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Three Closely Related Herpesviruses Are Associated with Fibropapillomatosis in Marine Turtles

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Green turtle fibropapillomatosis is a neoplastic disease of increasingly significant threat to the survivability of this species. Degenerate PCR primers that target highly conserved regions of genes encoding herpesvirus DNA polymerases were used to amplify a DNA sequence from fibropapillomas and fibromas from Hawaiian and Florida green turtles. All of the tumors tested ($n = 23$) were found to harbor viral DNA, whereas no viral DNA was detected in skin biopsies from tumor-negative turtles. The tissue distribution of the green turtle herpesvirus appears to be generally limited to tumors where viral DNA was found to accumulate at approximately two to five copies per cell and is occasionally detected, only by PCR, in some tissues normally associated with tumor development. In addition, herpesviral DNA was detected in fibropapillomas from two loggerhead and four olive ridley turtles. Nucleotide sequencing of a 483-bp fragment of the turtle herpesvirus DNA polymerase gene determined that the Florida green turtle and loggerhead turtle sequences are identical and differ from the Hawaiian green turtle sequence by five nucleotide changes, which results in two amino acid substitutions. The olive ridley sequence differs from the Florida and Hawaiian green turtle sequences by 15 and 16 nucleotide changes, respectively, resulting in four amino acid substitutions, three of which are unique to the olive ridley sequence. Our data suggest that these closely related turtle herpesviruses are intimately involved in the genesis of fibropapillomatosis. © 1998 Academic Press

INTRODUCTION

Green turtle fibropapillomatosis (GTFP), a neoplastic disease of green turtles that is characterized by the presence of epithelial fibropapillomas and internal fibromas, was first described in 1938 in green turtles (*Chelonia mydas*) from the Florida Keys (Lucke, 1938; Smith and Coates, 1938). Since then, GTFP has been reported in green turtles worldwide with increasing frequency (Williams *et al.*, 1994). Tumors are found on the skin, eyes, oral cavity, and carapace, as well as in visceral organs (Harshbarger, 1991; Jacobson *et al.*, 1989; Lucke, 1938; Norton *et al.*, 1990). Although the tumors are considered benign, the disease can be life-threatening; large cutaneous tumors can interfere with the turtles's locomotion, vision, swallowing, and breathing, and visceral tumors can be locally invasive and affect organ function.

Significant differences in the prevalence of GTFP have been observed between close geographic locations (Ehrhart, 1991; Herbst, 1994). During the past 15 years, prevalence rates of up to 50 and 90% have been documented in well-monitored areas of the Indian River, Florida, and Kaneohe Bay, Hawaii, respectively, while turtles in nearby reefs or on a neighboring island were free of disease (Balazs, 1991; Herbst, 1994). The disease appears to be associated with near-shore bays and lagoons that are accompanied by agricultural and urban development (Balazs, 1991; Ehrhart, 1994). In addition, fibropapillomatosis has recently been observed in olive ridley (*Lepidochelys olivacea*) and loggerhead (*Caretta caretta*) turtles (Herbst, 1994). The increased prevalence of GTFP, as well as its observation in other marine turtles, makes the identification of the responsible etiologic agent of paramount importance to the management of this disease and long-term survivability of these threatened and endangered species.

An infectious agent is strongly implicated in the etiology of GTFP (Herbst *et al.*, 1995, 1996; Jacobson *et al.*, 1991). Outbreaks of the disease ocurred in captive-reared turtles after contact with wild-caught turtles with GTFP (Herbst, 1994; Hoffman and Wells, 1991; Jacobson *et al.*, 1989) and, recently, fibropapilloma has been experimentally transmitted to green turtles using filtered cell-free tumor homogenates (Herbst *et al.*, 1995, 1996). These studies, and observations by others (Casey *et al.*,

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1997; Jacobson *et al.*, 1991), suggest that a virus is involved in the pathogenesis of this disease. However, attempts to isolate any virus in cell culture have been unsuccessful to date.

To investigate the possibility that a herpesvirus may be present in fibropapillomas from green turtles, we used PCR to amplify a highly conserved region of the herpesvirus DNA polymerase gene. This PCR approach was also used to identify closely related herpesviruses in fibropapillomas of loggerhead and olive ridley marine turtles. In addition, we determined the tissue distribution of the green turtle herpesvirus and used Southern blot analysis to quantitate the amount of viral DNA present in tumors.

