Analysis of 4.3 Kilobases of Divergent Locus B of Macaque Retroperitoneal Fibromatosis-Associated Herpesvirus Reveals a Close Similarity in Gene Sequence and Genome Organization to Kaposi’s Sarcoma-Associated Herpesvirus

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We previously identified retroperitoneal fibromatosis-associated herpesvirus (RFHV) as a simian homolog of Kaposi’s sarcoma-associated herpesvirus (KSHV) in a fibroproliferative malignancy of macaques that has similarities to Kaposi’s sarcoma. In this report, we cloned 4.3 kb of divergent locus B (DL-B) flanking the DNA polymerase gene from two variants of RFHV from different species of macaque with a consensus degenerate hybrid oligonucleotide primer approach. Within the DL-B region of RFHV, viral homologs of the cellular interleukin-6, dihydrofolate reductase, and thymidylate synthase genes were identified, along with a homolog of the gammaherpesvirus open reading frame (ORF) 10. In addition, a homolog of the KSHV ORF K3, the modulator of immune recognition-I, was identified. Our data show a close similarity in sequence conservation, gene content, and genomic structure between RFHV and KSHV which strongly supports the grouping of these viral species within the same RV-1 rhadinovirus lineage and the hypothesis that RFHV is the macaque homolog of KSHV.

The most recently discovered human tumor virus, Kaposi’s sarcoma-associated herpesvirus (KSHV), has been implicated in the pathogenesis of Kaposi’s sarcoma, primary effusion lymphoma, and Castleman’s disease (for a review, see reference 41). KSHV was assigned to the Rhadinovirus genus of the gammaherpesviruses based on similarities at the levels of nucleotide sequence, gene content, and genomic structure with the Rhadinovirus prototype, Herpesvirus saimiri (HVS) of the South American squirrel monkey (30). Like other gammaherpesviruses, KSHV has numerous genes with homology to cellular host genes which have been captured during virus evolution (37). Viral homologs of cellular genes contribute to the unique biological properties and pathogenic effects of different viral species and, in the case of KSHV, function in the disruption of antiviral responses, cytokine-regulated cell growth, apoptosis, and cell cycle control (29). As in other gammaherpesviruses, these cellular homologs cluster in a restricted number of divergent genomic loci, and the presence and orientation of these genes are uniquely characteristic of KSHV.

We have identified a simian homolog of KSHV in macaque retroperitoneal fibromatosis (36), a vascular fibroproliferative malignancy with morphological and histological similarities to Kaposi’s sarcoma (12, 45). A consensus degenerate hybrid oligonucleotide primer (CODEHOP) technique (35) utilizing PCR primers derived from highly conserved amino acid motifs within the herpesvirus DNA polymerase genes was used to amplify DNA sequences from retroperitoneal fibromatosis lesions of two macaque species. These sequences were identified as portions of the DNA polymerase genes of two closely related novel viruses, retroperitoneal fibromatosis-associated herpesvirus of Macaca mulatta (RFHVMM) and of Macaca nemestrina (RFHV Mn) (36). Subsequently, it was shown that these macaque species are also host to another closely related herpesvirus with similarity to KSHV, rhesus rhadinovirus (RRV) (11) and Macaca nemestrina rhadinovirus 2 (RV-2) (40). This virus has been alternatively named pig-tail rhadinovirus (26) and pig-tailed monkey rhadinovirus (4).

Phylogenetic analysis of the DNA polymerase sequences demonstrated that RFHVMM and RFHV Mn clustered together with KSHV within a lineage designated rhadinovirus 1 (RV-1), while RRV and M. nemestrina RV-2 cluster into a more distantly related lineage designated rhadinovirus 2 (RV-2) (40). Additional studies have identified viral species belonging to both the RV-1 and RV-2 lineages of KSHV-related viruses in a number of Old World primate host species, including African green monkeys, drills, mandrills, gorillas, and chimpanzees (13, 14, 20–22).

Classification of viral species within the RV-1 and RV-2 rhadinovirus lineages has been based mainly on comparisons of partial sequences of DNA polymerase genes. In this report, we cloned 4.3 kb of divergent locus B (DL-B) flanking the DNA polymerase gene of RFHVMM and RFHV Mn with the CODEHOP strategy. As in the corresponding DL-B locus in KSHV, viral homologs of the cellular interleukin-6 (vIL-6), dihydrofolate reductase (vDHFR), and thymidylate synthase
served amino acid sequence motifs, RHFG and DMGL, were chosen as targets.

KSHV, herpesvirus saimiri (HVS), and equine herpesvirus 2 (EHV2) (see Table 1) were identified, along with a homolog of the conserved gammaherpesvirus open reading frame (ORF) 10. In addition, a homolog of the KSHV ORF K3, the modulator of immune recognition-1 (MIR-1) (10), was identified. Our data show a close similarity between the nucleotide sequences, gene content, and genomic structure of RFHV and KSHV, suggesting important biological differences between the two rhadinovirus lineages.

 MATERIALS AND METHODS

Tissue samples. Retropertioneal fibromatosi tumor samples were obtained from a simian retrovirus 2-infected Mmne442N (MmuYN-91) provided by Riri Shibata, National Institutes of Health, Bethesda, Md. DNA isolation. Frozen tissue samples were quickly thawed in the presence of a standard protease K extraction buffer containing 0.1% sodium dodecyl sulfate by homogenization in a disposable homogenizer. Samples were digested at a standard proteinase K extraction buffer containing 0.1% sodium dodecyl sulfate (24)–16°C denaturing step, a 30-s to 70°C annealing step, and a 30-s to 72°C extension step for 40 cycles in a thermal gradient iCycler thermocycler (Bio-Rad).

