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An *in Vitro* Transformation Assay for SiSV-1 and HL23V Using Feline Embryonic Fibroblasts (40044)

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Simian sarcoma virus (SiSV-1) was first observed in and isolated from a fibrosarcoma of a woolly monkey (1-3), and has been shown to produce fibrosarcomas in newborn marmosets (2). Furthermore, several culture lines, including marmoset, human, cat, and rat cells were reported to be susceptible to transformation by this virus (3-5).

HL23V was isolated from the cultured peripheral blood and bone marrow leukocytes of a patient with acute myelogenous leukemia and was shown to be infectious for a wide variety of cell lines (6-8). An isolate of HL23V has the ability to induce tumors in marmosets and transform cultured marmoset cells (9) and has been shown to be a mixture of viruses related to both SiSV-1 and baboon endogenous virus (10-12). Two other primate type-C viruses used in this study, baboon endogenous viruses (BaEV) (13-15) and gibbon ape leukemia virus (GaLV) (16-18) have been shown to be nontransforming for a number of cultured cell lines.

In this report the focus-formation titer of SiSV-1 and HL23V on feline embryo fibroblast cells is compared to that using other commonly used cell lines, including; normal rat kidney; rat embryonic fibroblast; and several cultures of primate origin. Transformation by these viruses was found to be substantially more rapid and sensitive when FEF cells are used. A similar observation was recently reported by Sarma and Law (19).

Materials and methods. Cells and viruses. The various cell cultures and viruses used in this study are described in Table I. All cells were grown in McCoy's 5A modified media (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Reheis Chemical Company, Phoenix, Arizona) and carried in Falcon 3024 culture flasks at 37° and 5% CO_2 in a humid atmosphere. Cultures were trypsinized and passaged every 4-5 days as needed using a 0.25% trypsin-EDTA solution (Gibco, NY). Virus and cell stocks were stored in liquid nitrogen.

Transformation assay. Cells to be infected were seeded at 5×10^5 per 60 mm culture dish (Falcon 8083) or 7×10^4 per well in a 24 well Linbro Plate (Linbro Scientific, Inc., Hamden, CO). Cultures were then treated as follows: at 5-18 hr postseeding, fluid was replaced with 25 μg/ml DEAE-Dextran (Pharmacia, lot #2732), in McCoy's 5A unsupplemented medium, incubated at 37° for 30 min, and then rinsed twice with phosphate buffered saline (PBS, Int. Biological Lab., Inc., Rockville, MD). Serial tenfold dilutions of virus or control media (0.5 ml per 60 mm dish or 0.1 ml per Linbro well) were added to duplicate cultures for 1 hr at 37°. After virus adsorption, complete medium was added and cultures incubated. Twenty-four hours after infection, NRK-infected cells were changed to medium containing 5% serum, and other cells were refed with and maintained in regular growth medium. All cultures were carefully monitored to maintain a neutral pH. Foci were counted at the following times after infection of the indicated cell lines: feline embryonic cells, 3-4 days; NRK cells, 7-8 days; and all other cell lines, 7-10 days. After optimum focus development, all monolayers were rinsed with PBS, stained for 5 min with a mixture of giemsa

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TABLE I. CELLS AND VIRUSES.

