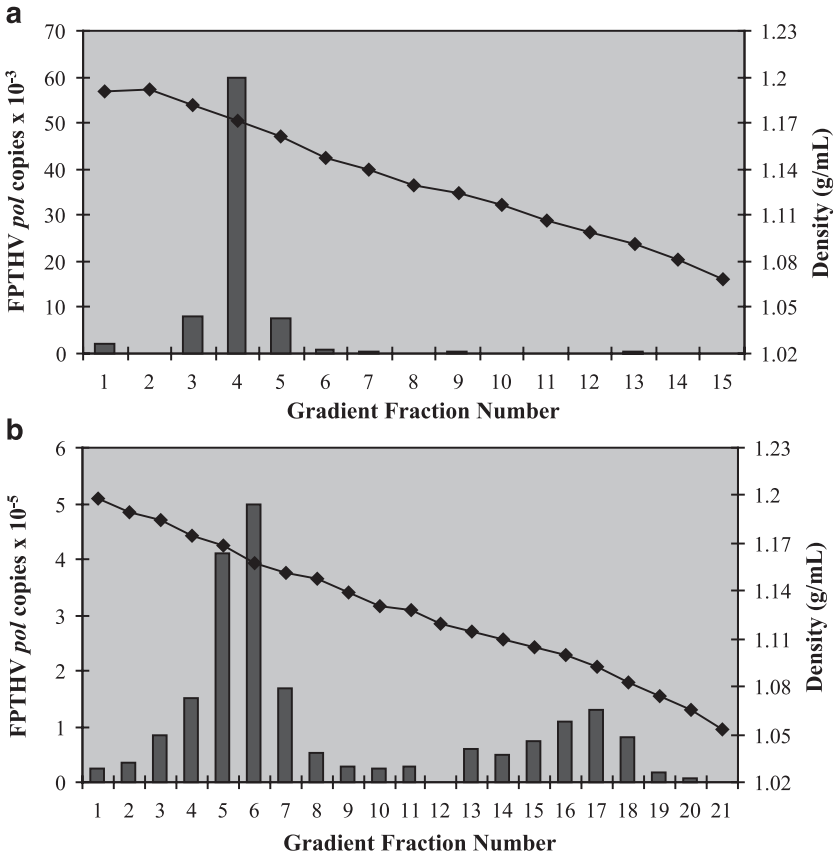

Fig. 2. (a) Isopycnic sucrose density gradient analysis of a pool of 11 adult *Ozobranchus* leeches (0.44 g of tissue). Bars [Y(1) axis] are absolute FPTHV *pol* DNA copies normalized to each whole gradient fraction, line [Y(2) axis] is fraction density (g/ml). (b) Sucrose gradient analysis of a homogenate of tissue from an eye fibropapilloma of a Hawaiian green turtle. Bars and line as above.

turtle were examined. The loads ranged from 1.3×10^4 to 7.7×10^4 copies, giving a mean of 4.4×10^4.
Eggs: DNA was prepared from three pools of leech eggs removed from three turtles: two free-ranging FP(+) and one free-ranging FP(−). They contained 730, 470, and 17 FPTHV *pol* copies, respectively.

Sucrose gradient analysis of adult leeches

Isopycnic sucrose density gradient analysis was utilized to determine whether the FPTHV *pol* copies in the leeches were associated with virus-like particles. Eleven adult leeches taken from a stranded FP(+) green turtle were rinsed in PBS, homogenized as a pool, then processed for gradient analysis as described below. DNA was prepared from a sample of each gradient fraction, and also from samples taken during processing. All of the preparations were analyzed with qPCR for FPTHV *pol* copy number (Table 1). The sample copy numbers revealed that most (95%) of the FPTHV *pol* copies in the original leech homogenate were associated with the initial low-speed centrifugation pellet, partitioning with nuclei, connective tissue, and cellular debris. However, of the copies that were loaded on the sucrose gradient, 96% partitioned into fractions with an average density of 1.17 g/ml, where enveloped viruses band (Fig. 2a). These results are consistent with those obtained from an earlier sucrose gradient analysis of a virus preparation from an FP eye tumor of a Hawaiian green turtle (Fig. 2b). In both cases, a small fraction of the total FPTHV DNA copies in the tissue were associated with particles of the density of enveloped viruses (2.5% in Fig. 2a; 1% in Fig. 2b). RNA was also prepared from two adult leeches removed at the same time from the same turtle as those prepared for the sucrose gradient in Fig. 2a, and from a pool of 22 mg of leech larvae (c. 40 larvae) removed at the same time from the same turtle as the pools in Fig. 1. When analyzed with qRT-PCR, all three leech RNA samples were negative for FPTHV *pol* (data not shown).