Long-range PCR amplification. High-molecular-weight DNA isolated from retropertioneal fibromatosis tissue of MmuYN-91 and spleen tissue from MmuYN-91 was used as the template in long-range PCR amplification of the region between the ORF 10 and TS genes of RFHV Mm and between the DNA polymerase and TS genes of RFHV Mm. The gene-specific PCR primers F6565 (5'-GAACTTTTGGCTCTTGGCCTATG-3'; ORF 10, RFHV Mm), R20432 (5'-CTTTGGCCCCCTGTGACCTCCGTGAT-3'; ORF 10, RFHV Mm), PolF1LR 5'-CCACCGTCCCAGACCAACGAAAGCG-3'; DNA polymerase, RFHV Mm), and TSR1LR 5'-GTCGCTCGTGAATCCCGTGAATACCAA-3'; TS, RFHV Mm) were designed with Oligonucleotide v5 software (Qiagen) with a standard length of 30 nucleotides to ensure high specificity and annealing temperature (see Fig. 1). Long-range PCR amplification was performed with the Expand Long Template PCR system (Roche) with the recommended protocol. PCR products were analyzed on a 1% agarose gel and purified with a QiAquick PCR purification kit (Qiagen).

DNA sequencing and sequence assembly. 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. Sequencing was performed on an ABI model 310 automated sequencer with Prism Big Dye terminator cycle sequencing ready reaction kit with AmpliTag DNA polymerase FS (Applied Biosystems). DNA sequences were assembled with Sequencher 4.0.8b10 (GeneCodes). Sequence redundancy was three- to fourfold.

Sequence and phylogenetic analysis. ORFs were identified in Sequencher and compared to existing sequences with Blast analysis of the NCBI nonredundant database. RFHV ORFs were named according to the nomenclature proposed for KSHV (37), with ORFs not found in HVS designated with an RF prefix, for example, RF3. Pairwise nucleotide and encoded amino acid alignments were performed with GenePro software (RiverSoft Scientific, Bainbridge Island, Wash.). Multiple sequence alignment was done with ClustalW (EMBL, Heidelberg, Germany). Phylogenetic analysis of amino acid sequences was done with

![Image](http://jvi.asm.org/)

**TABLE 1. Herpesviruses and sequence accession numbers**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Host</th>
<th>Gene(s)</th>
<th>Accession no.</th>
</tr>
</thead>
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<td>Pig-tailed macaque</td>
<td>DNA polymerase and partial flanks</td>
<td>AF204166</td>
</tr>
<tr>
<td>RFHV Mm</td>
<td>Rhesus macaque</td>
<td>DNA polymerase and partial flanks</td>
<td>AF005479</td>
</tr>
<tr>
<td>RRV</td>
<td>Rhesus macaque</td>
<td>CG</td>
<td>AF210726</td>
</tr>
<tr>
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<td>Human</td>
<td>CG</td>
<td>NC_003409</td>
</tr>
<tr>
<td>HVS</td>
<td>South American squirrel monkey</td>
<td>CG</td>
<td>NC_001350</td>
</tr>
<tr>
<td>A1Hv3</td>
<td>South American spider monkey</td>
<td>CG</td>
<td>NC_001987</td>
</tr>
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<td>Wildebeest</td>
<td>CG</td>
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<td>Cow</td>
<td>CG</td>
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</tr>
<tr>
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<td>CG</td>
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<td>CG</td>
<td>NC_00826</td>
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<tr>
<td>Lympocytovirus</td>
<td>Human</td>
<td>CG</td>
<td>NC_001345</td>
</tr>
</tbody>
</table>

*CG, complete genome (long unique region).
RESULTS

Partial inverse PCR amplification of ORF 10 sequences from RFHVMM. We have previously used the CODEHOP technique, described in reference 35, to clone the entire DNA polymerase genes and flanking sequences of the macaque rhadinoviruses RFHVMM and RFHVMM (40). At present, this is the only information available regarding the genetic make-up of these viruses. We have had to rely on PCR amplification for the characterization of these viruses because of the minimal copy number of the viral genomes within the available tissue samples and the inability to culture the viruses in vitro.

In an attempt to extend the sequences of these viral genomes into the region flanking the DNA polymerase gene, we initially employed a partial inverse PCR technique for cloning flanking sequences. Specific oligonucleotide primers, DRIPA and QIROB, were derived from opposite strands of the DNA sequence at the 3′ end of the RFHVMM DNA polymerase gene, as shown in Fig. 1. These primers were used to amplify circularized DNA templates obtained from partial restriction digests of DNA isolated from retroperitoneal fibrosarcoma tumor samples of MmuYN-91, essentially as described before (33). A 1.3-kb PCR fragment was obtained, cloned, and sequenced. Sequence analysis revealed that half of the fragment was identical to the previously determined DNA polymerase gene, and the other half contained new sequence derived from the downstream ORF 10 gene (see Fig. 1).

CODEHOP-based PCR amplification of sequences within the vTS genes of RFHVMM and RFHVMM. Although the inverse PCR technique resulted in new sequences flanking our original sequence, the amount of sequence was limited. Therefore, we pursued long-range amplification to obtain larger DNA fragments. Previous comparison of the nucleotide sequences of the DNA polymerase genes of KSHV and the two RFHV species suggested that the genomes of the macaque viruses would be similar to the KSHV genome (36, 40). Flanking the KSHV DNA polymerase gene is divergent locus B (DL-B), which contains a number of viral homologs of cellular genes that have been captured during the evolution of the virus (Fig. 1) (31). Because the presence and orientation of the genes within this region are uniquely characteristic of KSHV, analysis of this region within the genomes of RFHVMM and RFHVMM would allow a more definitive characterization of these viruses to determine their relationship to KSHV. The vTS gene was identified as a suitably conserved candidate target for cloning with the CODEHOP technique. Successful amplification of a portion of the RFHV vTS gene would provide the sequence information to prepare a TS-specific primer allowing long-range amplification between the vTS gene and known DNA polymerase/ORF 10 sequences within the RFHV genomes (Fig. 1).