RESULTS

Amplification of a herpesvirus DNA polymerase sequence

To identify and characterize a GTFP-associated herpesvirus, we used a nested PCR assay. DNA was extracted from fibropapillomas and normal skin and subjected to PCR using primers directed against the gene encoding herpesvirus DNA polymerase (VanDevanter *et al.*, 1996). A predicted band of approximately 250 bp was visualized by ethidium bromide staining of electrophoretically separated PCR products from five green turtle fibropapillomas (data not shown). No PCR products were amplified from uninvolved skin from two of these tumor-positive turtles. Direct nucleotide sequencing revealed that the PCR products from the five tumors were identical. A search of the GenBank database determined that the 237-bp sequence was most similar to bovine herpesvirus type I (BHV-1) and pseudorabies virus (PRV) DNA polymerases, to which it is 53% identical within a 183-bp region located between the primers (TGV and IYG) used for amplification (Fig. 1 nucleotides 301–483).

An additional sequence extending upstream from the TGV primer region was obtained using a seminested PCR assay with the upstream degenerate primer, DFA, and the downstream turtle-specific primer GTHV2 (refer to Fig. 1). Amplification products were detected and directly sequenced from fibropapillomas from Hawaiian and Florda green turtles. The Florida samples yielded a 483-bp sequence, excluding the 5′ degenerate primer, that contained five nucleotide changes encoding two predicted amino acid substitutions compared to the Hawaiian sequence (Fig. 1). An alignment of the amino acids encoded by the 483-bp DNA fragment from the Hawaiian and Florida turtle herpesviruses and homologous regions of other herpesviruses is shown in Fig. 2. The Hawaiian green turtle sequence is 59 and 55% identical by amino acid homology to herpes simplex virus type 1 (HSV-1) and Gallid-HV2 (Marek's disease virus), respectively, 48% identical to Epstein Barr virus (EBV), and 46% identical to Kaposi's sarcoma-associated herpesvirus (KSHV). A phylogenetic tree was constructed using the NEIGHBOR program of the PHYLIP package from the amino acid sequences contained in this conserved region of the DNA polymerase gene of several herpesviruses (Fig. 3). This analysis indicates that the turtle herpesviruses are a group of closely related viruses; conclusive assignment to a specific subfamily (alpha-, beta-, or gammaherpesvirinae) awaits further sequence information.

Detection of herpesvirus in fibropapillomas from loggerhead and olive ridley turtles

To determine if fibropapillomas from loggerhead and olive ridley turtles contain a herpesvirus, DNA extracted from six tumors (two loggerhead and four olive ridley tumors) was subjected to PCR using the seminested PCR assay with the degenerate DFA and specific GTHV2 primers. The sequence of the 483-bp fragment of the herpesvirus DNA polymerase gene from the two loggerhead turtles was identical to the sequence obtained from the Florida green turtles and differed from the Hawaiian green turtle sequence by five nucleotide changes and two predicted amino acid substitutions. All of the sequences obtained from the four olive ridley tumors were identical and differed from the Florida and Hawaii green turtle herpesviral DNA sequence by 15 and 16 nucleotide changes, respectively (Fig. 1). The nucleotide changes in the olive ridley sequence result in three unique amino acid substitutions compared to the Hawaiian and Florida green turtle sequences (Fig. 2). There is a phenylalanine (F) to tyrosine (Y) substitution that is common in the Florida green turtle and olive ridley sequences, while the alanine (A) to threonine (T) substitution is unique to the Florida green turtle sequences (Fig. 2). DNA isolated from five samples of normal tissue (heart, kidney, skin, and spleen) from two olive ridley turtles was subjected to the nested PCR assay using degenerate primers, and all of the samples were found to be negative (Table 1).

Tissue distribution of green turtle herpesvirus

To specifically amplify a region of the green turtle herpesvirus DNA polymerase gene, defined, nondegenerate primers were designed based on the sequence obtained above. The turtle HV-specific primers, GTHV1 and GTHV2, are located internal to the degenerate primer sites and amplify a 165-bp product. These primers allowed detection of femtogram quantities of cloned target DNA, pGTHV, diluted in 1 μg of control cellular DNA (data not shown). DNA was prepared from fibropapillomas collected from 10 Hawaiian and 2 Florida turtles and subjected to PCR using the GTHV1 and GTHV2 primers. Often several fibropapillomas were present on the turtles; therefore, we isolated DNA from two (5 turtles) or three (2 turtles) individual tumors obtained from Hawaiian turtles and

