*RESCUE OF THE DEFECTIVE MURINE SARCOMA VIRUS GENOME BY RADIATION-INDUCED LEUKEMIA VIRUS FROM C57BL MICE**

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In 1959 Lieberman and Kaplan[1] reported the isolation of a transmissible leukemogenic agent from radiation-induced lymphomas in C57Bl mice. Several subsequent studies investigated the mechanism of lymphoma induction with this virus, including the roles of radiation,[2–5] thymic dependency,[6–8] host susceptibility,[9, 10] and its natural occurrence in the C57Bl strain.[10, 11] However, precise virologic studies have been hampered by the inability to isolate and quantitate this virus *in vitro*. Virus activity as determined by lymphoma induction in mice requires a latent period of several months, although intrathymic inoculation of newborn C57Bl mice may shorten the latent period to three months.[8] Attempts to utilize the method of Hartley *et al.*[12] for the culture of mouse leukemia viruses in mouse embryo fibroblast (MEF) cells with the production of complement-fixing (CF) antigen as the endpoint were unsuccessful.

A possible new approach to the problem presented itself with the identification of two murine sarcoma viruses,[13, 14] both isolated from sarcomatous tumors arising in animals previously inoculated with Moloney leukemia virus (MLV). Hartley and Rowe[15] demonstrated that extracts of such sarcomas were capable of inducing altered foci in mouse embryo cultures, and that focus formation could be used to determine sarcoma virus titer. Their studies led them to conclude the agent was a defective sarcoma particle–leukemia virus complex similar to the Rous sarcoma–avian leukosis system. Huebner *et al.*[16] found that mouse sarcoma virus (MSV) induced nonproducer (NP) hamster tumors whose cells contained the defective mouse sarcoma genome, but not infectious sarcoma or leukemia virus and no mouse leukemia CF antigen. The defective sarcoma genome could be rescued from mixed cultures of hamster tumor NP cells and normal mouse embryo cells by the addition of one of a variety of mouse leukemia helper viruses. The resultant infectious mouse sarcoma virus had the serotypic specificities of the particular leukemia virus employed in the rescue, indicating the coating of the sarcoma genome by the leukemia virus envelope.

This study demonstrates that, under the proper conditions, the Kaplan radiation-induced leukemia virus (KLV) can act as a helper virus in the rescue of the hamster tumor cell MSV genome and that the Kaplan pseudotype can be quantitated by *in vitro* assay.

Materials and Methods.—Virus: Clarified 20% extract of lymphomatous thymus, spleen, and lymph node from C57Bl/Ka mice inoculated with radiation-induced leukemia virus was obtained from Dr. M. Lieberman, Stanford Univ., Palo Alto, Calif. The virus was serially passaged in newborn C57Bl/6 mice (Cumberland View Farms, Clinton, Tenn.) as a 10% cell-free (Millipore filter, 1.2 and 0.8 μ) thymus and lymph node extract. A primary thymic lymphoma was transplanted subcutaneously in weanling C57Bl/6 mice which developed large localized lymphomas in 14–18 days. A 10% clarified extract of second transplant subcutaneous lymphomas was made with Hanks' balanced salt solution (HBSS) containing 300 units penicillin-streptomycin/ml and 20%

tryptose phosphate broth. The stock KLV extract was stored at −70°C until used in the experiments.

Cell culture: Cultures of NP MSV hamster tumor cells were derived from a primary tumor induced by MSV. P_{25} cells employed in these experiments were shown to contain the defective MSV genome by the recovery of infectious MSV from mixed cultures of the hamster tumor cells and MEF cells infected with MLV. Primary MEF cultures were prepared from 16-day gestation C57Bl/6 embryos. Mixed cultures in 32-oz prescription bottles were made by seeding with 2×10^6 hamster tumor cells and 2×10^6 C57Bl MEF cells. Growth medium was Eagle's minimal essential medium (EMEM) with glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heated (56°C, 30 min) fetal bovine serum (Microbiological Assoc., Inc.).

After 24 hr the medium was discarded and the mixed cultures were inoculated with 1 ml of stock KLV lymphoma extract. The bottles were incubated at 37°C for one-half hour and medium was added. Maintenance medium for all experiments was EMEM with 5% fetal bovine serum. The cultures were fed twice weekly and passed when fully sheeted. The hamster tumor cells tended to overgrow the mouse cells, and additional primary C57Bl MEF cells were added to the cultures on days 4, 10, and 17. The mixed cultures were harvested on day 20 and 2×10^6 cells inoculated subcutaneously into newborn C57Bl/6 mice.

