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Contribution of the *gag* and *pol* Sequences to the Leukemogenicity of Friend Murine Leukemia Virus

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Friend murine leukemia virus (F-MuLV) is a highly leukemogenic replication-competent murine retrovirus. Both the F-MuLV envelope gene and the long terminal repeat (LTR) contribute to its pathogenic phenotype (A. Oliff, K. Signorelli, and L. Collins, J. Virol. 51:788–794, 1984). To determine whether the F-MuLV *gag* and *pol* genes also possess sequences that affect leukemogenicity, we generated recombinant viruses between the F-MuLV *gag* and *pol* genes and two other murine retroviruses, amphotrophic clone 4070 (Ampho) and Friend mink cell focus-inducing virus (Fr-MCF). The F-MuLV *gag* and *pol* genes were molecularly cloned on a 5.8-kilobase-pair DNA fragment. This 5.8-kilobase-pair F-MuLV DNA was joined to the Ampho envelope gene and LTR creating a hybrid viral DNA, F/A E+L. A second hybrid viral DNA, F/Fr ENV, was made by joining the 5.8-kilobase-pair F-MuLV DNA to the Fr-MCF envelope gene plus the F-MuLV LTR. F/A E+L and F/Fr ENV DNAs generated recombinant viruses upon transfection into NIH 3T3 cells. F/A E+L virus (F-MuLV *gag* and *pol*, Ampho *env* and LTR) induced leukemia in 20% of NIH Swiss mice after 6 months. Ampho-infected mice did not develop leukemia. F/Fr ENV virus (F-MuLV *gag* and *pol*, Fr-MCV *env*, F-MuLV LTR) induced leukemia in 46% of mice after 3 months. Recombinant viruses containing the Ampho *gag* and *pol*, Fr-MCF *env*, and F-MuLV LTR caused leukemia in 38% of mice after 6 months. We conclude that the F-MuLV *gag* and *pol* genes contain sequences that contribute to the pathogenicity of murine retroviruses. These sequences can convert a nonpathogenic virus into a leukemia-causing virus or increase the pathogenicity of viruses that are already leukemogenic.

Friend murine leukemia virus (F-MuLV) and Friend mink cell focus-inducing virus (Fr-MCF) are replication-competent type C retroviruses that induce a rapidly fatal erythroleukemia in susceptible strains of mice such as NIH Swiss or NFS/n (15, 17, 20, 21). Amphotropic clone 4070 (Ampho) is a replication-competent murine retrovirus that does not cause leukemia when inoculated into NIH Swiss or NFS/n mice (1, 12). To identify the viral sequences responsible for erythroleukemia, we constructed hybrid viral genomes between F-MuLV, Fr-MCF, and Ampho by using specific segments of molecularly cloned viral DNAs. Transfection of these hybrid DNAs into NIH 3T3 cells resulted in the production of recombinant retroviruses. The viruses generated in these experiments were assayed in vivo for their ability to cause leukemia.

We previously showed that the envelope genes and the long terminal repeat (LTR) sequences of F-MuLV and Fr-MCF contribute to the pathogenic phenotype of these viruses (13, 14). Recombinant viruses constructed with the F-MuLV or Fr-MCF *env* genes plus the F-MuLV or Fr-MCF LTR regions and the Ampho *gag* and *pol* genes cause erythroleukemia in NIH Swiss mice. However, even the most pathogenic of these recombinant murine leukemia viruses (MuLVs) do not cause leukemia as quickly or as frequently as do wild-type F-MuLV or Fr-MCF (Fig. 1). These results suggest that additional determinants of leukemogenicity lie within the F-MuLV and Fr-MCF *gag* and *pol* sequences (14). To test this hypothesis, we constructed three new hybrid viral genomes with the *gag* and *pol* genes of F-MuLV.

The complete viral genomes of F-MuLV, Fr-MCF, and Ampho were previously cloned (1, 11, 15). Restriction enzyme maps of these viral DNAs are displayed in Fig. 2.