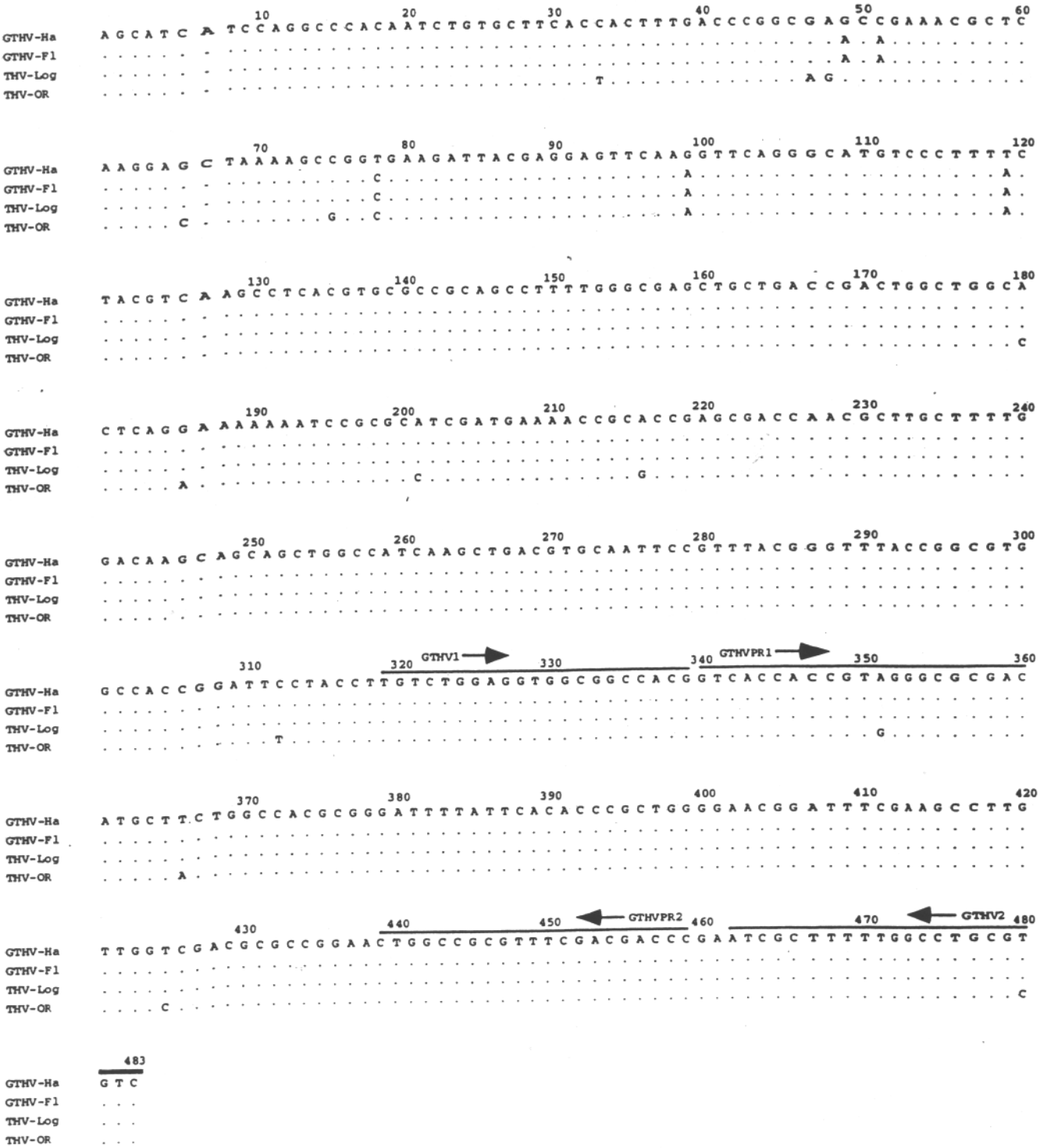

FIG. 1. Turtle herpesvirus DNA polymerase sequences. Comparison of the nucleotide sequences obtained from fibropapillomas from Hawaii green turtles (GTHV-Ha), Florida green turtles (GTHV-FI), loggerhead turtles (THV-Log), and olive ridley turtles (THV-OR). Turtle herpesvirus-specific PCR primers (GTHV1 and GTHV2) and primers used to generate hybridization probes (GTHVPR1 and GTHVPR2) are underlined. The dots indicate sequence identity with the first sequence in the alignment.

performed PCR with the turtle HV-specific primers. All of the fibropapillomas (n = 23) contained the turtle herpesvirus sequence (Table 1). In addition, 3 of the Hawaiian turtles had tumors in visceral organs (heart, lung, liver, and kidney), all of which contained viral DNA (Table 1). DNA isolated from skin biopsies from captive-reared and wild-caught green turtles were negative by PCR for the turtle herpesvirus sequence (Table 1).

