HELPER-INDEPENDENT MINK CELL FOCUS-INDUCING STRAINS OF FRIEND MURINE TYPE-C VIRUS: POTENTIAL RELATIONSHIP TO THE ORIGIN OF REPLICATION-DEFECTIVE SPLEEN FOCUS-FORMING VIRUS*

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Evidence recently obtained in two different murine leukemia virus systems has suggested that recombination between ecotropic and xenotropic viruses might be important in the leukemia induced by murine type-C RNA leukemia viruses. The Friend strain of spleen focus-forming virus (SFFV)[1] is a highly leukemogenic, replication-defective murine type-C virus, which was originally observed after continuous mouse-to-mouse passage of uncloned Friend leukemia virus stocks (1, 2). SFFV causes the rapid leukemic transformation of erythroid precursor cells in adult mice of susceptible strains (3), whereas cells of fibroblast derivation infected with this virus remain morphologically normal (4). Even though SFFV is unable to induce morphologic transformation of fibroblasts in cell culture, we recently devised a scheme by which we were able to clone this replication-defective virus free of replicating type-C virus in cell culture (5). Using SFFV-containing mouse and rat nonproducer cells derived in this manner, we analyzed the genetic composition of SFFV by molecular hybridization (6). These studies demonstrated that SFFV is a recombinant between Friend helper-independent ecotropic murine type-C leukemia virus (F-MuLV$_{eco}$) and genetic sequences highly related to mouse xenotropic virus. The possible role of the xenotropic viral sequences in SFFV in the leukemogenic potential of SFFV was discussed.

Independently, Hartley et al. (7) isolated from leukemic AKR thymoma cells several helper-independent viruses which have been extensively characterized and have been shown to be recombinants between ecotropic AKR virus and xenotropic virus involving recombination within the envelope (*env*) gene of each parental virus. These viruses were designated AKR-mink cell focus-inducing (MCF) viruses because they possessed the unique biological property of inducing foci of cytopathic effects when grown on mink lung cells. Using these AKR-MCF viruses as well as a similar virus derived from Moloney MuLV (Mol-MCF$_{83}$), we showed that the xenotropic-related genetic sequences in SFFV were homologous to those present in MCF viruses, and were likewise derived from the *env* gene of a murine xenotropic virus (8).

Although the precise role of the xenotropic *env* gene sequences contained in both SFFV and the AKR-MCF viruses in the leukemogenesis of these viruses remains unclear, definite parallels seem to exist between the F-MuLV system and the AKR-MuLV system. However, contrasts between the two systems also exist. First, the AKR-MCF viruses are helper-independent viruses, in contrast to the replication-defective recombinant SFFV. Although a replication-defective virus has been isolated from leukemic AKR thymic tissue (9), the molecular relationship

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[1] *Abbreviations used in this paper*: AMT 1504A, amphotropic virus 1504A; $_{eco}$, ecotropic; *env*, envelope; FLV, F-MuLV, Friend strain of murine leukemia virus; FRE, Fischer rat embryo cells; Fr-MCF, Friend strain of mink cell focus-inducing virus; F. V. A., anemic strain of Friend virus; KiSV, Kirsten murine sarcoma virus; MCF, mink cell focus-inducing; Mol, Moloney strain; MuLV, murine leukemia virus; NRK, normal rat kidney cells; PAG, pig anti-goat serum; R-MuLV, Rauscher strain of MuLV; SFFV, spleen focus-forming virus; SIRC, rabbit cornea cells.

between this virus (AKT8) and the helper-independent AKR-MCF viruses has not yet been elucidated. Second, mink lung cells infected with SFFV do not demonstrate cytopathic effects similar to those seen with the AKR-MCF viruses (8). Finally, whereas the AKR-MCF viruses have been implicated as potential leukemogenic agents in thymic leukemia, SFFV causes leukemic transformation of erythroid, not thymic cells. Thus, in spite of the parallels between the molecular composition of SFFV and the AKR-MCF viruses, biological differences between the two classes of xenotropic *env* gene recombinants exist and are as yet unexplained.

To more fully understand the potential relationship between the Friend erythroleukemia system and the AKR thymic leukemia system, we undertook to isolate other *env* gene recombinants between Friend ecotropic virus and endogenous murine xenotropic virus. We had previously noted that the leukemia which is produced by inoculation of high titers of cloned F-MuLV$_{eco}$ into newborn NIH Swiss mice is accompanied by a marked increase in levels of xenotropic viral RNA detected with cDNA$_{SFFV}$ (10). Therefore, we attempted to isolate viruses with a xenotropic host range which might be present in the spleens of preleukemic and leukemic NIH Swiss mice injected with F-MuLV. We report here that helper-independent *env* gene recombinants can be isolated from such spleen cells. These recombinants between F-MuLV and xenotropic virus, like the AKR-MCF recombinants, are able to produce cytopathic foci when grown on mink lung cells. The ability to isolate helper-independent MCF recombinants derived from F-MuLV further extends the similarity between the Friend and the AKR leukemia virus systems, and affords possibilities for further understanding the origin of the highly leukemogenic SFFV variant derived from such recombinational events.

Materials and Methods

Cell Lines

MOUSE CELLS. The mouse cell lines used in this study were SC-1, NIH 3T3, and BALB/c 3T3 mouse embryo fibroblast cells, which are permissive for MuLV$_{eco}$. They have been previously referenced (5, 6).

RAT CELLS. Rat cell lines used included normal rat kidney cells (NRK), Fischer rat embryo (FRE) cells, and XC cells, the origins of which have been previously referenced (5, 6). Rat cell lines nonproductively infected with SFFV (SFFV-FRE and SFFV-NRK CL 1) have been previously described (6).

