Localization of the Leukemogenic Determinants of SL3-3, an Ecotropic, XC-Positive Murine Leukemia Virus of AKR Mouse Origin

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SL3-3 is a potent leukemogenic retrovirus that closely resembles the non-leukemogenic virus Akv. Both viruses were isolated from AKR mice, have ecotropic host ranges, and form plaques in the XC assay. They differ at only 1 to 2% of the nucleotides in the viral genomes but differ markedly in virulence properties. SL3-3 induces leukemia in a high percentage of inoculated AKR, C3H, CBA, and NFS mice, whereas Akv does not induce disease in any of these strains. To determine which region of the genome accounts for the leukemogenic potential of SL3-3, we constructed recombinant genomes between molecular clones of SL3-3 and Akv. Recombinant, viral DNA genomes were cloned and then were transfected onto NIH 3T3 fibroblasts to generate infectious virus. The recombinant viruses were tested for leukemogenicity in AKR/J, CBA/J, and C3Hf/Bi mice. We localized the primary leukemogenic determinant to a 3.8-kilobase fragment of the SL3-3 genome containing the viral long terminal repeat, 5' untranslated sequences, *gag* gene, and 5', 30% of the *pol* gene. Reciprocal recombinants containing the equivalent region from Akv, linked to the *env* gene and the remainder of the *pol* gene from SL3-3, did not induce leukemia. We conclude that the primary virulence determinant of SL3-3 lies outside the region of the genome that encodes the envelope proteins gp70 and p15E.

SL3-3 is a potent leukemogenic retrovirus isolated from an AKR mouse (19, 28). SL3-3 induces leukemia in 100% of inoculated AKR/J mice after a mean latent period of approximately 80 days. It also induces disease in a high percentage of inoculated C3Hf/Bi, CBA/J, and NFS/N mice after a similar mean latent period (19). The genome of SL3-3 is comprised of three genes, *gag*, *pol*, and *env*, that encode the viral internal core, polymerase, and membrane envelope proteins, respectively. In addition, the genome contains noncoding sequences required for expression of the viral genes. All of these sequences function in viral replication. No transforming gene analogous to those of acute transforming retroviruses is present (28). What inherent property of the SL3-3 genome endows this virus with the ability to induce leukemia?

To approach this question, we compared the genome of SL3-3 with that of Akv virus. Akv is a non-leukemogenic retrovirus that can be found in all tissues of AKR mice from midgestation onward throughout life (37). Although SL3-3 is highly leukemogenic, it is very closely related to Akv. No differences can be detected by heteroduplex formation between molecular clones of the viral genomes (19). Only a few differences

are detectable by RNase T₁ fingerprinting analysis of the viral genomes, and several of these, at least, can be attributed to single nucleotide changes (28). Although the differences between SL3-3 and Akv are minimal, they must account for the difference in the leukemogenic potential of these viruses.

In earlier RNase T₁ fingerprinting studies of the viral genomes, we correlated the presence of specific oligonucleotides near the 3' end of the SL3-3 genome with the viral leukemogenic potential (28). The same oligonucleotides are found near the 3' ends of the genomes of several other leukemogenic retroviruses from AKR mice but are not present in the Akv genome (1, 27-29). These oligonucleotides were considered to be genetic markers for sequences near the 3' ends of viral genomes that confer leukemogenicity (27, 28). The location of these sequences correlates well with the results of similar studies with other murine viruses and avian viruses, results which also suggest that sequences near the 3' ends of the genomes are associated with virulence (22, 23, 36, 42). However, all studies of this type are limited, as they provide only indirect correlations between sequences near the 3' ends of the genomes and virulence.

We now report the results of studies designed to elucidate directly the region of the SL3-3 genome that accounts for the viral leukemogenic potential. By the use of molecular clones of SL3-3 and Akv, recombinant genomes were constructed between the viruses at specific restriction enzyme sites common to both viral genomes. Viruses derived by transfection of clones of the recombinant genomes were tested for leukemogenicity in mice.

MATERIALS AND METHODS

Enzymes and reagents. Restriction enzymes were from New England Biolabs, Bethesda Research Laboratories, and Boehringer Mannheim Corp. T4 DNA ligase and radiolabeled nucleotides were from New England Nuclear Corp. *Escherichia coli* DNA polymerase I was from New England Biolabs. DNase I was from Worthington Diagnostics. Calf serum was from Microbiological Associates. Low-melting-point agarose was from Bethesda Research Laboratories.

Growth of cells. *E. coli* strains LE392, HB101, and MBM7014 were maintained by serial passage in our laboratory in YT or Luria broth medium. *E. coli* containing the plasmids reported here was grown in medium containing 25 µg of tetracycline (Tc) per ml. λ phages were grown in *E. coli* LE392. Plasmids to be digested with *Xba*I were grown in *E. coli* MBM7014. NIH 3T3 fibroblasts were grown in Temin modified eagle medium or Dulbecco modified eagle medium containing 10% calf serum.

Mice. AKR/J and CBA/J mice were obtained from Jackson Laboratories. C3Hf/Bi mice were obtained from the University of Minnesota mouse colony. Mice have been maintained in our laboratory by brother-sister mating for several years. Experimental mice were weaned and segregated by sex at approximately 21 days of age.

Clones. Isolation of an infectious, leukemogenic clone, λSL3-95, of SL3-3 in the λ vector, Charon 4, was previously described (19). Isolation of an infectious clone of Akv in pBR322 was previously described. This clone was derived by subcloning the large *Pst*I fragment (see Fig. 1) of the Akv genome from λAKR-623 (21) into the *Pst*I site of pBR322.

