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# Inability of Kaplan Radiation Leukemia Virus To Replicate on Mouse Fibroblasts Is Conferred by Its Long Terminal Repeat

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**The molecularly cloned infectious Kaplan radiation leukemia virus has previously been shown to be unable to replicate on mouse fibroblasts (E. Rassart, M. Shang, Y. Boie, and P. Jolicoeur, J. Virol. 58:96–106, 1986). To map the viral sequences responsible for this, we constructed chimeric viral DNA genomes in vitro with parental cloned infectious viral DNAs from the nonfibrotropic (F$^-$) BL/VL3 V-13 radiation leukemia virus and the fibrotropic (F$^+$) endogenous BALB/c or Moloney murine leukemia viruses (MuLV). Infectious chimeric MuLVs, recovered after transfection of Ti-6 lymphocytes with these recombinant DNAs, were tested for capacity to replicate on mouse fibroblasts in vitro. We found that chimeric MuLVs harboring the long terminal repeat (LTR) of a fibrotropic MuLV replicated well on mouse fibroblasts. Conversely, chimeric MuLVs harboring the LTR of a nonfibrotropic MuLV were restricted on mouse fibroblasts. These results indicate that the LTR of BL/VL3 radiation leukemia virus harbors the primary determinant responsible for its inability to replicate on mouse fibroblasts in vitro. Our results also show that the primary determinant allowing F$^+$ MuLVs (endogenous BALB/c and Moloney MuLVs) to replicate on mouse fibroblasts in vitro resides within the LTR.**

The Kaplan strain of radiation leukemia virus (RadLV) was isolated after successive passages of cell extracts of T-cell lymphoma (thymoma) initially induced by fractionated doses of X rays (19, 20). This virus preparation was shown to have a very high leukemogenic potential and to lose its leukemogenicity when propagated on mouse fibroblasts in vitro (24). It was selectively thymotropic in vitro as well as in vivo (3, 4, 22). A lymphoid cell line (BL/VL3) established from thymomas induced by this highly leukemogenic, thymotropic, passaged RadLV was shown to produce a mixture of retroviruses which have conserved the same high leukemogenic potential, thymotropism, and inability to replicate on fibroblasts as the parental in vivo-passaged RadLV (3, 23). From BL/VL3 cells, we have recently molecularly cloned ecotropic retroviruses responsible for the high leukemogenic potential of BL/VL3 RadLV (31). As the parental BL/VL3 RadLVs, these cloned RadLVs were found to be highly leukemogenic, thymotropic, and unable to replicate on mouse fibroblasts in vitro (31). To map the region of the BL/VL3 viral genome harboring the sequences responsible for this inability to replicate on mouse fibroblasts, we constructed chimeric viral DNA genomes between parental cloned infectious DNAs of nonfibrotropic (F$^-$) BL/VL3 V-13 RadLV and fibrotropic (F$^+$) endogenous BALB/c or Moloney murine leukemia viruses (MuLVs).

## MATERIALS AND METHODS

**Cells and viruses.** The origins of NIH 3T3, BALB/3T3, SC-1, and SIM.R fibroblast cell lines and of the Ti-6 lymphoid cell line have been given elsewhere (16, 30). *Mus dunnii* cells (21) were obtained from Janet Hartley (National Institutes of Health). Fibroblasts were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.). Ti-6 lymphoid cells were maintained in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum (GIBCO). All cells were grown at 37°C in a humidified atmosphere containing 5% $CO_2$. Virus infection of fibroblasts was done in the presence of 8 μg of Polybrene per ml. Ti-6 lymphocytes were treated with 2 μg of Polybrene per ml for 24 h prior to virus infection.

**Construction of DNA recombinant.** The parental BL/VL3 V-13 (31), p75-2 (8), and Moloney (7, 35) infectious DNA molecules have been described. Recombinant p75-2 represents the genome of the endogenous N-tropic BALB/c MuLV except in its *gag* region, which is derived from the endogenous B-tropic BALB/c MuLV. This chimeric MuLV is now B tropic.

For subcloning, 5 to 10 μg of parental viral DNAs was cleaved with restriction endonucleases. The desired fragments were separated on 1 or 1.4% agarose gels and isolated by electroelution (8). These fragments were then ligated with T4 DNA ligase (Pharmacia P-L Biochemicals, Montreal, Quebec, Canada) at 20°C for 16 h in 0.01 ml of ligase buffer (50 mM Tris [pH 7.8], 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP) and used to transform competent *Escherichia coli* 1061. Colonies were screened by the method of Grunstein and Hogness (11), with $^{32}P$-labeled purified homologous viral DNA fragments. Positive clones were isolated, and plasmid DNA was extracted and molecularly analyzed with appropriate restriction endonucleases by agarose gel electrophoresis as described previously (8, 31).

**Preparation of viral DNA and hybridization procedures.** Unintegrated viral DNA was prepared from the Hirt supernatant of newly infected SIM.R cells as described previously (16, 29, 30). Genomic DNA from cells chronically infected with chimeric MuLVs was extracted as already described (16, 30). Restriction enzyme analysis of unintegrated or integrated viral DNA was done under the conditions recommended by the supplier (Boehringer Mannheim Biochemicals, Montreal, Canada; New England BioLabs, Inc., Beverly, Mass; Pharmacia P-L). The viral DNA was analyzed by agarose gel electrophoresis, transferred to nitrocellulose membranes by the method of Southern (36), and detected by

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hybridization with $^{32}$P-labeled DNA probes. The probes used were a gp70 ecotropic MuLV-specific fragment (2), a U3 BL/VL3-specific fragment corresponding to the U3 region of the BL/VL3 RadLV long terminal repeat (LTR) (31), and a U3 Moloney-specific fragment corresponding to the U3 region of the Moloney MuLV LTR. This *Pst*I-*Sac*I LTR Moloney fragment was isolated from subclone MM9R54 (33). The purified fragments were labeled by random oligohexamer primer extension (Pharmacia P-L), as described previously (9).

**Hybridization procedures.** Hybridization was performed as already described (30, 31). Briefly, after hybridization with the $^{32}$P-labeled probes (50% formamide–3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–Denhardt solution at 42°C), the filters were washed sequentially in 2× SSC for 20 min at room temperature, in 0.1× SSC–0.1% sodium dodecyl sulfate for 1 h at 60°C, and in 0.1× SSC for 5 min at room temperature. Membranes were air dried and exposed at −70°C to Kodak RP-Royal X-OMAT film with a Cronex Lighting-Plus intensifying screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

**Electroporation procedure.** The viral inserts from the recombinant DNA plasmids (20 μg) were excised from the vector by restriction endonuclease, phenol and chloroform extracted, ethanol precipitated, and ligated at a concentration of 20 μg/ml with T4 DNA ligase (Boehringer Mannheim, Canada). The ligated chimeric viral recombinant DNAs were phenol extracted, ethanol precipitated, and transfected into Ti-6 lymphoid cells by either electroporation (28, 31) or a DEAE-dextran (26) procedure. The presence of retroviruses in the culture supernatant was detected by the reverse transcriptase assay, as described previously (16, 30, 33).

