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Transfection of Molecularly Cloned Friend Murine Leukemia Virus DNA Yields a Highly Leukemogenic Helper-Independent Type C Virus

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Unintegrated viral DNA was isolated via the Hirt procedure from mouse fibroblasts newly infected with Friend murine leukemia virus (F-MuLV) clone 201, a biologically cloned helper virus isolated from stocks of F-MuLV complex. A physical map of the unintegrated in vivo linear viral DNA was generated for several restriction endonucleases. The supercoiled viral DNA was digested with *Eco*RI, which cleaved the viral DNA at a unique site. The linearized viral DNA was then inserted into λgtWES·λB at the *Eco*RI site and cloned in an approved EK2 host. Eight independent λ-mouse recombinants were identified as containing F-MuLV DNA inserts by hybridization with F-MuLV ^{32}P-labeled complementary DNA. One of the F-MuLV DNA inserts was 9.1 kilobases (kb) and had the same restriction enzyme sites as the unintegrated linear F-MuLV DNA. Six inserts were 8.5 kb; each lacked a single copy of the terminally redundant sequences of the unintegrated linear viral DNA. One insert was 8.2 kb and contained a 0.9-kb deletion. After digestion with *Eco*RI, one recombinant DNA preparation containing an 8.5-kb insert was infectious for NIH 3T3 cells. Undigested recombinant DNA was not infectious. The infectivity of the *Eco*RI-digested DNA followed multihit kinetics, indicating that more than one molecule was required to register as an infectious unit. The virus isolated from this transfection (F-MuLV-57) was NB-ecotropic, helper-independent, and formed XC plaques. Inoculation of this virus into newborn NIH Swiss mice induced leukemia and splenomegaly in >90% of animals within 3 to 4 weeks. The gross and microscopic abnormalities induced by F-MuLV clone 57 were identical to those seen with the original parent stocks of F-MuLV clone 201. These results indicate that this helper-independent F-MuLV can induce a rapid nonthymic leukemia in the absence of the spleen focus-forming virus.

For many years, the Friend virus complex, which is leukemogenic for adult as well as newborn mice, has been studied as a model system of retrovirus-induced leukemia (2). Friend disease is characterized by a short latent period and the development of hepato-splenomegaly, in marked contrast to the long latent period and thymic tumors which typify the disease induced by viruses of the Gross-AKR class. Most preparations of the Friend virus complex that are capable of inducing leukemia within a few weeks in susceptible mice have been found to contain a mixture of two types of virus: the spleen focus-forming virus (SFFV), which is a replication-defective virus, and a variety of helper-independent murine type C viruses (18). Although the SFFV component of these viral stocks is thought to be responsible for the development of leukemia in adult mice (18), the leukemogenicity of various isolates of the helper-independent type C viruses has been less clearly documented.

In the course of studies on the viruses present in different stocks of Friend virus complex, it was recently reported that a helper-independent virus, Friend murine leukemia virus (F-MuLV) clone 201, induced a rapid leukemia with anemia and hepato-splenomegaly when the virus was injected into newborn BALB/c or NIH Swiss mice (24). The onset of disease was particularly rapid (2 to 3 weeks) in the Swiss mice. It was suggested that the pathogenicity of this virus preparation resided in the helper-independent virus and not in any contaminating type C virus since the F-MuLV clone 201 had been purified through four cycles of endpoint biological cloning before testing. However, in the avian and mammalian retroviral systems, long-term per-

sistence of stable heterozygotes has been previously described in virus stocks subjected to careful biological cloning (12, 26); therefore, the possibility of a second virus contaminating F-MuLV clone 201 could not be rigorously excluded.

Molecular cloning with lambda or plasmid vectors in *Escherichia coli* hosts offers an approach to obtain the DNA of retroviruses in a biochemically pure form which is certain to be free of other potentially contaminating retroviral DNAs. We report here the molecular cloning in a lambda phage vector of an infectious form of the DNA of F-MuLV clone 201. The virus isolated after transfection of this DNA into NIH 3T3 fibroblasts retains the previously described rapid leukemogenicity in newborn NIH mice. Thus, the results indicate that the viral preparation obtained from the transfection of the molecularly cloned DNA copy of F-MuLV clone 201 induces the rapid onset leukemia.

MATERIALS AND METHODS

Mice. Newborn (<24-h-old) and adult (6- to 8-weeks-old) NIH Swiss mice were obtained from the National Institutes of Health Small Animal Facility, Bethesda, Md.

Viruses. F-MuLV clone 201 is an NB-ecotropic clone of Friend murine type C virus separated from the SFFV component of Friend virus complex by multiple endpoint dilutions. It was then passaged several times in mouse cells before use. The virus complex containing F-MuLV clone 201 was originally obtained from Charlotte Friend, and that complex represents what she has termed Friend virus anemic strain.

Cells. NIH 3T3 mouse embryo fibroblasts (9) were used in all transfection studies. NIH 3T3 embryo fibroblasts, BALB/c 3T3 embryo fibroblasts (9), and mink lung cells (ATCC) were used in the infection studies to define viral host range.

Isolation of low-molecular-weight DNA. One hundred seventy roller bottles (Corning, 490 mm^2) were seeded with approximately 2×10^6 NIH 3T3 cells each and allowed to grow to 90% confluence with Dulbecco-modified Eagle medium supplemented with 10% donor calf serum, penicillin, streptomycin, and L-glutamine (Flow Laboratories, Rockville, Md.). Each bottle was then inoculated in the presence of polybrene (4 μg/ml) (22) with approximately 5×10^7 XC PFU of F-MuLV clone 201 produced in NIH 3T3 cells. Twenty-four hours later, DNA was extracted from the cells by the Hirt procedure (7), yielding 24 mg of Hirt supernatant DNA.

Analytical electrophoresis. DNA samples were run on 0.5 to 0.7% (wt/vol) agarose gels made in 0.04 M Tris–0.02 M sodium acetate–0.0025 M EDTA (pH 8.1) attained with acetic acid (TGA buffer). Electrophoresis grade agarose was obtained from Bethesda Research Labs, Bethesda, Md. Slab gels were run at 35 V for 16 to 20 h at 25°C with a model 500 power supply from BioRad Laboratories and a horizontal gel apparatus from Bethesda Research Laboratories. Samples (50 to 100 μl) were adjusted to 6% sucrose in TGA plus 0.1% bromophenol blue as a marker dye and then loaded into individual wells in the agarose gel. Samples were then overlayed with TGA buffer and immediately electrophoresed. When UV light was to be used to visualize the electrophoresed DNA, ethidium bromide (0.5 μg/ml) was added to both the gel and the running buffer before electrophoresis.

