JOURNAL OF VIROLOGY, Feb. 1986, p. 701–705
0022-538X/86/020701-05$02.00/0

Vol. 57, No. 2

Nononcogenic Deletion Mutants of Herpesvirus Saimiri Are Defective for In Vitro Immortalization

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Received 9 August 1985/Accepted 15 October 1985

Herpesvirus saimiri L-DNA sequences between 0.0 and 4.0 map units (4.5 kilobase pairs) are required for oncogenicity; these sequences are not required for replication of the virus. To investigate the basis for the lack of oncogenicity of mutants with deletions in this region and to study the function of this region, we developed a reliable system for in vitro immortalization by herpesvirus saimiri. In contrast to peripheral blood lymphocytes from cotton-top tamarins (*Saguinus oedipus*) and owl monkeys (*Aotus* sp.), infection of peripheral blood lymphocytes from common marmosets (*Callithrix jacchus*) in vitro with herpesvirus saimiri consistently yielded continuously growing lymphoblastoid cell lines. Such cell lines were established using strains of herpesvirus saimiri from group A and group non-A, non-B; however, repeated attempts to immortalize common marmoset peripheral blood lymphocytes using strains from group B were not successful. Common marmoset cell lines immortalized by herpesvirus saimiri were $T12^+$, $T8^+$, $T4^-$, and $B1^-$, indicating that they were derived from suppressor/cytotoxic T lymphocytes. Cell lines could not be established using the nononcogenic mutants 11att and S4, both of which were derived from the group A strain 11 virus. Strain 11att has a spontaneous deletion and S4 has a constructed deletion in the 0.0 to 4.0 map unit region. Constructed strains which had these deleted sequences restored did immortalize common marmoset peripheral blood lymphocytes. Thus, the nononcogenic deletion mutants are defective for immortalization. This system should facilitate attempts to define the sequences responsible for immortalization and to determine their function.

Unlike other tumor virus systems, little is known at the molecular level about herpesvirus-induced oncogenic transformations. Herpesvirus saimiri offers a number of unique advantages for the study of oncogenic transformation in a herpesvirus system (for a review, see reference 7). T-cell lymphomas are induced rapidly and reproducibly by herpesvirus saimiri in a variety of New World primate species. Continuously growing lymphoblastoid cell lines have been derived from virus-induced lymphoma tissues, and these may be used to study the persistence of viral DNA and the genes required for the transformed phenotype. Unlike the human lymphotropic Epstein-Barr virus, herpesvirus saimiri causes a lytic infection in some monolayer cell lines, yielding infectious titers of $>10^7$/ml; this facilitates study of the infectious cycle, production of viral materials, and genetic analyses.

Replication-competent strains of herpesvirus saimiri with deletions in a 4.5-kilobase-pair (kbp) region of the viral genome have lost their oncogenic potential; restoration of the deleted DNA sequences restored the oncogenic potential to these virus strains (2, 3, 9). The genomic region required for oncogenicity is located in the left-most 4.5 kbp of L-DNA (Fig. 1). Comparison of different herpesvirus saimiri isolates has revealed a remarkable genetic variability in this region of the genome (12). The hypervariability in this region among different strains has allowed classification of herpesvirus saimiri into three groups: A, B, and non-A, non-B (12). Although the RNA products from this region in owl monkey kidney (OMK) cells lytically infected with strain 11 (group A) have been previously defined (8), the functional role of gene products from this region has yet to be elucidated.