**DNA sequence analysis.** Sequencing of the LTR of the BL/VL3-V-13 RadLV was performed on the infectious DNA molecule (31). Appropriate restriction fragments were treated with alkaline phosphatase (Boehringer Mannheim, Canada) and labeled at their 5′ end with 500 μCi of [γ-$^{32}$P]ATP (3,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) and 20 U of polynucleotide kinase (GIBCO-Bethesda Research Laboratories, Inc., Canada) as already described (25). The same fragments were also labeled by filling out the 3′ end with 50 μCi of one of the α-$^{32}$P-labeled deoxynucleoside triphosphates (3,000 Ci/mmol; Dupont, NEN Research Products) and the Klenow fragment of DNA polymerase I (Pharmacia P-L). End-labeled fragments were cleaved when necessary with appropriate restriction endonucleases and isolated by polyacrylamide gel electrophoresis, and the sequence was determined by the method of Maxam and Gilbert (25).

## RESULTS

**Characteristics of the parental MuLVs used to construct chimeric viruses.** Chimeric MuLVs were constructed with the viral genomes of BL/VL3 RadLV, BALB/c endogenous recombinant p75-2 MuLV, and Moloney MuLV. BL/VL3 RadLV (clone 13) is a molecularly cloned virus derived from the passaged RadLV (Kaplan strain) (31). It has been shown to be B tropic and highly thymotropic and to replicate poorly, if at all, on several mouse fibroblasts in vitro (nonfibrotropic, F$^-$) (31). The p75-2 recombinant MuLV was constructed from the N- and B-tropic BALB/c endogenous viruses (6, 8). It carries the *gag* region (*Pvu*I-*Hin*dIII) of the B-tropic MuLV, the remaining regions being derived from the N-tropic MuLV. This MuLV replicates efficiently on most mouse fibroblasts in vitro (fibrotropic, F$^+$). It is also B tropic (6), thus allowing the constructed chimeric MuLVs to be tested on *Fv-1*$^{b/b}$ cells, as the BL/VL3 RadLV. Moloney MuLV has been well characterized (10, 18) and is known to be NB tropic and to replicate very efficiently on mouse fibroblasts (fibrotropic, F$^+$). All three parental viruses are known to replicate efficiently on the C57BL/6 lymphoid cell line Ti-6 (31). Therefore, these parental viruses appeared to have sufficiently distinct replication potential on fibroblasts to identify the viral sequences responsible for the nonfibrotropism of BL/VL3 RadLV.

**Construction and characterization of chimeric viral genomes.** The construction of chimeric viral genomes from the parental viral DNAs of BL/VL3 RadLV and p75-2 MuLV was facilitated by the fact that the two genomes share several restriction endonuclease sites. The first recombinant, p75-2/VL3-PK, was constructed by ligating the *Pst*I-*Kpn*I LTR fragment (489 base pairs [bp]) of the BL/VL3 parental DNA with the complementary fragments of p75-2 (Fig. 1). The second recombinant, p75-2/VL3-XK, was constructed by ligating the *Xba*I-*Kpn*I p15E-LTR-containing fragment (1 kilobase pair [kbp]) of the BL/VL3 DNA with the complementary fragments of p75-2 (Fig. 1). The third recombinant, VL3/pBR7, was obtained by ligating the *Pst*I-*Pvu*I LTR-containing fragment (465 bp) of the p75-2 parental DNA with the complementary fragment of BL/VL3 RadLV DNA. The fourth recombinant, VL3/Mo-PK, was produced by ligating the *Pst*I-*Kpn*I LTR fragment (445 bp) of the parental Moloney MuLV DNA with the complementary fragments of BL/VL3 RadLV (Fig. 1). The *Pst*I site within the LTR of Moloney MuLV was constructed by site-specific mutagenesis, and the LTR fragment was isolated from clone MM9R54 (33) (Fig. 1). Finally, recombinant Mo/VL3-P was constructed by ligating the 3′ LTR of BL/VL3 (starting at the *Pst*I site) with the *Cla*I-*Pst*I fragment from clone MM9R54 and with the complementary fragments from a linear Moloney MuLV genome. Restriction enzyme analysis was performed on each recombinant DNA to verify the origin of each fragment. Each chosen recombinant had the expected structure (data not shown).

**Recovery and characterization of infectious chimeric MuLVs.** To recover infectious chimeric viruses, the viral inserts from p75-2/VL3-PK, p75-2/VL3-XK, and VL3/pBR7 DNAs were excised with *Hin*dIII and ligated with T4 DNA ligase to produce nonpermuted DNA molecules. The recombinant VL3/Mo-PK and Mo/VL3-P genomes were constructed as linear proviruses and did not require this process. The recombinant DNA molecules were then electroporated on Ti-6 lymphoid cells, and infectious viruses were recovered from the culture supernatant 15 to 20 days later. Titers of all viruses were determined by endpoint dilution on Ti-6 cells, utilizing the reverse transcriptase assay.

The recovered viruses were further characterized by analysis of either unintegrated viral DNA obtained by Hirt extraction of acutely infected SIM.R cells (recombinants p75-2/VL3-XK, p75-2/VL3-PK, and VL3/pBR7) or cellular DNA of chronically infected SIM.R cells (VL3/Mo-PK and Mo/VL3-P).

For analysis, the Hirt supernatant DNAs from p75/VL3-XK, p75/VL3-PK, and VL3/pBR7 MuLV-infected cells and appropriate control DNAs were cleaved with *Pst*I, *Kpn*I, or *Pst*I-*Kpn*I, since these sites allow us to distinguish the origin of each region of the genome. After *Kpn*I digestion, using a gp70 ecotropic MuLV-specific probe (2, 13), we could detect the presence of a 3.4-kbp fragment (*pol-env*) of RadLV origin (17, 31) in VL3/pBR7 MuLV DNA and a fragment of 3.9 kbp of p75-2 origin in p75-2/VL3-XK and p75-2/VL3 PK MuLV

