The Envelope Gene and Long Terminal Repeat Sequences Contribute to the Pathogenic Phenotype of Helper-Independent Friend Viruses

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Friend murine leukemia virus (F-MuLV) and Friend mink cell focus-inducing virus (Fr-MCF) are helper-independent murine retroviruses which induce a rapidly fatal erythroleukemia in NIH Swiss mice. Amphotropic clone 4070 (Ampho) is a murine retrovirus which does not cause leukemia in these animals. Mice inoculated with Ampho, an Fr-MCF/Ampho pseudotype, or F-MuLV developed leukemia in 0, 50, and 100% of animals, respectively. To identify the F-MuLV and Fr-MCF sequences responsible for leukemia, we constructed hybrid viral genomes between these viruses and Ampho, using subgenomic fragments of molecularly cloned viral DNA. Transfection of these hybrid viral DNAs into fibroblasts produces recombinant retroviruses. These new viruses are assayed in vivo for their ability to cause leukemia. Recombinant viruses constructed between the Ampho genome and the Fr-MCF envelope gene do not cause leukemia. Similarly, viruses constructed by using either the Fr-MCF long terminal repeat U3 region or the F-MuLV long terminal repeat U3 region and the remainder of the Ampho genome do not cause leukemia. However, if the Fr-MCF envelope gene plus the Fr-MCF U3 region are joined to Ampho, the resulting virus causes erythroleukemia in 14% of mice. Recombinant viruses made between the Fr-MCF envelope gene, the F-MuLV U3 region, and the remainder of the Ampho genome cause erythroleukemia in 38% of mice. This study demonstrates that both the envelope gene of Fr-MCF and the U3 regions of Fr-MCF and F-MuLV contain sequences which contribute to the leukemic phenotype of helper-independent Friend viruses.

The helper-independent Friend leukemia viruses include Friend murine leukemia virus (F-MuLV) and Friend mink cell focus-inducing virus (Fr-MCF). Each of these viruses is a replication-competent type C retrovirus which can induce a rapidly fatal erythroleukemia in susceptible strains of mice (28, 31). Previously, we molecularly cloned the genomes of these two viruses to ensure their purity (10, 22). In addition, we molecularly cloned the genome of an amphotropic MuLV, clone 4070 (Ampho) (9, 4). Ampho is also a replication-competent type C retrovirus, but Ampho does not induce leukemia in NIH Swiss or NFS/n mice (21, 23).

To identify the F-MuLV and Fr-MCF genetic sequences responsible for causing leukemia, we constructed hybrid viral genomes between the DNAs of F-MuLV or Fr-MCF and Ampho. These hybrid viral DNAs produce recombinant retroviruses upon transfection into NIH 3T3 cells. A leukemogenic recombinant virus (clone 1) has been generated in this manner, using a cloned DNA fragment containing the F-MuLV envelope gene and long terminal repeat (LTR) plus the entire Ampho genome (21). Clone 1 induces leukemia in 50% of animals within 8 to 12 weeks after inoculation of newborn mice. The leukemia caused by clone 1 is pathologically and histologically indistinguishable from F-MuLV-induced disease. Leukemogenic recombinants (5a25 virus) have also been constructed by using only the F-MuLV envelope gene plus the remainder of the Ampho genome (23). However, 5a25 virus produces a lower disease incidence (22%) and exhibits a longer latency period (up to 6 months) for leukemia than does clone 1. The prolonged latency period and lower disease incidence exhibited by 5a25 virus versus clone 1 imply that the F-MuLV LTR can increase the pathogenicity of viruses containing these sequences.

F-MuLV-, clone 1-, and 5a25-induced leukemias are associated with the generation of mink cell focus-inducing virus in host cells (21, 23, 28, 32). Fr-MCF can also cause erythroleukemia (28). Therefore, it is possible that the leukemia associated with F-MuLV is caused by the Fr-MCF that arises during F-MuLV infection. F-MuLV and Fr-MCF are genetically similar viruses. However, heteroduplex, restriction enzyme, and nucleotide sequence analyses indicate that the major differences between F-MuLV and Fr-MCF lie in the gp70 portion of their envelope genes. The *gag*, *pol*, *p15E*, and LTR regions of these viruses are closely related (15, 7a, 22). Therefore, the biological differences between Fr-MCF and F-MuLV are likely to be specified by envelope gene sequences. Alternatively, small differences in other viral sequences (e.g., the LTR region) could account for the functional differences between F-MuLV and Fr-MCF (15).