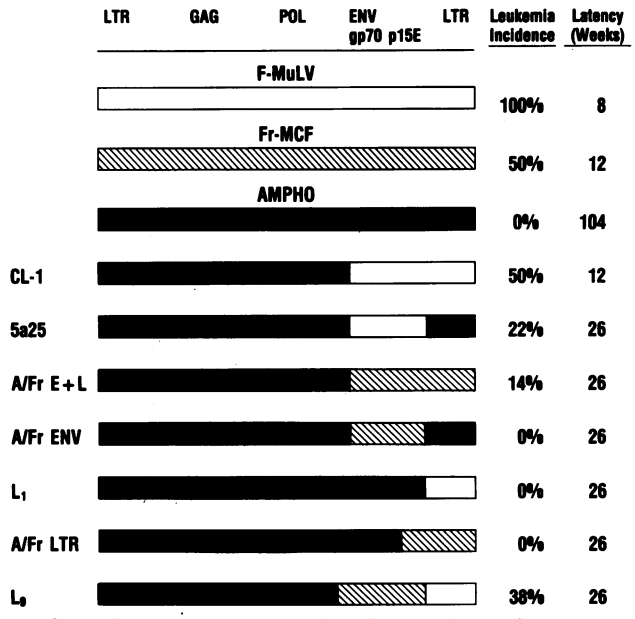

FIG. 1. Schematic representation of recombinant viral genomes constructed among F-MuLV, Fr-MCF, and Ampho DNAs (from reference 13). Maps refer to proviral DNA structure after reverse transcription and integration of the viral DNA into host cells. Leukemia incidence refers to NIH Swiss mice injected as newborns. Latency refers to the maximum time until disease. Many animals developed leukemia before the maximum latent period. No animals injected with Fr-MCF/Ampho or CL-1 developed leukemia between 12 and 26 weeks postinoculation.

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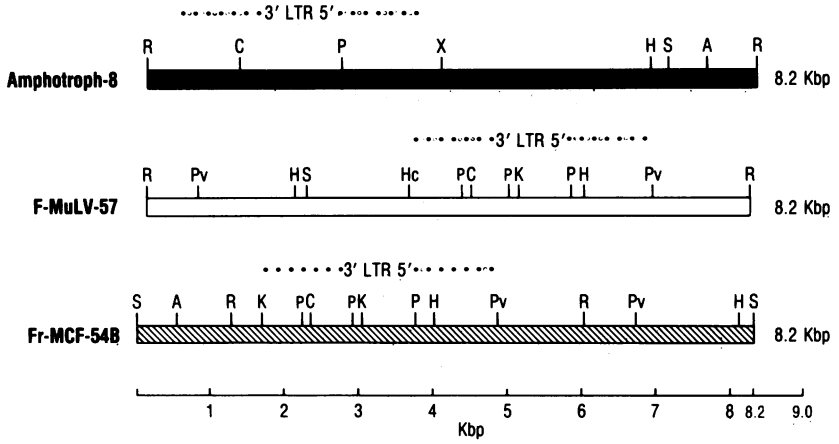

FIG. 2. Schematic representation of the restriction endonuclease maps of molecularly cloned F-MuLV, Fr-MCF, and Ampho viral DNAs. Abbreviations: A, *Acc*I; C, *Cla*I; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I; R, *Eco*RI; S, *Sph*I; X, *Xba*I.

Several enzyme sites are located in the same positions in all three viral DNAs. These common enzyme sites allow specific segments of viral DNA to be exchanged between homologous regions of the F-MuLV, Fr-MCF, and Ampho genomes. The *gag* and *pol* genes of F-MuLV cannot be subcloned from a single fragment of F-MuLV-57 DNA (Fig. 3). Instead, these sequences were isolated on two DNA fragments: *Cla*I-*Eco*RI and *Eco*RI-*Sph*I. These two DNAs were ligated together and cloned between the *Cla*I and *Sph*I sites of pBR322. This cloning yields a 5.8-kilobase-pair (kbp) *Cla*I-*Sph*I DNA fragment that contains the F-MuLV *gag* and *pol* genes and the F-MuLV LTR region.

