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Characterization of Sarcoma-Positive, Leukemia-Negative (S^+L^-) Human Cells Induced by the Feline Leukemia Virus Pseudotype of Moloney Sarcoma Virus (41221)

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Abstract. Human osteosarcoma clonal cells (TE-85 clone F-5) can be readily transformed by the feline leukemia virus pseudotype of Moloney sarcoma virus [Mo-MSV (FeLV)]. Among a number of foci isolated at limiting Mo-MSV (FeLV) dilution four clones of sarcoma-positive, leukemia-negative (S^+L^-) cells were selected and characterized. These clones were negative for infectious virus and type C virus particles; however, they contained a rescuable MSV genome and murine leukemia virus (MuLV)-specific antigen, such as the *gag* gene products, p15, p12, and p30, but lacked the *env* gene protein (gp 70). S^+L^- clones were all tumorigenic in nude mice. The Mo-MSV rescued by baboon type C virus (M-7), Mo-MSV(M-7), produced foci readily in human, NRK, and NIH 3T3 mouse embryo cells. Human cells transformed by Mo-MSV(M-7) were found to be virus producers; whereas Mo-MSV (M-7)-transformed NRK and NIH 3T3 mouse embryo cells were found to be S^+L^- cells. These rat- and mouse-transformed cells were negative for infectious virus but they contained a rescuable MSV genome and MuLV antigen.

Human cells can be transformed *in vitro* by murine sarcoma virus (MSV) and such cells usually contain both infectious virus and group-specific murine leukemia virus (MuLV) antigen (1–3). We have previously shown that nonproducer (NP) human cells could be isolated from human osteosarcoma (HOS) clonal cells transformed by the Kirsten strain of MSV (Ki-MSV). These NP cells produced neither infectious virus nor MuLV antigen, but they contained the MSV genome which could be rescued by superinfection with MuLV (4). Since the sarcoma-positive, leukemia-negative (S^+L^-) human cells were isolated from human amnion cells transformed by the feline leukemia virus pseudotype of Moloney sarcoma virus [Mo-MSV(FeLV)] (5), we have studied the interaction of Mo-MSV(FeLV) genome and human HOS cells.

We now report the isolation and characterization of S^+L^- human cells induced by Mo-MSV(FeLV). These human S^+L^- cells do not release infectious virus and are negative for type C virus particles. However, they do contain a rescuable MSV genome and express MuLV-specific antigens such as the *gag* gene products, p15, p12, and p30, but lacked the *env* gene protein (gp 70). S^+L^- human clones were all tumorigenic in nude mice. The MSV rescued by baboon type C virus (M-7), Mo-MSV(M-7), produced foci readily in human, NRK, and NIH 3T3 mouse embryo cells. Human cells transformed by Mo-MSV(M-7) were found to be virus producers; whereas Mo-MSV(M-7)-transformed NRK and NIH 3T3 mouse embryo cells were found to be S^+L^- cells.

Materials and Methods. The human osteosarcoma (HOS) cell line TE-85, clone F-5, used in this study has been described in detail (4). Its growth pattern is relatively regular and flat so that transformed foci are readily recognized. This line was found to be highly susceptible to transformation by Ki-MSV(4). Continuous cell lines of NRK, NIH 3T3 mouse embryo and a human skin fibroblast (PC-109) were also used. Cells were grown and maintained in Eagle's minimum essential medium (EMEM) with 10% fetal bovine serum (FBS), 2 m*M* glutamine, 100 units of penicillin, and 100 μg of streptomycin/ml (EMEM + 10% FBS).

The Mo-MSV(FeLV) pseudotype stock [supernatant fluids from a Mo-MSV(FeLV)-transformed Crandell's cat cell (CCC) clone P-521 line (6)] was kindly supplied by

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Dr. A. E. Frankel, National Cancer Institute, Bethesda, Maryland. Twenty-four-hour culture fluid harvests of P-521 cells yielded more than 10^5 focus-forming units of Mo-MSV(FeLV) per milliliter when assayed in feline embryo cells and contained a four-fold excess of Mo-MSV(FeLV) over FeLV (5). Mo-MSV(FeLV) has been shown to be capable of efficient infection of susceptible human cells (7). The M-7 baboon endogenous virus (8) isolated from a baboon (*Papio cynocephalus*) placenta, was grown in rhadbomyosarcoma (A-204) cells and had a titer of 10^6 focus-inducing units/ml when assayed on S^+L^- human amnion cells (6).