FPTHV gene expression in fibropapillomas and fibromas

Additional tissue samples were available from 19 fibropapillomas and fibromas that had previously been examined with qPCR for FPTHV DNA loads (Fig. 3a) (Quackenbush et al., 2001). RNA from these samples was assayed with qRT-PCR to determine the FPTHV *pol*

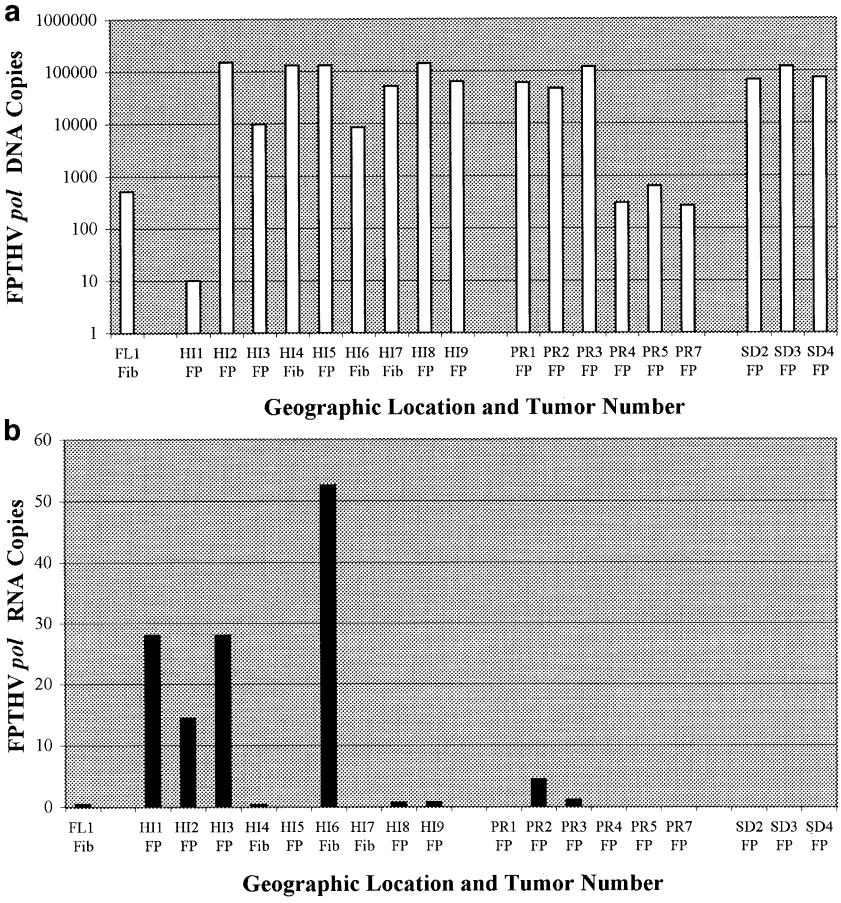

Fig. 3. (a) FPTHV *pol* DNA loads in FP tumors sampled from diverse geographic locations (Quackenbush et al., 2001). Copies are absolute per 50 ng total tumor DNA. FL = Florida, HI = Hawaii, PR = Puerto Rico, SD = San Diego, FP = Fibropapilloma (skin tumor), Fib = Fibroma (internal tumor). (b) FPTHV *pol* RNA loads in the same tumors analyzed above. Copies are absolute per 40 ng total tumor RNA. Abbreviations as above.