To assess the contribution of the *gag* and *pol* genes to virally induced leukemogenesis, the 5.8-kbp F-MuLV *Cla*I-*Sph*I fragment was joined to a segment of Ampho DNA, creating two new viruses (Fig. 3). The Ampho envelope gene and LTR region were isolated on two DNA fragments: *Sph*I-*Eco*RI and *Eco*RI-*Pst*I. These two DNAs were ligated together and cloned into a modified pBR325 plasmid vector (p325M$_1$P) between *Sph*I and *Pst*I sites. The resulting 3.7-kbp Ampho DNA contains the entire Ampho envelope gene and LTR region. The 5.8-kbp F-MuLV DNA segment was ligated to the 3.7-kbp Ampho DNA. The resulting 9.5-kbp hybrid DNA was cloned into p325M$_1$P between *Cla*I and *Pst*I sites. This hybrid viral DNA was designated F/A E+L. The 5' LTR and *gag* and *pol* genes of this DNA are derived from F-MuLV, whereas its *env* gene and 3' LTR are derived from Ampho. A second hybrid viral DNA, F/A ENV, was obtained from F/A E+L by subcloning an 8.2-kbp *Cla*I fragment into pBR322 (Fig. 3).

F/A E+L differs from F/A ENV in two ways. First, the F/A E+L viral genome exists in the normal proviral orientation, i.e., LTR-*gag*-*pol*-*env*-LTR. The F/A ENV genome exists as an incomplete provirus since it lacks a 3' LTR. Second, F/A E+L contains 1.3 kbp of redundant viral information located between its *Cla*I and *Pst*I sites. This segment of DNA includes the 3' LTR of Ampho and approximately 700 bp of Ampho *gag* sequences. F/A ENV possesses only one copy of each viral gene, including a single LTR derived from F-MuLV. Hybrid viral DNAs that exist in a proviral orientation can be transfected into fibroblasts without modification. The recipient fibroblasts produce recombinant virus that derives its U$_3$ sequences from the 3' LTR (5, 14). In contrast, hybrid viral DNAs that

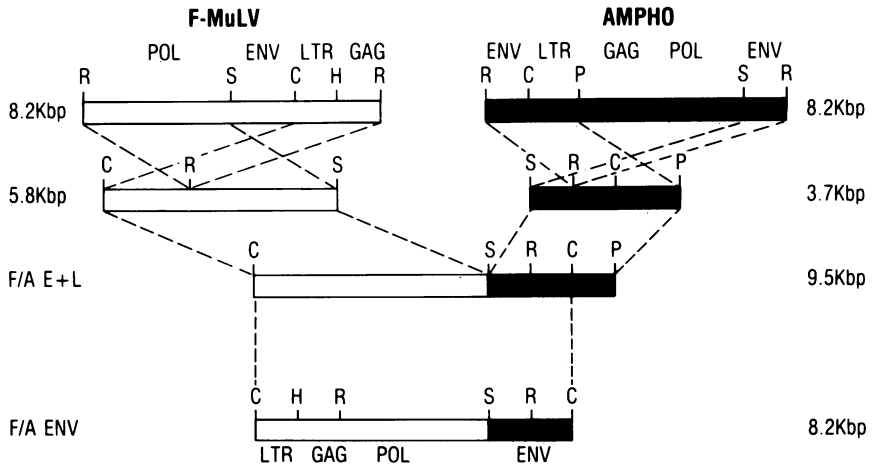

FIG. 3. Schematic representation of the molecular cloning of the recombinant viral genomes F/A E+L and F/A ENV. Each subgenomic viral DNA fragment was cloned into a plasmid vector and analyzed by restriction endonuclease mapping before being used to construct F/A E+L. Abbreviations are as described for Fig. 2. Symbols: ▭, F-MuLV; ■, Ampho.

do not possess LTRs at both ends or that are permuted in orientation relative to proviral DNA must be digested free from their vectors before transfection. Therefore, F/A E+L was transfected into NIH 3T3 cells while still attached to its plasmid vector. F/A ENV was digested free from pBR322 with *Cla*I before transfection. Both transfections yield XC-negative, Fv-1 NB-tropic, murine retroviruses that grow on murine (NIH 3T3 and BALB 3T3) and nonmurine (mink lung and HeLa) cells, i.e., amphotropic virus.

