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Herpesvirus Saimiri Strains from Three DNA Subgroups Have Different Oncogenic Potentials in New Zealand White Rabbits

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Herpesvirus saimiri is a primate tumor virus that induces acute T-cell lymphomas in New World monkeys. Strains of this virus have been previously classified into three groups on the basis of extreme DNA variability of the rightmost region of unique L-DNA. To compare the oncogenic potentials of various strains, we inoculated New Zealand White rabbits with viruses representing groups A, B, and C of herpesvirus saimiri. The results showed that a group C strain were highly oncogenic in New Zealand White rabbits; however, group A or B viruses were not oncogenic in these rabbits. Analysis of DNAs of tumor tissues and lymphoid cell lines established from tumors showed that the viral genome exists in circular episomal form. To identify which part of the genome of the group C strain is responsible for the highly oncogenic phenotype, group B-C recombinant strains were constructed by an efficient drug selection technique. Two group B recombinant strains in which the right-end 9.2 kilobase pairs of unique DNA is replaced by group C virus DNA were oncogenic in rabbits, indicating that the rightmost sequences contribute to the oncogenic properties of the group C strain. Oncogenicity of herpesvirus saimiri has been traditionally evaluated in New World monkeys; infection of rabbits with group C strain 484-77 offers a much more accessible animal model to study the mechanism of oncogenicity of this virus.

Herpesvirus saimiri was originally isolated from the blood of an apparently healthy squirrel monkey (20). The virus can induce acute lymphomas or lymphoid leukemias in several species of New World monkeys (21). Herpesvirus saimiri-transformed cells are T lymphocytes of the CD-8 class (14, 23, 25, 26).

The genome of herpesvirus saimiri consists of 110-kilobase-pair (kbp) unique sequences (L-DNA) flanked by tandem repeats (H-DNA) (2, 9). Deletion of the right-end sequences (Previous literature refers to this region as the leftmost region. The Eleventh Herpesvirus Workshop changed the orientation of the herpesvirus saimiri genome to align herpesvirus saimiri with the orientation of the genome of Epstein-Barr virus.) of L-DNA results in loss of oncogenicity (4, 8, 15, 29). The deletions map immediately left of the junction of H-DNA and L-DNA, and their sizes vary from 2 to 7 kbp. These mutants are entirely competent for lytic replication.

The genomes of various isolates of herpesvirus saimiri are fairly homologous, with the exception of the right-end oncogenic region. Surprisingly, the right-end 7-kbp stretch of L-DNA is extremely variable; no homology can be detected between some strains within the rightmost 2.5 kbp of L-DNA, even at low stringency, and there is only weak homology in the adjacent 4.5 kbp of DNA (19). On the basis of DNA variability, various isolates have been classified into DNA homology groups A, B, and C (group C was formerly called group non-A, non-B).

The variability of the genome correlates with important biological properties of the strains. This was first shown by studying in vitro immortalization of T cells by various virus isolates; group A and C strains can transform peripheral blood lymphocytes of common marmosets, whereas group B strains cannot (7, 31). Although group A (strains 11 and OMI) and B (S295C and SMHI) strains have been shown to induce lymphomas in various New World primates (4, 5, 8, 21, 29, 32), the oncogenic potential of group C strains has not been studied in any species. Moreover, the oncogenicities of strains representing all three groups have not been compared at all.

There are contradictory reports about the oncogenicity of herpesvirus saimiri in rabbits. Two laboratories have reported that a group B strain, S295C, can induce lymphomas with variable frequency in New Zealand White rabbits (3, 27). Another research group has reported lack of development of malignant disease in rabbits in a well-controlled experiment; they inoculated rabbits with a virus stock of strain S295C which was highly oncogenic in marmosets (5).

This study was designed to compare the oncogenic potentials of herpesvirus saimiri strains in rabbits. The data demonstrate that group C strain 484-77 is highly oncogenic but group B strains S295C and SMHI and group A strain 11 are not oncogenic in New Zealand White rabbits. Two group B-C recombinant viruses were constructed by cotransfection of cloned 484-77 DNA representing 15 kbp of the right-end region of L-DNA and virion DNA of strain S295C, followed by drug selection for recombinants. These two recombinant viruses, harboring right-end L-DNA sequences of 484-77, are also oncogenic in rabbits.

The state of viral DNA in tumor tissues in vivo has not been elucidated, although lymphoid cell lines established from tumors or immortalized in vitro by herpesvirus saimiri contain viral genomes as large circular episomal DNA molecules (13). This work shows that the viral genome is also circular in lymphoid tumor tissues of rabbits.

MATERIALS AND METHODS

Experimental animals. New Zealand White rabbits (7 to 8 weeks old, about 1.8 kg each) were obtained from Millbrook Rabbit Farms, Amherst, Mass. Inoculation of rabbits with

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herpesvirus saimiri and subsequent housing were done in a biocontainment level P3 animal facility. Rabbits were killed by exsanguination after anesthesia with 10 mg of xylazine per kg and 90 mg of ketamine per kg (body weight).

Histopathological studies. Samples of tissues collected for histopathological analysis were placed into 10% Formalin for routine histopathological processing. Five- to six-micrometer-thick sections of paraffin-embedded tissues were stained with hematoxylin and eosin and permanently mounted on glass slides for microscopic examination.

Virus stocks and cell cultures. Herpesvirus saimiri strains S295C and SMHI were originally obtained from Muthiah D. Daniel, and strains 11, 11-att, and 484-77 were from Ronald C. Desrosiers. The virus stocks used in this study were all from our earliest passages to avoid development of defective viruses. Viruses were propagated on monolayers of owl monkey kidney (OMK) cells grown in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics in 5% CO_2 in air at 37°C.

To obtain high-titer virus stocks, semiconfluent cultures of OMK cells grown in 10-cm-diameter petri dishes were infected with 1 ml of virus (about 10^6 PFU/ml) and incubated until all cells showed cytopathic effects (between 2 and 3 days). Cell debris was removed by centrifugation (1,500 × *g* for 10 min), and supernatant virus was concentrated by high-speed centrifugation (20,000 rpm in an SW28 rotor for 1 h). The virus pellet was suspended in 30 ml of phosphate-buffered saline, high-speed centrifugation was repeated, and the pellet was suspended in 1 ml of phosphate-buffered saline for each 20 ml of original culture volume. Virus titer was determined by endpoint titration of serial threefold dilutions of the virus by using OMK cells grown in 96-well microtiter plates. Virus stocks were stored at either −70°C in 20% sorbitol in phosphate-buffered saline or 4°C in phosphate-buffered saline and titrated again at the time of inoculation of rabbits. We observed no significant loss of titer of virus stocks during this experiment, even at 4°C.

Lymphoid cell lines were established by culturing lymphocytes from thymus and spleen tissues from strain 484-77-infected rabbits in RPMI 1640 medium supplemented with 20% fetal bovine serum, 20 U of human recombinant interleukin 2 (IL-2) per ml, and antibiotics. IL-2 was omitted from the medium after 3 months in culture. Cell lines were also established with comparable success without using IL-2.

Western immunoblotting. Polypeptides of purified herpesvirus saimiri virions (9) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose sheets by a semidry electroblotter (American Bionetics, Hayward, Calif.). Strips of nitrocellulose were sequentially incubated with preimmune serum or serum obtained after infection, with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase, and with color substrates of alkaline phosphatase; immunoglobulin G-alkaline phosphatase and color reagents were purchased from Promega Biotec, Madison, Wis. Size markers were purchased from Sigma Chemical Co. Dalton Mark VII, a mixture of seven proteins with sizes ranging from 14.2 to 66 kilodaltons (kDa); β-galactosidase (116 kDa); and α2-macroglobulin (180 kDa) were used.

