

## **Trans-Species Rescue of Defective Genomes of Murine Sarcoma Virus from Hamster Tumor Cells with Helper Feline Leukemia Virus\***

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**Abstract.** This report describes a trans-species rescue of defective MSV genome with helper leukemia virus derived from cats. This rescue was achieved by *in vitro* co-cultivation of hamster tumor cells with feline embryo cells in the presence of helper feline leukemia virus (FeLV), or by inoculation of tumor cells into FeLV-infected newborn cats.

The rescued focus-forming viruses produced foci in feline embryo cultures but not in cultures of mouse, rat, and hamster species. One isolate was tested and found to induce sarcoma in a kitten. Antigenic and viral interference studies indicated that the focus-forming virus has the viral envelope of FeLV. Virus stocks consisted of a mixture of focus-forming particles and a 1000-fold excess of helper FeLV. Virus assay patterns in feline embryo cultures with or without added helper FeLV indicated that this helper virus is required for the transformation of feline cells.

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Most strains of avian and murine sarcoma viruses are defective and are unable to produce their viral envelopes without assistance from closely related nondefective helper leukemia viruses.<sup>1-4</sup> Such defective sarcoma viruses are generally found in association with an excess of helper virus.<sup>1-5</sup> The resultant co-infection of homologous host cells with sarcoma and leukemia viruses permits the replication of the infectious progeny sarcoma virus which derives its viral envelope from the helper virus.<sup>1-4</sup> Thus, the sarcoma virus has the antigenic and host-range characteristics of the helper virus.<sup>1-4</sup>

Avian cells infected under conditions which only permit solitary infection with the defective sarcoma virus,<sup>1, 2, 6</sup> as well as heterologous mammalian cells transformed *in vivo* or *in vitro* by low or high multiplicity of avian or murine sarcoma viruses,<sup>7-10</sup> generally fail to show evidence of the release of infectious progeny sarcoma or leukemia viruses when usual tests for infectious virus are performed using cultures of the homologous host species. However, the presence in such cells of the sarcoma virus genome can be demonstrated by one or more procedures such as rescue of sarcoma virus by direct superinfection of transformed cells with helper virus,<sup>1-3, 11-13</sup> rescue of sarcoma virus genome by *in vitro* or *in vivo* association of tumor cells in the presence of helper virus,<sup>7-10</sup> detection of the release of labeled virus,<sup>13, 14</sup> detection of focus-forming virus with a very restricted host-

range,<sup>11, 15-18</sup> and by immunological identification of the presence of virus-specific antigens.<sup>7, 18-20</sup>

Feline leukemia (lymphosarcoma) is now known to be caused by a C-type RNA virus.<sup>24-26</sup> Geering *et al.*<sup>27</sup> recently reported a cross reaction in immunodiffusion test between the feline and murine leukemia viruses. Fischinger and O'Connor<sup>28</sup> recently described the encapsidation of the genome of defective Moloney murine sarcoma virus (Moloney MSV) within the viral envelope of competent FeLV by a physical technique. A focus-forming virus was derived by infecting cat cells with pellets obtained by cosedimentation of the two viruses at high centrifugal force. We report herein a successful trans-species rescue of defective MSV genomes of three strains of MSV by *in vitro* or *in vivo* association of MSV hamster tumor cells with feline cells in the presence of helper FeLV.

**Materials and Methods. Tissue cultures:** Cultured hamster tumor cells used in these studies were derived from hamster tumors induced by three strains of MSV: the Moloney MSV,<sup>29</sup> Kirsten MSV,<sup>30</sup> and Harvey MSV.<sup>31</sup> A cell line was established from third transplant passage Kirsten MSV hamster tumor, kindly provided by Dr. V. Klement. A clonal cell line<sup>32</sup> of Moloney MSV hamster tumor cells derived from HT-1 cells<sup>8</sup> and cultured Harvey MSV hamster tumor cells were kindly provided by Drs. K. Chang and R. H. Bassin, respectively.

