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Molecular Cloning and Characterization of Murine Leukemia Virus-Related DNA Sequences from C3H/HeN Mouse DNA

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Ten murine leukemia virus (MuLV)-related DNA sequences were isolated from C3H/HeN mouse genomic DNA by cloning of *Eco*RI fragments in a Charon 4A vector. Detailed restriction endonuclease maps of four of the clones were developed by using AKR MuLV [^{32}P]cDNA as a probe. C3H clone 14-9 contains approximately 7 kilobase pairs of MuLV-related DNA, one copy of an MuLV long terminal repeat-like sequence, and a region of flanking mouse DNA. C3H clones 34.2 and 36.1 contain approximately 2 kilobase pairs of MuLV-related DNA, one copy of a MuLV LTR-like sequence, and differing lengths of flanking mouse DNA sequences. C3H clone 8.13 was found to contain an insert of 5.7 kilobase pairs of MuLV-related DNA with two long terminal repeat-like regions and sequences which are partially homologous to AKv-1. Comparison of the restriction endonuclease cleavage maps of these C3H clones with maps recently developed for ecotropic and xenotropic MuLV DNAs indicates that C3H clone 14-9 corresponds to the 5'-terminal portion of a genomic DNA sequence related to xenotropic MuLVs, whereas C3H clones 34.2 and 36.1 correspond to the 3' terminal portions of genomic DNA sequences related to xenotropic MuLVs. Clone 8.13 represents a deleted, xenotropic MuLV-related provirus. C3H clones 14-9, 34.2, 36.1, and 8.13 provide defined DNA sequence probes with which to characterize the organization and expression of endogenous MuLV-related DNA sequences in the mouse genome.

Chromosomal DNA from mouse cells contains multiple copies of endogenous DNA sequences related to the RNA genome of murine leukemia viruses (MuLVs) (9). These MuLV-related DNA sequences give rise to several classes of MuLVs that differ in host range: ecotropic viruses that replicate only in murine cells, xenotropic viruses which replicate preferentially in cells from other species (22), and amphotropic viruses that replicate in both murine and nonmurine cells and have been found only in wild mice from California (3, 16, 25).

The relationship between the multiple endogenous MuLV-related DNA sequences and the MuLVs that they encode is only partially understood. Loci for the endogenous ecotropic MuLVs and inducible xenotropic MuLVs have been characterized genetically (18–20). However, these account for only a few of the 15 to 30 copies of MuLV-related sequences found in mouse DNA (4, 9). The majority of these endogenous MuLVs appear to be related to the xenotropic class of viruses. In spite of the evidence for numerous copies of this class of viruses by hybridization, most strains of mice contain only a single locus that has been genetically mapped by activation of infectious virus (20). This inducible xenotropic MuLV locus appears common to a variety of strains of mice, suggesting that unlike the ecotropic class of viruses, this locus was acquired before the establishment of inbred strains. The discrepancy between the biological experiments that indicate a single locus for infectious virus and the hybridization experiments that detect multiple copies suggest that the majority of proviruses are either transcriptionally repressed or defective proviruses.

Recombinant DNA techniques have been used to clone murine retroviral DNA from cells chronically infected with AKR-type MuLV (23). We report here the molecular cloning and initial characterization of 10 MuLV-related DNA sequences from the DNA of C3H/HeN mice. Restriction endonuclease mapping shows that several of the cloned sequences carry restriction enzyme cleavage sites characteristic of xenotropic MuLV DNAs. One of these isolates is an unusual MuLV-related DNA sequence containing two internal deletions. The cloned fragments have also been used to analyze the families of

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MuLV-related sequences in mouse DNA as well as their distribution in the DNAs of a variety of mouse strains (29).

