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Cloning of Endogenous Murine Leukemia Virus-Related Sequences from Chromosomal DNA of BALB/c and AKR/J Mice: Identification of an *env* Progenitor of AKR-247 Mink Cell Focus-Forming Proviral DNA

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Recombinant phages containing murine leukemia virus (MuLV)-reactive DNA sequences were isolated after screening of a BALB/c mouse embryo DNA library and from shotgun cloning of *Eco*RI-restricted AKR/J mouse liver DNA. Twelve different clones were isolated which contained incomplete MuLV proviral DNA sequences extending various distances from either the 5′ or 3′ long terminal repeat (LTR) into the viral genome. Restriction maps indicated that the endogenous MuLV DNAs were related to xenotropic MuLVs, but they shared several unique restriction sites among themselves which were not present in known MuLV proviral DNAs. Analyses of internal restriction fragments of the endogenous LTRs suggested the existence of at least two size classes, both of which were larger than the LTRs of known ecotropic, xenotropic, or mink cell focus-forming (MCF) MuLV proviruses. Five of the six cloned endogenous MuLV proviral DNAs which contained envelope (*env*) DNA sequences annealed to a xenotropic MuLV *env*-specific DNA probe; in addition, four of these five also hybridized to an ecotropic MuLV-specific *env* DNA probe. Cloned MCF 247 proviral DNA also contained such dual-reactive *env* sequences. One of the dual-reactive cloned endogenous MuLV DNAs contained an *env* region that was indistinguishable by *Alu*I and *Hpa*II digestion from the analogous segment in MCF 247 proviral DNA and may therefore represent a progenitor for the *env* gene of this recombinant MuLV. In addition, the endogenous MuLV DNAs were highly related by *Alu*I cleavage to the Moloney MuLV provirus in the *gag* and *pol* regions.

Based upon their host range, murine leukemia viruses (MuLVs) isolated from inbred mice have been divided into three classes: ecotropic, which infect only mouse cells; xenotropic, which grow mostly in heterologous cells; and mink cell focus-forming (MCF) viruses (19, 23), which are dual-tropic and replicate both in murine and nonmurine cells. Tryptic peptide (16–18), RNase T_1 oligonucleotide fingerprinting (41, 43), as well as heteroduplex mapping analyses (14, 15) have demonstrated that MCF MuLVs are recombinants which contain both ecotropic and xenotropic determinants in their envelope (*env*) region. The MCF MuLVs have been associated with the development of AKR thymic lymphomas based upon their appearance in late preleukemic thymuses and tumor tissues (23, 27) and their ability to accelerate the onset of lymphomas in AKR mice (16, 45).

Biological and biochemical studies have shown that MuLV-related sequences are present in mouse chromosomal DNA. Hybridization analyses indicate that there are at least 20 to 50 copies of endogenous MuLV-related sequences per haploid genome (1, 2, 7, 13, 20, 26, 46, 48). In general, inbred mouse strains carry no more than two or three loci for inducible ecotropic MuLVs (42) and no more than one or two for inducible xenotropic MuLVs (29). To study the relationship between the endogenous MuLV DNA sequences and the proviruses of infectious MuLVs, we have analyzed MuLV-reactive recombinant phages isolated from a BALB/c mouse embryo DNA library and from the shotgun cloning of *Eco*RI-restricted adult AKR/J mouse liver DNA. In this paper we report the characterization of 12 different long terminal repeat (LTR)-containing endogenous MuLV DNA clones with respect to their restriction maps, LTR structures, and *env* specificity.

MATERIALS AND METHODS

Cells, viruses, and DNA. MCF viruses were kindly provided by Janet W. Hartley (National Institutes of

Health [NIH], Bethesda, Md.) and included AKR-247, AKR-13, AKR L5, *Akv*-1-C36, AKR L3, *Akv*-1-C44-2, and *Akv*-1-C311. These viruses had been biologically cloned by limiting dilution. Single-passaged 5-iododeoxyuridine-induced BALB/c ecotropic and xenotropic MuLVs were generously provided by Christine Kozak (NIH). Unintegrated viral DNA was isolated by the Hirt procedure (24) 48 h after infection from mink lung cells (in the case of MCF and BALB/c xenotropic MuLVs) and SC-1 cells (in the case of BALB/c ecotropic MuLV) cocultivated with infected cells. Cells were grown in a Dulbecco-Vogt modification of Eagle minimal essential medium containing 10% heated calf serum.

Cloned proviral DNAs used in these studies included infectious AKR ecotropic 623 MuLV (30) and Moloney MuLV (MoMuLV) (kindly provided by Cha Mer Wei, Frederick Cancer Research Facility, Frederick, Md.). The latter clone consisted of circularly permuted unintegrated viral DNA cloned into the *Sal*I site of modified λgtWES.λB DNA vector arms. In addition, we used incomplete clones of NFS xenotropic MuLV proviral DNA containing 5′ flanking cellular DNA and viral DNA sequences extending to the *Eco*RI site at 6.7 kilobases (kb) in the *env* region (6) and a cloned 6.8-kb segment of MCF 247 proviral DNA lying between the *Eco*RI sites at map positions 0.1 kb (in the 5′ LTR) and 6.9 kb (in *env*) (28).

Screening of recombinant phages. The endogenous MuLV DNA clones were isolated from two sources. A BALB/c mouse embryo DNA library, kindly provided by Jonathan Seidman (NIH), was prepared by cloning fragments averaging 15 to 16 kb in size, selected from a partial *Hae*III digest of DNA from 14-day-old embryos according to the method of Maniatis et al. (31), into Charon 4A lambda arms (53) using *Eco*RI linkers. Of 150,000 phage plaques grown on *Escherichia coli* DP50 SupF, 95 hybridized to a generalized MuLV DNA probe ($MuLV^{gen}$) (described below), using the procedure described by Benton and Davis (3). Of the 95 reactive primary plaques, 40 were then subcloned.

The second source was clones containing segments of endogenous proviral DNA present in AKR/J mice (generously provided by Stephen P. Staal, The Johns Hopkins University, Baltimore, Md.). These were obtained by ligating 10- to 20-kb fragments from *Eco*RI-restricted adult liver DNA into λ Charon 4A DNA vector arms according to the method of Maniatis et al. (31). The recombinant DNA was packaged in vitro into infectious lambda phage particles as previously described (5). A total of 200,000 phage plaques were screened by in situ hybridization (3) using labeled AKR ecotropic MuLV cDNA. All 136 reactive primary plaques were subcloned. Subcloned phage particles were propagated, and DNA was isolated as previously described (22).

Recombinant phages containing MuLV-related sequences were screened further by in situ hybridization (3), using the various ^{32}P-labeled subgenomic proviral DNA probes described below (indicated in Fig. 1). Briefly, 2 μl of a phage stock solution was spotted on a lawn of *E. coli* DP50 SupF; after overnight incubation at 37°C, plaques were transferred to nitrocellulose filters and hybridized to the labeled DNA probes.

