JOURNAL OF VIROLOGY, May 1997, p. 4138–4144 Vol. 71, No. 5
0022-538X/97/$04.00+0
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Identification of Two Homologs of the Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) in Retroperitoneal Fibromatosis of Different Macaque Species

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Received 31 October 1996/Accepted 3 February 1997

Simian retroperitoneal fibromatosis (RF) is a vascular fibroproliferative neoplasm which has many morphological and histological similarities to human Kaposi's sarcoma (KS). Like epidemic KS in AIDS patients, RF is highly associated with an immunodeficiency syndrome (simian acquired immunodeficiency syndrome [SAIDS]) caused by a retrovirus infection. Recently, a new gammaherpesvirus, called Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8), has been identified in KS tumors, suggesting that KS has a viral etiology. Our previous experimental transmission studies and epidemiological data suggest that RF also has an infectious etiology. In order to determine whether a similar virus is also associated with RF, we have assayed for the presence of an unknown herpesvirus using degenerate PCR primers targeting the highly conserved DNA polymerase genes of the herpesvirus family. Here we provide DNA sequence evidence for two new herpesviruses closely related to KSHV from RF tissues of two macaque species, *Macaca nemestrina* and *Macaca mulatta*. Our data suggest that KSHV and the putative macaque herpesviruses define a new group within the subfamily *Gammaherpesvirinae* whose members are implicated in the pathogenesis of KS and KS-like neoplasms in different primate species.

Retroperitoneal fibromatosis (RF) was initially recognized as a disease syndrome in 1976 (10), and an epidemic of RF occurred in the colonies of several different macaque species at the Washington and Oregon Regional Primate Research Centers. This epidemic, which lasted for more than a decade, was characterized by a mean incidence rate of approximately 1% in all age groups and 10% in colony-born juveniles (25). RF consists of an aggressively proliferating mass of highly vascular fibrous tissue which is rapidly fatal (12). RF occurs predominantly near the peritoneum, covering the ileocecal junction and associated mesenteric lymph nodes (11). Many similarities have been noted between RF and Kaposi's sarcoma (KS) including the presence of the characteristic slit-like neovascular spaces and proliferating spindle-shaped cells of KS (12). RF is strongly associated with the presence of a type D retrovirus called simian retrovirus 2 (SRV-2) (4, 24), and diseased animals often develop a simian acquired immunodeficiency syndrome (SAIDS) that includes lymphoid depletion, weight loss, depressed immune functions, recurrent diarrhea, and chronic infections (26). Epidemiological data suggest an infectious etiology for RF, and experimental transmission studies have shown that intraperitoneal inoculations of suspensions of RF tissue can result in the transfer of the disease to naive monkeys (12).

Recently, a new gammaherpesvirus, alternately termed Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8), was discovered (7). Its presence in greater than 95% of KS tumors provides evidence that KSHV/HHV8 plays a role in the etiology of KS (7–9, 17, 22). Although KSHV/HHV8 has been found in other AIDS- and KS-associated malignancies, including body cavity-based non-Hodgkin's lymphoma (5, 6) and multicentric Castleman's disease (23), it is rarely detected in tissue samples from individuals without AIDS or KS. In situ studies have shown the presence of KSHV/HHV8 DNA in the characteristic spindle-shaped cells of KS and in the endothelial cells surrounding KS lesions (3). Sequence analysis of regions of the KSHV/HHV8 genome (7, 18) has demonstrated close similarities to herpesvirus saimiri (HVS) and Epstein-Barr virus (EBV), which are both members of the tumorigenic gammaherpesvirus subfamily. It has been suggested that KSHV/HHV8 be included within the *Rhadinovirus* genus of the gammaherpesvirus subfamily due to its similarity in sequence and genomic structure to HVS, the prototype for the *Rhadinovirus* genus (18).

In order to test the possibility that a herpesvirus plays a role in the pathogenesis of RF, we have developed a sensitive nested PCR assay to identify unknown members of the herpesvirus family in tissue extracts. The amino acid sequences of known herpesvirus DNA polymerases were obtained from the Protein Identification Resource database or derived from DNA sequences from the GenBank nucleotide database. Alignment of these sequences by using the CLUSTALW multiple alignment program (EMBL, Heidelberg, Germany) revealed three regions with conserved amino acid sequences which were specified by a relatively restricted number of codons. The conserved regions that we detected correspond to substrate binding sites within the DNA polymerases (2). Pools of oligonucleotide primers corresponding to the different possible coding sequences within these regions were prepared. The oligonucleotides derived from these regions are designated by letters corresponding to a 3- or 4-amino-acid sequence within the region, followed by an A or B indicating the