Construction of SL3-3 clones in pBR322. Subclones of the SL3-3 genome were constructed by digestion of about 2 µg of λSL3-95 DNA to completion with *Pst*I. This DNA was isolated and mixed with about 1 µg of *Pst*I-digested pBR322 DNA. The DNAs were then ligated and used to transform *E. coli* HB101 to Tc^r. Single colonies were chosen, recloned, and tested for the presence of viral sequences by hybridization to the nick translated, large *Pst*I fragment of the Akv genome isolated from pAKR-59 (20).

Construction of plasmids containing recombinant viral genomes. Akv or SL3-3 plasmid DNA (5 to 25 µg) was digested with the appropriate restriction enzymes as described above. Fragments were resolved by electrophoresis in gels comprised of 1% low-melting-point agarose containing 0.05% ethidium bromide. Fragments were excised and eluted into TE buffer (10 mM Tris-hydrochloride [pH 7.4]–1 mM EDTA) by heating to 65°C for 10 min. DNA was extracted with phenol-

chloroform and then was concentrated by spermine precipitation (14). It was resuspended in TE, and the concentration was determined by electrophoresis in agarose gels containing 0.4% ethidium bromide.

To assemble recombinant genomes, approximately equimolar concentrations of the appropriate fragments were mixed and ligated in a volume of 10 µl for 1 to 5 h at 13°C. Concatenate formation was monitored by removing small samples at various times during the ligation and electrophoresing the DNA on agarose gels with unligated fragments as size markers. When interfragment ligation was determined to be adequate, reactions were diluted to 100 to 200 µl and allowed to proceed overnight at 13°C to complete circularization of the plasmids (7).

Ligated DNAs were used to transform *E. coli* HB101 to Tc^r. Single colonies were picked and replated at low densities to assure cloning. Single colonies were again picked and grown to saturation in 10 to 200 ml of YT containing 25 µg of Tc per ml. Plasmid DNAs were isolated by scaling up the rapid boiling procedure of Holmes and Quigley (13). Plasmid DNAs were purified by RNase A digestion, followed by proteinase K digestion and phenol-chloroform extraction. DNA was concentrated by ethanol precipitation.

The structure of every recombinant genome used in this study was confirmed by restriction enzyme mapping of the isolated plasmid DNAs. Duplicates or, in some cases, triplicates of each construction were independently isolated and used to produce infectious virus.

Derivation of virus particles. Viruses were derived by transfection of NIH 3T3 fibroblasts with isolated plasmid DNAs, using the calcium phosphate-glycerol method (6). By analyzing the genomes of the resulting viral particles, using RNase T₁ fingerprinting, we previously showed that this technique produces viruses containing RNA genomes of the expected structure (20). Plasmids were digested with *Pst*I and then were religated under conditions favoring formation of concatenes. Plasmid DNA (1 µg) was mixed with 19 µg of salmon sperm DNA and transfected onto NIH 3T3 cells as described previously (19, 20). Fibroblast cultures were trypsinized, and the cells were diluted approximately 1:10 every third day. Production of virus particles was monitored regularly by assaying for reverse-transcriptase containing particles in the supernatant fluid (38). When the reverse transcriptase activity reached a maximum, the cells were permitted to grow to saturation density. Medium was changed daily. Virus was harvested between 10 and 25 days after transfection and collected by filtration through a 0.2-µm nitrocellulose filter. Small samples were stored at -70°C. Infectious titer of each stock was determined by duplicate, 10-fold serial dilutions of each stock on NIH 3T3 fibroblasts.

Leukemogenicity assays. Newborn mice were injected intraperitoneally with 0.1 ml of virus. Mice were injected before they were 72 h old, usually before they were 36 h old. All individual mice were monitored several times per week. Moribund mice were sacrificed and necropsied to determine whether they were lymphomatous. The gross pathology of every mouse scored as positive for disease in this report was typical of lymphoma, including enlargement of the thymus, spleen, mesenteric and peripheral lymph nodes, and liver.

RESULTS

High sequence homology, but different leukemogenic potentials of SL3-3 and Akv. The goal of our work is to define the sequences in the SL3-3 genome that account for the viral leukemogenic capability. Our approach is to determine which differences between leukemogenic SL3-3 virus and non-leukemogenic Akv virus affect virulence. We chose to use SL3-3 for these studies as it is the leukemogenic virus that is minimally different from Akv (22, 23, 27-29). The similarity of the two viruses minimizes the number of genomic differences that are potentially responsible for the difference in virulence.

An estimate of the number of differences between SL3-3 and Akv can be obtained by using several techniques to compare the viral genomes. RNase T₁ fingerprinting was previously used to estimate that a maximum of 50 to 70 nucleotides differences are present in the 8.2-kilobase (kb) viral RNA genomes (Haseltine et al., in R. Neth and R. Gallo, ed., *Modern Trends in Human Leukemia V*, in press). This observa-

tion indicates that they are approximately 99% homologous. These changes are scattered throughout the viral genome (28). Restriction enzyme mapping yields a similar estimate of the extent of homology between SL3-3 and Akv. Figure 1 shows a map of the relative positions of several 6-base-recognition-site restriction enzymes in the 8.8-kb viral DNA genome, relative to the viral genetic map. Isolations of the infectious, molecular clones used to perform the restriction analysis were previously reported (19-21). Of the 33 sites mapped in Fig. 1, 29 are common to both viruses. Four are unique to the SL3-3 genome. These unique sites are scattered throughout the SL3-3 genome (Fig. 1). If these four differences are due to changes at one of the six nucleotides in the recognition site of each restriction enzyme, then we can calculate that the viruses differ at 4 of the 198 nucleotides comprising the 33 restriction sites. Thus, by this method, the viral genomes are estimated to differ at approximately 2% of the nucleotides in the genomes.