OTHER CELLS. Cell lines permissive for xenotropic MuLV, namely mink lung fibroblasts (CCL 64) and rabbit cornea cells (SIRC; CCL 60), were obtained from American Type Culture Collection, Rockville, Md. In addition, dog thymus cells (CF2Th) were obtained from G. Todaro of the National Cancer Institute.

Cells were grown in Dulbecco's modified Eagle's medium with either 10% calf serum or 10% fetal calf serum, unless designated otherwise, and they were maintained in a Wedco incubator in a humidified 10% CO_2 atmosphere at 37°C. Each cell line was routinely assayed for mycoplasma species and was found to be negative.

Viruses Used

ECOTROPIC VIRUSES. F-MuLV clone 201, an NB-tropic clone of Friend murine type-C virus, was obtained from a preparation of the strain of Friend virus associated with severe anemia (F.V.A.) by three cycles of cloning on SC-1 cells in Microtest II culture plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), as previously described (5, 6). F.V.A. was provided by Dr. Charlotte Friend, Mt. Sinai School of Medicine, New York. Other cloned ecotropic viruses used included AKR-MuLV (derived from AKR 2B cells) and Mol-MuLV$_{eco}$. MuLV$_{eco}$ were grown on either SC-1 cells or NRK cells.

XENOTROPIC VIRUSES. Two strains of murine xenotropic virus, NZB-MuLV (11) and ATS-124 (12) were grown on mink lung cells.

MCF STRAINS. Two MCF strains of murine type-C virus, AKR-MCF 247 (7) and Mol-MCF_{83} (8) (previously designated Mol-$MuLV_{83}$), have been described.

OTHER VIRUSES. An amphotropic virus isolated from a feral mouse (AMT 1504A) (13) was propagated on mink or SC-1 cells. Kirsten murine sarcoma virus (KiSV) pseudotypes used in the interference studies were obtained by superinfecting KiSV-transformed nonproducer mink or NIH 3T3 cells with each of the above MuLV. Finally, the virus complex released from SFFV-FRE cells superinfected with F-MuLV, which contains a five- to sixfold excess of SFFV (D. H. Troxler, unpublished observations), was used to prepare $cDNA_{SFFV}$. AKR-MuLV, AMT 1504A, and AKR-MCF 247 were the kind gifts of Dr. Janet Hartley, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Mol-$MuLV_{eco}$ was kindly provided by Dr. Nancy Hopkins, Massachusetts Institute of Technology, Boston, Mass.

Virus Assays. Replicating type-C MuLV were assayed by: (*a*) reverse transcriptase assay of supernatant medium from infected cells by methods which have been previously described (5); (*b*) XC plaque assay (14), and (*c*) assay for cytopathic foci on mink lung fibroblasts, as previously described by Hartley et al. (7).

Mice. Newborn and 4–5-wk-old NIH Swiss mice were obtained from the National Institutes of Health Small Animal Facility, Bethesda, Md. Mice were housed in disposable plastic cages and given food and water ad libitum.

Co-Cultivation Techniques. Leukemic spleens were removed aseptically and passed through sterile gauze to make single cell suspensions. Cells were then treated with mitomycin C (Calbiochem, San Diego, Calif.) at a concentration of 25 μg/ml in darkness for 1.0 h at 37°C, after which 10^5–10^7 spleen cells were inoculated onto SC-1 cells or mink lung cells that had been seeded at a concentration of 10^5 cells/60-mm plate 24 h previously. The spleen cells were washed off the plate 24 h later, after which each culture was refed with fresh medium and assayed at weekly intervals for the presence of infectious MuLV by reverse transcriptase assays of a 24-h collection of supernatant medium. Cells were routinely transferred at weekly intervals if reverse transcriptase assay for replicating MuLV remained negative, and they were maintained for 4–6 wk before being discarded.

Interference Assays. The interference patterns of the MuLV reported herein were determined by quantifying focus formation induced by various MuLV pseudotypes of KiSV after inoculation onto either SC-1 or mink cells chronically infected with each MuLV. Each cell line was infected 24 h after seeding at 10^5 cells/60-mm plate in medium containing 5 μg/ml Polybrene. Media were changed at 3-day intervals, and plates were observed for KiSV-induced foci after 5–10 days. In the interference tests on SC-1 cells, the cells were switched to medium containing 3% fetal calf serum 3 days after infection in order to maintain the background SC-1 cell monolayer in a contact-inhibited state.

P12 Radioimmunoassay. Type-specific p12 radioimmunoassay was performed using Rauscher (R)-MuLV p12 obtained from Dr. Arup Sen, National Cancer Institute, and goat anti-Friend leukemia virus (FLV) antisera obtained from R. Wilsnack, Huntingdon Laboratories, Brooklandville, Md., courtesy of the Office of Resources and Logistics, Virus Cancer Program, National Cancer Institute. Extracts of purified viruses from infected mink or SC-1 cultures were used as competing antigens in the radioimmunoassay; these extracts were prepared by disrupting the virus in 1.0% Triton in phosphate-buffered saline, incubating for 30 min at 37°C, and then clarifying at 10,000 *g* for 10 min at 4°C. The second antibody used in these experiments was pig anti-goat (PAG) serum prepared as previously described (15). Further details of the competition radioimmunoassay are given in the legend to Fig. 1.

Tryptic Peptide Fingerprints of gp70. Tryptic peptide fingerprints of gp70s derived from F-MuLV, NZB-MuLV, and Friend (Fr)-MCF-1 were prepared as described in the legend to Fig. 2. The gp70 of each virus was prepared by phosphocellulose chromatography (16).