To prove that F/A E+L virus derives its U_3 sequences from the Ampho LTR, we analyzed the viral DNAs generated by F-MuLV, Ampho, and F/A E+L in NIH 3T3 cells. F-MuLV and Ampho each possess a *Pst*I site in their *gag* genes. F-MuLV also has a *Pst*I site in its U_3 region approximately 1.0 kbp distant from the *Pst*I site in *gag* (Fig. 2). The Ampho U_3 region does not contain a *Pst*I site. Since F/A E+L presumably derives its U_3 region from Ampho, the F/A E+L U_3 region should not contain a *Pst*I site. NIH 3T3 cells were acutely infected with F-MuLV, Ampho, or F/A E+L. Viral DNAs were isolated by the method of Hirt (7). The Hirt-extracted viral DNAs were digested with *Pst*I and analyzed by gel electrophoresis and Southern blotting. Hirt-extracted F-MuLV DNA exhibited a 1.0-kbp fragment that hybridized to an F-MuLV LTR probe (Fig. 4). No comparable restriction fragments were seen with either the Ampho or the F/A E+L viral DNAs. Since the F/A E+L U_3 region lacks a *Pst*I site, these sequences must be derived from the Ampho genome.

F/A ENV and F/A E+L were isolated, and 10^5 infectious units were inoculated into newborn (<24-h-old) NIH Swiss mice. Animals were monitored for signs of leukemia by periodic examinations of peripheral blood. Leukemic mice were sacrificed as their disease became evident. All remaining mice were sacrificed at 6 months of age. All of the F-MuLV-injected mice developed leukemia within 3 months.

TABLE 1. Autopsy results of MuLV-inoculated mice

Virus	Leukemia incidence (%)	Spleen wt range (g)[a]	Hematocrit range (%)[a]	Histologic diagnosis[b]	
				Erythroid	Lymphoid
F-MuLV	11/11 (100)	0.8–2.1	11–28	11	0
Fr-MCF/Ampho[c]	11/22 (50)	0.7–1.8	9–26	11	0
Ampho	0/17 (0)	0.09–0.18	40–50		
F/A E+L	4/20 (20)	0.8–1.0	17–40	1	3
F/A ENV	7/20 (35)	0.65–2.4	10–36	6	1
F/Fr ENV/Ampho[c]	7/15 (46)	0.75–1.2	18–32	7	0
None (control)	0/10 (0)	0.04–0.12	48–50		

[a] Ranges of spleen weights and hematocrits for animals inoculated with F-MuLV, Fr-MCF/Ampho, F/A E+L, F/A ENV, and F/Fr ENV/Ampho include only the diseased mice.

[b] Peripheral blood smears, cytospin preparations of spleen cell suspensions, and touch preparations of lymph nodes were analyzed at autopsy. Two diagnoses were evident from these studies: either erythroleukemia (erythroid) or splenic lymphoma with lymphoid leukemia (lymphoid).

[c] Viruses possessing the Fr-MCF envelope gene are phenotypically mixed with Ampho in vitro for 3 to 4 cell passages before inoculation into mice (17).