A combination of the following procedures was used to detect the replication of the virus in cultures as described previously (4) (a) a focus-forming assay for sarcoma virus; (b) an assay for CF antigen reactive with specific type C leukemia virus gs antigen; (c) an assay for virion-associated reverse transcriptase activity in tissue culture fluids; (d) examination by electron microscopy for the presence of virus particles. CF tests were carried out by the microtiter technique (4). Titers were recorded as the reciprocals of the highest dilution given 3+ to 4+ fixation of 1.8 units complement.

The MSV genome rescue experiment was carried out by superinfection of S^+L^- cells with helper type C virus. Supernatants from these cultures taken 14 days postinfection and were assayed on NRK, NIH 3T3 mouse embryo, and human skin (PC-109) cells.

For soft agar assay cell suspensions were diluted in 0.3% agar (Difco) in growth medium and added to 2 ml of a prehardened base containing 0.5% agar in growth medium. After 2 weeks, colony formation was measured with an inverted microscope.

Radioimmunoassays for Moloney-MuLV p15, p12, p30, and gp70 structural proteins as well as FeLV p30 and gp70 viral gene products were performed by methods previously described (9).

Transformed and untransformed cell lines were tested for their ability to produce tumors in NIH nude mice after subcutaneous inoculation with 5×10^6 cells.

Results. The Mo-MSV(FeLV) stock was filtered through a 0.45-μm membrane filter and inoculated into approximately 24-hr-old human cells. By Day 9, small discrete foci of transformed cells were observed (Figs. 1B and C). On Day 14, the titer was determined to be $10^{3.0}$ focus-forming units (FFU)/ml; and at a terminal dilution on the plate, a single focus was isolated by the cloning cylinder technique and subcultured. A number of focus-derived lines were established. A majority of the focus-derived lines continued to show the altered morphology (Fig. 1D) and contained FeLV gs antigens and demonstrated reverse transcriptase activity. However, two altered clones (FNR-85-1 and FNR-85-5) and two flat clones (FNR-85-2 and FNR-85-3) showed no evidence of virus production (Table I). Altered clones (FNR-85-1 and FNR-85-5) (Figs. 2A and D) contained spindle-shaped or stellar cells and three-dimensional clumps of round cells as well as many multinucleated giant cells which differed from the uninfected, control HOS cells; whereas flat clones (FNR-85-2 and FNR-85-3) were stable for eight subcultures (for 60 days) in their fibroepithelial-like morphology (Figs. 2B and C) resembling untransformed HOS cells (Fig. 1A). However, after 10 passages flat clones lost their stability and became morphologically altered. Attempts to detect FeLV gs antigen in these clones by the CF test were repeatedly unsuccessful. Assays for supernatant viral polymerase were also negative. Attempts to demonstrate cell-transforming activity by cell-free preparations of these focus-derived human cells were negative when assayed on human and NRK cells. Clonal lines were examined in thin section by electron microscopy for the presence of viral or type C virus particles. Repeated examination showed no evidence of viral production or maturation. All nude mice inoculated subcutaneously with morphologically altered clones developed tumors within 2 weeks. The tumors were poorly differentiated sarcomas. No tumors developed within 60 days in nude mice inoculated with untransformed HOS cells (Table I).

Since mammalian cells transformed by the genetically stable "S^+L^-" type Mo-MSV genome (10) maintain the MuLV group-specific gs-1 antigens in the absence of any detectable leukemia virus (6, 11, 12),