Purification of viral DNA, modified Southern blotting, and detection of circular genomes. Viral DNA was extracted from partially purified virus (see above). For screening recombinants, 1 ml of virus was pelleted in an Eppendorf centrifuge at 16,000 × *g* for 30 min. Virions were suspended in 0.1 ml of 50 mM Tris hydrochloride (pH 7.5)–10 mM EDTA–50 mM NaCl; pronase and SDS were added to final concentrations of 1 mg/ml and 1%, respectively. After 1 h of incubation at 37°C the viral DNA was extracted once with buffer-saturated phenol and once with a chloroform-isoamyl alcohol mixture (24:1). Sodium acetate was added to the aqueous phase (0.2 M final concentration), and the DNA was precipitated with 2.5 volumes of ethanol, pelleted by centrifugation (16,000 × *g* for 30 min), and suspended in TE buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA).

Electrophoresis of DNA fragments was performed in 1% agarose gels, and the DNA was blotted onto nitrocellulose by vacuum (18).

Circular and linear viral genomes were detected by lysis of 10^6 cells in situ in wells of a vertical 0.75% agarose gel with pronase-SDS, followed by electrophoresis, transfer to nitrocellulose by vacuum (18), and hybridization (11).

Hybridization of the filters was performed in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.02% polyvinylpyrrolidone–0.02% bovine serum albumin–0.02% Ficoll–15 mM sodium phosphate buffer (pH 6.5)–10% dextran sulfate–0.01% denatured and sonicated salmon sperm DNA–10 ng of denatured DNA probe labeled by nick translation (28) with [^{32}P]dATP to a specific activity of at least 10^8 cpm/μg.

To rehybridize the nitrocellulose filters, the radioactive probe was removed by gentle rocking in 70% formamide–0.1% SDS–10 mM sodium phosphate buffer (pH 7.0) at 80°C for 15 min, followed by a brief rinse in 0.1% SDS–10 mM phosphate buffer.

Cloning right-end sequences of L-DNA from strain 484-77 and description of clones of other strains. The genome structure of herpesvirus saimiri and various clones used in this study are shown schematically in Fig. 1. Restriction endonuclease sites were mapped by standard techniques and taken from published references (6, 10, 17). Plasmid vector pHyg (30; obtained from Bill Sugden) was linearized with *Bam*HI and subsequently treated with alkaline phosphatase. Virion DNA of strain 484-77 was partially digested with *Sau*3A, and the fragments were separated on a 15 to 40% sucrose gradient (SW41 rotor; 35,000 rpm for 8 h). DNA fragments of approximately 15 kbp were selected and ligated into the *Bam*HI site of pHyg. From the resulting library, one clone, designated pHyg484-15, was selected; pHyg484-15 hybridized with right-end probe pTp7.4 of the L-DNA of strain 11 (pTp7.4 contains a 7.4-kbp insert; 6); clone pHyg484-15 DNA and virion DNA were compared by cleavage with restriction endonucleases *Pst*I, *Sac*I, and *Taq*I and Southern hybridization (data not shown). The viral insert fragments of pHyg484-15, except for the two plasmid viral DNA junction fragments, comigrated with virion DNA. The results indicate that pHyg484-15 represents a continuous segment of the genome of strain 484-77 with no rearrangements and contains about 12 kbp of L-DNA plus at least 2 U of repetitive H-DNA.

A 2.5-kbp *Bam*HI-*Hin*dIII fragment was subcloned from pHyg484-15 into M13 SK plasmid DNA (Stratagene, Inc., La Jolla, Calif.). The subclone designated p484BH2.7 contains L-DNA sequences from map units 0.7 to 3.2.

A 2.2-kbp *Pst*I-*Sac*I fragment was also subcloned from pHyg484-15 into an M13 SK plasmid designated p484PS2.2; this clone represents L-DNA of strain 484-77 from map units 4.0 to 6.2.

A clone specific for group B viruses (pSBH1.2) contains 1.2 kbp of right-end L-DNA sequences of strain SMHI (Fig. 1); the cloning has been described earlier (19).

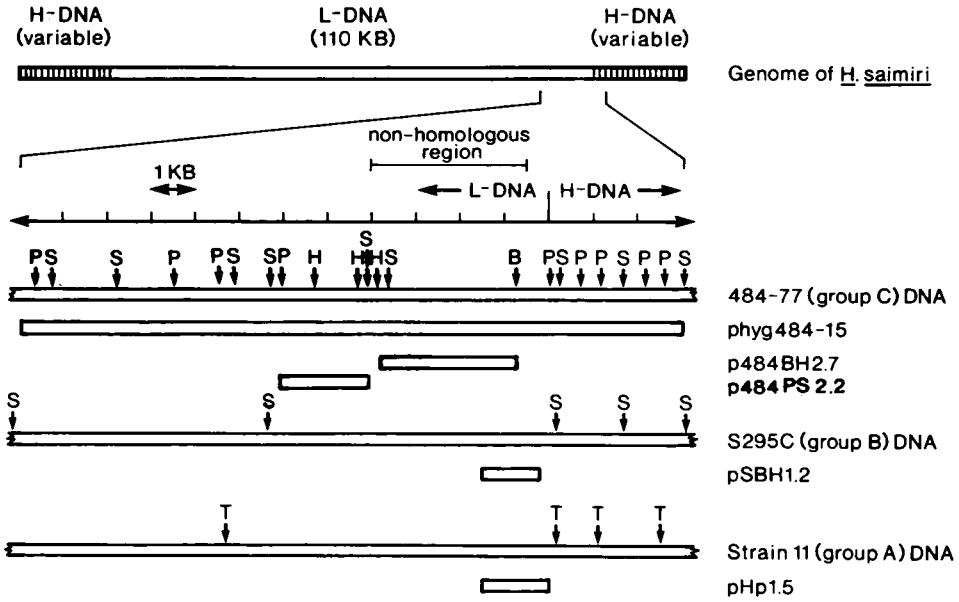

FIG. 1. Physical maps of DNAs of various herpesvirus saimiri strains and cloned fragments. Abbreviations: KB, kilobase; P, *Pst*I; S, *Sac*I; H, *Hin*dIII; B, *Bam*HI; T, *Taq*I.

A clone specific for group A viruses (pHp1.5) representing 1.5 kbp of right-end sequences of L-DNA of strain 11 was a gift from Ronald C. Desrosiers (6).