FIG. 1. Comparison of the restriction enzyme cleavage sites in the SL3-3 and Akv genomes with the viral genetic map. Digestions were performed by using molecular clones of the viral genomes. The SL3-3 genome is shown above the viral genetic map, and the Akv genome is shown below the viral genetic map. Restriction sites unique to the SL3-3 genome are shown by asterisks. No sites are unique to the Akv genome with the enzymes tested here. Positions of the viral genes are based on nucleotide sequencing studies of murine retroviruses (12, 20, 39). The position of the large *Pst*I fragment used to generate the pBR322 subclones is shown. The orientation and position of the 8.2-kb viral genome relative to the 8.8-kilobase pair DNA genome are also shown. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; H3, *Hind*III; Kp, *Kpn*I; Pl, *Pvu*I; Pv, *Pvu*II; Ps, *Pst*I; Sc, *Sac*I; Sa, *Sal*I; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I. The *Hind*III site in the SL3-3 is present in most isolates of Akv (2). However, it is missing in the clone of Akv used in this study (21).

Based on these two methods, we estimate that SL3-3 and Akv are identical at 98 to 99% of the nucleotides in the viral genomes. The few differences that occur are not large changes. Some of them, at least, are single nucleotide changes (19, 28). No insertions or deletions can be detected by heteroduplex, RNase T₁ fingerprinting, or restriction enzyme analyses (19). If any insertions or deletions are present, they must be very small, and they cannot disrupt the translational reading frames of the viral genes. How do such minimal differences, scattered throughout the viral genome, confer leukemogenicity on SL3-3?

Strategy used to localize determinants responsible for the leukemogenic potential of SL3-3. To localize the differences between SL3-3 and Akv that account for the difference in virulence, we constructed recombinants between the two viruses. The general approach was to construct viral genomes comprised partially of SL3-3 sequences and partially of Akv sequences, by linkage of restriction enzyme fragments of molecular clones of the viral DNA genomes. Most restriction enzyme sites are conserved between the viral genomes (Fig. 1). Ligation of fragments flanking either side of a specific site should generate a viral genome with a complete genetic complement and maintain the correct translational reading frame without introducing any insertions or deletions. As SL3-3 and Akv both replicate to similar titers on NIH 3T3 fibroblasts in tissue culture, we expected that recombinant viruses derived from them would also replicate well on these cells. Thus, the constructed, recombinant DNA genomes could be transfected onto NIH 3T3 cells, and infectious virus so produced could be tested for leukemogenicity in mice.

Details of the strategy we used are shown in Fig. 2. To simplify construction of recombinants, subclones of the viral genomes were constructed in the plasmid vector pBR322. These clones were derived by subcloning the large *Pst*I fragments of the SL3-3 and Akv genomes (shown in Fig. 1), from the previously isolated λ vector clones (19, 21), into pBR322. *Pst*I cleaves both viral genomes at equivalent locations. The resulting 8.2-kb fragments contain complete complements of the genetic information of the viruses. The organization of the viral genomes in the pBR322 subclones differs from that of the proviruses in the λ clones in that each contains only one copy of the viral long terminal repeat (LTR) sequences. LTRs are comprised of noncoding sequences that are required for several viral functions (40, 41). In each plasmid, the single LTR is split between the extreme ends of the viral inserts (Fig. 2). Most of the LTR region is adjacent to the 5' viral sequences, and the remaining nucleotides are

adjacent to the *env* gene at the 3' end of the genome.

To generate recombinant genomes, the viral plasmids were digested with the same restriction enzymes so that each plasmid was cleaved into two fragments of different sizes. The fragments were isolated by electrophoresis in agarose gels. Pairs of fragments were then joined by ligation to generate recombinant genomes comprised of sequences from each parental genome (Fig. 2). In each case, reciprocal recombinants were constructed. Plasmids constructed in this manner contain all the genetic information necessary for replication in *E. coli* and confer tetracycline resistance (Tc^r).

In studies of this type, it is essential to be certain that recombinant DNA preparations used for transfection are free of contaminating viral DNA fragments. For this reason, the recombinants generated by ligation of fragments of the SL3-3 and Akv genomes were used to transform *E. coli*. Single colonies containing recombinant genomes were selected by Tc^r. Several independently isolated Tc^r colonies were replated at low density and again selected for Tc^r. A single colony from each replating was chosen and used for isolation of plasmid DNA. The homogeneity of each recombinant plasmid preparation was verified by restriction enzyme analysis. Sufficient restriction enzyme heterogeneity exists between SL3-3 and Akv to verify unambiguously the structures of all recombinants constructed here (Fig. 1; see below).

To generate viral particles, isolated plasmid DNAs containing recombinant genomes were digested with *Pst*I to separate viral DNA from the plasmid vector DNA. The *Pst*I-treated DNA was then incubated with T4 DNA ligase under conditions that favored concatenation over circularization (7). Concatenates of the *Pst*I fragments contain proviral sequences with the viral coding region flanked by LTRs. This DNA was then used to transfect NIH 3T3 fibroblasts as previously described (19, 20). Transfected DNA generates the production of viral particles that infect the entire fibroblast culture. Virus was harvested from the supernatants of such cultures and used to inoculate mice.

Two assays were used to measure the leukemogenicity of the recombinants. One was acceleration of the onset of disease in AKR/J mice. AKR mice spontaneously develop lymphomas approximately 200 to 350 days after birth (5). Although the pathology of virally induced disease is similar to that of spontaneous disease, the two can be distinguished by a shortened latent period (19). Lymphomas occurring up to 150 days postinjection were scored as positive for viral induction. The second assay measured induction of lymphomas in strains of mice which

FIG. 2. Strategy used to construct recombinants and obtain viral particles. Recombinant plasmids were designated pRECAS, for recombinant between Akr and SL3-3. Details of the procedure are described in the text.

normally do not inherit this disease. CBA/J and C3Hf/Bi mice were used in the studies reported here.