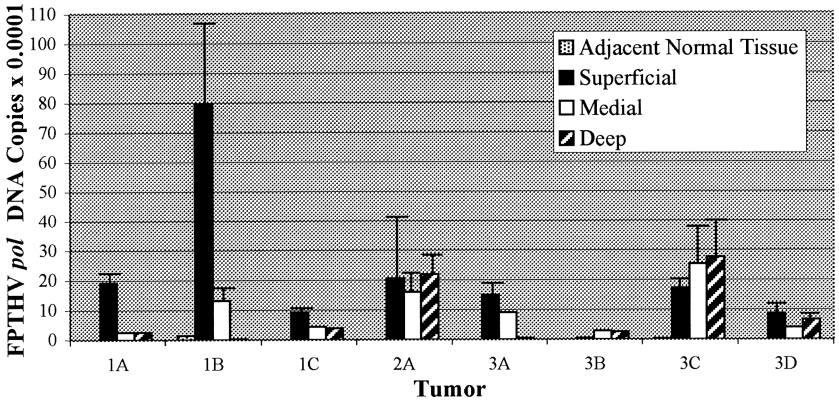

Fig. 4. FPTHV *pol* DNA loads in subsections of eight FP tumors from three Hawaiian green turtles. FPTHV copies are absolute per 50 ng total tumor DNA. Values given are means of four replicates; error bars indicate standard deviations. Note: because of the small size of tumor 1A, only one combined medial/deep sample was processed.

RNA copy load of each (Fig. 3b). Few copies of *pol* RNA were detected in any of these tumors. Because herpesvirus *pol* is part of the beta temporal expression group (expressed during productive or lytic infection), this result indicates that only a small minority (with one exception, <1%) of the FPTHV copies in these tumors were in the productive phase of infection at the time that the samples were taken.

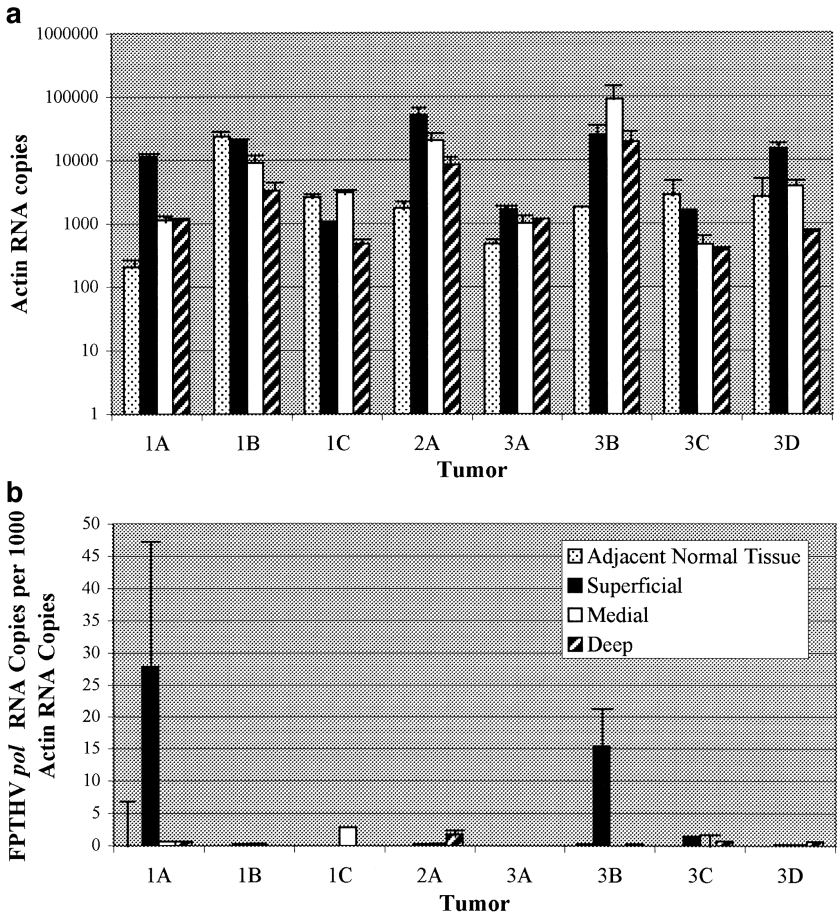

Fig. 5. (a) Actin RNA copies in the same tumor subsections as Fig. 4. Actin copies are absolute per 4 ng total tumor RNA. Values given are means of three replicates; error bars indicate standard deviations; color legend as in part (b). (b) FPTHV *pol* RNA copies normalized to the actin RNA copies above. Values given are the mean FPTHV *pol* copies for each sample divided by the mean actin copies for that sample. Error bars indicate the highest measured FPTHV load for each sample divided by the lowest actin load measured for that sample.

