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Characterization of Two Divergent Lineages of Macaque Rhadinoviruses Related to Kaposi's Sarcoma-Associated Herpesvirus

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We have cloned and characterized the entire DNA polymerase gene and flanking regions from Kaposi's sarcoma-associated herpesvirus (KSHV) and two closely related macaque homologs of KSHV, retroperitoneal fibromatosis-associated herpesvirus-*Macaca nemestrina* (RFHVMn) and -*Macaca mulatta* (RFHVMm). We have also identified and partially characterized the corresponding genomic region of another KSHV-like herpesvirus, provisionally named "*M. nemestrina* rhadinovirus type 2 (MneRV-2)," with close similarity to rhesus rhadinovirus (RRV). A sequence comparison of these four macaque viruses and two KSHV-like gammaherpesviruses recently identified in African green monkeys, Chlorocebus rhadinovirus types 1 and 2 (ChRV-1 and ChRV-2) reveals the presence of two distinct lineages of KSHV-like rhadinoviruses in Old World primates. The first rhadinovirus lineage consists of KSHV and its closely related homologs RFHVMn, RFHVMm, and ChRV-1, while the second more distantly related lineage consists of RRV, MneRV-2, and ChRV-2. Our findings raise the possibility of the existence of another human KSHV-like herpesvirus belonging to the second rhadinovirus lineage.

Kaposi's sarcoma-associated herpesvirus (KSHV) is postulated to be the infectious cause of Kaposi's sarcoma (KS) (for reviews, see references 5 and 19). Due to the strong similarities in sequence and gene organization with herpesvirus saimiri (HVS), the prototype of the gamma-2 (*Rhadinovirus*) genus of the gammaherpesvirus subfamily, KSHV has been classified as a human rhadinovirus (6, 13). We have previously identified DNA sequences related to KSHV in retroperitoneal fibromatosis (RF) lesions from two macaque species, the pig-tailed macaque (*Macaca nemestrina*) and the rhesus macaque (*Macaca mulatta*) (17). RF is a vascular fibroproliferative neoplasm with similarities to KS which was prevalent in the macaque colony in the Washington Regional Primate Research Center (WaRPRC) during the late 1970s and early 1980s (9, 10). The macaque KSHV-like sequences were identified using a novel consensus-degenerate hybrid oligonucleotide primer (CODEHOP) PCR technique (16), which was employed to detect unknown herpesvirus DNA polymerase genes. Phylogenetic analysis of the available sequence data suggested that the DNA polymerase fragments were derived from macaque homologs of human KSHV, with a unique genotype present in each macaque species. These macaque homologs were designated RF-associated herpesvirus-*M. nemestrina* (RFHVMn) and -*M. mulatta* (RFHVMm). Subsequently, an additional simian homolog of KSHV was identified in an *M. mulatta* from the New England Regional Primate Research Center, and approximately 10 kb of the viral genome, including the DNA polymerase and flanking regions, was sequenced (8). Because of its sequence similarity to KSHV and HVS, this new homolog was designated rhesus rhadinovirus (RRV) isolate H26-95. Another isolate of RRV, RRV-17577, was identified in a simian immunodeficiency virus-infected rhesus macaque with a lymphoproliferative disorder at the Oregon Regional Primate Research Center, and its complete genomic sequence was determined (20). Due to the similarities in gene sequence and genomic structure, it was concluded that RRV was the macaque homolog of KSHV (8, 20). However, comparison of the partial DNA polymerase sequences of RFHVMn and RFHVMm with the corresponding region of RRV suggested that the putative RFHVMn and RFHVMm were more closely related to KSHV than was RRV (4). More recently, DNA fragments from two distinct KSHV-like herpesviruses have been identified in African green monkeys, with one virus, designated *Chlorocebus* rhadinovirus type 1 (ChRV-1), more closely related to KSHV and the other virus, ChRV-2, more closely related to RRV (11).

In order to further characterize the macaque viruses and establish their evolutionary relationship to KSHV and other rhadinoviruses, we have cloned and sequenced the entire DNA polymerase gene and the upstream glycoprotein B and downstream open reading frame 10 (ORF 10) flanking regions from the genomes of RFHVMn, RFHVMm, and KSHV. We have also cloned and characterized the majority of the DNA polymerase gene and the downstream flanking ORF 10 region of a close variant of RRV identified in *M. nemestrina*. Using these sequences, we have determined a phylogenetic relationship between KSHV and the macaque rhadinoviruses.

Identification of an RFHVMn isolate from an RF tumor of *Mn*442N. In our original studies of RFHVMn, only archived samples of paraffin-embedded, formalin-fixed samples of RF tissue were available (17). Subsequently, a simian retrovirus type 2-infected *M. nemestrina* no. 442N (*Mne*442N) from the National Institutes of Health, Bethesda, Maryland, was diagnosed with RF (21), and small frozen tissue specimens were collected after necropsy (kindly provided by Riri Shibata). DNA was extracted and amplified by PCR using the CODEHOP technique with the SLYP1A upstream primer and the

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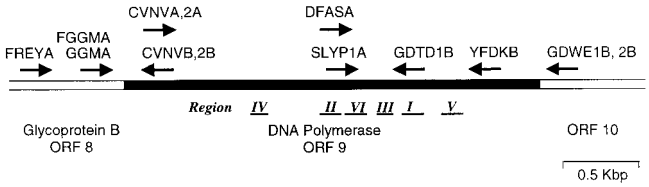

FIG. 1. CODEHOPs PCR cloning strategy. The expected linear arrangement of the glycoprotein B (ORF 8), DNA polymerase (ORF 9), and ORF 10 homologs in the KSHV-like gammaherpesviruses is indicated. The positions and orientation of CODEHOPs derived from conserved sequence motifs are shown. The name of the CODEHOPs indicates conserved amino acids within the motif, and the terminal A or B indicates sense or antisense orientation, respectively. The positions of the conserved DNA polymerase regions are indicated for reference (see reference 22).

GDTD1B downstream antisense primer (Fig. 1 and Table 1). This assay is similar to that described in the original identification of RFHVMn and RFHVMm (17), except that the DFASA primer was replaced with SLYP1A, which was designed with less degeneracy. A PCR amplification of 35 cycles (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C) was performed in 0.067 M Tris-HCl (pH 8.8), containing 4 mM $MgCl_2$, 0.016 M $(NH_4)_2SO_4$, 0.01 M 2-mercaptoethanol, and 100 μg of bovine serum albumin per ml (17). A PCR product of the expected size was obtained. This DNA fragment was purified from excess primers with a Qiagen spin column, cloned into the pCR2.1 T-cloning vector (Invitrogen), and sequenced on an ABI model 377 automated sequencer using Big Dye terminator technology. The DNA sequence obtained was 99.4% (463 of 466 bp) identical to the original published RFHVMn fragment from *M. nemestrina Mne*M78114 from the WaRPRC (17), suggesting that the NIH and WaRPRC macaques contained very similar but nonidentical isolates of RFHVMn (Table 2).

