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Classification of Herpesvirus Saimiri Into Three Groups Based on Extreme Variation in a DNA Region Required for Oncogenicity

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The leftmost 7 kilobase pairs of unique sequence L-DNA of herpesvirus saimiri was found to be highly variable among different strains as determined by restriction endonuclease analysis and blot hybridization. This region in one group of viruses (group A) showed only very weak hybridization with the DNA of two other groups. Similarly, a fragment of group B hybridized to DNA of its own group much more strongly than to group A. No homology was detected within a 1.2-kilobase-pair region between strain 11 (group A virus) and strain SMHI (group B) even under reduced stringency, and the adjacent 5.5-kilobase-pair segment of the region showed only a very weak intergroup hybridization. DNA of a third group of viruses (non-A, non-B) did not hybridize significantly with cloned fragments representing the leftmost 7-kilobase-pair region of either group A or group B. Since sequences in the highly variable region are required for the oncogenicity of the virus, these results raise interesting questions regarding the origin and function of this region of the genome.

Herpesvirus saimiri can be readily isolated from apparently healthy squirrel monkeys (*Saimiri sciureus*); the virus causes acute lymphomas and leukemias in other New World primates and also in at least two strains of rabbits (6, 26, 27, 29). The viral genome (M-DNA) is composed of unique sequence L-DNA (110 kilobase pairs [kbp]) flanked by multiple tandem repeats (H-DNA) (2, 15). The length of the genome is about 160 kbp as determined by electron microscopy (17). Comparison of virion DNA from different strains of herpesvirus saimiri by hybridization in solution has not revealed significant differences in nucleotide sequence (B. Fleckenstein, M. D. Daniel, C. Mulder, N. Berthelot, and P. Sheldrick, Abstr. Intl. Herpesvirus Workshop, Cambridge, United Kingdom, 1978, p. 48). However, with restriction endonucleases, DNA polymorphisms can be demonstrated easily (11, 16). This variability of restriction endonuclease sites within the genome has been presumed to represent random strain differences.

Nononcogenic variants of strains 11 and SMHI (11-att and SMHI-att, respectively) have been isolated (7, 28, 33). Detailed analysis of DNA from 11-att has revealed deletion of the leftmost 1.6 kbp of L-DNA (23), and a larger deletion has been found in the same region of SMHI-att (7). In vitro deletion of sequences in this area leads to loss of oncogenic potential of the virus (9; R. C. Desrosiers, A. Bakker, J. Kamine, R. D. Hunt, and N. W. King, submitted for publication). These results suggest strongly that left-terminal sequences of L-DNA are directly involved in oncogenesis, although they are not required for lytic growth or for establishment of latency in vivo (12, 14).

In this study, we show that the DNA region required for oncogenesis is hypervariable among different strains. Twenty strains of herpesvirus saimiri can be divided into three groups (A, B, and non-A, non-B) on the basis of DNA homology of this 7-kbp fragment. The leftmost 1.2-kbp segment does not show any detectable intergroup cross-hybridization, and the adjacent 5.5-kbp segment shows very weak hybridization between viruses of groups A and B.

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MATERIALS AND METHODS

Cells, viruses, and virion DNA. Owl monkey kidney (OMK) cells were grown in minimal essential medium supplemented with 10% fetal calf serum and antibiotics. Strains S295C and SMHI were obtained from M. D. Daniel, strain 11 was from B. Fleckenstein, and strain 11-att was from L. Falk. Other strains were isolated from peripheral blood of healthy squirrel monkeys of the New England Regional Primate Research Center colony (11). Viruses were propagated on OMK cells and purified as described previously (17). The viral DNA was extracted and purified from virions as published previously (24).

Molecular cloning. The construction of recombinant clones of strain 11 with pBR322 as the cloning vector has been described elsewhere (9). In this study three subclones of the leftmost 7 kbp of L-DNA were used. The map positions (Fig. 1) in the L-DNA of these viral DNA fragments cloned in pBR322 are as follows: pHp1.4, 0.1 to 1.5 kbp; pHp3.1, 1.5 to 4.6 kbp; pHp2.5, 4.6 to 7.1 kbp. (Map position 0 is the lefthand junction between H- and L-DNA.)

The leftmost 5.1-kbp fragment of L-DNA of strain SMHI (Fig. 1) was obtained by cleavage of the viral DNA with *Xma*I followed by a partial digestion with *Hin*dIII. The viral DNA was ligated into the *Xma*I-*Hin*dIII-cleaved and phosphatase-treated plasmid vector pNG2004 (provided by Nigel Grinther). Since *Xma*I (*Sma*I) cleaves only in herpesvirus saimiri H-DNA, and *Hin*dIII cleaves only in L-DNA, only H-DNA-to-L-DNA junction fragments can be efficiently ligated into the *Xma*I-*Hin*dIII-cleaved plasmid. Recombinant clones were selected by colony hybridization with the purified *Aos*I C fragment as a probe. A clone designated pSXH5.1 (Fig. 1) was selected and found (by mapping and Southern hybridization) to contain a 5.1-kbp insert of the leftmost segment from L-DNA plus a 0.2-kbp fragment of H-DNA; pSXH5.1 contained four *Hin*dIII fragments of L-DNA. The H-DNA-to-L-DNA junction *Xma*I-*Hin*dIII fragment was obtained by digestion of pSXH5.1 with *Hin*dIII followed by ligation. The resulting plasmid pSXH1.5 thus contained pNG2004 with a 1.5-kbp *Xma*I-*Hin*dIII insert.