Alignment of the available herpesvirus vTS homologs revealed the presence of two conserved amino acid motifs, RHFG and DMGL, which were separated by approximately 200 bp (data not shown). These motifs were chosen as CODEHOP targets, and RHFGA and DMGLB (antisense orientation) CODEHOPs were derived from these motifs. Thermal gradient PCR amplification was performed with these CODEHOP primer pools with DNA isolated from retroperitoneal fibrosarcoma tissues from MmuYN-91 and Mmu442N. Thermal gradient amplification empirically determines the optimal annealing temperature of CODEHOPs for amplification of unknown DNA templates by allowing multiple amplification reactions to proceed at different annealing temperatures. The PCR products obtained from amplification at different temperatures were analyzed by gel electrophoresis. An annealing temperature of ∼64.3°C (Fig. 2, lane 4) yielded a single strong PCR product of the predicted size (284 bp) from both DNA templates. These DNA products were chosen for analysis and sequenced directly with both of the TS CODEHOPs used in the amplification. A comparison of the sequences revealed a close similarity with the sequence of the KSHV vTS homolog, indicating that these PCR products were derived from the vTS genes of the closely related RFHVMM and RFHVMM (see below).

Long-range PCR amplification of divergent locus B located between DNA polymerase and vTS genes of RFHVMM and RFHVMM. To characterize the DL-B region of the RFHVMM genome, gene-specific primers were derived from the ORF 10 sequence obtained by partial inverse PCR (primer F6565) and the TS sequence obtained by CODEHOP PCR (primer R20432) (see Fig. 1). To characterize the RFHVMM genome, a DNA polymerase-specific primer was derived from our previous sequence of the DNA polymerase (PolF1LR), and a TS-specific primer (TSR1LR) was derived from the RFHVMM CODEHOP TS PCR product. Based on the relative positions of the DNA polymerase/ORF 10 and vTS genes within the KSHV genome, it was predicted that amplification of the RFHV region would yield a PCR product of approximately 5 kb (Fig. 1).

Long-range PCR amplification was performed on DNA
from MmuYN-91 and Mne442N tissues containing RFHVMM and RFHVMM viral DNA, respectively. A single 3.6-kb PCR product was obtained from the MmuYN-91 retroperitoneal fibromatosis tissue with the ORF 10 and TS primers, while a 4.1-kb product was obtained from the Mne442N spleen tissue with the DNA polymerase and TS primers. The DNA sequences of these products were obtained and analyzed by Blast for similarities to known sequences. Five ORFs were identified downstream of the DNA polymerase gene in both the RFHVMM and RFHVMM PCR products (see Fig. 3), including ORF 10, ORF RF2, a viral homolog of cellular IL-6; ORF 02, a viral homolog of dihydrofolate reductase (vDHFR); ORF RF3, a homolog of the ORF K3 modulator of immune response-1 (MIR-1); and ORF 70, a viral homolog of the thymidylate synthase (vTS). These ORFs have been named according to the HVS designations and their similarities to counterparts in the KSHV genome. ORFs not found in HVS were assigned labels beginning with RF (for retroperitoneal fibromatosis), similar to the convention used for naming KSHV ORFs, as described in Materials and Methods.

**Analysis of RFHV ORFs.** (i) **ORF 70 (vTS homolog).** Blast analyses of the sequences obtained from the RFHVMM and RFHVMM long-range PCR products identified sequences homologous to the 3’ end of the KSHV vTS gene, which was designated ORF 70 because of homology with the HVS ORF 70 vTS gene (Fig. 3). These sequences overlapped those obtained with the TS-specific CODEHOP primers described above, and a consensus was derived for each of the viral species. An alignment of the RFHVMM and RFHVMM ORF 70 vTS sequences with the homologous regions from other viral and cellular TS homologs is shown in Fig. 4A. The amino acids encoded by the RFHVMM and RFHVMM vTS sequences were 86% identical to each other and 74% identical to the C-terminal 170 amino acids of the 337-amino-acid KSHV vTS (Table 2). The RFHV vTS sequences were 69% and 66% identical to the corresponding regions of the RRV and HVS vTS sequences, respectively. Comparison of the RRV and KSHV vTS sequences revealed 74% identical residues, equivalent to that seen between RFHV and KSHV vTS sequences (Table 3).

Phylogenetic analysis of the TS sequence alignment in Fig. 4A (supplemented with outgroup sequences from the TS homologs from an insect, a yeast, and a plant) by the protein maximum-likelihood method produced a tree with an ln likelihood of $-2613.62538$, with bootstrap support as indicated on the nodes (Fig. 4B). In this tree, the RFHVMM and RFHVMM vTS sequences grouped together, as expected for virus variants obtained from closely related macaque host species. The RFHV vTS sequences grouped with the KSHV vTS sequence in a branch separate from RRV vTS and the other TS sequences. Although the bootstrap value for this branch pattern was 51%, the likelihood was significantly positive, $P < 0.01$. Maximum-likelihood, neighbor-joining, and parsimony methods with both protein and DNA sequences confirmed this branch pattern (data not shown). This pattern mirrors that obtained previously with DNA polymerase sequences, supporting the grouping of RFHV and KSHV together within the RV-1 lineage of Old World primate rhadinoviruses distinct from RRV (40). Interestingly, the vTS homologs of the alphaherpesviruses and the New World primate rhadinoviruses grouped distinctly with the mammalian and eukaryotic cellular TS homologs (Fig. 4B).