Cells	Designation	Source
Bat lung	CCL88	ATCC, Rockville, MD.
Fetal canine thymus	Cf2Th[a]	Naval Biological Laboratories, Oakland, CA.
Feline embryo fibroblast	FEF-1	Dr. P. Sarma, NIH, Bethesda, MD. (#D843)
Feline embryo fibroblast	FEF-2	Dr. S. Ruscetti, NIH, Bethesda, MD.
Feline embryo fibroblast	FEF-3	Dr. J. Whitman, HEM Research, Rockville, MD. (FEE-#6)
Feline embryo fibroblast	FEF-4	Naval Biological Laboratories, Oakland, CA. (FC113)
Feline embryonic tongue fibroblast	CCL176	ATCC, Rockville, MD.
Human embryo fibroblast	PTA-11	Dr. R. C. Gallo, Lab. of Tumor Cell Biol., NCI, Bethesda, MD.
Human embryo fibroblast	WHE-2	Dr. R. C. Gallo, Lab. of Tumor Cell Biol., NCI, Bethesda, MD.
Human foreskin fibroblast	HFF	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med. Center, Chicago, IL.
Marmoset skin fibroblast	HF	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med. Center, Chicago, IL.
Marmoset skin fibroblast	MFS	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med. Center, Chicago, IL.
Marmoset lung fibroblast	1283	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med. Center, Chicago, IL.
Rat embryo fibroblast (Fisher)	RE-1	Dr. R. Ting, Biotech, Rockville, MD. (#112)
Rat embryo fibroblast (Fisher)	RE-2	Dr. R. Ting, Biotech, Rockville, MD. (#810)
Normal rat kidney	NRK-1[b]	Dr. R. Ting, Biotech, Rockville, MD. (Clone #153-S)
Normal rat kidney	NRK-2[b]	Dr. R. Ting, Biotech, Rockville, MD. (Clone #13)
Normal rat kidney	NRK-3[b]	Dr. R. Ting, Biotech, Rockville, MD.
Normal rat kidney	NRK-4[b]	Dr. R. Bassin, NIH, Bethesda, MD. (#D173HC)
Viruses	**Designation**	**Source**
Baboon endogenous virus (M7)	BaEV[c]	Dr. G. Todaro, NIH, Bethesda, MD.
Gibbon ape leukemia virus	$GaLV_H$[d]	Dr. R. C. Gallo, Lab. of Tumor Cell Biol., NCI, Bethesda, MD.
Human leukemia virus (HL23V)	HL23V[e]	Drs. R. Gallagher and R. Gallo, NIH, Bethesda, MD.
Simian sarcoma virus	SiSV-1[f]	Drs. F. Deinhardt and C. Bergholz, Rush-Presbyterian-St. Lukes Med Center, Chicago, IL.

[a] Normal fetal canine thymus (Cf2Th) described by Nelson-Rees *et al.* (20).
[b] Normal rat kidney cells described by Duc-Nguyen *et al.* (21).
[c] Baboon endogenous virus (14) maintained in chronically infected dog thymus cells (20).
[d] Virus isolate ($GaLV_H$) (Gallo, R. C., Gallagher, R. E., Wong-Staal, F., Aoki, T., Markham, P. D., Schetters, H., Ruscetti, F., Valerio, M., Saxinger, W. C., Smith, R. G., Gillespie, D. H., and Reitz, M. S., Virology, in press, Feb. 1978), passed to and produced in bat lung cells (CCL88).
[e] HL23VI-1p10 (6–8) isolated and passed in dog thymus cells.
[f] SiSV (2) grown in marmoset cells and passed to and produced in bat lung cells (CCL88).

(Harelco, Phila., PA) and methanol (absolute) and rinsed with tap water. The focus forming titer of each virus was calculated from the average number of foci on cultures infected with the serially diluted virus.

Transformation assay under agar. Assays were done in cluster dishes (Falcon 3004, 35 mm × 4 wells per dish) in quadruplicate. Cells were seeded (3×10^5 per well) and allowed to incubate for approximately 18 h. Cultures were then infected and allowed to incubate for an additional 20 hr at 37° and 5% CO_2. At this time culture fluid was removed and the culture overlayed with 2.5 ml of 0.3% agar (Difco Lab., Detroit) dissolved in McCoy's 5A media supplemented with 15% fetal bovine serum. The cultures were carefully monitored for optimum focus formation for approximately 8 days.

Serum neutralization. Immune serum and virus were diluted in unsupplemented McCoy's 5A medium, mixed in equal

amounts, and incubated for 4 hr at 4° prior to adsorption to monolayers according to procedures described above. Goat anti-SiSV-1 serum raised against virus infected cells was obtained from Dr. C. Bergholz (Rush-Presbyterian-St. Lukes Medical Center, Chicago, IL.).

Viral interference assay. Cultures were infected as described above with sequential one to ten dilutions of the virus stock extending beyond the transforming endpoint of the virus. Infected cultures were subcultured at confluency three times and challenged using procedures described above with a known titer of transforming virus. A 90% reduction in the number of foci produced on the challenged cultures compared to that on control cultures was interpreted as being positive for interference.