Synthesis of cDNA Probes and Molecular Hybridization Experiments. The synthesis of $cDNA_{F\text{-}MuLV}$ from F-$MuLV_{201}$ grown on SC-1 cells has been previously described (6). $cDNA_{SFFV}$ was prepared from ^3H-labeled cDNA made from virus released from SFFV-FRE/F-MuLV, by methods previously described (6). The specificity of $cDNA_{SFFV}$ for SFFV and for murine xenotropic viruses and MCF viruses has been previously described (6, 8). The cDNA probes were labeled with [^3H]deoxycytidine triphosphate and had sp act of 2–5 × 10^7 cpm/μg DNA. RNA:[^3H]cDNA hybridizations were performed at 66°C in 0.75 M NaCl by methods which have been previously described (6), and hybridization was assayed with S1 nuclease and

analyzed as a function of RNA concentration and time (C_rt). Total cellular RNA and viral RNA were prepared as previously described (6, 17).

Results

Isolation of Mink-Tropic MuLV from F-MuLV-Induced Leukemic Spleen. To obtain F-MuLV-induced leukemic tissue, newborn NIH Swiss mice were inoculated intraperitoneally with $\cong 10^5$ XC plaque-forming units of F-MuLV$_{201}$ produced by infected SC-1 cells. As previously reported (10), most inoculated mice characteristically develop anemia, splenomegaly, and leukemia by 30–40 days of age. Furthermore, we had found in spleen tissue of these mice markedly increased levels of xenotropic-specific RNA detected with cDNA$_{SFFV}$ which paralleled the development of splenomegaly. However, previous attempts to isolate from these spleens MuLV which had a xenotropic host range were unsuccessful. In a continuation of these co-cultivation experiments, we have now isolated from the spleens of F-MuLV-inoculated NIH Swiss mice two different MuLV which are infectious for mink cells. The first isolate, designated Fr-MCF-1 (for reasons discussed below), was isolated from the slightly enlarged (0.39 g) spleen of an NIH Swiss mouse 27 days after inoculation of F-MuLV$_{201}$ by co-cultivation of mitomycin C-treated spleen cells with SC-1 cells and subsequent inoculation of filtered cell-free supernate from productively infected SC-1 cells onto mink lung cells. Reverse transcriptase activity was detected in the supernatant medium of infected mink cells after two transfers of this culture. The second isolate, designated Fr-MCF-2, was obtained from the overtly leukemic spleen (2.76 g) of an NIH Swiss mouse 35 days after inoculation of F-MuLV$_{201}$. In this instance, co-cultivation of mitomycin C-treated spleen cells directly with mink lung cells resulted in the appearance of reverse transcriptase activity in supernatant medium of these mink cultures after three weekly cell transfers. Subsequently, each isolate was purified by two cycles of end-point dilution on mink cells.

Mink cell cultures that became reverse transcriptase-positive after co-cultivation with spleen cells also demonstrated marked morphological changes consisting of rounding up and vacuolization of cells, failure to grow to normal saturation density, and detachment of many cells from the culture dish. Because of the resemblance of these cultures to mink cell cultures chronically infected with MCF-type MuLV (7), we analyzed the host range and growth properties of each of the MuLV isolates, as described in the next section.

Host Range and Growth Characteristics of MuLV Isolated from F-MuLV Spleens. MuLV have been classified into several categories based on their ability to infect and propagate in cell lines derived from various species (13). MuLV$_{eco}$, such as F-MuLV and AKR-MuLV, readily infect and propagate in mouse cells, but fail to infect mink cells. Xenotropic MuLV do not infect mouse cells but do infect heterologous cells such as mink and SIRC cells (11). MuLV with a broadened host range, which grow on both mouse and mink cells, are comprised of two different types of MuLV which have distinct interference and neutralization patterns: (*a*) a class of amphotropic MuLV isolated from wild mice, exemplified by AMT 1504A (13, 18); and (*b*) viruses that have acquired a broadened host range as a result of recombination between ecotropic and xenotropic MuLV involving the *env* gene (7). This category is made up of several recently described isolates, including the AKR-MCF viruses and similar isolates derived from Mol-MuLV (7, 8, 19).

TABLE I

Host Range and Biologic Properties of Various MuLV

Virus	Growth on: (Reverse transcriptase levels)*			Induction of:	
	SC-1	Mink	SIRC	XC plaques (on SC-1)	CPE‡ (on mink)
F-MuLV$_{201}$	666	<1	<1	Yes	N.R.§
NZB-MuLV	<1	340	120	N.D.‖	No
AMT 1504A	460	300	N.D.	No	No
AKR-MCF 247	400	320	N.D.	No	Yes
Fr-MCF-1	360	600	125	No	Yes
Fr-MCF-2	260	800	N.D.	No	Yes

Approximately 10^4–10^5 infectious units of each MuLV preparation were inoculated onto the designated cell lines 24 h after 1×10^5 cells had been seeded in 60-mm plates in medium containing 5 μg/ml Polybrene. Media were changed on day 3, and on day 6 cells were trypsinized and reseeded in 60-mm plates at a concentration of 3×10^5 cells per plate. Reverse transcriptase activity was determined in a 24-h collection of supernatant medium from infected cells 10 days post infection by methods previously described (5), and is expressed as cpm [^{3}H]TTP incorporated $\times 10^3$ per 8.0 ml of medium. The XC plaque-forming ability of each MuLV was determined by (*a*) direct XC assay on SC-1 cells and (*b*) co-cultivation of productivity infected SC-1 cells with XC cells. In addition, infected and control mink cell cultures were observed for the development of characteristic cytopathic effects (CPE) 5–7 days after infection. Such cultures were kept at 37°C in a Wedco incubator in a humidified atmosphere containing 5% CO_2. Virus preparations used were harvested from infected SC-1 cells, with the exception of NZB-MuLV which was obtained from infected CF2Th cells.