(ii) **ORF 10 homolog.** ORFs which encoded 411 amino acids that were 75% identical to each other and 46% identical to the KSHV ORF 10 were identified downstream of the DNA polymerase gene in the PCR fragments from RFHVMM and RFHVMM (Table 2). Blast (protein) analysis against the NCBI databases revealed similarities with the ORF 10 homologs of other gammaherpesviruses, including RRV (32% identity), HVS (23% identity), and Epstein-Barr virus (EBV) (12% identity, LF1 gene). Alignment of the amino acid sequences revealed the presence of numerous blocks of sequences conserved between the ORF 10 homologs of RFHVMM,
RFHVMM, KSHV, and RRV (Fig. 5A). The alignment showed much less sequence homology within the other ORF 10 homologs analyzed. A much stronger sequence similarity was detected between the ORF 10 homologs of RFHV and KSHV (46% identical residues) than was detected between the ORF 10 homologs of RRV and KSHV (34% identical residues) (Table 3).

A phylogenetic analysis was performed with the protein maximum-likelihood procedure, and a tree with an ln likelihood of \( -11551.5620 \) was obtained (Fig. 5B). Like the vTS sequences, the RFHVMM and RFHVMM ORF 10 sequences clustered together with the KSHV ORF 10 sequence in a lineage distinct from that of the macaque RRV. This clustering was well supported by the bootstrap analysis and was confirmed by neighbor-joining and parsimony methods. The extended branch lengths observed for the ORF 10 homologs of the nonprimate rhadinoviruses murine herpesvirus 68 (MHV68), bovine herpesvirus 4 (BHV-4), alcelaphine herpesvirus 1 (AHV1), and equine herpesvirus 2 (EHSV2) and the lymphocryptovirus EBV indicate the weak sequence conservation within the more distantly related members of this gene family; the amino acid identity between the ORF 10 homologs of the rhadinoviruses and lymphocryptoviruses was less than 20% (Table 2).

To search for possible functional motifs, iterated searching of the nonredundant NCBI protein database with PSI-Blast was performed. While no obvious sequence similarity was detected between the RFHV ORF 10 and genes of known function, a similarity was detected with the ORF 11 sequences of KSHV, RRV, and other gammaherpesviruses. Figure 5A shows an alignment of ORF 10 and 11 homologs in which few gaps were needed to align a number of conserved amino acid residues and motifs.

(iii) RFHV genome lacks an ORF 11 homolog. Downstream of ORF 10 in the KSHV genome is the 407-amino-acid ORF 11, which is homologous to the HVS ORF 11 and the EBV LF2 gene (37). ORF 11 homologs have been identified in analogous positions in all other gammaherpesviruses except BHV4, which lacks a homologous ORF 11 gene (49). In our initial long-range PCR amplification studies, the...
PCR fragment obtained from RFHVVm was approximately 2 kb shorter than the fragment size predicted from the KSHV genome (Fig. 1). Sequence analysis indicated that this difference in size was due to the absence of an ORF 11 homolog downstream of ORF 10. To determine whether this was a true characteristic of the RFHV genome, we obtained the sequence of the analogous fragment of the RFHVm genome, as described above. As shown in Fig. 3, the DL-B regions of both the RFHVm and RFHVVm genomes, like that of BHV4, lack an ORF 11 homolog.

(iv) ORF RF2 homolog (vIL-6). Blast analysis of the sequences obtained from the long-range PCR products from RFHVm and RFHVVm revealed the presence of a gene homologous to the ORF K2 gene of KSHV and the cellular cytokine IL-6. The ORF K2 gene has been identified previously as a viral homolog of IL-6 (vIL-6) (37). While the sequence similarity between the KSHV vIL6 and the cellular IL-6 is low (24%), the KSHV vIL6 has been shown to have many biological functions in common with cellular IL-6 (29). Consistent with the naming convention used for the KSHV genome, we have designated the vIL-6 homolog of RFHV ORF RF2. ORF RF2 is similar in genomic location and orientation to the KSHV ORF K2 gene (Fig. 3). A vIL-6 homolog, designated ORF R2, has been found in the macaque RRV genome (11).

The amino acid sequences of the RFHV vIL-6 homologs were aligned with the vIL-6 homologs from KSHV and RRV and the cellular human and macaque IL-6 sequences. Only 12 of more than 200 amino acids (<6%) were conserved between the cellular and viral IL-6 homologs. However, examination of the alignment revealed significant sequence similarity between the vIL-6 homologs of RFHV and KSHV which did not extend to either the vIL-6 homolog of RRV or the human and macaque cellular IL-6 homologs (Fig. 6A). Specific differences with RRV include the sequence length variations observed at the N and C termini, the cysteine at position 49, the potential N-linked glycosylation site at position 89, and four internal amino acid insertions present only in the RRV vIL-6. Overall, the vIL-6 homologs from RFHVVm and RFHVVm were 74% identical to each other and 35% identical to the KSHV vIL-6.

In contrast, the RRV vIL6 was only 14% identical to the KSHV vIL-6 homolog and 19% identical to the RFHV vIL-6 homologs (Tables 2 and 3). These relationships are shown graphically in the phylogenetic analysis obtained with protein maximum likelihood (Fig. 6B). The RFHVm and RFHVVm sequences clustered together with KSHV vIL-6 in a branch separate from the RRV and cellular IL-6 homologs. The long branch lengths between RRV and both the KSHV/RFHV vIL-6 and cellular macaque/human IL-6 clusters are indicative of the large sequence variation seen in the RRV sequence.

(v) ORF 02 homolog (vDHFR). Dihydrofolate reductase (DHFR) is a ubiquitous protein in both prokaryotes and eukaryotes that is responsible for de novo synthesis of purines and deoxythymidine monophosphate for DNA synthesis. Viral homologs of DHFR have been identified in a restricted number of gammaherpesviruses within the genus Rhadinovirus, including KSHV (37), macaque RRV (42), and HVS from the New World squirrel monkey (44). As shown in Fig. 3, a homolog of DHFR was detected flanking the vIL-6 gene within the PCR products obtained from RFHVm and RFHVVm in a position identical to that seen in KSHV. This differs from the situation in RRV and HSV, in which the vDHFR genes are located near the left ends of their respective genomes (Fig. 3). The RFHVm and RFHVVm vDHFR homologs were 208 amino acids in length and 84% identical to each other (Table 3).