One difficulty in working with the herpesvirus saimiri system has been the inability to develop a reliable in vitro immortalization assay following infection with the virus. The difficulty in achieving in vitro immortalization after infection with herpesvirus saimiri stands in marked contrast to its oncogenicity in vivo and to the ease with which in vitro immortalization is achieved with the closely related virus herpesvirus ateles. Herpesvirus ateles (6) induces T-cell lymphomas similar to those induced by herpesvirus saimiri in several New World primate species; immortalization of splenic and circulating lymphocytes from cotton-top tamarins, white-lipped tamarins, and common marmosets has been accomplished routinely with herpesvirus ateles (4). Early attempts to achieve in vitro immortalization of marmoset lymphocytes using herpesvirus saimiri were reported to be unsuccessful in spite of the successful immortalization of these cell populations with herpesvirus ateles (4). Similarly, Schirm et al. (15) failed in numerous attempts to immortalize peripheral blood lymphocytes (PBL) from four New World primate species (cotton-top tamarin, owl monkey, spider monkey, and tufted capuchin) using herpesvirus saimiri strains 11 and OMI; a single positive immortalization was achieved in this study using strain OMI and cotton-top tamarin PBL.

Another approach to achieving in vitro immortalization following infection with herpesvirus saimiri involved growing T cells in interleukin-2 (IL-2) before infection (5, 14). After infection, immortalization was assayed by continued cell growth in the absence of exogenously added IL-2. Successful immortalization using this assay has been reported for owl monkey PBL using strain S295-C (14) and for cotton-top tamarin PBL using strain 11 virus (5). However, this process is lengthy, with conversion to IL-2 independence occurring only over the course of several months. Furthermore, the frequency of conversion of these lymphocyte populations to IL-2 independence is sufficiently low that results of strain comparisons in this system are difficult to interpret (unpublished data).

Here we report the reproducible in vitro immortalization

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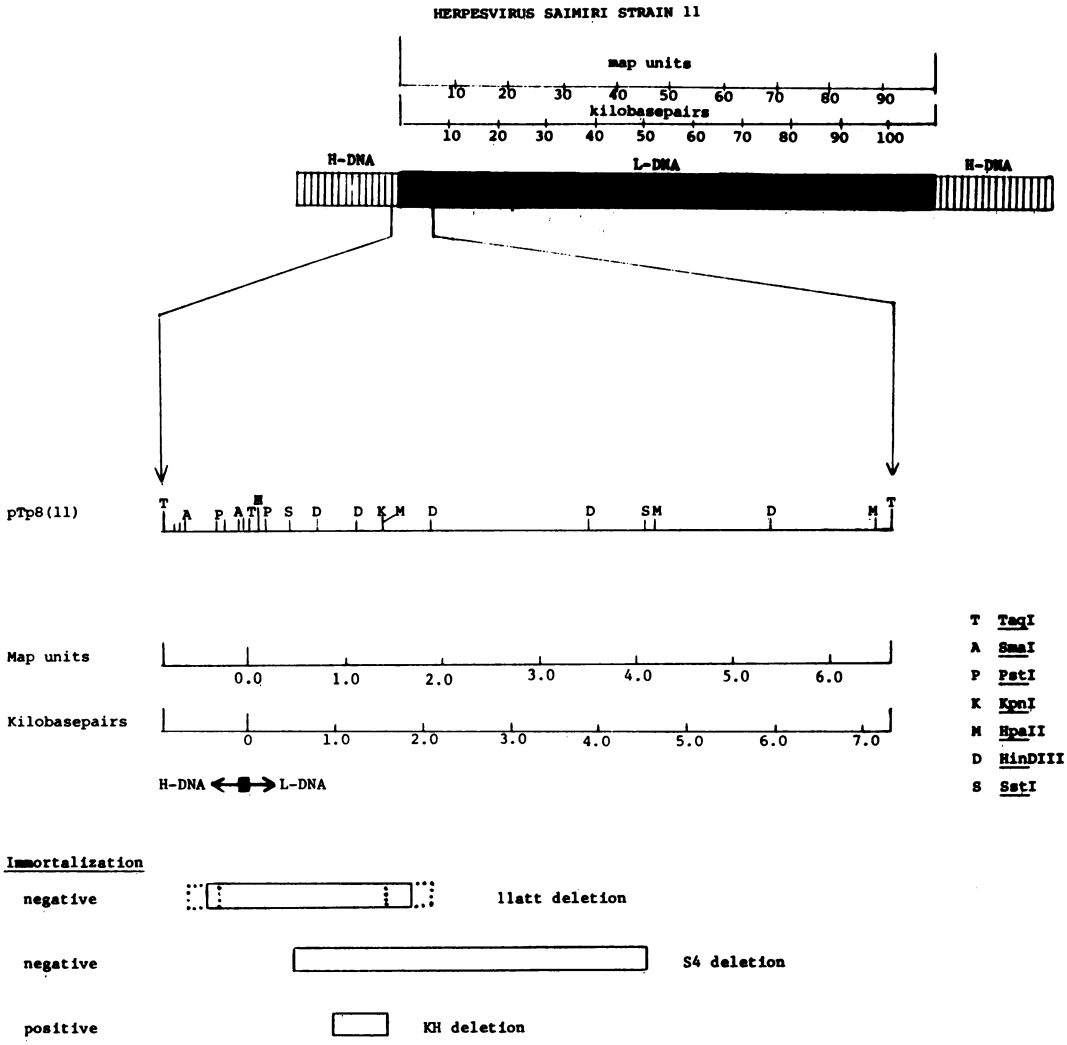