Advantages of this approach. Several important advantages are realized by using the approach shown in Fig. 2. One is that the recombinant genomes contain only a single LTR. Thus, both LTRs in progeny viral DNA genomes derived upon transfection are identical. If they were not identical, then we would need to be concerned that genetic rearrangements between the left and right LTRs do not occur. Another advantage is that the progeny recombinants are derived from cloned genomes. Thus, the DNA used for transfection is free of contaminating parental DNA. Additionally, the fidelity of the ligation reactions can be verified by restriction enzyme analysis of the cloned recombinant genomes. Verification that the original restriction site was regenerated during ligation assures that the proper translational reading frame was main-

tained. Finally, structures of the recombinant genomes can be verified by restriction enzyme analysis. Sufficient restriction enzyme heterogeneity exists between the SL3-3 and Akv genomes (Fig. 1) to confirm the structures of all of the recombinant genomes reported here. In addition to heterogeneity within the viral genomes, some of the plasmids used in this study were constructed containing additional heterogeneity by the inclusion of additional sequences outside the viral genomes (Fig. 3a). These sequences, which were derived from the λ vector, provided additional heterogeneity that was also used to confirm the structures of the recombinant genomes.

Figure 3a shows the structures of the parental SL3-3 and Akv plasmids used here. Isolation of Akv plasmid pAKR-59 was previously described (20). Relative to pAKR-59, the SL3-3 plasmids used here contain additional sequences derived from the original λ vector (Fig. 3a). The sole

FIG. 3. Detailed structures of parental and recombinant plasmids relative to the viral genetic map. All plasmids are shown arbitrarily linearized at the *Pst*I site in the viral LTR sequences (see Fig. 1). SL3-3 sequences are shown by the bold lines, Akv sequences are shown by the thin lines, and pBR322 and λ vector sequences are shown by the dashed lines. (A) Structures of the genomes of the SL3-3 and Akv containing plasmids used for the constructions. The sole reason for the inclusion of the λ sequences was to confirm the structures of the recombinant progeny. Positions of the restriction sites used for recombinant construction are shown. (B) Genetic map and structures of the genomes of the recombinant plasmids. The genetic map aligns with the viral sequences in all seven plasmids shown. The fragments exchanged in each of constructions I to IV are indicated. The line underneath each recombinant shows the portion of each genome derived from the appropriate SL3-3 plasmid. The remaining fraction of each is derived from pAKR-59. The SL3-3 sequences in plasmid structure IV are derived from pSL3-359. Those in the other plasmids are derived from pSL3-308. The structures of all of the recombinant plasmids were confirmed by restriction analysis (data not shown). Sufficient heterogeneity exists between the SL3-3 and Akv sequences, due to the inclusion of the λ sequences and the unique restriction sites (see Fig. 1), to verify the structure of all recombinants shown here. Abbreviations: Eco, *Eco*RI; Pst, *Pst*I; Sac, *Sac*I; and Xba, *Xba*I.

reason for the inclusion of these sequences was to confirm the structures of the recombinant genomes. As expected, the leukemogenicity phenotypes of the viral genomes in the plasmid subclones were identical to those of the original isolates. Viruses from the SL3-3 plasmids accelerate disease in 100% of recipient AKR/J mice, with a mean latent period of approximately 76 days (Table 1). This incidence and latent period are identical to those reported earlier for the λ vector clones of SL3-3 and the original biological isolates of the virus (19, 28). Similarly, induction of disease in CBA/J mice was equivalent to that reported earlier (19). No induction of disease occurred in AKR/J or CBA/J mice injected with Akv virus from the clones used here (Table 1).

Genomic structures and leukemogenic phenotypes of recombinant viruses. Restriction enzyme fragments from the SL3-3 and Akv plasmids that were used to generate recombinant genomes were selected because they divide the viruses into functionally and genetically distinct regions. Earlier studies correlated the presence of specific sequences near the 3' end of the SL3-3 genome with the leukemogenic phenotype (28). This portion of genome is comprised of the *env* gene and a noncoding region at the 3' end of the genome. The *env* gene encodes two peptides in the order NH₂-gp70-p15E-COOH (20). The noncoding region contains a short segment adjacent to the carboxyl terminal of the *env* gene containing the termination codon for the *env* gene, followed by a region of approximately 550 nucleotides comprising part of the LTR (12, 20, 39-41). Our initial recombinants were constructed to test whether sequences in this region were leukemogenic determinants.

The first pair of reciprocal recombinants was constructed to test whether sequences comprising the carboxyl half of p15E encode the leukemogenic phenotype. This was accomplished by exchanging *Xba*I-*Eco*RI fragments between the SL3-3 and Akv plasmids (Fig. 3B, groups I and II). The structures of the resulting pair of viral genomes are shown in Fig. 4 (groups I and II).

Viruses derived from the first recombinant genome (Fig. 4) are isogenic with Akv, except for the 0.5-kb region from SL3-3. This region encodes (i) the carboxyl half of the p15E portion of the *env* gene, (ii) a nontranslated, purine-rich sequence of 34 nucleotides, and (iii) the first 41 nucleotides of the LTR (12, 20). Viruses derived from this genome are not leukemogenic (Fig. 4; Table 2).