Distribution of FPTHV copies and expression in fibropapillomas

FPTHV DNA and RNA loads were measured in the superficial (dermis and epidermis), medial (center), and deep (stalk) sections of eight fibropapillomas. We hypothesized that more viral genomes and replicative gene expression would be present in the superficial layer of the tumor, where leeches feed.

As shown in Fig. 4, differences in viral DNA loads were observed among the tumor sections, but no consistent pattern of FPTHV genome distribution emerged. RNA was also prepared from each subsection and analyzed with qRT-PCR. In 94% of the cases (n = 31), the expression levels found in these subsections were similar to those in Fig. 3b: fewer than 85 copies of FPTHV *pol* RNA were present per 40 ng tumor RNA (c. 3000 cells). However, two tumor subsections contained more than 2000 copies of *pol* RNA, and both of these samples were taken from superficial tumor layers (Fig. 5b).

Actin normalization

To provide standardization for the RNA copy numbers generated in the qRT-PCR assays and to control for degradation of sample RNA before processing, a separate set of qRT-PCR assays was performed on every tumor RNA template discussed here, quantitating copy numbers of a *C. mydas* actin-like sequence (Fig. 5a). Because the actin qRT-PCR amplicon was the same length as the FPTHV *pol* qRT-PCR amplicon, it was anticipated that the actin copy number reported would be proportional to the quality of the RNA template for *pol* amplification. The RNA samples in which >2000 FPTHV *pol* copies were measured did not contain more actin copies than the samples that were negative for FPTHV *pol* (Fig. 5), so it is unlikely that template RNA quality was a factor influencing the results in Fig. 5b. However, there was variation in the actin RNA loads assayed in the different samples (range: 200–10 000 copies). It is unclear what proportion of this variability was due to sample degradation and what proportion was due to variation in actin expression among the heterogenous cell types examined.

Discussion

Candidate vector

Speculation that parasite organisms could play a role in marine turtle fibropapillomatosis (FP) dates back to the first reported cases (Aguirre et al., 1994; Choy et al., 1989; Smith and Coates, 1938). Since the discovery of FPTHV, conventional PCR studies have been performed, attempting to detect viral genes in candidate vector organisms (Lu et al., 2000b). However, because standard PCR does not provide quantitative data about the viral load in an organism, a positive result can be difficult to interpret in relationship to pathogenesis. In this study, the negative results obtained for the amphipods and bladder parasites are unequivocal, but the differences among the copy numbers observed in the blood flukes and barnacles versus the leeches required quantitation to interpret. The real-time fluorescence monitoring employed during the qPCR reaction increases the range of application and the power of PCR as an assay.

One drawback of the high sensitivity of PCR is the potential for misleading positive data resulting from trace molecular contamination. The quantitation of positive results in qPCR partially addresses this problem. For example, it could be suggested that the FPTHV copies found in this parasite panel (Fig. 1) were derived from external contamination with fibropapilloma or fibroma tissue, rather than from FPTHV copies actually within the parasites. Because the FPTHV load in fibropapilloma and fibroma tissue is now known (Quackenbush et al., 2001), some simple calculations can provide a sense of the relative reliability of a positive result. For example, if a blood fluke sample taken from an FP-positive turtle was contaminated such that 0.1% of the sample was actually fibropapilloma tissue, a copy number of 300–400 could be attributed entirely to this contamination. A leech sample with a FPTHV DNA copy number of 10^4, on the other hand, would have to have contained 10% fibropapilloma tissue for the positive result to be dismissed, and such a large mass of tumor would have been evident on visual inspection of the sample before processing.