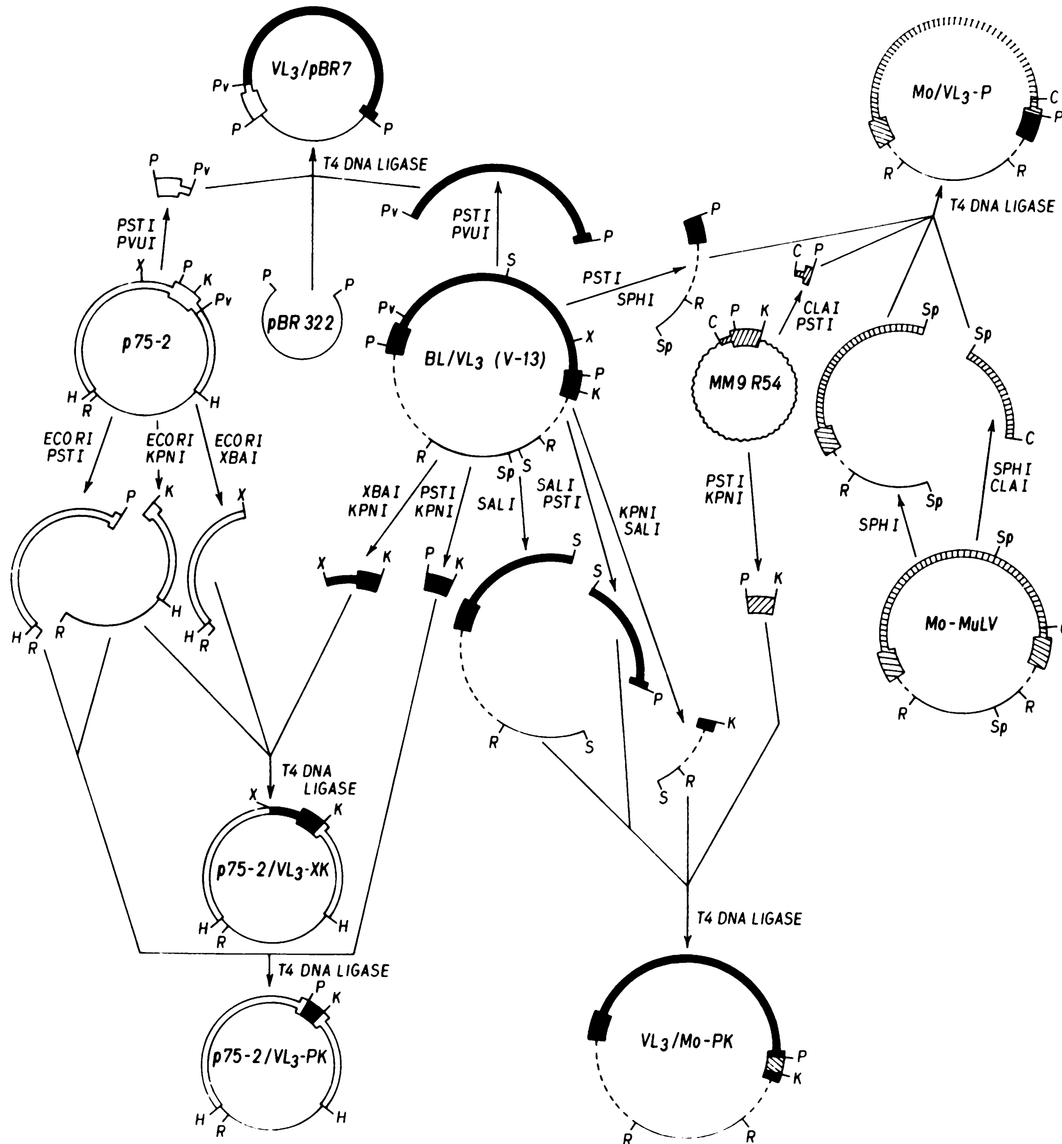


FIG. 1. Construction of the chimeric MuLVs. The 5.2-kbp *Eco*RI-*Pst*I and the 6.8-kbp *Kpn*I-*Eco*RI fragments from p75-2 and the complementary 489-bp *Pst*I-*Kpn*I fragment from BL/VL3 (V-13) were ligated with T4 DNA ligase and cloned in *E. coli* to generate p75-2/VL3-PK. The 4.7-kbp *Eco*RI-*Xba*I and the 6.8-kbp *Kpn*I-*Eco*RI fragments from p75-2 were ligated with the complementary 1-kbp *Xba*I-*Kpn*I fragment from BL/VL3 (V-13) to generate p75-2/VL3-XK. The 7.6-kbp *Pvu*I-*Pst*I fragment from BL/VL3 (V-13) was ligated with the complementary 465-bp *Pst*I-*Pvu*I fragment from p75-2 and cloned in *Pst*I-cleaved pBR322 to generate VL3/pBR7. The 8.8-kbp *Sal*I, the 4.1-kbp *Sal*I-*Pst*I, and the 1.5-kbp *Kpn*I-*Sal*I fragments of BL/VL3 (V-13) were ligated with the 445-bp *Pst*I-*Kpn*I fragment from Moloney MuLV, isolated from the mutagenized clone MM9R54 (33), to form VL3/Mo-PK. The 9.5-kbp *Sph*I, the 2.6-kbp *Sph*I-*Cla*I, and the 177-bp *Cla*I-*Pst*I fragments from Moloney MuLV were ligated with the 2.1-kbp *Pst*I-*Sph*I fragment from BL/VL3 (V-13) to generate Mo/VL3-P. Symbols: ■, viral DNA from BL/VL3 (V-13); □, viral DNA from p75-2; ▨, viral DNA from Moloney MuLV; – – –, cellular sequences flanking the provirus; ——, pBR322; ⌇⌇⌇, M13mp19 vector. Boxes represent LTRs. C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I; R, *Eco*RI; S, *Sal*I; Sp, *Sph*I; X, *Xba*I. Only relevant restriction sites are illustrated.

DNAs, as expected (Fig. 2A). The use of a probe specific for the LTR of BL/VL3 RadLV (31) allowed the detection of the chimeric viral DNAs containing the BL/VL3 RadLV LTR in their structure (p75-2/VL3-XK and p75-2/VL3-PK MuLVs) (Fig. 2B and C). After *Pst* digestion, the same probe confirmed the presence of two LTRs of the same origin on these chimeric viral DNAs. Viral DNA from VL3/pBR7 MuLV was not detected with this probe, as expected. Finally, we used a mixture of the 1.35-kbp (P15E-LTR) *Kpn*I fragments isolated from the two parental genomes as a probe to analyze the *Kpn*I-digested DNA from the three chimeric MuLVs and the two parental MuLVs. Since the U3 LTR of the parental BL/VL3 RadLV is larger than that of the parental p75-2 MuLV, we could document that chimeric p75-2/VL3-PK and p75-2/VL3-XK MuLV DNAs carry the U3 LTR of the parental BL/VL3 RadLV and that the U3 LTR of the VL3/pBR7 MuLV genome was derived from the p75-2 parental MuLV (Fig. 2D).

