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Genomic Variation of the Fibropapilloma-Associated Marine Turtle Herpesvirus across Seven Geographic Areas and Three Host Species†

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Fibropapillomatosis (FP) of marine turtles is an emerging neoplastic disease associated with infection by a novel turtle herpesvirus, fibropapilloma-associated turtle herpesvirus (FPTHV). This report presents 23 kb of the genome of an FPTHV infecting a Hawaiian green turtle (*Chelonia mydas*). By sequence homology, the open reading frames in this contig correspond to herpes simplex virus genes U_L23 through U_L36. The order, orientation, and homology of these putative genes indicate that FPTHV is a member of the *Alphaherpesvirinae*. The U_L27-, U_L30-, and U_L34-homologous open reading frames from FPTHVs infecting nine FP-affected marine turtles from seven geographic areas and three turtle species (*C. mydas*, *Caretta caretta*, and *Lepidochelys olivacea*) were compared. A high degree of nucleotide sequence conservation was found among these virus variants. However, geographic variations were also found: the FPTHVs examined here form four groups, corresponding to the Atlantic Ocean, West pacific, mid-Pacific, and east Pacific. Our results indicate that FPTHV was established in marine turtle populations prior to the emergence of FP as it is currently known.

Fibropapillomatosis (FP) of marine turtles is an emerging neoplastic disease associated with infection by a novel turtle herpesvirus, fibropapilloma-associated turtle herpesvirus (FPTHV) (17, 21, 30, 31). The prevalence of FP has increased over the past four decades, exceeding 50% in monitored areas of the Indian River Lagoon (Florida), Kaneohe Bay (Hawaii), and Moreton Bay (Australia) (2, 14, 19). Fibropapillomatosis is characterized by the presence of epithelial fibropapillomas; internal fibromas also develop in approximately 39% of terminal cases (38). The first reports were on green turtles (*Chelonia mydas*), but confirmed cases have now also arisen in the loggerhead (*Caretta caretta*) and olive ridley (*Lepidochelys olivacea*) species (30, 31). Since all seven species of marine turtles are either endangered or threatened with extinction (Convention on International Trade in Endangered Species of Wild Fauna and Flora, Appendix I), the additional stress of FP on these populations undermines their long-term survival.

Marine turtle fibropapillomatosis is associated with a herpesvirus infection (17, 21, 30, 31). Herpesvirus polymerase sequences have been detected by PCR in DNA from every tested fibropapilloma and fibroma reported to date (17, 21, 30, 31). In 79% of fibropapillomas and fibromas examined by real-time quantitative PCR, viral sequences were present at levels exceeding 10^4 copies per 100 ng of total tumor DNA, i.e., an average of one virus copy per tumor cell (31). The magnitudes of these loads indicate that viral gene products could be present in sufficient quantities to serve as driving forces for tumor development and/or maintenance. Viral polymerase gene expression has not been detected in fibropapillomas or fibromas, suggesting that infection is predominantly latent in tumors, comparable to human herpesvirus 8 in Kaposi's sarcoma lesions or gallid herpesvirus 2 in the tumors associated with Marek's disease of chickens (10, 32, 33, 36). This putative disease agent has been called the green turtle herpesvirus or the FPTHV. Because FPTHV provides a new model system with which to examine the emergence of a virus and virus-related oncogenesis, we have begun to sequence its genome as a basis for further study.

The apparent increase in prevalence of FP since the first reports in Florida (22, 34), combined with ongoing reports of outbreaks in new geographic areas, has raised speculation that FP emergence reflects a recent introduction of FPTHV into marine turtle populations. An alternative hypothesis is that FPTHV was already established in marine turtles when FP arose as the result of an interaction between the virus and additional environmental cofactors. The latter hypothesis is consistent with published observations that the disease is more prevalent in habitats that are proximal to agricultural and urban development (2, 12, 19). Turtle populations occupying more-pristine waters adjacent to the FP-enzootic areas listed above remain free of FP (2, 12, 19). The first objective of this study was to sequence a sufficiently large block of the FPTHV genome to compare FPTHV to the fully sequenced herpesviruses. The second was to align FPTHV sequences from various geographic locations and host turtle species. Convincing phylogenetic data have resulted from similar analyses of genetic diversity among geographic variants of human herpesvirus 8 (40, 41).