MATERIALS AND METHODS

Extraction of cellular DNA. Spleens from five weanling C3H/HeN mice were isolated and washed twice in cold RPMI-1640 medium (GIBCO Laboratories) and then forced through a wire mesh sieve (E-C Apparatus Corp.). The suspension was then filtered through nylon mesh bags or, with large amounts of tissue, was first filtered through two or three layers of sterile gauze. The cells were centrifuged at 1,500 × *g* for 10 min, washed once in RPMI-1640, and then suspended in 0.1 M NaCl–0.02 M Tris-hydrochloride (pH 7.5)–0.01 M EDTA (extraction buffer) in a final volume 20 times the volume of packed cells. The cell suspension was then made 0.5% (wt/vol) sodium dodecyl sulfate (SDS), 100 μg of proteinase K per ml, and 500 μg of RNase A per ml and incubated for 16 h at room temperature. DNA was extracted with an equal volume of phenol saturated with 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA and centrifuged, and the aqueous phase was extracted with an equal volume of phenol-chloroform (1:1). This step was repeated until there was no longer any material at the interphase. DNA was precipitated with 1/9 volume of 2 M sodium acetate (pH 5.5), and 2 volumes of cold ethanol and spooled out with a glass rod. After the DNA was dissolved in extraction buffer, proteinase K, RNase A, and SDS were added to the concentrations described above, the mixture was incubated at 37°C overnight, and the DNA was again extracted with an equal volume of phenol-chloroform until no material was present at the interphase. It was then precipitated, spooled, dissolved in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA, and dialyzed against the same buffer for 2 to 3 days with three changes of buffer. The ratio of optical density at 260 nm to that at 280 nm for the dialyzed DNA solutions was ≥1.8.

Murine leukemia virus. Ecotropic MuLV from AKR mice was prepared from an established cell line of AKR mouse embryos continuously producing ecotropic type C virus.

Enzymes. Restriction endonucleases were purchased from Bethesda Research Laboratories, New England Biolabs, or Boehringer Mannheim Corp. Avian myeloblastosis virus (AMV) reverse transcriptase, *Escherichia coli*, DNA polymerase I, DNase I, RNase I, and proteinase K were purchased from Boehringer Mannheim.

Preparation of 34S RNA from AKR ecotropic type C virus. Virus was harvested at 3- to 4-h intervals, banded in sucrose, and collected by centrifugation as described previously (18). Virus was then adjusted to 0.5% SDS, kept at room temperature until the solution cleared, and then made 20 mM Tris (pH 8.5), 10 mM EDTA, and 800 μg of DNA carrier per ml. The viral RNA was extracted with an equal volume of phenol, the interphase was extracted with phenol, and the two aqueous phases were extracted with phenol-chloroform (1:1). The aqueous phase was then precipitated with 1/9 volume of 2 M sodium acetate (pH 5.5) and 2 volumes of cold ethanol for 16 h at −20°C. High-molecular-weight RNA was purified by centrifugation over a 10 to 30% sucrose gradient for 4.5 h at 40,000 rpm in an SW41 rotor, and peak fractions were precipitated as above. RNA was centrifuged for 45 min at 40,000 rpm, dissolved in 10 mM Tris (pH 7.5–20 mM NaCl–1 mM EDTA–0.05% SDS, and heated to 80°C for 3 min to obtain 34S RNA. It was then centrifuged over a 10 to 30% sucrose gradient at 40,000 rpm for 8 h in an SW41 rotor. Peak fractions were collected, precipitated, and centrifuged as described above and dissolved in 10 mM Tris (pH 7.5–20 mM NaCl–1 mM EDTA for storage at −70°C.

Preparation of $cDNA_{AKR}$. Synthesis of $cDNA_{AKR}$ was performed in the following 200-μl reaction mixture: 2 μg of 34S AKR viral RNA; 200 μCi of [^{32}P]dTTP (dried down from a solution in 50% ethanol); 50 mM Tris (pH 8.1); 2 mM dithiothreitol; 7.5 mM $MgCl_2$; 200 mM each dATP, dCTP, and dGTP; 440 μg of calf thymus DNA primer (prepared by incubating 500 mg of calf thymus DNA with 1 mg of DNase I for 45 min at 37°C followed by phenol extraction, DEAE column chromatography, and ethanol precipitation of primer DNA); and 100 U of AMV reverse transcriptase (Boehringer-Mannheim). After 30 min at 37°C, the reaction was stopped by the addition of EDTA to 0.1 M and SDS to 0.05%, and the cDNA was extracted with an equal volume of phenol. After the addition of 100 μg of sonicated and denatured calf thymus DNA, the aqueous phase was adjusted to 0.4 M KOH and kept at room temperature for 16 h. The cDNA was neutralized with acetic acid, purified by chromatography over a 15-ml column of G-50 Sephadex (medium) equilibrated with 10 mM Tris (pH 7.5)–1 mM EDTA, and stored at −20°C.