For analysis of VL3/Mo-PK and Mo/VL3-P chimeric MuLV genomes, genomic DNAs of chronically infected

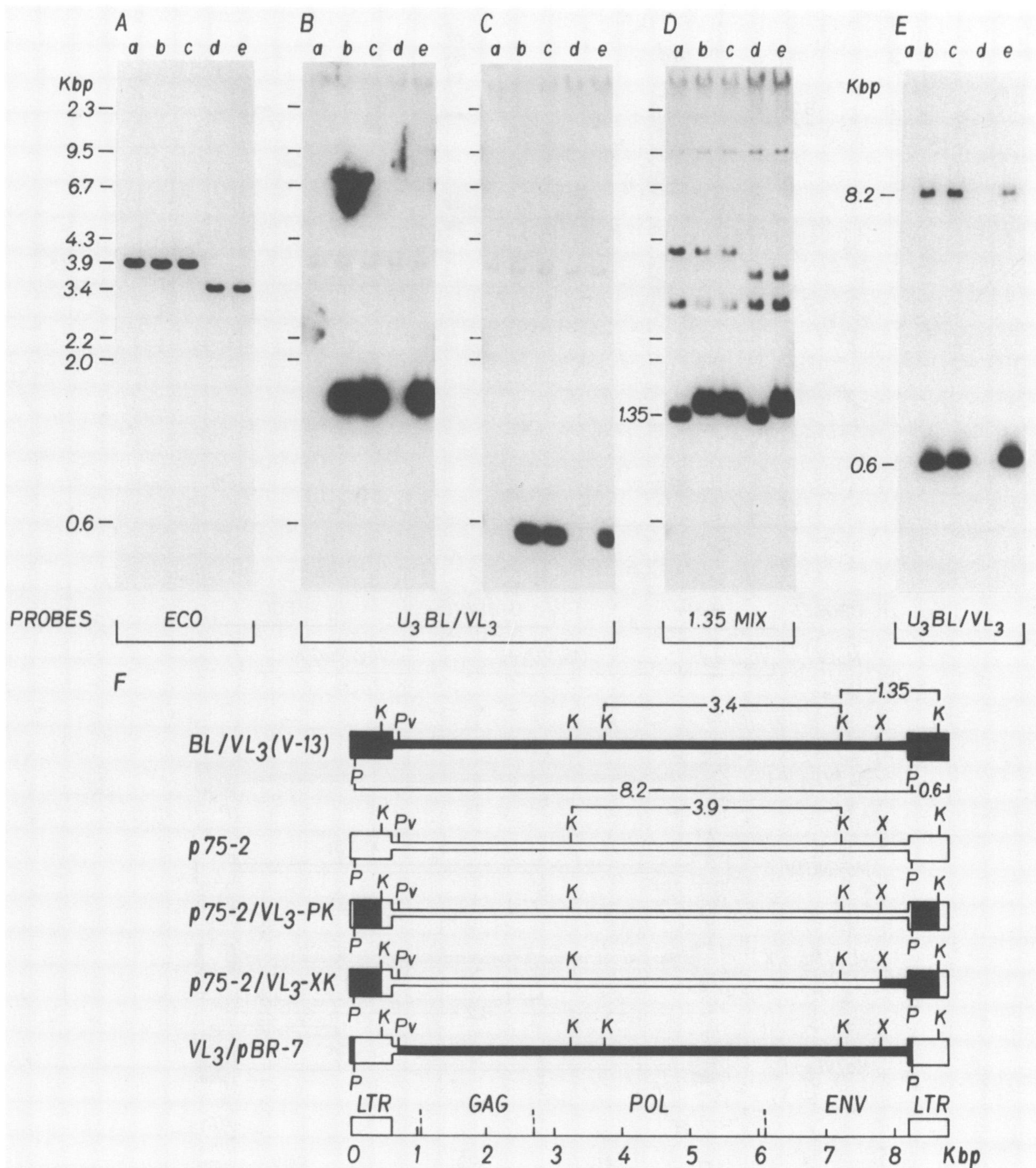


FIG. 2. Hybridization analysis of chimeric and control parental MuLV genomes. Infectious viruses recovered from transfected Ti-6 lymphoid cells were used to infect SIM.R cells at a multiplicity of infection of 1 in the presence of 8 μg of Polybrene per ml. After 48 h of incubation, Hirt supernatant DNA was extracted and digested with restriction endonucleases. The DNA fragments were separated by 1% agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with $^{32}$P-labeled DNA probes. Virus-specific DNA was detected by autoradiography. Hirt supernatant DNA from cells infected with p75-2 (lane a), p75-2/VL3-PK (lane b), p75-2/VL3-XK (lane c), VL3/pBR7 (lane d), and BL/VL3 (V-13) (lane e) MuLVs were digested with *Kpn*I (panels A, B, and D), *Pst*I (panel E), or *Pst*I-*Kpn*I (panel C) and hybridized with ecotropic MuLV-specific probe (panel A), U3 LTR BL/VL3 probe (panels B, C, and E), or a mixture of the 1.35-kbp *Kpn*I fragments isolated from the two parental MuLV DNAs (panel D), as described in Materials and Methods. (F) Partial restriction map of parental and chimeric MuLV genomes showing the sizes of the expected fragments. Open boxes indicate LTRs. Restriction sites: K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I; X, *Xba*I. Numbers are lengths in kilobase pairs. Only relevant restriction sites are illustrated.

