JOURNAL OF VIROLOGY, June 1983, p. 718–725
0022-538X/83/060718-08$02.00/0
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Vol. 46, No. 3

A 2.4-Kilobase-Pair Fragment of the Friend Murine Leukemia Virus Genome Contains the Sequences Responsible for Friend Murine Leukemia Virus-Induced Erythroleukemia

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Received 15 December 1982/Accepted 4 March 1983

Friend murine leukemia virus (F-MuLV) is a replication-competent, ecotropic, NB-tropic retrovirus which produces a rapidly fatal erythroleukemia in susceptible strains of mice. We previously molecularly cloned the entire F-MuLV genome. Transfection of this cloned DNA into NIH 3T3 mouse fibroblasts produces a virus with the same leukemia-inducing characteristics as F-MuLV. To identify which portion of the F-MuLV genome is responsible for causing leukemia, we made recombinant viruses between subgenomic fragments of F-MuLV DNA and another retrovirus—Amphotroph clone 4070. Amphotroph clone 4070 is a replication-competent, amphotrophic, N-tropic virus which does not produce any detectable malignancy in mice. A 2.4-kilobase-pair fragment of F-MuLV DNA was isolated. This DNA fragment encompassed approximately 700 base pairs from the 3′ end of the F-MuLV *pol* gene and 1.7 kilobase pairs of the *env* gene including all of gp70 and the N-terminal four-fifths of p15E. A molecularly cloned fragment of Amphotroph DNA was ligated to the 2.4-kilobase-pair F-MuLV DNA, and an 8.3-kilobase-pair hybrid F-MuLV-Amphotroph DNA was subcloned into a new plasmid (p5a25-H). Transfection of p5a25-H DNA into fibroblasts resulted in the production of a replication-competent, ecotropic, N-tropic retrovirus—5a25-H virus. Inoculation of this virus into newborn NIH Swiss mice caused leukemia within 4 to 6 months. The disease caused by 5a25-H was pathologically and histologically indistinguishable from the disease caused by F-MuLV. We conclude that the F-MuLV sequences needed to cause disease are contained in these 2.4 kilobase pairs of DNA.

Friend murine leukemia virus (F-MuLV) is a replication-competent type C retrovirus obtained from stocks of the Friend virus complex (F-MuLV plus spleen focus-forming virus). F-MuLV causes a rapidly fatal erythroleukemia upon inoculation into NIH Swiss or BALB/c newborn mice. This disease is characterized by severe anemia, circulating erythroblasts, massive hepatosplenomegaly, and normal thymus and lymph nodes (19). To insure that F-MuLV and not a contaminating virus was responsible for this disease, we previously purified the F-MuLV genome by molecular cloning. Transfection of this molecularly cloned F-MuLV DNA into NIH 3T3 mouse cells results in the production of an infectious virus which exhibits the same disease-producing characteristics as the biological clones of F-MuLV (9).

Our laboratory has attempted to identify the region of the F-MuLV genome which is responsible for causing erythroleukemia. To do this, we generated recombinant viruses between F-MuLV and another retrovirus—Amphotroph virus clone 4070 (Ampho). Ampho is a replication-competent virus isolated from a wild mouse (4, 11). Ampho grows on both murine and nonmurine cells, but it is functionally distinct from the dualtropic mink cell focus-forming (MCF) class of murine retroviruses. Ampho does not form foci on mink lung cells and does not produce any detectable malignancy in animals examined up to 18 months after inoculation of newborn mice.

Some F-MuLV-Ampho recombinants caused erythroleukemia. These pathogenic recombinants were generated with the entire Ampho genome and a 4.1-kilobase-pair (kbp) fragment of F-MuLV DNA. The disease produced by these recombinants is indistinguishable from the disease caused by F-MuLV. Therefore, the genetic sequences responsible for F-MuLV-induced disease must be contained in these 4.1 kbp of F-MuLV DNA (10). We have now extended this analysis by generating a new pathogenic F-MuLV-Ampho recombinant virus. The

genome of this new virus contains only 2.4 kbp of F-MuLV DNA.

MATERIALS AND METHODS

Mice. Newborn (<24-h-old) NIH Swiss mice were obtained from Microbiological Associates.

Viruses. F-MuLV clone 57, used in these studies, is a replication-competent, NB-ecotropic retrovirus obtained from the transfection of molecularly cloned F-MuLV DNA into NIH 3T3 mouse cells (19). The original isolate of the Friend virus complex used in cloning F-MuLV was obtained from C. Friend as the Friend virus anemic strain. Ampho is a replication-competent, FV-1 N, amphotrophic retrovirus obtained from the transfection of molecularly cloned Ampho DNA into NIH 3T3 mouse fibroblasts (2). The original Ampho isolate used to generate the Ampho DNA for cloning was supplied by Janet Hartley and Wallace Rowe.