TTP, thymidine triphosphate.

* cpm [^{3}H]TTP incorporated per 8.0 ml medium.

‡ CPE, cytopathic effects.

§ N.R., nonreplicating.

‖ N.D., not done.

To test the host range of the MuLVs isolated from F-MuLV spleens, a twice terminally-diluted isolate of each was directly inoculated onto several mouse and non-mouse cell lines, and virus replication was measured by reverse transcriptase assay. The results with the Fr-MCF viruses are shown in Table I. F-MuLV$_{201}$ grew on SC-1 cells, induced XC plaque formation, and failed to grow on mink cells. NZB-MuLV, a xenotropic virus, grew on mink and SIRC cells but not on SC-1 cells. AMT 1504A grew on both SC-1 and mink cells, and failed to induce XC syncytia. Mink cell cultures productively infected with either AMT 1504A or NZB-MuLV were morphologically indistinguishable from uninfected mink cells. AKR-MCF 247 grew on both mouse and mink cells, was XC negative, and induced typical MCF foci when grown on mink lung cells (7). Both of the MuLV isolated from F-MuLV leukemic spleens grew on SC-1 and mink cells, failed to induce XC plaques, and induced cytopathic foci on mink cells identical to those previously described for AKR-MCF isolates and Mol-MCF$_{83}$. In experiments not shown, the Fr-MCF isolates grew with similar efficiency on both BALB 3T3 and NIH 3T3 cells.

Thus, the two isolates of mink-infectious MuLV obtained from F-MuLV-leukemic spleen cells have a host range that includes both mouse cells and mink cells, they are XC negative, and they induce cytopathic foci when grown on mink cells. Because these viruses share unique biological properties with the AKR-MCF viruses described by Hartley et al. (7), we have designated these MuLV isolated from F-MuLV-induced leukemic spleens as Fr-MCF viruses.

TABLE II

Interference Properties of Fr-MCF Viruses

Challenge virus (KiSV pseudotype)	Percentage of focus induction relative to uninfected cells, seen on cells preinfected with:			
	SC-1 Cells			
	Uninfected	F-MuLV$_{201}$	Fr-MCF-1	AMT 1504A
F-MuLV$_{201}$	100	0*	[6]	49
AMT 1504A	100	100	100	0
Fr-MCF-1	100	[16]	0	48
Fr-MCF-2	100	[15]	0	52
	Mink cells			
	Uninfected	NZB MuLV	Fr-MCF-1	AMT 1504A
NZB MuLV	100	0	[0]	100
AMT 1504A	100	110	100	0
Fr-MCF-1	100	[0]	0	[9]
Fr-MCF-2	100	[0]	0	[11]

Interference studies were performed by inoculating various KiSV pseudotypes onto either SC-1 or mink cells which were infected with various cloned MuLV. Each cell line was seeded in 60-mm plates at 1×10^5 cells per plate in medium containing Polybrene (5 μg/ml), and inoculated 24 h later with $\geq 10^2$ focus-forming units of the KiSV pseudotypes. Media were changed every 3 days. Foci were scored at 10 days, and are expressed as a percentage of the number of foci seen with each KiSV pseudotype on preinfected as compared to uninfected cells.

* 0 = < than 0.02% of number of foci seen compared to uninfected cells.

Interference Pattern of Fr-MCF Isolates. In previous studies it was demonstrated that MCF-type viruses derived from AKR-MuLV and Mol-MuLV were interfered with completely by xenotropic viruses and partially by ecotropic viruses, but not at all by amphotropic MuLV 1504A, providing strong biologic evidence that the broadened host range of these MCF isolates had been acquired as a result of recombination between ecotropic and xenotropic MuLV within the *env* gene region (7, 8, 18). To determine whether or not the Fr-MCF isolates shared envelope properties with ecotropic, xenotropic, or amphotropic MuLV, we analyzed the interference pattern of each Fr-MCF isolate with regard to interference by ecotropic virus (F-MuLV), xenotropic virus (NZB MuLV), or wild mouse amphotropic virus (AMT 1504A). The results of these interference studies are shown in Table II.

In the interference assays on SC-1 cells, F-MuLV 201 was slightly interfered with by AMT 1504A. However, inoculation of KiSV/F-MuLV$_{201}$ onto SC-1 cells chronically infected with Fr-MCF-1 resulted in a significantly reduced number of foci compared to uninfected SC-1 cells. The number of foci seen on Fr-MCF-1/SC-1 was only 6% of that seen on SC-1 cells. Similarly, inoculation of KiSV/Fr-MCF pseudotypes onto SC-1 cells infected with F-MuLV$_{201}$ results in a diminished number of foci compared to SC-1 cells (reduction of foci, 15–16%). In the assays on mink cells, the Fr-MCF pseudotypes of KiSV failed to initiate any focus formation on mink cells infected with NZB-MuLV, but did induce focus formation on mink cells infected