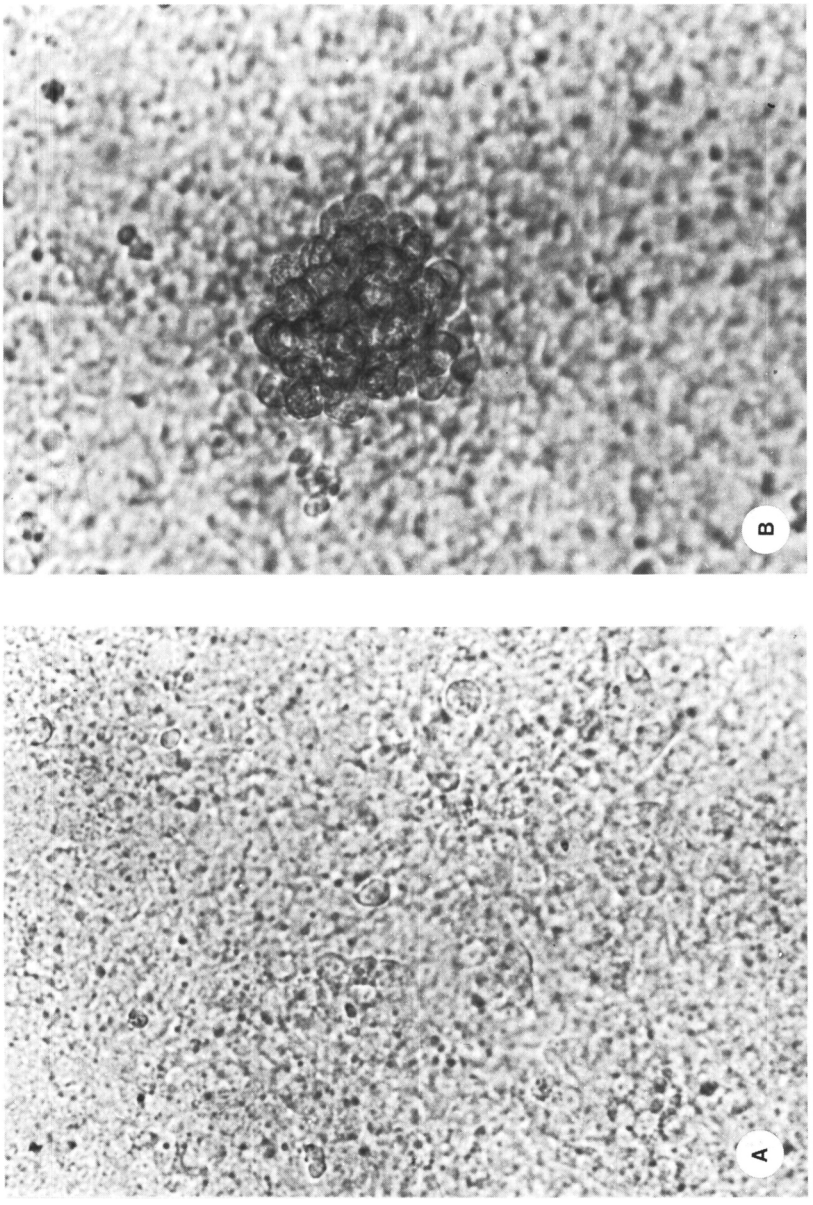


FIG. 1. Transformed foci induced by Mo-MSV(FeLV) in human osteosarcoma (HOS) cells. (A) Uninfected control cells (×60). (B) A focus induced by Mo-MSV(FeLV) on Day 9 after infection (×60). (C) Same as B at higher magnification (×120). (D) Foci in HOS cells (×32).