One question raised by this finding is how leeches acquire high FPTHV loads, because it is assumed that they feed primarily on blood, and FPTHV viremia has not been observed in FP(+) turtles (Lackovich et al., 1999; Lu et al., 2000a; Quackenbush et al., 1998, 2001). The acquisition of FPTHV DNA does appear to be related to feeding, because high copy numbers were present both in adult leeches and in leech larvae (which feed on the same host as their parent; Sawyer, 1986), but not in leech eggs. One possibility is that feeding leeches ingest enough FPTHV-infected epithelium to obtain the observed FPTHV DNA loads. Another is that leeches feeding on FPTHV-infected turtles consume blood during an FPTHV-viremic state that has not yet been observed. There is not presently a means of identifying FPTHV-infected FP(−) free-ranging turtles, and the captive animal transmission studies performed to date (Herbst et al., 1995, 1996; Jacobson et al., 1991) have not included monitoring of FPTHV loads in blood during the development of FP. Thus, it remains possible that FPTHV viremia does occur, but happens before the development of fibropapillomas, so that it is not detected in a visibly affected animal.

Our results demonstrating that leeches can carry high FPTHV DNA copy numbers and that 3% of these copies are associated with particles of the density of enveloped

viruses indicate that marine leeches of the genus *Ozobranchus* should be considered candidate vectors for FPTHV. Leeches are frequently observed on fibropapillomas and on the carcasses of animals that have died of FP (Herbst, 1994). There is evidence that leeches serve as vectors for other pathogenic agents, including hepatitis B virus in humans (Nehili et al., 1994; Wilken and Appleton, 1993), trypanosomes in European eels (Zintl et al., 2000), and several parasites in fish (Negm-Eldin and Davies, 1999). Another leech of the same genus studied here, *Ozobranchus shipleyi*, is known to serve as a vector for the blood protozoan *Haemogregarina nicoriae* to the turtle *Melanochelys trijuga thermalis* (Sawyer, 1986). Because no FPTHV *pol* transcripts were found in the leech RNA samples examined, we hypothesize that a vector role for the leech would be limited to mechanical transmission of FPTHV-infected epithelial tissue from one turtle to another.

One finding of particular interest was the presence of eight FPTHV(+) parasites of FP(−) turtles. These were one *Laeredius* blood fluke, two *Carettacola* blood flukes, two barnacles, one pool of leech eggs, and three adult leeches. One of these leeches carried the highest FPTHV copy number observed in this study [1.9×10^5 FPTHV *pol* copies per 100 ng DNA (c. 2×10^4 cells)]. As discussed above, it is difficult to dismiss a copy number of this magnitude as a contamination artifact. It thus seems likely that at least one of these FP(−) animals carried an inapparent FPTHV infection. Alternatively, the highly FPTHV(+) leech was in the process of exposing a naïve turtle to the virus at the time of collection.

Importantly, the results above implicating the leech as a candidate vector for FPTHV do not preclude the possibilities that other vector organisms exist or that the virus can be transmitted directly through seawater. On the contrary, waterborne transmission has been demonstrated among enveloped viruses of fish (Bowser et al., 1999), and another disease-associated herpesvirus of marine turtles has been shown to retain in vitro infectivity for 5 days in seawater at room temperature (Curry et al., 2000).

FPTHV infection appears predominantly latent in fibropapilloma cells

A variety of results from our laboratory and others indicate that the majority of FPTHV genomes in a visible fibropapilloma is not replicating. Every tested fibropapilloma and fibroma reported to date has been PCR-positive for FPTHV (Lackovich et al., 1999; Lu et al., 2000a; Quackenbush et al., 1998, 2001), yet herpesvirus-like intranuclear inclusions and ballooning cellular degeneration indicative of productive herpesvirus infection were observed in only 2% of naturally occurring fibropapillomas assayed by microscopy (Herbst et al., 1999). Even in transmitted tumors, which were available for monitoring throughout FP development, only 19% displayed these signs, and only in a minority of the tumor cells (Herbst et al., 1999). Next, sucrose gradient/qPCR analysis (Fig. 2b) demonstrated that only 1% of the FPTHV *pol* copies in a Hawaiian fibropapilloma was associated with particles of the density of enveloped viruses. The comparative qPCR and qRT-PCR results above (Fig. 3) demonstrate that the vast majority (with one exception, >99%) of the FPTHV copies in a fibropapilloma does not express the productive-phase gene DNA polymerase (*pol*). However, the success of FP transmission experiments using tumor homogenates (Herbst et al., 1995, 1996; Jacobson et al., 1991) does not seem consistent with a model of total FPTHV latency in tumors. Because the herpesvirus-like inclusions that have been observed in fibropapilloma tissue have all been found in the dermis and epidermis (Herbst et al., 1995, 1999; Jacobson et al., 1989, 1991), we decided to subdivide tumors and examine the different portions separately.