Cloning of the DNA polymerase gene and flanking regions of KSHV, RFHVMn, and RFHVMm. To further characterize and compare KSHV and the KSHV-like herpesviruses in macaques, we chose to clone and sequence the genomic regions containing the DNA polymerase genes using the CODEHOP PCR strategy (16). In gammaherpesviruses, such as HVS, the DNA polymerase gene (also known as ORF 9) is flanked upstream by the glycoprotein B gene (also known as ORF 8) and downstream by the homolog of the HVS ORF10 gene. We, therefore, designed CODEHOPs from blocks of conserved sequences in the DNA polymerase and flanking genes to use in PCR cloning, as shown in Fig. 1 and Table 1. The CODEHOPs were designed manually or with CODEHOP prediction software that we have written and made available at http://www.blocks.fhcrc.org/blocks/codehop.html as part of the BLOCKS database of the Fred Hutchinson Cancer Research Center, Seattle, Wash. The primers are named after the predominant sequence motif in the conserved block, where the terminal A or B designation indicates sense (A) or antisense (B) orientation.

DNA was isolated from RF tumor and spleen samples from *Mne*442N, described above, and from a simian retrovirus type 2-positive rhesus monkey (*M. mulatta*) (*Mmu*YN-91) provided by Harold McClure, Yerkes Regional Primate Research Center, Atlanta, Ga. (see reference 17). For KSHV, DNA was isolated from a human KS tumor sample (KS187) provided by David Koelle, Fred Hutchinson Cancer Research Center. The FREYA CODEHOP (sense orientation) derived from a conserved region in the 3′ end of the gammaherpesvirus glycoprotein B genes and the CVNVB CODEHOP (antisense) derived from a conserved region in the 5′ end of the herpesvirus DNA polymerase gene (Fig. 1 and Table 1) were used in initial PCRs to amplify the regions containing the 5′ end of the DNA polymerase gene and the 3′ end of the upstream flanking glycoprotein B gene. The PCR conditions were similar to those used above, except in some cases glycerol was added to a 4% final concentration. An aliquot (2 to 5%) of the initial PCR product was used as a template in a heminested PCR amplification with the upstream nested GGMA CODEHOP (sense orientation) and the downstream CVNVB CODEHOP (antisense). PCR products of the expected size were obtained from

TABLE 1. CODEHOPs

CODEHOPs (degeneracy)[a] in gene	Bias[b]	Viral sequence(s) cloned[c]	5′→3′ Sequence[d]
Glycoprotein B			
FREYA (32)	Gamma HV	RFHVMn/Mm, KSHV	TTTGACCTGGAGACTATGTTYMGNGARTAYAA
GGMA (128)	Gamma HV	RFHVMn/Mm, KSHV	ACCTTCATCAAAAATCCCTTNGGNGGNATGYT
FGGMA (128)	RRV-like		TTCATTAACTTTATAAAGAGTCCGYTNGGNGGNATG
DNA polymerase			
CVNVA (64)	Gamma HV	RFHVMn/Mm, KSHV	GACGACCGCAGCGTGTGCGTGAAYGTNTTYGGNCA
CVNV2A (64)	RRV-like	*Mne*RV-2	ACCGAGGACGGCACCAGCGTGTGCGTNAAYGTNTTY
CVNVB (64)	Gamma HV	RFHVMn/Mm	TAAAAGTACAGCTCCTGCCCGAANACRTTNACRCA
CVNV2B (64)	RRV-like		TAGAAATATACCTGTTGACGGAANACRTTNACRCA
SLYP1A (64)	All HV	RFHVMn/Mm, *Mne*RV-2, KSHV	TTTGACTTTGCCAGCCTGTAYCCNAGYATNAT
DFASA (256)	All HV	RFHVMn/Mm, KSHV	GTGTTCGACTTYGCNAGYYTNTAYCC
GDTD1B (64)	All HV	RFHVMn/Mm, *Mne*RV-2, KSHV	CGGCATGCGACAAACACGGAGTCNGTRTCNCCRTA
YFDKB (16)	Gamma HV	RFHVMn/Mm, *Mne*RV-2, KSHV	TTAGCTACTCCGTGGAGCAGYTTRTCRAARTA
ORF 10			
GDWE1B (8)	RRV-like	*Mne*RV-2	GAATCTACCCCGGTGAAAAGTCACCTCCCARTCNCC
GDWE2B (8)	Gamma HV	RFHVMn/Mm, KSHV	GAAGTGGCAGTTGGAGAGGCTGACCTCCCARTCNCC

[a] The degree of degeneracy, i.e., the number of individual primers in the pool, is given in parentheses. The terminal letter A or B in the CODEHOP primer name indicates sense or antisense orientation, respectively.

[b] Bias indicates the reliance on a specified subset of sequences for determination of the nondegenerate "clamp" region of the CODEHOP (see reference 16). Gamma HV, gammaherpes virus; HV, herpesviruses.

[c] RFHVMn/Mm, RFHVMm-RFHVMn.

[d] The positions of degeneracy are given. N = A, T, C, and G; R = A and G; Y = C and T; M = A and C. See Fig. 1 for the relative position of primers.

TABLE 2. Herpesviruses and sequences

Herpesvirus	Host	Gene and accession no.[a]		
		ORF 8 (GB)	ORF 9 (DNA Pol)	ORF 10
RFHVMn	Pig-tailed macaque	(AF204166)[b]	(AF005478),[c] (AF204166)[b]	(AF204166)[b]
RFHVMm	Rhesus macaque	(AF204168)[b]	(AF005479),[c] (AF204168)[b]	(AF204168)[b]
*Mne*RV-2:	Pig-tailed macaque	(AF204167)[b]	(AF204167)[b]	(AF204167)[b]
RRV	Rhesus macaque	(AF029302)	(AF029302)	(AF029302)
ChRV-1	African green monkey		CAB61753,[d] (AJ251573)[d]	
ChRV-2	African green monkey		CAB61754,[d] (AJ251574)[d]	
KSHV	Human	(AF204169)[b]	(AF204169)[b]	(AF204169)[b]
HVS	South American squirrel monkey	P24905, (X64346)	P24907, (X64346)	(X64346)
Ateline herpesvirus type 3	South American spider monkey	AAC95532, (AF083424)	AAC95533, (AF083424)	
Equine herpesvirus type 2	Horse	AAC55648	AAC13795	
EBV	Human	CAA24806, (V01555)	P03198, (V01555)	