Viral DNA polymerase assay. Culture fluids were monitored for viral DNA polymerase activity, using $(dT)_{15-18} \cdot (A)_n$ and $(dT)_{15-18} \cdot (dA)_n$ as primer-templates (7).

Results. Several cell lines were compared in their sensitivity to transformation by several primate type-C viruses. The results of one experiment are shown in Table II and are representative of data obtained in a number of experiments using the cell lines shown, as well as with several other human embryonic cell lines. Conditions needed for each virus and cell line to yield optimum transformation had been previously determined. The NRK-1 and RE-1 cell lines used in this study had been preselected for their sensitivity to transformation by SiSV-1 (R. Ting, personal communication). As seen in Table II, SiSV-1 or HL23V were able to induce foci in all of the cell lines tested, whereas $GaLV_H$ and BaEV gave no indication of transforming any of the tested cell lines as expected from previous reports (13–18). The viruses SiSV-1, HL23V, and $GaLV_H$ were able to replicate in all cell lines shown, whereas BaEV gave no indication of replicating in the NRK, RE, or FEF cells. This information was obtained by continued subculturing of infected cultures through at least five passages and assaying for the release of virus by testing for extracellular viral DNA polymerase activity.

TABLE II. COMPARISON OF THE TRANSFORMING TITER OF FOUR PRIMATE RNA TUMOR VIRUSES ON SEVERAL CELL LINES.

	Virus[a]			
Cell[a] (b)	SiSV-1 $\times 10^4$ ffu/ml	HL23V $\times 10^4$ ffu/ml	$GaLV_H$ ffu/ml	BaEV ffu/ml
FEF-1 (37)	11	4.2	0	0
NRK-1 (14)	5	1.5	0	0
RE-1 (3)	7	3.9	0	NT
PTA-11 (9)	0.1	ND	0	0
WHE-2 (14)	0.1	ND	ND	0
HFF (22)	1.2	0.9	0	0
HF (29)	0.1	0.02	0	0
1283 (22)	0.1	0.04	0	0
MFS (15)	ND	0.003	ND	ND

[a] Cells and viruses are described in Table I.
[b] Passage level.

In these preliminary studies, two observations were noteworthy. One was the difference in transforming titers of virus stocks when compared on the different cell lines. The other was the comparative rapidity of the assay when FEF-1 cells were used. In this regard, foci were completely developed and ready for counting by the third or fourth day after infection of FEF-1 cells, whereas all other cell lines required from 7 to 10 days to give maximum focus development.

To further test the apparently higher sensitivity of the FEF-1 cells to transformation by SiSV-1 and HL23V, a number of experiments were conducted comparing selected cell lines using standard stocks of virus. In Table III the transforming titer of virus on each cell line tested is expressed as a percentage of the titer obtained on FEF-1 cells used as a control in each experiment. Each percentage recorded represents an independent experiment. For example, in four independent experiments, SiSV-1 gave titers on NRK-1 cells 24, 50, 55, and 10% of those obtained using FEF-1 cells. Experiments were considered valid only when viral stocks gave the expected titer on the FEF-1 control cells, i.e., 10^4–10^5 ffu/ml for SiSV-1 and 10^3–10^4 ffu/ml for HL23V.

As mentioned above, the NRK-1 and RE-1 cultures were specifically selected because of their sensitivity to transformation by SiSV-1. This is apparent from the results shown in Table III where a number of NRK cultures and one other RE cell line was used.