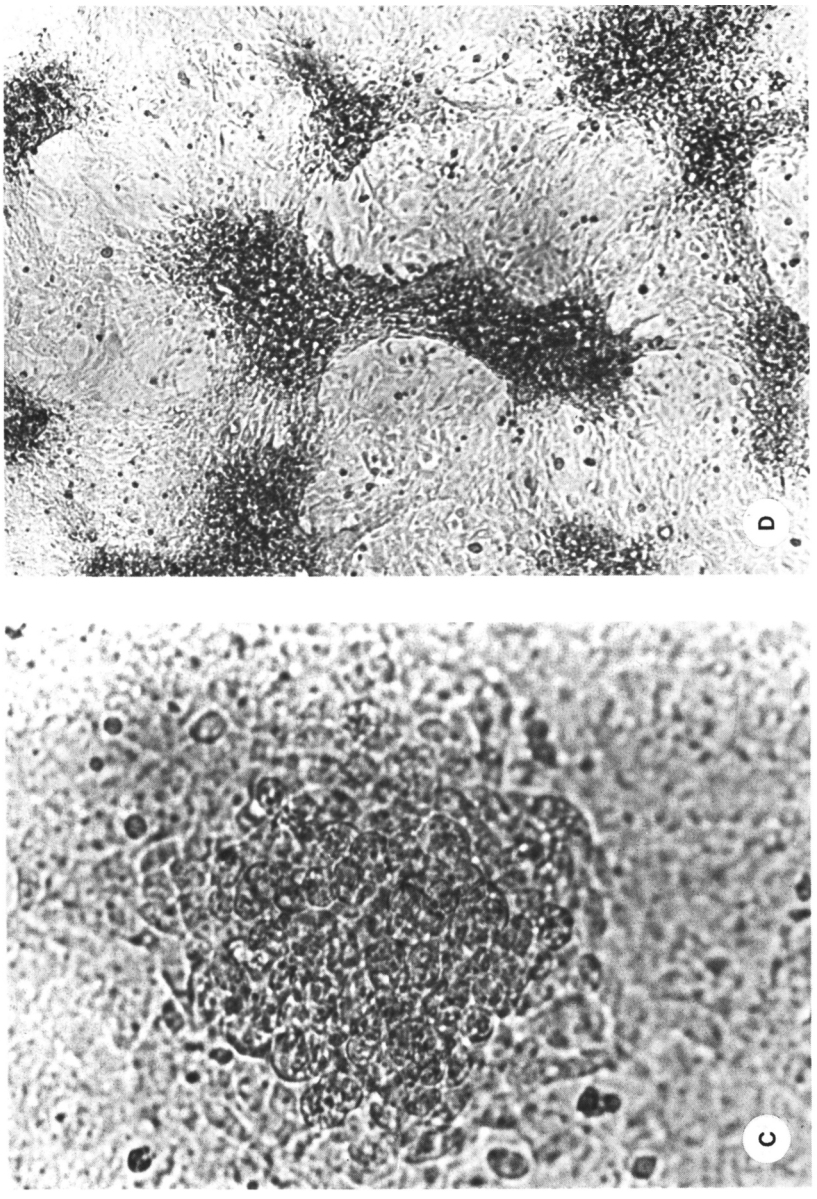


FIG. 1—*Continued.*

TABLE I. PROPERTIES OF CLONES OF S^+L^- HUMAN CELLS TRANSFORMED BY MO-MSV (FeLV)

Clonal line	Morphology	Saturation density[a] ($\times 10^5$/cm^2)	Type C virus particles	Reverse transcriptase[b]	MSV rescued by type C virus	Tumorigenicity in nude mice[c]
HOS	Flat	1.1	Negative	Negative	None	Negative
FNR-85-Cl 1	Transformed	2.6	Negative	Negative	Present	Positive
FNR-85-Cl 2	Flat	1.9	Negative	Negative	Present	Positive
FNR-85-Cl 3	Flat	1.8	Negative	Negative	Present	Positive
FNR-85-Cl 5	Transformed	2.8	Negative	Negative	Present	Positive

[a] Maximum number of cells obtained after initial planting 5×10^3 cells/cm^2 and then incubated at 36° under conditions where growth medium was changed every 3 days.

[b] RNA-dependent DNA polymerase activity was measured by incorporation of [^{3}H]thymidine triphosphate into acid-precipitable materials in 100 times concentrated culture supernatant. (4).

[c] Five million cells inoculated into each nude mouse.

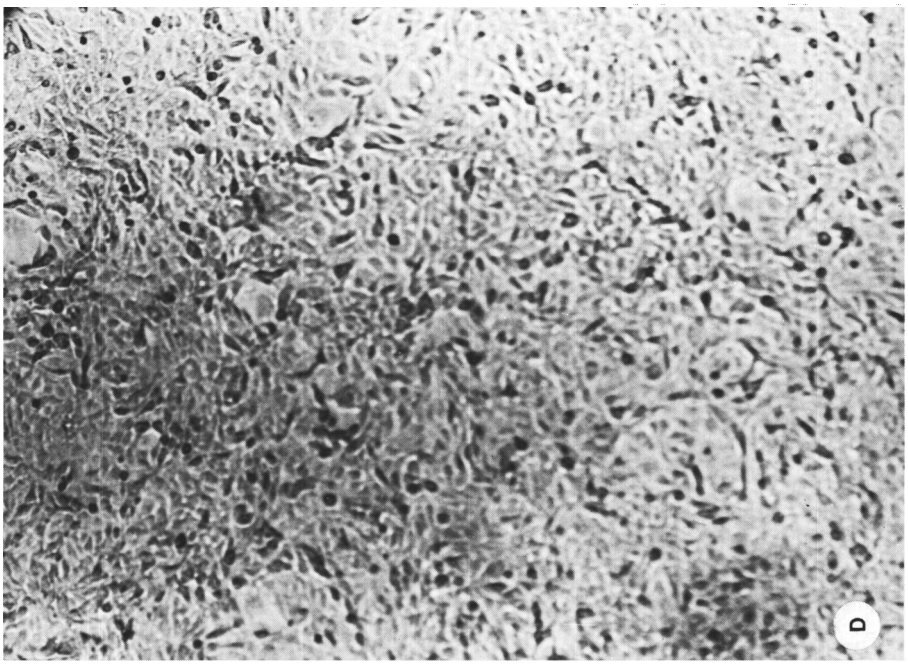
the presence of this antigen in clones of S^+L^- human cells would be evidence for the presence of the Mo-MSV genome. Therefore, expression of helper type C virus structural proteins in S^+L^- human clonal cells was examined utilizing radioimmunologic techniques (Table II). High levels of Moloney-MuLV *gag*-gene-coded p15, p12, and p30 proteins were found in all tested S^+L^- human clone cells. In contrast, no expression of Moloney-MuLV *env*-gene-coded gp70 glycoprotein nor FeLV structural antigens p30 and gp70 could be detected.