To analyze the role of the F-MuLV and Fr-MCF LTRs and the Fr-MCF envelope gene in leukemogenesis, we asked whether these genetic elements could convert Ampho into a leukemia-causing virus. Recombinant murine retroviruses were generated in vitro by using molecularly cloned subgenomic fragments of F-MuLV or Fr-MCF DNA and the remainder of the Ampho genome. These newly generated viruses were then tested in vivo for their ability to cause leukemia.

MATERIALS AND METHODS

Mice. Adult NIH Swiss or NFS/n mice were obtained from the Small Animal Facility of the National Institutes of Health, Bethesda, Md. Newborn mice (<24 h old) were bred in our own animal facility.

Viruses. F-MuLV clone 57 used in these studies is an NB-tropic ecotropic retrovirus obtained from the transfection of molecularly cloned F-MuLV DNA into NIH 3T3 cells (20). Ampho is an N-tropic amphotropic retrovirus obtained

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from the transfection of molecularly cloned Amphi 4070 DNA into NIH 3T3 fibroblasts (4). Fr-MCF is an NB-tropic dualtropic retrovirus obtained by endpoint dilution cloning from spleen cell extracts of leukemic NIH Swiss mice (32).

Cells. NIH 3T3 mouse embryo fibroblasts were used in all transfection studies (13). BALB/3T3 cells (13) and mink lung cells (American Type Culture Collection, Rockville, Md.) were a gift from Paul O'Donnell.

Viral DNAs. Isolation and molecular cloning of the entire F-MuLV, Fr-MCF, and Amphi genomes has been described previously (4, 20, 22). Briefly, form I circular viral DNA was isolated from Hirt extracts of acutely infected fibroblasts. This viral circular DNA was digested with a restriction enzyme that made a single cut in the viral genome. The resulting linearized viral DNA was permuted in its orientation relative to the *in vivo* viral genome. The linearized viral DNA was ligated into an appropriately cut plasmid vector, and the ligation mixture was transformed into a bacterial host. Bacterial clones containing the recombinant DNA were identified by Southern blot analysis. Restriction enzyme digests and agarose gel electrophoresis were carried out as described previously (20). DNA ligations, bacterial transformations, and plasmid DNA purifications were also carried out as described previously (20).

Transfections. Viral DNA transfections were performed by a modification of the technique of Graham and Van Der Eb (8). Reverse transcriptase levels and XC plaque assays were analyzed according to published procedures (27, 30).

Animal studies. Newborn NIH Swiss mice were inoculated by intraperitoneal injection of 0.2 ml of the viral stocks. Viral stocks were prepared either from chronically infected fibroblasts, as described previously (20), or from infected mice by homogenizing the spleens of these mice in phosphate-buffered saline (10% [wt/vol]) and removing the cell debris by low-speed centrifugation ($1,000 \times g$ for 10 min).

Histology. Peripheral blood smears and spleen cell suspensions were examined microscopically. Cytochrome preparations were made with a cytospin centrifuge. All slides were stained with dimethoxybenzidine and counterstained with Wright-Giemsa stain as described previously (21).

RESULTS

The strategy used to construct recombinant viral genomes between F-MuLV or Fr-MCF and Amphi depends on the presence of common restriction enzyme sites in the cloned DNAs of these viruses. These enzyme sites are used to exchange homologous segments of DNA between viral genomes. Figure 1 shows restriction enzyme maps of molecularly cloned F-MuLV, Fr-MCF, and Amphi DNA. Each DNA was approximately 8.2 kilobase pairs (kbp) in length and contained a single copy of the LTR region. All three viral DNAs were cloned at different restriction enzyme sites. Therefore, each viral genome was permuted in its orientation relative to the other two viral DNAs. Several restriction enzyme sites were shared by these DNAs (Fig. 1). A *Clal* site was present just upstream from the 3' LTR in all three DNAs. Similarly, an *EcoRI* site was present approximately 1.0 kbp upstream from the *Clal* site in the Fr-MCF and Amphi DNAs, and an *SphI* site was located 2.4 kbp upstream of the *Clal* site in all three viral DNAs. Nucleotide sequence analysis of the envelope genes of F-MuLV and Fr-MCF indicated that this *Clal* site was located in the carboxy-terminal one-fifth of the p15E gene, and the *EcoRI* site was located near the middle of the gp70 coding sequences. The *SphI* site was located near the C terminus of the *pol* gene, ca.