Cells. NIH 3T3 mouse cells were used in all transfection studies (5). BALB/c 3T3 mouse cells and mink lung cells clone 64 (American Type Culture Collection) were also used to define viral host range.

Viral DNAs. Isolation and molecular cloning of the entire F-MuLV and Ampho genomes has been described previously (2, 9). Restriction enzyme digests, agarose gel electrophoresis, and filter hybridization techniques were all performed as previously described (9). Molecular cloning techniques (DNA ligations, bacterial transformations, and plasmid DNA purifications) were also carried out as previously described (2, 9, 10).

Transfections. Viral DNA transfections were performed by a modification of the technique of Graham and Van Der Eb (3). For viral assays, XC plaque formation and reverse transcriptase levels were analyzed according to published procedures (13, 17). Viral infection of cell cultures and viral isolation by end-point dilution cloning were performed as previously described (9, 10).

Metabolic labeling of cells, immunoprecipitation, and gel electrophoresis. Metabolic labeling of cells and analysis of immune precipitates were carried out as previously described (16). Briefly, subconfluent monolayers of cells grown in 100-mm petri dishes were labeled with 300 μCi of [^{35}S]methionine (400 Ci/mmol) per plate. Extracts of cells were then preabsorbed with normal serum and *Staphylococcus aureus* to reduce nonspecific background precipitation and immunoprecipitated with specific antisera and *S. aureus*. The washed precipitates were electrophoresed on 7% sodium dodecyl sulfate-polyacrylamide gels, which were then fixed, flourographed, and exposed to X-ray film at −70°C.

Animal studies. Newborn NIH Swiss mice were inoculated by intraperitoneal injection of 0.2 ml of the viral stocks, using a 27-gauge needle. Viral stocks for inoculation were prepared from chronically infected NIH 3T3 cells by harvesting the culture fluid 18 h after feeding the confluent cells in a 100-mm petri dish. The culture fluid was then filtered through a 0.45-μm filter and placed on ice for up to 1 h before inoculation.

Peripheral blood and spleen cell suspensions were examined microscopically. Blood smears and cytocentrifuge preparations were allowed to air dry and were then stained with dimethyoxybenzidene or Wright-Giemsa stains, or both, as previously described (10).

RESULTS

Construction of recombinant viral genome. Our previous F-MuLV-Ampho recombinants were generated by transfecting a molecularly cloned 4.1-kbp F-MuLV DNA fragment into fibroblast cultures and then infecting those same cultures with Ampho. Recombinant viruses are reproducibly generated by this process, but the precise contribution of each viral DNA to the recombinant genome is unknown. We wished to create recombinants with defined genetic sequences donated from F-MuLV and Ampho. We hoped to create these precise recombinants by ligating specific pieces of F-MuLV DNA to the Ampho genome in vitro. These hybrid DNA molecules could then be tested for their ability to generate virus upon transfection into fibroblasts. To carry out this strategy, we first molecularly cloned the entire Ampho genome (2). The Ampho genome and the 4.1-kbp F-MuLV DNA fragment were then analyzed by restriction endonuclease digestions. Fig. 1 shows the restriction enzyme maps of the Ampho DNA and the 4.1-kbp F-MuLV DNA fragment. The 4.1-kbp F-MuLV DNA fragment encompasses the F-MuLV envelope gene, the long terminal repeat (LTR), and a portion of the N-terminal *gag* gene sequences between two *Hin*dIII sites (10). A *Cla*I site is located near the 3′ end of the F-MuLV envelope gene. This *Cla*I site divides the 4.1-kbp DNA fragment into two segments bound by *Hin*dIII and *Cla*I (H-C segments). The 2.4-kbp H-C segment contains primarily F-MuLV *env* gene information, whereas the remaining 1.7-kbp H-C segment contains p15E, the LTR, and *gag* gene sequences (see below). Each of these H-C segments was subcloned into new pBR322 vectors. The Ampho DNA also possesses a *Cla*I site near the 3′ end of its envelope gene.

We chose to exploit the finding of a common *Cla*I site in the genomes of F-MuLV and Ampho by using this site to join the 2.4-kbp F-MuLV H-C fragment to the Ampho genome. This construction would place F-MuLV *env* gene information in continuity with Ampho sequences coding for the remaining retroviral functions. To do this, we first eliminated the Ampho *env* sequences which lie immediately 5′ to the *Cla*I site by subcloning out the region between the Ampho *Cla*I site and the *Cla*I site in pBR322 (Fig. 1). The resulting plasmid contained 7.2 kbp of Ampho DNA extending from the *Cla*I site to an *Eco*RI site (C-E Ampho plasmid). We next inserted a *Cla*I DNA fragment containing the F-MuLV 2.4-kbp H-C fragment into the *Cla*I site of the C-E Ampho plasmid. Several recombinant clones were identified as containing the 2.4-kbp F-MuLV H-C fragment by hybridization to a nick-translated radiolabeled probe made from gel-purified 2.4-kbp H-C DNA. Four of these