Hybridization of DNA from gels. After electrophoresis, the DNA was transferred to nitrocellulose filter paper (Schleicher & Schuenll, Keene, N.H.) by the Southern blotting technique (17). Filters were next baked at 80°C for 2 h in vacuo and washed in preparation for hybridization as described elsewhere (17). The cellulose filters were placed into plastic bags designed for use with the Seal and Save (Sears) sealing apparatus before hybridization. Next, enough hybridization solution was added to fill the bags one-third of the way from the bottom, and approximately 10^7 cpm of ^{32}P-labeled probe was added. The bags were then sealed and placed in a 65°C water bath for 36 to 48 h. After hybridization the filters were again washed and dried as described before (17), then covered with Saran Wrap and placed in a film cassette with XRP-1 Kodak film and a single lightening plus Cronex intensifying screen (Du Pont Corp., Wilmington, Del.). Cassettes were kept at −70°C for from 24 h to 1 week before developing (20).

Synthesis of viral probes. Two forms of ^{32}P-labeled complementary DNA (cDNA) probes were prepared for hybridization. In one, cDNA was synthesized from 70S viral RNA obtained from F-MuLV (201) virions harvested from NIH 3T3 cells. An exogenous reverse transcriptase reaction with calf thymus DNA fragments as primer, avian myeloblastosis virus reverse transcriptase, actinomycin D, and [^{32}P]dCTP was carried out as previously described (21). The resultant cDNA was then extracted once with phenol and chloroform-isoamyl alcohol (24:1) and then passed over a 10-cm G-75 column. The fractions containing the first peak of radioactivity were pooled and adjusted to 0.5 M NaOH. After 2 h at 37°C, the preparation was neutralized with HCl and stored at −20°C for 1 day to 2 weeks before use. Immediately before hybridization reactions, [^{32}P]cDNA was heated to 80°C for 2 min and then quenched on ice. All hybridization reactions employed this cDNA probe unless otherwise stated. The other viral cDNA probe utilized in certain experiments was a 3′-selected [^{32}P]cDNA. The viral RNA utilized in the synthesis of this probe consisted of 4 to 10S RNA fragments obtained by sucrose gradient sedimentation of partially degraded 70S F-MuLV clone 201 RNA and subsequent passage over an oligodeoxythymidylic acid-cellulose column as described by Wang et al. (25). After isolation of this 3′ RNA, the [^{32}P]cDNA was prepared by the same method as described above except that the reverse transcriptase reaction was run for 75 min and the cDNA preparation was treated with alkali before passage over the G-75 column.

Restriction enzymes. *Eco*RI, *Kpn*I, *Hin*dIII, *Xba*I, and *Pvu*II were obtained from Bethesda Research Laboratories. Reactions were run in 50 to 100 μl in 1.5-ml Eppendorff plastic test tubes. Restriction enzymes were utilized at 10 to 20 U per μg of DNA substrate in the appropriate assay buffer specified for

each enzyme's use by Bethesda Research Laboratories. All reactions were run at 37°C for 2 h and then stopped by heating to 65°C for 15 min. Each reaction was run as a limit digestion; digestions were judged complete by the pattern of added marker DNA or the demonstration that no further digestion would occur if additional enzyme or longer reaction times were used. Double digestions were performed in two steps. After digestion with the first enzyme, the entire reaction mixture was adjusted to 0.2 M NaOAc, pH 5.0, and was precipitated with ethanol. The test tubes were then spun for 30 min in an Eppendorff model 5413 centrifuge, and the supernatants were decanted. The second restriction enzyme assay buffer was then added to the test tube, and the precipitated DNA was resuspended by gentle shaking. The second enzyme digestion then proceeded as with the first enzyme.

Preparative electrophoresis. A 20-mg amount of the Hirt supernatant DNA was loaded into a 1% agarose gel prepared as described above in a preparative horizontal slab gel apparatus (14) and electrophoresed at 60 V for 33 h at 4°C. Every 30 min after the start of electrophoresis the running buffer from the anode end of the gel (approximately 10 ml) was collected and changed with a model 7000 UltroRac fraction collector (LKB Bromma Co.). Fifty microliters of each 10-ml fraction was then tested in the analytical electrophoresis system by blotting and hybridization, and the fractions containing the form I circles, form II circles, and in vivo linear DNA molecules, respectively, were identified, pooled, and placed at 4°C until needed.

Molecular cloning. Bacteriophage λgtWES·λB, supplied by D. Tiemeier, was used as the cloning vehicle for F-MuLV DNA. Closed circular F-MuLV DNA was linearized by digestion with *Eco*RI, inserted at the *Eco*RI site of the λgtWES·λB arms, and amplified in the EK2 *E. coli* host (DP50 *sup*F) as previously described (5). F-MuLV recombinant plaques were identified by an in situ filter hybridization assay with F-MuLV [^{32}P]cDNA as previously described (5). All steps in the molecular cloning of the recombinant F-MuLV DNA were carried out under the conditions prescribed in the National Institutes of Health guidelines for recombinant DNA research. All packaging reactions and plating of recombinant plaques were carried out in the P-4 mobile containment laboratory at the National Institutes of Health. After the revision of the guidelines for recombinant DNA research in January, 1979, the remainder of the cloning work (growth of the F-MuLV-positive subclones in liquid media) was performed in a P-2 laboratory according to the new guidelines. Once the λ-F-MuLV recombinant clones were isolated and amplified in *E. coli* strain DP50, the DNA from the cloned recombinant phage was isolated by the methods of Sternberg et al. (19).

Transfection of the cloned recombinant F-MuLV. Transfection of DNA on NIH 3T3 cells was carried out as previously described (11). Titration of the F-MuLV isolates was performed as described in Fig. 9.

Inoculation of newborn (<24-h-old) NIH Swiss mice was accomplished by intraperitoneal injection of 0.2 cm^3 of the viral stocks utilizing a 27-gauge needle and a 1-ml tuberculin syringe. Viral stocks for injection were prepared from chronically infected NIH 3T3 cells by an 18-h harvest of 10 ml of media from a 100-mm petri dish and subsequent filtration through a 0.45-μm filter. Each viral stock was then diluted 1:5 in fresh media and placed on ice from 0.5 to 1 h before use.

Reverse transcriptase and XC plaque assays were performed as previously described (23).

RESULTS

Since the purpose of this study was to isolate potentially infectious F-MuLV DNA, the approach chosen was to find a suitable restriction endonuclease whose recognition sequence was present only once in the viral DNA. Cells newly infected with other retroviruses have been shown to contain closed circular DNA which is infectious, and it was reasoned that a similar closed circular form would exist for F-MuLV DNA (16). The potentially infectious circular viral DNA should therefore be clonable in its entirety by linearization with an enzyme which cleaved the DNA only once. Since it would also be necessary to compare any cloned DNA with known F-MuLV DNA, a preliminary restriction endonuclease map of the viral DNA was also derived.