To determine if the sensitivity to transfor-

TABLE III. SENSITIVITY OF SELECTED CELL CULTURES TO TRANSFORMATION BY SiSV AND HL23V RELATIVE TO FEF-1.[a]

Cell[b] (c)	Virus[b] SiSV-1 (%)	Virus[b] HL23V (%)
FEF-1 (30-45)	100	100
FEF-2 (3-5)[d]	68;63	74;75
FEF-3 (5)	3	6
FEF-4 (4-6)	ND	55;81
CCL-176 (17-20)	10;8	ND
NRK-1 (13-15)	24;50;55;10	17;33;33;38;38
NRK-2 (25-27)	0.5;0.3	16;6
NRK-3 (3-5)[d]	0.7;0.8	12;15
NRK-4 (137-142)	0.8;0.8	10;13;16;18
RE-1 (3-5)	62;45;65	150;47;93
RE-2 (4-6)	4	10;14
PTA-11 (9-12)	1.0;2.0;1.1	<1.0;<1.0
WHE-2 (12-21)	1.1;1.0;1.0	<1.0;<1.0
HFF (22-30)	11;25;11	22;42;39;19
HF (14-31)	3.6;1.2;1.1	0.5;0.7
1283 (20-25)	1.1;5.4;1.2	1.1;0.7
MFS (15-17)	ND	0.1;0.7

[a] The focus forming titer of each virus is expressed as a percent of that obtained using FEF-1 cells as the positive control in each experiment. Each percentage represents an independent experiment.

[b] Cells and viruses described in Table I.

[c] The range of passage levels used during the course of these studies.

[d] The passage level history is not known. Cultures were passaged the indicated number of times in our laboratory.

[e] ND: Not done.

mation observed for FEF-1 was generally for all feline cells, other feline cell lines were also included in our comparative transformation studies (shown in Table III). Of the five feline embryonic cell lines tested, two had a sensitivity to transformation by SiSV-1 and HL23V comparable to FEF-1.

The comparative transforming titer of SiSV-1 and HL23V on three human embryonic cell lines and three cell cultures of marmoset origin is also illustrated in Table III. With the exception of the human foreskin fibroblast (HFF) cells these cultures were approximately 100-fold less sensitive than FEF-1. The sensitivity of the HFF cell line to transformation by SiSV-1 and HL23V is substantially greater than several other primate cell lines that have been tested and has proven to be very useful in the study of these viruses.

In view of the apparent higher sensitivity of FEF cells to transformation by SiSV-1 and HL23V compared to other cell lines tested, it was important to rule out the possibility that the difference was due to nonspecific or secondary focus development. In this regard, the FEF-1 cells did not give any indication of spontaneous focus development under the conditions of our assay. Not only were numerous mock infected controls consistently negative, but also cultures treated with non-transforming viruses gave no indication of focus development (Table III). Furthermore, SiSV-1 induced focus development was completely neutralized by antiserum prepared in goats against SiSV-1 (Table IV). The virus produced by SiSV-1 transformed FEF cells retains its ability to induce foci on normal FEF cell lines (data not shown), however, it is unlikely that the development of secondary foci is responsible for the increased number of foci seen since under the conditions of our assay on FEF the optimum focus development coincides with the cells reaching confluency and both occurred very rapidly, i.e., by the third or fourth day after infection. Also, as shown in Table V, under the conditions of the focus assay used, it was not necessary to use an agar overlay to

TABLE IV. NEUTRALIZATION OF SiSV-1 INDUCED FOCI ON FEF-1 BY ANTI-SiSV-1 SERUM.[a]

Serum		Number foci[b]	% Reduction
None		77	0
Immune serum dilution	1:10	0	100
	1:100	0	100
	1:200	4	95
	1:300	54	30

[a] Viral stocks were preincubated with or without dilution of immune serum and assayed for transforming activity as described in *Materials and methods*.

[b] Average of duplicate cultures.

TABLE V. TRANSFORMATION OF FEF-1 CELLS WITH OR WITHOUT AGAR OVERLAY.

Virus[a]					
SiSV-1 × 10^4 ffu/ml[b]			HL23V × 10^2 ffu/ml[b]		
Agar		*No Agar*	*Agar*		*No Agar*
Day 3	*Day 8*	*Day 3*	*Day 3*	*Day 8*	*Day 3*
3.50	3.75	3.12	1.26	1.50	1.98

[a] Virus and cells are described in Table I.

[b] The virus titer was calculated from the average of quadruplicate dishes counted on the indicated number of days post infection.

avoid secondary or satellite focus development.

