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Genomes of Murine Leukemia Viruses Isolated from Wild Mice

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The genomes of murine leukemia viruses (MuLV) isolated from wild mice have been studied. Detailed restriction endonuclease maps of the 8.8-kilobase (kb) unintegrated linear viral DNAs were derived for five ecotropic and five amphotropic MuLV's from California field mice, for Friend MuLV, and for one ecotropic and one xenotropic MuLV from *Mus musculus castaneus*. In general, the California MuLV's were similar in their leftward 6 kb (corresponding to the leftward long terminal repeat [LTR], *gag*, and *pol*) and rightward 1 kb (7.8 to 8.8 kb, corresponding to p15E and the rightward LTR). For the region spanning 6.0 to 7.7 kb (which includes the sequences that encode gp70) the amphotropic MuLV's shared few enzyme sites with the ecotropic MuLV's, although the California ecotropic MuLV's were highly related to each other in this region, as were the amphotropic MuLV's. Cross-hybridization studies between amphotropic and California ecotropic MuLV DNAs indicated that they were not homologous in the region 6.3 to 7.6 kb; the California ecotropic viral DNAs cross-hybridized in this region to AKR ecotropic MuLV. When the California viral DNAs were compared with AKR ecotropic viral DNA, many differences in enzyme sites were noted throughout the genome. The U3 regions of the wild mouse LTRs showed partial homology to this region in AKR MuLV. The LTR of Moloney MuLV was highly related to that of the California MuLV's, whereas the LTR of Friend MuLV appeared to be a recombinant between the two types of LTRs. The *M. musculus castaneus* isolates were most closely related to ecotropic and xenotropic MuLV's isolated from inbred mice. One amphotropic MuLV DNA was cloned from supercoiled viral DNA at its unique *Eco*RI site in pBR322. Viral DNAs with one and two LTRs were isolated. After digestion with *Eco*RI, DNAs of both types were infectious. It is concluded that ecotropic and amphotropic MuLV's differ primarily in the region which encodes gp70.

The naturally occurring endogenous murine leukemia viruses (MuLV) have been classified into three groups principally on the basis of their host range: ecotropic, which infect only murine cells; xenotropic, which infect primarily heterologous cells (27); and amphotropic, which replicate efficiently in cells of both types (20, 38). Biological and serological data have suggested that the viral host range is determined principally by the viral envelope glycoprotein (gp70). A fourth class of MuLV, the mink cytopathic focus-forming (MCF) viruses (21), appear to be recombinant viruses between ecotropic MuLV's and xenotropic related sequences: they share the expanded host range of amphotropic viruses, but sera which neutralize amphotropic MuLV do not neutralize MCF viruses and vice versa. Amphotropic MuLV's have been isolated only from wild (feral) mice in California, whereas ecotropic and xenotropic MuLV's have been isolated from wild mice from many regions, as well as from inbred mice. Wild mouse ecotropic viruses have been shown to induce lymphomas and paralysis in susceptible mice, whereas the amphotropic viruses have induced only lymphomas (15).

The viral nucleic acids of ecotropic, xenotropic, and MCF MuLV's isolated from inbred mice (such as AKR) and from one wild Asian mouse *Mus musculus molossinus* have been studied in some detail (8, 33, 37, 41; S. K. Chattopadhyay, M. R. Lander, S. K. Gupta, E. Rands, and D. R. Lowy, Virology, in press). However, there has been relatively little analysis of the genomes of amphotropic or other wild mouse MuLV's (1, 5, 6, 28). In this report, we have developed restriction endonuclease maps for the

DNAs of several amphotropic and ecotropic wild mouse viruses, compared their sequence organization with that of other MuLV's, and molecularly cloned infectious amphotropic MuLV DNA.

MATERIALS AND METHODS

Viruses and unintegrated proviral DNA preparation. The designation and origin of the ecotropic, xenotropic, and amphotropic MuLV's are listed in Table 1. The ecotropic viruses were propagated in NIH 3T3 cells as described previously (37). The xenotropic viruses were propagated in *Mus dunii* cells (mice generously provided by T. C. Hsu, M. D. Anderson Medical Research Center, Houston, Tex.) and amphotropic MuLV's were grown in either *M. dunii* cells or *Mus caroli* cells, which are permissive for these viruses (Chattopadhyay et al., unpublished observation). Permissive cells were acutely infected with MuLV from chronically infected cells, and DNA was extracted from them by the Hirt procedure 16 to 18 h after infection as described previously (23, 37). DNA from Hirt supernatant was used as the starting material for restriction endonuclease digestion.

The precise location of each function of the viral genome has not yet been mapped on the viral DNA. Based on the data of other investigators (12, 16, 24, 25, 39, 43, 45), we have considered the viral genome to be divided as follows, going from left to right: 0 to 0.6 kilobases (kb), long terminal repeat (LTR); 0.5 to 1.0 kb, leader; 1.0 to 2.8 kb, *gag;* 2.9 to 5.8 kb, *pol;* 6.0 to 8.2 kb, *env;* 8.2 to 8.8 kb, LTR. The N terminus of mature gp70 is located at about 6.4 kb; its carboxy terminus maps at about 7.7 kb (C. Van Beveren and I. M. Verma, personal communication). p15E would map from approximately 7.7 to 8.2 kb.

Molecular hybridization. For the preparation of 70S RNA, xenotropic and amphotropic viruses were grown in *M. dunii* and *M. caroli* cells, respectively. Other details are described elsewhere (6). Single-stranded [^{32}P]cDNA probes were synthesized from viral RNA by using calf thymus oligomers as primers (46). Double-stranded [^{32}P]DNA probes were synthesized from molecularly cloned ecotropic MuLV DNA (31) by nick translation with *Escherichia coli* polymerase and DNase (34). In the text the probe referred to as 5′ + $R_{0.1}$ indicates the 4.6-kb *Sma*I fragment of AKR MuLV DNA which spans from 0.5 to 5.1 kb from the left end of the 8.8-kb linear viral DNA. It includes

TABLE 1. *MuLV's used in this study*

Class	Strain	Source (references)
Ecotropic[a]	1504E	Wild mouse embryo (no. 1504) from LaPuente (Calif.) duck farm (same as 1504 M in references 6 and 20)
	292E	Spinal cord extracts of spontaneously paralyzed wild mouse of Lake Casitas Squab Farm (Calif.) (15)
	4070E	Wild mouse embryo (no. 4070) from Lake Casitas Squab Farm (same as 4070M in references 6 and 20)
	1740E	Brain extract of spontaneously paralyzed wild mouse of LaPuente (15)
	4996E	Spleen extract of spontaneous lymphoma of wild mouse of Bouquet Canyon Squab Farm (Calif.) (15)
	Casitas brain-E	Brain extract of an eighth passage experimentally paralyzed NIH Swiss mouse inoculated initially with brain extract from a naturally paralyzed Lake Casitas mouse (same as Cas-Br-M in reference 20)
	Castaneus-E	Activated from tail tissue culture cell line of *M. musculus castaneus* by iododeoxyuridine treatment (this paper)
	Kyushu-E	Tail tissue culture cells of *M. musculus molossinus* (8)
	Friend-E	Friend eco clone 57 was used in this study; the original viral isolate was obtained from Charlotte Friend as the Friend virus anemic strain (14, 35)
Xenotropic[b]	Castaneus-X	Activated from tail tissue culture cell line of *M. musculus castaneus* (this paper)
	Kyushu-X	Tail tissue culture cells of a *M. musculus molossinus* (Chattopadhyay et al., in press)
	NFS-1X	Thymus of a 5.5-month-old NFS mouse (isolated by Janet Hartley; Chattopadhyay et al., in press)
Amphotropic[c]	1504A	Same as 1504E (20)
	292A	Same as 292E (15)
	4070A	Same as 4070E (20)
	1740A	Same as 1740E (15)
	1313A	Spontaneous lymphoma of a wild mouse from Hartz Mountain (Calif.) bird seed plant (15)

[a] All ecotropic viruses used in this study were grown in NIH 3T3 cells.
[b] All xenotropic viruses were grown in *M. dunii* tissue culture cell line developed from a tail (26).
[c] Amphotropic MuLV's were grown either in *M. caroli* or in *M. dunii* tail tissue culture cell line.

approximately 100 nucleotides of the LTR. Similarly, 3′ + $R_{0.5}$ is the 1.7-kb *Sma*I fragment of AKR MuLV DNA which spans from 7.0 to 8.7 kb from the left end of the viral DNA and includes about 500 nucleotides from the right LTR (see Fig. 7, Kyushu-E map; the AKR MuLV restriction endonuclease map is identical to the Kyushu-E map). Hybridizations were carried out in 0.6 M NaCl (4× SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C, using 5×10^6 to 10×10^6 cpm of viral probe. The lengths of the DNA cleavage products are given to the nearest 0.1 kb. Cleavage products smaller than 0.4 kb may not be detected by our technique.