To determine the tissue distribution of GTHV, PCR

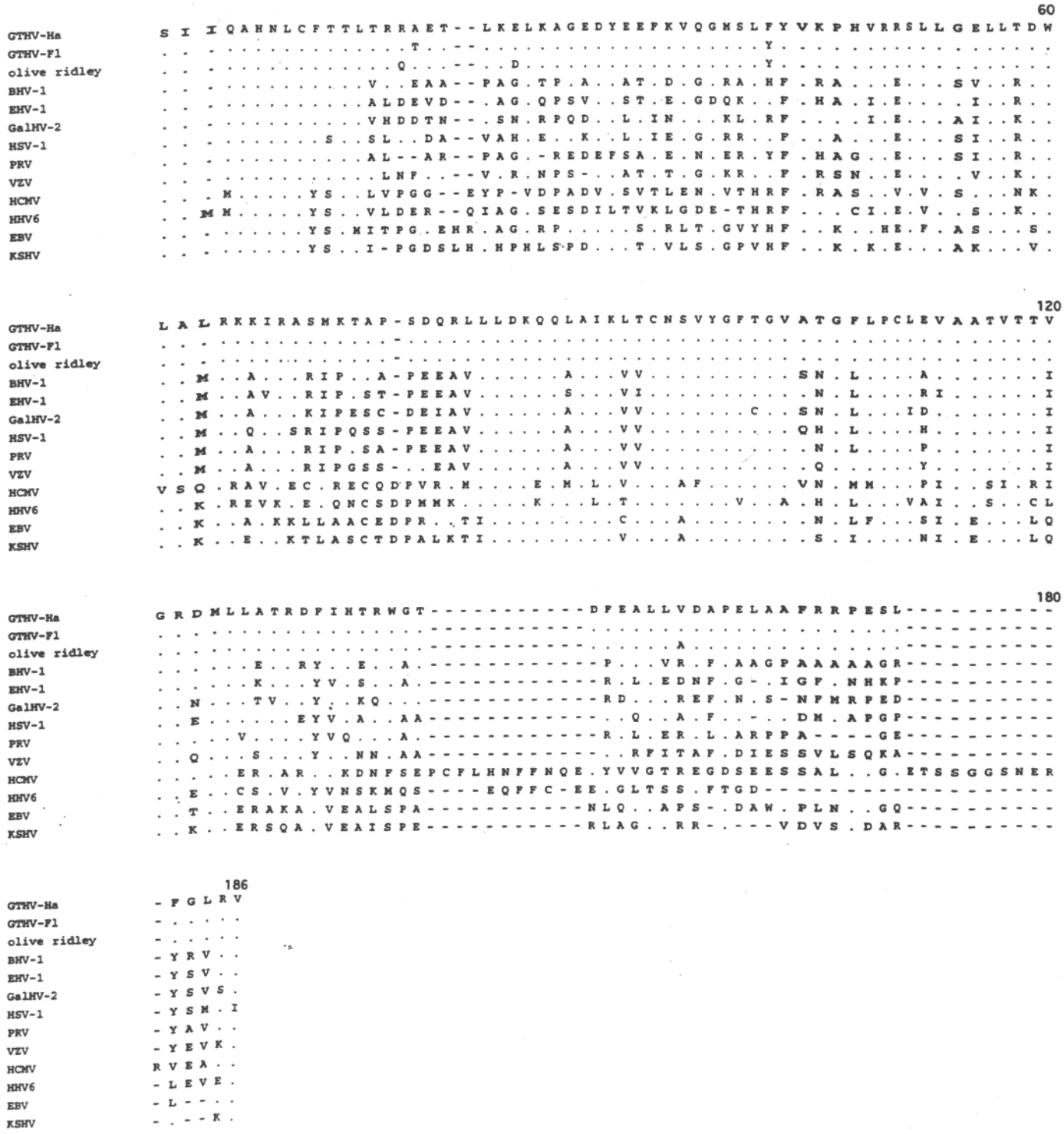

FIG. 2. Comparison of the amino acid sequences from the Hawaii green turtle (GTHV-Ha), Florida green turtle (GTHV-Fl), and the olive ridley turtle herpesvirus DNA polymerase genes with the corresponding region of other known herpesviruses: BHV-1 (Accession No. emb Z78205), EHV-1 (equine herpesvirus 1; Accession No. M86664), GalHV-2 (Accession No. L40431), HSV-1 (Accession No. emb X04771), PRV (Accession No. L24487), VZV (Varicella-Zoster virus, HHV-3; Accession No. emb X04370), HCMV (human cytomegalo virus, HHV-5, Accession No. M14709), HHV-6 (Accession No. emb X83413), HHV-7 (Accession No. U43400), EBV (Accession No. V01555), and KSHV (Accession No. U93872).