Construction of recombinant viruses. Approximately 2 μg of virion DNA and 20 μg of pHyg484-15 DNA were cotransfected into OMK cells grown in 100-mm-diameter plastic petri dishes by the DEAE-dextran technique (24). After about 2 weeks, a cytopathic effect which resembled that of herpesvirus saimiri-infected cultures was observed. To select for recombinants with hygromycin resistance, the virus obtained from the transfection was passaged three times in OMK cells in medium containing 0.3 mg of hygromycin per ml. After three passages, the virus titer was determined (about 10^4/ml), and OMK cells grown in 96-well microtiter plates were infected with about 1 PFU of a virus dilution per well. Hygromycin was omitted from the medium in this and subsequent propagations of the viruses. After about 10 days, a typical cytopathic effect was observed in 58 wells and the plates were chilled to 4°C and kept at 4°C for 1 day. The contents of the wells were pipetted up and down 10 times, and 2-μl portions were spotted onto nitrocellulose sheets. After 5 min of drying at room temperature, the sheets were sequentially laid onto Whatman 3MM filter papers saturated with 0.5 M NaOH–1.5 M NaCl for 5 min and filters saturated with 0.5 M Tris hydrochloride (pH 7.0)–1.5 M NaCl twice for 5 min each time and baked for 1 hour at 80°C. The filters were hybridized with ^{32}P-labeled cloned H-DNA (pH24; 1). This hybridization confirmed that the 58 positive wells contained herpesvirus saimiri sequences. After exposure, the repetitive probe was removed by 70% formamide and the filter was rehybridized with a strain 484-specific p484BH2.7 probe; this probe hybridized with 36 wells. Of the 36 positive viruses, 34 were subcultured in 24-well plates, and viral DNA was prepared from one-half of each culture. DNAs of the parental strains and the candidate recombinants were digested with *Sma*I and *Sac*I and analyzed by Southern blotting (data not shown). Five recombinants contained vector sequences, three were mixtures of two viruses, and three lost all pHyg484-15 sequences. Eighteen viruses contained both 484-77 and S295C right-end sequences. Five viruses, however, acquired the right-end region of strain 484-77 with concomitant loss of the colinear region of strain S295C. Two of these viruses were further subcultured by two cycles of endpoint dilution in hygromycin-free medium and analyzed by Southern blotting (see Fig. 4, legend).

RESULTS

Analysis of viral DNAs of strains involved in this study. It was important to identify the strains unambiguously for three reasons. To compare the oncogenic potentials of various strains of herpesvirus saimiri, it was necessary to identify them by restriction enzyme analysis and hybridization with group-specific DNA probes; the various probes are schematically depicted in Fig. 1. We were also concerned about spontaneous deletion mutants, since deletion of the right-end H-DNA–L-DNA junction sequences can result in loss of oncogenicity (4, 8, 15, 29). Also, previous work demonstrated that right-end DNA fragments of strains 11 and SMHI (groups A and B) have no detectable homology with strain 484-77; thus, either these sequences are deleted from 484-77 or the right end of 484-77 L-DNA is unique (19); the third goal of this experiment was to solve this problem.

Virion DNA was prepared from virus stocks and cleaved with restriction enzymes useful in identifying deletions (9, 15). Strain 11 and 11-att (group A) DNAs were digested with *Taq*I, strain S295C and SMHI (group B) DNAs were digested with *Sac*II, strain 484-77 (group C) DNA was digested with *Pst*I, and the DNA samples were analyzed by Southern blotting. The blot was sequentially hybridized with group-specific cloned DNA fragments from the right ends of the L-DNAs of strains 11 (pHp1.5; contains a 1.5-kbp insert), SMHI (pSBH1.2; contains a 1.2-kbp insert), and 484-77 (p484BH2.7; contains a 2.5-kbp insert) and finally with virion DNA of strain 484-77. The group-specific probes hybridized only with viral DNA bands from the corresponding virus (Fig. 2A to C), and the sizes of these bands (7.4 kbp for strain 11, 15 kbp for strains S295C and SMHI, and 6.2 kbp for strain 484-77) were as expected, with the exception of probe pHp1.5; this plasmid contains a small amount of H-DNA;

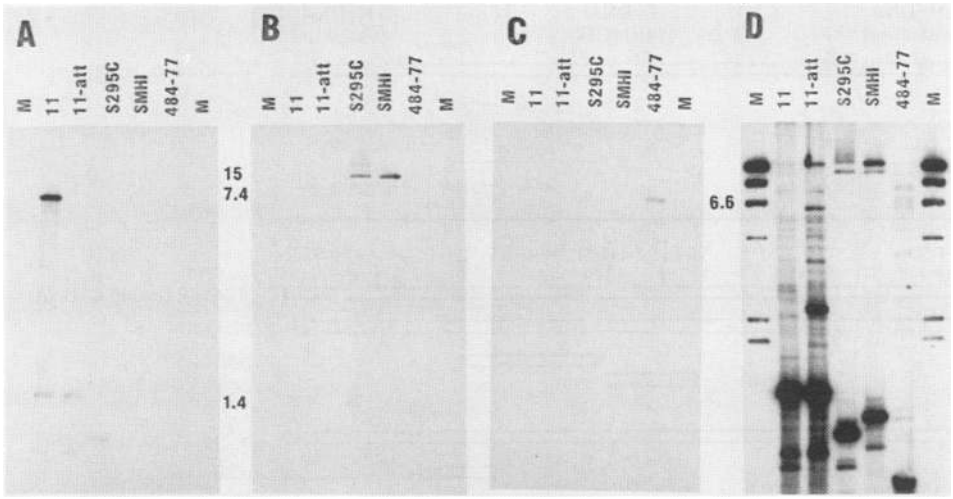

FIG. 2. Analysis of viral DNAs of strains for possible deletions and homology of right-end L-DNA region. Virion DNAs of strain 11 and 11-att DNAs were digested with *Taq*I, those of strain S295C and SMHI DNAs were digested with *Sac*II, that of strain 484-77 DNA was digested with *Pst*I, and lambda DNA (lanes M) was digested with *Hin*dIII. The blot was sequentially hybridized with cloned right-end DNA probe pHp1.5 of strain 11 (A), pSBH1.2 of strain SMHI (B), p484BH2.7 of strain 484-77 (C), and virion DNA of strain 484-77 mixed with lambda marker DNA (D). To rehybridize the same filter, the probe was removed by gentle rocking in 70% formamide at 80°C for 15 min. The numbers between panels represent sizes (in kilobase pairs) of viral DNA fragments; 7.4 and 1.4 kbp refer to lane 11 (A); 15 kbp refers to lanes SMHI and S295C (B); 6.6 kbp refers to lane 484-77 (C).

therefore, bands of 1.4 and 1 kbp also hybridized in lanes of strains 11, 11-att, and S295C (Fig. 2A). Although 11-att virus was derived from strain 11, no hybridization was observed, as expected (except with H-DNA [see above]), since 11-att DNA has a 2.0-kbp deletion of the right-end sequences. Figure 2D shows the patterns obtained after hybridization with virion DNA of 484-77; H-DNA fragments hybridized with much higher intensity than unique L-DNA fragments, as expected.

In conclusion, these viruses did not suffer sizeable deletions of the right-end L-DNA region. This analysis of viral DNAs of various strains also demonstrates that strain 484-77 contains a unique group-specific right-end fragment of at least 2.5 kbp with no detectable homology with group A and B strains.

High oncogenicity of strain 484-77 in New Zealand White rabbits. We inoculated New Zealand White rabbits with various doses of various strains of herpesvirus saimiri. Serum was taken before infection and thereafter to test seroconversion, and the animals were monitored for tumor development for up to 6 months. The fate of infected rabbits was very much dependent on the virus strain and virus dose (Table 1). No apparent disease was observed when rabbits received only 10^5 PFU of virus. When the rabbits were inoculated with 10^6 or 10^7 PFU of virus, seven of eight group C strain (484-77)-infected animals developed lymphoproliferative lesions. In contrast, neither group A nor group B virus caused any apparent symptoms or severe histopathological changes after inoculation with high doses of virus (Table 1). The lack of apparent disease in S295C-infected animals was surprising, since other investigators have reported that strain S295C can induce malignant lymphomas in rabbits in about 25% of infected animals (3, 27).