<table>
<thead>
<tr>
<th>RFHV ORF Location RFHVm/ RFHVmα</th>
<th>ORF size in amino acids (% identity with the RFHVm ORF)</th>
<th>Possible function</th>
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<tbody>
<tr>
<td>(v) ORF K3 in KSHV was not recognized as homologous to ORF 12 in HVS and thus was given a K designation in the original KSHV nomenclature (37). For this reason, the RFHV ORF was given the RF designation even though it is homologous to HVS ORF 12.</td>
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**TABLE 2. Putative RFHVm and RFHVVm gene products and comparisons with homologs present in other gammaherpesviruses**

<table>
<thead>
<tr>
<th>RFHV ORF Location RFHVm/ RFHVmα</th>
<th>ORF size in amino acids (% identity with the RFHVm ORF)</th>
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<td>(v) ORF K3 in KSHV was not recognized as homologous to ORF 12 in HVS and thus was given a K designation in the original KSHV nomenclature (37). For this reason, the RFHV ORF was given the RF designation even though it is homologous to HVS ORF 12.</td>
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**TABLE 3. Amino acid sequence comparison between the KSHV DL-B ORFs and homologous ORFs of RFHV and other gammaherpesviruses**

<table>
<thead>
<tr>
<th>Gamma-herpesvirus</th>
<th>ORF 9 (DNA polymerase)</th>
<th>ORF 10</th>
<th>ORF 11</th>
<th>ORF K2 (vIL-6)</th>
<th>ORF 02 (vDHFR)</th>
<th>ORF 70 (vTS)</th>
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<td>27</td>
<td>NP</td>
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</tr>
</tbody>
</table>

* NP, not present. * homologous genes located outside the DL-B region (see Fig. 1).
FIG. 5. Comparison of ORF 10 homologs. (A) Alignment of the gammaherpesvirus ORF 10 homologs and the distantly related ORF 11 homologs of KSHV and RRV. Residues conserved in four of the six ORF 10 homologs are highlighted to show ORF 10 conservation. Residues in the ORF 11 homologs which were conserved in two other ORF 10 or ORF 11 homologs are also highlighted to indicate the similarity between the ORF 10 and ORF 11 homologs. (B) Phylogenetic analysis with the protein maximum-likelihood method. ORF 10 homolog sequences from the Old World primate rhadinoviruses RFHVMn, RFHVMm, KSHV, and RRV, the New World primate rhadinoviruses HVS and AtHV3, the ungulate rhadinoviruses BHV4, AHV1, and EHV2, the murine rhadinovirus MHV68, and the lymphocryptovirus EBV (LF1 gene) were aligned with ClustalW and analyzed with the PROML program. Bootstrap values from 100 replica samplings and the scale for substitutions per site are provided. The ORF 11 homologs of KSHV and RRV were included as an outgroup.
2). They were similar in size to the 210-amino-acid KSHV vDHFR, which was 52% identical to the RFHV homologs. This similarity is greater than that seen between the RRV and KSHV vDHFR sequences (45%; Table 3). Furthermore, the RFHV and KSHV homologs contained a 17- to 24-amino-acid C-terminal region that was not found in either the cellular DHFR genes or the vDHFR homologs of HVS and RRV, all of which were 187 to 188 amino acids in length (Fig. 7A). Additionally, the RFHV and RRV sequences differed at two insertion-deletion positions, as indicated in Fig. 7A. Although the C-terminal regions of the RFHV and KSHV vDHFR homologs were highly conserved, little homology was seen with the KSHV C-terminal region except for a conserved ERP motif (Fig. 7A). Phylogenetic analysis revealed a clustering of the RFHV and KSHV vDHFR genes that was distinct from the RRV, HVS, and cellular DHFR genes (Fig. 7B).

(vi) ORF RF3 (MIR-1) homolog. ORFs encoding 233 and 232 amino acids were identified between the vDHFR and vTS genes of the RFHV and RFHV homologs compared to the vIL-6 of KSHV and RRV and the human (NP_000591) and rhesus macaque (L26028) cellular IL-6. Amino acids conserved between the KSHV and RFHV sequences are highlighted; a potential N-linked glycosylation site and a cysteine residue conserved between KSHV and RFHV are indicated with an asterisk and an open circle, respectively. The predicted signal peptide cleavage site for the vIL-6 homologs is shown (arrow) and corresponds to the cleavage site determined for human IL-6. The four major alphahelical regions (A, B, C, and D) and the minor helical region (E) determined for human IL-6 are indicated (47). The residues in sites II and III of KSHV vIL-6 which interact with the gp130 receptor subunit (see text) are indicated with θ and θ, respectively (7). The residues in site I which interact with the IL6R subunit are indicated with θ (23). The amino acid numbering is relative to the KSHV vIL-6 sequence. (B) Phylogenetic analysis of the alignment in A with the protein maximum-likelihood method. Bootstrap values from 100 replicate samplings and the scale for substitutions per site are provided.
distant sequence similarity was also noted with HVS ORF 12. An ORF RF3 homolog was not detected within the genome of the macaque RRV. Within the herpesviruses, all of the RF3-related ORFs were colocalized in analogous positions within their respective genomes.

Alignment of the RFHVNm and RFHVMMm RF3 genes with the other related sequences revealed a strong sequence similarity centered around a conserved cysteine and histidine motif within the N-terminal domain of these proteins. This motif belongs to the BKS (BHV4/KSHV/swinepox) subset of the C4HC3 PHD/LAP zinc finger motifs, as described before (31).