was performed on DNA extracted from several different organs from Hawaiian and Florida turtles using the GTHV1 and GTHV2 primers (Fig. 4, Table 1). On gross examination of each tissue there was no evidence of tumor. No viral sequences were detected in blood, brain, liver, or spleen. However, GTHV DNA sequences were detected from DNA isolated from the lung of two of four Hawaiian turtles and from the heart and kidney

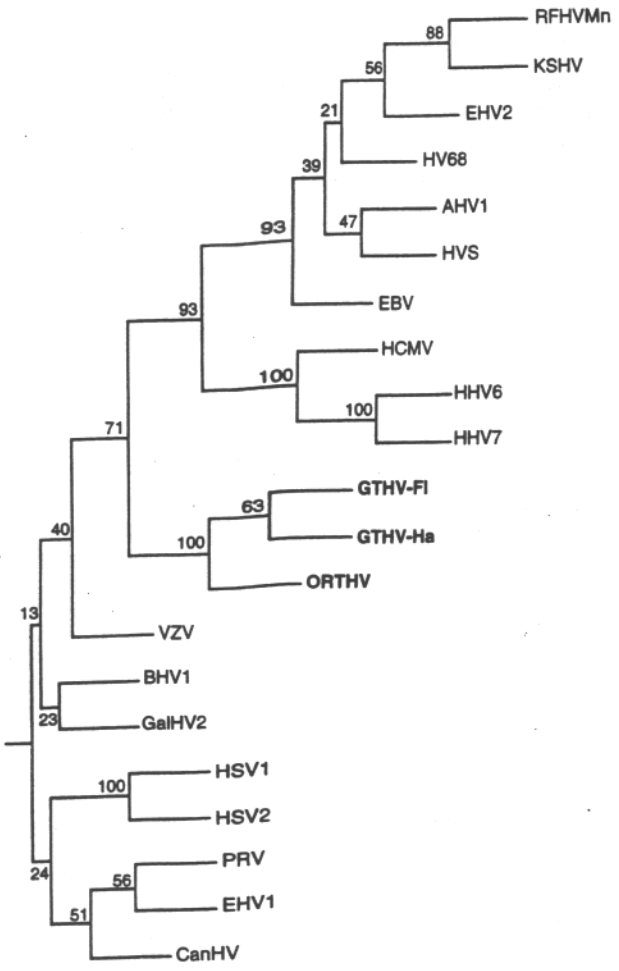

FIG. 3. Phylogenetic analysis of the turtle herpesvirus sequences (GTHV-Ha, GTHV-FI, and ORTHV) and the corresponding amino acid region from the DNA polymerase genes of other known herpesviruses. The rooted phylogenetic tree was generated with the NEIGHBOR program of the PHYLIP package. The numbers at each branch indicate the percent frequency of this grouping after 100 bootstrap evaluations. AHV-1 (alcelaphine herpesvirus 1; Accession No. AF005370), CanHV (canine herpesvirus 1; Accession No. emb X89500), EHV-2 (equine herpesvirus 2; Accession No. U20824), HV68 (murine gammaherpesvirus 68; Accession No. U97553), HSV-2 (HHV-2; Accession No. M16321), HVS (Saimirine herpesvirus 2; Accession No. M31122), and RFHVMn (retroperitoneal fibromatosis herpesvirus *Macaca nemestrina;* Accession No. AF005478).

from one of the Florida turtles. Herpesviral DNA was detected by PCR in three of four gonad samples. Four of seven normal skin samples collected from tumor-positive Hawaiian turtles were also found to contain viral DNA by PCR.

Southern blot analysis of THV in tumors

To quantitate the amount of herpesviral DNA present in fibropapillomas, Southern blot analysis was performed on DNA extracted from a tumor from each of the 12 green turtles, five fibromas, and, where available, from normal non-tumor-containing skin (7 green turtles) and from fibropapillomas from the 2 loggerhead and 4 olive ridley turtles. A representative Southern blot is presented in Fig. 5. GTHV DNA was not detected by hybridization in any of the normal non-tumor-containing skin samples. These results indicate that the amount of viral DNA in the four normal skin samples that were PCR-positive was below the level of detection by Southern blot hybridization. The GTHVpol probe hybridized to a band of approximately 11 kb in all of the *Bam*HI-digested tumor samples. By comparison to the copy-number standards, we estimated that viral DNA was present in the tumors on average at approximately two to five copies per cell.