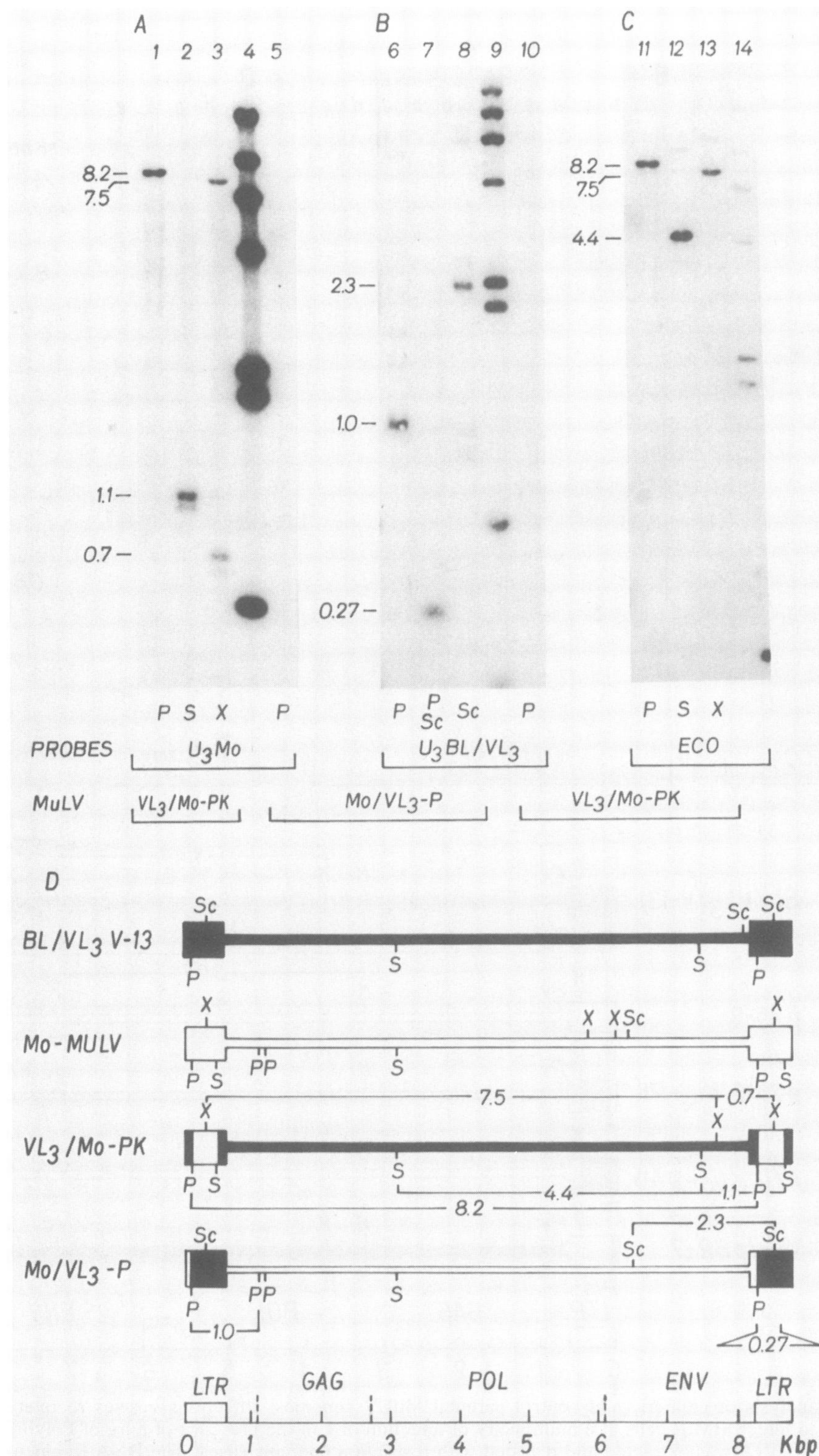


FIG. 3. Hybridization analysis of cellular DNA extracted from SIM.R cells chronically infected with VL3/Mo-PK or Mo/VL3-P chimeric MuLVs. The DNA from the cells infected with VL3/Mo-PK (lanes 1 to 3 and 10 to 13) was digested with *Pst*I (lanes 1, 10, and 11), *Sac*I (lanes 2 and 12), or *Xba*I (lanes 3 and 13). The DNA from cells infected with Mo/VL3-P (lanes 5 to 8) was digested with *Pst*I (lanes 5 and 6), *Sca*I (lane 8), or *Pst*I-*Sac*I (lane 7). The fragments were separated by electrophoresis in 1% agarose gels, transferred on nitrocellulose filters, and hybridized with the U3 LTR Moloney-specific (U3 Mo) (A) or BL/VL3-specific (U3 BL/VL3) (B) probe or with the gp70 ecotropic MuLV-specific probe (ECO) (C). The molecular weight markers are the $^{32}$P-labeled *Hin*dIII fragments of λ DNA (lanes 4, 9, and 14). Panels A (lanes 1 to 4) and C (lanes 11 to 14) come from the same filter, which was washed and rehybridized. (D) Partial restriction map of parental and chimeric MuLV genomes showing the sizes of the expected fragments. Open boxes indicate LTRs. Restriction sites: P, *Pst*I; S, *Sac*I; Sc, *Sca*I; X, *Xba*I. Numbers are lengths in kilobase pairs.

TABLE 1. Replication of chimeric and parental MuLVs on fibroblasts

| Virus | Reverse transcriptase activity ($10^3$ cpm) in the given cell line[a] | | | | | |
|---|---|---|---|---|---|---|
| | BALB/3T3 | SIM.R | *M. dunnii* | Mink | SC-1 | Ti-6 |
| p75-2 | 148 | 370 | 460 | 3 | 350 | 848 |
| BL/VL3(V-13) | 4 | 2 | 3 | 4 | 40 | 1,070 |
| Moloney | 800 | 500 | ND[b] | ND | 1,200 | 930 |
| VL3/pBR7 | 700 | 165 | 290 | 5 | 700 | 990 |
| p75-2/VL3-XK | 3 | 4 | 4 | 3 | 29 | 210 |
| p75/VL3-XK | 3 | 5 | 4 | 6 | 21 | 200 |
| VL3/Mo-PK[c] | 390 | 150 | ND | ND | ND | 1,500 |
| Mo/VL3-P[c] | 45 | 1.2 | ND | ND | ND | 780 |

[a] The reverse transcriptase assay was performed 20 days after infection on a fraction (1/10) of a suspended virus pellet from 8 ml of supernatant, as described before (31).
[b] ND, Not done.
[c] Assay done 10 days after infection.

SIM.R cells were used (Fig. 3). The analysis was carried out with restriction endonucleases which cleave within the provirus to facilitate interpretation. The viral DNA from VL3/Mo-PK MuLV-infected cells, cut with *Pst*I, *Sac*I, or *Xba*I, hybridized well with the U3 LTR Moloney-specific probe (Fig. 3, lanes 1 to 3) but did not hybridize with the U3 LTR BL/VL3-specific probe (Fig. 3, lane 10), indicating that the LTRs of the recombinant MuLV were derived from Moloney MuLV, as expected. The lengths of the internal fragments observed with this probe or with the gp70 ecotropic MuLV-specific probe were of a size expected for the chimeric molecule. An 8.2-kbp *Pst*I fragment was detected with both probes, as expected for a virus with the BL/VL3 coding region (Fig. 3, lanes 1 and 11). With the U3-specific Moloney probe, a 1.1-kbp *Sac*I fragment corresponding to a fragment from the 3′ end of *env* was detected (Fig. 3, lane 2). A 4.4-kbp *Sac*I and a 7.5-kbp *Xba*I fragment, corresponding respectively to the internal fragment of BL/VL3 genome and a novel chimeric fragment, was detected with the ecotropic MuLV-specific probe (Fig. 3, lanes 12 and 13). The viral DNA from Mo/VL3-P MuLV-infected cells hybridized with the BL/VL3-specific U3 LTR probe (Fig. 3, lanes 6 to 8) but not with the Moloney-specific U3 LTR probe (Fig. 3, lane 5), indicating that its LTR was derived from BL/VL3 RadLV. The length of the internal fragments observed with the BL/VL3-specific U3 probe confirmed that the genome of Mo/VL3-P had the expected structure: a 1.0-kbp *Pst*I fragment, corresponding to the 5′ LTR up to the *Pst*I site in the *gag* region of Moloney MuLV, was detected with this probe (Fig. 3, lane 6). After *Sca*I digestion, a fragment of 2.3 kbp could be detected (Fig. 3, lane 8), corresponding to a novel chimeric fragment from the *Sca*I site in Moloney *env* to the *Sca*I site in the BL/VL3 U3 LTR. These results indicated that the recombinant MuLV genomes had the expected chimeric structures and did not appear to have rearranged during their replication on the Ti-6 cell line in vitro.