The PHD/LAP motif family consists of a very general grouping of a number of proteins containing a C4HC3 motif, which has been implicated in DNA binding and chromatin-mediated transcriptional regulation (1, 38). A consensus sequence of highly conserved residues was determined for the BKS motif of RF3, K3, K5, Bo4, Bo5, and MK3 which consisted of C1-W-I-C2-X(2-4)-G-X(5-8)-C3-X-C4-X-G-X4-V-H5-X2-C6-L-X2-W-X3-S-X4-C7-X2-C8-X3-Y. Whereas the domains containing the zinc finger motif (amino acids 9 to 53, Fig. 5A) in RF3 from RFHVMMm and RFHVMMm were 98% identical, with only a single amino acid difference, Asp-Glu at position 19, they were only 70% identical to the domains found in K3 and K5 and approximately 40% identical to the domains present in the MK3, Bo5, and Bo4 homologs. Although HVS ORF 12 contained the conserved cysteine and histidine residues character-

**FIG. 7.** Comparison of viral and cellular DHFR homologs. (A) Amino acid sequence alignment of the vDHFR of KSHV, RFHVMMm, RFHVMMm, RRV, and HVS and comparison with the human (NP_000782) and mouse (NP_034179) cellular DHFR. Residues conserved between KSHV, RFHVMMm, and RFHVMMm are highlighted. An asterisk indicates the presence of an insertion or deletion difference with RRV. (B) Phylogenetic analysis of the alignment in A with the protein maximum-likelihood method. Bootstrap values from 100 replica samplings and the scale for substitutions per site are provided.
FIG. 8. Comparison of ORF RF3 homologs. (A) Amino acid sequence alignment of the MIR homologs of KSHV (K3 and K5), RFHV Mn (RF3Mn), RFHV Mm (RF3Mm), BHV4 (Bo5 and Bo4), MHV68 (MK3), HVS (ORF 12), and myxoma virus (MV-LAP; AAK00734). The BKS zinc finger domain is indicated, with the positions of the hydrophobic transmembrane domains and the conserved region (CR) in the C-terminal domain shown. Residues identical within the K3, K5, RF3Mn, and RF3Mm sequences are highlighted. A consensus sequence for the BKS zinc finger domain is shown. (B) Phylogenetic analysis of the complete ORFs with the protein maximum-likelihood method with the addition of the 5L protein of yaba-like disease virus (NP_073390). Bootstrap values from 100 replica samplings and the scale for substitutions per site are provided.
istic of the C$_4$H$_3$ BKS motif, it lacked many of the additional residues which were completely conserved in the other herpesvirus proteins within this domain (Fig. 8A).

Like the other viral homologs, the RF3 sequences contained two extended hydrophobic regions downstream of the zinc finger motif which were predicted to be membrane-spanning domains, as indicated in Fig. 8A. While the transmembrane domains and flanking regions (amino acids 55 to 156, Fig. 8A) of the RF3 homologs from RFHVMn and RFHVMm were 95% identical to each other, they were only 31% and 27% identical to the corresponding regions of KSHV K3 and K5, respectively. Only a few residues within this region were identical between the RF3, K3, and K5 sequences, as seen in Fig. 8A. The transmembrane domains and flanking regions of the other viral homologs were not well conserved and were aligned based on the positions of their hydrophobic regions. The C-terminal domains downstream of the second hydrophobic domain (amino acids 157 to 236) within the RF3 sequences from RFHVMn and RFHVMm were only 50% identical but contained a conserved region (CR) in which several sequence motifs were also conserved with the K3 and K5 sequences, for example, the NTRV motif (amino acids 156 to 159), the PY motif (amino acids 163 to 164), and the IEL motif (amino acids 180 to 182). The Asn and Pro at positions 156 and 163, respectively, were also highly conserved within the C-terminal domains of the other K3/K5-related homologs. The C-terminal domains of RF3, K3, and K5 also contained several G/N/D and N/D-G dipeptide motifs which were not found in the other homologous sequences. While the RF3 and K5 sequences terminated shortly after these dipeptide motifs, the C-terminal region of the K3 sequence extended for an additional 100 amino acids.

Phylogenetic analysis of the RF3 homologs with several different methods, including neighbor-joining and protein maximum likelihood, revealed an obvious clustering of RF3, K3, and K5 (see Fig. 8B) which was distinct from that of the other herpesvirus and poxvirus homologs. However, the K3 homolog did not cluster exclusively with the RF3 homologs, as expected from their homologous positions within their genomes. Instead, the branch pattern between K3, RF3, and K5 was inconsistent. In some analyses, K3 clustered with the RF3 homologs with the exclusion of K5, while in other trees K3 was clustered with K5 with the exclusion of the RF3 homologs. This was also evident from the low bootstrap score of 51 for this branch point in the protein maximum-likelihood analysis in Fig. 8B. The clustering of the two proteins from the myxoma and yaba-like disease virus poxviruses and the clustering of the two BHV4 homologs, Bo4 and Bo5, was consistent in the different analyses.

**DISCUSSION**

We and others have previously reported that a number of Old World primate species are each host to two distinct RV-1 and RV-2 lineages of KSHV-like rhadinoviruses (13, 14, 20–22, 40). While the complete sequences have been determined for the genomes of human KSHV and macaque RRV, the classification of these and other KSHV-like rhadinoviruses into the RV-1 and RV-2 lineages of rhadinoviruses has been based solely on comparison of DNA polymerase sequences. Sequence analysis of the complete genomes of KSHV and RRV has revealed numerous similarities in gene structure and content which support a close evolutionary relationship (11). However, the genetic content of these viruses is not identical, with numerous examples of genes which are present in one virus and absent in the other (42). It has not been clear whether these differences are simply because of speciation of the human and macaque viruses or whether they reflect important structural and functional properties of the two distinct viral lineages.

Identification of genetic properties unique to the RV-1 and RV-2 rhadinovirus lineages has been hampered by the absence of an identified RV-2 rhadinovirus from a human host and the difficulty in obtaining further genomic sequence from the other identified RV-1 rhadinoviruses from nonhuman hosts. Among the identified nonhuman RV-1 rhadinoviruses, the greatest amount of sequence information has been obtained for RFHV, the putative macaque RV-1 rhadinovirus, with sequence covering the entire DNA polymerase gene and flanking sequences. However, there has still been some uncertainty concerning the exact nature of the relationship between RFHV and the other macaque virus, RRV, because of the relatively small amount of sequence data and information on gene content available for RFHV.