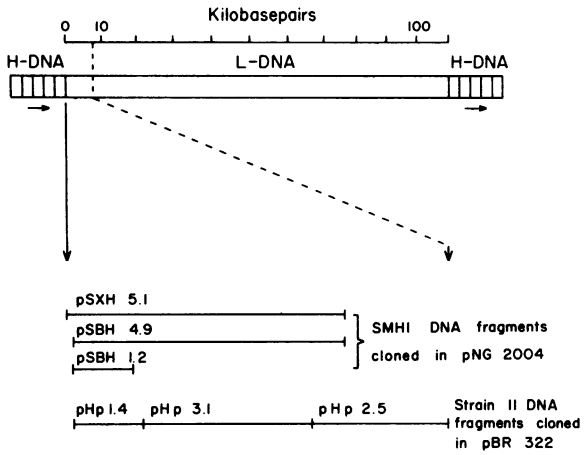

FIG. 1. Structure of herpesvirus saimiri DNA and the origin of recombinant plasmids used in this study.

The leftmost 0.2 to 5.1-kbp sequences of SMHI L-DNA were subcloned from pSXH5.1 by *Sma*I cleavage followed by partial BAL 31 digestion and ligation. One clone (pSBH4.9, Fig. 1) selected on the basis of its hybridization with L-DNA and non-reactivity with H-DNA was found to contain herpesvirus saimiri DNA sequences between 0.2 and 5.1 kbp.

The leftmost 0.3- to 1.5-kbp sequences of SMHI L-DNA were obtained by digestion of pSXH1.5 with *Sma*I followed by partial BAL 31 digestion. Clones were selected by colony hybridization to viral L- and H-DNA. One clone, pSBH1.2, was selected on the basis of its hybridization to L-DNA and lack of hybridization to H-DNA; pSBH1.2 was found to contain DNA sequences between 0.3 and 1.5 kbp on the herpesvirus saimiri SMHI L-DNA physical map (Fig. 1).

For large-scale preparation of cloned plasmid DNA, 1-liter cultures of *Escherichia coli* HB101 containing the appropriate plasmid were grown in LB medium, and plasmid DNA was prepared (9). The DNA was further purified in cesium chloride-ethidium bromide gradients. After the ethidium bromide was removed by isoamyl alcohol, the DNA was precipitated with ethanol, suspended in 10 mM Tris-hydrochloride (pH 7.6)–1 mM EDTA, and stored at 4°C.

Restriction endonucleases, gel electrophoresis, and blot hybridization. Restriction endonucleases were obtained from Promega-Biotec (Madison, Wis.), except *Aos*I, which was a generous gift from A. de Waard. Cleavage conditions were according to recommendations of the manufacturer. DNA fragments were subjected to electrophoresis through vertical agarose slab gels, stained in 1 μg of ethidium bromide per ml, and photographed over UV light. Lambda phage DNA digested with *Hin*dIII was used as a molecular size marker. DNA fragments in agarose gels were transferred to diazobenzyloxymethyl (DBM) papers (1) or nitrocellulose filters (35). Labeled DNA probes were obtained by nick repair (31) with ^{32}P-labeled dATP or dCTP (New England Nuclear Corp. or Amersham Corp.). Specific activities greater than 10^7 cpm/μg of DNA were usually obtained. Filters were hybridized in the presence of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate), 20 mM sodium phosphate buffer (pH 6.5), 10% dextran sulfate, 200 μg of calf thymus DNA per ml, Denhardt solution (0.02% bovine serum albumin, 0.02% Ficoll 500, and 0.02% polyvinylpyrrolidone), and 10^5 cpm of probe per ml at 42°C for 14 to 24 h. For low-stringency experiments, the formamide concentration was lowered to 30%, and the temperature was lowered to 20°C. After hybridization, filters were washed in several changes of 0.1% sodium dodecyl sulfate–0.1× SSC solution at 50°C; for lower-stringency washes, filters were washed under conditions identical to those used for their respective hybridization. For autoradiography, filters were exposed to Kodak X-Omat films and Du Pont intensifying screens. For sequential hybridizations of DBM papers, after hybridization and autoradiography, the first probe was removed by 0.5 N NaOH (4 h under gentle rocking at room temperature) followed by neutralization in four changes of 50 mM sodium phosphate buffer (pH 6.5). For sequential hybridizations, the original hybrids were removed from nitrocellulose filters by soaking in 70% formamide containing 10 mM sodium phosphate buffer (pH 6.5) and 0.1% sodium dodecyl sulfate at 70°C for 15 min, followed by a 1-min rinse in 0.1% sodium dodecyl sulfate–0.1× SSC. Filters were then prehybridized and hybridized as described above.

Dot-blot hybridization. The dot-blot hybridization method of Brandsma and Miller (4) was modified as follows: M-DNA of different strains in 5 μl of 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.6) was spotted on nitrocellulose sheets. The filters were placed onto Whatman no. 3 filter papers saturated with 0.5 N NaOH–1.5 N NaCl for 5 min, twice on 1.5 M NaCl–0.5 M Tris (pH 7.0) for 1 minute each, and once on 3× SSC for 2 min. After baking at 80°C for 1 h, the filter was incubated under pre-hybridization conditions, and the bound DNA was hybridized as described above.

RESULTS

Localization of a variable DNA fragment in strains 11 and SMHI. Physical maps of strains 11 and SMHI are available for several restriction endonucleases including *Sma*I and *Aos*I. *Sma*I cleaves each H-DNA unit several times, but does not cleave within L-DNA (2, 16). The available maps revealed some DNA polymorphism between strains in restriction endonuclease sites, and the strains were further compared by blot hybridization (Fig. 2). Virion M-DNA was cleaved with *Sma*I (to digest the repetitive H-DNA) followed by *Aos*I. DNA fragments were subjected to electrophoresis, transferred to DBM paper, and hybridized to probes as indicated in Fig. 2. *Aos*I-C of SMHI hybridized to M-DNA of strain 11 considerably less than did the other *Aos*I fragments of SMHI (Fig. 2A). *Aos*I-C (15 kbp) is located at the left end of SMHI L-DNA (Fig. 3). After autoradiography, the probe was removed by NaOH, and the filter was rehybridized with ^{32}P-labeled SMHI M-DNA. As expected, all SMHI bands hybridized in proportion to their sizes (Fig. 2B). However, SMHI M-DNA hybridized only very weakly to *Aos*I fragment F of strain 11 compared with, e.g., the slightly smaller *Aos*I-G of strain 11. *Aos*I-F is the leftmost 7-kbp fragment of strain 11 L-DNA (Fig. 3). Some other bands, e.g., *Aos*I-A, appeared to hybridize somewhat more strongly with homologous probe than with the heterologous one (Fig. 2). This could indicate that, in addition to the left-terminal 7 kbp of L-DNA, some slight sequence divergency occurs in some other regions of the genome too, although to a much lower degree than at the left end. We have compared these strains by cross-hybridization using restriction endonucleases *Eco*RI, *Sac*I, *Kpn*I, and *Bam*HI, but no substantial sequence divergence was found in the genome, except at the lefthand end of the L-DNA (data not shown).