Bacteria and bacteriophage strains. The EK2 certified vector Charon 4A (National Institutes of Health [NIH] catalog no. D-201-010) and its hosts *E. coli* DP50 *supF* (NIH catalog no. D-003-015) and K802 (*hsr*$^-$ *hsm*$^+$ *galK*$^-$ *su*II *lacY*$^-$ *md*$^-$) (NIH catalog no. D-TPG-001-015) were obtained through the Research Resources Branch, National Institute of Allergy and Infectious Diseases. *E. coli* strains LE392, NS428:N205 (λ*susA*11 *b2 red*3 *c*I857 *susS*7), NS433:N205 (λ*susE*4 *b2 red*3 *c*I857 *susS*7, and W3350 (*su*$^-$) were kindly provided by N. Sternberg.

Growth of vector and hybrid phage and isolation of DNA. Lambda Charon 4A or Charon 4A bearing MuLV-related DNA inserts were grown in *E. coli* DP50 *supF* or LE 392 cells by standard techniques (12). DNA was prepared by disrupting phage with 0.1 M NaCl–0.1 M EDTA–0.1% SDS followed by phenol and chloroform extractions. Phenol was removed by dialysis against 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA, and the DNA was pecipitated with ethanol when concentration required.

Construction of hybrid phage containing C3H DNA. Purified Charon 4A and C3H/HeN mouse DNA preparations (16 μg) were separately digested with 100 U of *Eco*RI in 90 mM Tris-hydrochloride (pH 8.0)–10 mM $MgCl_2$ for 1 h at 37°C, and the reaction was terminated by the addition of 1/10 volume of freshly prepared 1% diethylpyrocarbonate solution in ethanol. The reaction mixtures were then incubated at 37°C for 10 min followed by aspiration in a vacuum dessicator for 5 min. Samples of the *Eco*RI digests containing 7.1 μg of Charon 4A DNA and 1.5 μg of C3H/HeN mouse DNA were ligated with 0.2 U of T4 DNA ligase (Miles Laboratories, Inc.) in a 72.5-μl solution containing 86 mM Tris hydrochloride (pH 8.0), 33 mM $MgCl_2$, 25

mM dithiothreitol, and 0.125 mM ATP. After incubation for 60 h at 4°C, sample containing 5 μg of ligated DNA was packaged into infectious lambda particles as described by Enquist and Sternberg (13). Electrophoresis of samples of the *Eco*RI-digested and ligated DNA samples on 0.8% agarose gels confirmed the completeness of the digestion and ligation reactions. The efficiency of the in vitro packaging reaction was about 3×10^4 infectious particles per μg of ligated DNA.

Detection of recombinants bearing MuLV-related DNA sequences. The packaging reaction contained about 1.5×10^5 PFU per ml when plated on DP50 *supF* grown overnight in NZYDT broth (2) and contained no revertant lambda phage when plated on W3350. The complete packaging reaction was then plated on DP50 *supF* grown overnight in NZYDT broth containing 0.2% maltose at a dilution that yielded about 1,700 plaques per 10-cm petri plate. Nitrocellulose filters (Schleicher & Schuell Co.; BA85) were applied to each plate, and recombinant phage containing MuLV-related DNA sequences were located by the technique of Benton and Davis (1). Nitrocellulose filter replicas containing denatured phage DNA were incubated with AKR MuLV [^{32}P]cDNA. Plaques that hybridized with the AKR MuLV [^{32}P]cDNA probe (13 of about 170,000 screened) were suspended in 10 mM $MgSo_4$–0.1% gelatin–10 mM Tris-hydrochloride (pH 7.4) and recloned.