Previous qPCR results had shown that the average FPTHV DNA load in a fibropapilloma or fibroma was equal to or less than 12 copies per cell (Quackenbush et al., 2001); the qRT-PCR result above (Fig. 3) showed that the average RNA load was far lower. The localization experiment above (Fig. 4) followed up on these averages, analyzing a panel of tumor subsections with qPCR and qRT-PCR. In the majority of the tumors examined (75%, $n = 8$), FPTHV *pol* RNA levels were again less than 1% of the DNA levels. However, in two of the tumors, the superficial tumor section (the epidermis and dermis) carried 20-fold more actin-normalized FPTHV *pol* RNA copies than any of the other tumor sections, corresponding to 1–1.6% of the DNA copies in these sections (Figs. 4, 5b). The qRT-PCR assay cannot indicate whether these RNA copies are translated, but the TEM studies (Herbst et al., 1995, 1999; Jacobson et al., 1989, 1991) indicate that some virion-like particles are produced in the superficial fibropapilloma layer. Furthermore, the sucrose gradient analysis above suggests that some of these particles become enveloped, and the transmission studies (Herbst et al., 1995, 1996; Jacobson et al., 1991) indicate that some of the enveloped particles are infectious. The TEM studies, sucrose gradient analysis, transmission studies, and comparative qPCR/qRT-PCR analyses all contribute to an internally consistent model of fibropapillomas carrying small loci of productive FPTHV infection in the context of a background of latent infection. The TEM studies and sectional qRT-PCR analysis provide agreeing indications that these loci may reside in the superficial tumor layer.

Latent herpesvirus infections are associated with several other tumors; the best studied are human herpesvirus 8 in Kaposi's sarcoma of humans and oncogenic gallid herpesvirus 2 strains in Marek's disease lymphomas of chickens. In the case of Kaposi's sarcoma, human herpesvirus 8 is present in latent form in >96% of the tumor cells, while <1–3% of the cells appears to be productively infected at some stage of tumor development (Blasig et al., 1997; Dupin et

al., 1999; Katano et al., 2000). Marek's disease virus of domestic chickens is an alphaherpesvirus that exerts most of its pathology in cells that are infected but do not produce extracellular virions, while maintaining a few small loci of fully productive infection in the feather follicles of affected animals (reviewed by Venugopal, 2000). Testing of this model for FP/FPTHV awaits the sequencing of FPTHV latency-associated transcripts and the development of immunohistochemical tools for visualizing FPTHV in turtle tissue.

Materials and methods

Collection of samples

Parasites

Parasite collections were from free-ranging and stranded FP-affected and -unaffected green turtles (*C. mydas*) from Hawaii. Barnacles were collected from the skin, and amphipods from the oral cavity. Vascular trematodes were collected from arteries and blood clots; bladder trematodes were collected from the lumen of the urinary bladder. Leeches were collected from the surface of tumors by rinsing in fresh water (which induces detachment) and were placed in cryovials. Leech eggs were scraped from the skin. All parasites were stored at −70 °C and then rinsed in phosphate-buffered saline (PBS) before nucleic acid preparation.

Tumor subsections

To assess distribution of virus copies in skin tumors, fibropapillomas were collected from euthanized turtles that had been moribund with FP and were carefully sectioned into superficial (skin and dermis), medial, and deep sections. These were also stored at −70 °C before nucleic acid preparation.

Purification of virus and sucrose gradient density separation

Tissue homogenates were separated on isopycnic sucrose density gradients as described in Casey et al. (1997). Briefly, tissue was homogenized into 1× TNES (TNES = 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 5% sucrose) (Fig. 2a: 0.44 g or 11 leeches in 12 ml, Fig. 2b: 5 g FP eye tumor tissue in 30 ml), the homogenate was cleared at 10 000 × *g* for 30 min at 4 °C, the supernatant was filtered (0.45 micron), and then centrifuged for 2 h at 10 °C at 100 000 × *g* (25 000 rpm in a SW28 rotor) to pellet putative virus. The pellet was resuspended in 500 μl TNES/6 mM dithiothreitol and layered onto 38 ml of 15–60% sucrose density gradient. The gradient was centrifuged at 10 °C for 17 h at 100 000 × *g*.