The reciprocal recombinant is leukemogenic in both AKR/J and CBA/J mice (Fig. 4, Table 2). Viruses derived from this genome are isogenic with SL3-3, except for the 0.5-kb region from Akv. The virulence of this group shows that the

TABLE 1. Leukemogenicity tests of the SL3-3 and Akv plasmids

Virus	No. of isolates tested	Designation	Infectious titer (IU/ml)	AKR leukemogenicity				CBA leukemogenicity			
				No. of mice inoculated	No. of mice diseased	Incidence (%)	Mean latent period (days)	No. of mice inoculated	No. of mice diseased	Incidence (%)	Mean latent period (days)
Akv	1	PAKR-59	5×10^4	6	0	0		7	0	0	
SL3-3	2 ^a	PSL3-308	NT ^b	18	18	100	76	15	14	93	101
		PSL3-359	5×10^5								

^a Leukemogenicity data from both SL3-3 plasmids were pooled.

^b NT, Not tested.

FIG. 4. Relationship between leukemogenicity and the structure of the viral genome. Both the DNA genome, flanked by two LTRs, and the RNA genome are shown for each recombinant virus. The leukemogenicity of each structure is indicated. Each genome is shown aligned with the genetic map of the viral DNA genome. Shaded areas represent sequences derived from SL3-3; open areas show sequences derived from Akv.

primary leukemogenic determinant is not located in the region that encodes the carboxyl terminal of p15E through the first 41 nucleotides of the LTR region.

Another pair of reciprocal recombinants was constructed to test whether sequences in the remainder of the *env* gene and adjacent *pol* gene sequences might account for virulence. Substitutions in the gp70 portion of the *env* gene have been correlated with leukemogenicity of viruses from AKR mice (2, 3, 9, 11, 22, 23, 32, 43). Although no differences between SL3-3 and Akv are detectable in this region by heteroduplex or RNase T₁ fingerprinting analysis, the possibility remained that small, undetected changes in this region encoded the leukemogenic potential. To construct recombinants in this region, *SacI*-*EcoRI* fragments were exchanged between the parental plasmids (Fig. 3B, groups III and IV). The regions of the viral genomes exchanged in the second pair of recombinants included (i) the same sequences exchanged in the first pair of recombinant genomes, (ii) the remainder of the *env* gene, and (iii) the carboxyl 70% of *pol* gene (Fig. 4, groups III and IV).

Viruses that were comprised of the entire *env* gene and most of the *pol* gene from SL3-3 did not induce disease (Fig. 4; Table 2). Reciprocal recombinants, containing the equivalent region from Akv linked to the remainder of the genome from SL3-3, are leukemogenic in AKR/J, C3Hf/Bi, and CBA/J mice (Fig. 4, Table 2). This observation localizes the primary leukemogenic

determinant of the SL3-3 genome to a 3.8-kb fragment containing the LTR, 5' untranslated sequences, *gag* gene, and the amino terminal 30% of the *pol* gene. To the extent the SL3-3 genome is dissected in these studies, the same determinants function to accelerate disease in AKR/J mice and induce disease in C3Hf/Bi and CBA/J mice.

Infectious titer of the viral stocks. One explanation for the difference in leukemogenicity could be that the titers of the virulent and avirulent strains differ substantially. Table 1 shows that this is not the case. The infectious titer of each stock was quantitated by the technique of end-point dilution on NIH 3T3 fibroblasts (Table 1). No correlation was detected between titer and virulence.

Further support for this conclusion was obtained by assaying the virulence of dilutions of our SL3-3 stock. The 100-fold dilution of a 5×10^5 IU per ml SL3-3 stock did not significantly attenuate the leukemogenic potential (Table 3). These results argue that the leukemogenicity of our recombinants is due not to the number of infectious particles inoculated into mice, but to an inherent property of the recombinant genomes.

Detailed analysis of the leukemogenic potential of the recombinant viruses. The virulent recombinants accelerated disease in nearly 100% of recipient AKR/J mice; only 1 of 34 animals injected with these viruses (Table 2) failed to develop disease within 150 days postinjection.

TABLE 2. Leukemogenicity of recombinant viruses

Virus ^a	No. of isolates	Designation ^b	Infectious titer (IU/ml) ^c	AKR leukemogenicity			CBA leukemogenicity			C3H leukemogenicity					
				No. of mice inoculated	No. of mice diseased ^d	Incidence (%)	Mean latent period (days)	No. of mice inoculated	No. of mice diseased ^d	Incidence (%)	Mean latent period (days)	No. of mice inoculated	No. of mice diseased	Incidence (%)	Mean latent period (days)
I	3	PRECAS-25	5 × 10 ⁴	22	0	0		11	0	0		0	NT ^e	NT	
		PRECAS-26	5 × 10 ⁵												
		PRECAS-29	1 × 10 ⁷												
II	3	PRECAS-31	5 × 10 ⁶	28	28	100	106	13	7	54	103	0	NT	NT	
		PRECAS-33	1 × 10 ⁵												
		PRECAS-34	5 × 10 ⁴												
III	3	PRECAS-13	5 × 10 ⁶	22	0	0		10	0	0		7	0	0	
		PRECAS-14	1 × 10 ⁷												
		PRECAS-20	1 × 10 ⁸												
IV	2	PRECAS-6	5 × 10 ⁶	11	10	91	111	10	3	30	106	3	3	100	108
		PRECAS-8	5 × 10 ⁷												

^a Viral designations are the same as those used in Fig. 3 and 4.^b Each plasmid is an independent isolate, as described in the text. Those within each group (I to IV) have identical structures. Leukemogenicity data were pooled from all independent isolates of a particular structure.^c Infectious titer was determined by endpoint dilution on NIH 3T3 fibroblasts.^d All mice scored as positive for disease exhibited a gross pathology typical of lymphomas.^e NT, Not tested.

TABLE 3. Leukemogenicity of dilutions of SL3-3

Dilution	SL3-3 titer ^a	No. of mice		Incidence (%)	Mean latent period (days)
		Inoculated	Diseased		
0	5×10^5	8	8	100	73
10X	5×10^4	8	8	100	78
100X	5×10^3	6	6	100	81

^a The titer was determined by endpoint dilution of the undiluted viral stock on NIH 3T3 fibroblasts. The titer of the diluted stocks was calculated according to the appropriate dilution.