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TABLE 1. Primers used for amplification of compared FPTHV gB and U_L34 sequences

Primer name	Sequence
gB-A-F	CCGTCCGGCAATGATGAAAAA
gB-A-R	GTTGCAACTGCCGCCACTCCTG
gB-B-F	TTCCGCTACCGCCATCAAACACAACT
gB-B-R	ATTAACCCCCGACGGCACCACAAGAG
gB-C-F	TGCGCGTTATCCACTCTTCTTCCTAT
gB-C-R	TTTTTGGCCGCGACCCGTTTTC
gB-D-F	GTCAACAACACGCGAGCCAGAGC
gB-D-R	AAACCCCGCCGAACATAAAATACTTG
gB-E-F	CCTACTTGGGGTTGACGGAGAG
gB-E-R	GCCAGCGCCCCACCTACTAC
gB-F-F	GCTGGCCGGCGTGCTCAAT
gB-F-R	CTAGATACATACTGGCCGTGCTCGTC
UL34-A-F	CCTGAGCAAATTTCTGGACCTG
UL34-A-R	AATTTTCGCGGCTTCTCG
UL34-B-F	GGGCGGTTTTTGGGGGTCAG
UL34-B-R	ACTCAAGATCGCGGTCAGCAGA

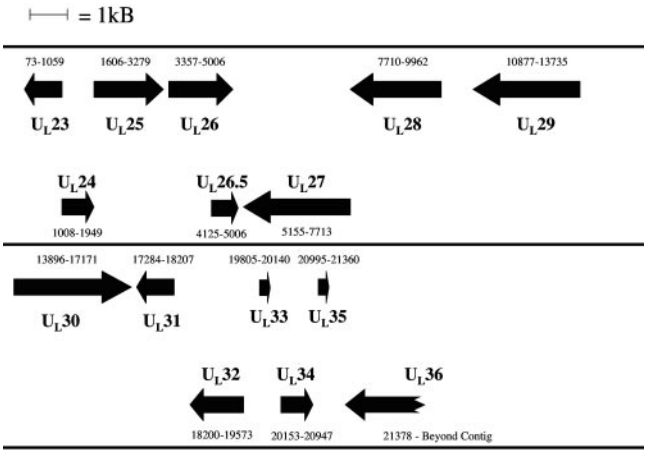

FIG. 1. Lengths, positions, orientations, and homologies of open reading frames in the FPTHV contig (GenBank accession number AF035003).

MATERIALS AND METHODS

Bacteriophage lambda library construction. Construction of an FPTHV λ library was performed as described in reference 18, from the eye tumor of an immature female green turtle collected at Maui, Hawaii. Genomic DNA was prepared and partially digested with MboI and size selected on a 5 to 20% potassium acetate gradient; fragments of 15 to 20 kb were cloned and packaged using the lambda DASH II/BamHI vector kit (Stratagene, La Jolla, Calif.) and Gigapak II XL packaging extract according to the manufacturer's instructions. Clones (10^6) were screened with a ^{32}P-labeled FPTHV *pol* sequence (pHaGTHV) (30).

Cloning and sequencing. (i) Lambda library. The fragments from eight positive λ clones were subcloned into the pCR-XL-TOPO vector (Invitrogen Corporation, Carlsbad, Calif.). Subclones were sequenced with an ABI 373A automated sequencer (Applied Biosystems, Inc., Foster City, Calif.) at the Biotechnology Resource Center at Cornell University. The SeqMan program from the Lasergene software package (DNASTAR, Inc., Madison Wis.) was utilized to assemble the sequence data into a contig of 23,055 bp.

(ii) Geographic and species comparisons. Nine FPTHV subclones, corresponding to six overlapping amplicons from the glycoprotein B (gB) open reading frame, one amplicon from the DNA polymerase (*pol*) open reading frame, and two overlapping amplicons from the U_L34 region, were PCR cloned from each of nine geographically diverse FP samples. The gB and U_L34 primers utilized are listed in Table 1; the *pol* primers (GTHV2 and GTHV3) were published previously (30). Amplification from *pol* utilized the original reaction conditions (30). Amplification with all the gB primer sets and also U_L34 set A was performed in a 100-μl volume containing 1 μg of target DNA, 20 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 2.5% dimethyl sulfoxide, 200 μM (each) deoxynucleoside triphosphates, 10 pmol (each) of primer, and 2.5 U of *Taq* DNA polymerase (Invitrogen). The mixture was incubated 5 min at 94°C and then subjected to 35 cycles of 94°C for 30 s, 57°C for 20 s, and 72°C for 20 s, followed by a hold of 5 min at 72°C. Amplification with the U_L34 primer set B used the same reaction mixture; thermal cycling began with a 5-min incubation at 94°C, followed by 40 cycles of 94°C for 30 s and 72°C for 30 s, and then a hold of 10 min at 72°C. Cloning, sequencing, and contig assembly were carried out as described above.

Sequence comparisons. Sequence comparisons, both pairwise and against published sequences, utilized the National Centers for Biotechnology Information Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequence alignments and phylogeny. Alignments of the nine FPTHV nucleotide sequence blocks were constructed by using a ClustalW alignment by the Lasergene software package (DNASTAR, Inc.). A phylogenetic tree was constructed with the NEIGHBOR algorithm of the Phylogeny Inference Package (PHYLIP) (http://evolution.genetics.washington.edu/phylip.html). Alignment of the amino-terminal domains of the U_L26-homologous open reading frames of FPTHV and other herpesviruses utilized the Jotun Hein algorithm of the MegAlign program from the Lasergene software package.