To determine the restriction endonuclease cleavage products for the viral DNAs, unintegrated proviral DNAs from these wild mouse viruses were digested with various enzymes, electrophoresed, transferred to nitrocellulose filters (40), and hybridized with [^{32}P]-cDNA synthesized from 70S RNA from at least one wild mouse virus with the same tropism. For the determination of restriction endonuclease maps for amphotropic viruses, probes synthesized from 70S RNA of 1504A and cloned AKR ecotropic viral DNA were used. Ecotropic AKR probes were also prepared by nick translation with molecularly cloned AKR proviral DNA (31) serving as substrate for this reaction.

Molecular cloning of 4070A viral DNA. Form I closed circular 4070A viral DNA was isolated from acutely infected NIH 3T3 cells by the Hirt procedure (23), and the Hirt supernatant DNA was fractionated by preparative agarose gel electrophoresis as previously described (35). The purified circles were linearized with *Eco*RI and then ligated together with *Eco*RI-digested pBR322 by using T4 DNA ligase. One microgram of this ligated DNA preparation was used to transform the RR-1 strain of *E. coli*, and these bacteria were plated onto agar plates containing antibiotic (10). Recombinant colonies containing amphotropic 4070A were screened by means of an in situ filter hybridization technique (19) employing a ^{32}P-labeled cDNA probe prepared from 70S 4070A RNA. Twenty-three hybridization-positive colonies were identified out of approximately 2,000 colonies examined. Nine of these colonies were picked and amplified in liquid culture for further analysis. The plasmid DNA was isolated from these cultures as previously described (9). Eight clones were found to contain a 4070A DNA insert in the plasmid DNA, and electrophoretic analysis revealed that three of these clones contained viral genomes with two copies of LTR, and five clones had only one LTR. In infectivity assays on mouse cells (see below), all three viral DNAs with two LTRs gave rise to amphotropic virus, whereas three of five clones with a single LTR were infectious.

Infectious DNA assay. Transfection of DNA onto NIH 3T3 cells was performed as previously described (30, 32) by using the calcium phosphate precipitation technique (17). Plasmid DNA was separated by *Eco*RI restriction, and calf thymus DNA (25 μg/ml) was used as carrier.

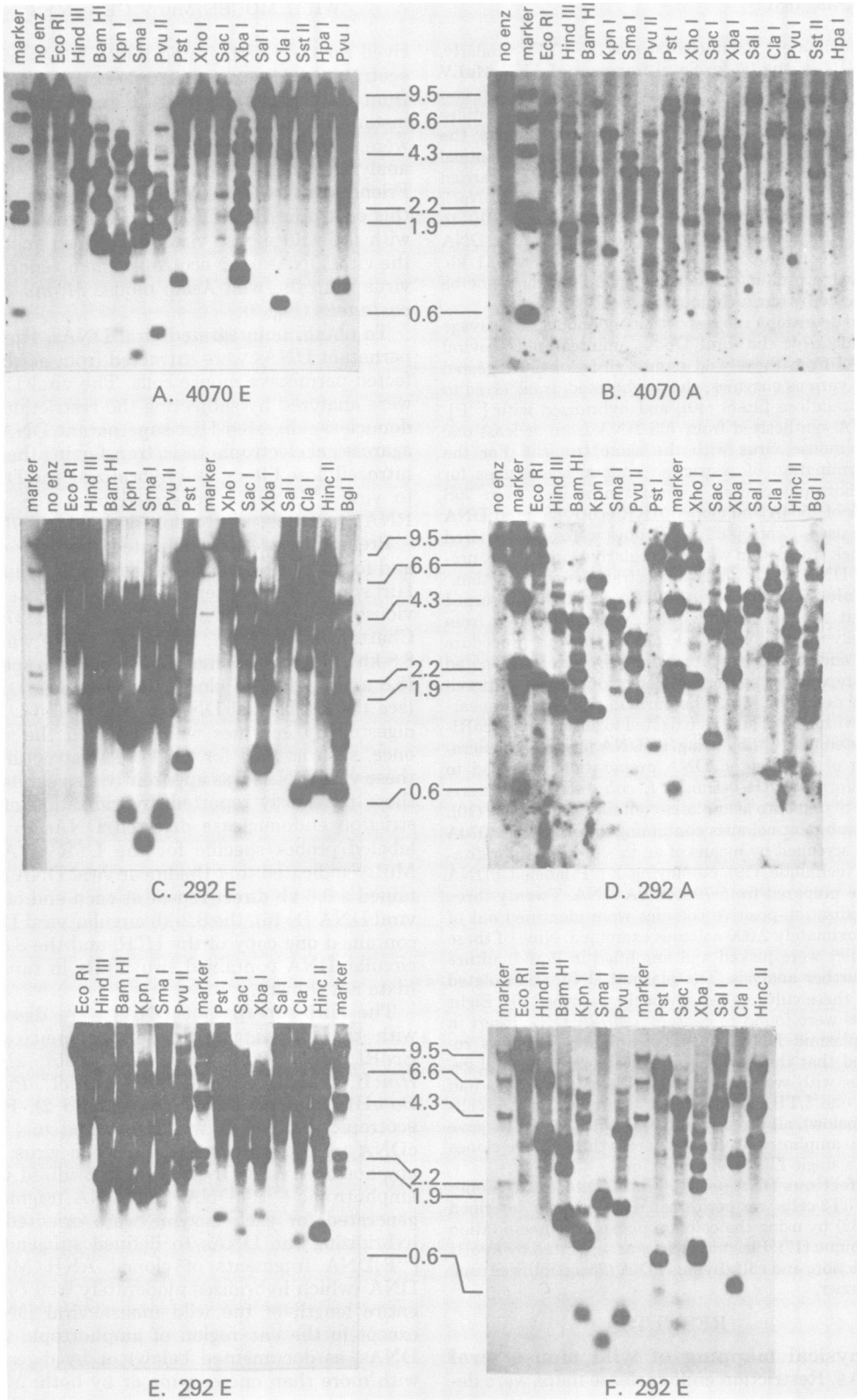
RESULTS

Physical mapping of wild mouse viral DNAs. Restriction endonuclease maps were derived for the unintegrated linear DNAs of five ecotropic and five amphotropic MuLV's isolated from California mice (Fig. 1 and 2). In some instances an amphotropic and an ecotropic virus were isolated from the same mouse; the viruses analyzed include four such pairs. A fine map of Friend MuLV (F-MuLV) was also derived since this ecotropic viral DNA shares some features with the wild mouse viruses. We also analyzed the viral DNAs of an ecotropic and a xenotropic virus from the wild Asian mouse *M. musculus castaneus* (Fig. 3).