Restriction enzyme digestion and agarose gel electrophoresis. Restriction endonucleases purchased from Bethesda Research Laboratories (Rockville, Md.) and New England Biolabs (Beverly, Mass.) were used according to the suppliers' instructions. Cloned DNAs (0.1 to 0.2 μg) or 5 μg of unintegrated Hirt supernatant proviral DNA was digested with 2 U of enzyme per μg of DNA at 37°C. Completeness of cleavage was monitored by digestion of lambda DNA added to an aliquot of the reaction mixture. For more than a single digestion, the cleaved DNA was ethanol precipitated, and the pellet was washed in 80% ethanol, air dried, and then resuspended in the reaction buffer of the second enzyme.

DNA restriction fragments were analyzed by horizontal agarose gel electrophoresis using 0.6 and 1.4% gels (20 by 25 cm) as previously described (25, 34). The standard marker mixture consisted of DNA fragments ranging in size from 23.5 to 0.07 kb and was prepared by combining the products of *Hin*dIII- and *Sma*I-cleaved lambda DNA and *Hpa*II- and *Hae*III-digested ϕX174 replicative form (RF) DNA with a mixture consisting of combined *Bam*HI, *Bam*HI plus *Hpa*II, and *Hin*f fragments of ^{32}P-labeled simian virus 40 DNA ranging in size from 5,226 to 24 base pairs (bp). DNA was transferred to nitrocellulose filters as described by Southern (44) after visualization by UV illumination of ethidium bromide-stained gels. The membranes were baked at 80°C for 2 h, hybridized, and washed as previously described (25). The nitrocellulose filters were air dried and exposed at −70°C to preflashed XR-5 xomat R (Kodak) film using intensifying screens.

DNA probes. ^{32}P-labeled AKR ecotropic MuLV cDNA was prepared as previously described (22). The generalized MuLV recombinant plasmid DNA probe ($MuLV^{gen}$) has been described previously (28, 33). The recombinant plasmid (pX_{env}) containing a 500-bp xenotropic MuLV *env*-specific DNA segment mapping 6.2 to 6.7 kb from the 5′ end of the NFS xenotropic proviral DNA clone has been recently described (6). The LTR DNA probe (33) consisted of a 600-bp *Kpn*I segment derived from a circularly permuted infectious clone of Harvey sarcoma virus containing three complete LTRs (9, 22). The construction of other recombinant plasmids containing subgenomic inserts from an infectious AKR ecotropic 623 proviral DNA clone (30) has been previously described. These include the 1.9-kb internal *Bam*HI segment, 1.9_{Bam} (1.8 to 3.7 kb) (33); the 2.7-kb *Sal*I-*Bam*HI fragment, $2.7_{Sal\text{-}Bam}$ (4.3 to 7.0 kb) (8); and the 500-bp ecotropic MuLV-specific *env* DNA segment, designated as pEc_{env} in this paper (6.5 to 7.0 kb) (8). Other subgenomic DNA fragments were isolated from cloned AKR MuLV DNA by gel elution and included the 0.9-kb *Bgl*II-*Bam*HI fragment ($0.9_{Bgl\text{-}Bam}$) (0.9 to 1.8 kb) and the 800-bp *Bam*HI-*Xba*I DNA segment, $0.8_{Bam\text{-}Xba}$ (7.0 to 7.8 kb). The map positions of the MuLV subgenomic DNA fragments used as probes are indicated in Fig. 1. DNAs were labeled by nick translation (32) and had specific activities of 6×10^7 to 13×10^7 cpm/μg.

RESULTS

Isolation and preliminary characterization of cloned endogenous MuLV-related DNAs. Recombinant phages containing MuLV-reactive sequences were isolated from a BALB/c mouse embryo DNA library (described above) and by shotgun cloning of size-selected (10- to 20-kb)

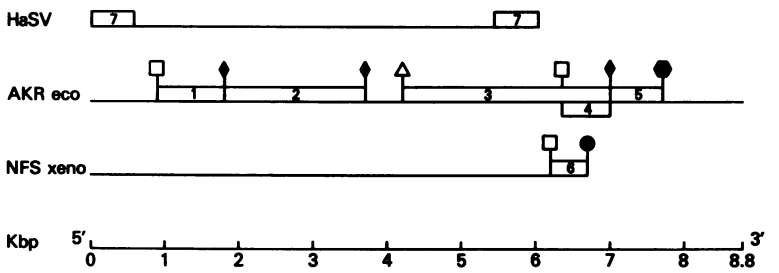

FIG. 1. Map locations of MuLV subgenomic DNA segments used as DNA probes. 1 = $0.9_{Bgl\text{-}Bam}$, 2 = 1.9_{Bam}, 3 = $2.7_{Sal\text{-}Bam}$, 4 = pEc_{env}, 5 = $0.8_{Bam\text{-}Xba}$, 6 = pX_{env}, 7 = LTR. The exact map position of each subgenomic fragment is indicated in the text. Restriction sites are *Bgl*II (□), *Bam*HI (◆), *Sal*I (△), *Xba*I (●), and *Eco*RI (●). HaSV, Harvey sarcoma virus; kbp, kilobase pairs.

*Eco*RI fragments of AKR/J mouse liver DNA. A total of 150,000 plaques isolated from the BALB/c library and 200,000 plaques from the shotgun-cloned AKR/J DNA were screened (3) with the $MuLV_{gen}$ and the AKR ecotropic MuLV cDNA probes, respectively. Then 40 of the 95 MuLV-reactive primary phage clones obtained from the BALB/c DNA library and all of the 136 primary phage plaques from the shotgun-cloned AKR/J mouse DNA were subcloned. The subcloned phage isolates were further characterized by the in situ hybridization technique (3) and subsequently subdivided based on their reactivity with ^{32}P-labeled LTR DNA. A total of 19 of the 40 BALB/c and 42 of the 136 AKR/J DNA clones hybridized to the LTR DNA probe; 16 of the 19 BALB/c and 7 of the 42 AKR/J DNA clones containing LTR-reactive sequences were selected for further study. Clones not containing LTR-reactive sequences will be described in a subsequent paper.

DNA was prepared from the 16 BALB/c and 7 AKR/J recombinant phage clones, digested with *Eco*RI or *Kpn*I, and hybridized to various ^{32}P-labeled subgenomic MuLV DNA segments after electrophoresis and transfer to nitrocellulose membranes (data not shown). Of the 23 endogenous LTR-containing MuLV DNA preparations examined, a total of 12 different DNA clones, consisting of 8 from BALB/c and 4 from AKR/J, were identified.