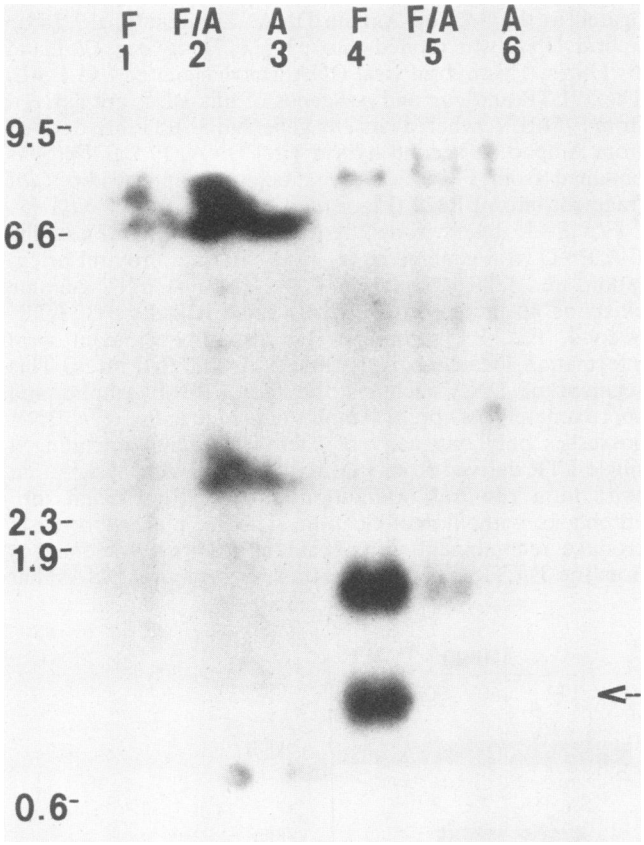

FIG. 4. Autoradiograph of a Southern blot analysis of viral DNAs extracted from NIH 3T3 cells infected with F-MuLV (lanes 1 and 4), F/A E+L (lanes 2 and 5), and Ampho (lanes 3 and 6). Hirt-extracted DNA (10 μg) was left uncut (lanes 1 through 3) or digested with *Pst*I (lanes 4 through 6) and electrophoresed through 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 a molecularly cloned F-MuLV LTR DNA fragment. The filter was washed until the level of radioactivity returned to background and was exposed to X-ray film at −70°C for 24 h. Note the 1.0-kbp band (arrow) in the *Pst*I-digested F-MuLV DNA (lane 4). Molecular size markers (in kbp) represent wild-type lambda DNA fragments digested with *Hin*dIII and electrophoresed in an adjacent lane.

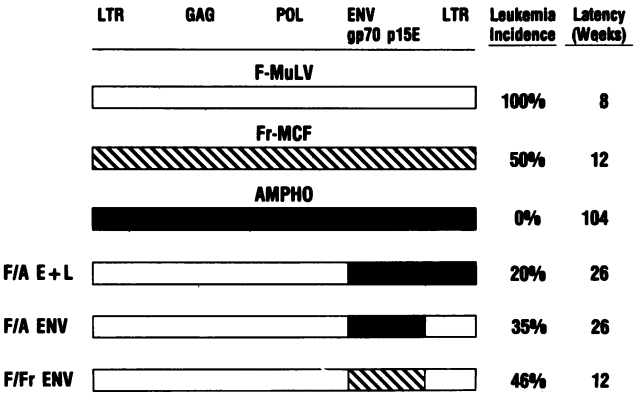

FIG. 5. Schematic representation of the hybrid viral DNAs constructed with the F-MuLV 5.8-kbp *Cla*I-*Sph*I DNA fragment. Maps refer to proviral DNA structure after reverse transcription and integration of the viral DNA into host cells. Leukemogenicity data are taken from Table 1. Latency refers to the maximum time until disease. Many animals developed leukemia before the maximum latency period. No animals injected with Fr-MCF/Ampho or F/Fr ENV/Ampho developed leukemia between 12 and 26 weeks post-inoculation.

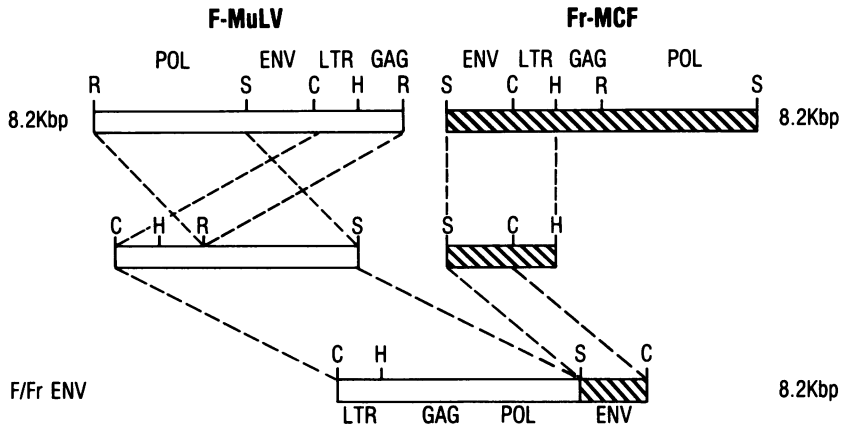

FIG. 6. Schematic representation of the molecular cloning of the recombinant viral genome F/Fr ENV. Each subgenomic viral DNA was subcloned into a plasmid vector and analyzed by restriction endonuclease mapping before being used to construct F/Fr ENV. Abbreviations are as described for Fig. 2.