Additional sequences used for geographic and host comparisons of the *pol* gene are Australian Green *pol* (AF299108), Australian Loggerhead *pol* (AF299107), Barbados Green *pol* (AF299110), Costa Rica Olive Ridley *pol* (AF049904), Florida Green *pol* (AF035004), and Hawaii Green 2 *pol* (AF035003 bases 15840 to 16322) (30).

The lung-eye-trachea disease virus (LETV) sequences used for comparisons below are $U_L26/U_L26.5$ (AY124578), U_L27 (AY124557), and a partial sequence of U_L30 (*pol*) (AAM95779) (5).

Additional herpesvirus sequences used in alignments below are the genome sequences of human herpesviruses 1 (NC001806), 3 (NC001348), 4 (NC001345), and 5 (NC001347), gallid herpesvirus 2 (NC002229), and equine herpesvirus 1 (NC001491) and partial sequences from gallid herpesvirus 1 (AB024414).

Nucleotide sequence accession numbers. The new sequences derived here have been submitted to GenBank. Accession numbers are as follows (in parentheses): U_L23–U_L26 contig (AF035003), Australian Green U_L27 (AY390402), Australian Loggerhead U_L27 (AY390403), Barbados Green U_L27 (AY390404), Costa Rica Olive Ridley U_L27 (AY390405), Florida Green U_L27 (AY390406), Hawaii Green 1 U_L27 (AY390407), Hawaii Green 2 U_L27 (AY390408), Puerto Rico Green U_L27 (AY390409), San Diego Green U_L27 (AY390410), Hawaii Green 1 *pol* (AY390420), Puerto Rico Green *pol* (AY390421), San Diego Green *pol* (AY390422), Australian Green U_L34 (AY390411), Australian Loggerhead U_L34 (AY390412), Barbados Green U_L34 (AY390413), Costa Rica Olive Ridley U_L34 (AY390414), Florida Green U_L34 (AY390415), Hawaii Green 1 U_L34 (AY390416), Hawaii Green 2 U_L34 (AY390417), Puerto Rico Green U_L34 (AY390418), and San Diego Green U_L34 (AY390419).

RESULTS

Sequence analysis of Hawaiian green turtle FPTHV. Sequence analysis of 23,055 bp of FPTHV (57.5% G+C content) determined that this virus is organized in the same manner as the *Alphaherpesvirinae* genera simplexviruses and Marek's disease-like viruses. The sequence block reported here contains open reading frames (ORFs) homologous to U_L23 through U_L36 (Fig. 1 and Table 2). Points of particular interest are discussed below (see Table S1 in the supplemental material).

U_L26. The 250 N-terminal residues of the predicted translation of the open reading frame at 3,357 to 5,006 bears 27 to 30% identity and 38 to 45% similarity to the comparison U_L26 (capsid maturation protease) genes in Table 2. Furthermore, an alignment of the amino-terminal domains of the U_L26-homologous primary sequences from FPTHV, herpes simplexvirus 1 (HSV-1), human herpesvirus 3 (varicella-zoster virus [VZV]), and human herpesvirus 5 (cytomegalovirus [CMV]) revealed that like VZV and CMV, FPTHV bears histidine residues in positions homologous to HSV-1 H_{61} and H_{148}.

TABLE 2. Homology of FPTHV open reading frames to those of other alphaherpesviruses

ORF	Strand	Length[a] (codons)	Identity (%)					Predicted function[b]
			HSV-1	MDV	VZV	ILTV	LETV	
U_L23	−	328	29	27	31	30		Thymidine kinase
U_L24	+	313	31	29	36	31		Membrane-associated protein
U_L25	+	557	33	30	28	31		Minor capsid protein; DNA packaging
U_L26[c]	+	549	27	30	29	29	33	Capsid maturation protease
$U_L26.5$	+	293	—[d]	—	—	—	26	Virion scaffolding protein
U_L27	−	852	29	30	27	31	40	Virion membrane glycoprotein B
U_L28	−	750	33	33	32	34		DNA cleavage and packaging
U_L29	−	952	34	29	32	33		Single-strand DNA binding protein
U_L30	+	1,091	45	43	44	42		DNA polymerase catalytic subunit
U_L31	−	307	27	29	28	25		Nuclear phosphoprotein
U_L32	−	457	29	30	33	29		DNA cleavage and packaging
U_L33	+	111	38	44	32	45		DNA cleavage and packaging
U_L34	+	264	25	25	23	—		Membrane-associated phosphoprotein
U_L35	+	121	—	—	—	—		Basic phosphorylated capsid protein

[a] Predicted length of primary sequence, counting from the first ATG codon.
[b] Predicted functions are those published for the homologous HSV-1 ORFs (reviewed in reference 39).
[c] U_L26 homology is restricted to the N-terminal 250 predicted amino acid residues.
[d] —, no significant similarity between the predicted amino acid sequences was found by pairwise BLAST. Blank space means comparative sequence data were not available.