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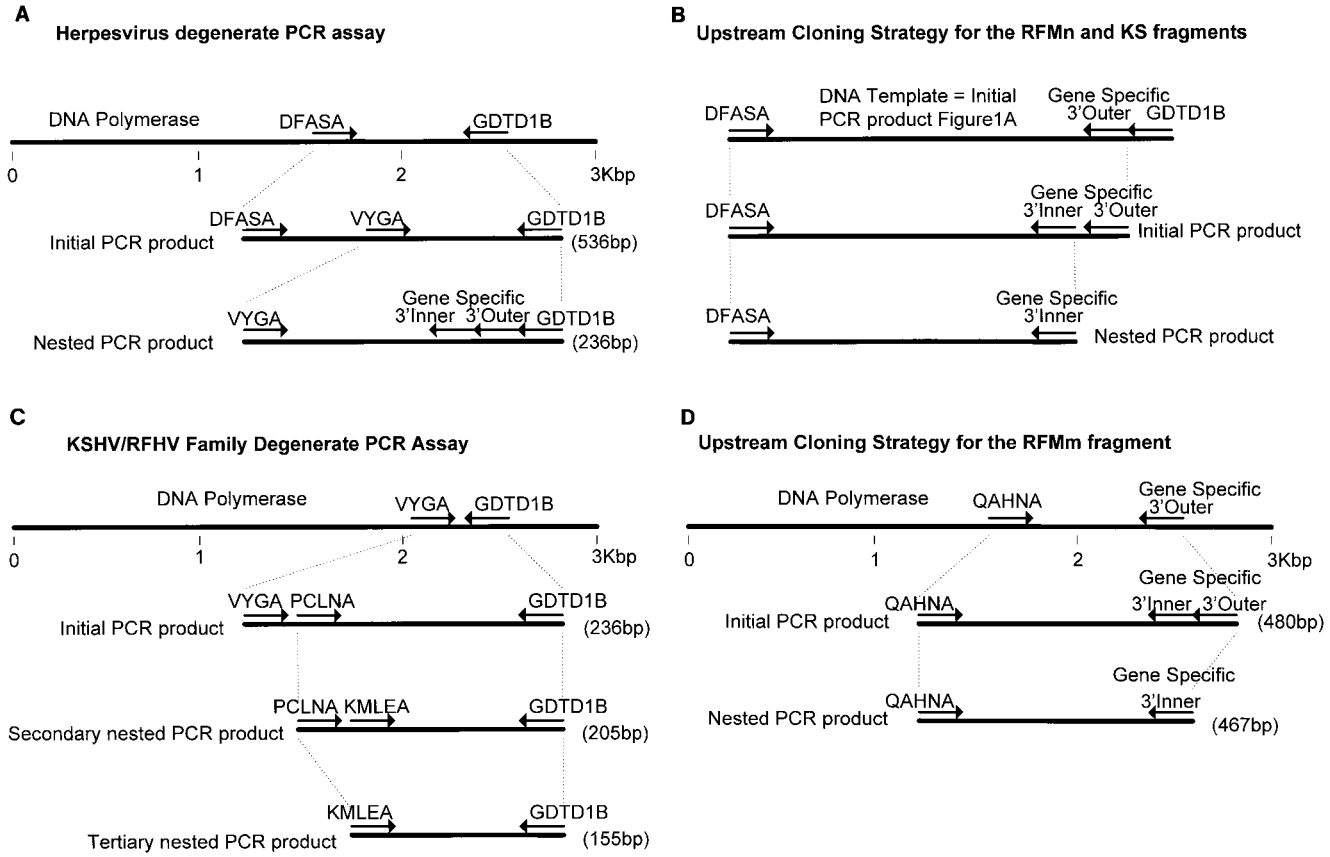


FIG. 1. Schematic diagram of the nested PCR assays. (A) Initial herpesvirus degenerate assay used to identify novel DNA polymerase sequences in the *M. nemestrina* RF and human KS samples. (B) Secondary degenerate-nondegenerate hybrid assay used to clone upstream DNA polymerase sequences from the *M. nemestrina* RF and human KS samples. (C) RFHV/KSHV family degenerate assay targeting sequences closely related to the *M. nemestrina* RF and human KS sequences. This assay was used to identify the DNA polymerase sequence in the *M. mulatta* RF sample. (D) Secondary degenerate-nondegenerate hybrid assay used to clone the upstream DNA polymerase sequences from the *M. mulatta* RF sample. The sequences and positions of the oligonucleotides are given in Table 1.

orientation of the primer; A oligonucleotides prime in the sense orientation and B oligonucleotides prime in the antisense orientation. A schematic diagram of the relative positions of the oligonucleotides and the manner in which they were used in the nested PCR assay is shown in Fig. 1A. The sequences of the oligonucleotide primers are given in Table 1.