To determine whether S^+L^- clones still harbored the Mo-MSV genome, cells were infected with helper type C baboon virus (M-7). Supernatants from infected cells were taken at 14 days, filtered, inoculated into the cells of variety of species, and examined for the presence of foci. Superinfection of M-7 virus readily rescued focus-forming MSV from these S^+L^- clones (Table III). The Mo-MSV rescued by M-7, Mo-MSV(M-7), produced foci on human, NRK, and NIH-3T3 mouse cells. Both human and NRK cells were transformed by Mo-MSV(M-7) at similar titers. Mo-MSV(M-7) was also able to transform NIH 3T3 mouse embryo cells.

Since baboon M-7 virus has been known not to replicate in and transform rat and mouse cells (13) Mo-MSV(M-7)-transformed NRK and Mo-MSV(M-7)-transformed NIH 3T3 mouse embryo cells were propagated and further characterized. The Mo-MSV(M-7)-transformed NRK and NIH 3T3 mouse lines were found to be S^+L^- cells. The transformed lines did not release infectious virus although cellular alterations were clearly observed but contained MuLV antigen. Culture fluids from these S^+L^- lines were negative when tested for supernatant reverse transcriptase activities and for focus formation on NRK and NIH 3T3 mouse cells. The presence of the sarcoma genome in S^+L^- cells could be readily detected by superinfection with helper type C Moloney-MuLV (Table IV). Supernatants from infected cultures taken at 7 days were passed through a 0.45-μm HA filter, inoculated into fresh NRK cells, and examined for the presence of foci. The Mo-MSV rescued by Moloney-MuLV, Mo-MSV(M-MuLV), produced foci readily in NRK cells (Table IV).

Discussion. The present results above show that S^+L^- human HOS clones can be isolated from transformed foci induced by a virus stock containing Mo-MSV(FeLV) in excess of helper FeLV. These clones did not release infectious virus or demonstrate type C virus particles. However, they contained a rescuable MSV genome and expressed MuLV antigens. Thus the S^+L^- human HOS cells described here closely resemble the S^+L^- human amnion cells induced by Mo-MSV(FeLV) (5). As previously reported, MSV has been shown to produce three classes of transformed cells (2, 4, 5). These are: (a) MSV producer cells that result from coinfection by MSV and MuLV, (b) MSV NP cells which are transformed by Ki-MSV in the absence of

FIG. 2. Comparison of the morphology of focus-derived clonal lines of Mo-MSV (FeLV)-transformed HOS cells (×32). (A) FNR-85 clone 1. Altered morphology. (B) FNR-85 clone 2. Flat morphology. (C) FNR-85 clone 3. Flat morphology. (D) FNR-85 clone 5. Altered morphology.