These two residues are known to be essential for U_L26 protease activity in HSV-1 (20). However, the remainder of the ORF ($U_L26.5$) does not align with the model alphaherpesviruses.

The U_L26 gene of LETV, another disease-associated herpesvirus of green marine turtles (5), shows greater homology to FPTHV U_L26 than the model viruses in Table 2. The primary sequence of LETV U_L26 is 33% identical and 48% similar to FPTHV U_L26, and the alignment between the two extends into the $U_L26.5$ area of the ORFs. LETV also bears the highly conserved histidine residues discussed above (see Table S2 in the supplemental material).

$U_L26.5$. The open reading frame at 4125 to 5006 is homologous by position (overlapping the 5′ end of U_L26) to the virion scaffolding protein genes of alphaherpesviruses. The sequence of these genes is not conserved across the alphaherpesvirus genera, so no significant similarities to the model alphaherpesviruses were detected (Table 2). The predicted amino acid sequence of this ORF, however, is 26% identical to the $U_L26.5$ homolog of LETV (5) (Table 2).

U_L29. The predicted amino acid sequence of the ORF at 13735 to 10877 bears herpesvirus single-stranded DNA binding protein homology, with 29 to 34% identity and 46 to 51% similarity to the model alphaherpesviruses (Table 2). This homology includes a putative conserved zinc finger motif at predicted positions 491 to 502 (**C**DL**C**DVETR**H**V**C**; Zn finger consensus sequence is **C**–X_{2-5}–**C**–X_{2-15}–**C**/**H**–X_{2-4}–**C**/**H**, where bold indicates conserved residues) (11), followed by a putative conserved DNA binding domain (**R**$_{795}$AE**K**VMQLQPILNGPHGYLLKR**F**HERL**F**PTN **K**AIS AMAF**W**NRAQNN**K**; the U_L29 DNA binding consensus motif is **K**/**R**–X_{2-3}–**K**/**R**–X_{9-10}–**F**–X_4–**F**–X_{1-3}–**K**/**R**–X_{10-12}–**W**–X_6–**R** (16). The U_L29 nucleotide sequence reported here is >99% identical to a FPTHV U_L29 sequence in GenBank (27).

U_L30. The previously published 482-bp FPTHV *pol* sequence (30) is a portion of this ORF, 13,896 to 17,171. This ORF is the most homologous to those of the comparison viruses, bearing 42 to 45% identity and 58 to 60% similarity in predicted primary sequence (Table 2). The predicted amino acid sequence of FPTHV U_L30 contains homologs to all of the nine domains highly conserved among herpesvirus *pol* genes (Fig. 2) (35). Of the 130 amino acid residues conserved among equine herpesvirus 1, VZV, HSV-1, gallid herpesvirus 2 (Marek's disease virus [MDV]), human herpesvirus 4 (Epstein-Barr virus), and CMV at these loci, FPTHV shares 123 (95%). Of the seven substitutions, four conserve the charge and approximate size of the consensus residue (98% identical or similar). Of an additional 83 residues shared by just the four alphaherpesviruses listed above, FPTHV shares 55 (66%); of the 28 substitutions, 22 are by similar residues (93% identical or similar). The *pol* nucleotide sequence reported here is 98% identical to an FPTHV *pol* sequence in GenBank (39); discrepancies between the two are concentrated at the 5′ end of the ORF, after the sequence encoding the ninth conserved polymerase domain.

U_L32. The predicted product of the open reading frame at 19573 to 18200 bears 29 to 33% identity and 41 to 49% similarity to the compared major envelope glycoproteins (Table 2). The homology includes an overall cysteine richness, highlighted by three highly conserved C–X_{2-3}–C sequences (C_{14}XVC, C_{281}XLC, and C_{360}DPXC, with X representing amino acids not shared with both HSV-1 and MDV); these may contribute to an overall zinc-binding motif (13).

U_L34. The open reading frame at 20153 to 20947 has 23 to 25% identity and 40 to 43% similarity to the U_L34 (membrane-associated phosphoprotein) genes of MDV, VZV, and HSV-1 in the central 150 residues of its predicted translation (Table 2). This conserved central region includes a motif of unknown function, L_{148}XFRF, which is highly conserved among alphaherpesviruses (13). Interestingly, the C-terminal 100-residue region of FPTHV U_L34 does not align with the C termini of any of the model viruses; however, in accordance with the hypothesized role of this domain in membrane anchoring, it is significantly (36%) hydrophobic, like the corresponding portions of HSV-1 (43%), VZV (38%), MDV (36%), and infectious laryngotracheitis-like viruses (ILTV) (38%). The N-ter-