500 bp upstream from the start of *env* (14, 15). Comparable nucleotide sequence data are not yet available for Amphi.

Amphi/F-MuLV LTR. To isolate the F-MuLV LTR, we first subcloned a 4.1-kbp *HindIII* fragment containing the F-MuLV LTR and envelope genes (Fig. 2). This *HindIII* fragment was then divided at the *Clal* site into two *HindIII*-*Clal* segments of 2.4 and 1.7 kbp, respectively. These segments were separated by agarose gel electrophoresis, and the smaller (1.7-kbp) fragment containing the LTR sequences was isolated by electroelution. The Amphi viral genome was also digested free from its pBR322 vector and isolated by electrophoresis. The F-MuLV 1.7-kbp LTR fragment cannot be ligated to the Amphi DNA, which has *EcoRI*-digested ends. Therefore, the Amphi DNA was digested with *Clal* to produce two *EcoRI*-*Clal* fragments. These two Amphi DNA fragments were ligated together with the F-MuLV LTR fragment into a pBR322 vector between the *HindIII* and *Clal* sites. Appropriate recombinants were identified by hybridization to nick-translated radiolabeled probes made from the F-MuLV LTR fragment and one of the two Amphi DNA fragments. Eight clones were found which hybridized to both probes. The plasmid DNA from these clones was analyzed by restriction enzyme digestions, and two of the clones proved to have the structure indicated in Fig. 2. These clones were designated L-1-1 and -2.

Amphi/Fr-MCF LTR. To isolate the Fr-MCF LTR sequences, we first cloned the Fr-MCF LTR and envelope gene between *SphI* and *HindIII* (Fig. 3). This DNA was digested with *EcoRI* to yield a 2.7-kbp fragment containing the Fr-MCF LTR plus the C-terminal half of gp70 and the entire p15E gene. Amphi DNA was isolated from its plasmid vector by digestion with *EcoRI* and then was cut with *Clal*. The two resulting Amphi DNA fragments were isolated by gel electrophoresis. The 7.2-kbp Amphi *Clal*-*EcoRI* fragment was mixed with the Fr-MCF LTR fragment and ligated into pBR322 between the *EcoRI* and *Clal* sites. Appropriate recombinants were screened by hybridization to nick-translated probes made from each of the desired viral DNA fragments. Eight hybridization-positive clones were picked and analyzed by restriction enzyme digestions. Five of these eight clones proved to have the structure indicated in Fig. 3 (A/Fr LTR-1 to -5).

Amphi/Fr-MCF envelope gene. The Fr-MCF envelope gene was contained in the *SphI*-*HindIII* 3.9-kbp DNA frag-

FIG. 1. Schematic representation of the restriction enzyme maps of molecularly cloned F-MuLV, Fr-MCF, and Amphi viral DNA. Note that all three viral DNAs were cloned at different restriction enzyme sites. Therefore, all three viral genomes were permuted in orientation relative to one another. Abbreviations: A, *AccI*; C, *Clal*; H, *HindIII*; Hc, *HincII*; K, *KpnI*; P, *PstI*; Pv, *PvuI*; R, *EcoRI*; S, *SphI*; and X, *XbaI*.