Restriction enzyme mapping of endogenous MuLV proviral DNAs. Restriction endonuclease maps of the 12 cloned endogenous MuLV proviruses were obtained by digesting the DNAs with a variety of restriction enzymes followed by hybridization to subgenomic MuLV DNA probes spanning the entire length of proviral DNA (indicated in Fig. 1). The restriction maps generated from these analyses as well as those previously published for MoMuLV (21), AKR ecotropic MuLV (28, 30, 46, 47), and NFS xenotropic MuLV (6, 11) proviruses are shown in Fig. 2. The map positions of restriction en-

TABLE 1. Map position of restriction sites present in endogenous cloned MuLV DNAs

Restriction site	Map position[a] within:			
	5' LTR	*gag*	*pol*	*env*
MuLV specific[b]				
*Kpn*I	0.5		3.35	7.35
*Sma*I	0.5		5.1	6.5
*Pst*I	0.1			
*Bgl*II		0.9	4.5	7.9
*Bam*HI			3.7	
*Sac*I			3.8	
*Pvu*II			4.3	
*Xho*I			4.5	
Xenotropic MuLV specific[c]				
*Bgl*II			2.25 (7/7)[d]	
			4.95 (2/5)	
*Sac*I			2.95 (4/5)	7.5 (2/2)
*Pst*I				7.5 (2/2)
*Pvu*II			4.75 (2/3)	6.05 (3/5)
				7.0 (2/2)
*Kpn*I			5.4 (1/5)	
*Eco*RI				6.9 (4/4)
Endogenous MuLV specific				
*Sac*I	0.2	1.0		
*Bgl*II	0.4			
*Kpn*I		1.25		6.9
*Xba*I		1.7	3.5	
			5.25	
*Sma*I			2.8	
			4.5	
*Pvu*II			4.4	
*Pst*I			4.6	
*Bam*HI				6.25[e]

[a] In kilobase pairs from the 5' terminus.
[b] Present in ecotropic and xenotropic MuLV proviruses (11, 38).
[c] Present only in xenotropic MuLV DNAs (11).
[d] The numbers in parentheses are the number of DNA clones containing the restriction site per the total number of DNAs containing sequences in that region.
[e] Present in several MCF MuLV proviruses (11, 28).

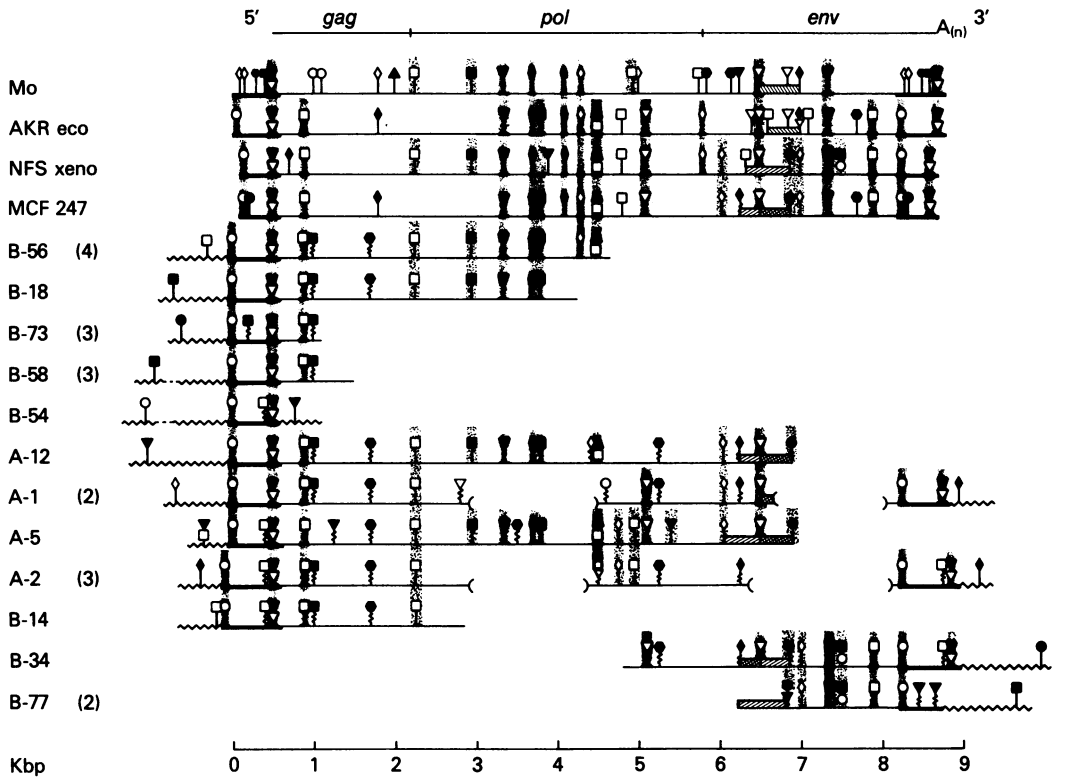

FIG. 2. Restriction endonuclease maps of cloned endogenous MuLV-related DNAs and MoMuLV, AKR ecotropic (eco) MuLV, NFS xenotropic (xeno) MuLV, and MCF 247 MuLV proviral DNAs. Restriction sites were mapped by blot hybridization using the ^{32}P-labeled MuLV subgenomic DNA probes described in the text and shown in Fig. 1. The structure of the genomic RNA is shown at the top of the figure. The numbers in parentheses after the clone designation indicate the number of identical isolates from BALB/c (B-) or AKR/J (A-) mouse DNAs. Heavy lines represent LTRs, wavy lines indicate flanking cellular DNA, and parentheses show the position of deleted DNA sequences. In the envelope region, ▨ = pX_{env} reactive, ▩ = pEc_{env} reactive, and ▨ = pX_{env} and pEc_{env} reactive. The location of the dual reactivity in *env* in MCF 247 proviral DNA is based upon its relationship to the analogous segment in the endogenous MuLV DNA of clone A-12. Shaded restriction sites are shared by all infectious MuLV proviruses, speckled restriction sites are present only in xenotropic MuLV proviral DNAs, and restriction sites on a "wiggled" stem are specific to the cloned endogenous MuLV segments. (The 6.25-kb *Bam*HI site mapped in *env* of several endogenous proviruses is also present in some MCF proviral DNAs [11, 28]). Map distances in kilobase pairs (kbp) are shown at the bottom of the figure. Restriction sites are *Sma*I (▽), *Kpn*I (▼), *Sac*I (■), *Xba*I (●), *Bgl*II (□), *Pvu*II (◇), *Bam*HI (◆), *Pst*I (○), *Eco*RI (●), and *Xho*I (△).

zyme sites shared by endogenous MuLV DNAs and known MuLV proviruses as well as those unique to endogenous MuLV proviral DNAs are presented in Table 1.