In the present study, we have added to the amount of available RFHV genetic information by determining 4.3 kb of contiguous sequence within the DL-B region of the RFHV genome. These sequences overlap the DNA polymerase sequences obtained previously (36, 40), extending the available sequence for both RFHV species to approximately 7.7 kb. Within this 7.7-kb genomic region, open reading frames encoding homologs of the KSHV glycoprotein B, DNA polymerase, ORF 10, vIL-6, vDHFR, ORF K3/MIR-1, and vTS genes were identified. These genes were present in the same order and relative positions as in the KSHV genome (37). However, unlike KSHV, the RFHV genomes lacked an ORF 11 homolog. Blast comparisons and phylogenetic analysis of each RFHV and RFHVM M ORF demonstrated the closest similarity to the corresponding ORF within the KSHV genome. These results strongly support the grouping of RFHV with KSHV within the RV-1 rhadinovirus lineage and provide evidence that RFHV and RFHVM are the rhesus and pig-tailed macaque homologs of KSHV.

Comparison of the macaque RFHV and RRV genomes within the region of the DL-B targeted in this study revealed important differences in the colinear organization and genetic content of the genomes. One such difference was the presence of the RF3 homolog of MIR-1/ORF K3 in RFHV which was absent from RRV. MIR-1 homologs have been identified in other herpesviruses, including BHV4, MHV68, and HVS, and in several poxviruses, including swinepox and myxoma virus (31, 46). MIR-1 and the closely related MIR-2 of KSHV, ORF MK3 of MHV68, and the MV-LAP protein of myxoma virus have been shown to function in immune evasion through downregulation of members of major histocompatibility complex class I (MHC-I) and other molecules involved in immune recognition (8, 9, 15, 16, 48). For this reason, the KSHV proteins have been termed modulators of immune response (MIR), while the MV-LAP protein have been described as endoplasmic reticulum-resident surface cellular receptor abductor proteins, called scrapins (15).
MIR-1 and MIR-2 function as membrane-bound E3 ubiquitin ligases which ubiquitinate the cytosolic tail of immune recognition proteins, targeting them for endolysosomal degradation (10, 25). The ability to impair host cytotoxic T-lymphocyte recognition of virus-infected cells is believed to be an important factor in the viral persistence characteristic of KSHV and other herpesviruses. The presence of the RF3 MIR homolog in RFHV and its absence in RRV demonstrates for KSHV and other herpesviruses. The presence of the RF3 MIR important factor in the viral persistence characteristic of cyte recognition of virus-infected cells is believed to be an
dation (10, 25). The ability to impair host cytotoxic T-lympho-
recognition proteins, targeting them for endolysosomal degra-
dation which ubiquitinate the cytosolic tail of immune
proteins (39). Although this region is conserved between the
MIR-1, MIR-2, and RF3 sequences, it is poorly conserved with
the corresponding regions of the other herpesvirus and poxvi-
residues, including three phenylalanine residues, conserved be-
etween the transmembrane domains of RF3, MIR-1, and
MIR-2. Even less conservation with other herpesvirus and pox-
virus proteins was noted across the transmembrane domains.

Only the RF3 transmembrane regions from RFHV Mm and
RFHV Mm were well conserved between each other, with 95%
amino acid identity between amino acids 80 and 145. Replace-
ment studies have shown that the transmembrane domains are
responsible for the target specificity of MIR activity (39). Thus,
the close similarity between the RF3 homologs of RFHV Mm
and RFHV Mm might reflect target similarities within the two
macaque species, while the differences between the RF3 and
MIR transmembrane domains could reflect the different spec-
icities required for interactions with macaque and human
immunoproteins. Experiments are under way to examine the
ubiquitin ligase activity and target specificity of macaque RF3.

Other differences in the genome structures of RFHV and
RRV were also evident from our analysis of the DL-B region.
Whereas the genomes of both RFHV and KSHV contain a
dHFTR gene immediately to the left and downstream of the
RF3/K3 homolog, the RRV dHFTR is positioned at the far
left end of the viral genome. The positioning of the RRV
dHFTR gene is similar to that seen in the New World primate
rhadinovirus HVS (see Fig. 3). Furthermore, the sequence of
the RRV dHFTR gene is quite distinct from the that of RFHV
and KSHV and groups phylogenetically more closely with the
HVS and mammalian cellular dHFTR homologs. In fact, the
RFHV and KSHV dHFTR sequences are more similar to
HVS dHFTR than to RRV dHFTR (Tables 2 and 3). Specific
differences include the presence of an additional amino acid at
position 45 in the sequences of human, mouse, HVS, and RRV
dHFTR homologs which is absent in the KSHV and RFHV
dHFTR homologs and the unique C-terminal extensions found
only in the KSHV and RFHV dHFTR homologs. These find-
ings suggest that the dHFTR homologs of KSHV and RFHV
were acquired from an ancestral host in a different acquisition
event than the dHFTR homologs of HVS and RRV. The
differences in the genomic location of the dHFTR genes fur-
ther support independent acquisition events. While the KSHV
and RFHV dHFTR genes are more closely related to each
other than to the other DHFR genes, they have evolved to
differ considerably from each other, especially when compared
to the minimal differences seen between the human and mu-
rine DHFR genes, which are 90% identical.