[a] Protein accession number is given; the nucleotide accession number is given in parentheses. The amino acid sequence was derived from the conceptual translation of the specified gene. Genes: GB, glycoprotein B; DNA Pol, DNA polymerase.
[b] This study.
[c] Partial sequences from reference 17.
[d] Partial sequences from reference 11.

the different DNA templates and were either sequenced directly or cloned prior to sequencing as described above. Gene-specific nested primers (sense orientation) derived from the sequences of the GGMA-CVNVB PCR products were used in subsequent nested PCRs with the gene-specific nested antisense primers derived from the original DFASA (or SLYP1A)-GDTD1B sequences determined previously to obtain a PCR product overlapping the two CODEHOP PCR products. Gene-specific nested sense primers derived from the sequence of the DFASA (or SLYP1A)-GDTD1B PCR product were used in nested PCR amplifications with the downstream YFDKB CODEHOP (antisense) (Fig. 1 and Table 1) to obtain the majority of the 3′ end of the DNA polymerase genes. Additional gene-specific nested sense primers derived from the sequence immediately upstream of the YFDKB region were used in nested PCR amplifications with the further downstream GDWE2B CODEHOP (antisense) to obtain the remainder of the 3′ end of the DNA polymerase and the 5′ end of the flanking ORF 10 gene. PCR products were either cloned and sequenced with vector-specific primers or sequenced directly with CODEHOP or gene-specific primers. Additional internal gene-specific primers were used to obtain overlapping sequences within the larger PCR products. Multiple PCR products and clones were sequenced in both orientations to avoid artifacts and *Taq* polymerase errors. Sequence assembly was done using Sequencher 4.0.5b10 (Gene Codes).

Characterization of the DNA polymerase and flanking regions of RFHVMn and RFHVMm. The nucleotide and encoded amino acid sequences of the overlapping PCR products spanning the DNA polymerase gene and flanking sequences obtained from RFHVMn isolate *Mne*442N (3,554 bp) and RFHVMm isolate *Mmu*YN-91 (3,540 bp) were aligned pairwise using GenePro software (Riverside Scientific, Bainbridge Island, Wash.) (data not shown). ORF analysis and BLAST similarity searches demonstrated that both sequences encode herpesvirus homologs of the C terminus of a glycoprotein B gene, an intact DNA polymerase gene, and the N terminus of a HVS ORF 10 gene homolog in the same linear order found in KSHV (18) and HVS (2). The 475-bp PCR fragment of the DNA polymerase gene of the RFHVMn isolate that we previously identified in the RF sample from *Mne*M78114 (17) aligned with the sequences from RFHVMn *Mne*442N isolate between nucleotides 2087 and 2562 with only a three-nucleotide difference (99.4% identity), as described above. The original 454-bp fragment of RFHVMm obtained from *Mmu*YN-91 (17) was present within the cloned genomic region of RFHVMm from the same source between nucleotides 2166 and 2619 (Fig. 2).

The gene and intergenic lengths within the cloned regions of RFHVMn and RFHVMm were identical, with the exception of a 14-bp region of heterogeneity within the ORF 9-ORF 10 intergenic region. The high degree of nucleotide similarity (83%) observed previously between the fragments of the DNA polymerase genes of RFHVMn and RFHVMm (17) was confirmed after analysis of the complete DNA polymerase sequences, which demonstrated 82% identical nucleotides over 3,039 bp (Table 3). Moreover, the 3′ region of the glycoprotein B genes and the 5′ ends of the ORF 10 gene homologs from these viruses also showed a high degree of similarity, with nucleotide identities of 90% across 331 bp of the glycoprotein B gene and 81% across 27 bp of the ORF 10 gene homolog (Table 3). Comparison of the encoded amino acid sequences showed even closer similarities between RFHVMn and RFHVMm, with 87 and 95% identical amino acid sequences within the DNA polymerase and C-terminal end of the glycoprotein B gene respectively (Fig. 3). Within the short region of the cloned ORF 10 gene fragments, 66% of the amino acids were identical (Table 3 and Fig. 4). These results validate the viral origin of our original PCR fragments and substantiate the inclusion of RFHVMn and RFHVMm within the gammaherpesvirus subfamily. The high degree of nucleotide and amino acid similarity between the RFHVMn and RFHVMm genotypes is consistent with their origin within two closely related species of macaque.

Identification and characterization of an *M. nemestrina* variant of RRV. During the process of amplifying the *Mne*442N DNA above with the SLYP1A and GDTD1B DNA polymerase CODEHOPs, a second PCR clone was identified which was 65% (306 of 466 bp) identical to the RFHVMn *Mne*442N sequence. A BLAST search of the GenBank DNA database with the new DNA sequence as a probe revealed a close similarity to the DNA polymerase gene of the RRV isolate H26-95 (accession no. AF029302) having 424 of 466 (91%) identical nucleotides. This suggested that we had identified a DNA polymerase fragment of an *M. nemestrina* homolog of RRV, which we have provisionally named "*M. nemestrina* rhadinovirus type 2 (*Mne*RV-2)," since it is the second rhadinovirus identified in *M. nemestrina* after RFHVMn. (After this manu-