Hybridization of DNA from new isolates to left-end fragments of strains 11 and SMHI. Since strains 11 and SMHI had been in culture for a considerable time, and could thus

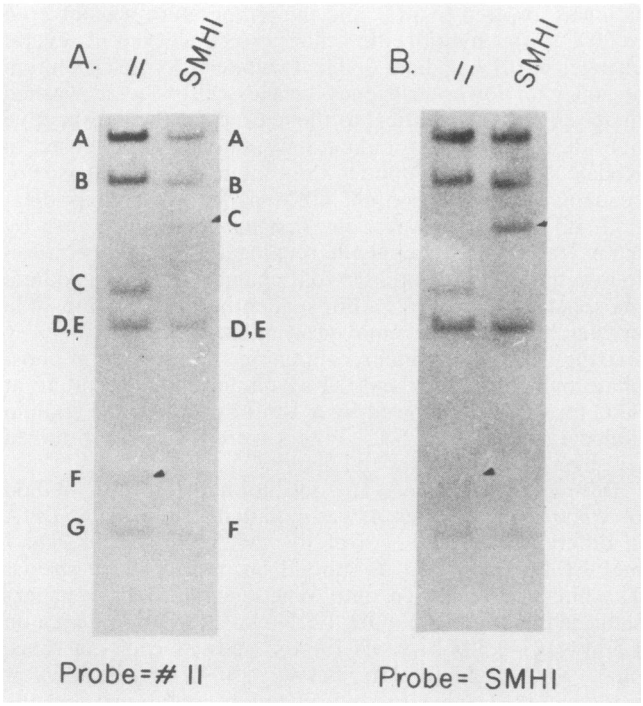

FIG 2. Hybridization of *Aos*I DNA fragments of strains 11 and SMHI with homologous and heterologous M-DNA probes. M-DNA (50 ng) of strains 11 and SMHI was successively cleaved with *Sma*I and *Aos*I and subjected to electrophoresis through a 0.7% agarose gel. After electrophoresis, the gel was stained with ethidium bromide, photographed over UV light, and transferred to DBM paper. (A) The filter was hybridized with ^{32}P-labeled strain 11 M-DNA and subjected to autoradiography. (B) The probe was removed with 0.5 N NaOH, and the filter was hybridized with ^{32}P-labeled M-DNA of strain SMHI.

have undergone rearrangements in vitro, we examined a number of recent isolates of herpesvirus saimiri to investigate whether the variations in nucleotide sequence observed between strains 11 and SMHI did also occur in strains freshly isolated from animals.

DNA from 11 strains of herpesvirus saimiri was sequentially cleaved with *Sma*I and *Aos*I (*Aos*I cleaved all strains within the left 20% of their L-DNA and not in H-DNA). A considerable size heterogeneity was observed among *Aos*I fragments of different strains (Fig. 3A). The DNA fragments were transferred to DBM paper and hybridized with *Aos*I fragment F of strain 11. As shown in Fig. 3B, the fragment hybridized strongly to bands of only six strains, which we will call group A viruses. After long exposures, the five other strains also showed a very weak hybridization (data not shown). The probe was eluted from the filter with NaOH, and the same filter was then rehybridized with SMHI *Aos*I-C fragment representing the leftmost 15 kbp of SMHI L-DNA.

No hybridization was observed between SMHI *Aos*I-C and those fragments that had shown strong hybridization to *Aos*I-F of strain 11 (Fig. 3B and C, lanes 3, 4, 5, 6, 7, and 10), although after prolonged exposure, a very weak hybridization was observed (data not shown). On the other hand, SMHI *Aos*I-C hybridized strongly to a fragment from all other viruses (Fig. 3C, lanes 1, 2, 8, 9, and 11) and also to a fragment of all group A viruses which comigrated with *Aos*I-C of strain 11. This fragment, 11 *Aos*I-C, mapped between 7 and 15 kbp (Fig. 2). These results indicated that diverged sequences were clustered within the first 7 kbp of L-DNA; all strains appear to have homologous sequences between 7 and 15 kbp.

Unfortunately, the transfer of DNA of this gel was not very efficient; a gradient of transfer efficiency was observed from left to right, especially in regard to the larger fragments. This resulted in bands of the leftmost lanes hybridizing significantly less than bands elsewhere, especially the rightmost lanes. This was observed both with the *Aos*I fragments used as probes (Fig. 3B and C) and with probes of other parts of the genome where no sequence divergence was observed (data not shown). This poor efficiency, however, did not effect the main conclusion of this experiment; the small left-terminal fragments of the DNA of group A strains do not appear to have homology to *Aos*I-C of strain SMHI.