The hamster tumor cells contained the MSV genome, but no detectable murine leukemia virus group complement-fixation antigens or infectious murine leukemia virus or MSV capable of replication in MEF cultures.<sup>8, 14, 18, 32</sup>

Secondary cultures of feline embryo fibroblasts (FEF) and NIH Swiss mouse embryo fibroblast (MEF) were used. The growth medium for these cultures and hamster tumor cells consisted of Eagle's minimum essential medium supplemented with 2 mM L-glutamine, 5-10% unheated fetal bovine serum, 5% tryptose phosphate broth and antibiotics (penicillin, 250 unit/ml; streptomycin, 250  $\mu$ g/ml; and fungizone, 25  $\mu$ g/ml). The cultures were grown in disposable Falcon Petri dishes in a humidified CO<sub>2</sub> incubator flushed with 5% CO<sub>2</sub> in air.

**Feline leukemia virus:** FeLV was derived from a cat with naturally occurring lymphosarcoma (Montgomery Animal Hospital, Rockville, Md.). Supernatants of cultured tumor cells containing 10<sup>6</sup> infectious units per milliliter of FeLV [complement-fixing antigen induction test<sup>33</sup>] and pleural fluid of this affected cat containing 10<sup>7</sup> infectious units per milliliter of virus were used.

**In vitro MSV genome rescue studies:** Hamster tumor cells were grown in mixed culture with secondary cultures of FEF similar to procedures already described<sup>7, 8</sup>; 5  $\times$  10<sup>6</sup> FEF and 5  $\times$  10<sup>8</sup> hamster tumor cells were planted in 5 ml of culture medium in each 60-mm disposable culture dish. Half the number of cultures in each group containing two to four mixed cultures and equal numbers of control FEF cultures and control hamster tumor cultures were immediately infected in suspension with 10<sup>6</sup> infectious units of FeLV. These cultures and parallel uninfected control cultures were maintained by medium replacements at 3 day intervals and by cell transfer at weekly intervals.

Culture supernatants of FeLV-infected and parallel uninoculated cultures were collected on the 4th, 8th, 12th, and 16th day after virus infection and clarified by filtration through Swinney filters fitted with 0.45  $\mu$  Millipore filters. The supernatants were tested for focus-forming virus by inoculation into secondary MEF and FEF cultures.

These cultures and control MEF and FEF cultures were maintained and observed for foci.<sup>4</sup> The culture media were replaced at 3-day intervals and the cells were serially transferred at approximately weekly intervals. The experiment was concluded 30 days after inoculation of culture supernatants.

**In vivo genome rescue<sup>8</sup>:** Kirsten MSV hamster tumor tissue culture cells were removed from monolayer bottle cultures by trypsinization. Three to five newborn

kittens from one litter were inoculated intramuscularly in the right thigh with 0.6 ml of the virus-hamster tumor cell mixture containing  $10^6$  infectious units of FeLV and  $2 \times 10^6$  hamster tumor cells. The animals were left with the mother and observed for tumor development.

When tumors reached sizes of 20 to 30 mm within 12 to 15 days, the animal was killed. The tumors, weighing 7–8 gm, were removed and tested for focus-forming virus by inoculation of clarified, cell-free 10% tumor extracts in MEF and FEF cultures.

**Virus stocks of recovered focus-forming viruses:** The focus-forming viruses rescued in these experiments were prepared into virus stocks as clarified culture supernatants of infected FEF cultures that showed confluent cell transformation within 5 to 8 days after inoculation of virus. In some experiments, cells and fluids were also collected, quick frozen, and thawed once and clarified. Virus stocks stored at  $-70^\circ\text{C}$  generally gave focus titer in FEF of  $2 \times 10^3$  to  $10^4$  per ml.