A single *Kpn*I restriction site and a single *Sma*I restriction site have been previously identified within the LTR of several MuLV proviral DNAs (11, 38). Both were also present in all but one of the LTRs associated with the cloned endogenous proviruses; clone B-77 lacked the *Sma*I site and contained two *Kpn*I sites (Fig. 2). Furthermore, a *Pst*I site located in the LTR of all MuLV proviruses except MoMuLV DNA was present in all 12 endogenous DNA clones examined. As shown in Fig. 2, the *gag*, *pol*, and *env* regions of the cloned endogenous MuLV DNAs contained several restriction sites previously reported to be highly conserved in known infectious MuLV proviruses (11, 38); the map positions of these MuLV-specific sites are indicated in Table 1. Restriction sites characteristic of ecotropic MuLV proviruses, such as *Bam*HI at 1.8 and 7.0 kb and *Xba*I at 7.7 kb, were not identified in the cloned endogenous MuLV DNAs. Restriction endonuclease sites unique to xenotropic MuLV proviruses, such as *Bgl*II and *Sac*I in *pol* and *Eco*RI, *Pvu*II, and *Sac*I in *env*, were also present in the endogenous proviral DNAs (Fig. 2 and Table 1). However, the *Pvu*II site at 5.8 kb, present in the proviruses of many

infectious ecotropic and xenotropic MuLVs, was absent in the endogenous DNA clones. In addition to the cleavage sites shared with proviruses of known infectious MuLVs, the cloned endogenous proviral DNAs contained several novel sites, such as *Sac*I at 1.0 kb, *Xba*I at 1.7 and 5.2 kb, and *Bam*HI at 6.25 kb (shown in Fig. 2). A *Bam*HI site has also been mapped at 6.25 kb in several MCF MuLV proviruses (11, 28).

Two endogenous MuLV proviral DNAs (A-1 and A-2) contained 1- to 2-kb deletions in *pol* and *env*. The size and location of the deleted sequences were determined by blot hybridization experiments and by heteroduplex mapping with a complete clone of AKR ecotropic MuLV provirus (A. S. Khan, N. G. Schmit, and C. F. Garon, unpublished data). Electron microscopy studies also indicated that the cloned endogenous MuLV proviral DNAs were colinear with cloned AKR ecotropic MuLV DNA in the *gag* and *pol* regions (A. S. Khan and C. F. Garon, unpublished data).

Detailed characterization of the *gag* and *pol* regions of endogenous MuLV proviral DNAs. The *gag* and *pol* regions of several cloned proviral DNAs were further characterized by digestion with *Alu*I and blot hybridization using the $0.9_{Bgl\text{-}Bam}$, 1.9_{Bam}, and $2.7_{Sal\text{-}Bam}$ subgenomic MuLV DNA probes (shown in Fig. 1). *Alu*I was used in these experiments because it recognizes a 4-bp DNA sequence and hence generates multiple cleavage products. As shown in Fig. 3A, all of the cloned endogenous proviral DNAs except A-5 and A-2 contained a 640-bp *Alu*I cleavage product which hybridized to labeled $0.9_{Bgl\text{-}Bam}$ DNA. Some of the endogenous proviruses containing the 640-bp *Alu*I fragment also contained a reactive 445-bp *Alu*I fragment. Neither of these two low-molecular-weight *Alu*I cleavage products was present in digests of AKR ecotropic, NFS xenotropic, or MoMuLV proviral DNAs. This result confirms the uniqueness of this region of the cloned endogenous proviruses as indicated by the presence of *Sac*I and *Xba*I restriction sites in the *gag* region (see Fig. 2). When the 1.9_{Bam} DNA was used as a hybridization probe to map *Alu*I digestion products, four shared reactive fragments (620, 570, 390, and 150 bp) were detected in several of the endogenous clones (Fig. 3B). Three of these fragments (620, 570, and 390 bp) comigrated with *Alu*I digestion products of MoMuLV proviral DNA. Figure 3C shows that there was much heterogeneity of nucleotide sequences in the cloned endogenous proviruses in the region extending 4.3 to 7.0 kb from the 5′ terminus. Only the 300-bp *Alu*I restriction fragment appeared to comigrate in clones A-1, A-12, A-2, and B-34 after hybridization to the $2.7_{Sal\text{-}Bam}$ DNA probe.

Specificity of the *env* region of cloned endogenous proviral DNA segments. We have previously reported the construction and use of a recombinant plasmid (designated as pEc_{env} in this paper) containing a 500-bp *Bgl*II-*Bam*HI DNA segment mapping between 6.5 and 7.0 kb from the 5′ end of the AKR ecotropic MuLV provirus; this fragment specifically hybridizes to ecotropic proviral DNAs but not to xenotropic proviruses (8). More recently we have identified and molecularly cloned an analogous 500-bp

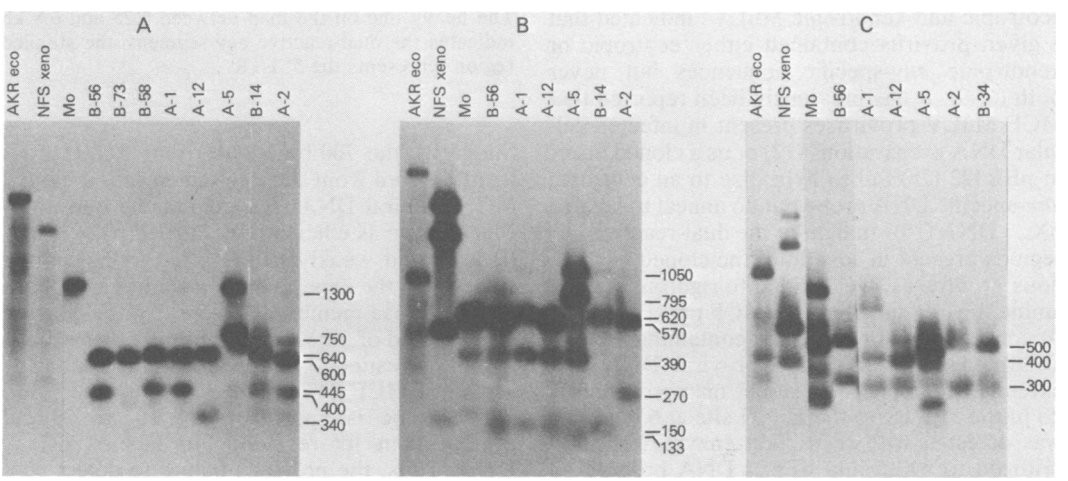

FIG. 3. Comparison of *Alu*I cleavage products generated from cloned endogenous MuLV DNAs and known MuLV proviruses. A 0.2-μg amount of digested DNA was electrophoresed through a 1.4% agarose gel (20 by 25 cm) at 50 V for 18 h, transferred to nitrocellulose filters, and hybridized as described in the text to (A) $0.9_{Bgl\text{-}Bam}$, (B) 1.9_{Bam}, and (C) $2.7_{Sal\text{-}Bam}$ MuLV DNA probes. The numbers on the right present DNA fragment sizes (bp) as determined from a standard DNA marker mixture (described in the text).