Analysis of DNA by gel electrophoresis and Southern blotting techniques. DNA was digested with restriction endonucleases under conditions specified by the suppliers, heated to 68°C for 15 min, and quickly cooled on ice. Samples were then electrophoresed into a 6-mm 1% agarose gel containing 20 mM sodium phosphate (pH 7.0), 2 mM EDTA, and 1 μg of ethidium bromide per ml by using a horizontal apparatus (Aquebogue Machine and Repair Shop, Aquebogue, N.Y.), and the DNA was transferred to nitrocellulose filter paper by the method of Southern (27). The filters were then placed in bags and incubated in hybridization buffer (Denhardt solution in 50% formamide) for 2 h. This solution was then replaced with 10 ml of hybridization buffer containing 10^7 cpm of AKR MuLV [^{32}P]cDNA and incubated at 24 h at 42°C. After hybridization, all filters were washed with rocking in 500 ml of Denhardt solution for 30 min at room temperature followed by 1 liter of 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 30 min at room temperature, 1 liter of 0.2× SSC–0.2% SDS for 1 h at 60°C, and finally with two changes of 250 ml of 0.1× SSC for 10 min each at room temperature. Filters were dried with a lamp and exposed to Kodak XR-1 film with a single intensifying screen for 1 to 5 days at −70°C.

Recloning to eliminate nonhybridizing mouse DNA sequences. To separate cloned MuLV-related DNA segments from nonhybridizing cloned C3H/HeN mouse DNA segments (see below), purified recombinant DNA preparations were digested with *Eco*RI and religated with T4 DNA ligase as described above. Ligated DNA preparations were transfected into K802 spheroplasts; 20 individual plaques were picked, and small-scale phage lysates were prepared. *Eco*RI digests of the small-scale lysate DNA were analyzed by agarose gel electrophoresis by a modification (O. Smithies, personal communication) of the technique of Cameron et al. (5). Examination of the agarose gel pattern of DNA fragments identified two recombinant phage isolates (designated 14-9 and 40-17) that had lost their nonhybridizing mouse DNA *Eco*RI segments, but retained the *Eco*RI fragment containing MuLV-related DNA sequences. These recombinant phages were plaque purified, and recombinant DNA was prepared from large-scale lysates as described above.

Preparation of in vitro nick-translated DNA. The DNA to be labeled (0.5 μg or less) was added to a buffer containing 50 mM Tris (pH 7.5), 10 mM magnesium sulfate, 1 mM dithiothreitol, 5.0 μg of bovine serum albumin, and 30 mM of each deoxynucleotide triphosphate minus the one or two labeled with α^{32}P. DNase was then added to a final concentration of 0.02 μg/ml, and the entire reaction mix was transferred to a tube containing 50 to 100 μCi of [^{32}P]dTTP or [^{32}P]dTTP and [^{32}P]dATP (lyophilized to dryness). After the addition of *E. coli* polymerase I to a final concentration of 640 U/ml, the 20-μl reaction was incubated at 14°C for 2 h. Synthesis was stopped by the addition of 25 μl of a solution of 20 mM trisodium EDTA, 2 mg of sonicated and denatured salmon sperm DNA per ml, and 0.2% SDS. The labeled DNA was purifed as described above.

Isolation of subgenomic DNA fragments. DNA from C3H clone 14-9 was digested with *Xba*I and *Bam*HI, and the fragments were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. Agarose containing the 4.2-kilobase-pair (kbp) band was then cut out of the gel, and the DNA was isolated by a butanol extraction method as described previously (21).

Heteroduplex analysis. Heteroduplex analysis was performed as previously described (26).

Biological and physical containment. These experiments employed the EK2 certified host vector system Charon 4A/DP50 *supF* and in vitro packaging requirements specified in the National Institutes of Health Guidelines for Recombinant DNA Research. Before January 29, 1980, all experiments involving recombinant phage were carried out under P2 physical containment conditions.