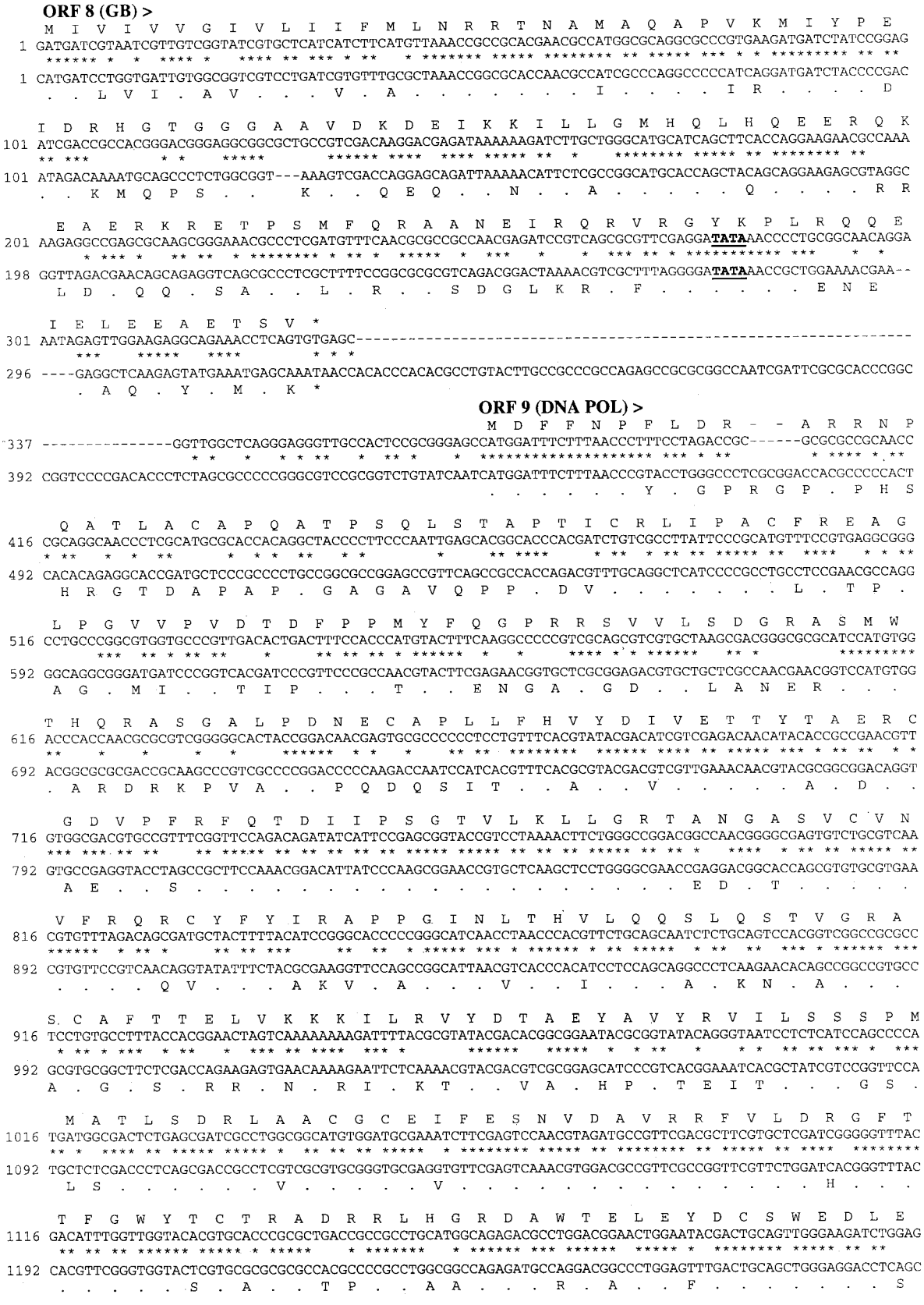

FIG. 2. Comparison of the nucleotide and encoded amino acid sequences of the 3′ end of the glycoprotein B gene (ORF 8), the entire DNA polymerase gene (ORF 9), and the 5′ end of the ORF 10 gene for RFHVMm (top line) and RRV (bottom line). Asterisks indicate the positions of identical nucleotide sequences. The positions of identical amino acids are indicated within the RRV sequence with a ·, and gaps are indicated with a -. The position of a potential TATA box in the promoter region of the DNA polymerase genes is indicated in boldface and underlined.