DNA segment (pX_{env}) from NFS-Th-1 xenotropic MuLV proviral DNA that recognizes *env* sequences in xenotropic proviruses but fails to anneal to ecotropic MuLV DNAs (6). These probes were used in blot hybridization experiments to characterize the envelope region of the cloned endogenous MuLV DNAs. As shown in Fig. 2, 6 of the 12 endogenous proviral DNAs contained *env* sequences. The *Eco*RI site at 6.9 kb, characteristic of the xenotropic (and MCF) *env* segments (11), was identified in four of these clones but was not detected in the two clones (A-1 and A-2) that contained deletions involving this region. Five of the six endogenous MuLV DNA preparations containing *env* sequences hybridized to the pX_{env} DNA probe; unexpectedly, four of the five also annealed to the labeled ecotropic *env*-specific DNA segment. The *env* region of clone B-77 had hybridization properties characteristic of xenotropic proviral DNA since it hybridized only to the pX_{env} DNA probe. The location of pX_{env}- and pEc_{env}-reactive sequences within the five *env*-containing endogenous proviral DNA clones was determined by blot hybridization experiments (data not shown) and is indicated diagrammatically in Fig. 2. In each of the five DNA clones except A-1 (which contained a deletion encompassing a portion of the dual-reactive *env* region), the pX_{env}-reactive DNA segment is bordered at its 3′ terminus by the 6.9-kb *Eco*RI site (see Fig. 2). The ecotropic *env*-reactive sequences are present in the region extending from the *Sma*I site at 6.5 kb to the *Eco*RI site at 6.9 kb except in clone B-34 in which the pEc_{env} reactivity is on the 5′ side of the *Sma*I site (Fig. 2).

Our analyses of proviral DNAs of infectious ecotropic and xenotropic MuLVs indicated that a given provirus contained either ecotropic or xenotropic *env*-specific sequences but never both (6, 8). It has previously been reported that MCF MuLV proviruses present in infected cellular DNA preparations (12) or as a cloned insert in pBR322 (28) fail to hybridize to an ecotropic *env*-specific DNA probe but do anneal to labeled pX_{env} DNA (28). In light of the dual-reactive *env* regions present in several of the cloned endogenous proviruses, we decided to rigorously reexamine the *env* specificity of MCF proviral DNA. Accordingly, a lambda clone containing the 6.8-kb fragment of MCF 247 proviral DNA that extends from an *Eco*RI site at map position 0.1 kb in the 5′ LTR to the *Eco*RI site at 6.9 kb (28) was digested with restriction enzymes and hybridized to pX_{env} and pEc_{env} DNA probes. As shown in Fig. 4, the 6.8-kb fragment released from λ-MCF 247 DNA after *Eco*RI digestion reacts strongly with the xenotropic *env* probe (lane 1) and hybridizes weakly to the labeled ecotropic env-specific DNA segment (lane 3).

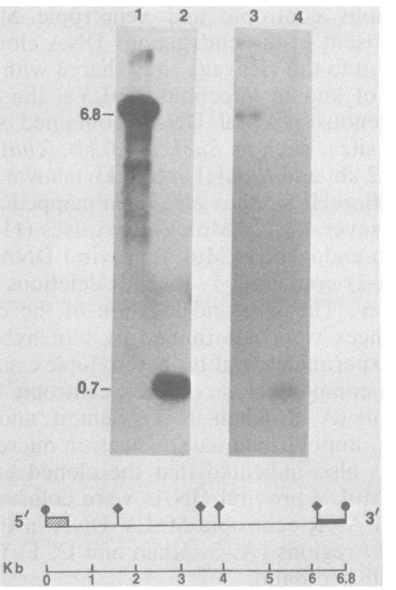

FIG. 4. Hybridization of *env*-specific DNA probes to cloned MCF 247 proviral DNA. A 0.2-μg amount of recombinant lambda DNA containing the 6.8-kb *Eco*RI segment of MCF 247 MuLV proviral DNA (28) was cleaved with *Eco*RI (lanes 1 and 3) and *Bam*HI plus *Eco*RI (lanes 2 and 4), electrophoresed through a 0.6% agarose slab gel (20 by 25 cm) at 40 V for 16 h, transferred to a nitrocellulose membrane, and hybridized to the pX_{env} (lanes 1 and 2) or the pEc_{env} (lanes 3 and 4) DNA probe. The filters were exposed for 2 days at −70°C to preflashed film using an intensifying screen. The sizes of the DNA cleavage products were determined from a standard DNA marker mixture described in the text. The locations of the *Eco*RI (●) and *Bam*HI (◆) sites in the cloned 6.8-kb MCF 247 DNA segment are shown in the diagram at the bottom. The heavy line on the map between 6.25 and 6.8 kb indicates the dual-reactive *env* segment; the stippled region represents the 5′ LTR.

Similarly, the 700-bp *Bam*HI plus *Eco*RI fragment derived from the *env* region of the cloned MCF proviral DNA (see map at the bottom of Fig. 4) anneals efficiently to labeled pX_{env} DNA (lane 2) and weakly to the pEc_{env} DNA probe (lane 4). In the experiment shown in Fig. 4, both nitrocellulose membranes were exposed for the same period of time. From this and other experiments, we estimate that the reactivity of the *env* region of MCF 247 proviral DNA to the pX_{env} DNA probe is approximately 15- to 20-fold greater than its reactivity to labeled pEc_{env} DNA. Thus, the previous failure to detect ecotropic *env* reactivity in MCF proviral DNA probably reflects a combination of insufficient exposure times and low concentrations of ecotropic *env* sequences in infected-cell (12) or recombinant plasmid (28) DNAs.

To more fully characterize the dual-reactive *env* region of the cloned endogenous MuLV DNAs, several of the proviral DNA preparations were digested with *Alu*I and hybridized to the ecotropic and xenotropic *env*-specific DNA probes. As shown in Fig. 5A, the pX_{env} DNA probe, as expected, did not anneal to either *Alu*I-restricted MoMuLV or AKR ecotropic proviral DNAs (lanes 1 and 2, respectively) but did react with at least three *Alu*I fragments of the NFS xenotropic provirus and MCF 247 proviral DNA (lanes 3 and 4, respectively). Each of the five *Alu*I-digested endogenous proviral DNAs contained at least two fragments which hybridized to labeled pX_{env} DNA (Fig. 5A, lanes 5 to 9), none of which comigrated with *Alu*I cleavage products of NFS-Th-1 xenotropic proviral DNA (Fig. 5A, lane 3). However, clones A-12 and A-5 (Fig. 5A, lanes 6 and 7) each contained three *Alu*I cleavage products that comigrated with *Alu*I fragments generated from cloned MCF 247 proviral DNA (Fig. 5A, lane 4). Furthermore, when the same cloned endogenous MuLV DNAs were cleaved with *Hpa*II and annealed to the pX_{env} DNA probe, clone A-12 (Fig. 5C, lane 6) contained three reactive digestion products (0.50, 0.25, and 0.20 kb) that comigrated with *Hpa*II fragments derived from MCF 247 proviral DNA (Fig. 5C, lane 4). Clone A-5 (Fig. 5C, lane 7) contained only a single pX_{env}-reactive 250-bp fragment, which was also detected in the *env* region of the MCF 247 provirus. Although *Hpa*II digestion of clone B-34 (Fig. 5C, lane 8) generated fragments that comigrated with cleavage products of MCF 247 proviral DNA, the pattern observed after *Alu*I digestion was quite different (cf. lanes 4 and 8, Fig. 5A).