**Assay of focus-forming virus:** The focus-forming viruses recovered in these experiments were assayed in secondary cultures of FEF planted in 60-mm Petri dishes. The cultures were infected in suspension. Culture media were replaced on the 3rd day with media containing reduced concentration of serum (5%). Foci of transformed cells were counted 6 days after virus inoculation with a Leitz inverted microscope.

**Oncogenicity in newborn kittens:** One litter of three newborn kittens was inoculated intramuscularly with 0.5 ml each of Moloney MSV-feline leukemia pseudotype virus. The animals were left with their mother and observed for tumor development. Animals which died were autopsied and tumors were collected for histopathological examination.<sup>36</sup>

**Test of virus stocks for defectiveness:** Stocks of MSV-feline leukemia pseudotype viruses were prepared by harvesting FEF culture fluids at 3 and 7 days after virus inoculation. These preparations were clarified by filtration and assayed in FEF cultures in 5-fold dilution steps. Parallel sets of inoculated FEF cultures were also simultaneously infected with  $10^6$  infectious units of FeLV to determine helper activity. The foci of transformed cells were counted after 6 days.

**Viral interference test:** The ability of FeLV to interfere with focus formation of the rescued MSV-feline leukemia pseudotype viruses was tested by a procedure similar to that described for murine leukemia viruses.<sup>35</sup> FEF infected with a high multiplicity of FeLV were maintained for 20 days to establish virus infection in the majority of the cells. The cells were then transferred to 60-mm plastic dish cultures at a concentration of  $5 \times 10^6$  cells in 5 ml of growth medium. Cultures were immediately separately infected with  $10^8$  focus-forming units of the MSV-feline leukemia pseudotype viruses. The sensitivity of FeLV-infected and control cultures to vesicular stomatitis virus was also determined by plaque assay.

**Virus neutralization test:** Antisera prepared in rabbits<sup>33</sup> against FeLV and in rats against MSV were tested for their capacity to neutralize the focus-forming viruses recovered in these experiments. FeLV antiserum was prepared by hyperimmunization of rabbits with intact FeLV. A pool of sera of MSV tumor-bearing rats containing a high titer of murine leukemia group-specific complement-fixing antibodies and virus neutralizing antibodies to the murine leukemia viruses of the FMR subgroup was kindly provided by Dr. Janet Hartley. Sera were heated at  $56^\circ\text{C}$  for 30 min before use. Equal parts of sera diluted 1:5 and virus dilutions were mixed and incubated at room temperature for 30 min and at  $4^\circ\text{C}$  for the following 30 min. The presence of focus-forming virus in the virus-serum mixtures was tested by inoculation into FEF cultures.

**Results.** The *in vitro* MSV genome rescue studies with three strains of MSV hamster tumor cells gave identical results. Of the various cell-free culture fluid samples of FeLV-infected and control cultures that were tested, only the serially collected samples derived from FeLV-infected mixed cultures produced foci of cell transformation in FEF cultures. Parallel MEF cultures inoculated with the same samples failed to show any visible changes. The foci consisted of refractile

spindle cells and round cells. The transformed cells tended to float off into the culture medium leaving gaps in the monolayer cultures. Between 10 to 20 foci were induced within six days of inoculation of 0.2-ml culture supernatants which indicated that between 50 to 100 focus-forming units were present per milliliter of culture medium.

Cell-free culture fluids of FEF cultures which showed confluent cell transformation effects induced foci in FEF but not in MEF cultures. In addition, foci of morphologically altered cells were observed in certain human fibroblast cultures inoculated with these preparations.<sup>37</sup> No visible effects were observed in secondary embryo fibroblast cultures of rat, hamster, and mouse species. The focus-forming virus could be serially propagated indefinitely in FEF cultures. Cell-free culture fluid preparations contained  $2 \times 10^3$  to  $10^4$  focus-forming units per milliliter. For identification purposes, the following provisional virus designations were given using the strain of MSV followed by FeLV within parentheses to signify isolation with FeLV helper: Moloney MSV(FeLV), Kirsten MSV(FeLV), and Harvey MSV(FeLV).