DISCUSSION

In this study we used a nested PCR assay with degenerate primers, designed to amplify a region of the DNA polymerase gene of herpesviruses that contains highly conserved amino acid motifs (Rose *et al.*, 1997; VanDevanter *et al.*, 1996), to identify herpesvirus sequences that are etiologically associated with fibropapillomatosis of marine turtles. This sensitive PCR assay has been used to amplify sequences of several known herpesviruses (VanDevanter *et al.*, 1996) and more recently was employed to describe two newly identified herpesviruses associated with simian retroperitoneal fibromatosis (Rose *et al.*, 1997). THV sequences were detected in all fibropapillomas, suggesting the presence of newly identified turtle herpesviruses that associate with these tumors. The sequence information and phylogenetic analysis presented here suggests that the turtle herpesviruses

TABLE 1

Detection of Turtle Herpesvirus in Tissues

	n	No. positive (%)
Tumor animals	18[a]	
Fibropapilloma	29	29 (100)
Fibroma	5	5 (100)
Blood	3	0 (0)
Brain	3	0 (0)
Gonad	4	3 (75)
Heart	6	1 (17)
Kidney	5	1 (20)
Liver	5	0 (0)
Lung	4	2 (50)
Skin	7	4 (57)
Spleen	3	0 (0)
Non-tumor animals	16[b]	
Skin	16	0 (0)
Kidney	1	0 (0)
Heart	1	0 (0)
Spleen	1	0 (0)

[a] 10 Hawaiian green turtles, 2 Florida green turtles, 2 loggerhead turtles, and 4 olive ridley turtles.

[b] 14 Hawaiian green turtles and 2 olive ridley turtles.

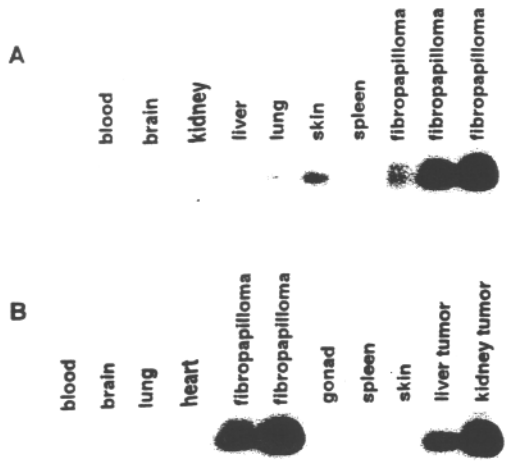

FIG. 4. Southern blot analysis of PCR products from turtle 12428 (A) and turtle 390 (B). One microgram of DNA isolated from various tissues was subjected to PCR amplification with turtle-specific herpesvirus primers (GTHV1 and GTHV 2). Amplification products were separated by electrophoresis, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled, turtle herpesvirus-specific probe (GTHVpol).

belong to a distinct group of viruses that may be more closely related to the alphaherpesviruses. However, further sequence information and the genome structure will be necessary for final classification.

The THV sequence was detected in all tested fibropapillomas and fibromas and was found to accumulate at approximately two to five copies per cell by Southern blot analysis. The presence of low levels of turtle herpesviral DNA, detected only by PCR, in some of the apparently normal lung, heart, kidney, and skin samples was not surprising, as tumors often develop in these organs and skin is the primary target tissue for tumor development. It is possible that very small tumors were present that were not visible grossly. Detection of PCR of viral DNA in some of the gonad samples from Hawaiian turtles was unexpected, as tumors have not previously been seen in this tissue (T. M. Work, unpublished data).

We also identified a herpesvirus DNA polymerase sequence in two fibropapillomas from loggerhead turtles and in four tumors from olive ridley turtles. The sequence of the 483-bp fragment of the herpesvirus DNA polymerase gene from loggerhead turtles was identical to the sequence from the Florida green turtles and differed from the Hawaiian green turtle sequence by five nucleotide changes and two predicted amino acid substitutions. The olive ridley sequence differs from the Florida and Hawaiian sequences by 15 and 16 nucleotide changes, respectively. These nucleotide changes result in four amino acid substitutions, three of which are unique to the olive ridley sequence. These results indicate that the Florida and Hawaiian green turtle and the olive ridley are distinct, but closely related, herpesviruses.

GTFP is a recently emerging disease and likely represents a new viral epizootic. The association of THV with tumors from green turtles, loggerhead turtles, and olive ridley turtles strongly implicates a herpesvirus as a critical factor in the pathogenesis of disease. The most compelling evidence to support the contention that these herpesviruses are the etiological agents of fibropapilloma is the presence of two to five copies of viral DNA per cell in tumors detected by Southern blot analysis. GTHV does not appear to be a common infectious agent in green turtles, as skin biopsies from tumor-free wild-caught and captive-reared turtles from Sea Life Park, Hawaii, and samples from two nontumored olive ridley turtles were negative by PCR. To investigate the pathogenesis of THV and definitively determine if these herpesviruses are the etiologic agents of marine turtle fibropapillomatosis will require propagation of these viruses and rigorous molecular characterization. Such efforts will likely be aided by the sequences described herein to serve as DNA probes to monitor virus propagation in cultured cells.