A great deal of variation was found in the sizes of left-terminal *Aos*I fragments, even within group A (Fig. 3B). We concluded that these fragments are indeed at the left

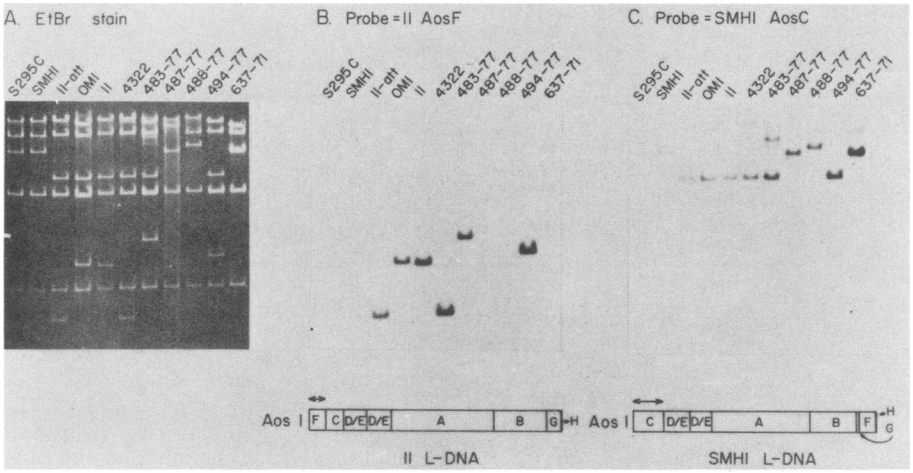

FIG. 3. Hybridization of *Aos*I DNA fragments of recent isolates of herpesvirus saimiri with left-end fragments of strains 11 and SMHI. M-DNA from 11 different strains was cleaved with *Sma*I and *Aos*I, the DNA fragments were separated on a 0.7% agarose gel (A) and transferred to DBM paper. The filter was hybridized with *Aos*I fragment F of strain 11 (B), the probe was removed, and the filter was rehybridized with *Aos*I-C of strain SMHI (C).

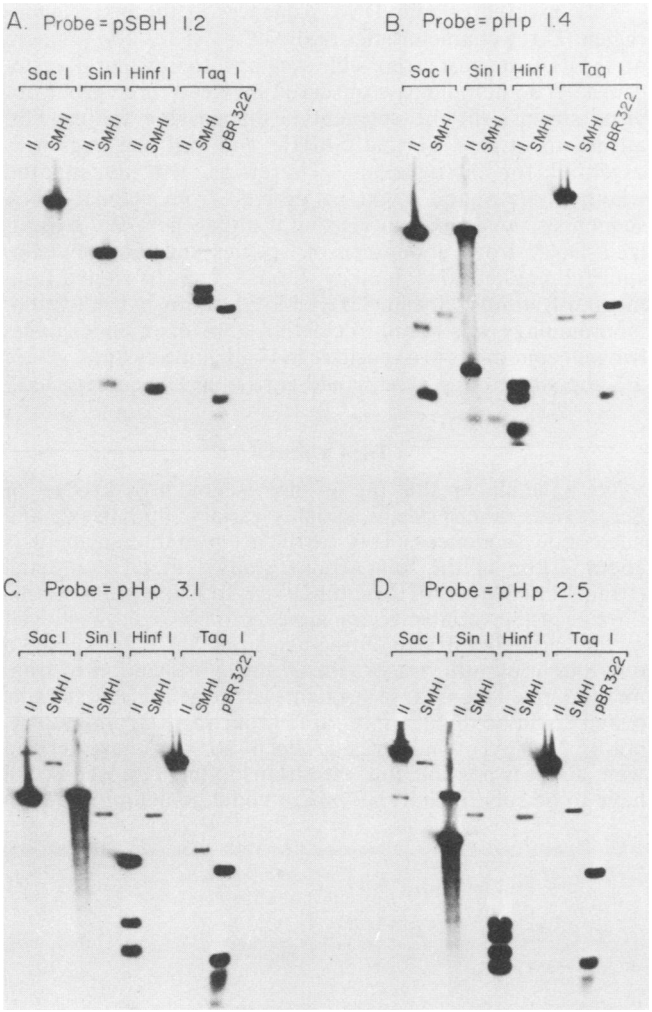

FIG. 4. Serial hybridization of *Sac*I, *Sin*I, *Hin*fI, and *Taq*I DNA fragments of strains 11 and SMHI with various probes. M-DNA (50 ng) from strains 11 and SMHI was digested with *Sac*I, *Sin*I, *Hin*fI, or *Taq*I as indicated. The DNA fragments were separated on a 1.2% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled plasmids as indicated, under conditions of low stringency (T_m, −50°C). After autoradiography the probe was removed and hybridized (T_m, −50°C) with the following probes: A, pSBH1.2 (strain SMHI); B, pHp1.4 (strain 11); C, pHp3.1 (strain 11); D, pHp2.5 (strain 11).

junction from H- to L-DNA, since (i) they hybridized to the left junction fragment of strain 11, and (ii) they did not migrate as a distinct band when the DNA was cleaved with *Aos*I alone; the two terminal fragments of L-DNA were then linked to H-DNA of variable length (1.4 to ca. 50 kbp) and visible only as a smear in the gel (data not shown).

Since the weak hybridization observed between the *Aos*I F fragment of strain 11 and the terminal fragments of the genome of the other five viruses could be explained by a small segment of H DNA attached to the *Sma*I-cleaved L-DNA, or by a weak homology in the terminal segment of L-DNA, or both, we further compared the DNA strains 11 and SMHI.