To obtain unintegrated viral DNAs, Hirt supernatant DNAs were extracted from newly infected permissive mouse cells. The viral DNAs were analyzed by subjecting the restriction endonuclease-digested Hirt supernatant DNAs to agarose gel electrophoresis, transferring them to nitrocellulose filters, and hybridizing the DNAs to viral [^{32}P]cDNA generated from 70S viral RNA or to molecularly cloned viral [^{32}P]DNA.

Preliminary results indicated that the sizes and forms of the wild mouse viral DNAs in the Hirt supernantants were analogous to those previously described for other MuLV's (16, 37, 48; Chattopadhyay et al., in press). There was an 8.8-kb unintegrated linear DNA and two species (8.8 and 8.2 kb) of closed circular viral DNAs (see the undigested DNAs in Fig. 1 and DNAs digested by enzymes which cleaved the virus once, such as *Sal*I for 292A). The structure of these viral DNAs was apparently also similar to those previously reported: hybridization of restriction endonuclease digested DNAs to ^{32}P-labeled probes specific for the LTR of AKR MuLV indicated that the linear viral DNA contained a 0.6-kb direct repeat at each end of the viral DNA (1, 16), the 8.2-kb circular viral DNA contained one copy of the LTR, and the 8.8-kb circular DNA contained two LTRs in tandem (data not shown).

The Hirt supernatant DNAs were digested with the following restriction endonucleases: *Eco*RI, *Pst*I, *Xho*I, *Sal*I, *Cla*I, *Xba*I, *Hpa*I, *Hinc*II, *Hin*dIII, *Kpn*I, *Sma*I, *Sac*I, *Pvu*II, *Bam*HI, *Pvu*I, and *Bcl*I (Fig. 1 and 2). Each ecotropic viral DNA was hybridized to [^{32}P]-cDNA from a wild mouse ecotropic virus; the amphotropic viral DNAs were hybridized with amphotropic [^{32}P]cDNA. The DNA fragments generated for each enzyme were oriented by hybridizing the DNAs to defined subgenomic [^{32}P]DNA fragments of cloned AKR MuLV DNA (which hybridizes moderately well to the entire length of the wild mouse viral DNAs, except in the *env* region of amphotropic viral DNAs, as documented below) or by digestion with more than one enzyme or by both. Many


marker
no enz
Eco RI
Hind III
Bam HI
Kpn I
Sma I
Pvu II
Pst I
Xho I
Sac I
Xba I
Sal I
Cla I
Sst II
Hpa I
Pvu I
9.5
6.6
4.3
2.2
1.9
0.6
A. 4070 E
B. 4070 A
Hinc II
Bgl I
C. 292 E
D. 292 A
E. 292 E
F. 292 E

cleavage products were oriented by hybridization of restriction endonuclease digested DNAs to two defined subgenomic viral DNA probes: a probe for the left side of the AKR viral DNA (the 4.6-kb *Sma*I fragment of AKR MuLV DNA which spans from 0.5 to 5.1 kb from the left end of the 8.8-kb linear viral DNA) and a probe for the right side (the 1.7-kb *Sma*I fragment of AKR

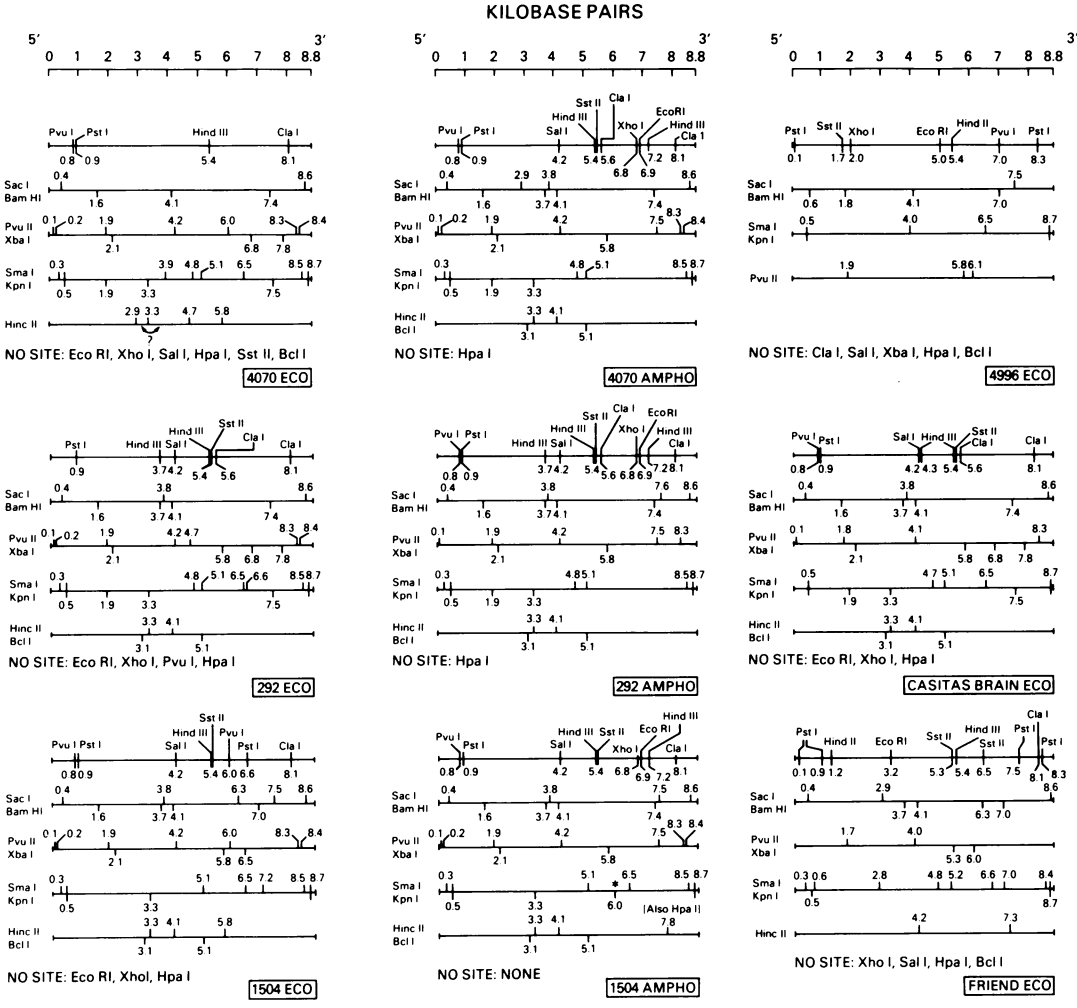


FIG. 2. *Restriction endonuclease maps of unintegrated linear ecotropic and amphotropic viral DNAs of wild mice. The maps for each virus were generated from blots similar to those shown in Fig. 1. The numbers of each restriction endonuclease cleavage site indicate the distance (in kb) of the site from the left (5') end of the viral DNA. The map of Friend ecotropic viral DNA is also included. The maps of 1740E and 1740A were identical to 292E and 292A, respectively. The map of 1313A was identical to 1504A, except that 1313A had an HincII site at 7.8 kb where 1504A has an HpaI site.*

FIG. 1. *Restriction endonuclease analysis of ecotropic (E) and amphotropic (A) MuLV DNAs from California mice. Hirt supernatant DNAs isolated from cells newly infected with the indicated virus were digested with the indicated restriction endonuclease, subjected to electrophoresis in 0.7% agarose gels at 40 V for 16 h, transferred to nitrocellulose filters (40), and hybridized to a viral [^{32}P]DNA as described in the text. Southern blots in various panels were hybridized to [^{32}P]DNA probe as follows: A and C, to 1504E cDNA probe; B and D, to 1504A cDNA probe; E, to a ^{32}P-labeled, nick-translated 5' (5' + $R_{0.1}$) viral DNA probe (the 4,600-bp SmaI fragment of AKR 623 cloned DNA; see Kyushu-E in Fig. 7); F, to a ^{32}P-labeled, nick-translated 3' (3' + $R_{0.5}$) viral DNA probe (the 1,700-bp SmaI fragment of AKR 623 cloned DNA; see Kyushu-E in Fig. 7). The lengths of the marker fragments (^{32}P-labeled, HindIII-digested λ DNA) are given in kb. Lanes with undigested DNA are labeled "no enz."*