Assay of tissue culture preparations and animal tumor extracts for MSV focus formation on MEF cultures was performed according to the method of Hartley and Rowe.[15] Plastic Petri dishes, 60 mm (Falcon Plastics, Los Angeles, Calif.), were seeded with 350,000 primary MEF cells and infected the following day with either 0.4 ml tissue culture virus or 0.1 ml 10% tumor extract. Titrations were performed in twofold dilutions and the titers expressed as the number of focus-forming units (ffu)/volume of inoculum. Cultures were maintained in 5% CO_2 humidified atmosphere at 37°C and foci were counted on days 5 and 7.

Antisera: Cell-free 10% extract of KLV-induced thymic lymphoma was injected subcutaneously into newborn C57Bl/6 mice in 0.05-ml amounts. The mice were bled orbitally 6 weeks post inoculation. Individual sera were titered for CF antibody to the homologous KLV lymphoma antigen, and the sera titering 1:80 or greater were pooled. Pooled sera at 1:10 dilution did not react with normal spleen, thymus, and lymph node pools from C57Bl/6 mice, nor with Rauscher, Moloney, or Friend leukemia tissue culture or tumor antigens. Borderline CF reactivity at 1:10 serum dilution was obtained with an antigen prepared from a transplantable Gross leukemia virus-induced rat lymphoma.

Antiserum from W/Fu × BNF_1 rats immunized with W/Fu rat leukemias induced by C58 mouse thymic tissue was obtained from Dr. Lloyd Old, Sloan-Kettering Institute for Cancer Research, New York. This serum demonstrated high-titered CF antibody to the group reactive antigens of Gross, AKR, Friend, Moloney, and Rauscher viruses. The serum also contained high-titered neutralizing antibody to Gross and AKR, but had eightfold lower titer to the Friend, Moloney, and Rauscher viruses.

Serum obtained from Fischer rats carrying subcutaneous transplants of virus-induced MSV (Moloney) sarcomas contained high-titered CF antibody to the group-reactive mouse leukemia antigens.[17] This serum had a tissue culture neutralization titer of 1:80 to 1:160 against Friend, Moloney, and Rauscher leukemia viruses and fourfold lower titer against AKR and Gross viruses.

Antiserum to AKR virus was pooled sera from Fischer rats carrying a transplantable lymphoma originally induced by a Swiss MEF tissue culture virus isolate from plasma of a nonleukemic AKR mouse. This pool demonstrated a neutralization titer of 1:160 against AKR virus and <1:20 against Rauscher, Moloney, Friend, and Gross viruses.[18]

Cell culture and animal neutralization tests were performed with a standard virus dose incubated with dilutions of immune and control sera for 30 min at room temperature and then inoculated into MEF cultures, or newborn C57Bl/6 mice intramuscularly.

MSV focus reduction tests for neutralizing antibody to the Rauscher, Moloney, Friend, Gross, and AKR strains of leukemia virus were performed by Dr. Janet W. Hartley, using the respective pseudotype viruses prepared by previously described methods.[16]

Results.—Production of infectious Kaplan leukemia-mouse sarcoma virus (KL-MSV): The conditions under which KLV could act as a helper virus for rescue of the defective MSV genome appeared quite restricted as compared to the other

mouse leukemia helper viruses. Hamster tumor cells containing the defective MSV genome (HTMSV cells) were suspended in KLV lymphoma extract and 2×10^6 cells were inoculated subcutaneously into newborn C57Bl mice; nodules formed at the site of inoculation but they did not contain infectious KL-MSV and subsequently regressed. HTMSV cells were inoculated directly into a primary KLV-induced thymic lymphoma and into subcutaneously transplanted KLV lymphomas; in neither case did sarcomas or infectious KL-MSV develop.