RESULTS

Characterization of phage bearing MuLV-related C3H DNA fragments. Three of the 13 original isolates gave rise to plaques which showed light and variable hybridization with the [^{32}P]cDNA probe and were not further characterized. The cloned C3H DNA segments were initially characterized by cleavage with *Eco*RI, separation of the DNA fragments on agarose gels, Southern transfer to nitrocellulose, and hybridization with AKR MuLV [^{32}P]cDNA. The sizes of hybridizing and nonhybridizing *Eco*RI DNA fragments observed for the remaining 10 original recombinant phage isolates are summarized in Table 1. Each recombinant DNA that contained hybridizing MuLV-related DNA segments also contained one or more nonhybridizing *Eco*RI DNA segments. This may be because the in vitro packaging system selectively pack-

TABLE 1. Characteristics of MuLV-related C3H/Charon 4A Recombinant DNAs

Clone no.	Sizes of *Eco*RI fragments (kbp)[a]	Description or interpretation
8.13[b]	**7.6**, 5.9	Deleted endogenous MuLV-related DNA
14.02	**11**, 6.2	See clone 14-9
25.02	**13**, **8.0**, 4.4	Deleted endogenous MuLV-related DNA
34.20	8.8, **6.8**, **4.3**	3′ MuLV-related sequences
36.10[b]	**12**, 2.7, 1.5, 0.9	3′ MuLV-related sequences
36.20	7.5, **7.0**, 2.3	Hybridizing region of at least 5 kbp
39.10	6.8, **4.8**, **4.6**, 2.4	Hybridizing region of about 4 kbp
40.20[b]	**14**, **8.4**, 2.7	See clone 40-17
42.00	7.6, **5.3**	Not further characterized
44.10	8.2, 4.6, 4.0, **2.8**	Contains at most 1.8 kbp of hybridizing DNA
14-9[c]	**11.4**	5′ MuLV-related sequence
40-17[b,d]	**13**	Unrelated to known MuLVs by restriction enzyme mapping
pIH1[e]	6.2	See clone 36.1

[a] Nonvector *Eco*RI fragments only; fragments which hybridize with AKR MuLV [^{32}P]cDNA probes are in boldface type.

[b] Phage lysates gave rise to two to three phage bands when phage were purified on CsCl gradients; data shown are for DNA from the middle phage band.

[c] Recloned from clone 14.02.

[d] Recloned from clone 40.20.

[e] *Eco*RI-*Bam*HI fragment subcloned into plasmid pBR322.

ages recombinant DNA molecules containing larger inserts (13). Alternatively, perhaps the C3H DNA fragments cloned had not been completely digested with *Eco*RI. Three of the 10 recombinant phage isolates (8.13, 36.10, and 40.20) gave rise to multiple phage bands on CsCl gradients when purified from large-scale phage lysates. The nonhybridizing *Eco*RI DNA fragments in two of the original isolates (14.02 and 40.20) were eliminated by a second cycle of cloning (see above) to yield the recombinant phage 14-9 and 40-17. The nonhybridizing fragments in clone 36.1 were eliminated by cloning a *Eco*RI-*Bam*HI subfragment into the plasmid pBR322.

We initially selected for more detailed study several of the MuLV-related C3H DNA clones containing large hybridizing *Eco*RI fragments. Detailed characterization of C3H clones 14-9, 34.2, 36.1, and 8.13 is described below. In addition, we performed a more limited characterization of several additional MuLV-related DNA segments from C3H DNA (Table 1). Finally, the different variants of clone 40-17 contained up to three tandem repeats of DNA sequences that hybridized with our AKR MuLV [^{32}P]cDNA probe. A detailed restriction enzyme cleavage map of clone 40-17 was developed (T. Bell, unpublished data) that indicates that clone 40-17 does not resemble any known MuLV DNA. The physical map and behavior of clone 40-17 will be described separately.