**Fibrotropism of chimeric MuLVs.** To identify the viral sequences responsible for the restricted replication of BL/VL3 RadLV on mouse fibroblasts, each chimeric MuLV was tested for its ability to replicate on mouse fibroblasts. Control parental and chimeric MuLVs were assayed on $Fv\text{-}1^{b/b}$ (BALB/3T3 and SIM.R) or $Fv\text{-}1^-$ (Sc-1) fibroblasts, on *M. dunnii* and mink (CCL-64) fibroblasts, and on permissive lymphoid cell line Ti-6. Virus replication was monitored by the reverse transcriptase assay at different times after infection. The parental BL/VL3 RadLV replicated very efficiently on Ti-6 cells and after a longer delay on SC-1 cells (Table 1), but was unable to replicate on four types of fibroblasts. The other parental p75-2 and Moloney MuLVs replicated efficiently on all mouse fibroblasts tested, but were restricted on mink fibroblasts, as expected for ecotropic MuLVs. In contrast, the chimeric p75-2/VL3-PK and p75-2/VL3-XK MuLVs were unable to replicate on BALB/3T3, SIM.R, and *M. dunnii* fibroblasts, although they replicated very efficiently on Ti-6 lymphoid cells and, after a longer delay, on SC-1 cells. The chimeric VL3/pBR7 MuLV replicated efficiently on all tested mouse fibroblasts. These results indicated that the LTR of MuLV, and not the p15E region, determined ability to replicate on mouse fibroblasts in vitro.

The VL3/Mo-PK recombinant MuLV had the same specificity as the parental Moloney MuLV toward replication on mouse fibroblasts, indicating that the addition of the Moloney LTR to the BL/VL3 RadLV was sufficient to make it fibrotropic. However, chimeric Mo/VL3-P MuLV did not show the same replication pattern as p75-2/VL3 PK or BL/VL3 RadLV; similarly to these two viruses, it could not replicate on SIM.R fibroblasts. However, in contrast to p75-2/VL3-PK or BL/VL3 RadLV, it was not restricted on BALB/3T3 cells and could replicate on these cells, although at a slower rate. Taken together, these results indicate that the primary determinant restricting the ability of BL/VL3 RadLV to replicate on mouse fibroblasts lies within its U3 LTR region. A given chimeric MuLV will replicate on permissive fibroblasts in vitro only if its genome harbors the U3 LTR region of a fibrotropic MuLV.

**Comparison of nucleotide sequences of the LTR from $F^-$ BL/VL3 (clone 13) RadLV and $F^+$ p75-2 MuLV.** Since the LTR region of BL/VL3 RadLV was found to be so critical in restricting its replication on mouse fibroblasts, it was of interest to compare its sequence with other sequences from $F^+$ MuLVs. The sequence of the BL/VL3 RadLV clone V-13 LTR was determined by the Maxam and Gilbert technique (25). The sequence of the p75-2 LTR has already been published (8) and is presented for comparison (Fig. 4). Sequences of both genomes are identical in U5 and almost identical in the R region. However, major differences (point mutations and base-pair deletions and insertions) are found in the U3 region, more specifically, in the enhancer region. The BL/VL3 V-13 enhancer contains sequences identical to those of the 99-bp repeat of p75-2 MuLV sequences frequently duplicated in tandem in several MuLVs (8, 12, 31, 34) and a large insertion. This enhancer is made of three perfect 43-bp repeats separated by 11 bp (Fig. 4B).

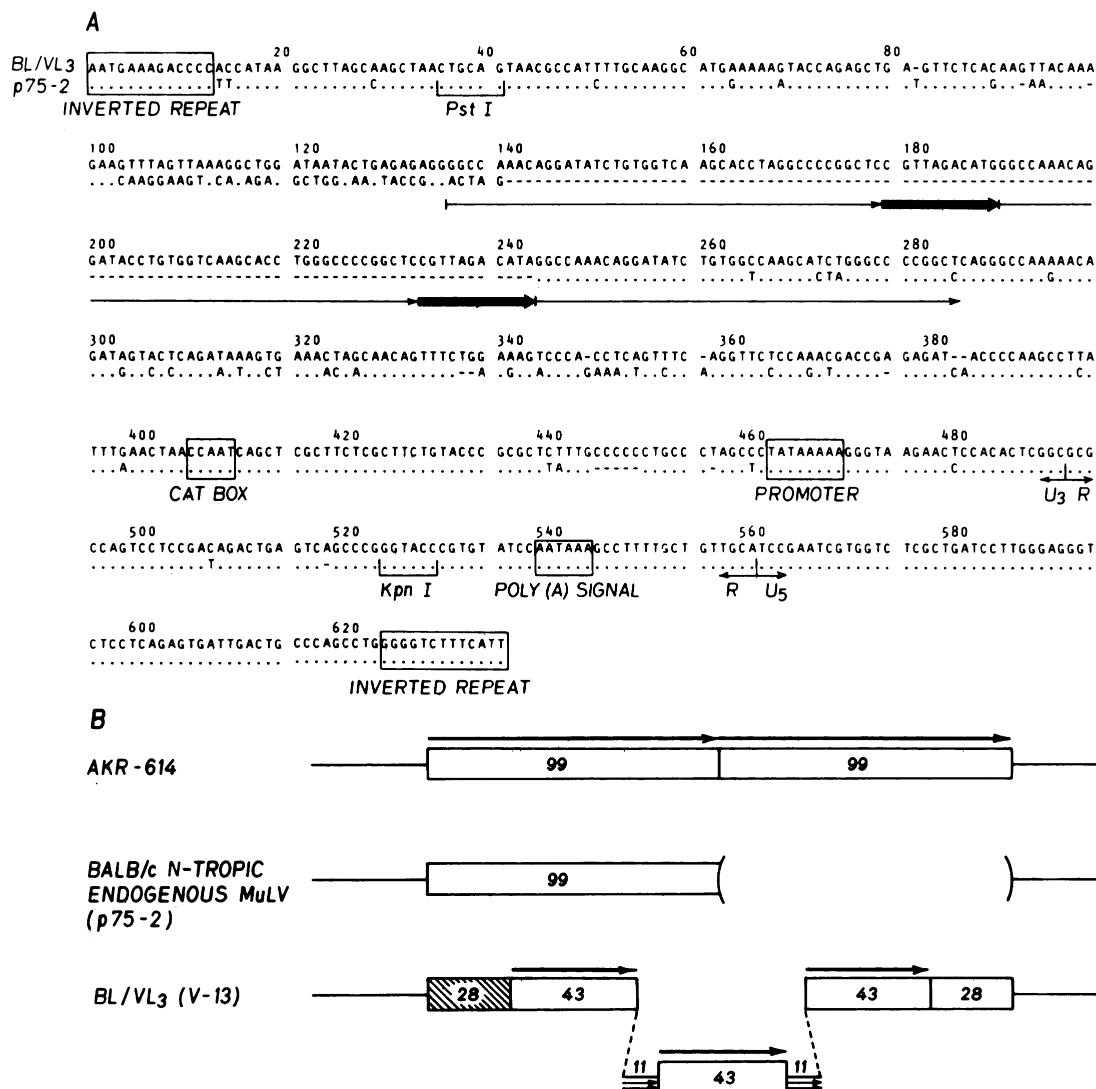

FIG. 4. (A) Nucleotide sequence of the LTR of the BL/VL3 (clone V-13) RadLV. For comparison, the same region of the genome of BALB/c N-tropic endogenous MuLV (8) is shown. The inverted repeats, CAT box, promoter, and poly(A) signal are boxed. A nucleotide identical to the one in BALB/c endogenous MuLV is indicated by a dot, and space left for an insertion in the corresponding region is indicated by a dash. The 43-bp (thin line) and 11-bp (thick line) direct repeats of BL/VL3 clone V-13 are indicated under the sequence by horizontal arrows. (B) Schematic representation of the U3 LTR tandem direct repeat structure of AKR-614 (12), N-tropic endogenous BALB/c (8) MuLVs, and BL/VL3 RadLV viral DNAs.