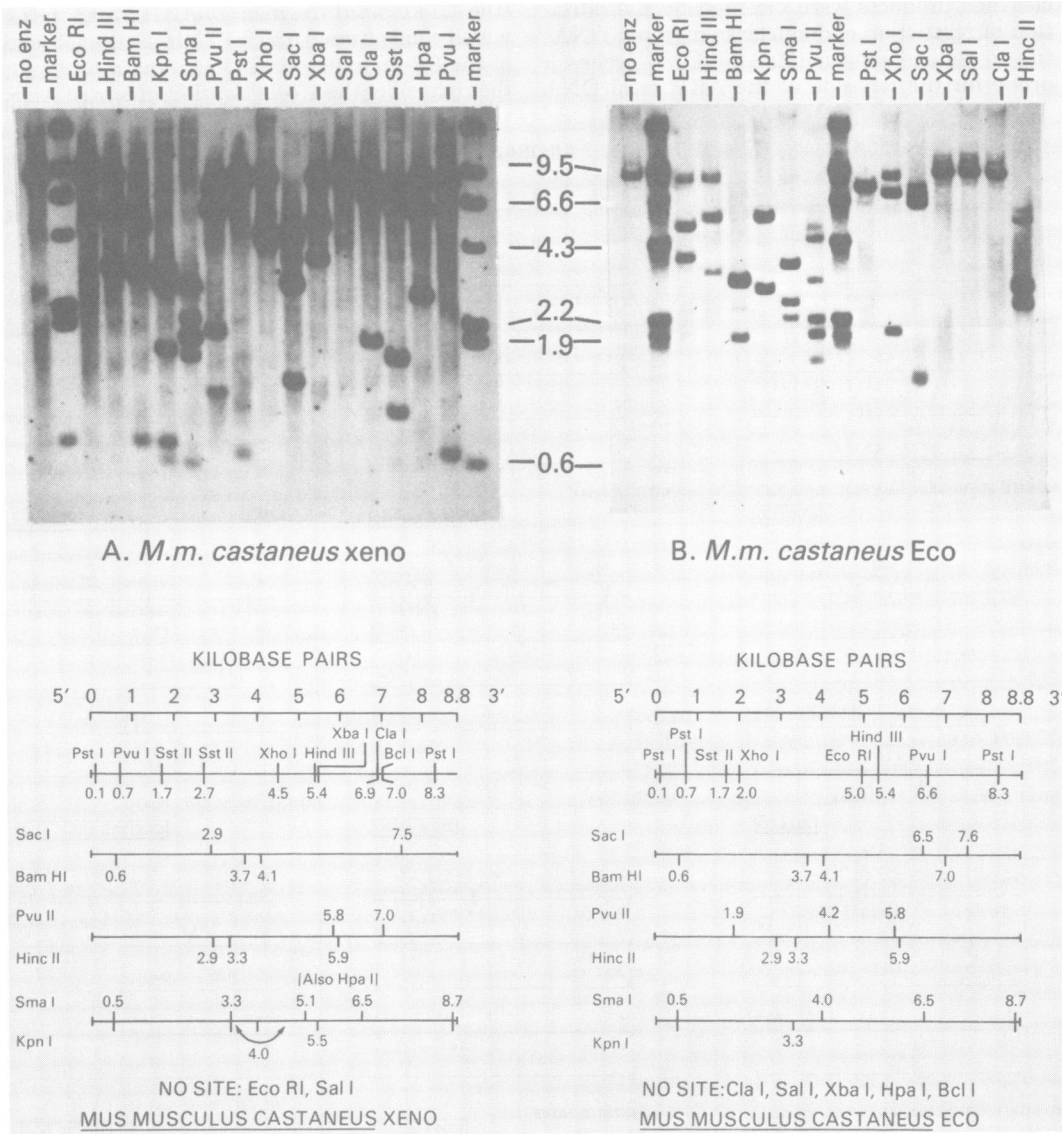


FIG. 3. *Restriction endonuclease analysis of ecotropic and xenotropic viruses isolated from M. musculus castaneus and the corresponding restriction endonuclease maps. Both blots were hybridized to ecotropic AKR-623 nick-translated probe. "Marker" indicates HindIII-digested, ^{32}P-labeled wild-type λ DNA. Other details are as in Fig. 1 and 2. When the xenotropic viral DNA was also hybridized to xenotropic [^{32}P]cDNA, no additional fragments were noted.*

MuLV DNA which spans from 7.0 to 8.7 kb from the left end of the viral DNA). (Here and in the subsequent discussion, the location of all sites on the viral DNA are given by their distance from the left end of the viral DNA.) The 5′ probe, which is designated 5′ + $R_{0.1}$, contains 0.1 kb of LTR; fragments at the extreme right end of viral DNA may, therefore, hybridize very faintly. The 3′-probe, designated 3′ + $R_{0.5}$, includes 0.5 kb of LTR and therefore will also hybridize to each left-end fragment in addition to those fragments at the 3′ end.

The hybridization of an ecotropic viral DNA (292E) to the 5′ + $R_{0.1}$ and 3′ + $R_{0.5}$ probes are shown in Fig. 1E and F, respectively. The *Cla*I cleavage products were analyzed as follows. Hybridization of 292E DNA to a full-length probe (Fig. 1C) indicated that three fragments had

been generated by *Cla*I digestion (5.6, 2.5, and 0.7 kb). The 5′ + $R_{0.1}$ probe hybridized strongly to the 5.6-kb fragment and faintly to the 0.7-kb fragment, indicating that the 5.6-kb fragment was located to the left of the 2.5-kb fragment, whereas the 0.7-kb fragment might be at the left or right end. If the 0.7-kb fragment were at the left end, the 3′ + $R_{0.5}$ probe would hybridize only to the 2.5- and 0.7-kb fragments, whereas this probe would hybridize to all three fragments if the 0.7-kb fragment were at the right end. The 3′ + $R_{0.5}$ probe hybridized to all three fragments, which established the left-to-right order of the fragments as 5.6, 2.5, 0.7 kb. The 5.6- and 0.7-kb fragments were further verified as terminal fragments, since *Cla*I digestion of the 8.8-kb circular form yielded, as expected, the 6.3-kb fragment seen most easily in Fig. 1F (the expected 5.7-kb fragment generated from digestion of the 8.2-kb circular form was not seen because it was superimposed on the 5.6-kb *Cla*I fragment). Other fragments were ordered in an analogous manner by digestion with two enzymes as required for this and the other viruses.