FIG. 2. Schematic representation of the molecular cloning of the recombinant viral genome L-1. Viral DNAs shown on lines 1 and 2 were molecularly cloned before their ligation into the hybrid viral genome shown on line 3. Abbreviations: C, *Clal*; H, *HindIII*; K, *KpnI*; P, *PstI*; R, *EcoRI*; and S, *SphI*.

ment (Fig. 3). This fragment was digested with *Clal*, and the resulting 2.9-kbp DNA was isolated. The 2.9-kbp *Clal* fragment contained 2.4 kbp of Fr-MCF DNA and 0.5 kbp of pBR322 sequences. The AmpHo *Clal*-*EcoRI* 7.2-kbp fragment (Fig. 3) was combined with the Fr-MCF *Clal* fragment, and these DNAs were ligated into pBR322 (Fig. 4). Appropriate recombinants were identified by hybridization to a radiolabeled Fr-MCF-specific envelope gene probe (22). One hybridization-positive recombinant was characterized by restriction enzyme analysis and found to have the structure shown in Fig. 4. This clone contained a 9.6-kbp hybrid viral DNA that contained both Fr-MCF and AmpHo envelope gene sequences. We eliminated the AmpHo envelope sequences from this hybrid by subcloning an 8.2-kbp DNA into pBR322 (Fig. 4, line 4). Three subclones were analyzed by restriction enzyme digestions and designated A/Fr Env-1 to -3.

AmpHo/Fr Env plus LTR. The Fr-MCF *SphI*-*HindIII* 3.9-kbp fragment contained the entire envelope gene and LTR of Fr-MCF (Fig. 3). The AmpHo *Clal*-*EcoRI* 7.2-kbp fragment contained the AmpHo LTR, *gag*, and *pol* genes plus the N-terminal half of the envelope gene (Fig. 5). These two DNAs were ligated together at their *SphI* sites and cloned into pBR322 to yield a 10.1-kbp hybrid viral genome. Appropriate recombinants were identified by hybridization to both the Fr-MCF and AmpHo DNA fragments. Restriction en-

FIG. 3. Schematic representation of the molecular cloning of the recombinant viral genome A/Fr LTR. Viral DNAs shown on lines 1 and 2 were molecularly cloned before their ligation into the hybrid viral genome shown on line 3. Abbreviations: C, *Clal*; H, *HindIII*; K, *KpnI*; P, *PstI*; R, *EcoRI*; and S, *SphI*.

FIG. 4. Schematic representation of the molecular cloning of the recombinant viral genome A/Fr Env. Viral DNAs shown on lines 1 and 2 were molecularly cloned before their ligation into the hybrid viral genome shown on lines 3 and 4. Abbreviations: C, *Clal*; H, *HindIII*; K, *KpnI*; R, *EcoRI*; and S, *SphI*.

zyme analysis of three hybridization-positive recombinants verified the genetic structure shown in Fig. 5, line 3. These recombinants were designated A/Fr E + L-1 to -3.

AmpHo/Fr-MCF Env plus F-MuLV LTR. The F-MuLV LTR was contained within a 1.5-kbp *Clal*-*HindIII* segment of the F-MuLV genome (Fig. 2). The Fr-MCF envelope gene was contained in a 2.4-kbp *SphI*-*Clal* DNA fragment (Fig. 3). The AmpHo LTR *gag* and *pol* genes were located between the *SphI* and *Clal* sites of the AmpHo *Clal*-*EcoRI* 7.2-kbp fragment (Fig. 6). Each of these DNA segments was digested free from its vector and isolated by electrophoresis and electroelution. All three fragments were ligated into pBR322 between the *Clal* and *HindIII* sites. Appropriate recombinants were identified by hybridization to probes made from both the Fr-MCF and F-MuLV DNA fragments. Recombinants that hybridized with both probes were further analyzed by restriction enzyme mapping. Two clones were identified which possessed the structure shown in Fig. 6. These recombinants were designated L-9-1 and -2.

If hybrid viral DNAs are joined at restriction sites which occupy the same position in both parental viral genomes, then the hybrid DNA will encode a complete virus. Transfection of these DNAs into fibroblasts will yield biologically competent virus. Two clonal isolates of each hybrid viral construct (L-1-1, L-1-2, A/Fr LTR-1, A/Fr LTR-3, A/Fr Env-3, A/Fr Env-4, A/Fr E + L-2, A/Fr E + L-3, L-9-1, and

FIG. 5. Schematic representation of the molecular cloning of the recombinant viral genome A/Fr E+L. Viral DNAs shown on lines 1 and 2 were molecularly cloned before their ligation into the hybrid viral genome shown on line 3. Abbreviations: C, *Clal*; H, *HindIII*; K, *KpnI*; R, *EcoRI*; and S, *SphI*.