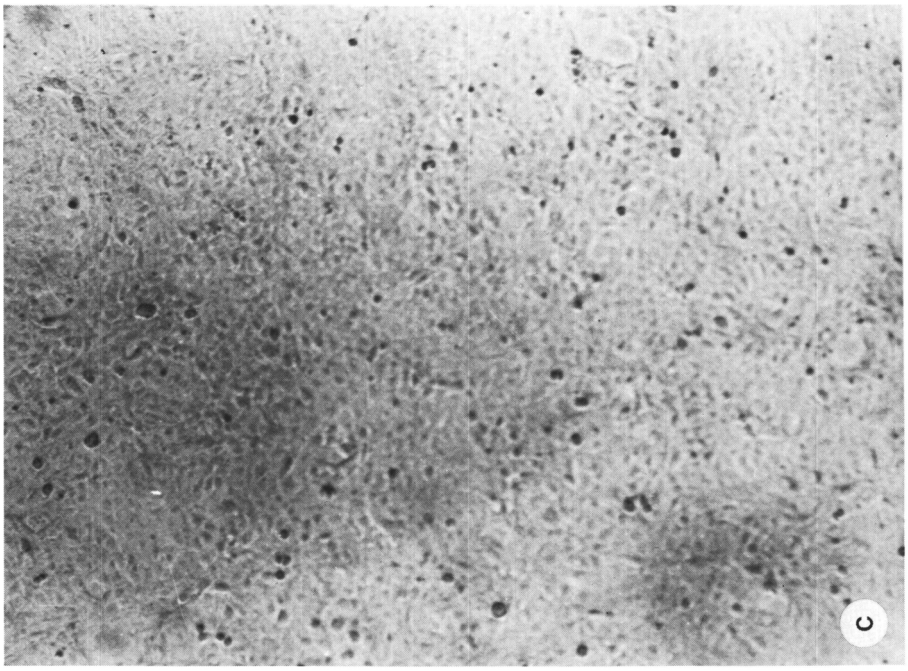


FIG. 2—*Continued.*

TABLE II. EXPRESSION OF HELPER TYPE C VIRUS STRUCTURAL PROTEINS IN CLONES OF S$^+$L$^-$ HUMAN CELLS TRANSFORMED BY MO-MSV (FeLV)

	Level of type C viral proteins (ng of protein per milligram of cell extract)					
	Moloney-MuLV				FeLV	
Cell line	p15	p12	p30	gp70	p30	gp70
HOS	<10	<10	<10	<20	<10	<20
FNR-85-Cl 1	320	280	450	<20	<10	<20
FNR-85-Cl 2	300	300	190	<20	<10	<20
FNR-85-Cl 3	640	560	490	<20	<10	<20
FNR-85-Cl 5	2200	1850	2330	<20	<10	<20

Note. Postmicrosomal fractions of detergent-disrupted cells were tested at twofold serial dilutions in homologous Moloney-MuLV p15, p12, p30, and gp 70 and FeLV p30 and gp70 radioimmunoassays as described under Materials and Methods. Results were calculated on the basis of the displacement of the complete competition curve for the unknown relative to that achieved with the corresponding purified viral antigen. Protein concentrations were determined according to Lowry *et al.* (21), using bovine serum albumin as standard.

MuLV and lack any evidence of virus production or MuLV antigen. The sarcoma genome in such cells can be rescued by superinfection with MuLV; (c) S$^+$L$^-$ cells which are transformed by Mo-MSV and do not release infectious sarcoma virus except upon superinfection with MuLV. S$^+$L$^-$ cells do release noninfectious virus particle (11) and contain MuLV antigen (12). These latter properties distinguish them from Ki-MSV NP cells. S$^+$L$^-$ transformants have been made in mouse (11) and human cells (5). However, S$^+$L$^-$ human amnion (5) cells as well as S$^+$L$^-$ human HOS cells described here are different from S$^+$L$^-$ mouse cells in that S$^+$L$^-$ human cells do not release detectable type C particles. NP human cells were isolated from foci induced by Ki-MSV in HOS cells (4) but attempts to isolate S$^+$L$^-$ cells from foci induced by Ki-MSV in HOS cells were unsuccessful. Thus, the success of isolating of S$^+$L$^-$ cells is dependent on the strain of MSV used in the experiment.