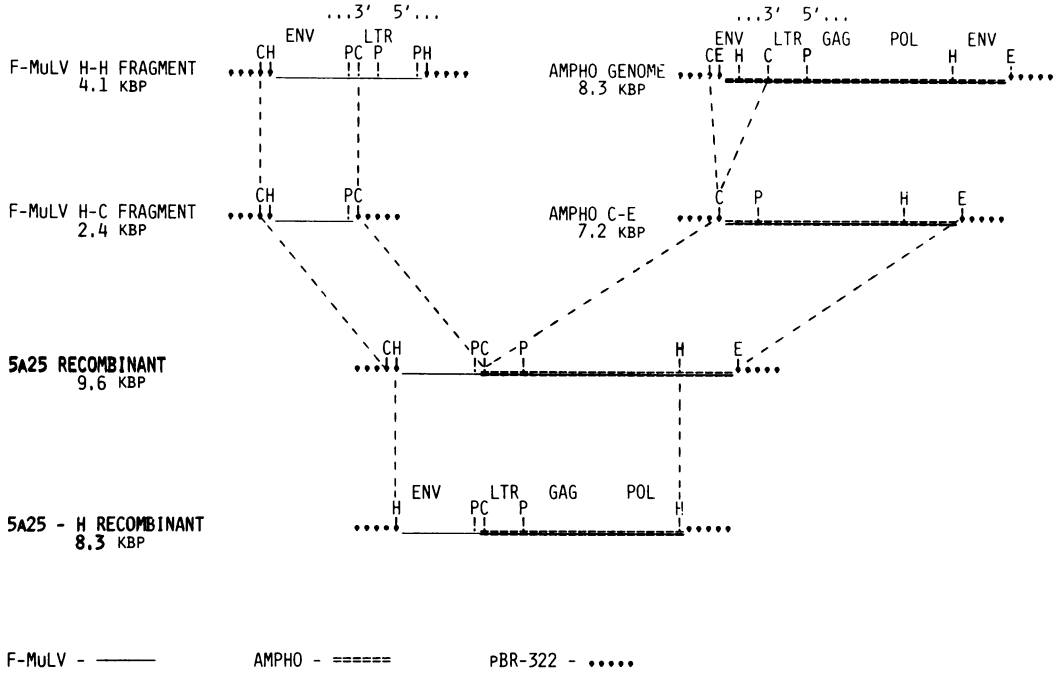

FIG. 1. Schematic model of strategy used to clone 5a25-H viral genome. The plasmid containing the F-MuLV H-H DNA fragment was digested with *Cla*I, and the 2.4-kbp F-MuLV H-C fragment was subcloned into a new plasmid. The plasmid containing the entire Ampho genome (8.3 kbp) was also digested with *Cla*I, and the sequences lying between the two *Cla*I sites were deleted. The remaining Ampho plasmid DNA was recircularized, using T4 DNA ligase to form the Ampho C-E plasmid which contained 7.2 kbp of viral DNA. The 2.4-kbp F-MuLV H-C fragment plus 5 bp of pBR322 DNA were digested out of their plasmid vector with *Cla*I and ligated into the unique *Cla*I site of the C-E plasmid. This recombinant is designated 5a25 and contains 9.6 kbp of viral DNA. 5a25 was digested with *Hin*dIII. An 8.3-kbp hybrid F-MuLV-Ampho DNA was recovered from this digestion and subcloned into a new plasmid, creating the 5a25-H recombinant. C, *Cla*I; H, *Hin*dIII; E, *Eco*RI; and P, *Pst*I. Each schematic drawing of a molecular clone represents the viral DNA insert and a short stretch of the flanking plasmid vector DNA. The remaining plasmid DNA regions are not shown.

clones were analyzed by digestion with *Hin*dIII to identify a recombinant plasmid which contained the H-C fragment in the same 3′–5′ orientation as the Ampho DNA (data not shown). One plasmid with the H-C fragment in this orientation was identified and designated p5a25 (Fig. 1).

The p5a25 viral insert was 9.6 kbp in length. Complete murine leukemia virus (MuLV) genomes contain approximately 8.3 kbp including a single copy of the LTR (18). The extra 1.3 kbp of MuLV information in p5a25 were derived from Ampho sequences located between the *Hin*dIII and *Eco*RI sites in the C-E Ampho plasmid (Fig. 1). This segment of Ampho DNA is analogous to a portion of the F-MuLV 2.4-kbp H-C DNA fragment. These 1.3 kbp of Ampho *env* gene DNA were not deleted before ligation of the F-MuLV H-C DNA into the C-E Ampho plasmid. Thus, p5a25 contained two sets of sequences spanning the same region of the MuLV genome. To eliminate these additional 1.3 kbp of Ampho DNA from our recombinant viral genome, we digested the p5a25 plasmid with *Hin*dIII and subcloned the resulting 8.3-kbp *Hin*dIII-*Hin*dIII viral DNA into a new pBR322 vector (Fig. 1). The resulting plasmid was designated p5a25-H.