DNA was extracted from formalin-fixed paraffin-embedded tissue samples obtained from nine different *Macaca nemestrina* RF lesions and a human KS lesion from an individual with AIDS. Paraffin was removed with xylene, and nucleic acids were extracted by using a standard proteinase K digestion protocol. The DNA samples were initially amplified with the primer pools DFASA and GDTD1B (Fig. 1A and Table 1). Amplifications were performed in 0.67 M Tris buffer (pH 8.8) containing 40 mM $MgCl_2$, 0.16 M $(NH_4)_2SO_4$, 0.1 M 2-mercaptoethanol, and 1 mg of bovine serum albumin per ml (14) for 35 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C). An aliquot (2 to 5%) of these amplification products (DFASA-GDTD1B) was then used as a template in a subsequent nested PCR with the primer pools VYGA and GDTD1B (Fig. 1A and Table 1). The products of the secondary nested PCR amplification were electrophoresed in a 2% agarose gel and visualized by irradiation with UV in the presence of ethidium bromide. Amplification products (VYGA-GDTD1B) of the predicted size (~236 bp) were detected in one *M. nemestrina* RF (RFMn) sample and the KS sample (data not shown; see Fig. 1A). The resulting fragments were cloned into pT7Blue (Novagen, Madison, Wis.) and sequenced with chain terminators (21). To obtain the nucleotide sequence extending upstream of the VYGA region to the initial upstream oligonucleotide, DFASA, a nested set of gene-specific nondegenerate oligonucleotide primers were derived from the complementary sequence of the RFMn and the human KS fragments (3′ inner and 3′ outer primers as indicated in Fig. 1A and Table 1). These gene-specific RFMn and KS primers were used in nested PCR amplifications with the upstream degenerate DFASA primer pool, as illustrated in Fig. 1B. The PCR products (DFASA-GDTD1B) from the initial RFMn and KS amplifications described above (Fig. 1A) were used as template DNA in these subsequent amplification reactions; the initial amplifications were done with either the degenerate DFASA primer pool and the specific RFMn 3′ outer primer with the RFMn (DFASA-GDTD1B) template or the DFASA primer pool and the KS 3′ outer primer with the KS (DFASA-GDTD1B) template (see Fig. 1B). Aliquots of these initial PCR products were used as templates in secondary nested reactions with the DFASA primer pool and the appropriate RFMn or KS 3′ inner primer. These upstream nested PCR products were analyzed by gel electrophoresis and were subsequently cloned into pT7Blue and sequenced. Multiple PCR products from each sample were cloned and sequenced to identify amplification artifacts. The nucleotide sequences of the upstream (DFASA-3′ inner primer) and downstream (VYGA-GDTD1B) PCR fragments derived from the RFMn

TABLE 1. Oligonucleotide primers

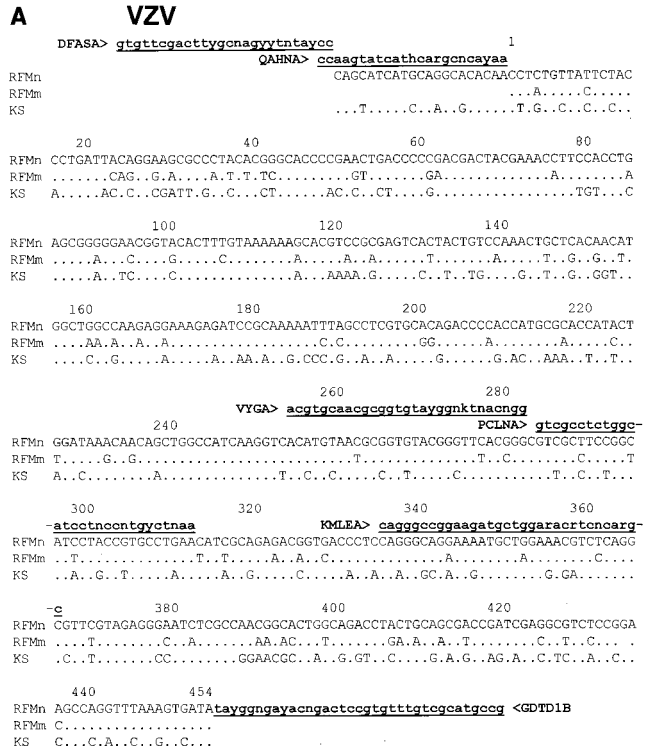
Primer	Orientation[a]	Nested position	Sequence (5′→3′)[b]	Sequence position (bp)[c]
Degenerate primer pools				
DFASA	+	5′ outer 1	GTGTTCGACTT [CT] GC [ACGT] AG [CT] [CT] T [ACGT] TA [CT] CC	
QAHNA	+	5′ outer 2	CCAAGTATCAT [ACT] CA [AG] GC [ACGT] CA [CT] AA	
VYGA	+	5′ inner 1	ACGTGCAACGCGGTGTA [CT] GG [ACGT] [GT] T [ACGT] AC [ACGT] GG	
PCLNA	+	5′ inner 2	GTCGCCTCTGGCATCCT [ACGT] CC [ACGT] TG [CT] CT [ACGT] AA	
KMLEA	+	5′ inner 3	CAGGGCCGGAAGATGCTGGA [AG] AC [AG] TC [ACGT] CA [AG] GC	
GDTD1B	−	3′ outer	CGGCATGCGACAAACACGGAGTC [ACGT] GT [AG] TC [ACGT] CC [AG] TA	
RFHVMn primers				
VASGA	+	5′ outer	CGTCGCTTCCGGCATCCTACC	283–303
ILPCA	+	5′ inner	GGCATCCTACCGTGCCTGAAC	293–313
PIEAB	−	3′ inner	CCGGAGACGCCTCGATCGGTC	434–414
PEARB	−	3′ outer	AACCTGGCTTCCGGAGACGCC	444–424
RFHVMm primers				
FVEGA	+	5′ outer	GTTTGTAGAGGGCATATCGCCAA	367–389
SPKDA	+	5′ inner	GAGGGCATATCGCCAAAAGAC	374–394
ASPDB	−	3′ inner	AACCTGGCGTCCGGGGAAGCG	444–424
KVIYB	−	3′ outer	GTATATCACTTTAAACCTGGC	457–437
KSHV primers				
SGILA	+	5′ outer	GCGTTGCCTCTGGCATACTG	282–301
CLNIA	+	5′ inner	CTGCCTTGCCTAAACATAGCG	299–319
IEASB	−	3′ inner	GGTGAGACGTCTATTGGCCT	432–413
EARFB	−	3′ outer	AATCGGGCGTCGGGTGAGACG	444–424