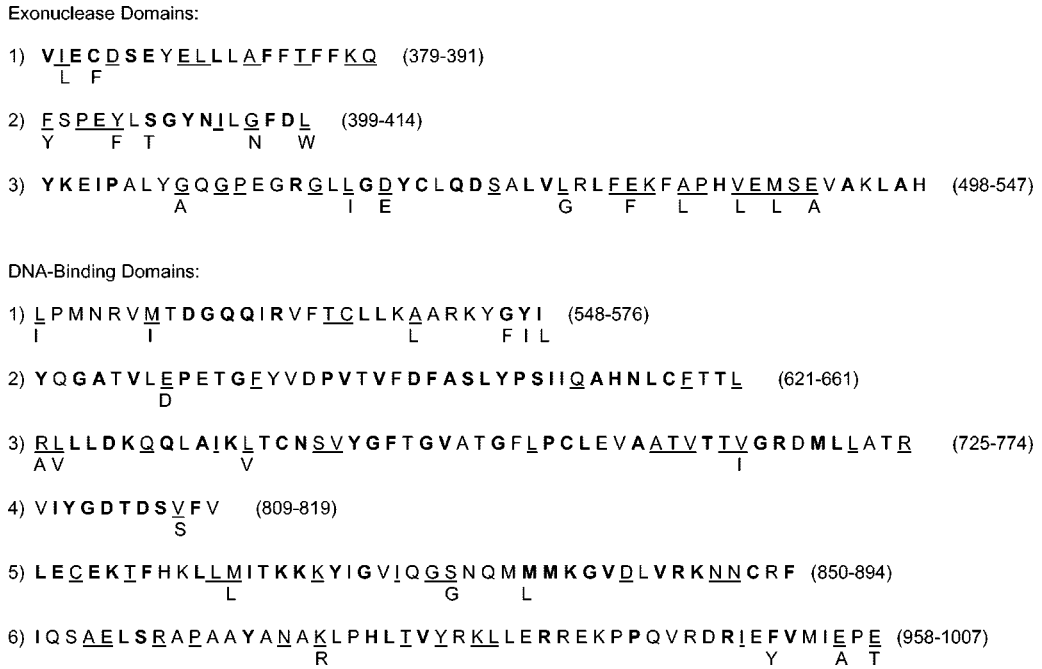

FIG. 2. Predicated amino acid sequences of FPTHV U_L30 in domains highly conserved among herpesvirus DNA polymerase catalytic subunits (35). Underline, amino acid positions conserved among equine herpesvirus 1, VZV, HSV-1, and Marek's disease virus of chickens. Bold, amino acid positions conserved among the four alphaherpesviruses listed above and also Epstein-Barr virus and CMV. When the sequence of FPTHV differs from either consensus, the consensus residue is provided below. The location of each domain in the 1,091-amino-acid predicted primary sequence of FPTHV *pol* is to the right.

minal region of the FPTHV U_L34 ORF does not align with those of MDV or VZV, and the ILTV U_L34 gene bears no significant similarity to the FPTHV homolog. It was because of this apparent variability in U_L34 sequences that the U_L34-U_L35 region was chosen for the FPTHV sequence diversity analysis described below.

Comparisons to *Alphaherpesvirinae* genera. The predicted primary amino acid sequences of the 13 complete FPTHV ORFs above were compared to those of 4 other alphaherpesviruses (Table 2). Each of these four comparison viruses is a founding member of one of the currently established genera (reviewed in reference 33): simplexviruses (HSV-1), varicelloviruses (VZV), Marek's disease-like viruses (MDV), and infectious laryngotracheitis-like viruses (ILTV) (infects chickens, pheasants, and peafowl). These comparisons discouraged assignment of FPTHV to any of these genera; the ORFs of FPTHV had similarly low homology to ORFs of all of the model viruses. (For comparison, within the *Simplexvirus* genus, cercopithecine herpesvirus 1 is 51.8 to 84.0% identical to HSV-1 and 47.3 to 84.8% identical to HSV-2 in the predicted amino acid sequences of its U_L23-U_L36 ORFs [28].) However, there is a partially characterized herpesvirus that appears from available sequence data to be more homologous to FPTHV than any of these four: the LETV, which also infects green marine turtles (*C. mydas*). The U_L27-homologous ORF of LETV is 40% identical to that of FPTHV (compared to the 31% identity between FPTHV and ILTV at this locus), and at the time of publication the $U_L26.5$-homologous ORF of LETV was the only sequence in GenBank with significant similarity (26% identical) to the putative FPTHV $U_L26.5$ discussed above. In addition, the published partial sequence of LETV U_L30 (5) is 57% identical to FPTHV U_L30 amino acid positions 751 to 809. This level of homology is also greater than those found for any of the four model alphaherpesviruses. The limited sequence data available thus suggest that FPTHV and LETV will eventually be grouped together in a new alphaherpesvirus genus.

Comparisons among FPTHV sequences from diverse marine turtles. Portions of the FPTHV open reading frames homologous to U_L27, U_L30, and U_L34, encompassing a total of 3,748 bp, were cloned and sequenced from fibropapillomas of nine geographically and genetically diverse FP-affected marine turtles. Greater than 96% nucleotide sequence conservation was found among the viruses infecting these animals (*C. mydas* from Hawaii, Florida, Australia, Puerto Rico, Barbados, and California, *C. caretta* from Australia, and *L. olivacea* from Pacific Costa Rica). However, there were sequence variations that correlated with geographic location. Figure 3 provides a summary of the nucleotide substitutions and deletions in the nine isolates analyzed. A phylogenetic tree based on the sum of the three sequences from each virus sample is shown in Fig. 4.