Mixed cultures of NP hamster tumor cells and C57Bl MEF cells infected with KLV were cultivated for 20 days, harvested, and 2×10^6 cells were transplanted subcutaneously into newborn C57Bl/6 mice. Tumors developed within 10 days in 100 per cent of the mice. However these mixed cell-induced tumors, transplanted as mince or inoculated as 10 per cent tumor extract in HBSS into newborn C57Bl mice, did not produce tumors. Therefore, from a second group of primary tumors, a tumor was harvested 8 days after the mixed culture cell transplantation and disrupted in a TenBroeck grinder with 10 per cent KLV lymphoma extract employed as diluent instead of HBSS. This homogenate was inoculated in 0.05-ml amounts subcutaneously into newborn C57Bl mice and induced tumors in 9/9 mice with a mean latent period of 18 days. By the fourth animal passage, cell-free tumor extracts made with KLV were highly oncogenic, and by the seventh animal passage cell-free extracts in HBSS caused tumors in 100 per cent of the mice with a mean latent period of 13 days. The passage history of the KL-MSV pseudotype is summarized in Table 1. Concentration and purification of the tumor extract on a gram equivalent basis according to the Moloney procedure,[19] together with intramuscular inoculation, shortened the mean latent period to 8 days.

Uninfected mixed cultures of HTMSV cells and C57Bl MEF cells were treated in the same fashion as cultures infected with KLV. Inoculation of 2×10^6 of these cells into newborn C57Bl/6 mice resulted in small soft nodules at the site of in-

TABLE 1

TUMOR PRODUCTION BY KL-MSV PSEUDOTYPE VIRUS IN NEWBORN C57BL MICE

Inoculum	Virus titer (ffu/0.1 ml)	Animal passage	Route of inoculation	Tumor incidence	Mean latent period (days)
HTMSV cells + C57 MEF + KLV*	$<10^0$	P_0	SQ	2/2	8
Clarified 10% P_0 tumor ext. in KLV†	$10^{0.5}$	P_1	SQ	9/9	18
Clarified 10% P_1 tumor ext.	—	P_2	SQ	5/6	18
Clarified 10% P_2 tumor ext.	—	P_3	SQ	6/7	13
Cell-free 10% P_3 tumor ext. in KLV†	$\geq 10^{2.6}$	P_4	SQ	18/19	14
Cell-free 10% P_4 tumor ext. in KLV†	$\geq 10^{2.6}$	P_5	SQ	11/11	15
Clarified 10% P_5 tumor ext.	$10^{2.2}$	P_6	SQ	13/13	17
Cell-free 10% P_6 tumor ext.	$10^{2.4}$	P_7	SQ	14/14	14
Purified‡ 6.6% P_7 tumor ext. in KLV†	$10^{3.2}$	P_8	IM	27/29	8
Cell-free 10% P_8 tumor ext. in KLV†	$10^{3.2}$	P_9	IM	31/31	9
Purified‡ 6.6% P_9 tumor ext.	—	P_{10}	IM	31/31	8
Cell-free 10% P_{10} tumor ext.	$10^{2.9}$	P_{11}	IM	14/14	10
Purified‡ 6.6% P_{11} tumor ext.	$>10^{3.5}$				

* Mixed cultures of hamster tumor cells and C57Bl MEF were inoculated with Kaplan leukemia virus, held for 20 days, and transplanted at a level of 2×10^6 cells per mouse.
† Tumors were homogenized in 10% extract of KLV-induced leukemic tissues instead of HBSS.
‡ According to the method of Moloney.[19]

oculation which gradually regressed. The nodules were not transplantable, nor did their extracts demonstrate any oncogenic activity in newborn mice.

Tumor pathology: The KL-MSV-induced tumors were hard, gritty, pale-gray masses that invaded the subcutaneous tissues of the back and neck and infiltrated the rib cage. Microscopically they were composed of sheets and whorls of fairly uniform spindle cells with pale acidophilic cytoplasm and elongate nuclei with coarsely clumped chromatin. Only rare giant cells were present. Scattered in the interstices of the tumor cells were lymphocytes and polymorphonuclear leukocytes. The tumors closely resembled those induced in Swiss mice by Huebner *et al.*[16] with other pseudotypes of MSV. They had the appearance of fibrosarcomas with little tendency toward the rhabdomyosarcomatous differentiation of Moloney sarcoma virus-induced tumors described by Perk and Moloney.[20] The morphology of tumors induced by higher animal-passaged virus remained unchanged. Tumors resulting from intramuscular inoculation resembled subcutaneous tumors but demonstrated a predisposition for infiltrating and invading muscle fibers.