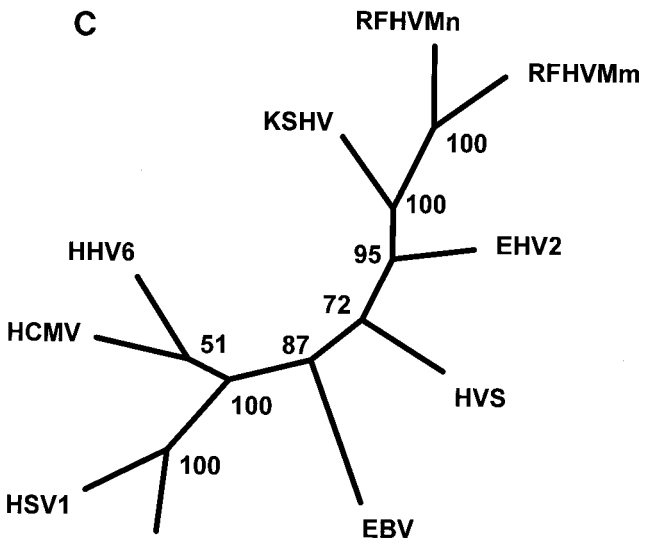
[a] +, sense; −, antisense.
[b] The positions of degenerate nucleotides are given with brackets surrounding the different nucleotide possibilities.
[c] Relative to sequence indicated in Fig. 2A.

and KS samples were combined (DFASA-GDTD1B). This yielded a 475-bp sequence for each sample after exclusion of the 5′ and 3′ sequences derived from the DFASA and GDTD1B oligonucleotides used in the amplification. The RFMn and KS nucleotide sequences are aligned in Fig. 2A.

In order to assay for a new herpesvirus in RF tumors obtained from a different species of macaque, *Macaca mulatta*, we developed a second degenerate PCR assay (KSHV/RFHV family degenerate assay; Fig. 1C) to identify sequences closely related to the *M. nemestrina* RF and human KS sequences. The degenerate oligonucleotides for this assay (PCLNA and KMLEA; sequences given in Table 1) were derived from regions of the RFMn and human KS sequences which were conserved between each other but not with other gammaherpesvirus sequences (Fig. 2). DNA was extracted from two frozen tissue samples from RF lesions of a single *M. mulatta*. A triple-nested PCR protocol was used to assay the *M. mulatta* RF samples, with VYGA and GDTD1B in the first amplification, PCLNA and GDTD1B in the second amplification, and KMLEA and GDTD1B in the third amplification reaction, as indicated in Fig. 1C. The PCR products of the final amplification step were analyzed on a 3% agarose gel and visualized by ethidium bromide fluorescence under UV illumination. A DNA fragment of 155 bp was identified which corresponded to the expected size for this PCR product. This fragment was cloned into the PT7Blue vector and sequenced. In order to clone the region upstream of the KMLEA region from the *M. mulatta* RF (RFMm) sample, a nested set of oligonucleotide primers was derived from the complementary sequence of this fragment (RFMm 3′ inner and 3′ outer primers, as indicated in Fig. 1D and Table 1). However, attempts to amplify the upstream region from the RFMm samples with the DFASA oligonucleotide pool and the RFMm 3′ inner and 3′ outer primers were unsuccessful. Therefore, an additional degenerate oligonucleotide pool (QAHNA; sequence given in Table 1) was derived from the RFMn and human KS sequences immediately downstream of the DFASA region, as indicated in Fig. 1D and 2A. QAHNA was designed to be able to amplify sequences closely related to the RFMn and human KS sequences but not other herpesvirus sequences. The QAHNA oligonucleotide pool was used in nested PCR amplifications initially with the RFMm 3′ outer primer and secondarily with the 3′ inner primer (Fig. 1D). A nested PCR product of the expected size (467 bp) was obtained, and this was cloned and sequenced. The sequence determined for this RFMm upstream sequence (QAHNA-3′ inner primer) was joined with the RFMm downstream sequence (KMLEA-GDTD1B) to give a 454-bp sequence after excluding the 5′ and 3′ sequences derived from the QAHNA and GDTD1B oligonucleotides. The RFMm nucleotide sequence was aligned with the RFMn and human KS sequences (Fig. 2A). The subsequent comparative analyses of these sequences were done using only the 3′ 454 bp in common among all three sequences (see nucleotide numbering in Fig. 2A). The positions of the degenerate oligonucleotide primers used to amplify the various sequences are shown in Fig. 2A.