Restriction mapping of F-MuLV DNA. To obtain a substrate for restriction endonuclease analysis of F-MuLV DNA and for its molecular cloning, virus released from F-MuLV clone 201-infected NIH 3T3 cells was used to newly infect NIH 3T3 cells. Unintegrated proviral DNA was isolated 24 h after infection by the Hirt extraction procedure. Initially, a small portion of this DNA preparation was electrophoresed in an analytical agarose gel, and the DNA forms were detected by the Southern transfer method with F-MuLV [^{32}P]cDNA prepared from F-MuLV clone 201 propagated in NIH 3T3 cells. Figure 1A shows an autoradiogram of the results of such a hybridization. Three bands of hybridization are observed, the most intense one migrating at a molecular weight of 9.0 kilobases (kb), and a lighter, broader band migrating more rapidly as if its molecular weight ranged from 5.2 to 3.5 kb, and a narrow band migrating at 7.6 kb. Based on its size and predominance, the 9.0-kb band, which was infectious, was assumed to represent the unintegrated linear form of proviral DNA (form III). The faster, broader migrating form was taken to represent the closed circular form of the molecule (form I circles). By using Hirt supernatant DNA from 140 roller bottles, these forms were separated from each other by preparative agarose gel electrophoresis. The presumed linear form (Fig. 1A, lane 2) was then digested with a variety of restriction enzymes, and the resulting digestion products were de-

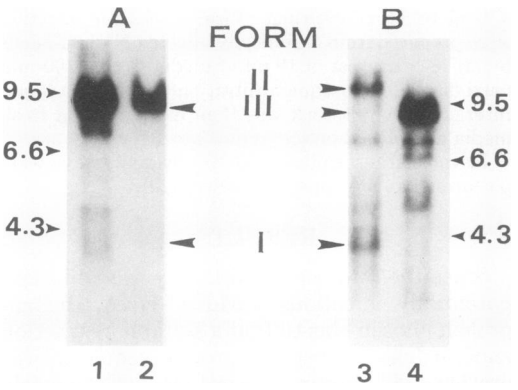

FIG. 1. *Southern blot analysis of electrophoresed Hirt DNA. (A) Hirt supernatant DNA isolated via the Hirt procedure from NIH 3T3 cells newly infected with F-MuLV clone 201 after electrophoresis on a 0.5% agarose gel at 35 V for 18 h and then blotted via the Southern transfer procedure and hybridized to F-MuLV [^{32}P]cDNA. Lane 1, 10 μg of the unfractionated Hirt supernatant DNA; lane 2, 0.5 μg of the in vivo linear DNA molecules isolated from the Hirt supernatant DNA by preparative electrophoresis. The bands of hybridized DNA corresponding to the form I circles, form II circles, and form III in vivo linear DNA molecules are identified by the appropriate arrows. (B) Lane 3, 5.0 μg of the Hirt DNA corresponding to the form I circles isolated by preparative electrophoresis from the unfractionated Hirt preparation and electrophoresed as in A. Some form II circles are evident in this autoradiogram due to nicking of the form I circles during the isolation procedure. Lane 4, 5.0 μg of the same form I circular DNA molecules after digestion with EcoRI enzyme at 20 U/μg of DNA at 37°C for 6 h and electrophoresed as in A. A fraction of the form I circles persists after restriction due to incomplete digestion of the form I circles, probably because of agarose contaminating the Hirt DNA fractions obtained from the preparative electrophoresis. The numbers at the sides of each autoradiograph indicate the location of HindIII-digested wild-type λ marker DNA fragments (in kilobases) run in an adjacent lane.*

tected by agarose slab gel electrophoresis and Southern transfer hybridization with the F-MuLV [^{32}P]cDNA. A summary of the cleavage products obtained with each of these enzymes is given in Table 1. *Eco*RI digestion yielded two fragments whose sum equaled 9 kb, indicating that this enzyme produced a single cut in the proviral DNA. Each of the other enzymes yielded three fragments after digestion, suggesting that they each cleaved the DNA twice. To test the hypothesis that the fastest migrating band in Fig. 1A represented closed circular DNA, it was digested with *Eco*RI. As shown in Fig. 1B, this digestion yielded a principal band which migrated at the same rate as the undigested linear form. This result confirmed that

TABLE 1. *Sizes of F-MuLV clone 201 DNA fragments obtained with restriction enzymes*[a]

Enzyme	Fragment size (kb)			Sum of the fragments
None	8.9–9.1			9.0
*Eco*RI	5.7	3.3		9.0
*Kpn*I	8.4	0.4	0.2	9.0
*Xba*I	5.5	2.8	0.7	9.0
*Hin*dIII	4.2	3.4	1.2	8.8
*Pvu*II	4.7	2.2	1.85	8.75
*Kpn*I-*Eco*RI[b]	5.5	2.8		
*Kpn*I-*Xba*I	5.1	2.6	0.7	
*Kpn*I-*Hin*dIII	4.2	3.2	0.7	
*Kpn*I-*Pvu*II	4.5	2.2	1.3	

[a] Size in kilobases of the DNA fragments obtained from F-MuLV clone 201 in vivo linear DNA molecules isolated from the Hirt supernatant DNA preparative electrophoresis and digested with *Eco*RI, *Kpn*I, *Xba*I, *Hin*dIII, *Pvu*II, or *Kpn*I plus *Eco*RI, *Kpn*I plus *Xba*I, *Kpn*I plus *Hin*dIII, and *Kpn*I plus *Pvu*II as described in the text. The products of these enzyme digests were then electrophoresed on 0.5% agarose gels at 35 V for 18 h and transferred to nitrocellulose filters via the Southern blotting technique and then hybridized to labeled F-MuLV [^{32}P]cDNA. The kilobase size of the fragments was obtained from a semilog plot of the relative migration distances of each fragment compared to *Hin*dIII-digested ^{32}P-labeled wild-type λ DNA run in the same gel.

[b] The 0.4- and 0.2-kb fragments were not visualized in this experiment, but the 0.4-kb fragment was visualized after the *Kpn*I-*Eco*RI digestions performed for the 3′ cDNA probe analysis (see Fig. 2).

the faster moving band represented a circular form of F-MuLV and that *Eco*RI digested F-MuLV proviral DNA at a single site.