Comparison of California MuLV DNA maps. When the restriction endonuclease maps of the California MuLV's were compared, several viruses of the same tropism were identical: 1740A and 292A, 1740E and 292E, and 1313A and 1504A, except that 1313A had an *Hinc*II site at 7.8 kb, whereas 1504A had an *Hpa*I site at this location. Because of these similarities, a representative picture of the various wild mouse isolates—except for 4996E, which was very different from other viruses—could be obtained by comparing the maps of only three pairs of viral DNAs: 4070E and A, 292E and A, and 1504E and A (Fig. 4). 4070E and A were isolated from one mouse, 292E and A were from a second mouse, and 1504E and A were from a third mouse. In the region 6.0 to 7.8 kb, the viruses of the same tropism were related to each other, but in this region the viruses of one host range differed significantly from those of the other. For the remainder of the genome, most viruses of both tropisms were highly related, although 4070E differed somewhat from the others in the 2.9- to 5.8-kb segment. The LTRs of all the

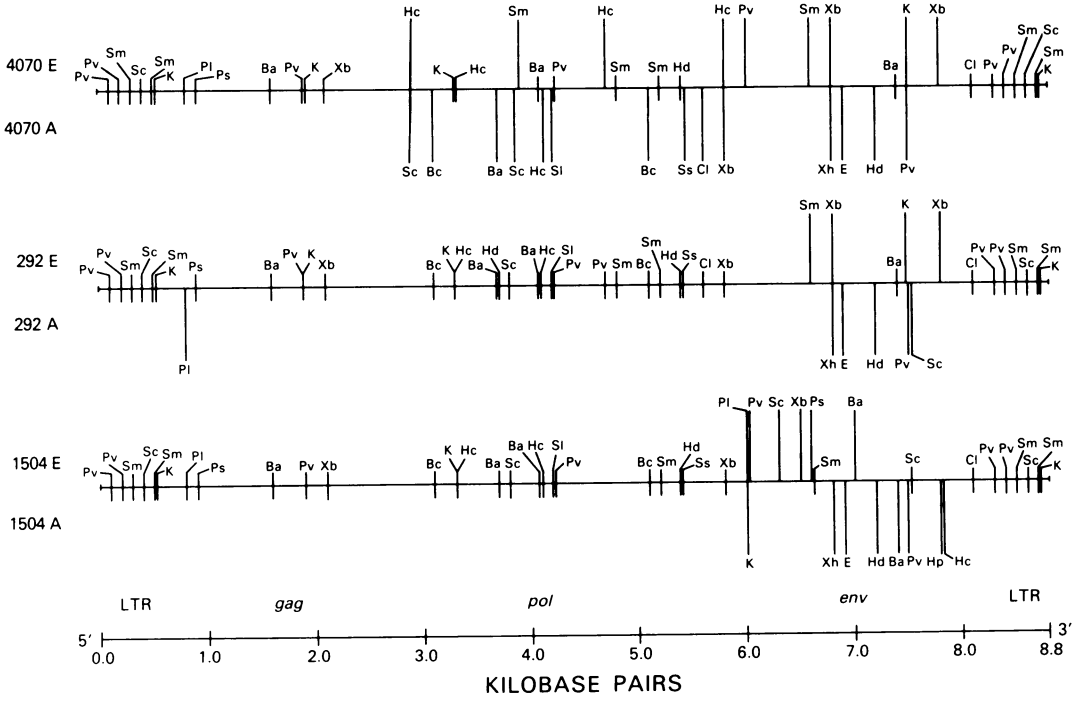


FIG. 4. *Analysis of the genomes of ecotropic and amphotropic MuLV's isolated from the California wild mice. Each pair was isolated from the same mouse. 1740E and 1740A were not included since they are identical to 292E and 292A, respectively. Common restriction endonuclease sites were indicated with short lines and the name of the enzyme indicated above. Enzyme sites that are different within the pair are indicated with longer lines. Abbreviations: E, EcoRI; Ps, PstI; Sm, SmaI; K, KpnI; PI, PvuI; Ss, SstII; Ba, BamHI; Hc, HincII; Hp, HpaI; Hd, HindIII; Sc, SacI; Sl, SalI; Pv, PvuII; Xh, XhoI; Cl, ClaI; Xb, XbaI; Bc, BclI.*

California viruses were identical (two *Pvu*II sites, two *Sma*I sites, and one site each for *Sac*I and *Kpn*I). Each viral DNA shared at least five of the six sites mapped within the 2.1-kb segment from 0.7 to 2.8 kb, which probably represents leader sequences and *gag*. If 4070E is not considered, each viral DNA shared at least 14 of the 19 sites mapped in the 2.8-kb segment spanning 2.9 to 5.8 kb, which probably corresponds to *pol*. Although 4070E was more divergent in this segment, it still shared 7 of the 17 sites mapped in this segment with 4070A. By contrast, 292E and 292A in this segment were identical to each other, as were 1504E and 1504A. The viral DNAs all contained a *Cla*I site at 8.1 kb, which may fall within p15E coding sequences.

As noted above, the greatest divergence among the California viral DNAs was found in the 1.8-kb segment 6.0 to 7.8 kb, which corresponds to most of *env* and probably includes all gp70-coding sequences. Of the 17 sites mapped in this region for the different viruses, only one (*Bam*HI at 7.4 kb) was present in all of them. However, viruses of the same tropism were more closely related to each other in this region; all amphotropic viral DNAs shared five of the nine sites mapped in this region, whereas 4070E and 292E shared five of six sites, and 1504E contained four of these six sites (plus two others not present in the other ecotropic viral DNAs). When the restriction endonuclease sites in the 6.0- to 7.8-kb segment of the California viruses were compared with those mapped for the Kyushu-type ecotropic viruses (37; see Kyushu-E in Fig. 7), only two sites were shared with the California ecotropic viruses (*Sma*I at 6.6 kb and *Xba*I at 7.8 kb), and only one site was shared with amphotropic viruses.

Homology among the mouse viral DNAs. The homology among various viral DNAs was first assessed by hybridizing ^{32}P-labeled 1504E, 1504A, 292E, and AKR-E to 1504E DNA which had been digested with each enzyme whose sites had been mapped. Significant differences in hybridization were noted for some *Kpn*I, *Sma*I, *Pvu*II, and *Sac*I fragments (Fig. 5). The 0.6-kb *Sma*I fragment (spanning 6.6 to 7.2 kb) and the 1.2-kb *Sac*I fragment (spanning 6.3 to 7.5 kb) did not hybridize to the 1504A probe. The 1.2-kb *Sma*I fragment (spanning 7.2 to 8.4 kb) hybridized poorly to this amphotropic probe. However, these fragments hybridized to all three ecotropic probes. These findings suggested that in the 6- to 8-kb region all of the ecotropic viral DNAs were related to each other, but that the amphotropic sequences in this region were highly divergent from the ecotropic sequences. These results were consistent with our earlier observation (7) that a probe specific for a portion of AKR ecotropic MuLV *env* (the 0.4-kb *Sma*I fragment located 6.5 to 6.9 kb from the left end of the AKR MuLV viral DNA) did not cross-hybridize with xenotropic, MCF, or amphotropic MuLV DNAs, but did hybridize to other eco-

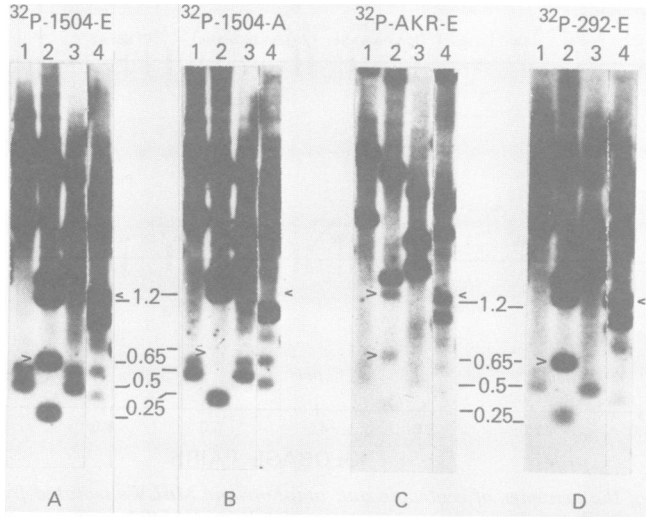


FIG. 5. *Restriction endonuclease digested 1504E DNA hybridized to amphotropic and ecotropic viral DNAs. Hirt DNA isolated from cells newly infected with 1504E virus was digested with KpnI (lane 1), SmaI (lane 2), PvuII (lane 3), or SacI (lane 4) and hybridized to a [^{32}P]cDNA probe from various viruses as indicated at the top of each panel. Arrows are given to emphasize certain fragments. Sizes of the fragments are given in kb.*

tropic DNAs. By contrast, the LTR fragments (0.5-kb *Kpn*I and *Pvu*II fragment, 0.4- and 0.2-kb *Sac*I fragments, and 0.25-kb *Sma*I fragment) hybridized well to all three wild mouse virus probes and less well to the AKR MuLV probe. These data on the LTR were consistent with the major differences noted between the restriction endonuclease sites mapped in the LTR of the California mouse viruses and the Kyushu viral LTR (see Fig. 7 and below).