Detailed analysis of heterologous regions of strains 11 and SMHI. To further map the extent of divergence within these leftmost sequences, M-DNA of strains 11 and SMHI was digested with restriction endonucleases known to cleave L-DNA frequently (*Sac*I, *Sin*I [*Ava*II], *Hin*fI, and *Taq*I). After electrophoresis, DNA fragments were transferred to nitrocellulose filters and sequentially hybridized with different DNA probes under low stringency (T_m, about −50°C). After autoradiography, the filters were washed under more stringent conditions (T_m, −20°C), followed by another autoradiography. Before hybridization with the next probe, the old probe was removed with 70% formamide at 70°C.

pSBH1.2 hybridized strongly to SMHI fragments (Fig. 4A), but failed to show any hybridization to fragments of strain 11 at low stringency hybridization (T_m, −50°C). Even highly overexposed films did not show any hybridization to bands of strain 11, and further decrease of stringency during hybridization resulted in unacceptable background (data not shown). In the reciprocal experiment, pHp1.4 (0.1 to 1.5 kbp of strain 11) strongly hybridized to DNA fragments of strain 11, but not to fragments of SMHI (Fig. 4B). Weak bands showing homology to pHp1.4 were identified as H-DNA by subsequent hybridization with ^{32}P-labeled H-DNA (data not shown); we have identified sequences with homology to H-DNA present in the first 0.2 kbp of L-DNA (unpublished results).

Cloned strain 11 fragments pHp3.1 and pHp2.5 (Fig. 4C and D) hybridized to DNA fragments of both strains, but the intensities of hybridization to bands of strain 11 were estimated to be 5 to 20 times stronger than to fragments of SMHI. By increasing the stringency of washes (T_m, −20°C), we were able to melt off the hybrids from SMHI bands, using conditions that did not affect the hybrids with strain 11 DNA fragments (data not shown).

We did not detect any heterogeneity between strains 11 and SMHI beyond the leftmost 7-kbp segment within the adjacent segment of L-DNA, as determined by hybridization to cloned *Kpn*I fragment B of strain 11, which maps between 1.5 and 15 kbp (data not shown).

Dot-blot hybridization of DNA from new isolates. The dot-blot hybridization results showed that the first 7-kbp segment of L-DNA was highly variable. To analyze the extent of homology more extensively, we employed dot-blot hybridizations of dilutions of DNA from 19 virus strains; most of these were freshly isolated from squirrel monkeys. These filters (Fig. 5) contained duplicate dot-blots with a known amount of purified M-DNA from each strain and five dot-blots with serial twofold dilutions of DNA from strains 11 and SMHI. The filters were sequentially hybridized to various cloned DNA fragments from strains 11 and SMHI. The extent of homology was estimated by comparing the intensities of spots of each strain to those of spots of the dilutions of DNA from strains 11 and SMHI on the autoradiogram.

DNA from five strains (Fig. 5C and D, slots 2, 3, 8, 10, and 16) hybridized at least as strongly to pHp2.5 and pHp3.1 as the spots containing a fourfold dilution of strain 11 DNA. We classified these strains as group A viruses. We also include strain OMI (Fig. 5 C and D, slot 4) in this group. In this experiment strain OMI showed only moderate homology to the strain 11 fragments. However, in other experiments (Fig. 3B and data not shown) OMI showed strong homology to strain 11. All other strains hybridized much more weakly and to variable extent to these two plasmids. Overexposure of these autoradiograms indicated that all strains have a very weak homology to these cloned fragments representing the genome of strain 11 between positions 1.5 and 7.0 kbp (data not shown).

Another set of four strains (Fig. 5A, slots 1, 5, 9, and 19) hybridized to SMHI fragment pSBH1.2 to more than 50% of the extent of hybridization of SMHI DNA. These strains

were classified as group B viruses. One strain (Fig. 5A, slot 6) hybridized much more weakly to pSBH1.2 and was tentatively classified as a group B virus. All other strains showed no homology to pSBH1.2

All B group viruses hybridized strongly to pSBH4.9 as expected (Fig. 5B, slots 1, 5, 9, and 19). Some other strains (group A viruses) also hybridized weakly to this fragment; these strains had not shown any hybridization with pSBH1.2 (Fig. 5A and B, slots 2, 3, 8, 10, and 16). pSBH4.9 contained a larger SMHI L-DNA fragment that included the viral sequences in pSBH1.2; therefore, we concluded that there was a weak homology between different groups in the region mapping between positions 1.5 and 5.1 kbp.

DNA from seven strains (Fig. 5A, B, C, and D, slots 7, 12, 13, 14, 15, 17, and 18) hybridized so weakly to the cloned fragments mentioned above that this could only be detected on highly overexposed autoradiograms. Thus, these strains could not be placed into groups A or B. We do not know yet whether these seven viruses show intergroup homology in this region of the genome; therefore, we tentatively classify these strains as non-A, non-B viruses, and we conclude that there are at least three groups of herpesvirus saimiri based on the homology of the left-terminal region of their L-DNA.

The observed variations in DNA homology was not due to loss of DNA from the filter, since all strains hybridized to about equal extent to *Kpn*I-E, a fragment from the right end of the L-DNA of strain 11 (data not shown).

The results of Fig. 5 and other similar experiments are summarized in Table 1, which shows the approximate extent of homology of all strains to the left-terminal fragments of strains 11 and SMHI.

No conserved cellular DNA sequences in the heterologous region. Three characteristics of the leftmost 1.2-kbp segment of L-DNA suggest a possible analogy to retroviral oncogenes: (i) no homology is observed between three groups of virus strains, (ii) the segment is dispensable for growth, atleast in strains 11 and SMHI, and (iii) the region is essential for oncogenesis. Therefore, we investigated whether this region might be derived from cellular DNA sequences, analogous to retroviral oncogenes. We hybridized DNA from a number of species including human, squirrel monkey, owl monkey, mouse, etc., to cloned fragments of strains 11 and SMHI by Southern hybridization. No homology was found to cellular DNA even under nonstringent conditions in a sensitive hybridization system, where a 1-kbp single-copy gene would easily have been detected.