To further orient the restriction enzyme sites in the F-MuLV DNA, two experiments were performed. First, the linear DNA was digested with both *Kpn*I and *Eco*RI, and the resulting fragments were analyzed by Southern transfer hybridization. The results are shown in Table 1. Both of the *Eco*RI fragments migrated more rapidly when they were also digested with *Kpn*I, which indicated that each *Eco*RI fragment contained a *Kpn*I site. Since *Kpn*I alone cleaved the DNA into one large fragment (8.4 kb) and two small fragments (0.4 and 0.2 kb), these results indicated that *Kpn*I digested the original linear form at each end of the molecule. Secondly, the linear F-MuLV DNA was digested with *Eco*RI and *Eco*RI-*Kpn*I, and the cleavage products were analyzed by hybridization to a [^{32}P]cDNA probe prepared from the 3′ end of F-MuLV clone 201 RNA. The results are shown in Fig. 2. Since other unintegrated linear retroviral DNAs have been found to contain direct terminal duplications of the 3′ and 5′ ends of the viral RNA (8,

15), both *Eco*RI fragments would be expected to hybridize with the 3′ cDNA probe; this predicted result was obtained. After digestion with *Kpn*I–*Eco*RI, only the large fragment hybridized to this 3′ cDNA. The results are consistent with *Kpn*I cleaving in the terminal redundancies at a site corresponding to the portion of the redundancy derived from the 5′ end of the viral RNA and allow the assignment of the larger *Eco*RI fragment to the 3′ portion of the viral genome. By similar analyses with *Kpn*I, *Eco*RI, and each of the three other enzymes noted in Table 1, a physical map of the F-MuLV 201 genome was obtained. This map is shown in Fig. 3.

Cloning of F-MuLV 201 DNA. To obtain a linear form of the viral DNA with known sequences at either end of the molecule for molecular cloning, the form I circular DNA isolated from the Hirt preparation was cleaved with *Eco*RI. As demonstrated in Fig. 1B, this digestion opened the circular DNA into a linear form with *Eco*RI-specific sequences at both ends. This DNA was then ligated to the *Eco*RI arms of the λgtWES·λB DNA and packaged into λ-phage virions. This process of molecular cloning is schematically shown in Fig. 4. The bacteriophage produced in this reaction were then amplified in *E. coli* strain DP-50 and plated onto agar culture dishes. Approximately 50,000 putative recombinant bacteriophage particles were produced, as evidenced by plaque formation on the agar plates. Eighteen of these plaques were preliminarily identified as likely F-MuLV recombinant clones by hybridization with F-MuLV cDNA. These 18 plaques were picked from the agar plates, amplified in DP-50 again, and retested by hybridization for the presence of F-MuLV DNA. Eight of these subclones proved to be true λ-F-MuLV recombinants. These eight subclones were then propagated to a high density in liquid culture media, and the bacteriophage were isolated by isopycnic banding on CsCl gradients. The isolated phage were then lysed, and their DNA was purified.

Analysis of the recombinant F-MuLV-bacteriophage DNA. The purified λ-F-MuLV DNA preparations were digested with *Eco*RI to cleave the F-MuLV DNA insert from the λDNA and then analyzed by electrophoresis (see Fig. 5). Since the F-MuLV DNA used to generate the recombinant clones was obtained from *Eco*RI-cleaved form I circles, the linear recombinant F-MuLV DNA should map with restriction enzymes as a permutation of the linear proviral DNA; the pattern of cleavage products of the cloned DNA restriction fragments should therefore be compared with the predicted map of the linear DNA (see Fig. 4). Since others (8, 15, 27) have found two forms of closed circular

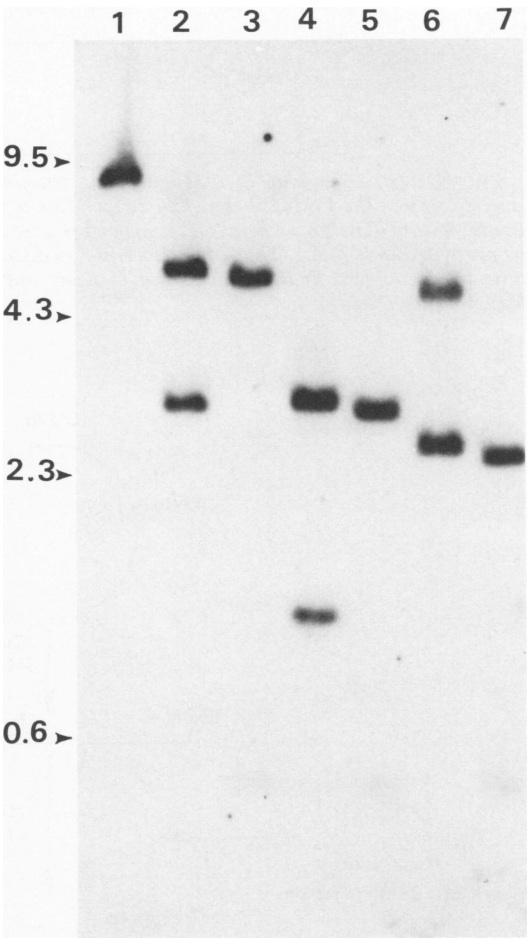

FIG. 2. *Hybridization of DNA fragments to 3′ F-MuLV probe. Autoradiograph of the in vivo linear F-MuLV clone 201 DNA isolated from the Hirt DNA by preparative electrophoresis and digested with no enzyme, lane 1; EcoRI, lane 2; EcoRI plus KpnI, lane 3; HindIII, lane 4; HindIII plus KpnI, lane 5; XbaI, lane 6; and XbaI plus KpnI, lane 7, and then electrophoresed for 18 h at 35 V on a 0.5% agarose gel. After electrophoresis the DNA fragments were transferred to nitrocellulose filter paper, hybridized to a 3′ F-MuLV [^{32}P]cDNA and developed as described in the text. The numbers at the right of the autoradiogram indicate the location of HindIII-digested wild-type λ marker DNA fragments (in kilobases) run in an adjacent lane.*

DNA in their preparations of proviral DNA which correspond to the viral genome and differ by the presence of one versus two copies of the repeated terminal sequences, we expected to find one or two size classes of cloned recombinant F-MuLV DNA. However, we found three different size recombinant F-MuLV DNA inserts: one clone of 9.1 kb (the same size as the linear

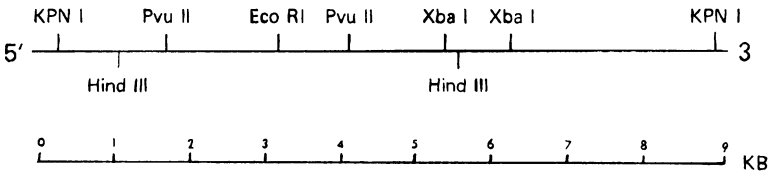

FIG. 3. *Restriction map of F-MuLV clone 201 linear DNA. Schematic representation of the restriction enzyme map of the F-MuLV clone 201 in vivo linear DNA molecules using EcoRI, KpnI, XbaI, HindIII, and PvuII. Relative distances between enzyme sites were obtained from the analysis of restriction fragment sizes as presented in Table 1. The 3'-5' orientation is deduced from the autoradiograph of the in vivo linear DNA after digestion with EcoRI-KpnI, HindIII-KpnI, and XbaI-KpnI, and subsequent hybridization to the 3' F-MuLV probe (Fig. 2).*