Isolation of KL-MSV in cell culture: At the time the KLV-infected mixed cultures were harvested for animal inoculation, a portion of the cells was also concentrated 6× in supernatant fluid, briefly sonicated to disrupt the cells, and 0.4 ml of a 1:2 dilution inoculated into secondary C57Bl MEF cultures. No focus-forming activity was detected. Likewise, P_0 tumor extract in HBSS failed to induce foci. However, the P_0 tumor which was homogenized in KLV extract induced a few typical MSV foci. Focus-forming titers increased with animal passage level, the addition of Kaplan leukemia helper virus, and viral concentration by the Moloney procedure (Table 1).

Preliminary data indicated that focus formation by the Kaplan leukemia pseudotype of MSV followed a two-hit dose response curve similar to that described by Hartley and Rowe for Moloney's MSV.[15] Twofold dilutions initially provided a linear ffu response, but at higher dilutions the ffu tended to fall with the square of the dilution. The addition of KLV helper virus to dilutions of KL-MSV extract restored linearity to the dose response curve.

Characterization of KL-MSV pseudotype: Unlike the Moloney sarcoma virus, the host range of the Kaplan leukemia pseudotype was quite specific. Tumors were readily induced by cell-free extracts in 100 per cent of newborn C57Bl mice within 14 days but no tumors have developed in newborn NIH strain Swiss mice in the 150 days of observation to date. Titration of KL-MSV extract (tumor $dose_{50}$ of $10^{2.6}$/0.05 ml in newborn C57Bl mice) in newborn BALB/c mice resulted in the development of a few tumors but the TD_{50} was $<10^0$/0.05 ml, indicating this strain was less susceptible than C57Bl. These findings are in general accordance with Kaplan's observations[10] on the host range of the C57Bl radiation-induced leukemia virus.

Studies *in vitro* of cell susceptibility to KL-MSV paralleled the animal studies. KL-MSV virus with a ffu titer of $10^{3.2}$/0.4 ml in C57Bl/6 MEF cells had a titer of $10^{2.1}$/0.4 ml in BALB/c MEF and produced no foci in Swiss, DBA/2, RF, or C3H/He MEF cultures. The virus demonstrated approximately 2 $\log_{10}$ less infectivity in C57L, C57Br, and C58 MEF than in C57Bl/6 MEF cultures. Secondary mouse embryo cultures prepared from C57Bl/6 mice obtained from five sep-

arate colonies, C57Bl/Ka mice (obtained from Dr. Lloyd Law, NIH), and C57Bl/10 mice showed little variation in susceptibility to KL-MSV.

In an effort to determine whether the selective susceptibility of C57Bl MEF to KL-MSV was a reflection of virus adaptation during serial passage in C57Bl mice, parallel titrations of the following virus preparations were performed simultaneously in secondary cultures of C57Bl MEF and Swiss MEF cells: KL-MSV C57Bl cell culture harvests, KL-MSV C57Bl tumor extracts, Moloney leukemia (ML)-MSV grown in Swiss MEF cultures, and extracts of tumors induced by ML-MSV that had been adapted to C57Bl mice through 25 serial tumor transplantations (Table 2). Several KL-MSV preparations had ffu titers of $10^{2.4}$ to $10^{2.8}$/0.1 ml in C57Bl

TABLE 2

COMPARATIVE TITERS OF KL-MSV AND ML-MSV IN C57BL MEF AND SWISS MEF CULTURES*

Virus preparation	C57Bl MEF	Swiss MEF
KL-MSV C57Bl P_3 tumor ext. in KLV	$>10^{2.6}$/0.1 ml	$<10^{0}$/0.1 ml
KL-MSV C57Bl P_4 tumor ext.	$10^{2.4}$/0.1 ml	$<10^{0}$/0.1 ml
KL-MSV C57Bl P_4 tumor ext. in KLV	$10^{2.8}$/0.1 ml	$<10^{0}$/0.1 ml
KL-MSV C57Bl cell culture pool	$10^{1.6}$/0.4 ml	$<10^{0}$/0.4 ml
KL-MSV C57Bl cell culture pool	$10^{2.1}$/0.4 ml	$<10^{0}$/0.4 ml
ML-MSV C57Bl P_3 tumor ext.	$10^{2.9}$/0.1 ml	$10^{3.0}$/0.1 ml
ML-MSV C57Bl P_{25} tumor ext.	$10^{2.5}$/0.1 ml	$10^{2.4}$/0.1 ml
ML-MSV Swiss cell culture pool	$10^{1.9}$/0.4 ml	$10^{2.2}$/0.4 ml
ML-MSV Swiss cell culture pool	$10^{3.6}$/0.4 ml	$10^{3.5}$/0.4 ml