Clinical disease in 484-77-infected rabbits developed about 2 weeks after infection, with nasal and ocular discharges, dyspnea, diarrhea, and loss of appetite. All rabbits had clumps of lymphoblastoid cells in the peripheral blood and infiltrates of atypical lymphocytes in various organs. In some terminally ill rabbits, severe necrotizing ischemia was apparent in the skin, especially of the ears, toes, and nose, as a result of infarction. Internal organs were massively infiltrated with multiple intravascular and parenchymatous foci of atypical lymphocytes. Four animals died, and three were sacrified in extremis between 19 and 25 days after infection.

To test whether apparently healthy animals seroconverted and to confirm that viruses were successfully inoculated, preimmune and immune sera (1 to 3 months after infection) were analyzed for antibodies to virion proteins by Western blotting. Figure 3 shows examples of Western blot assays; a somewhat fuzzy band above the 116-kDa marker (estimated at about 125 kDa) was consistently present in blots incubated with sera of infected animals; this band was absent or much weaker in blots incubated with preimmune sera. Seroconversion was apparent in all of the animals tested, regardless of the virus strain used for inoculation. We did not identify the 125-kDa virion protein of strain 484-77 in terms of its relation to known strain 11 proteins. This antibody response can be due to limited replication of the virus or merely to an antibody response to the 125-kDa protein present in the virions of the inoculum.

Construction of S295C–484-77 recombinants. Although the rabbit experiments showed that group B viruses are not

TABLE 1. Comparison of New Zealand White rabbits inoculated with strains of herpesvirus saimiri

Virus strain	DNA group	No. of malignancies/no. of deaths caused by:		
		10^5 PFU	10^6 PFU	10^7 PFU
11	A	0/2	0/2	0/2
11-att[a]	A	0/2	0/2	0/2
SMHI	B	0/3	0/2	ND[b]
S295C	B	ND	0/3	0/5
484/77	C	0/2	2/3	5/5
Rec1	B-C	ND	ND	2/2
Rec2	B-C	ND	ND	2/2

[a] Strain 11-att is a nononcogenic mutant.
[b] ND, Not done.

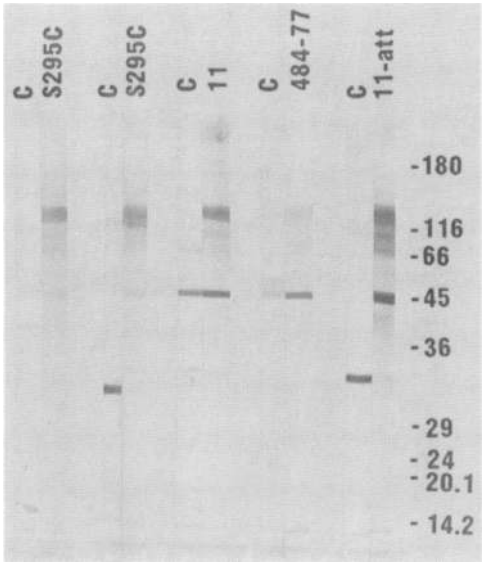

FIG. 3. Western blot analysis of sera of infected rabbits. Polypeptides of purified herpesvirus saimiri virions (9) were separated by SDS-polyacrylamide gel electrophoresis, and proteins were electroblotted onto nitrocellulose sheets. Strips of nitrocellulose were incubated with preimmune serum (lanes C) or serum obtained 3 months after infection (the lanes are marked with the names of various strains of herpesvirus saimiri), except for 484-77; the animal infected with this strain was sacrificed in a moribund state 22 days after infection. The nitrocellulose strips were then incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G and color substrates of alkaline phosphatase. The sizes (in kilodaltons) of the markers are indicated on the right (a Dalton Mark VII kit consisting of seven proteins of 14.2 to 66 kDa; β-galactosidase [116 kDa], and α-macroglobulin [180 kDa] was obtained from Sigma).

oncogenic in rabbits, whereas a group C virus, 484-77, is highly oncogenic in rabbits, it remained unknown which region of the genome of strain 484-77 is responsible for the oncogenic phenotype. Since the right-end sequences are significantly diverged among the different virus groups, whereas the rest of the genome is homologous (19), it seemed most likely that the right-end region is involved.

Recombinants were obtained by cotransfection of cloned 484-77 right-end DNA and purified virion DNA of strain S295C into OMK cells (for details, see Materials and Methods). The resulting virus was passaged three times in 0.3 mg of hygromycin per ml. This concentration of the drug inhibits replication of herpesvirus saimiri in OMK cells (M. Medveczky, unpublished data). Hygromycin was omitted from the medium for subsequent propagation and cloning of viruses. Preliminary analysis of cloned viruses showed that five viruses (designated Rec1 to Rec5) lost all vector and right-end S295C sequences and acquired the right-end region of strain 484-77. The 29 other viruses either contained vector or S295C right-end sequences or were mixtures of two viruses.

Two of the five viruses (Rec1 and Rec2) were selected for further characterization; to avoid contaminants, Rec1 and Rec2 viruses were cloned again by two cycles of endpoint dilutions in OMK cells. To determine the sequence composition and stability of the genomes of the recombinants, viral DNA was analyzed by Southern blotting.

Figure 4A shows sequential hybridizations of *Sac*I-digested DNA samples of the two recombinant viruses and the two parental viruses. The first probe (p484BH2.7) was a clone from the right end of the L-DNA of strain 484-77 (0.7 to 3.2 map unit L-DNA region; 0 is the right junction between L-DNA and H-DNA, and a map unit is 1 kbp). This 484-77-specific probe hybridized with one fragment, *Sac*I-*a*, of about 3.7 kbp in lanes containing 484-77 and those containing the two recombinants; no hybridization was with S295C DNA observed. Therefore, *Sac*I fragment *a* of 484-77 was acquired by the recombinants.

The second 484-77 probe (p484PS2.2; 4.0 to 6.2 map units) hybridized strongly with three comigrating bands of 484-77 and the recombinants (a 2.2-kbp fragment labeled as *Sac*I-*c* of 484-77 [Fig. 4A]) and with a much larger band of about 7.0 kbp of S295C (*Sac*I-*a*). Therefore, *Sac*I fragment *c* of 484-77 was present in the recombinants, whereas the 7.0-kbp S295C *Sac*I fragment *a* was lost during recombination (the 3.7-kbp bands from the previous hybridization were also visible because of incomplete stripping).

The third probe was a gel-purified *Sma*I fragment of pHyg484-15; this probe contained about 12 kbp of L-DNA of strain 484-77, less than 1 U of H-DNA and plasmid DNA. This probe hybridized with four fragments of the recombinants (Fig. 4A), which all comigrated with 484-77 fragments: *Sac*I-*a*, *Sac*I-*c*, *Sac*I-*f*, and *Sac*I-*g* of 484-77 (*Sac*I-*g* of 484-77 is a 0.3-kbp fragment and ran out of this gel but was detected in other experiments). Therefore, the genome of the recombinants contains at least these four *Sac*I fragments (*a*, *c*, *f*, and *g*) of 484-77. Three bands (*Sac*I fragments *b*, *d*, and *e*; 2.5, 1.8, and 1.1 kbp, respectively) were present only in 484-77 DNA. Since these three bands were absent in the recombinants, one of these 484-77 fragments was probably involved in the recombination event, as confirmed by further analysis. Two S295C fragments (*Sac*I-*a* and *Sac*I-*b*) were present only in S295C DNA and were absent in the recombinants; however, a unique *Sac*I fragment of about 5.5 kbp was detected in the DNAs of both recombinants (the largest bands in the REC lanes). The creation of these unique 5.5-kbp fragments of the recombinants is consistent with a recombination event between *Sac*I-*b* of S295C DNA and *Sac*I-*b* of pHyg484-15 DNA (Fig. 4A, map). A band of 1.4 kbp was present in all four lanes; this fragment is from the repetitive H-DNA (the probe contained a small amount of H-DNA).