p5a25-H contained an entire MuLV genome consisting of 2.4 kbp of F-MuLV DNA and 5.9 kbp of Ampho DNA. A limited restriction enzyme analysis of p5a25-H is presented in Fig. 2. Lanes 1, 2, and 3 indicate that the p5a25-H viral DNA insert is similar in size to the DNAs of F-MuLV and Ampho. Lanes 4, 5, and 6 contain p5a25-H DNA cut with both *Hin*dIII and a second enzyme. The restriction map shown in Fig. 1 is consistent with this analysis and confirms the identity of the p5a25-H viral insert as a recombinant between the F-MuLV H-C fragment and the Ampho C-E DNA. The 2.4-kbp F-MuLV H-C fragment has been sequenced (R. Friedrich, W. Koch, and G. Hunsmann, Conference on RNA Tumor Viruses, Cold Spring Har-

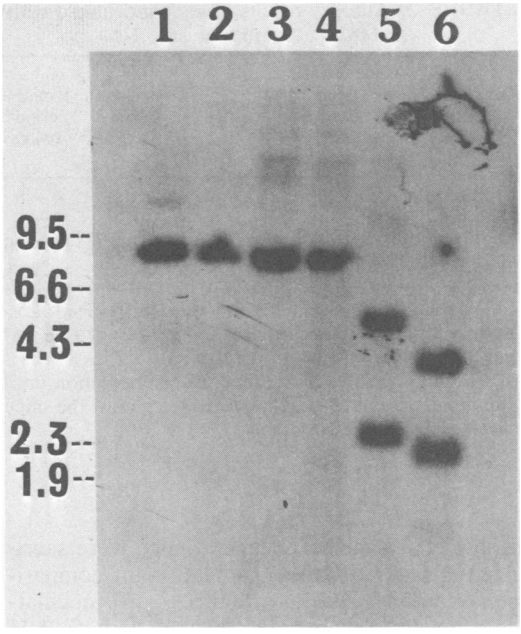

FIG. 2. Autoradiograph of a restriction enzyme analysis of molecularly cloned retroviral DNAs. Plasmid DNAs containing either the F-MuLV, Ampho, or 5a25-H genomes were digested with the restriction enzymes indicated below and electrophoresed on a 0.5% agarose gel at 35 V for 18 h. The gel was then Southern blotted onto nitrocellulose filter paper. The filter was hybridized with a nick-translated probe made from a mixture of F-MuLV and Ampho genomic DNAs, washed to remove the excess label, and then exposed to X-ray film for 18 h. The complete F-MuLV and Ampho DNAs are inserted into their plasmid vectors at *Eco*RI sites (2, 9). The 5a25-H viral DNA is inserted into its plasmid vector at a *Hin*dIII site (see text). Lane 1, F-MuLV-containing plasmid cut with *Eco*RI. Lane 2, Ampho-containing plasmid cut with *Eco*RI. Lanes 3 through 6, p5a25-H cut with *Hin*dIII, *Hin*dIII plus *Eco*RI, *Hin*dIII plus *Cla*I, and *Hin*dIII plus *Pst*I, respectively. Note the similar migration distance of the single band of viral DNA in lanes 1 through 4. Viral DNA bands can be seen at 5.6 and 2.5 kbp in lane 5, and at 4.4 and 2.2 kbp in lane 6. A 1.4-kbp viral DNA band is also present in lane 6 but is poorly visualized on this exposure. The numbers at the side of the autoradiograph indicate the location of *Hin*dIII-digested wild-type lambda DNA fragments (in kilobases) run in an adjacent lane.

bor Laboratory, Cold Spring Harbor, N.Y. 1982, p. 71). These sequences span approximately 700 base pairs (bp) from the 3′ end of the F-MuLV *pol* gene, the entire gp-70 coding region, and approximately four-fifths of the p15E gene (6). The Ampho genome has not been sequenced. However, by analogy to the F-MuLV DNA sequence and restriction map, the 5.9 kbp of Ampho DNA in p5a25-H should encode the remaining one-fifth of p15E, the R peptide, the LTR, the *gag* gene, and the majority of the *pol* gene sequences. If these sequences are contained in the Ampho DNA in p5a25-H, then p5a25-H should contain a complete MuLV genome. Transfection of the p5a25-H viral DNA into fibroblasts should result in the production of a complete retrovirus.

Generation and characterization of 5a25-H virus. The recombinant viral DNA insert contained in p5a25-H was released from its pBR322 vector by digestion with *Hin*dIII. The resulting DNA preparation was transfected into NIH 3T3 cells, using the technique of Graham and Van Der Eb (3). The recipient 3T3 cells were passaged three times and then tested for virus production by XC plaque formation. Multiple experiments of this kind have repeatedly yielded an XC-positive virus preparation. This virus preparation was designated 5a25-H.