Discussion. A number of cell lines were compared for efficiency of transformation by SiSV-1 and HL23V. The assay was consistently more rapid and sensitive with feline embryonic cells than with rat (NRK and RE) or several human and marmoset cell lines. The results obtained with FEF-1 cells are apparently not unique to one specific feline embryonic cell line since two other cultures gave comparable results. Feline embryonic cells have been previously reported to be four times more sensitive than marmoset cells to transformation by feline sarcoma virus (22).

The basis for the enhanced sensitivity of feline embryonic cells to transformation by SiSV-1 and HL23V is not known, but it is likely related to relative differences in susceptibility to infection by the viruses, rather than a difference in expression of viral transforming information. This is implied by the data in Table VI, which demonstrates that the titers of both the transforming and nontransforming components of SiSV-1 are shifted proportionally downward on human embryonic cells (PTA-11) compared to FEF-1 cells.

The mechanisms responsible for the observation that feline and rat cells are more sensitive than are the other cells studied to infection and/or transformation by some type-C viruses are not known, but a contributing factor could be that they both contain inducible endogenous viral information (23).

Both SiSV-1 and HL23V behaved in a similar manner in transformation experiments with respect to time needed for maximum focus development and morphology of the induced foci. The foci induced by HL23V or SiSV-1 were densely packed, stellate arranged, and fusiform (Fig. 1). On the other hand, foci induced on feline embryonic cells by feline sarcoma virus, are reported to have a distinctly different morphology (less dense, flat, round, or fusiform [22]). Another similarity between the viruses (Table VI) is that the non-transforming components of both SiSV-1 and HL23V are present at an approximately two logs higher titer than the transforming component. The same is also true for SiSV-1 tested on PTA-11 cells. A possible difference between the two viruses (SiSV-1 or HL23V) was, however, seen in their transformation behavior on rat cells compared to that on FEF cells (Table III). With the exception of the NRK-1 cultures, HL23V was consistently more efficient in its ability to transform the rat cell lines relative to FEF-1 than was SiSV-1.

In conclusion, transformation assays using FEF cells offers several advantages over other commonly used cell lines. The rapidity of focus development, i.e., 3–4 days versus 7–10 days, could facilitate progress in experiments involving the use of large numbers of focus assays. Also, the increased sensitivity of the cells to infection by type-C viruses could be critical when low levels of viral activity may be present. Finally, FEF cells have also been found to be target for a mixed cell syncytial (24, 25) assay with XC cells (our personal observations). This, therefore, permits the assay and study

TABLE VI. TITER OF TRANSFORMING AND NONTRANSFORMING ACTIVITIES OF SiSV-1 AND HL23V.[a]

		Initial SiSV-1 dilution							Initial HL23V dilution				
Cell[b] (c)	Activity	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
FEF-1 (35)	Transformation[d]	+	+	+	+	0	0	0	+	+	+	0	0
	Interference[e]					+	+	0				+	+
PTA-11 (10)	Transformation[d]	+	+	0	0	0							
	Interference[e]			+	+	0							

[a] Serial tenfold dilutions of each virus was used to infect cell cultures. Transformed cultures were noted and all cultures were subcultured three times, before challenging with a known transforming titer of virus.

[b] Cells and viruses are described in Table I.

[c] Passage level.

[d] + indicates cultures containing viral induced foci; 0 indicates no transformation.

[e] + indicates at least 90% reduction of foci in challenged cultures compared to control cultures, i.e., positive interference; 0 indicates transformation comparable to control cultures, i.e., no interference.