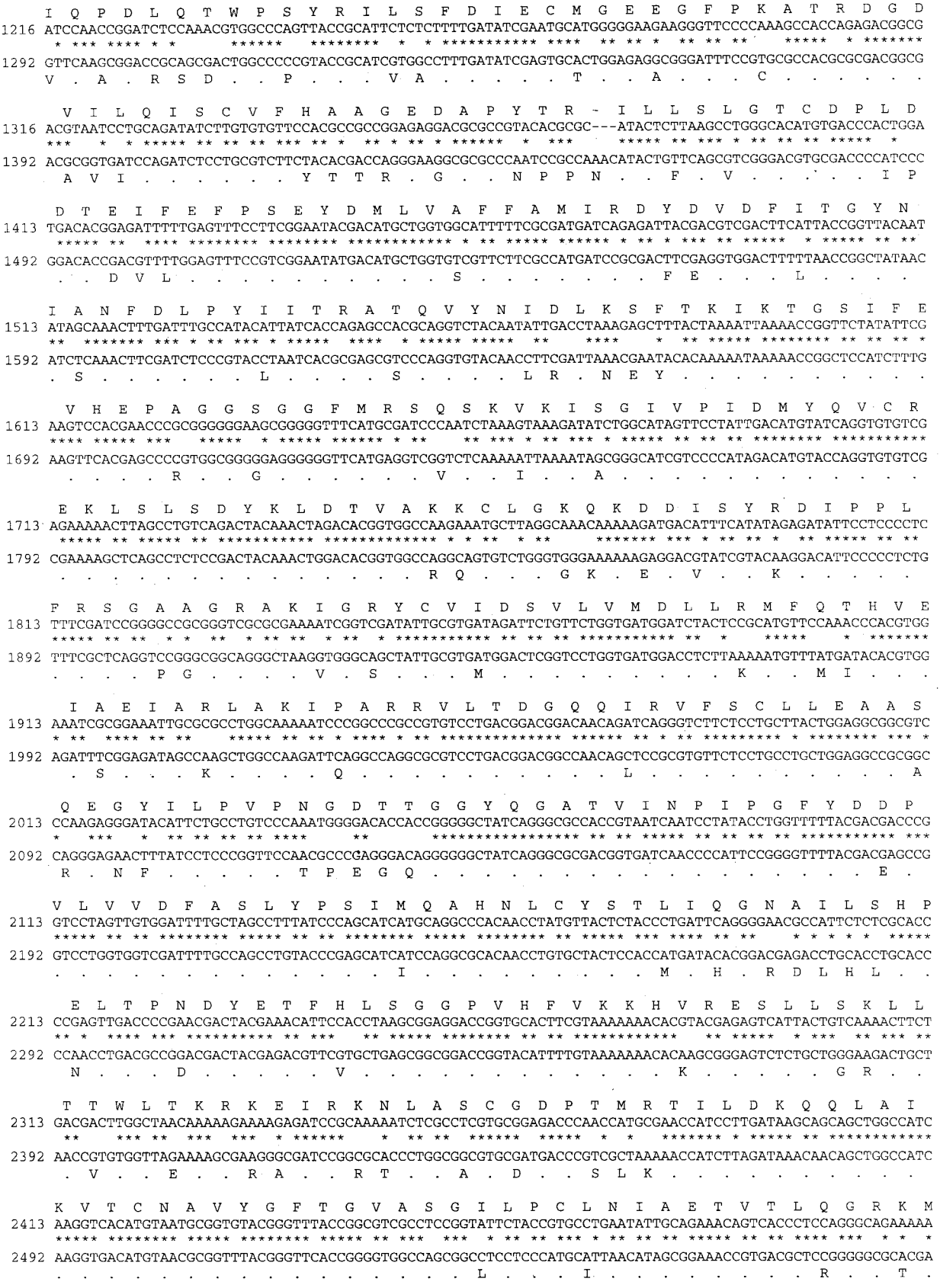

FIG. 2—*Continued.*

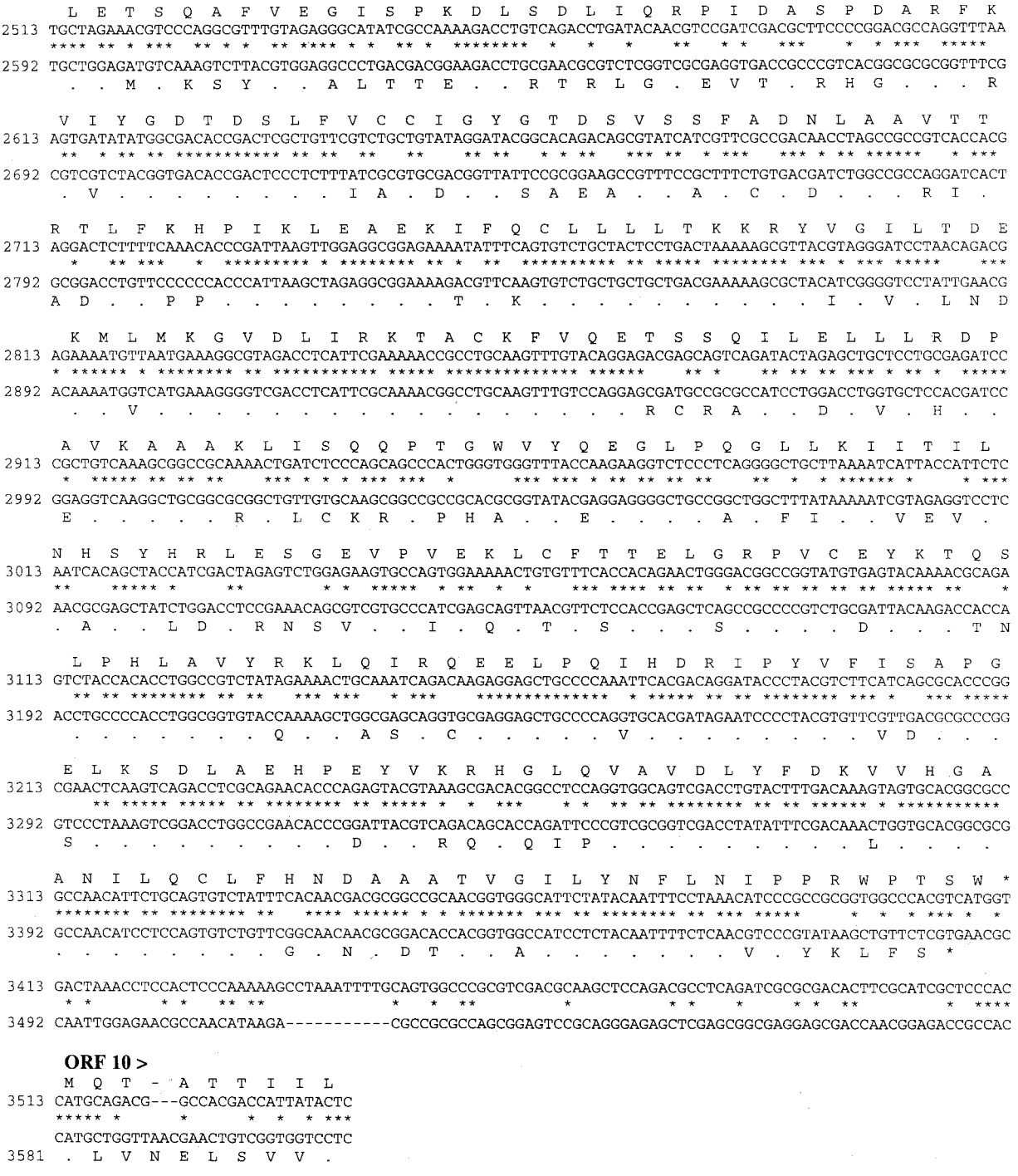

FIG. 2—*Continued.*

script was submitted for publication, two reports describing the existence of a virus similar to *Mne*RV-2 were published. The virus was alternatively designated pig-tailed rhadinovirus (12) and pig-tailed monkey rhadinovirus (3).

To compare RFHVMn and RFHVMm to RRV and its *Mne*RV-2 homolog, we utilized the same scheme described above for cloning and characterizing the DNA polymerase gene and flanking regions of *Mne*RV-2. To facilitate this, derivatives of the upstream and downstream CODEHOPs used above, i.e., FGGMA, CVNV2A, CVNV2B, and GDWE1B, were designed with a bias for RRV-like sequences (Table 1 and Fig. 1). Using these primers in amplification reactions with an *Mne*442N DNA template, PCR clones were obtained extending from CVNV2A to GDWE2B, which includes the majority of the DNA polymerase gene and the downstream ORF 10 flanking region. We were unable to obtain clones with FGGMA and CVNV2B which would contain the 5′ end of the DNA polymerase and the 3′ end of the glycoprotein B gene.

TABLE 3. Comparison of the nucleotide and amino acid sequences of the 3′ end of the glycoprotein B gene, the entire DNA polymerase gene, and the 5′ end of the ORF 10 homolog of primate gammaherpesviruses

Percent nucleotide identity[a]	Percent amino acid identity[a]						
	KSHV	RFHVMn	RFHVMm	*Mne*RV-2[b]	RRV	HVS	EBV
KSHV		60, 75, 67	60, 74, 67	ND, 69, 22	54, 66, 22	35, 60, 44	26, 53, ND
RFHVMn	60, 69, 62		95, 87, 66	ND, 68, 33	53, 67, 30	33, 59, 22	35, 53, ND
RFHVMm	64, 68, 62	90, 82, 81		ND, 69, 33	53, 66, 22	32, 59, 11	36, 53, ND
*Mne*RV-2	ND, 67, 42	ND, 67, 46	ND, 67, 42		ND, 94, 80	ND, 63, 33	ND, 55, ND
RRV	60, 66, 42	61, 66, 44	62, 65, 46	ND, 90, 73		43, 61, 33	24, 52, ND
HVS	49, 57, ND	50, 56, ND	48, 57, ND	ND, 57, ND	52, 57, ND		31, 53, ND
EBV	43, 58, ND	45, 57, ND	45, 57, ND	ND, 61, ND	45, 59, ND	42, 52, ND	

[a] Identity values are given for the partial sequence of ORF 8 (glycoprotein B gene), the complete sequence of ORF 9 (DNA polymerase gene) and the partial sequence of the ORF 10 homolog, respectively, as described in the text. ND, not determined.
[b] Identity values derived from the partial sequences of ORF 9 (DNA polymerase gene) and ORF 10 (CVNV-GDWE) fragment, as described in the text.