The host range and growth characteristics of 5a25-H virus were assayed by infection of NIH 3T3 cells, BALB/c 3T3 cells, and mink lung cells. 5a24-H virus grew on murine cells but did not grow on mink cells (Table 1). This ecotropic host range must be derived from the F-MuLV genome since Ampho grows equally well on murine or mink cells. 5a25-H was an XC plaque-forming virus which exhibited single-hit kinetics

TABLE 1. Host-range growth characteristics of 5a25, F-MuLV, and Ampho[a]

Virus	Growth titers		
	NIH 3T3 cells	BALB/c 3T3 cells	Mink lung cells
F-MuLV	10^5 XC	10^5 XC	$<10^{0.3}$ RT
Ampho	10^4 RT	10^2 RT	10^4 RT
5a25-H	10^5 XC	10^2 XC	$<10^{0.3}$ RT

[a] Host range of each viral isolate was ascertained by inoculation of serial dilutions of virus into 60-mm culture dishes containing 10^5 NIH 3T3, BALB/c 3T3, or mink lung cells. Murine cells were assayed by the XC test, and the results are presented as PFU/ml. Reverse transcriptase (RT) titers were performed with 5 ml of freshly harvested medium obtained from confluent cells (in 60-mm culture dishes) fed with 5 ml of medium 18 h before harvest. Viral stocks were diluted in log dilutions onto 60-mm plates as noted above. When the cells in the plates had reached confluence, 30% of the cells were subcultured to a new 60-mm plate and grown again to confluence. At that time, the reverse transcriptase assays were performed. Plates that were negative for viral reverse transcriptase were subcultured in a similar way two more times and retested for reverse transcriptase to ensure that correct endpoint titers were obtained. Ten plates of mink cells were assayed at each viral dilution.

for XC plaque formation (Fig. 3). This property must also derive from the F-MuLV genome since Ampho does not form XC plaques. Therefore, the properties of ecotropism and XC plaque formation must be contained within the 2.4-kbp F-MuLV H-C fragment used to generate the 5a25-H viral genome. 5a25-H grows to higher titers on NIH cells than on BALB/c cells and is therefore Fv-1 N-tropic. This property must be derived from the Ampho genome since our Ampho isolate is N-tropic whereas our F-MuLV is NB-tropic.

Leukemogenicity. 5a25-H virus was harvested from chronically infected NIH 3T3 cells and used to inoculate newborn NIH Swiss mice (see above). Additional mice were similarly inoculated with F-MuLV and Ampho. Beginning 4 weeks after inoculation, all mice were tested for the presence of leukemia by weekly examinations of peripheral blood smears and hematocrits. Of the F-MuLV-inoculated mice, 100% developed leukemia by 8 weeks of age and were sacrificed and autopsied (Table 2). None of the Ampho-infected mice has developed any signs of leukemia to date (1 year after inoculation).

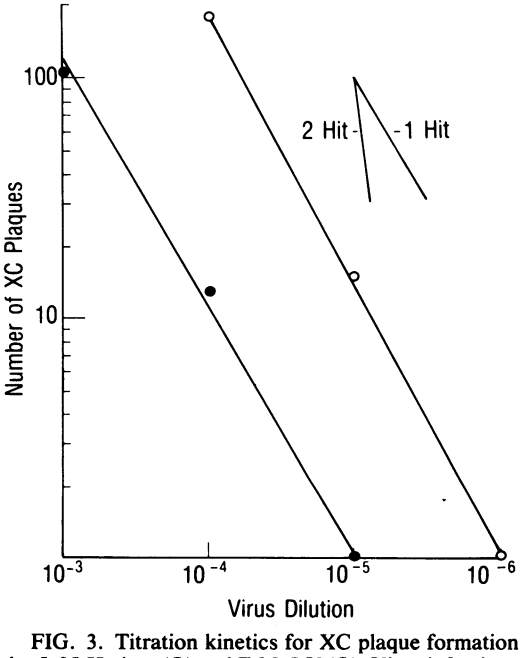

FIG. 3. Titration kinetics for XC plaque formation by 5a25-H virus (○) and F-MuLV (●). Virus infections were performed with filtered (0.45-μm filter) 18-h supernatant fluids from confluent cells (in 100-mm petri dishes) chronically infected with 5a25-H or F-MuLV. Virus dilutions were prepared as serial 10-fold dilutions and applied in the presence of Polybrene (Aldrich Chemical Co.) to 60-mm dishes seeded the previous day with 10^5 NIH 3T3 cells per dish. XC plaque assays were performed 6 days after infection.