FIG. 1. Localization of herpesvirus saimiri DNA sequences required for immortalization. Herpesvirus saimiri contains a 110-kbp stretch of unique sequence DNA called L-DNA (light, 36% G+C). This is flanked at each end by a variable number of 1.4-kbp H-DNA repeat units (heavy, 71% G+C). The bottom portion of the figure shows on an expanded scale the location of the deletions in the mutants used in this study. These deletion mutants have been described previously (2, 3, 9). The region of uncertainty in the 11att deletion is indicated by the dotted lines.

of common marmoset PBL using strain 11 and other group A strains of herpesvirus saimiri in a simple assay system without the use of IL-2. Nononcogenic deletion mutants derived from strain 11 did not immortalize common marmoset PBL. This assay system should prove useful in further dissection of left-end L-DNA sequences necessary for oncogenic transformation.

Virus stocks were prepared in OMK cells (line 637), and the titers were determined titered by limiting dilution. Preservative-free heparinized blood samples were obtained from New World primates housed at the New England Regional Primate Research Center. Mononuclear leukocytes were banded over a Ficoll-sodium diatrizoate solution (density, 1.0745 g/cm^3) and washed twice with complete RPMI 1640 medium with 10% fetal calf serum before use.

For immortalization attempts, 3.0×10^6 PBL were incubated with 1 ml of a freshly prepared stock of herpesvirus saimiri at a multiplicity of infection of approximately 5. Virus was adsorbed for 2 h at 37°C. Three milliliters of RPMI 1640 with 20% fetal calf serum was added, and the infected cells were transferred to a 25-cm^2 culture flask and incubated at 37°C at a slight angle (about 15° from horizontal) in a CO_2 incubator with the caps loose. For the next 3 weeks, 2 ml of RPMI 1640 with 20% fetal calf serum was added weekly. After this time, the medium was completely replaced every 3 to 4 days until the cells could be expanded or until the culture was discarded. The medium was replaced by pelleting any suspended cells in the culture supernatant, resuspending the pelleted cells in fresh medium, and adding this back to the original flask.

Consistent with previous studies, infection of PBL from cotton-top tamarins with herpesvirus saimiri strain 11 did not yield any continuously growing lymphoblastoid cell lines. Varying the tamarin donor, the serum concentration in the medium, the cell concentration, and the angle of the flask in the incubator did not seem to affect these negative results. Parallel attempts using herpesvirus ateles were frequently successful. The use of uninfected and infected cell monolayer cultures as a feeder layer also did not alter the outcome. Failure to generate continuously growing cell lines

was also observed in similar experiments using owl monkey PBL and herpesvirus saimiri strain 11. On two separate occasions the infection of single-cell suspensions and explant cultures prepared from cotton-top tamarin thymus and spleen with strain 11 also did not yield any continuously growing cell lines. Lymphoid cells growing in the presence of IL-2 (Electro-Nucleonics, Inc., Fairfield, N.J.) were established from three different cotton-top tamarins under conditions similar to those described previously (5, 14); infection with herpesvirus saimiri strain 11 for as long as 6 months failed to yield any IL-2-independent cell lines.