ORF analysis and BLAST similarity searches of the 2,708-bp fragment of *Mne*RV-2 demonstrated that this sequence was most closely related to the analogous region of the RRV genome. Pairwise alignments of the *Mne*RV-2 and RRV nucleotide sequences (data not shown) revealed a 90% identity within the DNA polymerase genes and 73% identity within the 5′ end of the ORF 10 gene (Table 3). Analysis of the encoded amino acid sequences demonstrated 94% identity within the DNA polymerase gene and 80% identity within the ORF 10 gene fragment (Table 3 and Fig. 4). Analysis of gene and intergenic length heterogeneity across the homologous genomic regions of *Mne*RV-2 and RRV indicated a close similarity. These results confirmed that *Mne*RV-2 is the *M. nemestrina* counterpart of RRV.

Sequence comparison of RFHVMm-RFHVMn, RRV–*Mne*RV-2, and KSHV. The alignment of the sequences of the two gammaherpesviruses, RFHVMm and RRV, both identified in *M. mulatta* (rhesus), is shown in Fig. 2. The alignment of the *M. nemestrina* herpesviruses, RFHVMn and *Mne*RV-2, is not shown due to space considerations. Both pairs of viruses from each host macaque species showed significant similarities and distinct differences in nucleotide and encoded amino acid sequences throughout the 3′ glycoprotein B fragment, the entire DNA polymerase gene, and the 5′ ORF 10 gene fragment. The nucleotide sequences of RFHVMm and RRV were only 65% identical within the DNA polymerase gene, 62% identical within the fragment of the glycoprotein B gene, and 46% identical within the downstream ORF 10 gene fragment (Fig. 2 and Table 3). Comparison of the sequences of RFHVMn and *Mne*RV-2 showed almost identical similarities. The encoded amino acid sequences of RFHVMm and RRV were 66, 53, and 22% identical within the DNA polymerase gene, the partial glycoprotein B gene fragment, and the ORF 10 gene fragments, respectively. This level of sequence similarity is greater than that seen between either of these viruses and HVS (Table 3). While the lengths of the gene and intergenic regions of RFHVMn and RFHVMm were quite similar, heterogeneity of length was detected between RFHVMn and *Mne*RV-2 and between RFHVMm and RRV.

BLAST similarity searches demonstrated that the RFHVMm and RFHVMn sequences were more similar to the analogous sequences of KSHV than were the sequences of RRV or *Mne*RV-2 (data not shown). Tabulation of sequence identity from pairwise alignments of the 3′ glycoprotein B fragment, the entire DNA polymerase gene, and the 5′ ORF 10 gene fragment obtained from the isolate KS187 of KSHV with the other viral sequences confirmed these results. (The DNA sequence of the KS187 isolate identified here varied from the previously published sequence of the BC-1 isolate of KSHV (18) at two positions within the 3′ glycoprotein B region, with one amino acid change, and at four positions within the DNA polymerase gene, with no amino acid changes). At the nucleotide level, sequence similarities between KSHV and the two pairs of macaque viruses, RFHVMm and RFHVMn and RRV and *Mne*RV-2, were comparable (68 to 69% and 66 to 67%, respectively). However, the encoded amino acid sequences of KSHV were identical with RFHVMm and RFHVMn at 74 to 75% of the positions within the DNA polymerase, at 60% of the positions within the 3′ glycoprotein B fragment, and at 67% of the positions within the 5′ ORF 10 gene fragment (Table 3). This contrasts with similar comparisons of KSHV to RRV and *Mne*RV-2, where only 66 to 69% identical amino acids are present within the DNA polymerase gene, 54% identical amino acids are present within the glycoprotein B fragment (RRV only), and 22% identical amino acids are present within the short ORF 10 gene fragment (Table 3). Analysis of the G-C content of the DNA polymerase and flanking regions showed that RFHVMn (57%) and RFHVMm (54%) have values similar to KSHV (54%), while *Mne*RV-2 (61%) and RRV (60%) have slightly higher values (Table 4). However, while KSHV shows some suppression of CpG dinucleotides within this region (0.86), the macaque rhadinoviruses did not, with values of 1.13 to 1.17. This contrasts with the extensive suppression of CpG in HVS and Epstein-Barr virus (EBV) (Table 4).

A comparative alignment of the amino acid sequence of the KSHV DNA polymerase with the DNA polymerases of RFHVMm, RFHVMn, *Mne*RV-2 (partial), and RRV is shown in Fig. 3. This alignment shows the high degree of conservation across all of the sequences, substantiating their inclusion within the family of KSHV-like herpesviruses. It also shows that all five DNA polymerases are highly conserved across the six conserved nucleotide polymerase domains that play important functional roles in polymerase activity (see reference 22). Length heterogeneity is seen between the pairs of macaque DNA polymerases (RFHVMm and RFHVMn and RRV and *Mne*RV-2) and the human KSHV DNA polymerase at two amino acid insertion and/or deletion positions (positions 87 to 88 and 421 to 422) and at the C-terminal end of the proteins. While the sequence lengths of the RFHVMm and RFHVMn