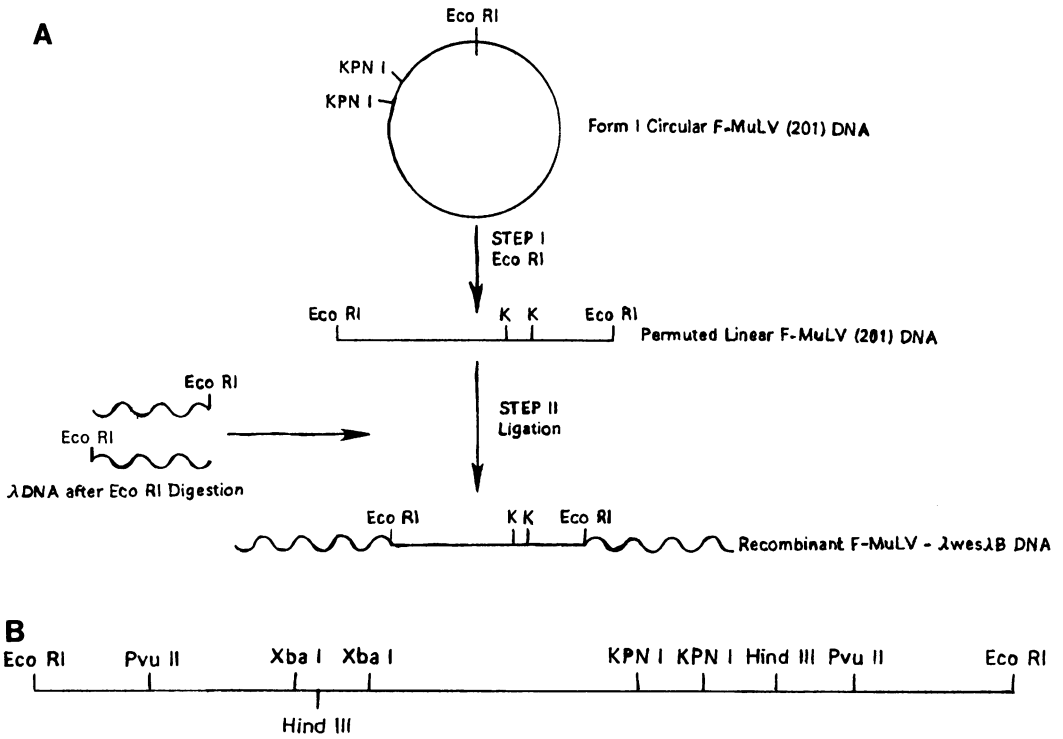

FIG. 4. *Molecular cloning procedure. (A) The schematic model for the molecular cloning of F-MuLV clone 201 DNA begins with the isolation of form I circular DNA corresponding to the linear DNA from the low-molecular-weight Hirt DNA. These molecules have a single EcoRI restriction enzyme site. Step I involves the cleavage of these circular molecules with EcoRI enzyme, which then generates a permuted linear F-MuLV clone 201 DNA molecule with EcoRI sequences at both ends of the molecule. These permuted linear DNA molecules are then mixed with the EcoRI arms of λgtWES·λB DNA, which also have EcoRI-specific sequences at one end of each molecule. In step II, the permuted F-MuLV DNA and the EcoRI-digested λgtWES·λB DNA molecules are ligated together at their EcoRI-specific ends, generating the λ-F-MuLV recombinant DNA. (B) The restriction enzyme map of the permuted linear F-MuLV clone 201 DNA molecule is shown with EcoRI, XbaI, HindIII, PvuII, and KpnI as predicted from the actual restriction enzyme map of the in vivo linear DNA molecules of F-MuLV clone 201 (Fig. 3). The permuted map was generated from the in vivo linear map by joining the two ends of the in vivo linear map, forming a circular map, and then opening this circularized map at the EcoRI site, forming a new (permuted) linear map.*

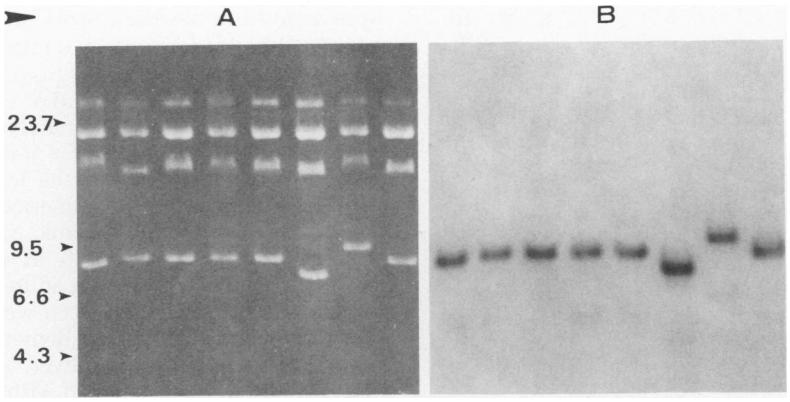

FIG. 5. *EcoRI digest of λ-F-MuLV recombinant DNAs. (A) Photograph under UV light of the DNA from the eight F-MuLV-positive subclones of the recombinant λgtWES·λB bacteriophage after digestion of 1-μg samples with EcoRI and electrophoresis on a 0.5% agarose gel containing 0.5 μg of ethidium bromide per ml. The numbers at the right of the photograph indicate the location of HindIII-digested wild-type λ marker DNA fragments (in kilobases) run in an adjacent lane. (B) Autoradiograph of hybridization with F-MuLV [^{32}P]cDNA of DNA from gel in A. Southern transfer of DNA and hybridization techniques were employed as in Fig. 1.*

proviral DNA), six clones of 8.5 kb, and one clone of 8.2 kb. Further analysis of these three size classes by digestion with the four other restriction enzymes has shown that the largest recombinant clone (9.1 kb) meets all of the predictions made from the permuted map of the linear proviral DNA, whereas the six clones at 8.5 kb contain the sequences of the large (8.4-kb) *Kpn*I fragment of the linear viral DNA obtained from mouse cells while missing the 0.4- and 0.2-kb *Kpn*I fragments which represent the 0.6-kb direct repeats noted in murine retrovirus DNA (3, 27). All six clones which contain the 8.5-kb insert have the same restriction enzyme pattern. The 8.2-kb clone is missing 0.9 kb from a central portion of the molecule (see Fig. 6 and 7).