A similar sequential hybridization strategy was used to further analyze the recombinants; the DNAs of the viruses and pHyg484-15 were digested with *Pst*I. The pHyg probe with no viral insert hybridized only with pHyg484-15, but no signal was obtained in lanes containing the recombinants and a recombinant reisolated from a tumorous rabbit (Fig. 4B). We estimate that our technique is sensitive enough to detect pHyg sequences if 1% or more of the viral DNA molecules would have integrated vector sequences. These results show that the recombinant viruses contain no vector sequences detectable by our method. This is an important point, since pHyg contains the promoter of the thymidine kinase gene of herpes simplex virus type 1 (30); this promoter could enhance the expression of a herpesvirus saimiri gene involved in transformation if pHyg would integrate adjacent to such a herpesvirus saimiri gene.

The second probe was a group B-specific probe representing 0.3 to 1.5 map units of SMHI DNA (S295C and SMHI belong to the same DNA subgroup; 19). This group B-specific probe hybridized only with *Pst*I fragment *a* of S295C DNA and not at all with the DNA of the recombinants, proving that the recombinant viruses lost the rightmost region of S295C L-DNA during recombination.

In contrast, the 484-77-specific p484BH2.7 probe (0.7 to 3.2 map units) hybridized with 6.2-kbp *Pst*I fragments *a* of

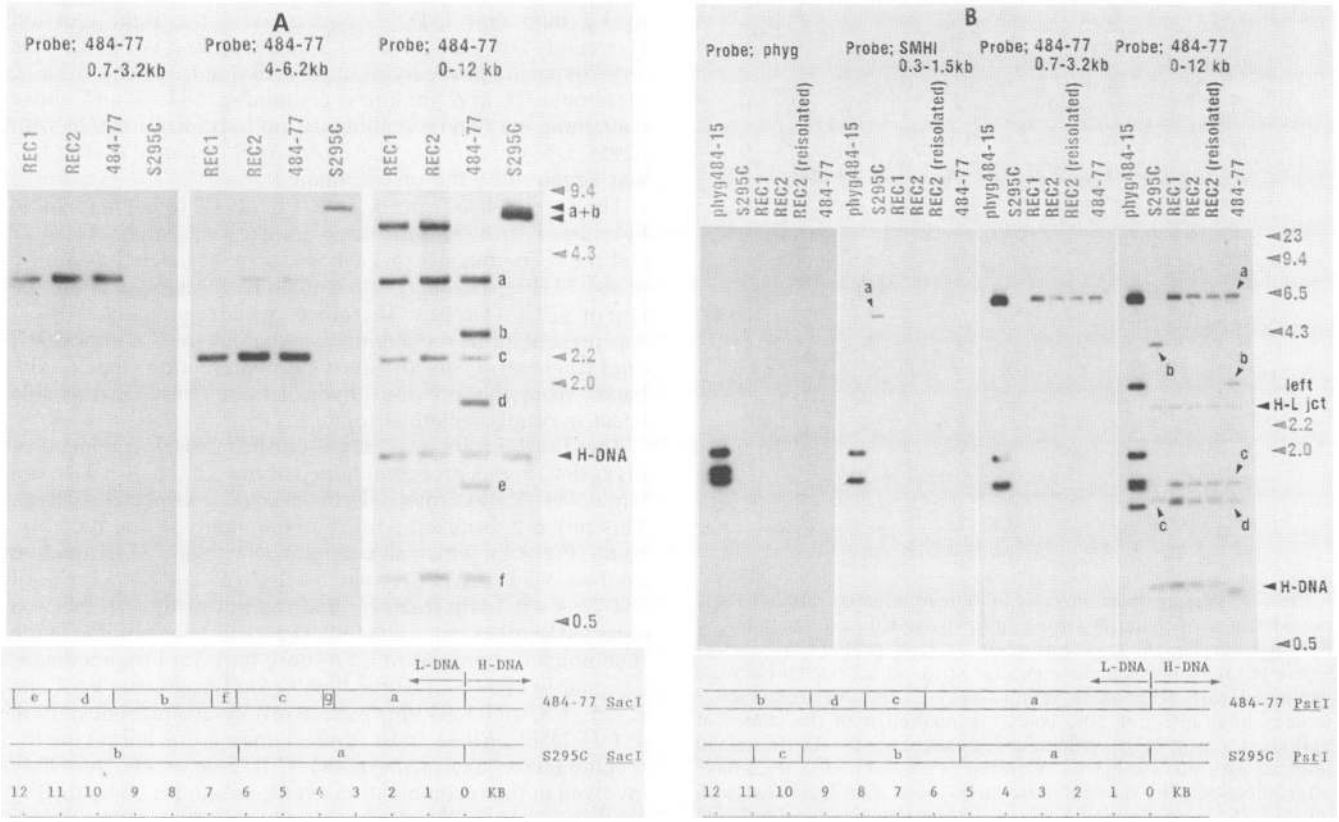

FIG. 4. Southern blot analysis of recombinant viruses. (A) Virion DNAs of strains Rec1, Rec2, 484-77, and S295C were digested with *Sac*I. (B) pHyg DNA and virions DNAs of strains Rec1 and Rec2, Rec2 vius recovered from a T-cell line established from a tumorous mesenterial lymph node, 484-77, and S295C were digested with *Pst*I. Hybridization was done with the probe indicated above each autoradiogram. kb, Map units in kilobases. To rehybridize the same filter, the probe was removed by gentle rocking in 70% formamide at 80°C for 15 min. The numbers at the right represent the sizes (in kilobase pairs) of marker DNA fragments (bacteriophage lambda DNA cleaved with *Hin*dIII). Restriction enzyme maps of the right-end 12-kbp L-DNAs of strains 484-77 and S295C are shown below the panels; DNA fragments are marked with letters on the physical maps as well as on the corresponding autoradiograms.

484-77, Rec1, and Rec2 DNAs but not at all with S295C DNA (Fig. 4B). Therefore, the recombinants acquired at least 6.2 kbp of 484-77 L-DNA.

When the same blot was hybridized with the 12-kbp 484-77 right-end probe (the *Sma*I fragment of pHyg484-15 was also used as a probe for Fig. 4A), three comigrating *Pst*I fragments, *a*, *c*, and *d* (6.2, 1.6, and 1.4 kbp, respectively), were present in lanes containing recombinants and strain 484-77, indicating that the recombinants contain at least 9.2 kbp of L-DNA from 484-77. *Pst*I-*c* of S295C (about 1.4-kbp fragment) was present in the recombinants and S295C, suggesting that recombination occurred further to the right of S295C *Pst*I-*c*. All of these data are consistent with a recombination event between *Pst*I-*b* of S295C and *Pst*I-*b* of 484-77. A band above the 2.2-kbp marker (about 2.7 kbp) was present in the genomes of all of the viruses; this band is probably the left-end H-L junction fragment which contains H-DNA sequences; thus, its hybridization signal is due to H-DNA sequences in the probe. An approximately 0.9-kbp fragment (the smallest bands of the gel, just above the 0.5-kbp marker) is repetitive DNA (again, its hybridization is due to H-DNA sequences in the probe).