To compare the homology of the 6- to 8-kb region more systematically, we also hybridized radiolabeled amphotropic and ecotropic MuLV DNA to cloned AKR MuLV DNA which had been digested with one or two enzymes. The results of these hybridizations for those fragments mapping in the 6- to 8-kb region have been tabulated diagrammatically in Fig. 6. Under the conditions of hybridization employed, there was no detectable hybridization between amphotropic DNA and the AKR MuLV DNA in the 1.1-kb fragment spanning from 6.5 to 7.6 kb. Incomplete homology was present to the left and right of this segment. By contrast, 1504E hybridized moderately well to this entire segment.

DNA maps of F-MuLV and *M. musculus castaneus* viruses. The restriction endonuclease maps of the ecotropic and xenotropic viruses isolated from *M. musculus castaneus* (Fig. 3 and

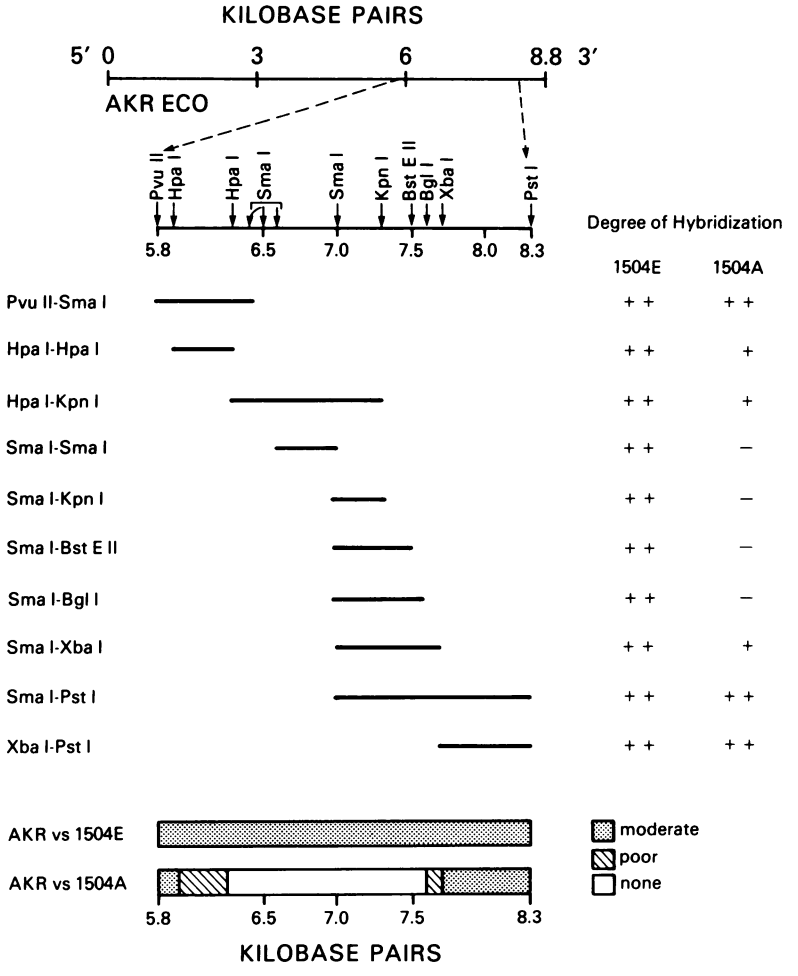


FIG. 6. *Region of heterogeneity between ecotropic AKR MuLV DNA and amphotropic (1504A) viral DNA. Schematic representation of hybridization between the ecotropic AKR MuLV restriction endonuclease fragments shown on the left (in the region 5.8 to 8.3 kb from the left end of the viral DNA) and 1504E or 1504A [^{32}P]cDNAs. These results are summarized schematically at the bottom of the figure. The degree of hybridization was defined qualitatively as indicated at the bottom of the figure. The three SmaI sites from 6.4 to 6.6 kb have been identified by R. Repaske and M. Martin (personal communication).*