The biological experiments described in the remainder of this paper were carried out with the DNA from one of the six 8.5-kb recombinant clones (clone 57).

Transfection of the recombinant F-MuLV DNA. The purified recombinant DNA was digested with *Eco*RI to cleave the F-MuLV DNA insert from the λDNA, and then the entire reaction mixture (λgtWES·λB restriction fragments plus the F-MuLV DNA insert) was transfected into NIH 3T3 fibroblasts by $CaCl_2$ precipitation (4). By using 1 μg of total DNA (phage plus F-MuLV) per 35-mm culture dish, infectious virus was consistently produced, as evidenced by both XC plaque and reverse transcriptase assays, which became positive within 5 to 7 days of the transfection. Attempts to transfect the purified recombinant DNA directly into NIH 3T3 fibroblasts without first digesting the F-MuLV insert with *Eco*RI were unsuccessful. Figure 8 shows the titration curve obtained for one of the successful transfection experiments with clone 57. The graph indicates that the transfection of the F-MuLV recombinant DNA follows two-hit kinetics, although in the same experiment, transfection with molecularly cloned integrated AKR-MuLV DNA (data not shown) and infection with both F-MuLV clone 201 and the virus obtained from a successful transfection (viral clone 57) all followed single-hit kinetics. These results suggest that more than one copy of the F-MuLV DNA must enter a target cell, and they must be free from the constraints of flanking bacteriophage DNA to yield a productively transfected cell. Attempts to transfect the recombinant DNAs containing the 9.1-kb and the 8.2-kb inserts were unsuccessful.

Characterization of the transfection-generated virus. The virus produced by the transfection of the recombinant F-MuLV DNA (viral clone 57) was characterized biologically in comparison with our original F-MuLV (clone 201) isolated by endpoint dilution. The host range of both viruses was tested on NIH 3T3, BALB/c 3T3, and mink lung cells. Both viral isolates replicated on both mouse cells with single-hit kinetics and with equal efficiency, and neither virus was able to replicate in mink cells (Table 2). These results indicate that viral clone 57 is an NB-tropic, ecotropic, XC plaque-forming virus, as is F-MuLV clone 201.

Finally, the in vivo effects of viral clone 57

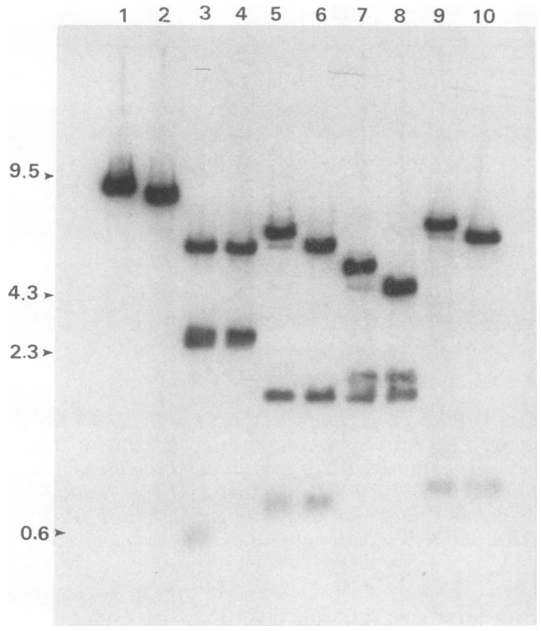

FIG. 6. *Restriction endonuclease analysis of recombinant F-MuLV DNA. Autoradiograph of the F-MuLV recombinant DNA large size class (9.1-kb insert), lanes 1, 3, 5, 7, 9; middle size class (8.5-kb insert), lanes 2, 4, 6, 8, 10. One microgram of recombinant DNA was loaded into each lane of a 0.5% gel after digestion with EcoRI, lanes 1 and 2; EcoRI plus KpnI, lanes 3 and 4; EcoRI plus XbaI, lanes 5 and 6; EcoRI plus HindIII, lanes 7 and 8, EcoRI plus PvuII, lanes 9 and 10. Electrophoresis was at 35 V for 18 h. DNA was transferred to a nitrocellulose filter by the Southern technique and hybridized to F-MuLV [^{32}P]cDNA as in Fig. 1. Note that the middle size clonal isolate in lane 4 is missing the most rapidly migrating DNA fragment (0.6 kb) contained in the large size clonal isolate (lane 3). The numbers at the right of the autoradiograph indicate the location of HindIII-digested wild-type λ marker DNA fragments (in kilobases) run in an adjacent lane.*

were assayed in newborn Swiss mice. Newborn mice (<24 h old) were injected intraperitoneally with 0.2 cm^3 of a filtered virus preparation. Both F-MuLV clone 57 and F-MuLV clone 201 induced leukemia in approximately 90% of the mice 3 to 4 weeks after the injections. The pathology and histology of the leukemia seen with each virus were also identical. Clone 57-inoculated mice became anemic, with hematocrits ranging from 14 to 36% at 4 weeks. All affected mice (28/30) developed hepato-splenomegaly with an average spleen weight of 1.4 g (normal mouse, 0.09 g) and demonstrated leukemic infiltration of their liver, spleen, bone marrow, and peripheral blood with an undifferentiated hematopoietic blast cell (see Fig. 9). Clone 201-inoculated mice also became anemic at 4 weeks with hematocrits ranging from 22 to 34%. Eighty-three percent (10/12) of the mice developed hepato-splenomegaly, with an average spleen weight of 1.2 g and revealed the same undifferentiated blasts infiltrating their liver, spleen, bone marrow, and peripheral blood (Fig. 9 and Table 2).