Fractions (2 ml) were collected and the density of each was determined by calculation from measured refractive index (digital refractometer, Reichert ABBE Mark II, AO Scientific Instruments, DIV Warner-Lambert Tech, Inc., Buffalo, NY). Fractions were stored at −80 °C.

Preparation of DNA

From parasite tissue

Twenty-five milligrams of tissue were homogenized into 200 μl of lysis buffer (0.1 M EDTA, 0.05 M Tris, pH 8), then treated with 100 μg RNAse A 15 min at 22 °C, then with 400 μg proteinase K and 0.1% SDS 2 h at 56 °C. The Qiagen Miniprep Blood Kit "spin" protocol was followed to extract DNA from the resulting homogenate, with the addition of 20–30 min of "soaking" period between application of elution buffer to the column and centrifuging the column to elute the DNA.

From sucrose gradient fractions

Sucrose gradient fractions were thawed in a cold water bath and diluted with 1 volume PBS to reduce the sucrose content of the preps to the specifications of Qiagen Miniprep Blood Kit columns. Each was treated for 5 min at room temp with 100 μg RNAse A, then for 10 min at 56 °C with 400 μg Qiagen protease, and 200 μl Qiagen Buffer AL. The resulting homogenate was then processed on Qiagen miniprep columns as described above.

Real-time quantitative PCR

qPCR was performed as in Quackenbush et al. (2001). Briefly, 50 ng of sample DNA were assayed in each 25 μl of TaqMan reaction, containing 0.25 μl of each 100 μM primer and 1 μl of 5 μM probe synthesized by Applied Biosystems (Foster City, CA):

turtle 5′-pol 5′ACTGGCTGGCACTCAGGAAA3′
turtle 3′-pol 5′CAGCTGCTGCTTGTCCAAAA3′
turtle pol probe 5′-[6FAM]-CGATGAAAACCGCACC-GAGCGA-[TAMRA]-3′

and 12.5 μl of the Applied Biosystems Universal PCR Master Mix (contains AmpliTaq Gold DNA polymerase, AmpErase uracil *N*-glycosylase (UNG), TaqMan buffer, and deoxynucleoside triphosphates). Thermal cycling was performed in an ABI 7700 Quantitative PCR instrument (Applied Biosystems): 2 min at 50 °C to activate UNG and 10 min at 95 °C to activate AmpliTaq Gold, followed by 40 cycles of 15 s at 95 °C and 1 min at 62 °C. Copy number calculation was by standard regression fit of the samples to a user-supplied standard curve with the supplier's software (SequenceDetector version 1.6). These "standard" samples were dilutions of pHaGTHVpol (Quackenbush et al., 2001), a clone of a previously published 482 bp FPTHV *pol* PCR product in the commercial vector Bluescript (Stratagene, La Jolla, CA). A solution of the plasmid in buffer TE (2.0×10^8 template copies/μl,

calculated from the OD_{260} of the prep) was stored at −20 °C, and fresh dilutions in diethylpyrocarbonate (DEPC)-treated water were prepared from this stock for each qPCR plate.

Preparation of RNA

RNA was purified by the RNAzol extraction procedure (Tel-test, Inc., Friendswood, TX). Tissue samples (20–60 mg) were homogenized in glass tissue grinders on ice in 1 ml of RNAzol; RNA was extracted and pelleted from the homogenate according to the manufacturer's protocol.

Real-time quantitative RT-PCR

qRT-PCR was performed with the TaqMan EZ RT-PCR Kit and ABI 7700 quantitative PCR instrument (both Applied Biosystems). The sample and standard (see below) RNAs were first treated with 1 unit/μg RNA of RQ-1 RNAse-free DNAse (Promega, Madison, WI). Forty nanograms of each RNA were loaded in a total volume of 50 μl containing 1 × TaqMan EZ Buffer, 3 mM manganese acetate, 300 μM dATP, dCTP, and dGTP, 600 μM dUTP, 5 units rTth DNA Polymerase, 0.5 units AmpErase UNG, 0.1 μl of each 100 μM primer, and 1 μl of 5 μM probe (the same primers and probe were used for qRT-PCR as for qPCR, above). Thermal cycling began with holds of 2 min at 50 °C to activate UNG, 30 min at 60 °C for reverse transcription, and 5 min at 95 °C to deactivate UNG. Forty cycles of 20 s at 94 °C and 1 min at 62 °C followed. Copy number calculation was again with standard regression fit by the supplier's software (as above).