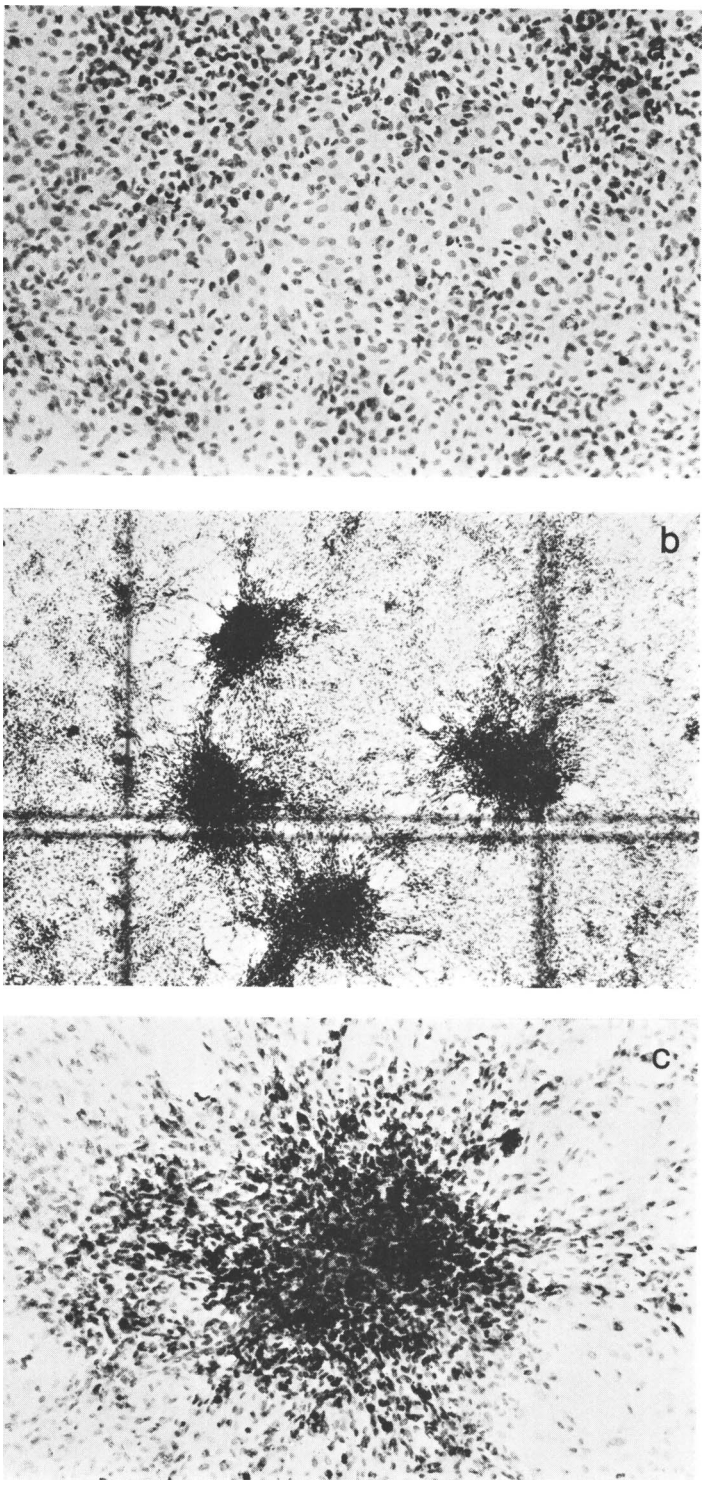

FIG. 1. Normal and HL23V transformed feline embryonic fibroblast cells. FEF-1 were infected with HL23V virus and stained with giemsa methanol as described in *Materials and methods*. Uninfected FEF-1 cells, ×410 (a), transformed FEF-1 cells, ×120 (b), and ×410 magnification (c).

of transforming and nontransforming virus titers on the same culture. Rat cell lines have been used in XC syncytial assay with these and other mammalian viruses (7, 25) but have not been suitable, in our hands, for use with these viruses.

Summary. Feline embryo fibroblast cells were found to be very sensitive to transformation by the primate RNA tumor viruses, simian sarcoma virus (SiSV-1) and HL23V, a virus isolated from the leukemic myeloblasts of an AML patient. *In vitro* transformation of feline embryo fibroblast cell cultures with these viruses was 2 to 100 × more efficient than when performed on the normal rat kidney (NRK) or rat embryo fibroblast cell lines commonly used for SiSV-1 transformation studies. Several cell lines of marmoset and human origin were also observed to be less sensitive to transformation by these viruses. Furthermore focus development on feline embryonic fibroblast cultures required only 3–4 days compared to 7–10 days needed using rat or primate cultures.

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