In contrast to the above results, PBL from common marmosets consistently yielded continuously growing lymphoblastoid cell lines in the absence of exogenously added IL-2 when infected with herpesvirus saimiri strain 11. By 2 to 3 weeks after infection with this virus, the cells became elongated and attached to the surface of the flask. Growth and transformation became evident as cells began to round up and detach from the surface 4 to 6 weeks after infection. The cells were pleomorphic at these early stages. By 8 weeks in culture, as cells multiplied, they became more uniform in appearance and the concentration was maintained at 0.5×10^6 to 1.0×10^6 cells per ml. Established cell lines grew in suspension mainly as small clumps or larger cell aggregates of 100 cells or more, similar in appearance to tumor cell lines established from herpesvirus saimiri-induced lymphomas.

Immortalization attempts using herpesvirus saimiri strain 11 were initiated in duplicate on five separate dates. Continuously growing lymphoblastoid cell lines were obtained from all 10 cultures (Table 1). Immortalization of common marmoset PBL was also achieved with other group A viruses, strains OMI and 494-77 (Table 1). Successful immortalization was also accomplished after infection with non-A, non-B strains 484-77 and 488-77. Continuously growing cell lines were not obtained when virus infection was omitted from the protocol. The success rate in these experiments (16 of 16 for group A and 4 of 4 for group non-A, non-B) provided strong evidence for the reproducibility of the assay and indicated that this system could be used to compare the capacity of various strains of herpesvirus saimiri to induce lymphocyte immortalization. In contrast to the results obtained with virus strains from group A and group non-A, non-B, repeated attempts to immortalize common marmoset PBL using group B strains have not been successful (Table 1). For each negative outcome, the same pool of PBL yielded continuously growing lymphoblastoid cell lines in parallel flasks when infected with other herpesvirus saimiri strains.

TABLE 1. In vitro immortalization of common marmoset lymphocytes by strains of herpesvirus saimiri

Virus group[a]	Virus strain	Immortalization (no. of immortalized cell lines established/no. of attempts)[b]
A	11	10/10
A	494-77	4/4
A	OMI	2/2
B	S295-C	0/2
B	302-78	0/2
B	SMHI	0/2
Non-A, non-B	484-77	2/2
Non-A, non-B	488-77	2/2

[a] As classified by Medveczky et al. (12) according to the degree of homology of sequences at the left end of L-DNA.

[b] Pools of PBL from three common marmosets (Cj213-81, Cj412-82, and Cj473-80) were used for most of these immortalization attempts. Pools of PBL from two different common marmosets (Cj313-80 and Cj314-80) were used in four of the strain 11 immortalizations. Establishment of cell lines using strain 11 was achieved in duplicate on five separate dates. For each negative group B immortalization, positive immortalization was achieved in parallel flasks with other herpesvirus saimiri strains.

TABLE 2. Phenotypes of common marmoset PBL lines

Line[a]	Days post-infection	% PBL expressing lineage-restricted antigens[b]			
		T12	T4	T8	B1
2	120	NT[c]	8	80	1
	140	70	0	41	0
4	120	91	4	87	1
	291	88	0	68	2
5	112	55	1	87	1
	283	70	0	61	1
6	112	88	0	75	2
7	106	79	0	66	0
8	83	84	0	54	3
	254	97	0	87	0
12	46	89	30	57	1
	96	96	0	80	1
	119	67	0	69	0
13	46	90	27	56	0
	119	93	21	73	3
	167	82	2	36	0
15	104	95	8	56	0
18	39	95	16	81	6
	68	97	4	93	0

[a] In vitro infections were performed with herpesvirus saimiri strain 11 in all cases except for line 8 (where strain 494-74 was utilized), line 15 (where strain KH was utilized), and line 18 (where strain OMI was utilized).