This disease incidence clearly localizes the primary leukemogenic determinant to the LTR, 5' untranslated sequences, *gag*, or 5', 30% *pol* sequences. However, the mean latent periods for disease onset were 105 and 111 days for the recombinant viruses in groups II and IV, respectively. The increase in the latent periods of the recombinants suggests that the virulence of these isolates was slightly attenuated. A *t* test was performed to compare the mean latent period of disease induction in animals receiving SL3-3 with that in animals receiving the viruses in group I (Fig. 4; Tables 1 and 2). This analysis showed that the difference in latent periods of 78 versus 105 days is significant ($P < 0.001$). Similarly, comparison of the group IV latent period of 111 days with that of SL3-3 showed that it is also significantly longer ($P < 0.001$). Comparison of the different latent periods for the two different structures of leukemogenic recombinants, 105 versus 111 days, fails to demonstrate a significant difference, $P > 0.2$.

Induction of disease in C3Hf/Bi mice by the recombinant viruses occurs with an equivalent incidence and mean latent period to SL3-3 (Table 2) (19, 28). However, a larger number of these mice must be tested before statistical conclusions are made. Induction of disease in CBA/J mice by the recombinant viruses occurs with an equivalent latent period but a lower incidence compared with SL3-3 (Tables 1 and 2). Because the mice used in the studies reported here were monitored for only 5 months after inoculation, it is likely that both of the disease parameters will increase as the surviving mice are monitored for extended times.

These leukemogenicity studies show that the primary leukemogenic determinant endows viruses with a potency for disease induction approaching that of SL3-3 in AKR/J, C3Hf/Bi, and CBA/J mice. The slight, but statistically significant, attenuation of the virulence of the recombinants, apparent at least in some strains, raises the possibility that the SL3-3 genome contains other sequences that do not confer virulence by themselves but slightly increase the potency when the primary leukemogenic determinant is present. It is possible that the small number of differences between SL3-3 and Akv affects leukemogenicity in multiple ways.

DISCUSSION

We constructed a series of recombinants between SL3-3 and Akv to determine which region of the SL3-3 genome accounts for the viral leukemogenic potential. Specifically, the recombinants reported here were constructed to test whether the genes at the 3' end of the viral genome encode the leukemogenic determinants. Earlier evidence indirectly correlated the presence of certain sequences in this region with the viral leukemogenic potential (1, 19, 20, 27, 28). RNase T₁ fingerprinting studies demonstrated the presence of two oligonucleotides near the 3' end of the SL3-3 genome that are not present in Akv (28). The same oligonucleotides are also present in a number of other leukemogenic retroviruses derived from AKR mice (1, 27, 28). These oligonucleotides were proposed to be genetic markers for sequences near the 3' ends of the viral genomes that confer virulence (27, 28).

One of these markers is located in the *env* gene of SL3-3. This gene is translated as a polypeptide that is proteolytically processed into two mature peptides, gp70 and p15E (8, 16, 24). We previously determined the complete nucleotide sequence of the Akv *env* gene and proposed a structure by which it associates with membranes (20). In this model, gp70 is located completely on the outer side of the lipid portion of the membrane and is covalently attached to p15E by disulfide links (30). The latter peptide crosses the lipid portion of the membrane and anchors the complex to it. The *env* gene oligonucleotide marker for leukemogenicity alters the coding sequence of p15E at the point at which it emerges on the cytoplasmic side of the lipid component of the membrane (20).

The second oligonucleotide marker for leukemogenicity maps closer to the 3' end of the SL3-3 genome, in the noncoding LTR region. A single base change of G to A, 65 nucleotides from the 5' end of the LTR, is characteristic of SL3-3 and other virulent isolates (27, 28; J. Lenz, R. Crowther, A. Sheldon, R. Patarca, D. Celander, and W. Haseltine, unpublished data).

The first pair of reciprocal recombinants (Fig. 4; Table 2) was constructed to test whether sequences surrounding the oligonucleotide

marker in p15E encode the leukemogenic phenotype. However, leukemogenicity tests clearly showed that the leukemogenic potential was not located in this region (Fig. 4; Table 2).

Therefore, another set of recombinants was constructed to test whether the leukemogenic phenotype is encoded elsewhere in the *env* gene or in the adjacent *pol* gene sequences. Considerable evidence exists correlating the presence of substitutions in the *env* gene of various leukemogenic viruses from AKR mice with virulence (2-4, 9, 22, 23, 32, 43). However, SL3-3 and Akv are both ecotropic and form plaques in the XC assay, properties that are associated with the viral *env* genes (28). No differences between SL3-3 and Akv are detectable by heteroduplex analysis or RNase T₁ fingerprinting in this region, but the possibility remained that small, undetected changes were present that affected virulence (19, 28). Leukemogenicity tests with the recombinant viruses clearly showed, however, that the leukemogenic potential of SL3-3 relative to Akv was determined by sequences outside the 3' coding region (Fig. 4; Table 2). Therefore, it is clear that the primary leukemogenic determinant of SL3-3 lies entirely outside the region of the genome that encodes the *env* proteins gp70 and p15E.