Figure 5B shows the reactivity of the ecotropic *env*-specific DNA probe to *Alu*I-digested cloned endogenous proviral DNAs. Besides annealing, as expected, to cleaved MoMuLV and AKR ecotropic proviruses (lanes 1 and 2, respectively), the labeled pEc_{env} DNA segment hybridized to a single *Alu*I restriction fragment generated from each of the endogenous proviral DNAs (lanes 5 to 8) except clone B-77 (lane 9). Furthermore, the 0.63-kb pEc_{env}-reactive *Alu*I fragment present in clones A-12 and A-5 (lanes 6 and 7) comigrated with an *Alu*I digestion product of MCF 247 proviral DNA (lane 4). This 0.63-kb *Alu*I fragment also hybridizes to the pX_{env} DNA probe (Fig. 5A). On the basis of similar *Alu*I and *Hpa*II digestion patterns and the dual-reactive nature of their *env* regions, MCF 247 proviral DNA appears to have been generated by a recombinational event involving an endogenous provirus corresponding to clone A-12.

Analysis of the LTR present in the cloned endogenous MuLV proviral DNAs. The size and internal organization of the LTRs associated with different cloned endogenous proviral DNAs were examined and compared with analogous segments present in the proviruses of known infectious ecotropic, xenotropic, and MCF MuLVs. With the exception of MoMuLV, all infectious MuLV proviruses examined contain a single *Kpn*I and a single *Pst*I restriction site in the LTR. The MoMuLV LTR contains the *Kpn*I site but is lacking the *Pst*I site (49, 50). Since *Kpn*I and *Pst*I restriction sites were also present

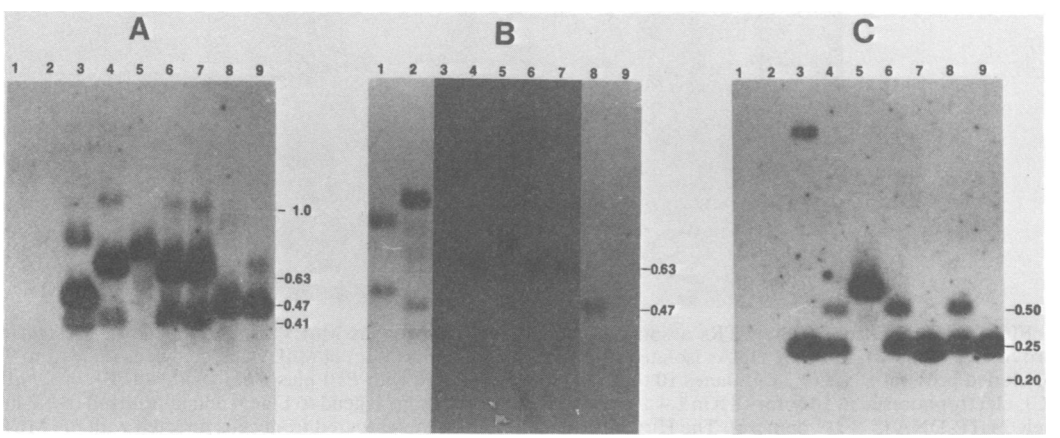

FIG. 5. Hybridization of *env*-specific DNA probes to *Alu*I- and *Hpa*II-digested MuLV proviral DNAs. A 0.20-μg amount of λ-MoMuLV (lane 1), λ-AKR ecotropic 623 MuLV (lane 2), λ-NFS xenotropic MuLV (lane 3), and λ-MCF 247 MuLV (lane 4) proviral DNAs and 0.2 μg of endogenous MuLV DNA clones A-1 (lane 5), A-12 (lane 6), A-5 (lane 7), B-34 (lane 8), and B-77 (lane 9) were digested with *Alu*I (A and B) or *Hpa*II (C), electrophoresed through 1.4% agarose slab gels (20 by 25 cm) at 50 V for 18 h, and transferred to nitrocellulose membranes as described in the text. The filters were then hybridized to the pX_{env} (A and C) or the pEc_{env} (B) DNA probe and exposed to X-ray film for 1 week as described in the text.