The alignment of the nucleotide sequences in Fig. 2A revealed strong similarities between the DNA fragments isolated from the RFMn, RFMm, and human KS samples. The two macaque sequences were 83% identical across the 454-bp alignment. Furthermore, the RFMn and RFMm sequences were 71 and 69% identical to the human KS sequence, respectively (Table 2). Searches of the GenBank database by using the BLAST Web server demonstrated that these three sequences were most similar to the DNA polymerases of the gammaherpesvirus subfamily (data not shown). However, as shown in Table 2, the two macaque RF and human KS se-





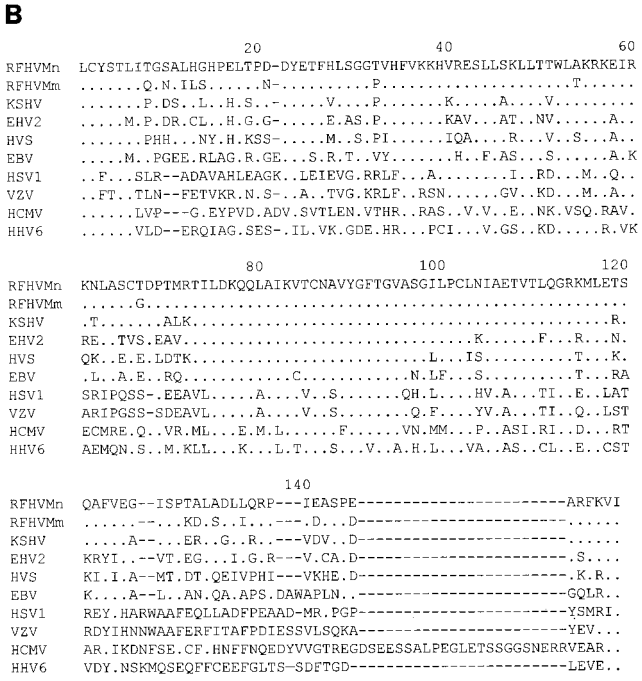


FIG. 2. Herpesvirus-like DNA polymerase sequences identified by degenerate PCR assays. (A) Comparison of the nucleotide sequences obtained from RF lesions of two macaque species, *M. nemestrina* and *M. mulatta*, and from a KS lesion of a human immunodeficiency virus-infected individual. Underlined sequences indicate the positions of the degenerate primers, with the redundant positions indicated as n (A, C, G, and T), r (A and G), y (C and T), h (A, C, and T), and k (G and T) (see also Table 1). The sequences of the positive strand primers (>) and the inverse complement of the negative strand primer (<) are given. Dots indicate identity with the first sequence in the alignment. (B) Alignment of the amino acid translations of sequences in panel A with analogous regions from the DNA polymerase sequences of several known herpesviruses: EHV2 (GenBank accession no. U20824), HVS (GenBank accession no. M31122), EBV (GenBank accession no. V01555), herpes simplex virus type 1 (HSV1; GenBank accession no. X04771), varicella-zoster virus (VZV; GenBank accession no. M14891), human cytomegalovirus (HCMV; GenBank accession no. M14709), and human herpesvirus 6 (HHV6; GenBank accession no. M63804). (C) Phylogenetic analysis of the amino acid alignments shown in panel B.