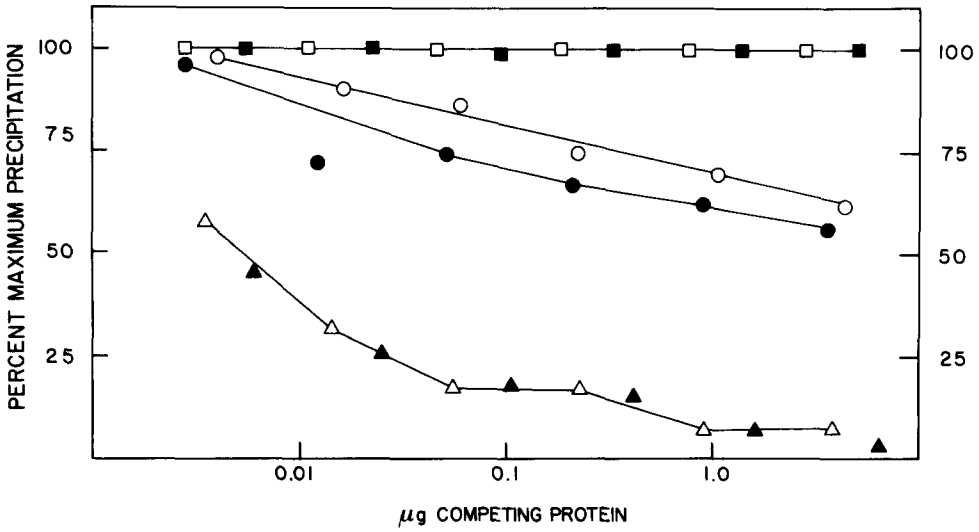


FIG. 1. p12 Radioimmunoassay. Double antibody p12 radioimmunoassay was performed in 0.05-ml reaction mixtures using techniques which have been detailed in a previous publication (17), with the order of addition of reaction components as well as the incubation time and temperature unchanged. ^{125}I-labeled Rauscher strain (R)-MuLV p12 (≅ 50,000 cpm) was used as labeled antigen for each assay, and a 1:6,400 dilution of goat anti-Friend leukemia virus (FLV) antisera was used as primary antibody. The second antibody was PAG, which was used at a 1:8 dilution. In the absence of competing antigen, the primary antibody precipitated ≅ 10,000 cpm of the ^{125}I-labeled R-MuLV. ^{125}I radioactivity present in the pelleted precipitate was counted in an LKB gamma scintillation counter, as previously described (17). The results of the competition assay are plotted as percent precipitation of the maximum counts precipitated in the absence of competing antigen vs. micrograms of competing antigen. Triton-disrupted preparations of MuLV used to compete in this assay include: AKR-MuLV (□); NZB-MuLV (■); Mol-MuLV$_{eco}$ (●); Mol-MCF$_{83}$ (○); F-MuLV$_{201}$ (▲); Fr-MCF-1 (△).

with AMT 1504A. However, there was a significant reduction in foci on AMT 1504A-infected mink cells inoculated with Fr-MCF pseudotypes of KiSV compared to uninfected mink cells. The reason for this partial interference of Fr-MCF viruses by AMT 1504A is currently under study.

These results indicate that the Fr-MCF viruses are completely interfered with by xenotropic virus and partially by F-MuLV$_{eco}$, and provide biologic evidence that the Fr-MCF isolates have acquired envelope properties derived from both ecotropic and xenotropic virus. Similar results have been previously reported for AKR-MCF viruses and Mol-MuLV$_{83}$ (7, 8).

Radioimmunoassay of Fr-MCF p12. We next undertook to analyze the molecular composition of the Fr-MCF viruses. To further determine whether or not each Fr-MCF isolate was derived from F-MuLV$_{201}$, we performed a type-specific p12 radioimmunoassay (20) in which it was possible to distinguish F-MuLV from other murine ecotropic and xenotropic viruses. In the type-specific assay which we used, R-MuLV p12 was substituted for F-MuLV p12, since these two MuLV have p12 molecules which are virtually indistinguishable in a type-specific radioimmunoassay. The results of the competition assay are shown in Fig. 1. F-MuLV$_{201}$ competed completely with labeled R-MuLV p12, whereas AKR-MuLV and NZB-MuLV failed to compete at all. In addition, helper-independent xenotropic virus derived from an NIH Swiss mouse, ATS-124, failed to compete in this assay (data not shown). Both Mol-MuLV$_{eco}$

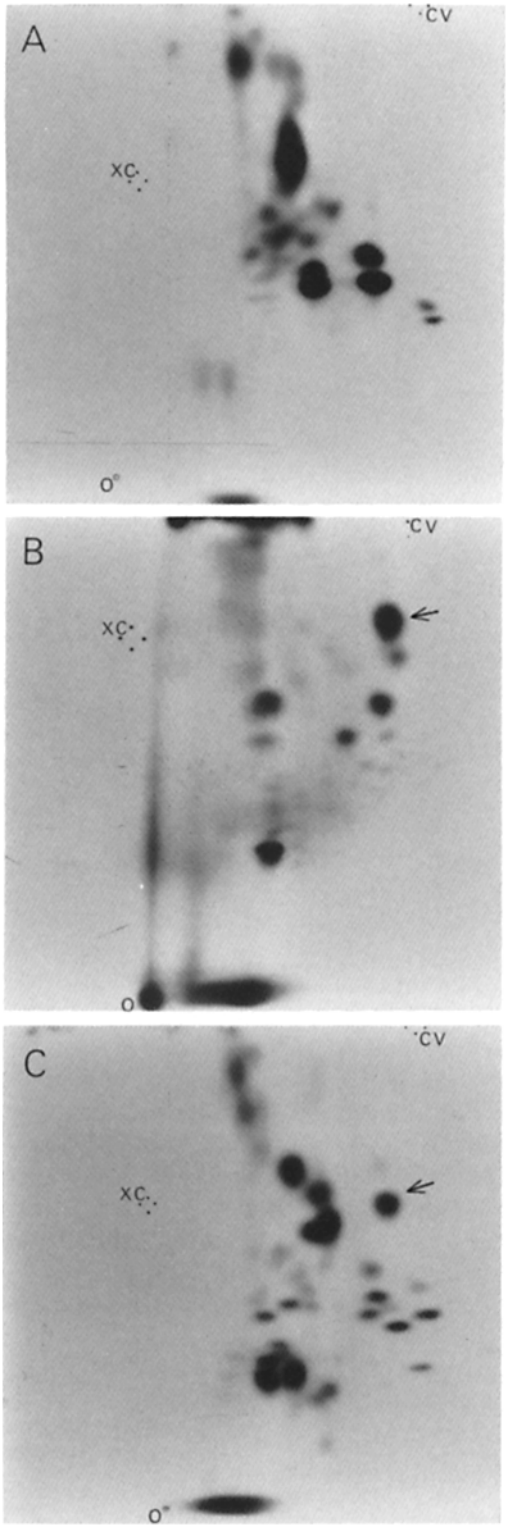
and Mol-MCF$_{83}$ partially competed (to a level of ≅50–60%), but had p12 molecules which were clearly distinguishable from that of F-MuLV$_{201}$. Finally, Fr-MCF-1 competed with a pattern which was identical to that of F-MuLV$_{201}$, and was clearly distinguishable from AKR-MuLV, NZB-MuLV, and Mol-MuLV. These results demonstrate that the p12 of Fr-MCF-1 is fully cross-reactive with that of F-MuLV$_{201}$, and they further indicate that the genetic region of Fr-MCF which encodes for p12, located at the 5′ end of the genome (21), was derived from F-MuLV. A competition curve similar to that of Fr-MCF-1 was obtained using Fr-MCF-2 as competing antigen.