In conclusion, the blot hybridization provided evidence that the rightmost 9.2 kbp of strain 484-77 DNA is present in both recombinants, with concomitant loss of the corresponding right-end region of strain S295C and loss of all vector sequences, and the genomes of the two recombinants are indistinguishable. It seems very likely that the recombination occurred between homologous colinear fragments of S295C and 484-77.

Two recombinant S295C viruses harboring right-end sequences of 484-77 also oncogenic in rabbits. Two rabbits were inoculated with 10^7 PFU of interstrain recombinant Rec1, and two rabbits were inoculated with Rec2. All four infected rabbits died or were sacrificed in extremis between 19 and 24 days after clinical signs appeared which were identical to those seen in 484-77-infected rabbits (Table 1). Viruses were recovered from two rabbits infected with Rec2. Southern hybridization of a viral DNA of a Rec2 virus reisolated from mesenteric lymph node lymphoid cells revealed no rearrangement of the viral DNA compared with the DNA of the input virus (Fig. 4B).

Establishment of tumor cell lines. Several lymphoid cell lines were established from thymus and spleen tissues of a strain 484-77-infected rabbit. Initially, single lymphocytes were seen mixed with large clumps of lymphoblastoid cells. After about 3 months, most single cells disappeared and the cultures started to grow very well without IL-2 as large clumps. The two cell lines involved in this study were in culture in IL-2-free medium for 6 months at the time of the analysis of the viral genome. Although the two cell lines were cultured initially in IL-2, it seems that IL-2 is not

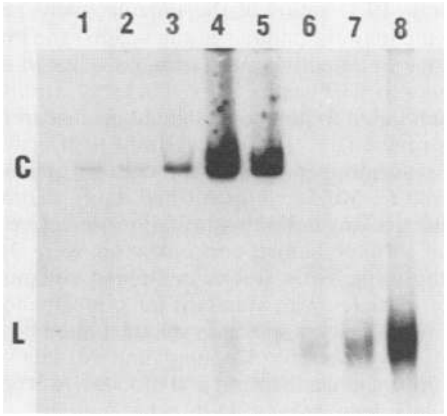

FIG. 5. Analysis of tissues of 484-77-infected rabbits and tumor-derived cell lines for circular viral genomes. Cell suspensions prepared from tissues and 10^6 cells were lysed with pronase-SDS in situ in wells of a vertical 0.75% agarose gel, followed by electrophoresis, transfer to nitrocellulose, and hybridization with cloned H-DNA (11). Lanes 1, lymph node; 2, spleen; 3, thymus (all from a 484-77-infected rabbit); 4, thymus-derived cell line 484T; 5, spleen-derived cell line 484S; 6 to 8, 2, 10, and 50 copies of 484-77 virion DNA per cell equivalent. C, Circular genome; L, linear genome.

necessary since several cell lines were established from 484-77-, Rec1-, and Rec2-infected animals recently without using IL-2. Despite numerous attempts, we were unable to establish cell lines from strain 11-, 11-att-, SMHI-, and S295C-infected rabbits with or without the aid of IL-2.

Circular viral genome in tumor cells and tumor-derived cell lines. Herpesvirus saimiri-transformed monkey lymphoid cell lines carry the viral genome in episomal circular form (13). To examine the state of the viral genome in vivo, we removed lymphoid tissues of sacrificed rabbits and analyzed the cells by the gel technique of Gardella et al. for separation of circular and linear herpesvirus DNAs (11). After electrophoresis, viral DNA was detected by transfer to nitrocellulose and hybridization with a repetitive H-DNA probe of herpesvirus saimiri strain 11 (1; the repetitive DNA was homologous among the strains and was present in all of the transformed cells tested). Figure 5 shows a typical assay; all lymphoid organs of a 484-77-infected animal (lanes 1 to 3) contained 1 to 5 copies of circular viral DNA per cell (copy number was estimated by comparing the intensities of hybridization of known amounts of virion DNA run in lanes 6 to 8).

The results of DNA gels are summarized in Table 2. Circular DNA was detected reproducibly in the lymphoid tissues of six 484-77-infected rabbits. Of these six rabbits, three were found dead and tissues were collected at 4 to 16 h (estimated) postmortem, and two moribund animals were sacrificed and tissues were collected immediately. One rabbit died more than 36 h before samples were taken, and no circular DNA was detected. The lack of circular viral DNA in this rabbit was probably due to partial autolysis of tissues, since viral DNA was still detectable, but the DNA was degraded and hybridized as a smear on a Southern blot (data not shown). As in 484-77-infected animals, circular viral DNA was detected in lymphoid tissues of Rec1- and Rec2-infected rabbits.

We also tested the physical state of viral DNA in two lymphoid cell lines established from a 484-77-infected rabbit. The cell lines were in tissue culture for 6 months at the time of DNA analysis. About 100 and 50 copies of circular genomes per cell were present in thymus and spleen cell lines, respectively (Fig. 5, lanes 4 and 5).

TABLE 2. Viral DNA in New Zealand White rabbits inoculated with strains of herpesvirus saimiri

Virus strain	DNA group	No. of rabbits with viral DNA/total[a] at:		
		14 Days	19–28 Days	3–6 Months
11	A	ND	ND	0/4
11-att	A	0/2	0/2	0/4
SMHI	B	ND	ND	0/4
S295C	B	0/2	2/2	0/6
484/77	C	ND	7/7	0/1
Rec1	B-C	ND	2/2	ND
Rec2	B-C	ND	2/2	ND

[a] Viral DNA was analyzed as described in the legend to Fig. 4, except in one case in which viral DNA in an animal infected with strain 484-77 was detected by Southern blotting. ND, Not done.

Circular viral genomes transiently present in lymphoid tissues of rabbits infected with group B strain S295C. To test whether viral DNA could be detected in lymphoid cells of animals infected with strains which did not kill rabbits, two strain S295C-infected rabbits and two strain 11-att-infected animals were sacrificed 28 days after infection. Circular viral genomes were present in all lymphoid organs tested from the two S295C-infected animals (Fig. 6). Thymus tissue from this animal contained only a very small amount of viral DNA; the corresponding circular DNA band was clearly