FIG. 6. Schematic representation of the molecular cloning of the recombinant viral genome L-9. Viral DNAs shown on lines 1 and 2 were molecularly cloned before their ligation into the hybrid viral genome shown on line 3. Abbreviations: C, *Clal*; H, *HindIII*; K, *KpnI*; R, *EcoRI*; and S, *SphI*.

L-9-2) were transfected into NIH 3T3 cells. The L-1, A/Fr LTR, A/Fr E+L, and L-9 plasmids each encode a retroviral genome in the normal proviral orientation (i.e., LTR *gag pol env* LTR). These plasmid DNAs were transfected without modification. The A/Fr Env plasmid contains a retroviral genome that is permuted in orientation relative to a normal provirus. Permuted retroviral DNAs do not efficiently direct virus production in transfected cells unless they are digested free from their vectors before transfection. Therefore, the A/Fr Env plasmid DNA was digested with *SphI* before transfection. Under these transfection procedures, all 12 hybrid viral DNAs produced virus. No virus was obtained when each of the subgenomic DNA fragments used to construct the hybrid viral DNAs was separately transfected into NIH 3T3 cells.

The L-1, A/Fr LTR, A/Fr E+L, and L-9 viral DNAs possessed LTR sequences from both Amphi and either F-MuLV or Fr-MCF (Fig. 2, 3, 5, and 6). In each case, the 5' LTR was donated by Amphi and the 3' LTR was donated by either F-MuLV or Fr-MCF. Even et al. (7) have shown that this type of hybrid retroviral DNA generates virus which derives its U3 sequences from the 3' LTR of the construct. Therefore, viruses derived from the L-1, A/Fr LTR, A/Fr E+L, and L-9 constructs should obtain their U3 sequences from F-MuLV or Fr-MCF. To conclusively establish the origin of the U3 sequences in these viruses, we analyzed the viral DNAs generated by L-1, A/Fr LTR, A/Fr E+L, or L-9 virus in fibroblasts. F-MuLV, Fr-MCF, and Amphi each possess a *PstI* site in their *gag* genes. F-MuLV and Fr-MCF also possess a *PstI* site in their U3 regions. The Amphi U3 region does not contain this site. Each of our recombinant viruses should possess both of these *PstI* sites. Therefore, *PstI* digestion of the DNA generated by these viruses should yield an approximately 1.0-kbp fragment that hybridizes with F-MuLV or Fr-MCF. NIH 3T3 cells were acutely infected with L-1, A/Fr LTR, or Fr-MCF virus. Viral DNA was extracted by the method of Hirt (11). *PstI* restriction analysis of the Hirt-extracted viral DNAs and hybridization with an F-MuLV probe revealed a 1.1-kbp fragment in each DNA (Fig. 7). Similar restriction analysis and hybridization performed on cellular DNA from fibroblasts chronically infected with F-MuLV, A/Fr E+L, or L-9 virus showed a similar *PstI* fragment. Fibroblasts chronically infected with

Amphi do not exhibit this *PstI* fragment (data not shown). Therefore, the L-1, A/Fr LTR, A/Fr E+L, and L-9 viruses must derive their U3 regions from F-MuLV or Fr-MCF.

In vitro growth properties. The tissue culture properties of the L-1, A/Fr LTR, A/Fr Env, A/Fr E+L, and L-9 viruses are listed in Table 1. The growth characteristics of Amphi, Fr-MCF, and F-MuLV are listed for comparison. Amphi is an Fv-1 N-tropic, amphotropic, XC-negative virus. F-MuLV is an Fv-1 NB-tropic, ecotropic, XC-positive virus. Fr-MCF is an Fv-1 NB-tropic, dualtropic, XC-negative virus. Amphi-, eco-, or dualtropic and XC activity are MuLV tissue culture properties specified by the viral envelope gene. Fv-1 tropism is determined by sequences within the *gag* gene (12). Since all of our recombinant viruses derive their *gag* genes from Amphi, they should exhibit Fv-1 N-tropism (i.e., grow better on NIH 3T3 cells than on BALB/3T3 cells). All of the recombinants derive their envelope sequences from either Amphi or Fr-MCF. Therefore, they should exhibit either amphi- or dualtropic (i.e., grow on either murine or nonmurine cells), and all of the recombinants should be XC negative. The recombinant viruses exhibited their predicted tissue culture properties (Table 1). Thus, the in vitro characteristics of the recombinants were consistent with the genetic structure of the hybrid viral DNAs presented in Fig. 2 to 6.