quences were more similar to each other than to any other herpesvirus sequence. Figure 2B shows an alignment of the amino acid sequences encoded by the 454-bp sequences of the two macaque RF and human KS fragments and the corresponding sequences from other herpesvirus DNA polymerases. The RFMn and RFMm amino acid sequences were 90% identical to each other (Table 2). The human KS sequence was 84 and 83% identical to the RFMn and RFMm sequences, respectively. The similarities between these three amino acid sequences and the DNA polymerase sequences of the gammaherpesviruses (equine herpesvirus 2 [EHV2], HVS, and EBV) ranged from 60 to 71% (Table 2). Phylogenetic studies of the amino acid alignments were performed by using the PHYLIP (University of Washington, Seattle) package based on distance matrices obtained by the maximum likelihood approach and unweighted pair group method by arithmetic averaging (UPGMA) analyses. These studies grouped the KS and two macaque RF sequences in a separate cluster within the gammaherpesvirus subfamily (Fig. 2C). Statistical bootstrap evaluation of this phylogenetic tree strongly supported the branch point that separates the newly defined cluster containing the RF- and KS-derived sequences from the other gammaherpesviruses. Identical tree topologies were obtained by using neighbor-joining analysis of the protein distance matrices and by a parsimony method, as implemented in the PHYLIP package. The phylogenetic analysis presented in this study is in close agreement with the known clustering of herpesviruses into alpha, beta, and gamma subfamilies, even though only partial fragments of the DNA polymerases were studied.

Analysis of the 454-bp sequences using GenePro software (Riverside Scientific, Bainbridge Island, Wash.) showed that the G+C mononucleotide frequencies of the KS and two macaque RF sequences were quite similar to each other and ranged from 51 to 56%. These values fall midway between the high G+C content found in the same regions of EBV and EHV2 (64 to 65%) and the low G+C content found in HVS (40%), as indicated in Table 3. The CpG dinucleotide frequen-

TABLE 2. Nucleotide and amino acid sequence identities within the cloned DNA polymerase region of different herpesviruses

Herpesvirus[a]	Subfamily	% Nucleotide (amino acid) sequence identity		
		RFHVMn	RFHVMm	KSHV
RFHVMn	Gamma		83 (90)	71 (84)
RFHVMm	Gamma	83 (90)		69 (83)
KSHV	Gamma	71 (84)	69 (83)	
EHV2	Gamma	68 (67)	63 (65)	68 (71)
HVS	Gamma	59 (64)	60 (63)	62 (67)
EBV	Gamma	64 (63)	57 (60)	62 (62)
HSV1	Alpha	53 (46)	52 (43)	53 (46)
HSV2	Alpha	53 (46)	53 (44)	53 (46)
VZV	Alpha	45 (43)	42 (41)	48 (45)
HCMV	Beta	53 (41)	45 (38)	49 (40)
HHV6	Beta	46 (41)	44 (38)	48 (41)

[a] HSV1, herpes simplex virus type 1 (GenBank accession no. X04771); EBV, GenBank accession no. V01555; HSV2, herpes simplex virus type 2 (GenBank accession no. M14793); VZV, varicella-zoster virus (GenBank accession no. M14891); HCMV, human cytomegalovirus (GenBank accession no. M14709); HHV6, human herpesvirus 6 (GenBank accession no. M63804).

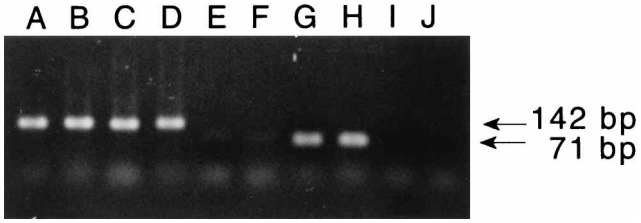


FIG. 3. Representative PCR screen for the prevalence of RFHVMn and RFHVMm sequences. Results obtained from PCR analysis of RF samples from *M. nemestrina* monkeys 2, 3, 4, 7 (lanes A to D, respectively), 1 (lane I), and 5 (lane J) and two samples from the same *M. mulatta* monkey (lanes G and H) are shown. Results obtained with peripheral blood lymphocytes from two unaffected SRV-2-negative *M. nemestrina* monkeys are shown in lanes E and F. Samples were assayed using nested PCR specifically targeting the RFHVMn DNA polymerase sequence (which yields a 142-bp PCR product [lanes A to F]) and the RFHVMm DNA polymerase sequence (which yields a 71-bp PCR product [lanes G to J]). The RFHVMn assay used RFHVMn primers VASGA and PEARB in the initial PCR amplification and primers ILPCA and PIEAB in the nested amplification. The RFHVMm assay used RFHVMm primers FVEGA and KVIYB in the initial amplification and primers SPKDA and ASPDB in the nested amplification (see Table 1 for primer sequences).

cies determined from the KS and two macaque RF sequences were also quite similar to each other, with values close to those expected from their mononucleotide compositions (ratios of observed to expected ranged from 0.91 to 1.13; Table 3). These values are more similar to the ratios observed in this region of the DNA polymerase of alphaherpesviruses and betaherpesviruses (0.99 to 1.37) than to those of other gammaherpesviruses, particularly HVS, which are deficient in CpG dinucleotides (0.28 to 0.73) (13). As indicated in Table 3, the G+C and CpG values for this defined region of the known herpesvirus DNA polymerases are consistent with the values obtained from the entire polymerase genes from these viruses and reflect the values obtained from the whole viral genomes.