DISCUSSION

We have shown that the leftmost segment of L-DNA of herpesvirus saimiri strains is highly variable both in size and nucleotide sequences. This extremely variable segment is about 7 kbp in the four strains studied in greater detail (strains 11, OMI, SMHI, and S295C). In many other strains the size of the variable region appears to be larger, as judged by the variability in size of fragments that show homology to only one group of viruses. Based on hybridization to fragments derived from these segments of the genome, all strains tested could be divided into three groups, A, B, and non-A, non-B (Table 1). The latter group is not yet characterized well, and it is possible that the strains in this group may still have to be subdivided; thus, there could be even more than

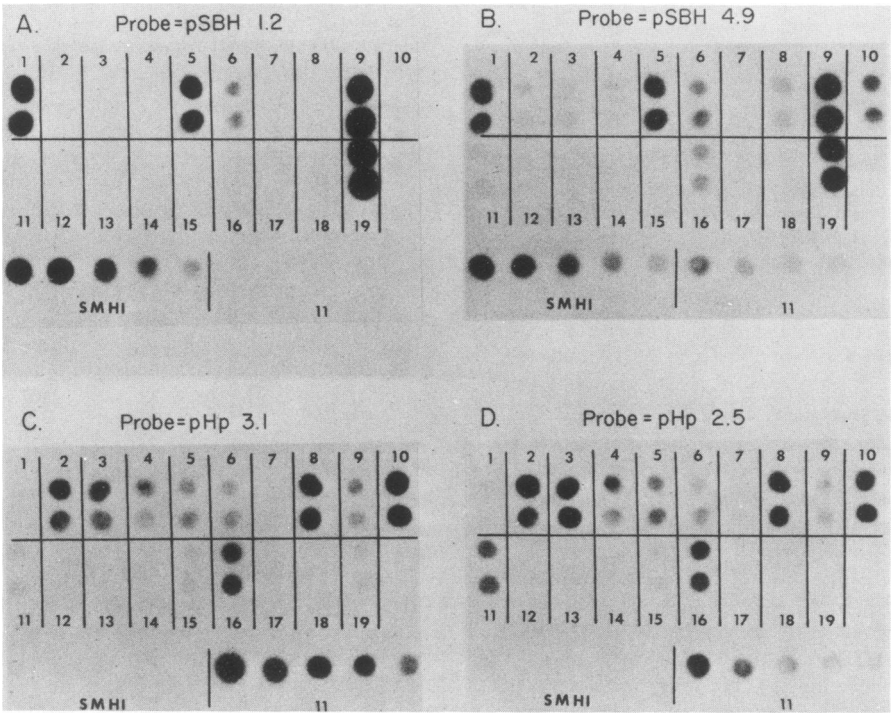

FIG 5. Dot-blot hybridization of M-DNA from 19 strains. M-DNA (50 ng) of 19 different strains was spotted in duplicate on a sheet of nitrocellulose. Twofold serial dilutions (50 to 3.125 ng) of DNA from strains 11 and SMHI were also spotted as standards. The DNA was denatured and fixed to the filter (see the text), and the filter was successively hybridized with cloned DNA fragments from strains SMHI (A and B) and 11 (C and D) as indicated. The following strains are coded by numbers above the dots on the top row and below the dots on the bottom row: 1, SMHI; 2, 11; 3, 11-att; 4, OMI; 5, 24-76; 6, 29-76; 7, 197-71; 8, 254-71; 9, 302-78; 10, 417-78; 11, 483-77; 12, 484-77; 13, 487-77; 14, 487-80; 15, 488-77; 16, 494-77; 17, 505-77; 18, 637-71; 19, S295C.

TABLE 1. Classification of herpesvirus saimiri strains

Strain	Reference	Origin[a]	Extent of hybridization[b] to left-end fragments of:		Group
			SMHI	11	
11	13	NK	<6	100	A
11-att	33	NK	<6	100	A
4322	14	NK	<6	100	A
OMI	20	NK	<6	50	A
254-71	11	Peru	<6	>50	A
417-78	11	NK	12	>50	A
494-77	11	Colombia	6	>50	A
483-77	11	Colombia	<6	25	A
SMHI	26	NK	100	<6	B
S295C	27	NK	100	<6	B
24-76	11	Bolivia	100	12	B
302-78	11	NK	100	6	B
29-76	11	Bolivia	12	<6	B
197-71	11	Guyana	<1	<6	N[c]
484-77	11	Colombia	<1	<1	N
487-77	11	Colombia	<1	<1	N
487-80	11	Bolivia	<1	<1	N
488-77	11	Colombia	<1	<6	N
505-77	11	Colombia	<1	<1	N
637-71	11	Peru	<1	<1	N

[a] NK, Not known.
[b] The approximate extent of hybridization is derived from the results of several experiments such as that shown in Fig. 5.
[c] N, Non-A, non-B virus.

three groups of herpesvirus saimiri strains based on homology in this region. No homology was found at all between cloned fragments representing the leftmost 1.2 kbp of strain 11, a group A virus, and SMHI, a group B strain, and only very weak homology was found in the adjacent 5.5-kbp segment. Only sequencing the appropriate segments of the genomes will inform us whether the leftmost 1.2-kbp segments of the three groups of viruses are at all related.

Variability in restriction endonuclease cleavage sites appears to be a common feature of all herpesviruses. In herpes simplex virus DNA heterogeneity in restriction endonuclease sites appears to be mainly clustered in small regions (hot spots) of the genome, but those variable regions still display tight homology by hybridization (5). Similar DNA polymorphisms have been described for Epstein-Barr virus (3, 18, 32), equine herpesvirus (36), cytomegalovirus (19), varicella-zoster virus (30), and herpesvirus tamarinus (10). In herpesvirus saimiri, the extent of DNA polymorphisms appears to be considerably higher than in the other herpesviruses mentioned; all epidemiologically unrelated strains examined show multiple variations in the ca. 200 recognition sites tested; e.g., we have not yet found two such strains with identical *Sac*I or *Hpa*II fragment patterns (11, 16). As shown in this study, despite these DNA polymorphisms, the rightmost 93% of the L-DNA is nearly completely homologous among all strains. However, contrary to all other herpesviruses known, a sizable segment of the herpesvirus saimiri genome, ca. 7% of the unique sequences, has little or no homology among three groups of strains; this large difference in nucleotide sequences and even large deletions in this region have no apparent effect on the in vitro propagation of the virus (7, 9, 23) or the in vivo establishment of latency (12, 14). Such properties have not been demonstrated in any other herpesvirus.