* Expressed as focus-forming units/unit volume.

cells but produced no foci in Swiss MEF cultures when tested undiluted. The ML-MSV preparations, whether grown in Swiss MEF cells or adapted to the C57Bl host, showed equal infectivity in either cell culture. This suggests that host adaption or "modulation" of the Kaplan sarcoma virus was not primarily responsible for its selective growth in C57Bl MEF, since ML-MSV serially passed in C57Bl mice showed no selectivity for C57Bl MEF.

In animal experiments the Friend, Moloney, and Rauscher MSV pseudotypes produced sarcomas in both C57Bl and Swiss mice; the Gross pseudotype in Swiss but not C57Bl mice; and the Kaplan pseudotype in C57Bl but not Swiss mice. Since factors other than virus adaption to the host seem to be responsible in large part for the host specificity noted in these studies, the range of host susceptibility suggests that KL-MSV is distinct from the other pseudotypes.

Fifty per cent of seven-day-old C57Bl mice inoculated with P_5 KL-MSV extract made with HBSS developed subcutaneous tumors within three weeks but all tumors regressed. Twelve of twelve sera collected from these mice four weeks post inoculation had CF antibody titers of 1:20 to 1:80 against Kaplan lymphoma extract, indicating a serotypic relationship between KLV and KL-MSV. The sera did not react with control C57Bl spleen-thymus extract.

A serum pool obtained six weeks after neonatal inoculation of C57Bl mice with cell-free extract of KLV-induced thymic lymphoma had a CF antibody titer of 1:80 against KLV antigen. This serum had a very narrow spectrum of reactivity; it did not demonstrate significant cross reaction at 1:10 dilution with other mouse leukemia CF antigens (*vide supra*), nor with extracts of MSV rat tumor or mouse tumors induced by Moloney pseudotype of MSV. Differential centrifugation experiments indicated that KLV and KL-MSV tumor extracts contained both

soluble and sedimentable CF antigens when assayed with group reactive rat antibody.[17] However, the KLV mouse antibody was only reactive with the sedimentable (20,000 × *g*, 1 hr), virion-associated antigens. The mouse serum did not react at 1:10 dilution with mouse hepatitis virus, PVM, Reo-3, GD-VII, Sendai, LCM, polyoma, rat virus, mouse adenovirus, K virus, or normal C57Bl spleen-thymus control antigens. A final dilution of 1:10 of this serum did not neutralize 50 ffu of KL-MSV in C57Bl cultures, but a 1:20 serum dilution incubated with 10 TD_{50} of KL-MSV and inoculated into newborn C57Bl mice resulted in a 77 per cent reduction of tumor incidence by day 36. Control sera from normal C57Bl mice had no neutralizing activity.

The anti-C58 thymus rat serum obtained from Dr. Old which contained high-titer group reactive mouse leukemia CF antibody completely neutralized 10 TD_{50} of KL-MSV at a serum dilution of 1:160 in animal experiments. In cell culture this serum completely neutralized 30 ffu of KL-MSV at a 1:160 dilution. In previous tests the rat serum at this dilution completely neutralized Gross and AKR leukemia viruses, but not Friend, Rauscher, or Moloney viruses.[18] Two other rat sera were also used in *in vitro* neutralization studies: anti-MSV (Moloney) rat serum with neutralization titers of 1:80 to 1:160 against Friend, Moloney, and Rauscher viruses, and anti-AKR rat serum with neutralization titers of 1:160 against AKR virus and <1:20 against Gross, Friend, Moloney, and Rauscher viruses. Neither serum at a dilution of 1:20 demonstrated neutralizing activity against KL-MSV. Although KLV contains CF antigen common to the mouse leukemia virus group, the neutralization data suggest that the Kaplan agent is not identical to Friend, Moloney, Rauscher, or AKR leukemia viruses. The lack of specific high-titered neutralizing sera for the Gross and Kaplan viruses hinders more definitive characterization.