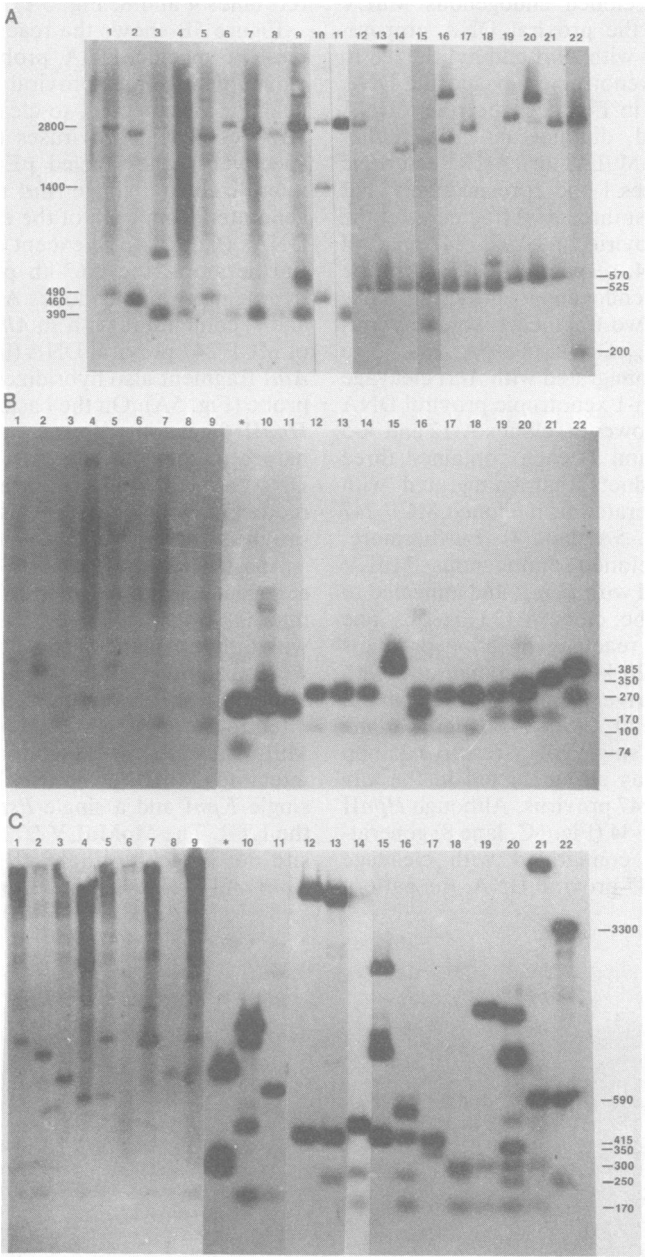

FIG. 6. Comparison of the LTRs associated with cloned endogenous MuLV DNAs and those present in proviruses of known MuLVs. DNAs isolated from Hirt supernatants (5 μg) of MuLV-infected cells (lanes 1 to 9) or cloned proviral DNA (0.2 μg) (lanes 10 to 22 and *) was digested with *Pst*I plus *Kpn*I (A), *Alu*I (B), or *Hpa*II (C), electrophoresed at 50 V for 18 h in 1.4% agarose as described in the legend to Fig. 5, and hybridized to ^{32}P-labeled LTR DNA (5×10^7 cpm/μg). The Hirt supernatant DNAs were prepared from cells infected with the MCF MuLVs, AKR-247 (lane 1), AKR-13 (lane 2), *Akv*-1-C36 (lane 3), AKR L5 (lane 4), AKR L3 (lane 5), *Akv*-1-C44-2 (lane 6), and *Akv*-1-C311 (lane 8), with BALB/c xenotropic MuLV (lane 7), and with BALB/c ecotropic MuLV (lane 9). The cloned proviral DNAs examined include AKR ecotropic MuLV 623 (lane 10), NFS xenotropic MuLV (lane 11), MoMuLV (lane *), and endogenous MuLV DNA clones B-56, B-73, B-58, B-54, A-1, A-12, A-5, B-14, A-2, B-34, and B-77 in lanes 12 to 22, respectively. The autoradiogram of the cloned DNAs (lanes 10 to 22) was exposed overnight, whereas the Hirt supernatant DNAs (lanes 1 to 9) were exposed for 1 week.

in the LTRs of all of the cloned endogenous MuLV DNAs, double digestion with the two enzymes was carried out in a series of blot hybridization experiments using the ^{32}P-labeled LTR DNA to characterize sequences located between these two sites. Three LTR-reactive DNA fragments resulted from *Kpn*I plus *Pst*I digestion of cloned AKR ecotropic proviral DNA (Fig. 6A, lane 10): the 2.8-kb fragment contained LTR and *gag*-reactive sequences, the 1.4-kb segment contained LTR and 3′ flanking cellular DNA sequences, and the 490-bp fragment contained exclusively LTR sequences located between the *Kpn*I and *Pst*I sites. The size of this internal *Kpn*I plus *Pst*I LTR fragment (490 bp) corresponded to that expected from the nucleotide sequence of the AKR ecotropic MuLV LTR (51). The NFS xenotropic MuLV provirus contained an internal 390-bp *Kpn*I plus *Pst*I LTR segment as well as a 2.8-kb *gag*-reactive segment containing LTR sequences (Fig. 6A, lane 11). The smallest *Kpn*I plus *Pst*I LTR-reactive DNA fragment in BALB/c xenotropic and BALB/c ecotropic MuLV proviral DNAs (Fig. 6A, lanes 7 and 9, respectively) comigrated with the 390-bp segment in NFS xenotropic MuLV DNA. A comparison of the internal LTR DNA fragments present in various MCF MuLV proviruses (lanes 1 to 6 and 8) indicated the existence of segments corresponding in size to ecotropic or xenotropic proviral DNAs except in MCF 13 (lane 2), which contained a 460-bp *Kpn*I plus *Pst*I LTR DNA segment, intermediate in size between the fragments generated from ecotropic and xenotropic proviruses. Some MCF MuLV DNA preparations contained internal LTR segments of two sizes, such as 460 and 390 bp in MCF L5 (lane 4) and 490 and 390 bp in MCF L3 (lane 5), presumably reflecting heterogeneity in the preparations with respect to the 70- to 100-bp direct repeat region of the LTR (38-40).

The cloned endogenous MuLV DNAs yielded three different-sized *Kpn*I plus *Pst*I internal LTR DNA segments (525 bp [Fig. 6A, lanes 12 to 18], 570 bp [lanes 19 to 21], and 200 bp [lane 22]), none of which were present in ecotropic, xenotropic, or MCF proviral DNAs. The cloned endogenous proviral DNAs were also digested with *Alu*I (Fig. 6B, lanes 12 to 22) or *Hpa*II (Fig. 6C, lanes 12 to 22) and hybridized to labeled LTR DNA after electrophoresis and transfer to a nitrocellulose membrane. In both experiments, the LTR-reactive fragments did not comigrate with digestion products derived from proviruses of known infectious MuLVs (lanes 1 to 11).

DISCUSSION

In this communication we describe the molecular cloning and biochemical characterization of 12 different incomplete endogenous MuLV proviruses containing LTRs that were isolated from BALB/c and AKR/J mouse DNAs. Of the numerous MuLV-reactive recombinant phage clones, approximately 50% of the BALB/c and 33% of the AKR/J had an associated MuLV LTR. The genomic organization of all of the endogenous MuLV DNAs except clone B-54 (which consists of an LTR flanked on both sides by cellular sequences) was similar to that of known integrated retroviral DNAs (i.e., cell DNA-LTR-*gag*-*pol*-*env*-LTR-cell DNA). The cloned endogenous MuLV DNAs contained 14 of the 16 highly conserved restriction sites (Table 1 and Fig. 2) previously reported to be present in the proviruses of infectious ecotropic and xenotropic MuLVs isolated from inbred mice (11, 38). In addition, several restriction sites unique to the cloned endogenous proviral DNAs, such as the *Xba*I sites a 1.7 and 5.25 kb, the *Sac*I site at 1.0 kb, and the *Bam*HI site at 6.25 kb, were also identified. Although the proviral DNA segments present in the different clones were closely related to one another on the basis of similar restriction sites, they were clearly located at different loci in mouse chromosomal DNA as indicated by the distinctive flanking cellular sequences associated with each clone (Fig. 2).

The LTRs associated with the cloned endogenous proviral DNAs (except B-77) resemble the LTRs of known infectious MuLVs in that they contain characteristic *Pst*I, *Sma*I, and *Kpn*I restriction sites. However, when the sizes of the endogenous LTRs were analyzed by digesting the proviral DNAs with *Pst*I plus *Kpn*I, the cloned proviruses (except B-77) could be divided into at least two classes, both of which contained internal LTR segments that were larger than those present in the infectious MuLV DNAs. Heterogeneity in the size of LTRs associated with MuLV proviral DNAs has previously been attributed to a difference in the size of directly repeating units (75) bp in MoMuLV [49, 50] compared with 101 bp in AKR ecotropic MuLV [51]) or to the presence or absence of the direct repeat (4, 28). The LTRs of the cloned endogenous proviral DNAs are larger than those in ecotropic and xenotropic MuLV proviruses; whether this reflects additional alterations of the direct repeat region is being studied. Furthermore, although the two classes of endogenous LTRs contain characteristic 100- and 170-bp internal *Alu*I fragments (Fig. 6B), each obviously contains unique members as judged by the heterogenous *Hpa*II cleavage patterns (Fig. 6C).