DISCUSSION

The clone 57 viral isolate described in this report is the progeny virus from a transfection of molecularly cloned F-MuLV DNA. As such, it has been derived from a biochemically pure viral DNA molecule and cannot be contaminated by any other complete or defective retrovirus particles. Clone 57 is both replication competent and pathogenic causing virtually 100% of inoculated newborn mice to become leukemic within 3 to 4 weeks. These characteristics are not unexpected since the progenitor viral stocks of F-MuLV clone 201 which were used to prepare the F-MuLV DNA for molecular cloning also contain a helper-independent MuLV and possessed the same property of rapid leukemo-

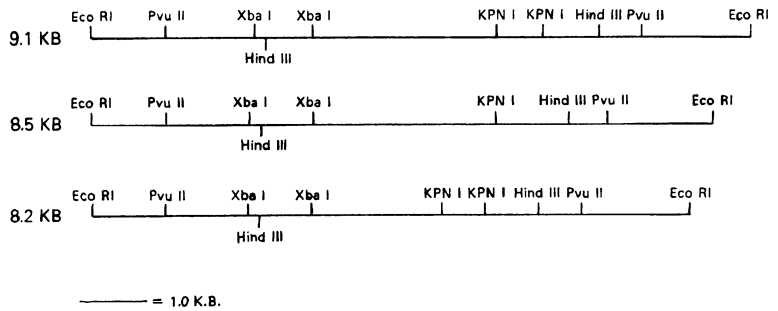

FIG. 7. *Schematic representation of the three different size classes of the recombinant F-MuLV DNA clones using EcoRI, KpnI, XbaI, HindIII, and PvuII restriction enzymes. Relative distances (in kilobases) between the restriction enzyme sites were obtained from the sizes of the restriction fragments generated by double digestion of the recombinant DNA with EcoRI and each of the other four enzymes.*

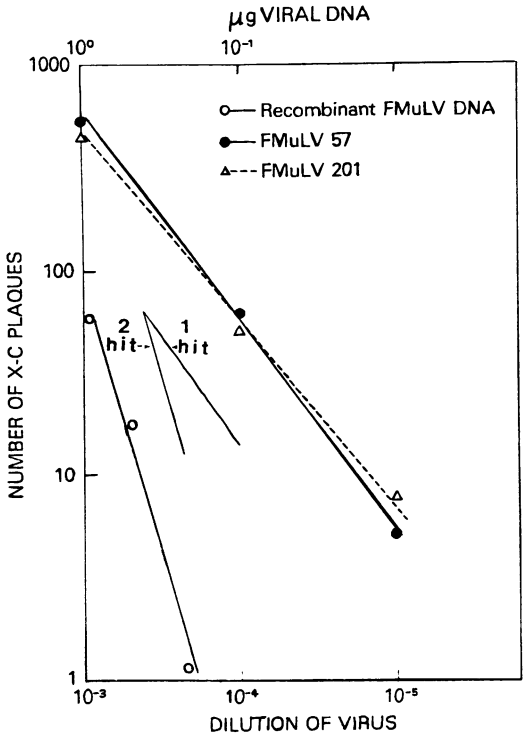

FIG. 8. *Titration kinetics for the transfection of F-MuLV recombinant DNA and the infection of F-MuLV clones 201 and 57. EcoRI-digested recombinant DNA was transfected into 35-mm dishes seeded the previous day with 3 × 10⁵ NIH 3T3 cells per dish. The following day, the cells from each dish were trypsinized and plated into three 60-mm dishes. Virus titrations were performed with filtered (0.45-μm) 18-h supernatant fluids from confluent dishes chronically infected with F-MuLV clone 201 and 57. Virus dilutions were applied in the presence of polybrene to 60-mm dishes seeded the previous day with 10⁵ NIH 3T3 cells per dish. The XC plaque assay was performed 6 days after the transfections or infections.*

genicity. Clone 201 virus was isolated from stocks of Friend virus complex (SFFV-F-MuLV) by endpoint dilution. The fact that clone 57 possesses all of the biological properties observed with clone 201 is further proof that clone 201 is free from contamination with SFFV. If the leukemogenic properties observed with clone 201 were due to a replication-defective virus such as SFFV, then the molecularly cloned helper-independent virus obtained from this stock would not be leukemogenic.

We cannot rule out the possibility that the viral stock obtained from the transfection of molecularly cloned F-MuLV DNA has picked up another murine virus during its passage in the NIH 3T3 fibroblasts and that this other virus could be responsible for the pathogenic properties of the clone 57 viral stock. We consider this possibility to be highly unlikely since numerous other viral DNAs have been transfected into this same cell line and have not demonstrated these biological properties (rapid induction of splenomegaly and leukemia) upon subsequent inoculation of the progeny virus into newborn mice. Nonetheless, studies are under way to further test this hypothesis by the transfection of molecularly cloned F-MuLV DNA into rat embryo cells, which would not be expected to harbor any potentially contaminating murine viruses.

It should be noted that the disease produced in newborns by this molecularly cloned F-MuLV closely resembles the early descriptions of Friend disease produced in adult mice with the unpurified Friend complex (SFFV plus F-MuLV) (13), i.e., anemia, hepato-splenomegaly, normal lymph nodes, and unenlarged thymus. This suggests that the pathological properties of this particular Friend viral complex may reside at least as much within the F-MuLV component of the complex as the SFFV component.

A significant but unexpected finding was that recombinant clone 77, which was the same length as the infectious in vivo linear F-MuLV DNA and contained the same restriction enzyme sites, was not infectious, whereas recombinant clone 57, which was about 0.6 kb smaller, was infectious. The restriction enzyme analysis of clone 57 DNA indicates that the missing 0.6 kb lie in the region of the recombinant F-MuLV DNA that should represent the terminal sequences of the in vivo linear DNA (Fig. 3). Both Hsu et al. (8) and Shank et al. (15) have shown that the terminal sequences of avian retroviral DNA are redundant, with the identical sequences appearing at either end of the molecule. Although the redundancy is about 0.3 kb for avian viral DNA, it is about 0.6 kb for murine viral DNA (3, 5, 27). Apparently the recombinant F-MuLV DNA used to generate clone 57 has only one copy of these sequences as evidenced by a single *Kpn*I restriction site which lies in the redundancy versus the two copies (two *Kpn*I sites) of these sequences seen in the in vivo linear DNA molecules.

Obviously, the missing sequences in clone 57 do not contain any genetic information required for infection, since clone 57 is infectious in transfection experiments. However, these 0.6 kb may have an important biochemical purpose in allowing the expression of the viral genome. The titration curves for the transfection of the recombinant F-MuLV DNA indicated two-hit kinetics for the transfection of the DNA, whereas the