These results localize a key determinant in the leukemogenic process to a 3.8-kb fragment that encodes the SL3-3 LTR, 5' untranslated sequences, *gag* gene, or 5', 30% of the *pol* gene. Thus, the primary leukemogenic determinant is located either in noncoding sequences or in the coding region at the 5' end of the viral genome. Of these sequences, only those in the noncoding region at the 3' end of the viral RNA genome were previously correlated by indirect techniques with leukemogenicity (28). Whether the leukemogenic determinant is located in the noncoding sequences, the *gag* gene, or the 5' portion of the *pol* gene, it must perform an important function in one or more of the steps involved in viral leukemogenesis. These steps include establishment of general viremia, interaction with the host immune system (17, 18), specific infection of critical target cells (4, 26), induction of proliferation, and malignant outgrowth of the tumor cells. It is unlikely that the virulent and avirulent isolates differ at the stage of inducing general viremia. High titers of non-leukemogenic Akv virus occur in all tissue of AKR mice throughout life (37). It seems likely that the virulence determinant functions by allowing a different interaction with the host immune system, by conferring tropism for critical cells, or by permitting a specific interaction at the molecular level with potential leukemogenic factors in the host pretumor cell.

A comprehensive hypothesis for the role of

viruses from AKR mice in leukemogenesis must account for the observation that the Akv genome can acquire a leukemogenic phenotype by the substitution of sequences located outside the *env* gene. The simplest explanation is that the primary leukemogenic determinant confers virulence independently of the nature of the *env* sequences. However, indirect evidence correlates the presence of substitutions in the *env* gene with virulence of viruses from AKR mice (2, 3, 4, 9, 22, 23, 32, 43). The possibility remains that SL3-3 and our leukemogenic recombinants acquire *env* substitutions after infection of the mouse. Such changes might increase the virulence of a virus beyond the level conferred by the primary leukemogenic determinant alone. One possibility is that our virulent isolates have a greater capacity to acquire new *env* sequences. However, if the leukemogenic determinant confers such a capacity, it must do so from a distant position in the genome.

These results also raise the question of whether an equivalent determinant functions during disease induction by other murine leukemia viruses and by leukemia viruses isolated from other species. Many different retroviruses that cause tumors have been isolated from mice (5, 10, 25, 33, 34). Isolates of veterinary significance include feline, bovine, and gibbon ape leukemia viruses. The virus associated with adult T cell leukemia, HTLV, may also belong to this class (15, 31, 35).

We are currently constructing new sets of higher resolution recombinant genomes to analyze further the leukemogenic determinants. When these are coupled with DNA sequencing studies, we hope to be able to identify at the level of single nucleotides the viral determinants responsible for leukemogenicity.

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LITERATURE CITED

1. Buchhagen, D. L., F. S. Pedersen, R. L. Crowther, and W. A. Haseltine. 1980. Most sequence differences between the genomes of Akv virus and a leukemogenic Gross A virus passaged *in vitro* are located near the 3' terminus. *Proc. Natl. Acad. Sci. U.S.A.* 77:4359-4363.
2. 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.
3. Chattopadhyay, W. K., M. R. Lander, S. Gupta, E. Rands, and D. R. Lowy. 1981. Origin of mink cytopathic focus-forming (MCF) viruses: comparison with ecotropic and xenotropic murine leukemia virus genomes. *Virology* 113:465-483.
4. Cloyd, M. W. 1983. Characterization of target cells for