Detailed characterization of C3H clone 14-9 DNA. A restriction endonuclease cleavage map was prepared by digesting C3H clone 14-9 DNA with various restriction endonucleases, separating the DNA fragments by agarose gel electrophoresis, transferring the DNA fragments to nitrocellulose filters (27), and hybridizing the DNA fragments to AKR MuLV [^{32}P]cDNA. A sample of the results obtained for C3H clone 14-9 is shown in Fig. 1, which is interpreted as follows. *Eco*RI digestion (lane 2) generated a single hybridizing DNA fragment of 11.4 kbp. Digestion with *Eco*RI and *Xho*I (lane 3) produced two hybridizing fragments of 9.1 and 2.3 kbp. Digestion with *Xho*I alone (lane 4) yielded only two DNA fragments of about 29 and 12.8 kbp, both of which hybridized with AKR MuLV [^{32}P]cDNA. Since *Xho*I does not cut the arms of Charon 4A (10), the 2.3-kbp hybridizing fragment must be attached to the right arm of the vector DNA (10.5 kbp), and the 9.1-kbp hybridizing fragment must be attached to the left arm of the vector DNA (19.8 kbp). Cleavage with *Eco*RI and *Sal*I (lane 5) produced two hybridizing fragments of 8.7 and 2.7 kbp. Since digestion with *Sal*I alone generates only two fragments of approximately 28 and 16 kbp, both of which hybridize with AKR MuLV [^{32}P]cDNA (data not shown), the single *Sal*I site in the hybridizing fragment is located 0.4 kbp to the left of the single *Xho*I site.

Cleavage of 14-9 DNA with *Eco*RI and *Pst*I (Fig. 1, lane 6) yielded two hybridizing fragments: a 4.8-kbp fragment, which from other experiments were determined to be generated by *Pst*I, and a 2.2-kbp fragment, which ended at an *Eco*RI site. Digestion with *Eco*RI, *Pst*I, and *Sal*I (lane 7) yielded two hybridizing fragments of 4.4 and 2.2 kbp and a small fragment of approximately 0.6 kbp whose hybridization was barely detectable on the original autoradiogram. These results locate the 4.8-kbp *Pst*I fragment with respect to the single *Sal*I site and strongly suggest that the 2.2-kbp hybridizing *Pst*I-*Eco*RI fragment is located at the right end of the cloned insert. In addition, there is clearly about 4.2 kbp of DNA in the cloned 14-9 insert that does not hybridize strongly with our AKR MuLV [^{32}P]cDNA probe. All of our restriction endonuclease cleavage mapping results are consistent with the interpretation that the nonhybridizing