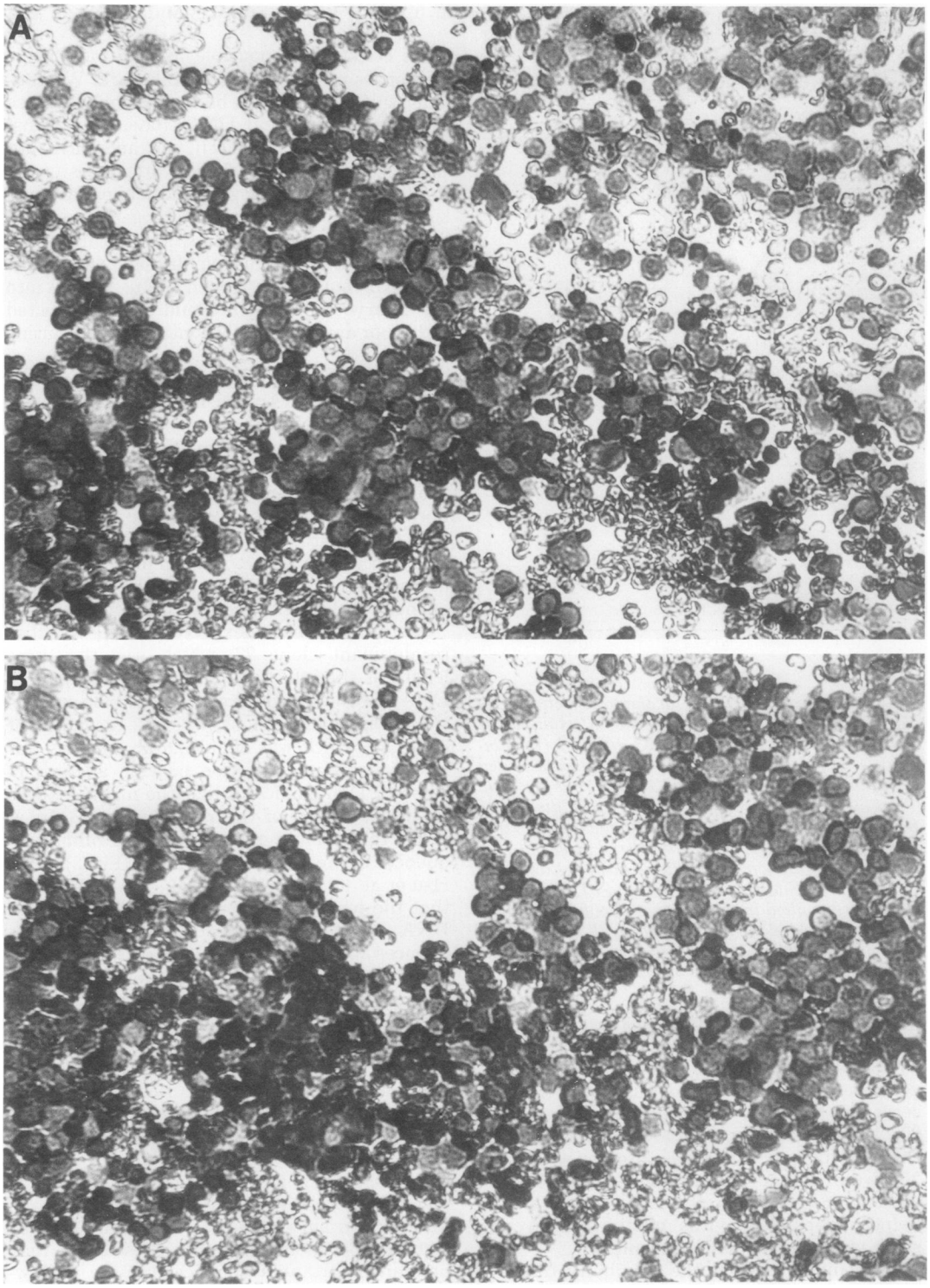

FIG. 9. *Photomicrographs from spleen touch preparations of leukemic mice. (A) Touch preparation of enlarged spleen from NIH Swiss mouse 4 weeks after inoculation with F-MuLV clone 201 (magnification, × 400). (B) Touch preparation of enlarged spleen from NIH Swiss mouse 4 weeks after inoculation with F-MuLV clone 57 (magnification, × 400).*

TABLE 2. *Comparison of the biological properties of viral clone F-MuLV (201) versus F-MuLV (57)*[a]

Viral clone	Host range titer (PFU/ml)			Leukemogenic properties			
	NIH 3T3 cells	BALB/c 3T3 cells	Mink lung cells	Latency period (wk)	Leukemia	Splenomegaly	Hematocrit (%)
F-MuLV 201 biological isolate	$10^{6.0}$	$10^{6.2}$	$<10^{0.3}$	3-4	10/12 (83)[b]	1.2	22-34
F-MuLV 57 molecularly cloned isolate	$10^{6.1}$	$10^{5.9}$	$<10^{0.3}$	3-4	28/30 (94)	1.4	14-36

[a] Host range of each viral isolate was ascertained by inoculation of dilutions of each virus into 60-mm culture dishes containing 10^5 NIH 3T3, BALB/c 3T3, or mink lung cells. Mouse cells were assayed for virus by the XC test. Titration followed single-hit kinetics on both mouse cell lines. Titers for mink cells were also determined by reverse transcriptase assay. Leukemogenic properties: The latency period observed from the time of inoculation of newborn NIH Swiss mice until the appearance of gross disease as evidenced by palpable splenomegaly or death or both.

[b] Leukemia is expressed as the percentage of inoculated animals developing identifiable disease by 4 to 5 weeks postinoculation, when the animals were sacrificed and autopsied and spleen touch preparations were made to assess for leukemic infiltration. Splenomegaly, expressed in grams, is the average weight of the spleens of the leukemic mice only (normal spleen = 0.09 g). Hematocrits were obtained by ophthalmic puncture immediately before sacrifice of the animals (normal hematocrit = 42 to 46%).

progeny virus exhibited single-hit kinetics for the infection of susceptible cell lines. Furthermore, it was not possible to productively transfect the recombinant F-MuLV DNA unless it had first been cut out of its bacteriophage vector DNA. These data strongly suggest that more than one molecule of clone 57 DNA participates in the generation of this XC plaque-forming virus. It is not yet clear how this interaction might occur, but several possibilities may be considered. Since the missing 0.6 kb represent redundant sequences, it would be possible for a recombinational event to generate a complete full-sized 9.1-kb F-MuLV DNA molecule from two 8.5-kb molecules. Alternatively, the permuted orientation of the recombinant F-MuLV DNA may preclude the full expression of the viral genome; therefore, the recombinant DNA must either recircularize and then relinearize in the proper orientation or dimerize with a second recombinant F-MuLV DNA molecule to form one complete F-MuLV DNA molecule in the original in vivo linear orientation (Fig. 4 and 7). Either of these alternative explanations would account for the need to cut out the F-MuLV DNA insert from its phage vector DNA to be biologically active, whereas the final hypothesis would also account for the observed multihit transfection kinetics with recombinant F-MuLV DNA.

Our results indicate that a single viral DNA molecule (F-MuLV DNA) contains all of the information necessary for both the replication of a murine type C virus and the development of a rapid leukemia in the murine system. The existence of virtually unlimited quantities of this DNA in a highly pure form via molecular cloning makes it possible to begin a detailed genetic analysis of the leukemic functions within this genome. A variety of techniques, including marker rescue (1) and in vitro mutagenesis (6), can now be employed to characterize these virus-specific functions.

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