Several experiments strongly suggest that the highly variable left end of herpesvirus saimiri is required for the oncogenic property of the virus. Deletion of DNA sequences in this region was found in two nononcogenic variants, 11-att and SMHI-att (7, 23). Deletion of sequences in vitro in the same region also resulted in loss of oncogenicity (9; Desrosiers et al., submitted for publication). In different cell lines derived from tumor-bearing animals, DNA sequences of the left terminal 15 kbp as well as the right terminal 15 kbp have been found always preserved, but parts of the central 80-kbp segment of the L-DNA region are often deleted (8, 21, 34). Therefore, this central 80-kbp region of the genome appears nonessential for maintenance of the transformed state.

It is not clear what the origin of this variable segment is. It does not represent a contiguous stretch of cellular DNA, since we have not found evidence for homology to cellular DNA. It cannot be excluded that in multiple recombinational events different virus strains would have incorporated different mosaics of short stretches of cellular DNA. This possibility, although very unlikely, cannot be ruled out, since our preliminary results indicate a very weak homology under nonstringent hybridization conditions between both pSXH4.9 and pHp1.5 on the one hand and squirrel monkey DNA on the other hand. These cellular sequences probably represent moderately repeated sequences. No such homology was found to DNA from other species including owl monkeys, a related monkey species. A more plausible explanation for the great sequence variability of this segment could be an extreme divergence from a common ancestral viral sequence, a sequence which is apparently not required for lytic viral infection and, therefore, is possibly not conserved. However, this would not explain why (i) the virus retained the sequence at all, and (ii) this seemingly freely diverged sequence has retained its oncogenic function.

There appears to be a correlation between virus groups and geographical origins of animals from which some of the recent virus isolates have been obtained. As shown in Table 1, all B group virus strains with known origin are isolated from Bolivian squirrel monkeys; all such A group viruses are derived from animals from Colombia and Peru (two countries with a long common border). These data, however, should be interpreted with caution, since we have no good records of the origins of more than one-third of the monkeys used in this study.

A difference in oncogenic potential between two strains of group A viruses (strains 11 and OMI) versus two strains of group B viruses (strains SMHI and S295C) has been observed by several investigators in at least three monkey species, cottontopped and common marmosets and owl monkeys. For example, strains 11 and OMI cause tumors in common marmosets, whereas strains SMHI and S295C have been reported to be nononcogenic for these animals (25, 37). At this stage, we have no evidence to link this biological difference to the differences in nucleotide sequences between group A and B viruses; several other explanations for differences in oncogenicity cannot be excluded, e.g., these four viruses have been isolated many years ago and have been passaged in tissue culture for a long period and thus could have become slightly attenuated.