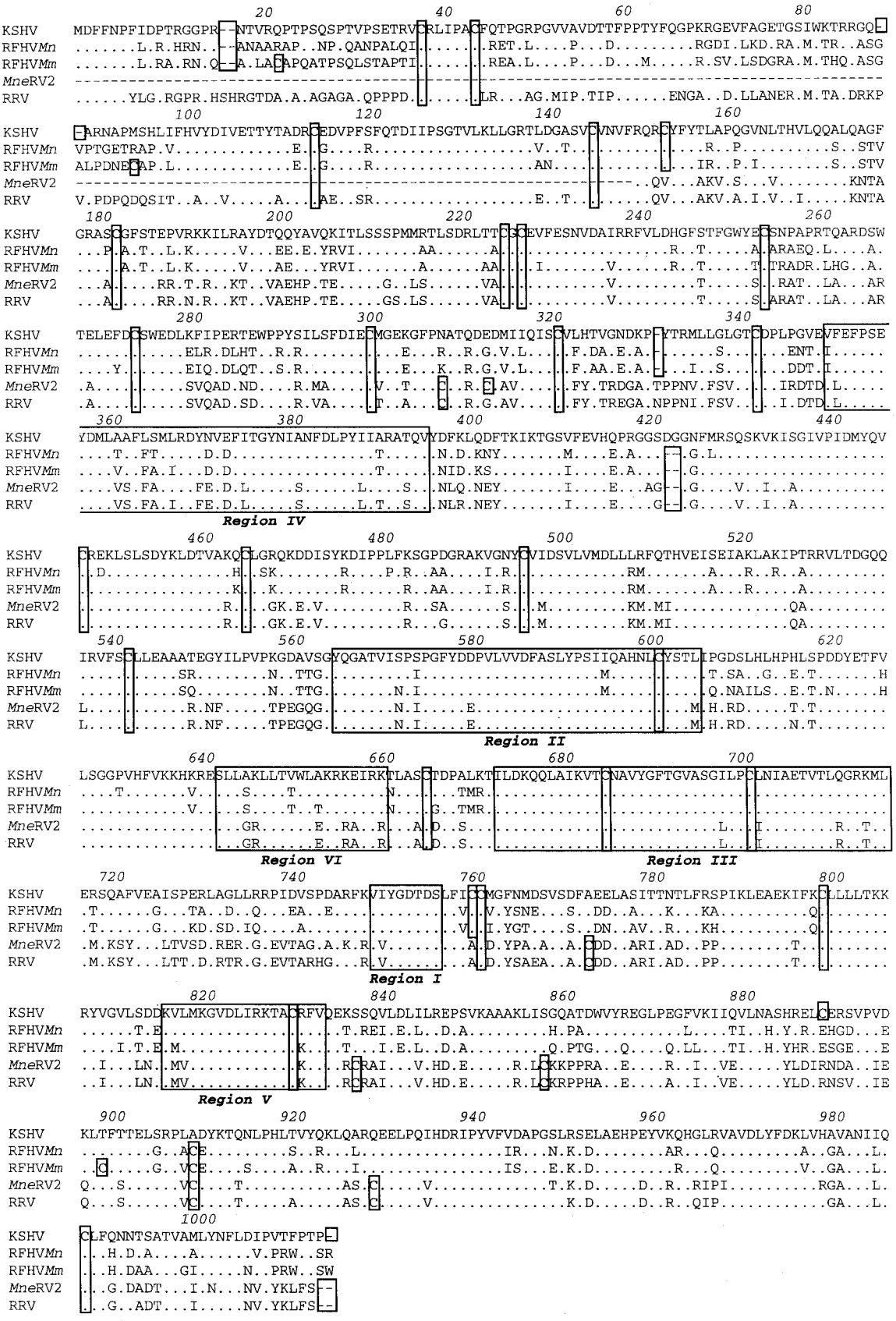

FIG. 3. Amino acid sequence comparison of the DNA polymerases of the KSHV-like gammaherpesviruses. The positions of gaps and cysteine residues are boxed, and the general nucleotide polymerase conserved regions are indicated (see reference 22). Positions with identities with the KSHV sequence are shown as a ·, and the unidentified N-terminal region of *Mne*RV-2 is indicated. The numbering refers to the KSHV sequence. The source of the sequences is given in Table 2.

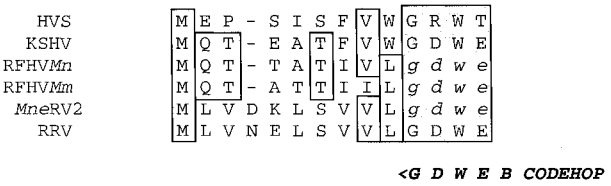

FIG. 4. Comparison of the amino acid sequences encoded within the 5′ end of the cloned ORF 10 homologs of HVS and the KSHV-like gammaherpesviruses. Highly conserved amino acids are boxed, and the position of the GDWE sequence motif from which the ORF 10 CODEHOPs were derived is highlighted (shaded area), with known sequences in uppercase and presumed sequences in lowercase.

polymerases are otherwise identical to that of KSHV, the RRV and *Mne*RV-2 polymerases differ at two other insertion and/or deletion positions (positions 16 to 17 and 330 to 331). Further analysis of the amino acid conservation within the DNA polymerase genes shows additional similarities between RFHVMm and RFHVMn and KSHV that are not found with RRV–*Mne*RV-2. For example, the sequences across the conserved DNA polymerase regions I and III (Fig. 3) are identical between KSHV and RFHVMn and RFHVMn, while RRV and *Mne*RV-2 have amino acid differences at numerous positions. Analysis of the cysteine residues in Fig. 3 shows more conservation between the sequences of KSHV and RFHVMm and RFHVMn than between RRV and *Mne*RV-2 and either KSHV or RFHVMm and RFHVMn. In fact, several cysteine residues found in the C-terminal region of the RRV–*Mne*RV-2 DNA polymerase are conserved only between RRV–*Mne*RV-2 and HVS. Analysis of the amino acid alignments across the partial glycoprotein B sequence (data not shown) and across the short region obtained from ORF 10 (Fig. 4) shows that KSHV sequences align more closely with those from RFHVMm-RFHVMn than from RRV–*Mne*RV-2.

Phylogenetic analysis of the DNA polymerases. Phylogenetic analyses were performed on the amino acid sequences of the DNA polymerases shown in Fig. 3 with the addition of other gammaherpesvirus polymerases, as indicated in Table 2. Multiple alignments were performed using ClustalW, and gapped positions were removed. Figure 5 shows a maximum parsimony analysis (Phylip package; University of Washington, Seattle) of these sequence alignments with the tree file displayed with TreeView (15). Bootstrap analysis, performed with the programs Seqboot, Protpars, and Consense from the Phylip package, strongly supported all the branching patterns as indicated. Neighbor-joining analysis gave an equivalent topology. Recently, portions of the DNA polymerases of additional KSHV-like gammaherpesviruses have been identified in African green monkeys with a CODEHOP strategy similar to that previously described (17). Sequence analysis of the derived PCR products demonstrated that African green monkeys have a homolog of RFHVMm-RFHVMn, designated ChRV-1, and a homolog of RRV–*Mne*RV-2, designated ChRV-2 (11). The available amino acid sequences from the African green monkey DNA fragments were used in parsimony analyses with the corresponding sequences from the other gammaherpesviruses, and the relative branch points of the ChRV-1 and ChRV-2 sequences were added to the phylogenetic tree obtained from the entire DNA polymerase sequences (Fig. 5). From these analyses, it is evident that macaques and African green monkeys contain two distinct lineages of KSHV-like rhadinoviruses. The first lineage, which we have designated rhadinovirus genogroup type 1 (RV-1), consists of RFHVMn, RFHVMm, ChRV-1, and human KSHV, which cluster together on one branch of the tree. The second lineage, which we have designated RV-2, consists of RRV, *Mne*RV-2, and ChRV-2, which cluster together on a different branch more closely connected to the branch containing HVS, the prototype gamma-2 herpesvirus, and herpesvirus ateles type 3 (1), both rhadinoviruses of New World monkeys. These branching patterns demonstrate a distinct differentiation between the two groups of KSHV-like viruses and the prototypes of the gamma-1 *Lymphocryptovirus* genus, EBV, and the gamma-2 *Rhadinovirus* genus, HVS. Phylogenetic analysis of the available sequences derived from the glycoprotein B amino acid sequences (Table 2) confirmed this topology (data not shown).