**In vivo genome rescue studies:** Tumors measuring 20 to 30 mm were observed in all inoculated newborn kittens within 15 days of inoculation of the Kirsten MSV hamster tumor cells-FeLV mixture. Clarified cell-free 10 per cent extracts of such tumors readily induced foci in FEF cultures but not in MEF cultures. Such tumor preparations contained between 3 to  $8 \times 10^3$  focus-forming units per milliliter.

**Induction of sarcoma in kittens:** The three kittens inoculated with Moloney MSV(FeLV) died on the 20th day after virus inoculation. One kitten had a mass measuring 2 cm at the site of inoculation. Histopathological examination indicated that the growth was a myxosarcoma with abundant mesenchymal cells.<sup>36</sup> A metastatic tumor was also found in the kidney of this kitten.

**Detection of FeLV:** Virus stocks of Moloney MSV(FeLV), Kirsten MSV(FeLV), and Harvey MSV(FeLV) were found to contain three logs excess of FeLV over the cell-transforming virus. In virus-assay experiments in FEF cultures, FeLV was detected by the complement-fixation antigen induction test<sup>33</sup> in dilutions up to  $10^{-5}$ . However, foci of transformed cells were observed only in dilutions up to  $10^{-2}$ .

**Defectiveness:** Virus stocks of Moloney MSV(FeLV) and Kirsten MSV(FeLV) prepared as clarified culture fluids of FEF cultures at three and seven days after virus inoculation gave similar and defective virus titration patterns in FEF cultures (Table 1). Co-infection of parallel inoculated cultures with FeLV increased the virus titer and converted the titration pattern to a theoretical one-hit curve.

**Virus neutralization test:** Rabbit antiserum to FeLV ( $\log_{10}$  neutralization index  $>2$ ) reduced the focus titer of approximately 500 focus-forming units of Kirsten MSV(FeLV) and Moloney MSV(FeLV) by 95 to 98 per cent whereas antisera to Moloney MSV and control normal sera failed to neutralize the virus.

**Viral interference test:** FEF cultures chronically infected with FeLV were fully resistant to challenge with  $10^3$  focus-forming units of Kirsten MSV(FeLV), Moloney MSV(FeLV), or Harvey MSV(FeLV). However, the same cultures were fully susceptible to plaque formation by vesicular stomatitis virus.

TABLE 1. Defective focus titration patterns of Moloney MSV(FeLV) and Kirsten MSV (FeLV) and the requirement of an added helper feline leukemia virus for transformation of feline embryo fibroblasts.

Virus sample	Virus dilution	Moloney MSV(FeLV)					
		Without Helper			With Helper		
		No. Foci		Titer	No. Foci		Titer
Collected 3rd day	1:10	C	C		C	C	
	1:50	20	26	$1.15 \times 10^3$	C	C	
	1:250	3	1	$5 \times 10^2$	40	38	$9.75 \times 10^3$
	1:1250	0	0		8	10	$1.26 \times 10^4$
	1:6250	0	0		2	1	$9.38 \times 10^3$
	1:31250	0	0		0	0	
Collected 7th day	1:10	C	C				
	1:50	C	C				
	1:250	35	32	$8.38 \times 10^3$	C	C	
	1:1250	2	2	$2.5 \times 10^3$	15	18	$2.06 \times 10^4$
	1:6250	0	0		3	4	$2.19 \times 10^4$
	1:31250	0	0		0	0	
Virus sample	Virus dilution	Kirsten MSV(FeLV)					
		Without Helper			With Helper		
		No. Foci		Titer	No. Foci		Titer
Collected 3rd day	1:10	C	C		C	C	
	1:50	24	26	$1.25 \times 10^3$	C	C	
	1:250	1	1	$2.5 \times 10^2$	62	60	$1.53 \times 10^4$
	1:1250	0	0		16	10	$1.63 \times 10^4$
	1:6250	0	0		3	2	$1.56 \times 10^4$
	1:31250	0	0		0	0	
Collected 7th day	1:10	C	C		C	C	
	1:50	C	C		C	C	
	1:250	32	28	$7.5 \times 10^3$	C	C	
	1:1250	3	2	$3.1 \times 10^3$	32	30	$3.88 \times 10^4$
	1:6250	0	0		6	7	$4.06 \times 10^4$
	1:31250	0	0		2	0	$3.13 \times 10^4$

C, foci confluent.