[b] Data are expressed as percent cells staining positively as determined by flow cytometry. Phenotyping of PBL from freshly drawn common marmoset blood demonstrated: T12, 62 ± 6%; T4, 30 ± 7%, T8, 15 ± 7%; B1, 3 ± 2%.

[c] NT, Not tested.

The phenotypes of many of the in vitro-immortalized cell populations were determined by staining with monoclonal antibodies and fluorescence-activated cell sorter analysis. The monoclonal antibodies utilized in these studies included the pan-T-cell antibody anti-T12, the T8 (suppressor/cytotoxic)-specific antibody 21 Thy2D3, and the B-cell-specific antibody anti-B1. These reagents were provided by S. Schlossman, Dana-Farber Cancer Institute (Boston, Mass.). The T4 (helper/inducer)-specific antibody 94b1-1 was provided by R. Winchester, New York University School of Medicine. In a screening of numerous monoclonal antibodies, these antibodies were found to react effectively with the appropriate determinants on common marmoset PBL (11). Cells were banded onto a Ficoll-sodium diatrizoate solution to remove nonviable cells; banded cells were then washed in Hanks balanced salt solution and incubated in equal portions of 5×10^5 cells for 20 min with the noted monoclonal antibody at 4°C. These cells were then washed in Hanks balanced salt solution and incubated in fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Tago, Burlingame, Calif.) for 20 min at 4°C. After each sample was washed in phosphate-buffered saline and fixed with a 1% Formalin solution, cytofluorographic analysis of cell populations was performed on a fluorescence-activated cell sorter (FACS-1; Becton Dickinson and Co., Mountainview, Calif.).

As shown in Table 2, these studies demonstrated that the established cell populations expressed the pan-T-cell antigen T12 but not the B-cell-specific antigen B1 and were therefore T lymphocytes. The distribution of T4-bearing

TABLE 3. Nononcogenic deletion mutants of herpesvirus saimiri are defective for in vitro immortalization

Virus strain[a]	Oncogenicity[b]	Deletion[c]	Immortalization (no. of immortalized cell lines established/ no. of attempts)[d]
11	+		10/10
11att	−	−0.5 to +1.6	0/4
S4	−	+0.4 to +4.1	0/4
11att-pTP8	+		4/4
S4-pT7.4	+		3/4
KH	+	+0.9 to +1.35	4/4

[a] These strains have been previously described (2, 3, 9).
[b] Previously determined in owl monkeys (3) or in common marmosets (see text).
[c] In map units of L-DNA. The region of uncertainty in the 11att deletion is shown in Fig. 1.
[d] A pool of PBL from three common marmosets (Cj213-81, Cj412-82, and Cj473-80) was used for these immortalization attempts. For each immortalization failure with 11att and S4, immortalization was achieved in parallel flasks with other herpesvirus saimiri strains. Immortalization attempts were done in duplicate on different dates.

(helper/inducer) and T8-bearing (cytotoxic/suppressor) T cells within these populations changed in a reproducible manner over time in every line studied (Table 2). A significant number of $T4^+$ cells were present in these populations up to 50 days after infection of the common marmoset lymphocytes with herpesvirus saimiri. By 80 days after infection few or no $T4^+$ cells were detected; cells in these populations were predominantly $T8^+$. In addition to the immortalized cell lines established with group A viruses shown in Table 2, cell lines established using strains 484-77 and 488-77 (group non-A, non-B) had the same phenotype, i.e., $T12^+$ $T8^+$ $T4^-$ $B1^-$.

Most of the experiments represented in Table 1 were performed with pools of PBL from three common marmosets (Cj213-81, Cj412-82, and Cj473-80). We investigated whether cells from each of these animals were equally susceptible to immortalization by herpesvirus saimiri strain 11. PBL of Cj473-80 appeared to be refractory to immortalization by herpesvirus saimiri strain 11, while those of the other two marmosets were readily immortalized.