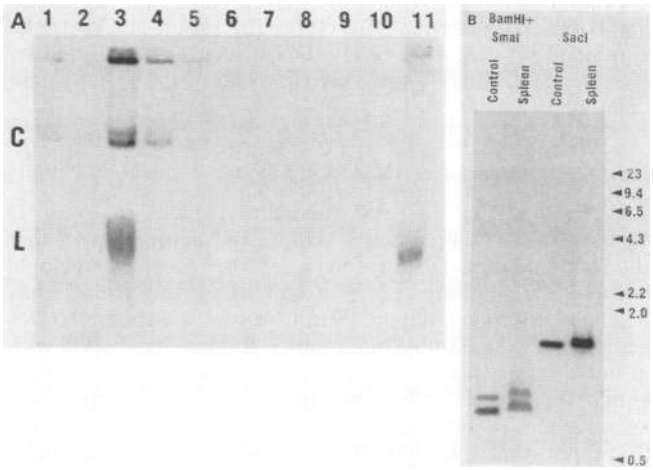

FIG. 6. Analysis of tissues of S295C- and 11-att-infected rabbits for viral genomes. (A) Cell suspensions prepared from tissues and 10^6 cells were lysed with pronase-SDS in situ in wells of a vertical 0.75% agarose gel, followed by electrophoresis, transfer to nitrocellulose, and hybridization with cloned H-DNA (11). Lanes: 1, lymph node; 2, thymus; 3, spleen (all from an S295C-infected rabbit); 4, thymus; 5, lymph node (both from a second S295C-infected rabbit); 6 to 10, tissue samples from two 11-att-infected rabbits; 11, two copies of 484-77 virion DNA per cell equivalent. C, Circular genome; L, linear genome. (B) Cellular DNA was isolated from spleen cells of the rabbit infected with strain S295C (circular DNA is shown in Fig. 5A, lane 3). A 10-μg sample of S295C-infected rabbit spleen DNA (spleen) and 0.5 ng of S295C virion DNA (control, about one copy per cell equivalent) was digested with either *Bam*HI-*Sma*I or *Sac*I and analyzed by Southern blotting by using a pHyg484-15 probe labeled by nick translation. The numbers at the right represent the sizes (in kilobase pairs) of marker DNA fragments (bacteriophage lambda DNA cleaved with *Hin*dIII).

visible on the original X-ray film but was very faint on the photograph (Fig. 6A, lane 2).

However, no viral DNA was detected in 11-att-infected animals 28 days after infection (Fig. 6A). Thus, strain 11-att, which has a 2.2-kbp deletion of the right end of L-DNA (15), is unable to infect or unable to transform lymphoid cells as do strains 484-77 and S295C.

Viral DNA was not detected in rabbits 14 days after infection (Table 2). Similarly, no viral DNA was detected (strains S295C, SMHI, 11-att, and 11) at 3 months after infection or later (Table 2).

The possibility still remained that circular genomes found in lymphoid cells of rabbits infected with strain S295C are generated by infection with defective particles. Defective virus particles contain defective genomes; the right end of L-DNA (10 to 90% of L-DNA) is absent in the DNA of defective particles obtained by serial passages at high multiplicities of infection (M. Koomey and C. Mulder, personal communication). To test the possibility of defective genomes, DNA was isolated from spleen cells of an S295C-infected rabbit and analyzed by Southern blotting (circular viral DNA of a sample of this spleen cell suspension is shown in Fig. 6A, lane 3). The blot was hybridized with a pHyg484-15 probe which contains about 12 kbp of L-DNA from the right end and H-DNA. Figure 6B shows no significant differences between the patterns of bands containing virion DNA and bands containing S295C-infected rabbit spleen DNA; a large *Bam*HI-*Sma*I fragment (expected size, about 35 kbp) and two *Sac*I fragments of about 6.8 and 7 kbp and supermolar bands in the 0.7- to 1-kbp range were detected. The faint large bands are fragments representing the right end of L-DNA and the supermolar bands representing H-DNA fragments. Therefore, the rabbit spleen tissue contained no defective S295C DNA such as that found by Koomey and Mulder, since the right end of L-DNA was not deleted.

DISCUSSION

We cloned the right-end region of L-DNA of group C strain 484-77 of herpesvirus saimiri and demonstrated that the right end of L-DNA (at least 2.5 kbp) has no detectable homology with group A or B strains; thus, strain 484-77 has a unique right-end region. Strain 484-77 was highly oncogenic in New Zealand White rabbits. Rabbits inoculated with group C strain 484-77 died of a lymphoproliferative disease similar to that described in earlier reports of herpesvirus saimiri-induced lymphomas in marmosets (21). This is the first report that a group C strain, 484-77, is oncogenic in any animal.

Unexpectedly, only strain 484-77 was oncogenic; strains from group A or B were not oncogenic. This is surprising, since group A strain 11 and group B strains S295C and SMHI have been previously shown to induce lymphomas in New World primates (4, 5, 21, 32). We excluded trivial possibilities as to why the group A and B viruses were not oncogenic. (i) The animals developed an antibody against a 125-kDa virion protein after infection, indicating that inoculation was successful. This antibody response may be due to limited replication of the virus, or it may be due simply to an antibody response to the protein present in the virions of the inoculum. (ii) No deletion was detected in the oncogenic DNA region of the strains.

It is not very likely that the differences in oncogenicity among different strains are due to noninfectious defective particles, although no direct assays, e.g., determination of the particle-to-PFU ratios of the inocula, were performed. The following indirect findings argue against the importance and existence of detective interfering particles in our rabbit experiments. (i) The same virus stocks of strains 11 and SMHI which failed to induce malignant disease in this study have been previously used successfully to transform common and cottontopped marmoset cells in vitro (31; D. DeGrand and C. Mulder, unpublished data), demonstrating that the inocula are not transformation defective. (ii) The titers of all viruses before concentration were 10^5 to 10^7 PFU/ml; thus, the virus stocks contained sufficiently high numbers of particles with standard infectious genomes. (iii) Furthermore, the rabbit spleen tissue contained no defective viral DNA, as found by Koomey and Mulder (personal communication), since the right end of L-DNA of S295C was detectable in spleen cells of an infected animal.

Two laboratories have reported that group B strain S295C can induce lymphomas in 15 to 50% of New Zealand White rabbits (3, 27). However, similar to our results, investigators from two other laboratories have observed lack of oncogenicity of S295C virus in New Zealand White rabbits (5; R. W. Honess, personal communication). There is no clear explanation for the contradictory results. Since the various laboratories have purchased the rabbits from different breeding sources, it is possible that not only the virus strain but the strain of rabbits is important in determining the oncogenicity of herpesvirus saimiri.

Differences in oncogenicity between 484-77 and S295C can be due to two possible alternative mechanisms; (i) both viruses can immortalize rabbit cells in vivo, but 484-77 immortalized cells are more oncogenic, or (ii) the two strains may differ in their abilities to immortalize rabbit cells. No matter which alternative is the correct hypothesis, we can safely conclude that the various strains representing three groups of herpesvirus saimiri are naturally occurring host range mutants in terms of oncogenicity in New Zealand White rabbits.

Drug selection was useful in the construction of desired B-C recombinants. Strain 484-77 sequences were present in about 60% of cloned viruses after three passages in hygromycin-containing medium, followed by one passage in hygromycin-free medium. The B-C recombinants with no vector sequences (about 15% of all recombinants) were very stable; the genomes of Rec1 and Rec2 viruses did not rearrange or lose 484-77 DNA after five passages in tissue culture. Furthermore, a recombinant was reisolated from tumors of rabbits with no apparent rearrangements of the viral genome.