## DISCUSSION

Using chimeric MuLVs constructed in vitro from parental genomes of nonfibrotropic and fibrotropic MuLVs, we mapped the viral determinant involved in the inability of BL/VL3 Kaplan RadLV to replicate in mouse fibroblasts within its U3 LTR. This ecotropic passaged BL/VL3 RadLV is unique among retroviruses in its inability to replicate in mouse fibroblasts in vitro. To the best of our knowledge, it is the only representive member of its class among the ecotropic MuLV family of retroviruses. Among other mouse retroviruses, the wild-type mouse mammary tumor virus, which induces mammary carcinoma (27), and one of its variants, which induces T-cell leukemia (5), have also been found to be unable to replicate in vitro on mouse fibroblasts. The data presented here clearly indicate that the determinant of the inability of BL/VL3 RadLV to replicate on mouse fibroblasts resides within its LTR, most likely within its U3 region, which is remarkably distinct from that of other murine retroviruses.

Comparison of the LTR sequence of BL/VL3 V-13 RadLV with that of p75-2 BALB/c N-tropic MuLV revealed differences scattered throughout the U3 region. In addition, the BL/VL3 V-13 sequence has an insertion of 5C immediately upstream of the promotor. This insertion is unlikely to be involved in the inability of this virus to replicate in mouse fibroblasts since other fibrotropic primary RadLVs (31) also present this insertion. The most striking change within the U3 LTR of BL/VL3 RadLV is the presence of a large insertion in its enhancer. This structure has a direct repeat of 43 bp repeated three times and spaced by a stretch of 11 bp also repeated (Fig. 4B). This 43-bp repeat is unique to the passaged BL/VL3 RadLV, although some of the isolates only contain two 43-bp repeats instead of three (15, 31). Nevertheless, all isolates that harbored two or three 43-bp

repeats in the U3 LTR were unable to replicate in mouse fibroblasts (31). This specific enhancer is likely to contribute to the inability of BL/VL3 RadLVs to replicate in mouse fibroblasts. However, the construction of finer chimeric molecules will be needed to determine the exact region of the LTR involved in this restriction.

Since most MuLVs replicate efficiently on fibroblasts, our data also indicate that this fibrotropism phenotype is conferred by the LTR. Studies on the variant BL/VL3 RadLV were instrumental in uncovering this specific determinant. Indeed, the addition of LTR sequences from a fibrotropic MuLV was sufficient to give to BL/VL3 RadLV the ability to replicate on mouse fibroblasts. Considering that the determinants of thymotropism (8, 32), preferential replication of oncogenic mink cell focus-forming MuLVs on SC-1 cells over NFS mouse embryo cells (14), and disease specificity (1, 7, 37, 38) have previously been mapped within the same region of the genome (LTR), our finding that the same region governs the ability of MuLV to replicate on fibroblasts was not unexpected. It extends the function of this structure and emphasizes its role in virus replication in different cell lines.

We used two distinct parental sequences to construct chimeric MuLVs with BL/VL3 RadLV, namely, a derivative of the endogenous BALB/c N-tropic MuLV and the Moloney MuLV. The BL/VL3 genome under the control of endogenous BALB/c MuLV LTR (VL3/pBR7) or Moloney MuLV LTR (VL3/Mo-PK) was equally active in replicating in mouse fibroblasts, indicating that these LTRs were functionally identical for this phenotype. However, the endogenous BALB/c MuLV genome (p75-2/VL3-PK) and the Moloney MuLV genome (Mo/VL3-P) under the control of the BL/VL3 LTR behaved somewhat differently. The p75-2/VL3-PK MuLV was as restricted as the parental BL/VL3 RadLV, while the Mo/VL3-P chimeric MuLV was not restricted on all fibroblasts; in fact, it replicated relatively well, but after a longer delay, on BALB/3T3 cells (Table 1). This result indicates that the *gag-pol-env* sequences from endogenous BALB/c and Moloney MuLVs are distinct and influence the primary determinant of nonfibrotropism within the BL/VL3 LTR. This influence could be exerted in *trans* through one of the proteins encoded by these genes. Proteins of endogenous and Moloney MuLVs are known to have several amino acid differences (12, 34). Alternatively, this effect could be caused by *cis*-acting elements. To our knowledge, this is the first time that other MuLV sequences have been shown to modify the effect of an LTR determinant in vitro.

We previously reported that the restriction of RadLV on mouse fibroblasts was not at the membrane level, but rather occurred after the synthesis of unintegrated linear or supercoiled viral DNA (31). Our present data confirm that this restriction is indeed intracellular. It could be operating at the integration level itself, where the LTR is the critical viral structure used for the colinear integration of the provirus. Alternatively, the restriction could be at the transcription level, the BL/VL3 promotor/enhancer region within the LTR being inactive in mouse fibroblasts. This last model is certainly compatible with the known role of LTR in transcription of retroviruses. A block later during the virus cycle, at the translation or assembly level, cannot be ruled out at this moment, but this appears less likely considering that MuLV LTR has never been shown to be involved in these steps.

The mechanisms by which LTR blocks replication of BL/VL3 RadLV has not been uncovered by our present work. But it clearly appears that the interaction of this BL/VL3 LTR with cell factors is distinct from that of other fibrotropic MuLVs. Thus, the cellular factors which usually interact with fibrotropic MuLVs do not seem to interact with the BL/VL3 LTR. The specific structure of BL/VL3 LTR appears to require an interaction with distinct and possibly specific factors. These putative factors could be activators and would then be present in permissive Ti-6 cells and absent in most mouse fibroblasts. Alternatively, these factors could be repressors, being present in fibroblasts and absent in Ti-6 lymphoid cells. Further work will be needed to characterize these putative cellular factors involved in the LTR-mediated restriction of BL/VL3 RadLV.