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LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U.S.A. **74**:5350–5354.
2. **Bornkamm, G., H. Delius, B. Fleckenstein, F. J. Werner, and C. Mulder.** 1976. Structure of *Herpesvirus saimiri* genomes; arrangement of heavy and light sequences in the M-genome. J. Virol. **25**:154–161.
3. **Bornkamm, G., H. Delius, U. Zimber, J. Hudewentz, and M. A. Epstein.** 1980. Comparison of Epstein-Barr virus strains of different origin by analysis of viral DNAs. J. Virol. **35**:603–618.
4. **Brandsma, J., and G. Miller.** 1980. Nucleic acid spot hybridization: rapid quantitative screening of lymphoid cells for Epstein-Barr virus DNA. Proc. Natl. Acad. Sci. U.S.A. **77**:6851–6855.
5. **Buchman, T. G., B. Roizman, G. Adams, and B. H. Stover.** 1978. Restriction endonuclease fingerprinting of herpes simplex virus DNA; a novel epidemiological tool applied to a nosocomial outbreak. J. Infect. Dis. **138**:488–489.
6. **Daniel, M. D., L. V. Melendez, R. D. Hunt, N. W. King, M. Anver, C. E. O. Fraser, H. H. Barahona, and R. B. Baggs.** 1974. *Herpesvirus saimiri*. VII. Induction of malignant lymphoma in New Zealand white rabbits. J. Natl. Cancer Inst. **53**:1803–1807.
7. **Daniel, M. D., D. Silva, J. M. Koomey, C. Mulder, B. Fleckenstein, R. Tamulevich, N. W. King, R. D. Hunt, P. Sehgal, and L. A. Falk.** 1979. *Herpesvirus saimiri*: strain SMHI modification of oncogenicity, p. 395–396. *In* D. S. Yohn, B. A. Lapin, and I. Blakeslee (ed.), Advances in comparative leukemia research. Elsevier/North Holland Publishing Co., New York.
8. **Desrosiers, R. C.** 1981. *Herpesvirus saimiri* DNA in tumor cells—deleted sequences and sequence arrangements. J. Virol. **39**:497–507.
9. **Desrosiers, R. C., R. L. Burghoff, A. Bakker, and J. Kamine.** 1984. Construction of replication-competent *Herpesvirus saimiri* deletion mutants. J. Virol. **49**:343–348.
10. **Desrosiers, R. C., and L. A. Falk, Jr.** 1981. *Herpesvirus tamarinus* and its relation to herpes simplex virus. J. Gen. Virol. **56**:119–130.
11. **Desrosiers, R. C., and L. A. Falk.** 1982. *Herpesvirus saimiri* strain variability. J. Virol. **43**:352–356.
12. **Falk, L. A., P. A. Schaffer, J. Wright, F. Deinhardt, L. Wolfe, and M. Benyesh-Melnick.** 1976. Experimental infection of marmoset and squirrel monkeys with attenuated HVS, p. 336–338. *In* J. Clemmesen and D. S. Yohn, (ed.), Comparative leukemia research 1975. S. Karger, Basel.
13. **Falk, L. A., L. G. Wolfe, and F. Deinhardt.** 1972. Isolation of *Herpesvirus saimiri* from blood of squirrel monkeys (*Saimiri sciureus*). J. Natl. Cancer Inst. **48**:1499–1505.
14. **Falk, L., J. Wright, F. Deinhardt, L. Wolfe, P. A. Schaffer, and M. Benyesh-Melnick.** 1976. Experimental infection of squirrel and marmoset monkeys with attenuated *Herpesvirus saimiri*. Cancer Res. **36**:707–710.
15. **Fleckenstein, B., G. W. Bornkamm, and H. Ludwig.** 1975. Repetitive sequences in complete and defective genomes of *Herpesvirus saimiri*. J. Virol. **15**:398–406.
16. **Fleckenstein, B., and C. Mulder.** 1980. Molecular biological aspects of *Herpesvirus saimiri* and *Herpesvirus ateles*, p. 799–812. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
17. **Fleckenstein, B., and H. Wolf.** 1974. Purification and properties of *Herpesvirus saimiri* DNA. Virology **58**:55–64.
18. **Heller, M., T. Dambaugh, and E. Kieff.** 1981. Epstein-Barr virus DNA. IX. Variation among viral DNAs from producer- and nonproducer-infected cells. J. Virol. **38**:632–648.
19. **Huang, E., S. Huong, G. Tegtmeier, and C. Alford.** 1980. Cytomegalovirus: genetic variation of viral genomes, p. 332–346. *In* P. Palese and B. Roizman (ed.), Genetic variation of viruses. New York Academy of Sciences, New York.
20. **Hunt, R. D., F. G. Garcia, H. H. Barahona, N. W. King, C. E. O. Fraser, and L. V. Melendez.** 1973. Spontaneous *Herpesvirus saimiri* lymphoma in an owl monkey. J. Infect. Dis. **127**:723–725.
21. **Kaschka-Dierich, C., F. J. Werner, I. Bauer, and B. Fleckenstein.** 1982. Structure of nonintegrated circular *Herpesvirus saimiri* and *Herpesvirus ateles* genomes in tumor cell lines and in vitro-transformed cells. J. Virol. **44**:295–310.
22. **Keil, G., I. Muller, B. Fleckenstein, J. Koomey, and C. Mulder.** 1980. Generation of recombinants between different strains of *Herpesvirus saimiri*, p. 145–161. *In* M. Essex, G. Todaro, and H. zur Hausen (ed.), Viruses in naturally occuring cancers. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Koomey, J. M., C. Mulder, R. L. Burghoff, B. Fleckenstein, and R. C. Desrosiers.** 1984. Deletion of DNA sequences in a nononcogenic variant of *Herpesvirus saimiri*. J. Virol. **50**:662–665.
24. **Laufs, R., and B. Fleckenstein.** 1973. Purification of *Herpesvirus saimiri* DNA. Bibl. Haematol. **39**:457–461.
25. **Laufs, R., and B. Fleckenstein.** 1973. Susceptibility to *Herpesvirus saimiri* and antibody development in Old and New World monkeys. Med. Microbiol. Immunol. **158**:227–236.
26. **Melendez, L. V., M. D. Daniel, R. D. Hunt, and F. G. Garcia.** 1968. An apparently new herpesvirus from primary kidney cultures of the squirrel monkey (*Saimiri sciureus*). Lab. Anim. Care **18**:374–381.
27. **Melendez, L. V., R. D. Hunt, M. D. Daniel, F. Garcia, and C. E. O. Fraser.** 1969. *Herpesvirus saimiri*. II. Experimentally induced malignant lymphoma in primates. Lab. Anim. Care **19**:378–386.
28. **Melendez, L. V., D. Jackman, C. E. O. Fraser, and M. D. Daniel.** 1974. *In vitro* attenuation of *Herpesvirus saimiri* by serial passage in dog fetal lung continuous cultures, p. 353–360. *In* G. de-The, M. A. Epstein, and H. zur Hausen (ed.), Oncogenesis and herpesviruses, vol. 2. International Agency for Research on Cancer, Lyon.
29. **Rangan, S. R. S., L. N. Martin, F. M. Enright, and W. P. Allen.** 1976. *Herpesvirus saimiri*-induced malignant lymphoma in rabbits. J. Natl. Cancer Inst. **57**:151–156.
30. **Richards, J. C., R. W. Hyman, and F. Rapp.** 1979. Analysis of the DNAs from seven varicella-zoster virus isolates. J. Virol. **32**:812–821.
31. **Rigby, P., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling DNA to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–252.
32. **Rymo, L., T. Lindahl, and A. Adams.** 1979. Sites of sequence variability in Epstein-Barr virus DNA from different sources. Proc. Natl. Acad. Sci. U.S.A. **76**:2794–2798.
33. **Schaffer, P. A., L. A. Falk, and F. Deinhardt.** 1975. Brief communication: attenuation of *Herpesvirus saimiri* for marmosets after successive passage in cell culture at 39°C. J. Natl. Cancer Inst. **55**:1243–1246.
34. **Schirm, S., I. Muller, R. C. Desrosiers, and B. Fleckenstein.** 1984. *Herpesvirus saimiri* DNA in a lymphoid cell line established by in vitro transformation. J. Virol. **49**:938–946.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
36. **Studdert, M., T. Simpson, and B. Roizman.** 1981. Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. Science **214**:562–564.
37. **Wright, J., L. A. Falk, L. G. Wolfe, J. Ogden, and F. Deinhardt.** 1977. Susceptibility of common marmosets (*Callithrix jacchus*) to oncogenic and attenuated strains of *Herpesvirus saimiri*. J. Natl. Cancer Inst. **59**:1475–1478.