We also attempted to estimate the number of cells immortalized after exposure to virus. Common marmoset PBL were exposed to herpesvirus saimiri strain 11, and various numbers of cells in individual wells of Linbro trays were monitored for the outgrowth of immortalized cells. The results of this experiment indicated that 10^4 to 10^5 PBL per well were necessary to observe outgrowth of immortalized cells, suggesting that the efficiency of immortalization was between 0.01 and 0.001% when measured in this way.

Using this reliable in vitro immortalization assay, we were then able to test the immortalizing capacity of nononcogenic deletion mutants derived from strain 11 virus (Table 3). The 11att and S4 replication-competent deletion mutants (2, 9) did not immortalize common marmoset PBL. Virus strains in which the deleted sequences were replaced had been constructed previously (3). These strains, 11att-pTP8 and S4-pT7.4, efficiently immortalized common marmoset PBL. These results demonstrate that the nononcogenic deletion mutants 11att and S4 are defective for in vitro immortalization.

We also tested the in vitro immortalizing potential of another deletion mutant strain (KH) derived from the parental oncogenic strain 11 (2). The KH strain of herpesvirus saimiri was recently found to induce lymphoma in common marmosets (R. Desrosiers and N. King, unpublished data). Although the deletion in KH falls within the 11att and S4 deletions, the KH strain retained the capacity to immortalize common marmoset PBL (Table 3).

The results presented here demonstrate the ease with which common marmoset lymphocytes can be immortalized by certain strains of herpesvirus saimiri. Consistent with previous results which indicated that T cells were specific targets of infection and transformation by herpesvirus saimiri (13, 14, 16), all established cell lines in the present study were positive with the pan-T-cell reagent anti-T12. All established cell lines were also $T8^+$ $T4^-$ $B1^-$, suggesting that the $T8^+$-cell population of common marmosets is the major target cell for in vitro immortalization by herpesvirus saimiri. These findings appear to be similar to those obtained with owl monkey cells converted to IL-2 independence by herpesvirus saimiri strain S295C, in which the cells were $OKT11a^+$ (pan-T-cell marker), $Leu\text{-}5^+$ (pan-T-cell marker), $Leu\text{-}3a^-$ (T4-cell marker), and $B1^-$ (14); reagents for measuring the T8-cell population were not available to the investigators in this previous study.

The results presented here for group A and group B viruses are also consistent with the limited information in the literature regarding in vivo oncogenicity of herpesvirus saimiri in common marmosets. Although strain 11 induces lymphomas in common marmosets (17), attempts to induce disease in common marmosets with strain S295C have generally not been successful (1, 10). Successful direct immortalization by herpesvirus saimiri was probably not achieved by other investigators in the past simply because common marmoset PBL and group A or group non-A, non-B viruses were not used in those studies. The inability to induce immortalization of cotton-top tamarin and owl monkey PBL directly by herpesvirus saimiri remains unexplained at this time.

This new immortalization assay should be extremely useful in furthering our understanding of the function of left-end L-DNA sequences. Since the KH deletion mutant was able to immortalize common marmoset PBL, sequences between 0.9 and 1.35 map units must not be essential for immortalization (Fig. 1). This suggests that the region immediately leftward from 0.9 map units or immediately rightward from 1.35 map units may contain the sequences essential for immortalization and oncogenicity. In OMK cells lytically infected with herpesvirus saimiri strain 11, $poly(A)^+$ RNA transcripts initiate at 4.4 and 2.0 map units, run through this region, and terminate at 0.1 map units (8). However, in herpesvirus saimiri-induced lymphomas and in tumor cell line 1670, these $poly(A)^+$ RNAs were not detected at all (8). What were detected in tumor cells and in tumor cell line 1670 were four specific small RNAs 70 to 140 nucleotides in length (8; S. Murthy, J. Kamine, and R. Desrosiers, manuscript in preparation). Transcript mapping and analysis of the effects of other deletions and mutations on immortalization may provide evidence for the importance of these small RNAs.

This work was supported by Public Health Service grant 31363 from the National Cancer Institute and by grant RR00168 from the Division of Research Resources.

We thank Martha Elliott and Prabhat Sehgal for assistance in obtaining blood samples.

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