Expression of Moloney-MSV genetic information in clones of human cells transformed by the S$^+$L$^-$ strain of Moloney-MSV was presented (Table II). Moloney-MSV originated by recombination between replication-competent Moloney-MuLV with a subset of cellular sequences (2, 9, 14, 15). The *gag* gene helper viral sequences

TABLE III. RESCUE AND HOST RANGE OF MO-MSV RESCUED FROM S$^+$L$^-$ CLONES BY SUPERINFECTION WITH M-7 VIRUS

		Virus titers (FFL/ml) of rescued MSV assayed[a]		
Clonal cell	M-7 superinfection	Human PC-109[b]	Rat NRK	Mouse NIH 3T3
FNR-85-Cl 1	Yes	2.0×10^4	3.2×10^4	1.2×10^2
	No	0[c]	0	0
FNR-85-Cl 2	Yes	2.2×10^4	2.5×10^4	1.1×10^2
	No	0	0	0
FNR-85-Cl 3	Yes	2.0×10^4	2.2×10^4	1.2×10^2
	No	0	0	0
FNR-85-Cl 5	Yes	2.4×10^4	3.4×10^4	1.0×10^2
	No	0	0	0

[a] M-7 virus was inoculated into 1-day-old human S$^+$L$^-$ clones. Supernatant from infected S$^+$L$^-$ cells were taken at 14 days, filtered, inoculated into various cells, and examined for the presence of foci.
[b] Human skin fibroblasts (PC-109):
[c] 0 indicates that the foci were not detected in 0.2 ml of undiluted filtered virus preparation.

TABLE IV. RESCUE OF MSV GENOME FROM MO-MSV(M-7) TRANSFORMED NRK AND MO-MSV(M-7) TRANSFORMED NIH 3T3 MOUSE EMBRYO CELLS

Cell	Moloney-MuLV superinfection	Induction of foci after inoculation into NRK cells[a]
Mo-MSV(M-7)/NRK[b]	Yes	>200[c]
	No	0
Mo-MSV(M-7)/NIH 3T3[b]	Yes	>200
	No	0

[a] Moloney-MuLV was inoculated into (1-day-old MSV(M-7) transformed NRK- and MSV(M-7)-transformed NIH 3T3 mouse embryo cells. Supernatants from these infected cells were taken at 7 days, filtered, inoculated into NRK cells, and examined for the presence of foci.

[b] These cells contained MuLV antigen.

[c] Average number of foci/plate; 0 indicates no foci in two petri dishes inoculate with undiluted virus.

present in the S$^+$L$^-$ strain of Moloney-MSV are known to be expressed at high levels in infected cells (2, 9, 15). However, expression of these sequences does not seem to be related to malignant transformation (16–19). Because the translational product of the transforming gene of Moloney-MSV has not been identified, expression of Moloney-MuLV *gag*-gene-coded proteins in cells transformed by S$^+$L$^-$ Moloney-MSV is a convenient genetic marker to determine presence and active transcription of the sarcoma virus genome.

It has been shown that a M-7 virus isolated from baboon placental tissue had helper activity as demonstrated by rescue of Mo-MSV from S$^+$L$^-$ human amnion cells (5) and the host range study of the rescued Mo-MSV(M-7) indicated that Mo-MSV-(M-7) was able to replicate in and transform human and dog cells but was unable to transform mouse, rat, and cat cells (13). However, evidence is presented here to show that Mo-MSV rescued from human S$^+$L$^-$ HOS clones by superinfection with M-7 virus, Mo-MSV(M-7), transformed human, rat, and mouse cells. As shown in Table 2, focus formation by Mo-MSV(M-7) was comparably efficient in NRK and human skin cells. In fact, NRK cells were a little more sensitive. NIH 3T3 mouse embryo cells were also sensitive even though about 200-fold less sensitive compared to human and NRK cells. The ability of the rescued Mo-MSV(M-7) to transform NRK and mouse cells had not been reported (13). Thus, the present results indicated that a M-7 virus can penetrate into rat and mouse cells as shown by Schnitzer *et al.* (20) in their host range study with VSV(M-7).

In addition, it is interesting to note that both Mo-MSV(M-7)-transformed NRK and NIH 3T3 mouse lines were found to be S$^+$L$^-$ cells (Table IV). These results indicate that a M-7 virus can be able to penetrate into rat and mouse cells but is unable to replicate on these cells; restriction of M-7 virus probably occurs only after penetration. These data indicate also that S$^+$L$^-$ cells can be isolated from rat and mouse cells by means of a simple Mo-MSV(M-7) virus infection and subcultivation. Most of the reported MSV S$^+$L$^-$ or NP transformants have been isolated either by cylinder techniques (4, 5, 10) or by agar suspension cultures (11) using an endpoint dilution technique.

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