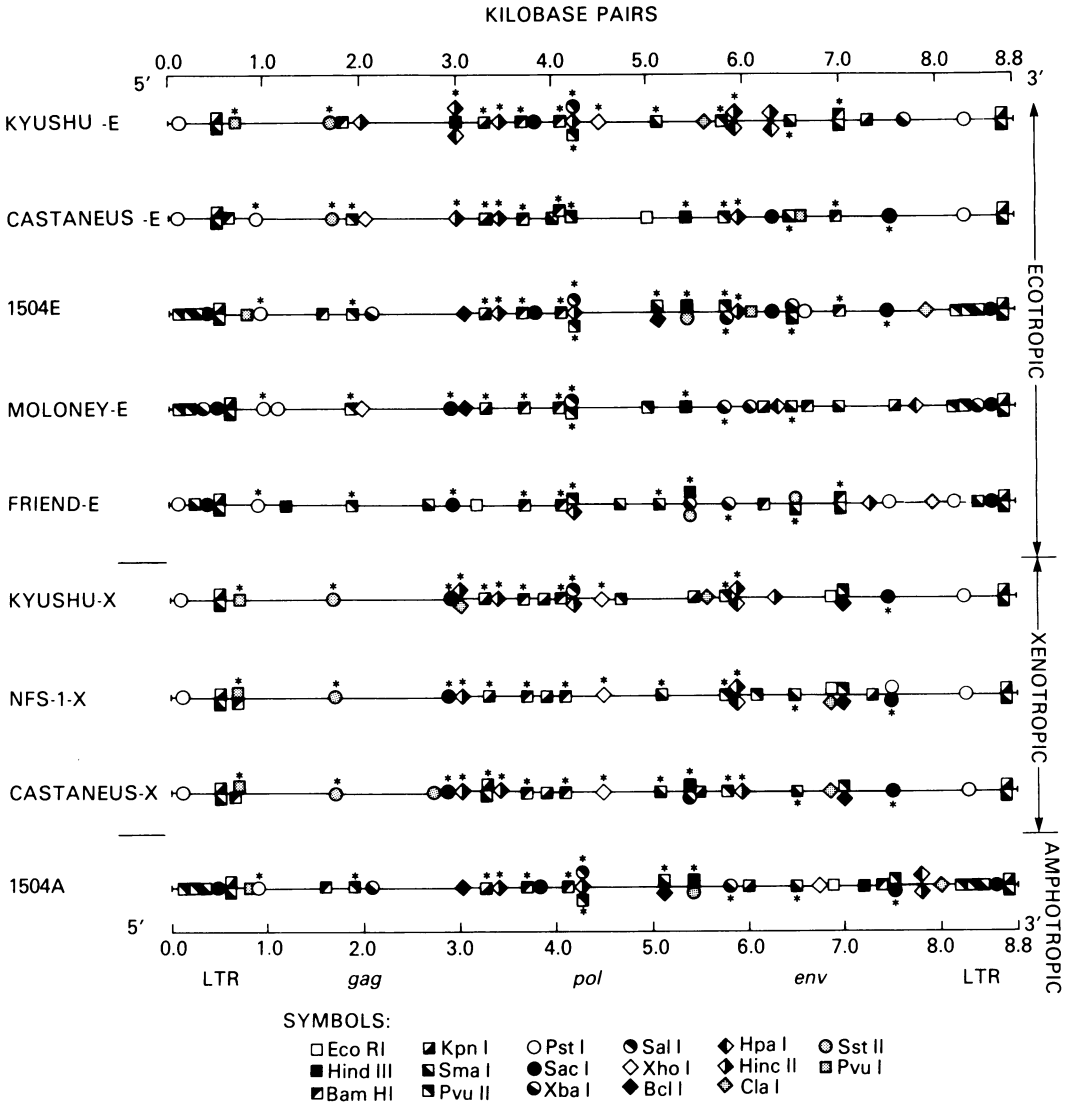


FIG. 7. *Linear maps of representative ecotropic, xenotropic, and amphotropic viruses of inbred and wild mouse strains. The viruses are grouped according to their tropism. Each symbol corresponds to an enzyme as indicated in the figure. An asterisk indicates that the site is present in four or more of the viruses in the figure. Moloney ecotropic map was adapted from Gilboa et al. (16). Kyushu-E is an ecotropic virus isolated from M. musculus molossinus. This map is virtually identical to ecotropic viruses of inbred strains of mice (the strains and isolates analyzed are: Akv-1, two isolates; Akv-2, two isolates; C58, three isolates; C3H/Fg, three isolates; C3H/HeN, one isolate; PL, three isolates; F/St, one isolate; BALB/c, one isolate). Castaneus-E is an ecotropic virus isolated from M. musculus castaneus.*

7) were identical to each other in the LTR and similar in 3.2 to 5.8 kb (6 of 14 sites), but they shared few sites (1 of 5) in segments 1.0 to 3.0 kb and 6.0 to 8.0 kb (2 of 9). Their degree of divergence throughout their genomes were therefore somewhat greater than that of the California MuLV's, but similar to that previously observed for ecotropic and xenotropic MuLV's isolated from Kyushu and inbred mice (Chattopadhyay et al., in press).

In general, the *M. musculus castaneus* ecotropic and xenotropic viral DNA maps were more closely related to the viruses isolated from Kyushu and inbred mice than to the viruses isolated from California mice. The LTR of the *M. musculus castaneus* viruses had the same three enzyme sites as viruses from Kyushu and inbred mice. Despite their closer relationship to

the Kyushu-type viruses, the *M. musculus castaneus* viral DNAs did share a rare site with the California viruses that was absent in the Kyushu-type viruses, such as *Pst*I at 0.9 kb, *Pvu*II at 1.9 kb, *Hind*III at 5.3 kb, and *Sac*I at 6.3 kb in the *M. musculus castaneus* ecotropic viral DNA and *Hin*dIII at 4.3 kb in the *M. musculus castaneus* xenotropic viral DNA.

The *M. musculus castaneus* xenotropic viral DNA shared many sites with a previously mapped NFS xenotropic viral DNA (Chattopadhyay et al., in press; NFS-1 in Fig. 7). In *env*, the *M. musculus castaneus* xenotropic viral DNA lacked the *Eco*RI site at 6.9 kb noted in all other previously mapped xenotropic viral DNAs, but it contained the two other sites (*Pvu*II at 7.0 and *Bcl*I at 7.0 kb) found in *env* of all other xenotropic viral DNAs as well as *Cla*I at 7.0 kb also noted in several xenotropic viral DNAs. The 6- to 8-kb region (*env*) of the *M. musculus castaneus* ecotropic virus had an enzyme pattern which from 6.4 to 7.4 kb was different from all other ecotropic viral DNAs. However, this viral DNA also hybridized, though poorly, to the type-specific probe (6.5 to 6.9 kb) of AKR ecotropic MuLV (7; data not shown).

When F-MuLV DNA was compared with other viral DNAs (Fig. 7), several interesting features were noted. The viruses mapped previously do not allow a clear conclusion as to its origin, as is also true of Moloney MuLV (Fig. 7). The F-MuLV viral DNA had some short segments closely related to one type of virus and others related to other types, suggesting that F-MuLV may be the product of several recombinational events. For example, the left side of the F-MuLV LTR appeared to be derived from a Kyushu-like LTR (a *Pst*I site and no *Pvu*II sites), whereas the right side of its LTR was similar to that of the California MuLV's (two *Sma*I sites, one *Xba*I site, and one *Kpn*I site). In the highly type-specific region of *env* (6.6 to 7.0 kb), F-MuLV contained the same three sites as Kyushu MuLV. The *Bam*HI site at 6.2 kb has been noted previously only in MCF viral DNAs, and the *Pst*I site at 7.5 kb has been found in some xenotropic and AKR MCF DNAs (Chattopadhyay et al., in press). However, the *Pst*I site at 0.9 kb and the *Hin*dIII site at 5.5 kb were found only in the California viruses and the *M. musculus castaneus* ecotropic virus, whereas the *Cla*I site at 8.1 kb was noted only in the California viruses and in one AKR MCF virus.

Molecular cloning of amphotropic 4070A MuLV DNA. For further detailed biological and biochemical comparisons of amphotropic genomes with those of other viruses, it would be highly desirable to have unlimited quantities of amphotropic viral DNA. Therefore, the closed circular forms of 4070A MuLV DNA were molecularly cloned at their unique *Eco*RI site in the plasmid vector pBR322. As reported for other helper-independent ecotropic viruses, pBR 4070A recombinant DNAs were isolated with 8.2- and 8.8-kb 4070A DNA inserts; these DNAs represented the larger and smaller circular forms (two and one LTR, respectively) permuted at the *Eco*RI site (Fig. 8). Restriction endonuclease digestion of the cloned viral DNAs yielded the fragment sizes and order predicted from the Hirt supernatant viral DNA analysis described above, except that the *Cla*I site located 5.6 kb from the left end of the linear viral DNA was missing in each of the six clones which were examined. After digestion with *Eco*RI, DNAs with either one or two copies of the LTR were infectious; their infectivity appeared to be similar to that of cloned F-MuLV DNA (35). When the viral DNA was not digested with *Eco*RI, no infectious virus was recovered.

The viruses isolated from transfection of *Eco*RI-digested cloned DNAs were characterized biologically as amphotropic: they were XC negative and replicated efficiently on mouse, mink, and NRK cells. When Hirt supernatant DNA isolated from cells newly infected with an isolate derived from transfection with a clone with one LTR, the restriction enzyme analysis was identical to that of the uncloned viral DNA, except that the *Cla*I site which was absent in the cloned DNA was also missing in the Hirt DNA. The viral DNA of MuLV derived from transfection of an 8.8-kb clone also contained only one LTR at each end of the linear viral DNA in the Hirt supernatant (data not shown).

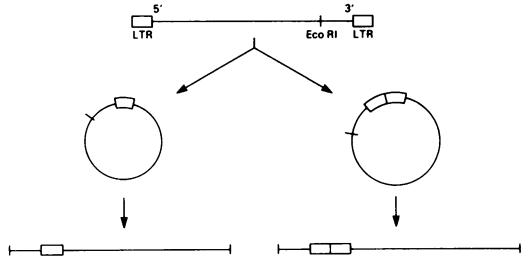


FIG. 8. *Schematic diagram of molecular cloning of 4070A virus and the clones obtained. The top line indicates the linear form of viral DNA with an EcoRI site at 6.9 kb from the 5' end. The two types of circular forms present in the Hirt DNA are shown in the middle portion. The virus was cloned in pBR322 at its unique EcoRI site. Clones circularly permuted at the EcoRI site were of two size classes due to the presence of one or two copies of LTR, as shown in the bottom part. Plasmid DNA is not shown.*

DISCUSSION

We have previously generated detailed restriction endonuclease maps of ecotropic, xenotropic, and MCF viruses isolated from inbred mice and from the wild mouse strain *Mus musculus molossinus* (37; Chattopadhyay et al., in press). The maps of these ecotropic viral DNAs were virtually identical. By contrast, we and others have found that each xenotropic or MCF genome was unique, even those of viruses isolated from the same strain (8, 33; Chattopadhyay et al., in press).