For this set of standards, a portion of the plasmid GTHV7 (kindly provided by S. Quackenbush) was subcloned into the commercial vector pGEM-7Zf(+) (Promega). It was confirmed by sequencing (Cornell University BioResource Center DNA sequencing facility) that the 1585 bp subcloned portion (corresponding to Genbank accession number AF035003 bases 14877–16475) encompassed the 86 bp qPCR target. The insert was transcribed from the vector's T7 start site using the MAXIscript T7 Kit (Ambion, Inc., Austin, TX); the product was precipitated and redissolved according to the manufacturer's protocol. The resulting solution of transcribed RNA in buffer TE (10 mM Tris, 1 mM EDTA, pH 7) contained 7.8×10^{11} copies/μl (calculated from the OD_{260} of the prep). A set of serial tenfold dilutions of the solution in carrier RNA (10 μg/ml calf liver RNA) was prepared, and the appropriate dilutions were used as standards in the qRT-PCR plates.

Control reactions lacking reverse transcriptase were run for the panel of geographically diverse fibropapilloma and fibroma RNAs in Fig. 3b. Before DNAse treatment, up to 300 DNA copies could be detected in 40 ng of the RNA preps. After DNAse treatment, these values dropped to below 40 copies (data not shown). DNAse incubation time was extended for the tumor section RNAs in Fig. 5b.

Actin normalization for qRT-PCR

Two actin-like sequences were PCR-amplified from Hawaiian *C. mydas* DNA using the following primers:

TurtleActinF1: TGT GAT GGT KGG WAT GGG YCA GAA
TurtleActinR1: TCG GCT GTG GTG GTG AAG CT

The primer sequences were chosen from areas of near-identity in the published sequences of leghorn chicken (*Gallus gallus*) beta actin mRNA (GenBank accession L08165) and a snapping turtle (*Chelydra serpentina serpentina*) actin-like sequence (accession AF541916). Two products, 1078 and 471 bp, were generated and sequenced. The longer sequence appeared to be part of a beta actin-like gene including an intron, and the shorter sequence appeared to be part of an actin-like pseudogene. The two sequences have been submitted to GenBank (accession numbers AY37353 and AY37354). The longer product was cloned into a commercial vector (pCR2.1-TOPO, Invitrogen, Carlesbad, CA) and the insert was transcribed from the vector's T7 start site using the MAXIscript T7 Kit (Ambion, Inc.); the product was precipitated and redissolved according to the manufacturer's protocol. The resulting solution of transcribed RNA in buffer TE (10 mM Tris, 1 mM EDTA, pH 7) contained 7.3×10^{10} copies/μl (calculated from the OD_{260} of the prep). A set of serial tenfold dilutions of the solution in DEPC-treated water was prepared, and the appropriate dilutions were used as standards in the qRT-PCR plates. The oligonucleotides used for actin qRT-PCR were designed in the second exon of the putative actin sequence. Divergence between this sequence and the putative pseudogene (including a 6 bp deletion in the pseudogene where the forward primer would anneal) was sufficient that a PCR reaction using the forward and reverse primers with a cloned pseudogene template was unsuccessful (data not shown).

Turtle actin F: CACAGATCATGTTTGAGACCTT
Turtle actin probe: FAM-GAATGGCTACGTACATGG-CTGGGGTGT-TAMRA
Turtle actin R: GCATACAGGGACAACACAGCC

Acknowledgments

The authors thank Bob Rameyer, Shawn K. K. Murakawa, and Shandell Eames for their continuing research support, and Barbara Tefft at the Cornell University Image Lab for her kind assistance with Fig. 1. RJG was supported by a fellowship from a NIH-NIEHS training grant, 2T32 ES07052-26.

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