TABLE 2. Autopsy results of mice inoculated with F-MuLV, Ampho, or 5a25-H[a]

Virus	Incidence of leukemia (no. leukemic/no. injected)	Spleen wt (g)	Hematocrit (%)	Latency period[b] (wks)
F-MuLV	12/12	0.65 – 2.2	28 – 11	4 – 8
Ampho	0/20			26
5a25-H	5/20	0.80 – 1.7	22 – 14	16 – 26
None	0/20	0.06 – 0.14	48 – 50	26

[a] Spleen weights and hematocrits for the F-MuLV- and 5a25-H-inoculated mice only represent the diseased animals.

[b] Latency period is the time after inoculation until the development of obvious disease or until the mice were sacrificed for autopsy.

Half of the Ampho-inoculated mice were sacrificed at 6 months after inoculation for comparison of autopsy results with the 5a25-H-inoculated animals. The mice inoculated with 5a25-H virus were sacrificed as they developed disease. The earliest leukemia observed in these mice occurred 4 months after inoculation. The remaining mice were sacrificed at 6 months after inoculation. Of the 5a25-H mice, 25% developed erythroleukemia within this 6-month period. The pathological features of this disease (i.e., hepatosplenomegaly, severe anemia, and normal thymus and lymph nodes) were indistinguishable from the disease caused by F-MuLV. Microscopic examinations of peripheral blood smears and cytospin preparations of spleen cells were also indistinguishable between F-MuLV- and 5a25-H-diseased mice. Using a combination of benzidene and Wright stains, we identified hemoglobin in the cytoplasm of numerous blast cells. The majority of the nucleated cells in the peripheral blood and >90% of the spleen cells were judged to be erythroblasts.

Viruses recovered from 5a25-H-diseased mice. To examine the viruses present in the diseased tissues of mice infected with 5a25-H, we prepared spleen homogenates from two mice with severe leukemia. The spleen homogenates were used to infect NIH 3T3 fibroblasts (see above). After three cell passages, the fibroblasts were assayed for XC and reverse transcriptase activities. Both spleen homogenates produced XC-positive, polymerase-positive virus preparations. One of these virus preparations was end point diluted and used to infect new NIH 3T3 fibroblasts. Two types of virus were obtained from this experiment, an XC-positive virus (Cl-A) and an XC-negative, polymerase-positive virus (Cl-D). We attempted to infect mink lung cells with each of these viruses. Only Cl-D was

able to infect the mink cells, as evidenced by a positive polymerase assay. Cl-D virus obtained from the infected mink cells was able to infect new NIH 3T3 cells. Cl-A is most likely an isolate of the 5a25-H virus which was initially used to inoculate the mice. Cl-D appears to be a dualtropic retrovirus and must have been generated in the infected animals.

The gp70 precursor proteins of ecotropic and dualtropic murine leukemia viruses (MuLVs) are distinguishable from one another on the basis of their electrophoretic mobility in acrylamide gels (14, 16). If the 5a25-H-infected mice contain both ecotropic and dualtropic viruses, then two distinct viral gp70 precursor proteins should be identifiable in murine cells infected with spleen homogenates from these animals. NIH 3T3 cells infected with the spleen homogenate from a leukemic mouse inoculated with 5a25-H exhibited two gp70 precursor proteins (Fig. 4, lanes 4 and 5). The more rapidly migrating gp70 precursor (gPr80) in these cells was precipitated by an anti-gp70 antisera raised against Moloney MCF virus (lane 5). NIH 3T3 cells infected with 5a25-H exhibited only a single gp70 precursor protein (gPr85), which was not recognized by the anti-MCF virus gp70 antisera (Fig. 4, lanes 1 and 2).

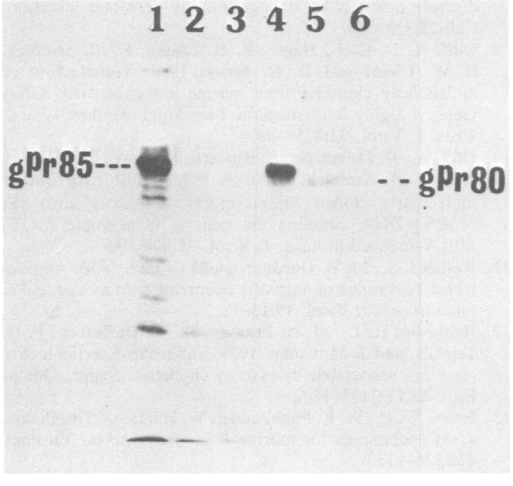

FIG. 4. Autoradiograph of sodium dodecyl sulfate gel containing immunoprecipitates of metabolically labeled NIH 3T3 cell extracts after immunoprecipitation with antiviral sera. Metabolic labeling was carried out for 30 min with [^{35}S]methionine. Lanes 1 through 3, Precipitates from cells chronically infected with 5a25-H virus. Lanes 4 through 6, precipitates from cells infected with a spleen homogenate obtained from a mouse with leukemia induced by 5a25-H virus. Cell extracts were immunoprecipitated with goat anti-Rauscher MuLV gp70 antiserum (lanes 1 and 4), goat anti-Moloney MCF virus gp70 antiserum absorbed with Rauscher MuLV (lanes 2 and 5), or normal goat serum (lanes 3 and 6). Immunoprecipitates were then electrophoresed on a 7% sodium dodecyl sulfate-polyacrylamide gel. The gel was dried and exposed to X-ray film.