DISCUSSION

FPTHV classification. Previous studies of FPTHV have been hampered by the very limited set of available sequence data. The contig presented here will partially relieve this constraint, since it comprises 15% of the predicted FPTHV genome (135 to 175 kb, based on the size of the fully sequenced alphaherpesviruses). The order, orientation, and sequence homology of the ORFs presented here confirm that FPTHV is a member of the *Alphaherpesvirinae*, strengthening the conclu-

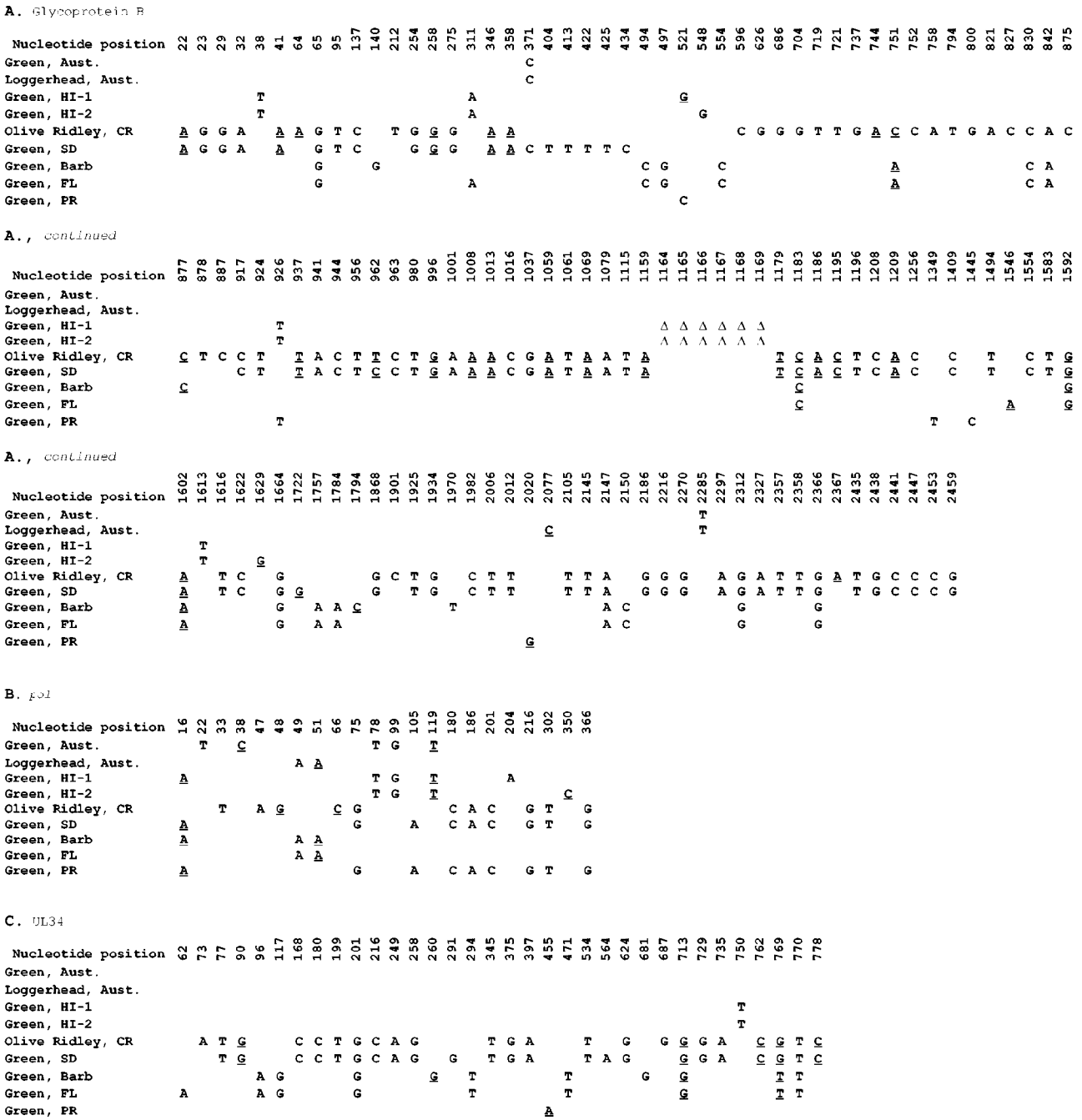

FIG. 3. Positions and identities of nucleotide substitutions among the FPTHV geographic variants. Substitutions that alter the identity of the encoded amino acid are underlined. Source species and geographic abbreviations: green, *C. mydas*; loggerhead, *C. caretta*; Olive Ridley, *L. olivacea*; Aust., Australia; HI-1, Hawaii, turtle one of two; HI-2, Hawaii, turtle two of two (same source as contig); CR, Costa Rica; SD, San Diego; Barb, Barbados; FL, Florida; PR, Puerto Rico.