Our results suggest that the DNA sequences detected in the macaque RF and human KS lesions are derived from DNA polymerase genes of distinct but closely related herpesviruses of the gammaherpesvirus subfamily. The putative viruses in the RF tissues from *M. nemestrina* and *M. mulatta* would, therefore, correspond to previously undescribed herpesviruses, tentatively called retroperitoneal fibromatosis herpesviruses (RFHVMn and RFHVMm, respectively), and the virus in human KS tissue would correspond to the recently described KSHV/HHV8 (7). A comparison of our sequence from the KS sample with the recently published sequence of the KSHV/HHV8 genome (20) confirms our identification of the KSHV DNA polymerase gene.

TABLE 3. G+C mononucleotide and CpG dinucleotide frequencies in the DNA polymerase genes of different herpesviruses

Herpesvirus	Subfamily	% G+C content[a]	CpG ratio[b]
RFHVMn	Gamma	55.9	0.96
RFHVMm	Gamma	51.1	1.13
KSHV	Gamma	54.4	0.91
EHV2	Gamma	64.8 (63.6)	0.71 (0.75)
HVS	Gamma	39.9 (34.9)	0.28 (0.33)
EBV	Gamma	64.4 (61.8)	0.73 (0.70)
HSV1	Alpha	67.6 (65.8)	0.99 (1.06)
VZV	Alpha	41.5 (42.2)	1.37 (1.15)
HCMV	Beta	60.2 (59.9)	1.27 (1.23)
HHV6	Beta	46.0 (40.9)	1.00 (1.12)

[a] The nucleotide frequencies from the known herpesviruses are derived from the region analogous to that shown in Fig. 2A. The frequencies obtained from the entire polymerase genes are given in parentheses.

[b] The CpG ratio is the observed frequency/expected frequency ratio taking into consideration the mononucleotide composition. The frequencies obtained from the entire polymerase genes are given in parentheses.

To determine the prevalence of RFHVMn and RFHVMm DNA polymerase-like sequences in different macaque tissues and the KSHV/HHV8 polymerase-like sequences in KS lesions, we established three nested, nondegenerate PCR assays, each specifically targeting one of the viral sequences. The 5′ inner and outer nondegenerate oligonucleotide primers used were derived from each of the three sequences shown in Fig. 2A (see Table 1). These oligonucleotides were paired with the downstream 3′ inner and outer oligonucleotides previously derived from each sequence, as described above. The 5′ and 3′ outer primer pairs were used in the initial PCR amplification, and an aliquot (1 to 2%) of the product of that reaction was used in a second nested amplification reaction with the 5′ and 3′ inner primer pairs for each of the three assays.

DNA was extracted from KS lesions of seven individuals with KS, from RF samples of 40 different *M. nemestrina* monkeys, and from 2 RF samples from a single *M. mulatta* monkey. All DNA samples were shown to be amplification competent, as nonnested PCR assays identifying either human or macaque beta-globin were positive. The nested PCR amplifications were performed essentially as described above. Nested amplification products were visualized after electrophoresis in a 2% agarose gel. KSHV DNA polymerase-like sequences were detected in all seven KS samples (data not shown). Out of the 40 RF samples from *M. nemestrina* monkeys, RFHVMn DNA polymerase-like sequences were detected in 9. Representative results are shown in Fig. 3, lanes A to D. The four PCR fragments shown in lanes A to D were cloned and sequenced. These fragments had sequences identical to the corresponding sequences in Fig. 2A. Additional paraffin samples were available from three of the RFHVMn-positive RF tissues (corresponding to lanes A, B, and D in Fig. 3). The presence of RFHVMn DNA in these additional samples was confirmed in a subsequent PCR. The two samples of the *M. mulatta* RF tissue were shown to have RFHVMm DNA polymerase-like sequences (Fig. 3, lanes G and H) by using the RFHVMm nested PCR assay. The 31 *M. nemestrina* samples that were

negative in the RFHVMn PCR assay were also negative in the RFHVMm PCR assay (representative negative results for two monkeys are shown in Fig. 3, lanes I and J). We have also tested nearly half of these negative samples in the KSHV/RFHV family-specific degenerate PCR assay which was used to detect the *M. mulatta* RF sequence described above (see Fig. 1C). These assays were also negative (data not shown). DNA was extracted from a variety of tissues, including thymus, bone marrow, spleen, salivary gland, liver, mesenteric lymph node, ileocecal junction, duodenum, kidney, and gonads, from four *M. nemestrina* monkeys naturally infected with SRV-2 (16). These tissue samples were assayed for the presence of RFHVMn sequences and were found to be negative (data not shown). Nine of the RF samples from the *M. nemestrina* monkeys were randomly tested for the presence of SRV-2 by PCR (28). All of these samples were positive, confirming previous observations that all RF lesions are associated with SRV-2 (1). We have assayed for the presence of RFHVMn sequences in the DNA extracts of peripheral blood leukocytes isolated from 20 normal, SRV-2-negative, colony-born *M. nemestrina* monkeys from the Washington Regional Primate Research Center. These samples were assayed several times in duplicate and were uniformly negative (see Fig. 3, lanes E and F, for representative negative results) with the exception of one sample which gave an inconsistent and weak signal. We are currently establishing studies with larger numbers of animals to further investigate the prevalence of RFHV sequences in normal monkey populations.