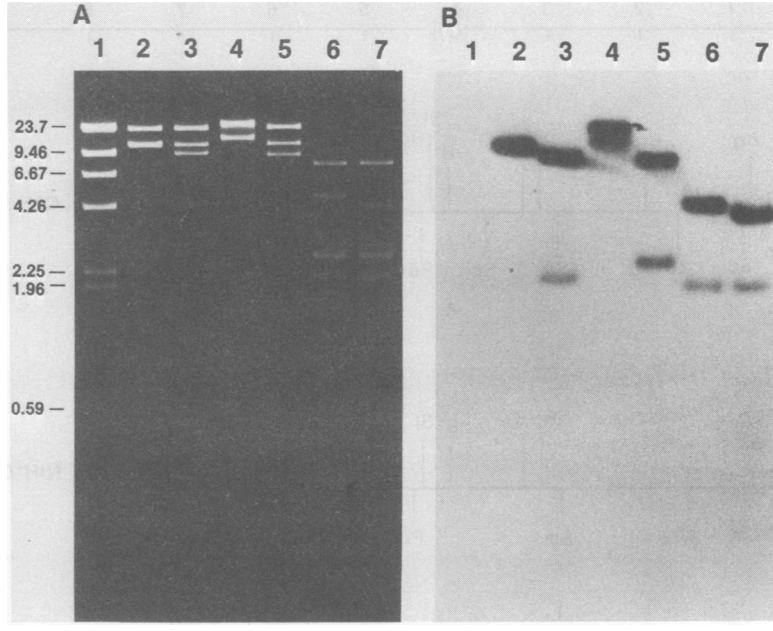


FIG. 1. Restriction endonuclease digestion of C3H clone 14-9 DNA. (A) Photograph of ethidium bromide-stained gel under UV light. Lane 1 shows the positions of the 23.7-, 9.46-, 6.67-, 4.26-, 2.25-, 1.96-, and 0.59-kbp fragments in an *Hin*dIII digest of wild-type λ DNA used as molecular weight markers in this and subsequent gels. Subsequent lanes show fragments of clone 14-9 DNA after digestion with the following: 2, *Eco*RI; 3, *Eco*RI and *Xho*I; 4, *Xho*I; 5, *Eco*RI and *Sal*I; 6, *Eco*RI and *Pst*I; 7, *Eco*RI, *Pst*I and *Sal*I. (B) Autoradiograph of hybridization with AKR MuLV [^{32}P]cDNA of DNA fragments transferred from the gel in A.

region of cloned 14-9 DNA is located at the left end of the cloned insert. Using the *Xho*I, *Sal*I, and *Pst*I cleavage sites located above as points of reference, we used digestion with additional combinations of restriction enzymes to develop the physical map of C3H clone 14-9 shown in Fig. 3.

Comparison of C3H clone 14-9 and NIH clone 621. Lowy et al. (23) cloned several integrated AKR MuLV-like DNAs from the DNA of NIH 3T3 cells productively infected with ecotropic AKR MuLV. NIH clone 621 differed from the infectious DNA segments they isolated (e.g., NIH clone 623) in that it was not infectious and contained only a single copy of MuLV long terminal repeat (LTR)-like sequences, and the MuLV-related DNA sequence ended at an *Eco*RI site located approximately 7 kbp from the

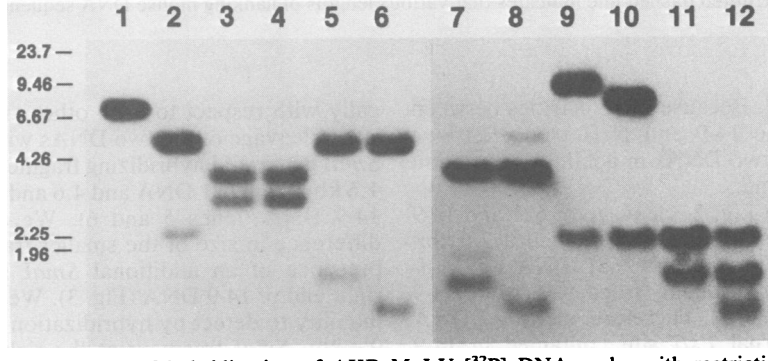
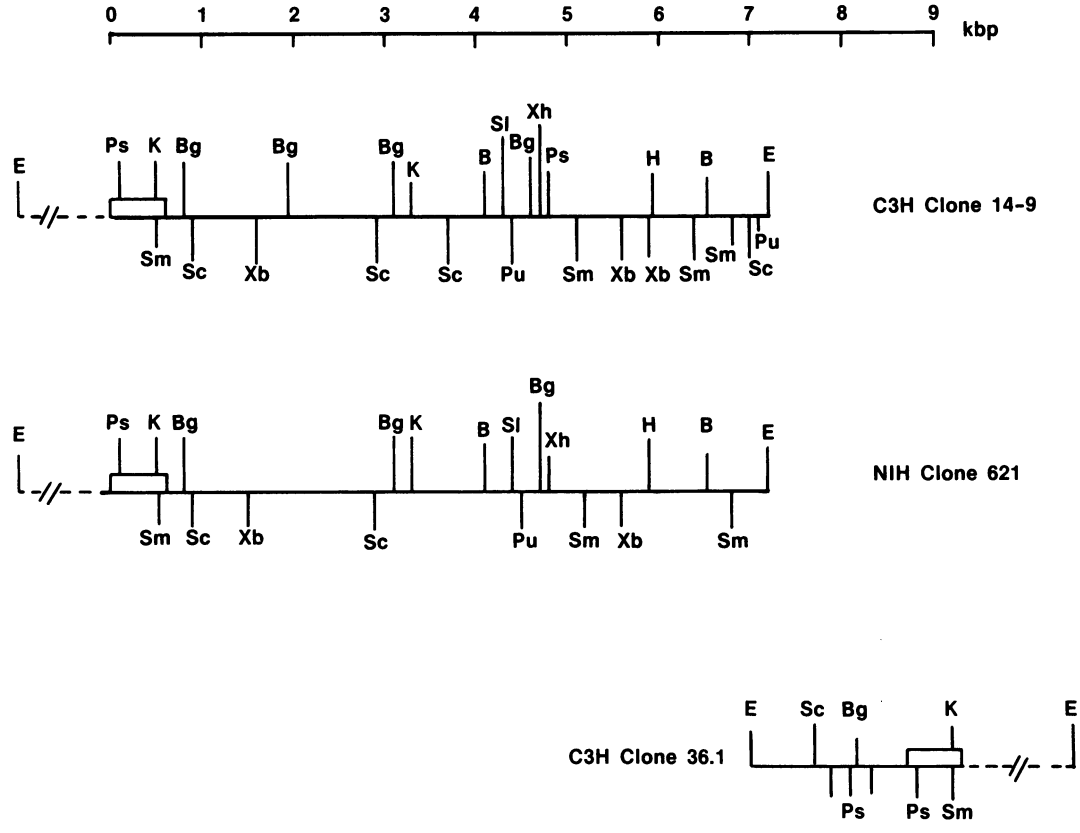