In the present study, we have developed restriction endonuclease maps for several wild mouse viral DNAs, compared these genomes by cross-hybridization techniques, and molecularly cloned an infectious amphotropic MuLV genome. Four pairs of amphotropic and ecotropic California viruses were compared. These viruses were more homogeneous than ecotropic and xenotropic viruses isolated from Kyushu, *M. musculus castaneus,* or inbred mice. Each pair had been isolated from a single mouse. 292E and 1740E were identical, as were 292A and 1740A, although the 292 and the 1740 viruses were isolated from mice in different trapping areas. Similarly, 1504A and 1313A were isolated from mice trapped in different areas, but their DNA maps were almost identical. Except for the segment 6.0 to 8.0 kb, 292E and 292A were virtually identical to each other, as were 1504E and 1504A and 1740E and 1740A. These results strongly suggest that the non-*env* sequences of these ecotropic MuLV's were initially derived from the amphotropic virus through recombination with endogenous ecotropic *env* sequences (or vice versa). Since the only detectable difference between the maps of these three virus pairs resided in the *env* region, these data imply that different in vitro biological activities of the two viruses as well as the different pathology which they induce in susceptible mice is a function of these differences in *env*.

Restriction endonuclease mapping and cross-hybridization studies have both shown that different viral tropisms are associated with major sequence diversion in a segment of *env*. The length of this segment depends to some extent on the viruses being compared, but it has consistently included at least the 6.6- to 7.1-kb region (7). In the *env* region, all amphotropic viruses shared four enzyme sites (*Xho*I at 6.7, *Eco*RI at 6.9, *Hin*dIII at 7.2, and *Pvu*II at 7.5 kb). The *Eco*RI site at 6.9 kb was also found in all xenotropic viral DNAs except for the *M. musculus castaneus* xenotropic virus (Chattopadhyay et al., in press). However, the other three amphotropic sites were not found in virus of any other tropism. All xenotropic viruses contained a *Pvu*II site at 7.0 and a *Bcl*I site at 7.0; these sites were not found in amphotropic or ecotropic MuLV's.

The ecotropic *env* region has been found to be much more heterogeneous, even among only the California mice (Fig. 2), than amphotropic or xenotropic *env* (Fig. 7). Only one restriction endonuclease site (*Sma*I at 6.5 kb) was common to all ecotropic viruses in the 6- to 8-kb segment. Nonetheless, the *env* region showed cross-hybridization among all ecotropic viruses, indicating that the different ecotropic *env* genes were still closely related and that the restriction enzyme site heterogeneity had exaggerated the degree of divergence to some extent. A comparison of the tryptic peptide patterns of the gp70's of wild mouse ecotropic viruses and AKR virus is consistent with our findings (4, 13).

The cross-hybridization experiments between amphotropic and ecotropic MuLV DNAs showed that there was a 1.3-kb region of nonhomology in *env* extending from 6.3 to 7.6 kb from the left end of the viral DNA. This segment includes the 0.6-kb region of nonhomology noted previously between xenotropic and ecotropic MuLV's (7; Chattopadhyay et al., in press), but the region of nonhomology between amphotropic and ecotropic MuLV is larger.

With the exception of *env,* whose homology correlates well with the tropism of the virus, ecotropic, xenotropic, and amphotropic virus genomes cross-hybridize under stringent conditions, which implies that all of these viral DNAs have a similar structural organization. Nonetheless, various degrees of homology have been noted in the non-*env* segments of the viral DNAs. Outside of the tropism region, viruses isolated from the same strains of mice were usually more related to each other than to viruses isolated from other strains. When the restriction endonuclease maps of these viruses were compared according to regions, some other generalizations have been noted.

The viruses isolated from Kyushu and from inbred mice were similar, and they will be referred to collectively as Kyushu type. There appear to be two predominant patterns for the U3 region of the LTR. One was found in the Kyushu and *M. musculus castaneus* xenotropic and ecotropic viruses, which are both of Asian origin. The other was found in the California mouse viruses, with the U3 region of Moloney MuLV being closely related to this type (Fig. 7). F-MuLV appears to be a recombinant between these two types in this region. In the U5 region all the viruses contained the same *Sma*I and

*Kpn*I sites. Relatively few sites have been found in the 0.7- to 2.8-kb region, which probably correspond to the leader sequence and *gag;* however, no sites were common to all viruses isolated from the three types of wild mice. For example, the *Sst*II site at 1.7 kb was found only in Kyushu and *M. musculus castaneus* viruses, whereas the *Pvu*II site at 1.9 kb was found only in *M. musculus castaneus* and California viruses. Many sites from 3.0 to 5.8 kb, probably corresponding to *pol,* were shared by viruses isolated from all three mouse types; in cross-hybridization studies, this region often appears to hybridize more extensively than does *gag*. These results are consistent with immunological studies which have found greater homology among the reverse transcriptases of MuLV's than among the *gag* proteins (2, 42, 44).

The cloning of infectious viral DNA from circular viral DNAs of helper-independent MuLV's has been reported previously (3, 35). Although earlier cloning efforts have resulted in the isolation of apparently full-length circularly permuted molecules with one or two copies of the LTR, only the molecules with one copy of the LTR were infectious in one study (35), whereas only molecules with two copies of the LTR were infectious in the other report (3). For the molecularly cloned amphotropic MuLV DNA, three of five DNAs with LTR and all three DNAs with two LTRs were infectious, which strongly suggests that the lack of infectivity of some apparently full-length viral DNAs was the result of minor structural lesions in the DNA, rather than because one form is not infectious. Cloned DNAs of avian sarcoma virus with one or two LTR copies have also been infectious (22). In this regard, it may be significant that the cloning of amphotropic MuLV DNA was associated with the loss of one of the *Cla*I sites which was present in the viral DNAs in the Hirt supernatant from which the viral DNA was cloned. The loss of this *Cla*I site was also found in amphotropic MuLV derived from transfection of the cloned viral DNA. The loss of an *Xba*I site was also noted during the molecular cloning of the Friend spleen focus-forming virus (29). In both of these instances, the viral DNAs were infectious despite these alterations in nucleotide sequence, but other mutations might decrease or abolish the infectivity of the DNA.

The cloned amphotropic viral DNA will permit more detailed analysis of the relationship between this virus and other MuLV's. Liquid hybridization data have produced conflicting results when endogenous amphotropic sequences have been measured in cell DNAs of inbred mice (1, 6). The use of rigorously defined probes from the cloned amphotropic viral DNA may help to determine definitively whether amphotropic sequences are endogenous to inbred mice. This viral DNA can also be used to construct recombinants with F-MuLV DNA to explore the leukemogenic activity of the latter virus. The oncogenic information of F-MuLV has recently been localized to the *env* LTR segment (36). Since amphotropic virus does not induce F-MuLV type disease, the recombinants should help to define further the viral gene sequences of F-MuLV which are required for this oncogenic activity. This study has revealed sequence differences between the LTRs of amphotropic MuLV and F-MuLV which might be significant biologically. Another possible clue to the pathogenic property of F-MuLV suggested by the F-MuLV DNA map may lie in finding of *Bam*HI and *Pst*I sites at 6.2 kb and 7.5 kb, respectively. These sites, which flank the nonecotropic sequences of MCF viruses, have been found in MCF viruses (Chattopadhyay et al., in press), which have also been isolated from spleens of mice infected with ecotropic F-MuLV (47); these sites were not present in Kyushu ecotropic MuLV's. If the generation of MCF viruses were important pathogenetically, it might be speculated that F-MuLV is highly pathogenic in part because sequences around these sites homologous to endogenous MCF sequences may facilitate the formation of MCF viruses.

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