MATERIALS AND METHODS

Tissues and DNA isolation

Tissues were obtained from 10 Hawaiian green turtles, 2 Florida green turtles, 2 Florida loggerhead turtles (tumors from loggerhead turtles were kindly provided by Barbara Schroeder, National Marine Fisheries Service), and 6 olive ridley turtles. Biopsies of normal skin were collected from captive-reared turtles from Sea Life Park Hawaii, and from tumor-free wild-caught Hawaiian turtles. A green turtle embryo fibroblast cell line, GTEF (Moore *et al.*, 1998), was kindly provided by Melody Moore (U.S. Geological Survey,

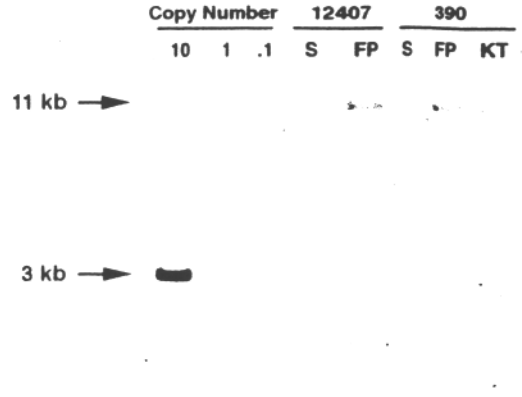

FIG. 5. Southern blot analysis of turtle herpesvirus in fibropapillomas (FP), kidney tumor (KT), and normal skin (S). The GTHVpol plasmid was used to determine the copy number of herpesvirus in each tissue. GTHVpol plasmid DNA equivalent to 10, 1, and .1 copies/cell and 10 μg of DNA isolated from tissues were digested with *Bam*HI, subjected to electrophoresis on a 1% agarose gel, and transferred to nitrocellulose. The blot was crosslinked with UV light and hybridized with a ^{32}P-labeled GTHVpol probe.

Madison, WI). Tissues were homogenized in TE (10 mM Tris–HCl, pH 8.0, 50 mM EDTA, pH 8.0), and 100 μg/ml of proteinase K and 0.1% sodium dodecyl sulfate (SDS) were added. After overnight incubation at 37°C, the cellular DNA was extracted three times with phenol:chloroform:isoamyl alcohol (25:24:1), two times with chloroform:isoamyl alcohol, ethanol precipitated, resuspended in H_2O, and quantitated.

Consensus sequence PCR amplification

Amplification of a portion of the herpesviral DNA polymerase gene was performed with nested primers targeted to highly conserved amino acid motifs and modified, as previously described (VanDevanter *et al.,* 1996). The nested PCR assay using degenerate primers was performed in 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 2.5% DMSO, 100 pmol of each primer, and 2.5 units of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD). One microgram of DNA was added to each PCR in a total volume of 100 μl, which was then overlaid with oil. All samples were subjected to one cycle of 3 min at 94°C, 2 min at 60°C, and 1 min at 72°C, followed by 30 s at 94°C, 1 min at 46°C, and 30 s at 72°C for 45 cycles and 7 min at 72°C for 1 cycle. The oligonucleotides used for PCR were as follows: (1) DFA 5′ GAYTTYGCNAGYYTNTAYCC 3′, (2) ILK 5′ TCCTGGACAAGCAGCARNYSGCNMTNAA 3′, (3) KG1 5′ GTCTTGCTCACCAGNTCNACNCCYTT 3′, (4) TGV 5′ TGTAACTCGGTGTAYGGNTTYACNGGNGT 3′, and (5) IYG 5′ CACAGAGTCCGTRTCNCCRTADAT 3′ (VanDevanter *et al.,* 1996). The degenerate primers DFA, ILK, and KG1 were used in the first round PCR. One microliter of the first-round PCR was used as the template for the second-round nested PCR with the TGV and IYG degenerate primers. PCR products were gel purified with a Qiaex II Gel extraction kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's instructions. Automated sequencing with the TGV and IYG primers was done with an ABI 373A automated sequencer (Applied Biosystems, Inc. Foster City, CA) at the Biotechnology Resource Center at Cornell University. The design of new primers corresponding in location to the degenerate primers, TGV and IYG, was based on the sequence obtained above and included *Bam*HI and *Eco*RI restriction sites. These primers were used in a second round of PCR to amplify the 237-bp turtle herpesvirus pol region from DNA isolated from a fibropapilloma. The PCR products were digested with *Bam*HI and *Eco*RI, cloned into pBluescript(SK$^-$) (Stratagene, LaJolla, CA), and sequenced for verification. The plasmid clone pGTHVpol was subsequently used as a probe for hybridization and as a viral copy-number control.