Discussion.—The rescue of the mouse sarcoma virus genome by Kaplan leukemia virus represents a symbiotic association of the two components, which are separately "defective" in the test system employed. The NP hamster tumor cell contains the murine sarcoma viral genome which is defective in that it cannot produce free infectious virus or induce foci in MEF cultures,[16] apparently due to its inability to direct the synthesis of an outer envelope. Kaplan leukemia virus is "defective" in that *in vitro* viral replication has not been demonstrated by utilization of the mouse embryo culture-CF antigen production system of Hartley *et al.*[12] Repeated attempts to demonstrate CF antigen indicative of viral growth in KLV-infected C57Bl, Swiss, and BALB/c secondary MEF cultures were unsuccessful. KLV infection of cultures of embryonic C57Bl thymus, lung, heart, liver, spleen, or skin and muscle likewise failed to induce CF antigen. The apparent inability of KLV to replicate to detectable levels in this system is not shared by the other mouse leukemia viruses. Gross, Moloney, Friend, Rauscher, AKR, and several wild strains of mouse leukemia have all been shown to replicate in MEF cells.[12, 18] Attempts to demonstrate KLV antigen in infected C57Bl MEF cultures by indirect immunofluorescence utilizing KLV C57Bl antiserum or CF group-reactive rat antisera provided negative or equivocal results. Perhaps Kaplan virus replicates very poorly in C57Bl MEF cultures, but is capable of inducing synthesis of sufficient viral envelope to coat and complete the defective MSV genome in mixed hamster tumor cell-MEF cultures.

Even in the presence of complete infectious focus-forming sarcoma virus of the Kaplan pseudotype, the associated KLV helper virus still did not replicate well. The early animal passages of KL-MSV tumor extract required the addition of more KLV to maintain oncogenicity for the mouse and to induce foci in cell cultures. C57Bl MEF cultures inoculated with dilutions of KL-MSV extract that produced foci by day 7 were found to contain CF antigen by day 21 when tested against KLV mouse antiserum; however, cultures inoculated with KL-MSV extract at a twofold dilution greater than that which produced foci failed to yield KLV CF antigen. This indicates the helper virus is not present in excess of the sarcoma virus, as is the case for the other MSV pseudotypes, or is unable to replicate in the absence of the MSV genome.

Attempts to serially pass cell harvests of KL-MSV-infected C57Bl MEF cultures resulted in the loss of approximately 1 $\log_{10}$ of focus-forming activity per passage. After two to three passages of KL-MSV as sonicated 10× MEF cell packs, foci were no longer produced.

Definitive identification of the coat of KL-MSV as that of KLV is difficult to provide, but preliminary studies of the KL-MSV pseudotype have elucidated the following points: (1) KL-MSV-induced sarcomas have a histologic appearance very similar to those induced by other pseudotype viruses, suggesting that the phenotypic expression of the MSV genome is the same regardless of the envelope; (2) the susceptibility of the C57Bl strain and resistance of the Swiss strain to KL-MSV differentiates this pseudotype from the other mouse leukemia–sarcoma complexes and indicates that the host range of susceptibility is related to the helper virus; (3) KLV antivirion antiserum from C57Bl mice contains CF antibody and low-level neutralizing antibody to KL-MSV; and (4) anti-C58 thymus rat serum with high-titer neutralizing antibody to Gross and AKR leukemia viruses contains high-titer neutralizing antibody to Kaplan sarcoma virus.

Summary.—Unlike most mouse leukemia viruses, radiation-induced leukemia virus from C57Bl mice has not been shown to replicate *in vitro*. Mixed cell cultures of mouse embryo fibroblasts and hamster tumor cells containing the defective (noninfectious) murine sarcoma virus genome were inoculated with extracts of radiation-induced leukemic tissues and the cells were subsequently transplanted into newborn C57Bl mice. The resultant transplantable tumors yielded infectious, sarcomagenic murine sarcoma virus that could be quantitated *in vitro*. This virus exhibited the host range of pathogenicity and immunologic reactivity of C57Bl radiation-induced leukemia virus, indicating the sarcoma genome carried the envelope of the leukemia virus.

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