Studies by others have detected KSHV/HHV8 sequences in KS lesions at frequencies approaching 95 to 98%, while the frequency in normal individuals not infected with HIV is less than 1% (7–9, 17, 22). The 100% prevalence of KSHV/HHV8 DNA polymerase sequences that we detected in the KS lesions is consistent with these previous findings. Although we did not detect RFHV sequences in tissues other than RF, only 14 of the 45 RF samples had detectable viral sequences. It is not clear whether this is due to an absolute lack of RFHV sequences in the negative RF samples or whether the amount of virus in some samples is below the limit of detection in our assay (less than 10 copies with standard dilution assays). Additionally, it is not known whether the virus load is heterogeneous within the tumor. Initial RF lesions can vary from 1 to 4 cm in diameter, while in later stages the tumor mass can often encase the intestines and lymph nodes and cover the walls of the abdominal cavity (25). It is possible that the virus may only be localized to particular sites within the tumor mass, thus making representative sampling a problem. In vitro studies have linked the proliferation of tumor cells derived from KS lesions to the autocrine and paracrine activities of several cytokines and growth regulatory factors, including interleukin-6, tumor necrosis factor alpha, basic fibroblast growth factor, and oncostatin M (reviewed in reference 15). Similarly, studies with cells derived from RF lesions have linked the expression of interleukin-6 with cellular proliferation in RF (19, 27). Thus, RF tumors could result from a cellular activation initiated by infection with RFHV which leads to increased cytokine expression and the cytokine-driven proliferation of tumor cells. These RF tumor cells may or may not retain RFHV genomic sequences. We are currently attempting to delineate the role of RFHV in the pathogenesis of RF.

It has been previously suggested that KSHV/HHV8 be included within the *Rhadinovirus* genus of the *Gammaherpesvirinae* subfamily due to its similarity in sequence to the prototype of the *Rhadinovirus* genus, HVS, which is found in the New World saimiri squirrel monkey (18). Our data from the DNA polymerase sequences of RFHVMn, RFHVMm, and KSHV/HHV8 demonstrate a very close evolutionary relationship among these three viral species which infect Old World primates. The phylogenetic clustering of the RFHVMn, RFHVMm, and KSHV/HHV8 sequences and the additional similarities detected in G+C mononucleotide and CpG dinucleotide frequencies differentiate these viruses from HVS, EBV, and other members of the gammaherpesvirus subfamily (Table 3). The presence of the expected frequency of CpG dinucleotides within the DNA polymerase fragments suggests that RFHVMn, RFHVMm, and KSHV/HHV8 genomes remain latent in nondividing cells, in contrast to HVS and EBV which are deficient in CpG dinucleotides and remain latent in proliferating lymphoblastoid cells (13). Preliminary sequence analyses of other regions of the RFHVMn and RFHVMm genomes support the DNA polymerase findings (unpublished data). Based on these data, we suggest that the putative RFHVMn and RFHVMm and the human KSHV/HHV8 define a new group within the subfamily of *Gammaherpesvirinae*. The exact hierarchical position of this group within the gammaherpesvirus subfamily and the formal nomenclature for these putative herpesviruses remain to be determined. We are presently characterizing larger regions of the RFHVMn and RFHVMm genomes to allow definitive classification of these putative herpesviruses. The identification of three distinct but closely related viruses in different primate species (*Homo sapiens*, *M. nemestrina*, and *M. mulatta*) implies that these viruses have evolved from a common ancestral virus that was present before primate speciation occurred. Although the possibility exists that KSHV/HHV8 has been introduced into the human population by transmission from nonhuman primates, the association of KSHV/HHV8 with KS in different geographically distinct human populations suggests that this could not have been a recent event. The distribution of RFHV/KSHV variants in different primate species in the wild needs to be studied to directly address this question. The apparent association of three closely related herpesviruses with similar malignancies occurring in different primate species strengthens the link between disease pathogenesis and these viruses. Thus, our studies provide further evidence for a viral etiology of KS and KS-like neoplasms and support the proposal that RF in macaques would be a relevant animal model for the study of human KS. In this model, the evaluation of antiviral compounds targeted to the distinct DNA polymerases of the putative herpesviruses identified in this study may lead to therapeutic treatment of KS-like neoplasms.

T. M. Rose and K. B. Strand contributed equally to this work.

This work was supported by grants from the National Center for Research Resources (National Institutes of Health) (RR00166), the M. J. Murdock Charitable Trust, and the Corixa Corporation, Seattle, Wash.

We acknowledge William Morton and George Todaro for their support and Yibing Wang for technical assistance.

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