In vivo pathogenic properties. Newborn NIH Swiss mice were inoculated with L-1, A/Fr LTR, A/Fr Env, A/Fr E+L, or L-9 viruses. Newborn mice were also inoculated with F-MuLV, Fr-MCF, or Amphi. Fr-MCF does not cause leukemia when injected by itself into newborn mice. However, if

FIG. 7. Southern blot analysis of viral DNAs, showing an autoradiograph of viral DNA obtained from NIH 3T3 cells acutely infected with Fr-MCF (lanes 1 and 2), A/Fr LTR (lanes 3 and 4), or L-1 virus (lanes 5 and 6). Viral DNA was digested with *PstI* (lanes 1, 3, and 5) or uncut (lanes 2, 4, and 6) and electrophoresed on a 0.8% agarose gel at 35 V for 18 h. DNA was transferred to nitrocellulose paper by Southern blotting and hybridized to a nick-translated radiolabeled probe made from molecularly cloned F-MuLV DNA. The filter was washed until the level of radioactivity returned to background and then was exposed to X-ray film at -70°C for 24 h. Note the 1.1-kbp band (arrow) seen in all three *PstI*-digested viral DNAs. Molecular size markers (in kbp) represent wild-type lambda DNA fragments digested with *HindIII* and electrophoresed in an adjacent lane.

TABLE 1. Virus host range and growth characteristics

Virus	Reverse transcriptase activity (IU/ml) ^a in:			XC fusions
	NIH 3T3 cells	BALB/3T3 cells	Mink lung cells	
F-MuLV	10 ⁵	10 ⁵	<1	+
Fr-MCF	10 ⁴	10 ⁴	10 ³	—
Ampho	10 ⁴	10 ¹	10 ⁴	—
L-1	10 ⁴	10 ²	10 ⁴	—
A/Fr LTR	10 ⁴	10 ²	10 ⁴	—
A/Fr Env	10 ⁴	10 ¹	10 ³	—
A/Fr E+L	10 ⁴	10 ²	10 ³	—
L-9	10 ⁴	10 ²	10 ³	—

^a The host range of each viral isolate was ascertained by a reverse transcriptase assay. Viral stocks were diluted in serial log dilutions onto 60-mm culture dishes containing 10⁵ NIH 3T3, BALB/3T3, or mink lung cells. The cells were allowed to grow to confluence and were passed 1:3 into fresh culture dishes. When the second passage reached confluence, the cells were fed with 5 ml of fresh media and incubated for 18 h. All 5 ml of media was removed and used to determine reverse transcriptase activity. Plates that were negative for viral reverse transcriptase were passaged twice more and reassayed to ensure that correct endpoint titers were obtained. IU, Infectious units.

Fr-MCF is pseudotyped with either F-MuLV or Ampho it is highly leukemogenic (28). Fr-MCF may require pseudotyping to achieve viral titers adequate to cause disease. This hypothesis is consistent with in vitro infectious center assays which show that Fr-MCF and A/Fr Env are 50 to 100 times less efficient at spreading infection than F-MuLV or Ampho (unpublished data). Therefore, Fr-MCF and each of the recombinants that contain the Fr-MCF envelope gene (A/Fr Env, A/Fr E+L, and L-9) were injected into mice as pseudotypes with Ampho.