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## LITERATURE CITED

1. **Chatis, P. A., C. A. Holland, J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1983. Role of the 3' end of the genome in determining disease specificity of Friend and Moloney murine leukemia viruses. Proc. Natl. Acad. Sci. USA **80**:4408–4411.
2. **Chattopadhyay, S. K., M. R. Lander, E. Rands, and D. R. Lowy.** 1980. Structure of endogenous murine leukemia virus DNA in mouse genomes. Proc. Natl. Acad. Sci. USA **77**:5774–5778.
3. **Declève, A., M. Lieberman, J. N. Ihle, P. N. Rosenthal, M. L. Lung, and H. S. Kaplan.** 1978. Physico-chemical, biological and serological properties of a leukemogenic virus isolated from cultured RadLV-induced lymphomas of C57BL/Ka mice. Virology **90**:23–35.
4. **Declève, A., C. Sato, M. Lieberman, and H. S. Kaplan.** 1974. Selective thymic localization of murine leukemia virus-related antigens in C57BL/Ka mice after inoculation with radiation leukemia virus. Proc. Natl. Acad. Sci. USA **71**:3124–3128.
5. **Dekaban, G. A., and J. K. Ball.** 1984. Integration of type B retroviral DNA in virus-induced primary murine thymic lymphomas. J. Virol. **52**:784–792.
6. **DesGroseillers, L., and P. Jolicoeur.** 1983. Physical mapping of the *Fv-1* tropism host range determinant of BALB/c murine leukemia viruses. J. Virol. **48**:685–696.
7. **DesGroseillers, L., and P. Jolicoeur.** 1984. Mapping the viral sequences conferring leukemogenicity and disease specificity in Moloney and amphotropic murine leukemia viruses. J. Virol. **52**:448–456.
8. **DesGroseillers, L., E. Rassart, and P. Jolicoeur.** 1983. Thymotropism of murine leukemia virus is conferred by its long terminal repeat. Proc. Natl. Acad. Sci. USA **80**:4203–4207.
9. **Feinberg, A. P., and B. Volgelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
10. **Gross, L.** 1970. Oncogenic viruses. Pergamon Press, Inc., Elmsford, N.Y.
11. **Grunstein, M., and D. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72**:3961–3965.
12. **Herr, W.** 1984. Nucleotide sequence of AKV murine leukemia virus. J. Virol. **49**:471–478.
13. **Herr, W., and W. Gilbert.** 1983. Somatically acquired recombinant murine leukemia proviruses in thymic leukemias of AKR/J mice. J. Virol. **46**:70–82.
14. **Holland, C. A., J. Wozney, P. A. Chatis, N. Hopkins, and J. W. Hartley.** 1985. Construction of recombinants between molecular clones of murine retrovirus MCF 247 and Akv: determinants of an in vitro host range property that maps in the long terminal repeat. J. Virol. **53**:152–157.
15. **Janowski, M., J. Merregaert, J. Boniver, and J. R. Maisin.** 1985. Proviral genome of radiation leukemia virus: molecular cloning of biologically active proviral DNA and nucleotide sequence of

its long terminal repeat. J. Virol. **55**:251–255.

16. **Jolicoeur, P., and E. Rassart.** 1980. Effect of *Fv-1* gene product on synthesis of linear and supercoiled viral DNA in cells infected with murine leukemia virus. J. Virol. **33**:183–195.
17. **Jolicoeur, P., E. Rassart, and P. Sankar-Mistry.** 1983. Strong selection for cells containing new ecotropic recombinant murine leukemia virus provirus after propagation of C57BL/6 radiation-induced thymoma cells in vitro or in vivo. Mol. Cell. Biol. **3**: 1675–1679.
18. **Jolicoeur, P., N. Rosenberg, A. Cotellessa, and D. Baltimore.** 1978. Leukemogenicity of clonal isolates of murine leukemia viruses. J. Natl. Cancer Inst. **60**:1473–1476.
19. **Kaplan, H. S.** 1964. The role of radiation in experimental leukemogenesis. Natl. Cancer Inst. Monogr. **14**:207–220.
20. **Kaplan, H. S.** 1967. On the natural history of the murine leukemias: presidential address. Cancer Res. **27**:1325–1340.
21. **Lander, M. R., and S. K. Chattopadhyay.** 1984. A *Mus dunni* cell line that lacks sequences closely related to endogenous murine leukemia viruses and can be infected by ecotropic, amphotropic, xenotropic, and mink cell focus-forming viruses. J. Virol. **52**:695–698.
22. **Lieberman, M., A. Declève, and H. S. Kaplan.** 1978. Rapid in vitro assay for thymotropic, leukemogenic murine C-type RNA viruses. Virology **90**:274–278.
23. **Lieberman, M., A. Declève, P. Ricciardi-Castagnoli, J. Boniver, O. J. Finn, and H. S. Kaplan.** 1979. Establishment, characterization and virus expression of cell lines derived from radiation- and virus-induced lymphomas of C57BL/Ka mice. Int. J. Cancer **24**:168–177.
24. **Lieberman, M., O. Niwa, A. Declève, and H. S. Kaplan.** 1973. Continuous propagation of radiation leukemia virus on a C57BL mouse embryo fibroblast line with attenuation of leukemogenic activity. Proc. Natl. Acad. Sci. USA **70**:1250–1253.
25. **Maxam, A., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**: 499–560.
26. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethyl amino ethyl dextran. J. Natl. Cancer Inst. **41**:351–357.
27. **Moore, D. H., C. A. Long, and A. B. Vaidya.** 1979. Mammary tumor viruses. Adv. Cancer Res. **29**:347–417.
28. **Potter, H., L. Weir, and P. Leder.** 1984. Enhancer-dependent expression of human K immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc. Natl. Acad. Sci. USA **81**:7161–7165.
29. **Rassart, E., L. DesGroseillers, and P. Jolicoeur.** 1981. Molecular cloning of B- and N-tropic endogenous BALB/c murine leukemia virus circular DNA intermediates: isolation and characterization of infectious recombinant clones. J. Virol. **39**:162–171.
30. **Rassart, E., P. Sankar-Mistry, G. Lemay, L. DesGroseillers, and P. Jolicoeur.** 1983. New class of leukemogenic ecotropic recombinant murine leukemia virus isolated from radiation-induced thymomas of C57BL/6 mice. J. Virol. **45**:565–575.
31. **Rassart, E., M. Shang, Y. Boie, and P. Jolicoeur.** 1986. Studies on emerging radiation leukemia virus variants in C57BL/Ka mice. J. Virol. **58**:96–106.
32. **Rosen, C. A., W. A. Haseltine, J. Lenz, R. Ruprecht, and M. W. Cloyd.** 1985. Tissue selectivity of murine leukemia virus infection is determined by long terminal repeat sequences. J. Virol. **55**:862–866.
33. **Savard, P., L. DesGroseillers, E. Rassart, Y. Poirier, and P. Jolicoeur.** 1987. Important role of the long terminal repeat of the helper Moloney murine leukemia virus in Abelson virus-induced lymphoma. J. Virol. **61**:3266–3275.
34. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukemia virus. Nature (London) **293**:543–548.
35. **Shoemaker, C., S. Goff, E. Gilboa, M. Paskind, S. W. Mitra, and D. Baltimore.** 1980. Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc. Natl. Acad. Sci. USA **77**:3932–3936.
36. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
37. **Stocking, C., R. Kollek, U. Bergholz, and W. Ostertag.** 1986. Point mutations in the U3 region of the long terminal repeat of Moloney murine leukemia virus determine disease specificity of the myeloproliferative sarcoma virus. Virology **143**:145–149.
38. **Vogt, M., C. Haggblom, S. Swift, and M. Haas.** 1985. Envelope gene and long terminal repeat determine the different biological properties of Rauscher, Friend, and Moloney mink cell focus-inducing viruses. J. Virol. **55**:184–192.