sion of several previous studies based on single FPTHV genes (17, 31, 39). However, we have found that FPTHV does not seem to belong to any of the existing alphaherpesvirus genera. None of the four genus-founding viruses had greater homology to FPTHV than any of the others. The order and orientation of FPTHV U_L23–U_L26 are identical to those of the herpes simplexviruses; but, like VZV and MDV, FPTHV lacks a recognizable origin of replication site adjacent to U_L29. The regions of FPTHV *pol* homologous to the nine highly conserved herpesvirus *pol* domains (35) showed extensive homology to the overall herpesvirus consensus (98% of predicted residues identical or similar) but significant variation from the additional alphaherpesvirus-specific consensus (7% neither identical nor similar). These unique features in the context of extensive homology indicate that FPTHV may belong to an as-yet-uncharacterized alphaherpesvirus genus. This finding clearly is

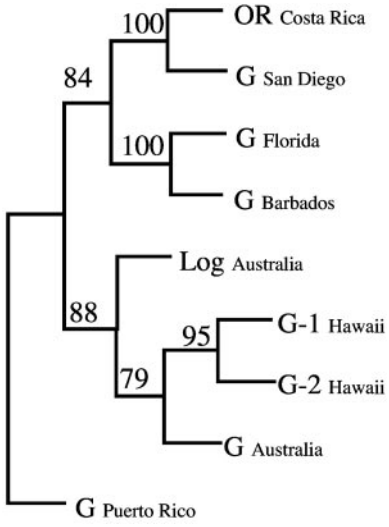

FIG. 4. Phlyogenetic tree of FPTHV geographic and host species variants generated for the total compared sequence block by NEIGHBOR (PHYLIP). Bootstrap values for each node are provided. Compared regions correspond to FPTHV contig (AF035003) positions 5186 to 7667, 15840 to 16242, and 20072 to 20932 (3.7-kB total). Species abbreviations: G, green; OR, olive ridley; log, loggerhead.

not surprising, since there are no completely sequenced model herpesviruses of reptiles. Many reptile herpesviruses exist; known hosts include marine turtles (FTPHV, LETV, gray patch disease virus), freshwater turtles (Pacific pond turtle herpesvirus, Painted turtle herpesvirus, Argentine turtle herpesvirus), snakes (Boa herpesvirus, Indian cobra herpesvirus, banded krait herpesvirus, Siamese cobra herpesvirus), and lizards (green lizard herpesvirus) (reviewed in reference 33). New reptile herpesvirus genera are expected to emerge as the set of available sequence data grows.

FPTHV sequence variation. Among human herpesviruses, distinct genetic variants have been found to partition in geographically defined populations (8, 9, 25, 26, 29). This herpesvirus phylogeography is most extensively characterized in human herpesvirus 8 (HHV-8) (the Kaposi's sarcoma-associated herpesvirus), for which four geographic clades have been identified (40, 41). Based on these findings and early observations that FPTHV polymerase variants were found in different geographic regions (30, 31), we carried out a genetic analysis to determine whether there are variants of FPTHV corresponding to the host species or geographic location. For phylogenetic inference among FPTHVs, the U_L27, U_L30, and U_L34 loci were selected as potentially variable (based on comparisons to other alphaherpesviruses [Table 2]). The inclusion of portions of three different ORFs was also intended to control for variation in the rates of evolution of individual genes, a phenomenon observed in other alphaherpesviruses (24).

FPTHV variants: by host geography, not species. Four FPTHV variant groups were revealed by this analysis: the Florida and Barbados sequences form an Atlantic Ocean group, the Hawaii sequences form a mid-Pacific group, the Australia sequences form a west Pacific group, and the Costa Rica and California sequences form an east Pacific group (Fig. 4). Figure 3 provides a summary of the substitutions and deletions that differentiate the clades.

The identities of the four geographic clades revealed here are fairly clear-cut. The Atlantic group displays a 0.98% divergence from the west Pacific and mid-Pacific groups. The west Pacific and mid-Pacific FPTHVs are quite similar in sequence (0.42% total divergence), but the mid-Pacific group sequences are distinguished by a 6-bp in-frame deletion at positions 1164 to 1169 in the glycoprotein B sequence (Fig. 3). The Atlantic, west Pacific, and mid-Pacific groups collectively differ extensively from the east Pacific group, ranging from 2.8 to 3.1% nucleotide substitutions. The east Pacific group had the highest internal nucleotide variability of the four; the east Pacific group may separate into multiple groups when a more comprehensive analysis includes additional sequences from FP cases in Pacific Mexico and Baja, Calif. The Puerto Rico variant did not cluster with any of the geographic groups defined here; it could belong to a separate Caribbean group. Extending this analysis to include a larger sampling size and more variable genome regions may clarify the relationship of this variant to the others.