Peripheral blood smears were periodically evaluated for the presence of leukemia cells. Mice were sacrificed and autopsied as they developed signs of leukemia. After 6 months, all remaining mice were sacrificed. F-MuLV and the Fr-MCF/Ampho pseudotype caused leukemia in 100 and 50% of mice, respectively (Table 2). None of the mice injected with Ampho developed disease. Similarly, no leukemias were found among the mice injected with recombinant viruses containing only the Fr-MCF envelope gene or only the U3 regions of Fr-MCF or F-MuLV (A/Fr Env/Ampho, A/Fr LTR, or L-1). However, recombinant viruses that contained both the Fr-MCF envelope gene and either the Fr-MCF or F-MuLV U3 region did cause disease: 14% of mice

TABLE 2. Autopsy results of virus-inoculated mice^a

Virus	Leukemia incidence (%)	Spleen wt range ^b (g)	Hematocrit range ^b (%)
F-MuLV	8/8 (100)	0.80–1.92	17–28
Fr-MCF/Ampho	6/12 (50)	0.70–2.20	13–32
Ampho	0/16 (0)	0.22–0.28	44–50
L-1	0/12 (0)	0.12–0.26	42–52
A/Fr LTR/Ampho	0/91 (0)	0.15–0.23	44–50
A/Fr Env/Ampho	0/15 (0)	0.09–0.19	46–50
A/Fr E+L/Ampho	3/21 (14)	0.72–1.10	23–33
L-9/Ampho	6/16 (38)	0.62–1.72	16–34
None	0/10 (0)	0.09–0.18	48–50

^a Two viral isolates derived from independent molecular clones of each hybrid viral genome were injected into separate litters of newborn mice. No significant differences were noted between litters inoculated with viruses representing the same DNA constructs. The results express the cumulative disease incidence for both groups of animals.

^b Spleen weights and hematocrits for animals inoculated with F-MuLV, Fr-MCF/Ampho, A/Fr E+L/Ampho, and L-9/Ampho include only the diseased mice.

inoculated with the A/Fr E+L/Ampho pseudotype and 38% of mice injected with the L-9/Ampho pseudotype developed leukemia within 6 months. All of the leukemias caused by the A/Fr E+L and L-9 viruses exhibited similar pathological and histological characteristics: enlarged livers and spleens with infiltration of erythroblasts, severe anemia, circulating erythroblasts, and normal lymph nodes and thymus. These pathological findings are identical to the disease characteristics of F-MuLV- or Fr-MCF-induced leukemia.

DISCUSSION

Our previous studies have indicated that both the envelope gene and the LTR of F-MuLV contain important determinants for the induction of leukemia. The F-MuLV envelope gene by itself conveys leukemogenicity to the Ampho genome. The F-MuLV LTR influences the rate and incidence of disease caused by Ampho/F-MuLV recombinant viruses. The present study indicates that the F-MuLV and Fr-MCF LTRs by themselves cannot transfer leukemogenicity to Ampho (L-1 and A/Fr LTR recombinants) (Fig. 8). However, in our earlier studies, we used the entire F-MuLV LTR. In this study, only U3 sequences were transferred to Ampho. It is possible that sequences needed to cause leukemia reside in the LTR U5 region. We consider this possibility unlikely since several groups of investigators have shown that the U3 LTR sequences of F-MuLV and other murine leukemia viruses contribute to the leukemogenic phenotype of these viruses (3, 5, 6, 17).

The present study also demonstrated that the Fr-MCF envelope gene was ineffective at converting Ampho into a leukemogenic virus (A/Fr Env recombinant) (Fig. 8). This is surprising since the F-MuLV envelope gene can transfer leukemogenicity to Ampho. These studies point out the complex nature of virus-induced leukemia. F-MuLV is ap-

IN VITRO GENERATED RECOMBINANT MuLV DNA

	LTR	GAG	POL	ENV gp70 p15E	LTR	Leukemia Incidence	Latency (Weeks)
F-MuLV							
						100%	8
Fr-MCF							
						50%	12
AMPHO							
						0%	104
CL-1						50%	12
5a25						22%	26
A/Fr E + L						14%	26
A/Fr ENV						0%	26
L ₁						0%	26
A/Fr LTR						0%	26
L ₉						38%	26

FIG. 8. Schematic representation of all recombinant viral genomes. Leukemogenicity data are taken from Table 2 and references 21 and 23. Latency refers to the maximum time until disease. Many animals developed leukemia before the maximum latent period.

parently needed in the leukemogenic process to generate Fr-MCF in host cells. The F-MuLV envelope gene contains sufficient information to promote the generation of MCF virus (23). However, once Fr-MCF is made, other viral genes in addition to the Fr-MCF envelope gene are needed to cause leukemia.