These results demonstrate that the Fr-MCF viruses contain genetic information that was derived from F-MuLV$_{eco}$ inoculated into NIH Swiss mice from which each Fr-MCF virus was isolated. Furthermore, along with biological evidence that the Fr-MCF viruses have host range and interference properties derived from both ecotropic and xenotropic virus, the p12 data make it highly probable that these viruses arose by recombination between F-MuLV$_{eco}$ and an endogenous xenotropic virus.

Tryptic Peptides of gp70s of F-MuLV and Fr-MCF. To further document that the Fr-MCF viruses, like SFFV and AKR-MCF viruses, are *env* gene recombinants, we compared the tryptic fingerprints of gp70s isolated from F-MuLV$_{201}$, Fr-MCF-1, and NZB-MuLV, using the techniques described in the legend to Fig. 2. The results of this tryptic peptide analysis are shown in Fig. 2. The gp70 maps of the parental F-MuLV$_{201}$ (2 A) and a representative xenotropic virus, NZB-MuLV (2 B) can be readily distinguished. The spot indicated with an arrow in the map of NZB-MuLV gp70 is identical to that reported by Elder et al. (22, 23) to be specific for xenotropic mouse viruses. As shown in Fig. 2 C, the gp70 map of Fr-MCF-1 contains this spot, it has retained some of the F-MuLV specific peptides, and it has lost other peptides present in F-MuLV gp70. These results provide strong evidence that Fr-MCF-1 is an intragenic *env* gene recombinant between F-MuLV$_{eco}$ and a mouse xenotropic virus.

Hybridization of cDNA$_{F\text{-}MuLV}$ to the Fr-MCF Isolates. To obtain additional biochemical evidence that the Fr-MCF viruses were derived by recombination between F-MuLV$_{eco}$ and endogenous xenotropic virus, we analyzed the genomic RNA of each Fr-MCF virus by molecular hybridization using two distinct cDNA probes, cDNA$_{F\text{-}MuLV}$ and cDNA$_{SFFV}$. In the molecular hybridization experiments described below, we have used total cellular RNA as a source of RNA for F-MuLV grown on NRK cells to serve as a more appropriate control for cellular RNA from SFFV-NRK nonproducer cells. However, in the experiments with helper-independent ecotropic and MCF viruses, we used 70s viral RNA instead of cellular RNA from infected cells. In each

FIG. 2. Fingerprint analysis of Fr-MCF-1 gp70 tryptic peptides. The gp70s of F-MuLV grown on SC-1 cells, NZB virus grown on dog cells, and Fr-MCF-1 grown on mink cells was isolated by phosphocellulose chromatography (16). Each protein was labeled with ^{125}I using the chloramine T method (29), and was subjected to sodium dodecyl sulfate-acrylamide gel electrophoresis by the procedure of Cross and Fields (30) on discontinuous sodium dodecyl sulfate gels. The ^{125}I-gp70 bands were identified by autoradiography, eluted with diphenylcarbamyl chloride-treated trypsin, (Sigma Chemical Co., St. Louis, Mo.) (DCC)-trypsin (31) and subjected to two dimensional peptide analyses on cellulose thin layer plates. Each map had ≅ 50,000 cpm applied, and spots were identified by autoradiography on Kodak X-Omat X-ray film. "O" indicates origin, and the dotted upper circles indicated the dye markers xylene cyanol (XC) and crystal violet (CV) included in each sample run. Electrophoresis was conducted from left (+) to right (−) in 30% formic acid at 20°C, and ascending chromatography in butanol-pyridine-acetic acid-water (15:10:3:12), pH 6.0. (A), F-MuLV gp70; (B), NZB-MuLV gp70; (C), Fr-MCF-1 gp70.