DISCUSSION

5a25-H virus is a recombinant MuLV artificially constructed and produced in vitro with pieces of molecularly cloned F-MuLV and Ampho DNAs. This recombinant virus caused a lethal erythroleukemia upon inoculation into newborn NIH Swiss mice. The disease caused by 5a25-H was pathologically and histologically indistinguishable from the disease caused by F-MuLV. Since the Ampho isolate used in these studies does not cause leukemia, the genetic sequences responsible for the erythroproliferative disease caused by 5a25-H must be contained within the F-MuLV DNA fragment used to construct the 5a25-H genome.

The diseases caused by 5a25-H and F-MuLV were not identical. F-MuLV caused leukemia within 8 weeks of inoculation and affected 90 to 100% of the injected mice. 5a25-H caused leukemia in only 25% of the injected mice and required an incubation period of 4 to 6 months before disease became apparent. It is unlikely that these differences are due to chance mutations which arose during the cloning of p5a25-H since two parallel but independently isolated F-MuLV-Ampho DNA constructs (2a13 and 2a25) also produced virus with the same pathogenic potential as 5a25-H. Our previous F-MuLV-Ampho recombinant viruses caused disease in 40 to 50% of inoculated mice with a latency period of 6 to 8 weeks (10). The F-MuLV DNA fragment used in those studies was the 4.1-kbp H-H fragment (Fig. 1). This DNA contained the 2.4 kbp of *env* gene sequences used in the present study plus an additional 1.7 kbp of F-MuLV DNA. These additional 1.7 kbp of F-MuLV sequences encode the C-terminal one-fifth of p15E, the R peptide, the LTR, and a portion of the N-terminal *gag* gene sequences. Although this 1.7-kbp region of the F-MuLV genome is not required to construct a leukemogenic virus, these sequences may influence the growth characteristics and pathogenic potential of F-MuLV. In the case of avian leukosis virus, differences between the U3 regions of different avian leukosis virus isolates have been shown to affect the ability of those viruses to grow and to cause disease (8, 12, 20). In 5a25-H, the C-terminal one-fifth of p15E, the R peptide, and the entire LTR region are encoded by Ampho sequences. Thus, the increased latency period or decreased incidence of leukemia (or both) caused by 5a25-H versus F-MuLV or our earlier recombinants may be due to functional differ-

ences between F-MuLV and Ampho encoded in this 1.7-kbp region of their genomes.

F-MuLV and 5a25-H share *env* gene sequences. In the case of 5a25-H, these sequences are contained in the 2.4-kbp H-C fragment of F-MuLV DNA (Fig. 1). This DNA fragment encompasses the entire gp70 coding region and the N-terminal four-fifths of p15E. In addition, the H-C fragment contains 700 bp of F-MuLV DNA which lie immediately 5′ to the gp70 coding sequences. By analogy to the published sequence of Moloney murine leukemia virus, these 700 bp may encode specific polypeptides (18). It is therefore possible that the leukemia-inducing capacity of the F-MuLV H-C fragment is not due to the *env* gene sequences present in this DNA. Instead, an as yet unidentified gene contained in the 700 bp lying upstream from *env* may be needed to cause leukemia. We consider this possibility to be unlikely in light of the genetic analysis of another murine erythroleukemia virus, spleen focus-forming virus. Using molecularly defined deletion mutants of spleen focus-forming viral DNA, Linemeyer et al. (7) have shown that the spleen focus-forming viral envelope gene sequences are needed to cause leukemia.

Although it is clear that the 2.4-kbp F-MuLV H-C fragment contributes to the development of erythroleukemia, the mechanism of leukemogenesis remains unknown. The 5a25-H viral gp70 or p15E proteins encoded by these sequences may act to stimulate the proliferation of erythroid precursor cells. Alternatively, 5a25-H virus may not be the direct cause of leukemia. Instead, 5a25-H may produce disease by inducing the formation of a pathogenic MCF virus. This hypothesis is consistent with the fact that dualtropic virus was recovered from the diseased tissues of mice inoculated with 5a25-H. Previous studies from our laboratory have implicated a dualtropic virus (Friend-MCF virus) in the pathogenesis of F-MuLV-induced erythroleukemia (14, 15). Leukemias caused by other ecotropic MuLVs (e.g., Moloney and AKR) have also been associated with the formation of dualtropic viruses (1, 21). If MCF viruses are responsible for the leukemias associated with F-MuLV and 5a25-H, then the 2.4-kbp F-MuLV H-C fragment may be needed to participate in the recombinational event which creates these leukemogenic viruses.