Our studies show that RFHV, like KSHV and RRV, con-
tains a viral homolog of the cellular IL-6 in a conserved ori-
entation within the DL-B region. Of all the herpesviruses iden-
tified to date, only these KSHV-like rhadinoviruses contain a
IL6 homolog (31, 37). While our studies show that the IL-6
homologs of RFHV and KSHV have a conserved gene struc-
ture and sequence, the RRV IL-6 has diverged considerably,
with substantial changes in amino acid sequence and three
internal insertions or deletions with respect to the other IL-6
and cellular IL-6 homologs. These structural differences sug-
gest the possibility of functional differences. Cellular IL-6 is
one of a family of related cytokines which has been implicated
in the pathogenesis of several B-cell neoplasias (18), and the
presence of a IL6 homolog in KSHV suggests a potential role
for this protein in the induction and progression of Kaposi's
sarcoma and the B-cell neoplasias associated with KSHV.

Whereas cellular IL-6 requires the presence of the nonsig-
naling receptor subunit (IL-6R) and the signaling receptor su-
ubunit gp130 for signal transduction, the KSHV IL-6 can
interact and signal directly through the ubiquitous gp130 re-
ceptor subunit in the absence of IL-6R (28). Recent results
suggest that the gp130-specific signaling by KSHV IL-6 plays
an important role in bypassing the antiviral effects of interferon
(6). Crystallographic studies of the KSHV IL6-gp130 extra-
cellular receptor complex have identified major sites of protein
interaction which are primarily hydrophobic (7). The site II
interface of KSHV IL-6 is dominated by Trp41, Trp44, and
Trp134, while the site III interface is dominated by Leu165,
Tyr166, Trp167, and Phe171. These interactions are predicted
to allow two molecules of IL-6 to bind to two gp130 subunits
in the absence of the IL-6R subunit. Most of these residues are
conserved within the RFHV Mm and RFHV Mm IL-6 ho-
omologs, suggesting similarities in receptor binding (Fig. 6A).

Interestingly, very few of these hydrophobic residues are
conserved within the RRV IL-6. In RRV, Trp41 is replaced by
a negatively charged glutamic acid and Trp167 is replaced by
a positively charged arginine. This is similar to the situation
with the cellular IL-6 homologs, which lack the conserved
hydrophobic residues in the site II and site III domains and
instead have small polar residues in these positions. The lack of
hydrophobic residues available for site II and III binding sug-
gests that, like cellular IL-6, RRV IL-6 may not bind and
signal directly through interactions with gp130, but instead
could require the presence of the IL-6R subunit. Studies
with RRV IL-6 support this hypothesis. Antibodies directed
against the IL-6R subunit completely inhibit RRV IL-6-in-
duced proliferation of B9 mouse cells (17). This contrasts with
the situation with KSHV IL-6, where antibodies against gp130
inhibited IL-6 activity while antibodies against IL-6R did not
rhadinoviruses HVS and ateline herpesvirus 3 and the unguulate rhadinovirus EH2V, in which the vTS genes are located much further downstream at the right end of the genome (2, 3, 43). While TS homologs are also present in the human and simian varicella-zoster virus homologs (as shown in Fig. 4A and B), the genome structures of these alphaherpesviruses are not analogous to the rhadinovirus genomes and cannot be easily compared. Three separate clusters of the herpesvirus vTS homologs were evident from our phylogenetic analysis: (i) the Old World primate and unguulate rhadinoviruses vTS homologs, (ii) the human and monkey varicella-zoster virus-related alphaherpesvirus and the mammalian TS homologs, and (iii) the New World primate rhadinovirus and lower eukaryote TS homologs. This suggests that at least three separate acquisitions of cellular TS genes into the herpesviridae may have occurred. A second possibility is that the sequence and genomic location differences between the TS homologs may derive from a combination of different mutational rates and translocations between the two genomic terminal regions (27).

In contrast to KSHV and RRV, RFHV lacks an ORF 11 homolog downstream of ORF 10. This was confirmed by sequencing this region in both RFHV-Mm and RFHV-Mn species. Although the presence of an ORF 11 homolog is a conserved feature within most gammaherpesviruses, the unguulate rhadinovirus BHV4 also lacks an ORF 11 homolog (49). Analysis of the ORF 11 homologs of other gammaherpesviruses with repetitive PSI-Blast searches of the NCBI databases revealed a distant similarity to the gammaherpesvirus ORF 10 homologs (see alignment in Fig. 5A). This similarity, though weak, suggests a possible functional overlap between ORFs 10 and 11. However, no function has yet been attributed to either ORF 10 or 11, nor have obvious similarities been detected with proteins of known function. ORFs 10 and 11 do not appear to be required for viral replication, as both gene homologs (LF1 and LF2) are deleted in the prototypical EBV variant which replicates within the B95-8 marmoset cell line (34). Our results indicate that sometime after host speciation and the evolutionary split between RFHV and KSHV, the RFHV lineage lost the ORF 11 homolog within the DL-B region. Future cloning studies will reveal whether or not an ORF 11 homolog is alternatively present within the remainder of the uncharacterized RFHV genome.

Our studies demonstrate a strong similarity in sequence conservation, genetic complement, and colinear organization of genes within the DL-B region of the genomes of macaque RFHV and human KSHV. These findings strongly support the hypothesis that RFHV is the macaque equivalent of KSHV and substantiate the grouping of these viruses together within the RV-1 lineage of Old World primate rhadinoviruses. Conversely, the differences detected between the sequence conservation, genetic complement, and colinear organization of the macaque RFHV and RRV genomes support the grouping of these two macaque viruses into distinct RV-1 and RV-2 rhadinovirus lineages, respectively. However, the ultimate comparison of these macaque rhadinoviruses awaits the complete sequencing of the RFHV genome. Even though the RV-1 and RV-2 lineages of rhadinoviruses are more closely related to each other than to other members of the Herpesviridae, our findings suggest that the two macaque rhadinovirus lineages have diverged considerably over time, yielding differences in gene content, organization, and structure that would provide the basis for biological differences between the members of these two viral lineages. No evidence of interlineage recombination between the macaque RV-1 and RV-2 rhadinoviruses was detected, even though mixed infections were identified in numerous individual macaques.

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