A
CV
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C
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TABLE III

Hybridization of $cDNA_{F\text{-}MuLV}$ and $cDNA_{SFFV}$ to RNA from Various MuLV

Source of RNA	Hybridization with:			
	$cDNA_{F\text{-}MuLV}$		$cDNA_{SFFV}$	
	cpm	*% hybrid*	*cpm*	*% hybrid*
Cellular RNAs				
F-MuLV/NRK	855	100	24	3
SFFV-NRK C1 1	375	43	727	100
NZB-MuLV/CF2Th	496	58	624	86
Viral RNAs				
$Mol\text{-}MuLV_{eco}$*	428	50	36	5
$Mol\text{-}MCF_{83}$‡	435	51	636	88
AKR-MuLV*	333	39	30	4
AKR-MCF #247‡	325	38	565	78
Fr-MCF-1‡	785	92	736	101
Fr-MCF-2‡	802	94	720	99

Hybridization of $cDNA_{F\text{-}MuLV}$ and $cDNA_{SFFV}$ to either cellular RNA or viral RNA was performed as described in Materials and Methods, using saturating amounts of cellular or viral RNA. 2,000 trichloroacetic acid-insoluble cpm of $cDNA_{F\text{-}MuLV}$ and 1,500 trichloroacetic acid-insoluble cpm of $cDNA_{SFFV}$ were used in each assay, and the values shown reflect the average of duplicate points. S1 conditions were as previously described (6). The S1 resistant cpm and percent hybridization shown above represent corrected values for 100% hybridization to F-MuLV/NRK (for $cDNA_{F\text{-}MuLV}$) and to SFFV-NRK C1 1 (for $cDNA_{SFFV}$), and 0% hybridization to RNA from uninfected cells. Absolute cpm hybridized with each probe to uninfected cellular RNA were: $cDNA_{F\text{-}MuLV}$ to dog cellular RNA, 49 cpm; to NRK cellular RNA, 130 cpm; $cDNA_{SFFV}$ to dog cellular RNA, 30 cpm; to NRK cellular RNA, 147 cpm.

* Virus grown on SC-1 cells.

‡ Virus grown on mink cells.

hybridization assay, saturating amounts of viral or cellular RNA were used.

Since F-MuLV can be distinguished from most other MuLV by molecular hybridization with $cDNA_{F\text{-}MuLV}$ under stringent conditions (D. H. Troxler and E. M. Scolnick, unpublished observations), the final extent of hybridization of $cDNA_{F\text{-}MuLV}$ to the Fr-MCF isolated could provide additional evidence that these viruses were in fact derived from F-MuLV. The results of hybridization of a representative $cDNA_{F\text{-}MuLV}$ to a variety of ecotropic, xenotropic, and MCF strains of MuLV, including the Fr-MCF isolates, are shown in Table III. $cDNA_{F\text{-}MuLV}$ hybridizes to RNA of rat cells producing F-MuLV to a value of 100%, whereas it hybridizes only 58% to RNA from cells producing xenotropic virus (NZB-MuLV), 50% to viral RNA from both ecotropic and MCF-type Mol-MuLV isolates, and 38% to RNA from AKR-MuLV or AKR-MCF 247. As shown in Table III, $F\text{-}MuLV_{cDNA}$ hybridizes >90% to viral RNA from both of the Fr-MCF viruses, indicating that much of the genetic information contained in these viruses was derived from F-MuLV.

Hybridization of $cDNA_{SFFV}$ to Fr-MCF Viruses. Hybridization of $cDNA_{SFFV}$ to the Fr-MCF isolates was of interest because $cDNA_{SFFV}$ hybridized to both AKR- and

Moloney-derived MCF viruses, but did not hybridize to each parental ecotropic MuLV. The results of molecular hybridization experiments using $cDNA_{SFFV}$ are also shown in Table III. As previously reported, $cDNA_{SFFV}$ hybridizes well (100%) to cellular RNA from NRK cells nonproductively infected with SFFV, to RNA from cells infected with xenotropic NZB virus (86%), and to viral RNA from two MCF-type viruses, $Mol\text{-}MCF_{83}$ (88%) and AKR-MCF 247 (78%); it does not, however, hybridize to ecotropic F-MuLV, Mol-MuLV, or AKR-MuLV. As shown in Table III, $cDNA_{SFFV}$ hybridizes completely (100%) to RNA from the Fr-MCF viruses. These results suggest that the Fr-MCF isolates, like other MCF-type viruses, contain genetic sequences derived from the *env* gene region of xenotropic virus, and thus provide biochemical evidence that the Fr-MCF viruses arose by recombination between F-MuLV and xenotropic *env* gene sequences. Furthermore, the results with both cDNA probes suggest that Fr-MCF viruses potentially contain most, if not all, of the genetic information that is present in the highly leukemogenic SFFV.

Discussion

Two biologically dissimilar MuLV systems, the AKR-thymic leukemia system and the Friend erythroleukemia system have recently been found to exhibit certain biochemical parallels, suggesting that recombination between ecotropic and endogenous xenotropic virus might be important in the leukemia induced by murine type-C viruses (6–8). An analysis of parallels and contrasts between these two systems seems to be a potentially rewarding approach toward elucidating the fundamental molecular events involved in the pathogenesis of leukemia.

In the AKR system, thymic leukemia is the predominant disease (24), and extensive insights have recently been gained with regard to the virological aspects of AKR leukemia (7, 25). A new class of viruses which has the capacity to induce cytopathic foci on mink cells (AKR-MCF viruses) has been isolated from preleukemic and leukemic thymus tissue of AKR mice, and from similar tissues from NIH Swiss mice that are partially congenic for the AKv-1 locus (7). The AKR-MCF viruses, some of which accelerate leukemia in AKR mice (J. Hartley, personal communication), have been extensively characterized and have been shown to be *env* gene recombinants derived from two different parental viruses, N-tropic ecotropic AKR virus and a murine xenotropic virus (7, 23, 26). Although the most thoroughly studied MCF viruses are replicating, helper-independent viruses, a replication-defective MCF variant, the AKT8 virus, has been isolated from AKR leukemic cells (9). The precise relationship of this virus to the replicating AKR-MCF has not yet been elucidated; however, the AKT-8 virus appears to have an enhanced transforming capacity for mink lung cells.