By 6 months, 20% of the F/A E+L mice and 35% of the F/A ENV mice developed leukemia. None of the Ampho-injected mice became ill (Table 1). Thus, the addition of the F-MuLV 5′ LTR *gag* and *pol* sequences to the Ampho *env* gene and 3′ LTR creates a weakly pathogenic MuLV (F/A E+L). If both LTRs and the *gag* and *pol* genes are derived from F-MuLV and only the *env* gene is supplied by Ampho, the resulting virus (F/A ENV) is more strongly pathogenic (Fig. 5).

The preceding experiments indicate that the F-MuLV *gag* and *pol* sequences can convert a nonpathogenic virus (Ampho) into a leukemogenic virus. We next asked whether these sequences could affect the pathogenicity of a virus that was already able to cause leukemia. L_9 is a leukemogenic virus constructed from segments of F-MuLV, Fr-MCF, and Ampho DNAs (Fig. 1). L_9 derives its 5′ LTR *gag* and *pol* genes from Ampho, its *env* gene from Fr-MCF, and its 3′ LTR from F-MuLV (14); 38% of mice injected with L_9 developed leukemia within 6 months. To determine whether the F-MuLV *gag* and *pol* genes would alter the pathogenicity of L_9, the 5.8-kbp F-MuLV *Cla*I-*Sph*I fragment was joined to an *Sph*I-*Cla*I fragment of Fr-MCF DNA. The resulting 8.2-kbp hybrid DNA was cloned into pBR322 at the *Cla*I site and designated F/Fr ENV (Fig. 6). F/Fr ENV derives its 5′ LTR *gag* and *pol* sequences from F-MuLV, its *env* gene from Fr-MCF, and its 3′ LTR from F-MuLV. Thus, F/Fr ENV and L_9 possess the same *env* genes and 3′ LTRs but differ in their 5′ LTR *gag* and *pol* sequences (Fig. 5).

F/Fr ENV DNA was digested free from its plasmid vector and transfected into NIH 3T3 cells. An XC-negative, Fv-1 NB-tropic, mink cell focus-inducing virus (F/Fr ENV) was recovered from the transfected cells. Newborn NIH Swiss mice were inoculated with 5×10^4 infectious units of F/Fr ENV virus and periodically monitored for leukemia; 46% of these animals developed leukemia within 3 months (Table 1). F/Fr ENV virus induced leukemia more quickly than did L_9 virus.

In comparisons of the pathogenicity of F/A E+L versus that of Ampho and that of F/Fr ENV versus that of L_9 (Fig. 5), it is evident that the F-MuLV 5′ LTR *gag* and *pol* genes increase the pathogenicity of MuLVs that normally derive these sequences from Ampho. Therefore, at least one determinant of F-MuLV-induced leukemias must be encoded in the 5′ LTR *gag* and *pol* genes. We believe that the *gag* and *pol* genes are more likely to encode this determinant than the 5′ LTR for two reasons. (i) The 5′ LTR only contributes U_5 sequences to its progeny virus (5, 6, 19). U_5 sequences do not encode proteins or contain enhancer elements that might increase the rate of viral RNA transcription (9, 18). (ii) Several investigators have found that *gag* and *pol* genes of other murine and avian retroviruses can influence the pathogenicity of those viruses (3, 8, 16).

We and others have shown that viral sequences responsible for leukemia are encoded in the *env* gene and 3′ LTR regions of MuLVs (2, 4, 8, 10, 14). The present study indicates that *gag* and *pol* sequences harbor additional determinants of viral pathogenicity. It is unclear how each of these determinants contributes to disease, but several points concerning virally induced leukemia are evident: (i) the sequences responsible for leukemia are present in multiple locations throughout the MuLV genome (*gag pol env* and 3′ LTR), (ii) these sequences can affect both the rate of leukemogenesis and the overall incidence of leukemia (Fig. 5), and (iii) the effects of these sequences on viral pathogenicity are additive. F/A E+L is more pathogenic than Ampho, and F/A ENV is more pathogenic than F/A E+L. Similarly, L_9 is more pathogenic than Ampho, and F/Fr ENV is more pathogenic than L_9 (Fig. 5). In each case, a series of hybrid viral DNAs were constructed. As each succeeding construct derives more of its sequences from a leukemogenic virus (F-MuLV or Fr-MCF), the resulting viruses become more pathogenic.

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