Surprisingly, only 15% of recombinants contained pHyg vector DNA after two passages in hygromycin-free medium. Presumably, pHyg sequences were present during drug selection, but vector sequences were lost during passages in hygromycin-free medium. Figure 7 illustrates a model which is consistent with our data and explains the loss of vector sequences integrated into H-DNA. During homologous recombination between colinear segments of the L-DNAs of pHyg484-15 and S295C, the new virus would lose the right end of S295C DNA and acquire the right-end sequences of 484-77; plasmid sequences (hyg) are positioned at the right end of the genome. The result is a recombinant L-DNA with S295C H-DNA at the left terminus and two units of 484-77 H-DNA and pHyg at the right terminus. During DNA replication, the genome would circularize and concatemeric forms of the genome would be cleaved into head-full unit-length genomes. Since cleavage of the viral DNA can probably occur at any one of the about 30 H-DNA units,

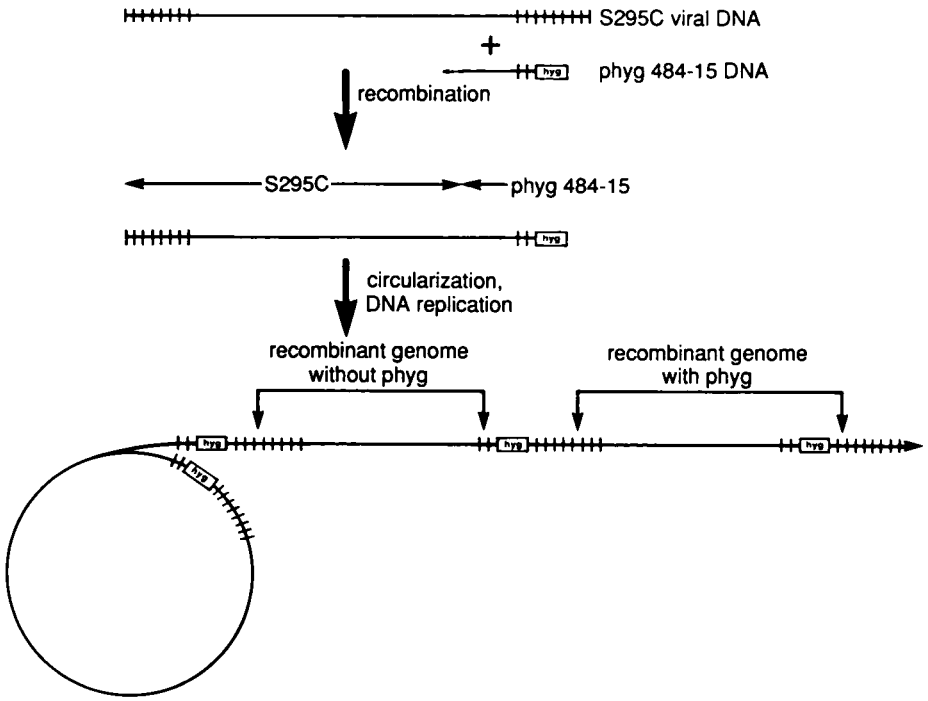

FIG. 7. A suggested model to explain the segregation of pHyg during virus replication.

some molecules would be packaged into virions with pHyg; other molecules would be packaged without pHyg. Therefore, vector sequences integrated in H-DNA are much less stable than 484-77 sequences integrated in L-DNA. An alternative model is also consistent with the data. Recombination between viral DNA and some of the plasmids via homologous sequences (thereby eliminating the hygromycin resistance gene) could have occurred. However, replication and passage of this recombinant virus should be inhibited by the drug. Passage of this virus is possible only if the cell is also infected with a helper virus which expresses the hygromycin gene. More work is required to understand the details and mechanisms involved.

Two B-C recombinants in which the leftmost 91% of the L-DNA is derived from S295C and the rightmost 9% is from 484-77 were oncogenic in rabbits. The significance of this result is that the gene(s) contributing to the oncogenicity of strain 484-77 probably maps within the rightmost 9.2 kbp of L-DNA. Potential candidates of the rightmost region include small RNAs and at least one mRNA. Four strain 484-77-specific small RNAs are detectable in the rabbit or primate tumor cell lines, and these small RNAs can be immunoprecipitated by an antibody to the 2,2,7-trimethyl cap found in U-like RNA small RNA polymerase II transcripts (S. Whitaker, P. Geck, and P. Medveczky, submitted for publication). Although the right-end L-DNA regions of strains 484-77 and 11 are significantly diverged, small RNAs have been described in marmoset cell lines transformed by strain 11 (P. Medveczky and C. Mulder, unpublished data; 22). The strain 11-encoded small RNAs are also products of RNA polymerase II with conserved enhancer, promoter, and termination signals characteristic of U RNAs of mammalian cells (16). It is unknown whether these U-like RNAs of strains 11 and 484-77 are functionally equivalent and whether they play any role in oncogenic transformation.

We found a potentially important transcript in 484-77-transformed cells; a 1.2-kb polyadenylated virus-specific DNA is also transcribed in rabbit tumor cells from the rightmost region of the viral genome (P. Geck, M. Medveczky, S. Whitaker, and P. Medveczky, manuscript in preparation). No such polyadenylated RNA is transcribed by strain 11; although OMK cells lytically infected by strain 11 contain two late viral mRNAs from the right-end region (12), these RNAs are not found in tumor cells (P. Medveczky and C. Mulder, unpublished data; 12). Further work is required to clarify whether any of these viral RNAs is responsible for the highly oncogenic phenotype of strain 484-77.

Virion DNAs of lymphotropic herpesvirus are linear. We failed to detect linear genomes of herpesvirus saimiri in lymphoid tissues of rabbits or tumor-derived cell lines. In addition, we were unable to recover infectious virus from most of the cell lines and rabbit tissues by cocultivation with OMK cells (data not shown); thus, rabbit lymphocytes do not support permissive infection of herpesvirus saimiri. We did not attempt to induce virus production. In contrast, established lymphoblastoid cell lines transformed by Epstein-Barr virus or herpesvirus saimiri contain multiple copies of latent genomes which are circular episomes (13). This is the first report that lymphoid cells of herpesvirus saimiri-infected animals carry mainly circular genomes. As expected, cell lines established from 484-77-infected rabbits also contain circular genomes. Therefore, it is likely that the circular genomes in tissues represent transformed lymphocytes. The numbers of copies per cell in the two established cell lines are at least 10 times higher than in tissues; 50 to 100 copies versus 1 to 5 copies. The increase of viral DNA copies can be due to amplification of viral DNA in cell culture. Alternatively, only about 10% of the cells of the tissue samples are infected, and each infected cell carries 50 to 100 copies of viral DNA; thus, no amplification takes place during culturing of these tumor cells.

We have shown in a previous study that the appearance of covalently closed circular DNA of herpesvirus saimiri is associated with immortalization; infection of common mar-

moset peripheral blood lymphocytes with herpesvirus saimiri was followed by increased cell growth and cell clumping, and the appearance of circular genomes was observed in those cultures, which later were found to be immortalized (31). In contrast, no immortalization and no circular DNA was observed in lymphoid cells infected with deletion mutant 11-att (31). We have demonstrated that strain S295C infection results in transient appearance of circular forms of DNA in lymphoid tissues, indicating that our inoculum of strain S295C can infect rabbit cells and establish genomes as efficiently as strain 484-77. No experiments were performed to detect viral DNA at 28 days postinfection in rabbits infected with strains 11, 11-att, and SMHI. Therefore, it is possible that these three strains cannot infect rabbits, thereby accounting for lack of oncogenicity.

The oncogenicity of herpesvirus saimiri has been studied mainly in New World monkeys. Infection of rabbits with the highly oncogenic group C strain 484-77 offers an alternative and much more accessible animal model for investigators who have no animal facilities to house primates and wish to study mechanisms of oncogenicity induced by this virus. Further work is required to locate precisely within the right-end region of the gene(s) that contributes to the oncogenicity of strain 484-77.

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