In the F-MuLV system, a highly leukemogenic replication-defective viral component, SFFV (2), has been cloned free of helper virus and has also been shown to be an *env* gene recombinant virus derived from $F\text{-}MuLV_{eco}$ and xenotropic virus (5, 6, 8). SFFV causes the rapid transformation of erythropoietin-responsive stem cells (3, 27), resulting in splenomegaly and polycythemia (28), even when inoculated into mice after being pseudotyped in cell culture by helper viruses other than F-MuLV (D. H. Troxler, R. A. Steeves, and E. M. Scolnick, unpublished observations). Furthermore, when appropriate pseudotypes of SFFV are inoculated onto mink lung cells, MCF-

like foci have not been observed (8). Thus, whereas in the AKR system, recombination between AKR virus and xenotropic *env* gene sequences has given rise to helper-independent MCF viruses which are apparently leukemogenic for thymic cells, in the Friend system, recombination between F-MuLV and xenotropic *env* genetic information has, up until now, been associated only with the replication-defective SFFV which is leukemogenic for erythroid and not thymic cells and which does not induce MCF effects when grown on mink cells. From the data available on the AKR-MCF viruses and SFFV, it has not thus far been possible to ascertain which specific biochemical and biological properties of each of these recombinant viruses can be related to their different leukemogenic effects.

In the current studies, we sought to isolate recombinants between F-MuLV$_{eco}$ and endogenous xenotropic virus which might be more analogous to the MCF-viruses that have been obtained from AKR leukemic tissues. We report in this paper the isolation of two helper-independent viruses which share unique biological properties with the helper-independent MCF viruses derived from AKR mice. The Fr-MCF viruses grow on both mouse cells and mink cells, induce typical MCF cytopathic foci when grown on mink cells, cross-interfere completely with xenotropic virus, and have *env* gene products (gp70s) which, based on tryptic peptide analysis, are derived from both F-MuLV and a xenotropic virus parent. Furthermore, like other MCF viruses, RNA of the Fr-MCF viruses hybridizes well to cDNA$_{SFFV}$, a probe for the non-F-MuLV portions of SFFV. Thus, the isolation of these helper-independent *env* gene recombinants between F-MuLV and xenotropic virus from NIH Swiss mice inoculated with F-MuLV further extends the similarities between the AKR and the F-MuLV systems.

Studies are currently in progress in our laboratory to determine whether or not Fr-MCF viruses are leukemogenic for newborn and adult mice, either alone or phenotypic mixtures with F-MuLV$_{eco}$. Apart from their leukemogenic properties, the most potentially useful application of the Fr-MCF viruses that we have described herein is their use in studies designed to investigate the origin of the highly leukemogenic SFFV. Although replication-defective, SFFV has been shown in previous studies to be a recombinant between F-MuLV and xenotropic *env* gene sequences, and molecular hybridization analysis of the Fr-MCF viruses presented in this report has demonstrated that these viruses likewise contain xenotropic *env* gene sequences which are homologous to those in SFFV. Thus, a plausible hypothesis for the generation of SFFV would be that F-MuLV first underwent recombination with xenotropic *env* gene sequences to give rise to a helper-independent recombinant virus similar to the Fr-MCF viruses that we have described. In this model, a replicating MCF-type virus would be an intermediate stage in the formation of SFFV, since SFFV might have been generated by a further genomic modification (such as a deletion) of the RNA genome of the replicating recombinant virus. On the other hand, it is possible that SFFV might have arisen from an *env* gene recombinational event which occurred between F-MuLV and a xenotropic virus, but which for unknown reasons resulted in the formation of replication-defective virus. The isolation of different Fr-MCF viruses and an analysis of their leukemogenic properties might give clues as to which of the above hypotheses is true. In either case, a detailed biologic and biochemical characterization of the various xenotropic *env* gene recombinant viruses derived from F-MuLV (including different Fr-MCF isolates as well as SFFV) might contribute to an understanding of the specific genetic region(s) and gene product(s) which are coded for by these viruses

and which might be directly involved in the leukemic transformation of specific target cells.

Summary

Recent studies have indicated that both the replication-defective spleen focus-forming virus (SFFV) in the Friend virus complex and the helper-independent mink cell focus-inducing (MCF) viruses derived from AKR-murine leukemia virus (MuLV) are *env* gene recombinants between ecotropic virus and xenotropic virus. In an attempt to isolate additional *env* gene recombinants between Friend murine leukemia virus (F-MuLV) and xenotropic virus, we have inoculated cloned ecotropic F-MuLV into newborn NIH Swiss mice and analyzed MuLV released from preleukemic and leukemic spleens of infected mice. Two helper-independent MCF strains of F-MuLV have been isolated. Like the previously described AKR-MCF viruses, the Friend MCF viruses are *env* gene recombinants between an ecotropic virus (F-MuLV) and a mouse xenotropic virus, as shown by host range, interference pattern, and tryptic peptide analysis of the gp70s of these MuLV. Furthermore, RNA from the Friend MCF viruses hybridizes completely to $cDNA_{SFFV}$, a nucleic acid probe which detects that portion of SFFV which was not derived from F-MuLV. The ability to isolate replicating MCF viruses derived from F-MuLV further strengthens the parallels between the Friend erythroleukemia system and the AKR thymic leukemia system. Finally, the potential relationship of helper-independent *env* gene recombinants between F-MuLV and xenotropic virus to be highly leukemogenic SFFV is discussed.

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