Fr-MCF/Ampho recombinants only cause leukemia when both the Fr-MCF envelope gene and either the Fr-MCF or F-MuLV U3 regions are included in the viral genome (A/Fr E+L and L-9 recombinants) (Fig. 8). Interestingly, L-9 virus, which possesses F-MuLV U3 sequences, is more leukemogenic than A/Fr E+L, which contains Fr-MCF U3 sequences. This observation is consistent with the relative pathogenicity of F-MuLV and Fr-MCF. F-MuLV induced leukemia in 100% of inoculated newborn mice, whereas an Fr-MCF/Ampho pseudotype caused leukemia in 50% of injected mice (Table 2). The molecular basis for this difference may lie in the fact that the F-MuLV U3 region possesses two copies of a 65-bp direct repeat element. The Fr-MCF U3 region has only one copy of these sequences (15). As in other MuLVs, the U3 region direct repeat sequences may contain enhancer elements which increase the transcription of F-MuLV genes in infected cells (2, 18). Recombinant viruses containing two copies of these sequences may transcribe their genomes more effectively than viruses containing only one set of these sequences (16).

It is unclear how both the Fr-MCF envelope gene and the U3 region contribute to leukemogenesis. Two mechanisms consistent with our results may be considered. The Fr-MCF envelope may be needed to direct viral infection into target cells that Ampho cannot penetrate. Once infected, these cells may be transformed via LTR-mediated activation of cellular genes (19). Alternatively, the Fr-MCF envelope glycoprotein itself may stimulate hematopoietic cell proliferation. In this model, the U3 region provides specific enhancer sequences which increase the expression of the Fr-MCF envelope gene in appropriate target cells.

We favor the second hypothesis for two reasons. First, Ampho pseudotypes of spleen focus-forming virus cause erythroleukemia. These pseudotypes are coated entirely by Ampho envelope glycoproteins (29). Therefore, Ampho must be able to penetrate appropriate target cells for spleen focus-forming virus-induced leukemia. Second, Fr-MCF envelope proteins are invariably expressed in erythroleukemia cell lines transformed by the helper-independent Friend viruses. If the Fr-MCF envelope is needed only to allow penetration of specific target cells, then once these cells are infected they should no longer require the expression of viral envelope genes. Erythroleukemia cell lines induced by infection with spleen focus-forming virus or F-MuLV continue to express spleen focus-forming virus gp52 or Fr-MCF gp70 even after years in culture (1, 24). Many of these same cell lines cease to express viral *gag* and *pol* genes (1). These results imply that continued expression of viral envelope genes is needed for leukemia cell growth.

The leukemia produced by clone 1 (F-MuLV *env* + LTR/Ampho *gag* + *pol*) resembles F-MuLV-induced disease more closely than the leukemia caused by 5a25 virus (F-MuLV *env*/Ampho *gag*, *pol*, LTR) (23). However, even clone 1 does not cause leukemia as rapidly or with the same incidence as F-MuLV (21). Similarly, the A/Fr E+L and L-9 recombinant viruses do not induce leukemia at the same rate as Fr-MCF. Therefore, additional determinants of leukemogenicity must be present in the F-MuLV or Fr-MCF *gag* and *pol* genes. Robinson et al. (26) describe pathogenic avian leukosis virus recombinants that contain oncogenic determi-

nants in their *gag* or *pol* genes. We are currently testing whether the addition of the F-MuLV *gag* and *pol* genes to the A/Fr E+L and L-9 constructs will increase the pathogenicity of viruses obtained from these hybrid DNAs. It is not surprising that a complex biological process such as virally induced leukemia is affected by multiple determinants in the MuLV genome. To cause leukemia, an MuLV must replicate in its host, penetrate specific target cells, integrate into the genome of those cells, and alter the normal growth properties of the cell. The efficiency of each of these functions will influence the ability of an MuLV to cause disease. Clonal isolates of F-MuLV and other highly leukemogenic MuLVs (e.g., AKR-MCF 247 and SL3-3) have been selected for their rapid and efficient induction of leukemias (10, 25). These pathogenic isolates may have maximized their ability to cause leukemia by acquiring advantageous mutations throughout their genomes.

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