- MCF viruses in AKR mice. *Cell* 32:217-225.
5. Cloyd, M. W., J. W. Hartley, and W. P. Rowe. 1980. Lymphomagenicity of recombinant mink cell focus-inducing murine leukemia viruses. *J. Exp. Med.* 151:542-552.
 6. Copeland, N. G., and G. M. Cooper. 1979. Transfection by endogenous and exogenous murine retrovirus DNAs. *Cell* 16:347-356.
 7. Dugaiczky, A., H. W. Boyer, and H. M. Goodman. 1975. Ligation of EcoRI endonuclease-generated DNA fragments into linear and circular structures. *J. Mol. Biol.* 96:171-184.
 8. Famulari, N. G., D. L. Buchhagen, H.-D. Klenk, and E. Fleissner. 1976. Presence of murine leukemia virus envelope proteins gp70 and p15(E) in a common polypeptide of infected cells. *J. Virol.* 20:501-508.
 9. Famulari, N. G., and K. J. English. 1981. *env* gene products of AKR dual-tropic viruses: examination of peptide maps and cell surface expression. *J. Virol.* 40:971-976.
 10. Gross, L. 1970. *Oncogenic viruses*, 2nd ed. Pergamon Press, Oxford.
 11. Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe. 1977. A new class of murine leukemia viruses associated with the development of spontaneous lymphomas. *Proc. Natl. Acad. Sci. U.S.A.* 74:789-792.
 12. Herr, W., V. Corbin, and W. Gilbert. 1982. Nucleotide sequence of the 3' half of Akv. *Nucleic Acids Res.* 10:6931-6944.
 13. Holmes, D. S., and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* 114:193-197.
 14. Hoopes, B. C., and W. R. McClure. 1981. Studies on the selectivity of DNA precipitation by spermine. *Nucleic Acids Res.* 9:5493-5504.
 15. Kalyanaraman, V. S., M. G. Sarngadharan, Y. Nakao, Y. Ito, T. Aoki, and R. C. Gallo. 1982. Natural antibodies to the structural core protein (p. 24) of the human T-cell leukemia (lymphoma) retrovirus found in sera of leukemia patients in Japan. *Proc. Natl. Acad. Sci. U.S.A.* 79:1653-1657.
 16. Karshin, W. L., L. J. Arcement, R. C. Naso, and R. B. Arlinghaus. 1977. Common precursor for Rauscher leukemia virus gp69/71, p15(E), and p12(E). *J. Virol.* 23:787-798.
 17. Lee, J. C., and J. N. Ihle. 1981. Chronic immune stimulation is required for Moloney leukemia virus-induced lymphomas. *Nature (London)* 289:407-409.
 18. Lee, J. C., and J. N. Ihle. 1981. Increased responses to lymphokines are correlated with preleukemia in mice inoculated with Moloney leukemia virus. *Proc. Natl. Acad. Sci. U.S.A.* 78:7712-7716.
 19. Lenz, J., R. Crowther, S. Klimenko, and W. Haseltine. 1982. Molecular cloning of a highly leukemogenic, ecotropic retrovirus from an AKR mouse. *J. Virol.* 43:943-951.
 20. Lenz, J., R. Crowther, A. Straceski, and W. Haseltine. 1982. Nucleotide sequence of the Akv *env* gene. *J. Virol.* 42:519-529.
 21. Lowy, D. R., E. Rands, S. K. Chattopadhyay, C. F. Garon, and G. L. Hager. 1980. Molecular cloning of infectious integrated murine leukemia virus DNA from infected mouse cells. *Proc. Natl. Acad. Sci. U.S.A.* 77:614-618.
 22. Lung, M. L., J. W. Hartley, W. P. Rowe, and N. H. Hopkins. 1983. Large RNase T₁-resistant oligonucleotides encoding p15E and the U3 region of the long terminal repeat distinguish two biological classes of mink cell focus-forming type C viruses of inbred mice. *J. Virol.* 45:275-290.
 23. Lung, M. L., C. Hering, J. W. Hartley, W. P. Rowe, and N. Hopkins. 1980. Analysis of the genomes of mink cell focus-inducing murine type C viruses: a progress report. *Cold Spring Harbor Symp. Quant. Biol.* 44:1269-1274.
 24. Naso, R. C., L. J. Arcement, W. L. Karshin, G. A. Janjoom, and R. B. Arlinghaus. 1976. A fucose-deficient glycoprotein precursor to Rauscher leukemia virus gp69/71. *Proc. Natl. Acad. Sci. U.S.A.* 73:2326-2330.
 25. Nowinski, R. C., and E. F. Hays. 1978. Oncogenicity of AKR endogenous leukemia viruses. *J. Virol.* 27:13-18.
 26. O'Donnell, P. C., E. Stockert, Y. Obata, and L. J. Old. 1981. Leukemogenic properties of AKR dualtropic (MCF) viruses: amplification of murine leukemia virus-related antigens on thymocytes and acceleration of leukemia development in AKR mice. *Virology* 112:548-563.
 27. Pedersen, F. S., R. L. Crowther, E. F. Hays, R. C. Nowinski, and W. A. Haseltine. 1982. Structure of retroviral RNAs produced by cell lines derived from spontaneous lymphomas of AKR mice. *J. Virol.* 41:18-29.
 28. Pedersen, F. S., R. L. Crowther, D. Y. Tenney, A. M. Reimhold, and W. A. Haseltine. 1981. Novel leukemogenic retroviruses isolated from cell line derived from spontaneous AKR tumor. *Nature (London)* 292:167-170.
 29. Pedersen, F. S., and W. A. Haseltine. 1980. Analysis of the genome of an endogenous ecotropic retrovirus of the AKR strain of mice: micromethod for detailed characterization of high-molecular-weight RNA. *J. Virol.* 33:349-365.
 30. Pinter, A., and E. Fleissner. 1977. The presence of disulfide-linked gp70-p15(E) complexes in AKR murine leukemia virus. *Virology* 83:417-422.
 31. Poesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo. 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. U.S.A.* 77:7415-7419.
 32. Quint, W., W. Quax, H. van der Putten, and A. Berns. 1981. Characterization of AKR murine leukemia virus sequences in AKR mouse substrains and structure of integrated recombinant genomes in tumor tissues. *J. Virol.* 39:1-10.
 33. Rapp, U. R. 1983. Kinetics of expression of infectious ecotropic, xenotropic, and mink cell focus-forming murine leukemia virus after 5-iododeoxyuridine induction of cells from high and low leukemia mouse strains. *J. Virol.* 45:755-765.
 34. Rapp, U. R., and G. J. Todaro. 1978. Generation of oncogenic type C viruses: rapidly leukemogenic viruses derived from C3H mouse cells *in vivo* and *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 75:2468-2472.
 35. Reitz, M. S., B. J. Poesz, F. W. Ruscetti, and R. C. Gallo. 1981. Characterization and distribution of nucleic acid sequences of a novel type C retrovirus isolated from neoplastic human T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 78:1887-1891.
 36. Robinson, H. L., B. M. Blais, P. N. Tsichlis, and J. M. Coffin. 1982. At least two regions of the viral genome determine the oncogenic potential of avian leukosis viruses. *Proc. Natl. Acad. Sci. U.S.A.* 79:1225-2229.
 37. Rowe, W. P., M. W. Cloyd, and J. W. Hartley. 1980. Status of the association of mink cell focus-forming viruses with leukemogenesis. *Cold Spring Harbor Symp. Quant. Biol.* 44:1265-1268.
 38. Roy-Burman, P., M. Dougherty, B. K. Pal, H. P. Charman, V. Klement, and M. B. Gardner. 1976. Assay for type C virus in mouse sera based on particulate reverse transcriptase activity. *J. Virol.* 19:1107-1110.
 39. Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe. 1981. Nucleotide sequence of Moloney murine leukemia virus. *Nature (London)* 293:543-548.
 40. Temin, H. M. 1981. Structure variation and synthesis of retrovirus long terminal repeat. *Cell* 27:1-3.
 41. Temin, H. M. 1982. Function of the retrovirus long terminal repeat. *Cell* 28:3-5.
 42. Thomas, C. Y., and J. M. Coffin. 1982. Genetic alterations of RNA leukemia viruses associated with the development of spontaneous thymic leukemia in AKR/J mice. *J. Virol.* 43:416-426.
 43. Yoshimura, F. K., and M. Breda. 1981. Lack of AKR ecotropic provirus amplification in AKR leukemic thymuses. *J. Virol.* 39:808-815.