ACKNOWLEDGMENTS

We thank Douglass Lowy and Edward Scolnick for their thoughtful discussions and Paul Marks and Richard Rifkind for their advice and support in performing these experiments.

These studies were supported in part by the National Cancer Institute (PO A31768-02) and (AO8748), the American Cancer Society (CH-68D), and the Bristol Myers Cancer Grant Program.

LITERATURE CITED

1. **Chattopadhyay, S. K., M. W. Cloyd, D. L. Linemeyer, M. R. Lander, E. Rands, and D. R. Lowy.** 1982. Cellular origin and role of mink cell focus-forming viruses in murine thymic lymphomas. Nature (London) **295**:25–31.
2. **Chattopadhyay, S. K., A. I. Oliff, D. L. Linemeyer, M. R. Lander, and D. R. Lowy.** 1981. Genomes of murine leukemia viruses isolated from wild mice. J. Virol. **39**:777–791.
3. **Graham, F. L., and A. J. Van Der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
4. **Hartley, J. W., and W. P. Rowe.** 1976. Naturally occurring murine leukemia viruses in wild mice: characterization of a new "amphotropic" class. J. Virol. **19**:19–25.
5. **Jainchill, J. L., S. A. Aaronson, and G. J. Todaro.** 1969. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. J. Virol. **4**:549–553.
6. **Koch, W., G. Hunsmann, and R. Friedrich.** 1983. Nucleotide sequence of the envelope gene of Friend murine leukemia virus. J. Virol. **45**:1–9.
7. **Linemeyer, D. L., S. K. Ruscetti, J. G. Menke, L. H. Evans, and E. M. Scolnick.** 1982. Envelope gene sequences which encode the gp52 protein of spleen focus-forming virus are required for the induction of erythroid cell proliferation. J. Virol. **43**:223–233.
8. **Neel, B. G., W. S. Hayward, H. L. Robinson, J. Fang, and S. M. Astrin.** 1981. Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. Cell **23**:323–334.
9. **Oliff, A. I., G. L. Hager, E. H. Chang, E. M. Scolnick, H. W. Chan, and D. R. Lowy.** 1980. Transfection of molecularly cloned Friend murine leukemia virus DNA yields a highly leukemogenic helper-independent type C virus. J. Virol. **33**:475–486.
10. **Oliff, A., D. Linemeyer, S. Ruscetti, R. Lowe, D. R. Lowy, and E. M. Scolnick.** 1980. A subgenomic fragment of molecularly cloned Friend murine leukemia virus (F-MuLV) DNA contains the gene(s) responsible for F-MuLV-induced disease. J. Virol. **35**:924–936.
11. **Rasheed, S., M. B. Gardner, and E. Chan.** 1976. Amphotropic host range of naturally occurring wild mouse leukemia viruses. J. Virol. **19**:13–18.
12. **Robinson, H. L., M. N. Pearson, D. W. DeSimone, P. N. Tsichlis, and J. M. Coffin.** 1979. Subgroup-E avian leukosis virus associated disease in chickens. Symp. Quant. Biol. **XLIV**:1133–1142.
13. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
14. **Ruscetti, S., L. Davis, J. Fields, and A. Oliff.** 1981. Friend MuLV-induced leukemia is mink cell focus-inducing (MCF) virus associated and is genetically restricted in mice expressing endogenous xero-related envelope viral genes. J. Exp. Med. **154**:907–920.
15. **Ruscetti, S., J. Fields, L. Davis, and A. Oliff.** 1982. Factors determining the susceptibility of NIH Swiss mice to erythroleukemia induced by Friend murine leukemia virus. Virology **117**:357–365.
16. **Ruscetti, S. K., D. Linemeyer, J. Feild, D. Troxler, and E. M. Scolnick.** 1979. Characterization of a protein found in cells infected with the spleen focus-forming virus that shares immunological cross-reactivity with the gp70 found in mink cell focus-inducing virus particles. J. Virol. **30**:787–798.
17. **Scolnick, E. M., E. Rands, S. A. Aaronson, and G. J. Todaro.** 1970. RNA dependent DNA polymerase in five RNA viruses: divalent cation requirements. Proc. Natl. Acad. Sci. U.S.A. **67**:1789–1796.
18. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981.

Nucleotide sequence of Moloney murine leukaemia virus. Nature (London) 293:543–548.
19. **Troxler, D. H., and E. M. Scolnick.** 1978. Rapid leukemia induced by cloned Friend strain of replicator murine type-C virus. Virology 85:17–27.
20. **Tsichlis, P. N., and J. M. Coffin.** 1979. Role of the C region in relative growth rates of endogenous and exogenous avian oncoviruses. Symp. Quant. Biol. XLIV:1123–1132.
21. **Van der Putten, H., W. Quint, J. Van Raaj, E. R. Mardag, I. M. Verma, and A. Berns.** 1981. M-MuLV-induced leukemogenesis: integration and structure of recombinant proviruses in tumors. Cell 24:729–739.