Despite the large number of endogenous MuLV proviruses detected in mouse DNA by solution and blot hybridization analyses, the number of inducible loci for infectious MuLVs is

low (36, 42). Our analyses of the 12 different LTR-containing endogenous MuLV proviruses point to several structural features that could potentially affect their expression as infectious virus particles. For example, the proviruses in clones A-1 and A-2 have 1- to 2-kb deletions in both the *pol* and the *env* regions (Fig. 2) and would therefore be incapable of encoding an infectious virus. As pointed out above, all of the endogenous LTRs (except clone B-77) are larger than the LTRs associated with known infectious MuLVs as measured by the size of the internal *Pst*I-*Kpn*I fragment (Fig. 6A). Several cloned endogenous LTRs also contained a unique *Bgl*II restriction site. Furthermore, the LTR present in clone B-77 contained an additional *Kpn*I site and was lacking the highly conserved *Sma*I site characteristic of the LTRs of known infectious MuLV proviruses (Fig. 2). If these alterations in LTR structure affect the integrity of the direct repeat or the RNA initiation/processing signals, the adjacent proviral DNA might be rendered defective.

Restriction sites characteristic of ecotropic proviruses of endogenous origin, such as *Bam*HI at 1.8 and 7.0 kb and *Xba*I at 7.7 kb (38), were not detected in any of the cloned endogenous proviruses. Furthermore, the endogenous MuLV DNAs were not closely related to AKR ecotropic proviral DNA as judged by the absence of comigrating *Alu*I restriction fragments generated from various defined regions of the endogenous proviruses (Fig. 3). However, an intriguing relationship was observed between the cloned endogenous MuLV DNAs and the provirus of MoMuLV. MoMuLV was isolated from a transplanted tumor (Sarcoma 37) carried in BALB/c mice (35); it cannot be induced from mouse cells nor has it been subsequently recovered from any other tumor. Our results clearly indicate that the cloned endogenous MuLV DNAs are related to the MoMuLV provirus in the region extending 1.8 to 3.7 kb from the 5′ terminus (Fig. 3B). The dissimilarity of the *Alu*I digestion patterns of other regions of MoMuLV proviral DNA compared with analogous segments of the cloned endogenous proviruses suggests that MoMuLV proviral DNA most likely is a recombinant MuLV.

The cloned endogenous MuLV DNAs contain several restriction sites characteristic of xenotropic MuLV proviruses (11), such as *Sac*I at 2.95 and 7.5 kb and *Eco*RI at 6.9 kb (see Table 1 for a more complete listing). Furthermore, five of the six cloned endogenous DNAs which contained an envelope region also hybridized to the pX_{env} DNA probe. Of the five cloned endogenous proviruses containing *env*-specific sequences, only B-77 hybridized exclusively to labeled pX_{env} DNA (Fig. 2). However, the *env* region of clone B-77 was distinct from known infectious xenotropic MuLV proviruses in having a *Kpn*I site at 6.9 kb. Clone B-77 was also unique in containing two *Kpn*I sites in the LTR (Fig. 2). The other four clones contained segments that annealed to both envelope-specific DNA probes (discussed below) and could thereby be distinguished from known xenotropic proviruses. No endogenous MuLV DNA clone was isolated which reacted only to the pEc_{env} DNA probe.

The *env* region of cloned endogenous MuLV provirus A-12 contains a 700-bp *Bam*HI-*Eco*RI dual-reactive segment that is also present in MCF 247 proviral DNA. A *Bam*HI-*Eco*RI fragment of this size has been identified in the *env* region of several MCF proviral DNAs (11, 28) as well as in integrated recombinant viral DNAs in AKR lymphomas (37) or MoMuLV-induced tumors (52) and in AKR mouse chromosomal DNA (10). *Alu*I and *Hpa*II digestions of MCF 247 and clone A-12 endogenous proviral DNAs generate comigrating cleavage products with similar reactivities with the xenotropic and ecotropic *env*-specific DNA probes. It is very likely, therefore, that the endogenous provirus corresponding to clone A-12 in AKR mouse DNA contributed a portion of its *env* region during the generation of MCF 247 MuLV. Most of the clone A-12 provirus is not present in MCF 247 DNA, since the latter does not contain restriction sites unique to the endogenous MuLV DNA, such as *Sac*I at 1.0 kb, *Xba*I at 1.7 and 5.25 kb, or *Pvu*II at 4.4 kb (Fig. 2). Furthermore, the LTR in MCF 247 DNA can be distinguished from the LTR associated with clone A-12 based upon the size of the internal *Pst*I plus *Kpn*I LTR segment (Fig. 6A) and by the absence of an *Eco*RI site in the LTR of clone A-12 (see Fig. 2). Recombination must have occurred between the *Xba*I site at 5.25 kb and the *Bam*HI site at 6.25 kb in the A-12 provirus (see Fig. 2) since the former site is absent in the MCF 247 provirus, whereas the latter site is present in both A-12 and MCF 247 DNAs but absent from the AKR ecotropic proviral DNA.

The experiment presented in Fig. 5 indicates that three other cloned endogenous MuLV proviral DNAs contain dual-reactive *env* segments. One of these (clone A-5) was similar to clone A-12 and MCF 247 proviral DNA by *Alu*I digestion but could be distinguished after *Hpa*II cleavage (Fig. 5C). The difference in this region is also reflected in the absence in clone A-5 DNA of the *Bam*HI site at 6.25 kb (Fig. 2) found in MCF 247 and clone A-12. It should be noted that several MCF proviral DNAs also lack the 6.25-kb *Bam*HI site (11), raising the possibility that the *env* segment present in clone A-5 might be incorporated into this group of MCF proviruses.

Although we have previously reported that the *env* region of MCF 247 proviral DNA mapping between 6.25 and 6.9 kb consists exclusively of xenotropic *env*-reactive sequences (28), the results described above clearly indicate that this segment also shows a small degree of reactivity with the pEc_{env} DNA probe. This is in contrast to the *env* specificity in the NFS-Th-1 provirus which shows no evidence of dual reactivity. The reactivity of MCF 247 proviral DNA to labeled pX_{env} DNA is 15 to 20 times greater than to pEc_{env} DNA, explaining in part previous failures to demonstrate the dual *env* reactivity characteristic of MCF proviruses. This cross-reactivity could simply reflect the presence of a small portion of the ecotropic *env*-specific segment in the MCF *env* region or could be due to the existence of a unique MCF envelope which shares a greater homology with the xenotropic *env* region and is only distantly related to the ecotropic envelope. Nucleotide sequencing of this MCF 247 proviral DNA segment should explain the differential reactivity observed.

Assuming that the cloned MuLV proviruses constitute a representative sample of the endogenous proviral DNAs present in the mouse genome, we must conclude that endogenous proviruses with purely xenotropic MuLV *env* specificity, such as clone B-77, make up a small fraction of the endogenous pX_{env}-reactive proviral DNAs. A majority of the endogenous proviruses hybridizing to labeled pX_{env} DNA also anneal to the pEc_{env} DNA probe and therefore do not contain *env* regions identical to those of any infectious xenotropic MuLV thus far studied. This may explain the discrepancy between the large number of pX_{env}-reactive sequences detected in mouse DNA by blot hybridization experiments (M. D. Hoggan, personal communication) and the genetic evidence that most mouse strains contain only one locus for inducible xenotropic virus (29).

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