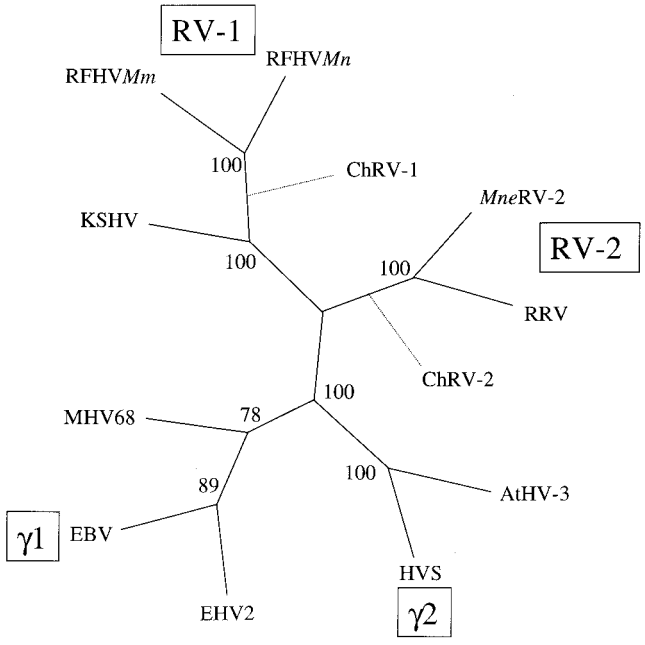

FIG. 5. Phylogenetic tree of the gammaherpesviruses. Phylogenetic trees were generated by maximum-parsimony analysis using the amino acid sequences of the DNA polymerases shown in Fig. 3 and Table 2. The branch points of the ChRV-1 and ChRV-2 sequences were derived from analyses of smaller sequence sets due to limited sequence data and are indicated with dotted lines. Bootstrap values derived from 100 replicates are shown for each branch point. The EBV (gamma-1) and HVS (gamma-2) prototypes (γ1 and γ2) and the proposed rhadinovirus genogroups RV-1 and RV-2 are indicated.

TABLE 4. G+C mononucleotide and CpG dinucleotide frequencies in the DNA polymerase genes and flanking regions of primate gammaherpesviruses

Herpesvirus	No. of nucleotides analyzed (bp)	% G+C	CpG ratio[a]
KSHV	3,640	54	0.86
RFHVMn	3,554	57	1.14
RFHVMm	3,540	54	1.13
*Mne*RV-2	2,712[b]	61	1.17
RRV	3,623	60	1.17
HVS	3,477	35	0.36
EBV	3,413	62	0.69

[a] The CpG ratio is the observed frequency/expected frequency of the CpG dinucleotides, given the mononucleotide composition.

[b] Derived from the partial sequence of the DNA polymerase and ORF 10 (CVNV-GDWE) fragment.

Although it has been suggested that RRV is the macaque homolog of the human KSHV (8, 20), our sequence data dem-

onstrate that the RFHVMn-RFHVMm lineage is more closely related to KSHV than the RRV–*Mne*RV-2 lineage. This relationship is supported both by the general nucleotide and amino acid sequence conservation across the DNA polymerase and flanking glycoprotein B and ORF 10 genes, as well as by the conservation of gaps and insertions and specific amino acid residues within the DNA polymerase genes themselves. These data confirm our earlier results, which were based on limited sequence information (4, 17). Our analysis also suggests that the putative ChRV-1 gammaherpesvirus is the African green monkey homolog of KSHV, while ChRV-2 is a more distant relative within the RRV–*Mne*RV-2 lineage. Confirmation of these relationships awaits the sequencing and characterization of the entire genomes of RFHVMn, RFHVMm, ChRV-1, and ChRV-2. It is expected that the genomes of RFHVMn-RFHVMm and ChRV-1 will be more similar to KSHV than the genomes of RRV–*Mne*RV-2 and ChRV-2.

Our data clearly show that macaques are host to two distinct lineages of gammaherpesviruses that are both related to the human KSHV. We have shown that members of both viral lineages can naturally coinfect the same host animal. The significant nucleotide and amino acid differences between the members of these lineages suggest that they have separately evolved over a long period of time. The evidence that viral species belonging to both gammaherpesvirus lineages are also found in African green monkeys suggests that the presence of two lineages of KSHV-like viruses could be a common phenomenon in Old World primates. The identification of two distinct lineages of rhadinoviruses in Old World primates further suggests the possibility that a human homolog of KSHV in the RRV–*Mne*RV-2–ChRV-2 lineage might exist. We are currently using modified versions of CODEHOPs derived from the DNA polymerase sequences to assay for the presence of such an additional human herpesvirus.

The close nucleotide and amino acid sequence similarities between the members of the two lineages of KSHV-like herpesviruses in macaques and African green monkeys could lead to potential problems in molecular and serological assays identifying these viruses. As readily seen in Fig. 2, strong possibilities exist that oligonucleotide primers designed for one virus would cross-react with the other virus in molecular assays. Similarly, cross-reactivity in serological assays could occur, due to the large segments of the viral genes from the two lineages which contain identical amino acid sequences. On the other hand, the significant differences between the sequences of the two lineages underline the conclusion that these two lineages have separately evolved over a long period of time and may have important functional and pathological differences. One such biological difference is evident in the ability of viruses from the RV-2 lineage to be easily transmitted and passaged in vitro (8, 20), whereas this has proven to be difficult with KSHV and other members of the RV-1 lineage. Differences have also been noted in virus transmission in vivo where herpesviruses of the RV-2 lineage appear to be much more ubiquitous within populations than those of the RV-1 lineage (6, 8, 19, 20; unpublished observations). Thus, caution should be exercised in comparing biological models developed with herpesviruses of the RV-2 lineage with the viral infection and disease pathogenesis caused by KSHV and its homologs in the RV-1 lineage.

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