This portion of our study succeeded in paralleling an earlier analysis of geographic clades among HHV-8 variants through sequencing of three PCR amplicons from each (40, 41). Intriguingly, the HHV-8 studies found only 1.5% sequence variation between the two most divergent geographic clades, whereas the east and west Pacific FPTHV sequence groups diverge by 4.6%, including 45 consistent substitutions that alter the identity of the encoded amino acid. This more-extensive divergence could reflect greater isolation among turtle populations than human ones.

A surprising finding of this analysis was the strikingly high degree of sequence conservation among FPTHVs infecting different marine turtle species. Only eight nucleotide substitutions in these loci separate the Australian green and loggerhead FPTHVs. The Costa Rica olive ridley variant, likewise, was substantially similar to FPTHVs infecting green turtles in the same geographic area, although there is one string of 21 contiguous substitutions (3 nonsynonymous) unique to the olive ridley sample. Whether this represents species-specific selection or defines another geographic group as discussed above remains to be investigated. It was initially expected that host species would be at least as important a factor as geography for virus sequence variation. The possibility remains that interspecies FPTHV differences exist but are clustered in genes which have not yet been sequenced. However, the idea that the same virus could infect multiple marine turtle species is not implausible. Reptilian lineages have been found to diverge from one another at significantly (perhaps eightfold) lower rates than mammalian ones (1, 4). Most of the seven marine turtle species are able to mate and produce viable hybrid offspring (6, 15, 37), indicating that they share substantial genetic similarity. Further FPTHV sequencing and a larger cross-species sample set will be required to resolve this issue.

Dating FPTHV in marine turtles. The existence of consistent geographic sequence variations in FPTHV make it clear that this infection was established in marine turtle populations prior to the start of the current FP epizootic 70 years ago. We hypothesize that FPTHV is in fact an ancient virus that was established in marine turtle populations prior to the rise of the

Isthmus of Panama (circa 3 million years ago). However, it is currently difficult for a number of reasons to determine the evolutionary "age" of FPTHV. The slow divergence of reptilian lineages mentioned above complicates estimates of the timing of virus introduction to a population and raises questions about the validity of applying evolutionary clock analyses developed for mammalian lineages. Further uncertainties complicate the development of evolutionary clocks for herpesviruses specifically. For example, the VZV nucleotide substitution rate appears to be an order of magnitude lower than that of HSV-1, likely due to the lower replication rate and/or longer latency period of VZV (26). Nothing is currently known about the rate of FPTHV replication or length of latency in marine turtles. As a result of these complications and the lack of complete genome data, it is unlikely that any meaningful estimate of the duration of this virus-host relationship can be made at this time.

FPTHV geographic variation: the cause of FP variation? It is tempting to hypothesize that the regional sequence variations uncovered here are involved in the observed geographic variation in FP outcome (reviewed in reference 14). The existence of various geographic FPTHV clades opens the possibility that viral genetic factors are involved in, for example, the high FP mortality rate observed in the Indian River Lagoon in Florida and not in the San Diego Bay in California (23). On the other hand, local variations in FP incidence and severity have been observed even within the geographic clade areas studied here: FP is nearly absent along the Florida coast adjacent to the Indian River Lagoon (12). It thus seems likely that environmental factors, particularly water pollutants, play a role in FP pathogenesis. However, a number of characteristics of the marine turtle host complicate the differentiation of virus genetic versus environmental factors in FP. One complication concerns the largely unknown but far-reaching migration patterns of marine turtles: juveniles spend six or more years in a pelagic (ocean-faring) state, sometimes traveling between continents, and efforts to track their movements and risk factors during this time are just beginning (3). In addition, there are not yet precise genetic definitions of the turtle stocks nesting at different sites.

It has been suggested that FPTHV infection is acquired when turtles return to their natal feeding-nesting sites (38). The phylogeographic data presented here would appear to be in agreement with this hypothesis, since nesting site was the greatest variable observed. LETV, another herpesvirus of marine turtles, has been shown to be highly stable in salt water, suggesting that FPTHV infection could be waterborne (7). Waterborne infection would be expected to be more efficient in embayments where turtles congregate than in the open ocean. Similarly, the coastal organism *Ozobranchus*, a marine leech that parasitizes turtles, has recently been shown to harbor FPTHV DNA associated with particles of the density of enveloped viruses; this leech thus may act as a vector for FPTHV (10).

In summary, we have presented evidence that FPTHV, a factor in the etiopathology of marine turtle fibropapillomatosis, is an alphaherpesvirus but not a member of any of the currently established genera. Phylogenetic analysis indicates that FPTHV is long established as a marine turtle virus, carrying sequence variations unique to the geographic locations of its hosts. The 23-kb FPTHV contig presented here will serve as a basis for further work modeling virus-associated tumorigenesis and eventually for managing the FP syndrome in endangered turtle populations.

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