**Discussion.** The experiments reported by Fischinger and O'Connor<sup>28</sup> and those described herein indicate that although MSV and FeLV are derived from divergent species they can nevertheless interact both *in vitro* and *in vivo* under the conditions described to yield pseudotypes of MSV with FeLV envelope. Our observations on viral titration patterns indicate that Moloney MSV(FeLV) as well as Kirsten MSV(FeLV) viruses are defective in the same way as the original Moloney MSV. We found that the pseudotype viruses contained an excess of FeLV which was easily detected in virus assay experiments.

Viral neutralization tests established that the viral envelope of the isolated pseudotype viruses we recovered were identical to FeLV and not MSV. In addition, the induction of viral interference by FeLV to the isolated pseudotype viruses and the host-range characteristics of this virus also established that the viral coat and host-range characteristics of the pseudotype virus are those of feline leukemia virus.<sup>1-3, 35</sup>

We have observed that culture antigens of feline leukemia virus-infected FEF cultures give little or no reaction in complement-fixation test versus pooled sera of MSV tumor-bearing rats containing group-specific CF antibodies to the viruses of the murine leukemia-sarcoma group.<sup>8, 34</sup> On the other hand, preliminary experiments have shown that cell antigens of FEF cultures infected with a high dose of the MSV-FeLV pseudotype viruses react in complement-fixation test with the

same MSV rat serum in dilutions up to 1:8, but not with an antiserum prepared in guinea pigs with purified group-specific antigen No. 1 component of the murine leukemia-sarcoma virus group.<sup>38, 39</sup> Studies are in progress to determine if the complement-fixation reaction observed is due to a component of the group-specific antigens of the viruses of the murine leukemia-sarcoma group.

These experiments indicate that different strains of MSV are identical in their biological behavior in being able to furnish genomes for encapsidation with FeLV envelope. The rescue studies have also been recently confirmed with additional field isolates of FeLV.

Fischinger and O'Connor<sup>28</sup> provided evidence to indicate that focus-forming viruses similar to those described here can be recovered by infecting cat cells with FeLV-defective MSV interval pellets. The virus isolated induced foci and propagated in cell cultures of cat,<sup>28</sup> dog and human species<sup>40</sup> but not in mouse species, and appeared to have the viral envelope of FeLV.

We have found that murine and avian leukemia and sarcoma viruses do not share common antigens and do not interact to form pseudotype viruses by the viral genome rescue techniques.<sup>41</sup> Whether they will do so by physical techniques<sup>28</sup> remains to be determined.

The interaction between MSV and FeLV and their immunological relatedness<sup>27</sup> indicates that these viruses may have a common origin. It is conceivable that biologically related, but as yet undetected RNA viruses of the C-type may be involved etiologically in cancers of other species including man.

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*Abbreviations:* MSV, murine sarcoma virus; FeLV, feline leukemia virus; Moloney MSV, Moloney strain murine sarcoma virus; Kirsten MSV, Kirsten strain murine sarcoma virus; Harvey MSV, Harvey strain murine sarcoma virus; Moloney MSV(FeLV), pseudotype of Moloney strain murine sarcoma virus with the envelope of feline leukemia virus; FMR subgroup, Friend, Moloney, and Rauscher leukemia virus subgroup of murine leukemia viruses; MEF, mouse embryo fibroblasts; FEF, feline embryo fibroblasts; GS-1, group-specific antigen #1; MEM, Eagle's Minimum Essential Medium.

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