Additional sequences were obtained by primary amplification with the consensus primers DFA and KG1 as described above, but with a total of 35 cycles. One microliter of the first-round PCR was used as the template for the secondary amplification with the DFA upstream primer and a turtle herpesvirus downstream-specific primer, GTHV2 (see below). This second-round PCR was performed as described above, but with a total of 35 cycles and an annealing temperature of 60°C. The PCR products were purified and directly sequenced with the GTHVPR2 primer as described above.

GTHV-specific PCR amplification and analysis of PCR products

One microgram of DNA isolated from various tissues was subjected to PCR amplification with turtle-specific herpesvirus primers (GTHV1: 5′ TGTCTGGAGGTGGCGGCCACG 3′; GTHV2: 5′ GACACGCAGGCCAAAAAGCGA 3′, location of primers underlined in Fig. 1). The PCR mixture consisted of 20 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 2.5% DMSO, 200 μM each dNTP, 10 pmol of each primer, and 2.5 units of *Taq* DNA polymerase (GiBCO BRL). All samples were denatured at 94°C for 5 min and then amplified for 30 s at 94°C, 30 s at 62°C, 30 s at 72°C for 35 cycles followed by 5 min at 72°C for 1 cycle. Fifteen microliters of each PCR amplification reaction was separated on a 2% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled turtle-specific probe (GTHVpol). The GTHVpol probe was amplified by PCR from a gel-purified fragment corresponding to the turtle DNA polymerase region from the pGTHVpol plasmid clone using the GTHVPR1 (5′GTCACCACCGTAGGGCGCGA3′) and GTHVPR2 (5′GGGTCGTCGAAACGCGGCCA3′) primers and labeled with [^{32}P]dCTP using a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. The GTHVPR1 and GTHVPR2 primers are located internal to the turtle herpesvirus specific primers, GTHV1 and GTHV2 (location depicted in Fig. 1). The sensitivity of the turtle-specific PCR assay using GTHV1 and GTHV2 primers was tested on serial 10-fold dilutions of pGTHVpol in 1 μg of control green turtle DNA (GTEF cell line) (Moore *et al.,* 1998).

Southern blot analysis

The pGTHVpol plasmid was used to determine the copy number of herpesvirus in each tissue. GTHVpol plasmid DNA equivalent to 10, 1, and 0.1 copies/cell and 10 μg of DNA isolated from tissues were digested with *Bam*HI, subjected to electrophoresis on a 1% agarose gel, and transferred to nitrocellulose. The blot was crosslinked with UV light and prehybridized at 37°C in 50% formamide, 1 M NaCl, 0.5% Sarkosyl, 50 mM Pipes (pH 6.8), 100 μg/ml calf thymus DNA, 10 mM EDTA, and 5× Denhardt's. Hybridization was carried out for 36 h with a ^{32}P-labeled GTHV-specific probe. The blot was washed in 2× SSC and 0.1% SDS at room temperature

and then in 0.2× SSC and 0.1% SDS at 45°C. The blot was dried and placed on X-ray film at −80°C.

Phylogenetic analysis

Phylogenetic trees were generated with the PHYLIP package (Felsenstein, 1995) based on a CLUSTAL multiple sequence alignment (DNAStar Inc., Madison, WI) of herpesvirus DNA polymerase gene sequences. Any position in any amino acid sequence that contained an inserted gap (see Fig. 2) was eliminated from the phylogenetic analysis in order to analyze amino acid substitutions and exclude addition/deletion events (McGeoch *et al.*, 1995). The phylogenetic tree in Fig. 3. was generated by the neighbor-joining method with the NEIGHBOR program of the PHYLIP package using a Dayhoff PAM 250 distance matrix (calculated with the PROTDIST program). Bootstrap analysis (100 replications) was performed with the SEQBOOT and CONSENSE programs of the PHYLIP package.

Nucleotide sequence Accession numbers

The sequences described in this paper have been deposited with the GenBank database and assigned the following Accession numbers: GTHV-Hawaiian, AF035003; GTHV-Florida, AF035004; Loggerhead HV, AF035005; Olive Ridley HV, AF049904

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