FIG. 2. Autoradiograph of hybridization of AKR MuLV [^{32}P]cDNA probe with restriction endonuclease fragments of NIH clone 621 (23) and C3H clone 14-9 DNAs. The numbers at the right of the autoradiograph indicate the size of *Hin*dIII-digested wild-type λ marker DNA fragments in kbp. In each pair of lanes the digest of NIH clone 621 DNA is on the left and the digest of C3H clone 14-9 DNA is on the right. Lanes: 1 and 2, *Eco*RI-*Pst*I digests; 3 and 4, *Eco*RI-*Kpn*I digests; 5 and 6, *Eco*RI-*Sma*I digests; 7 and 8, *Eco*RI-*Xba*I digests; 9 and 10, *Eco*RI-*Xho*I digests; 11 and 12, *Eco*RI-*Bgl*II digests.



Bg = *Bgl*II	K = *Kpn*I	Sl = *Sal*I
B = *Bam*HI	Ps = *Pst*I	Sm = *Sma*I
E = *Eco*RI	Pu = *Pvu*II	Xb = *Xba*I
H = *Hind*III	Sc = *Sac*I	Xh = *Xho*I

FIG. 3. Restriction endonuclease maps of cloned MuLV-related cellular DNAs. NIH clone 621 was isolated and partially mapped by Lowy et al. (23). The more detailed map was derived from data in Fig. 2 plus additional restriction endonuclease digestions. The boxed regions indicate DNA sequences similar to LTR regions in viral DNAs. The interrupted dashed line indicates that various lengths of flanking mouse DNA sequences are present in the clones.

LTR sequence. Because of similarities between our C3H clone 14-9 and NIH clone 621, we compared the two DNAs in detail by restriction enzyme mapping.

As shown in Fig. 2, cleavage of 621 and 14-9 DNAs with *Eco*RI and *Pst*I yielded a 7.0-kbp hybridizing fragment from 621 DNA and 4.8- and 2.2-kbp hybridizing fragments from 14-9 DNA (lanes 1 and 2). Therefore, clone 621 DNA lacks the internal *Pst*I site contained in 14-9 DNA. Digestion with *Eco*RI and *Kpn*I yielded apparently identical hybridizing fragments of 3.7 and 2.7 kbp from both 621 and 14-9 DNAs (lanes 3 and 4). Thus, the *Kpn*I sites within the hybridizing region of the two clones are located identically with respect to each other and the *Eco*RI site. Cleavage of the two DNAs with *Eco*RI and *Sma*I generated hybridizing fragments of 4.6 and 1.6 kbp from 621 DNA and 4.6 and 1.1 kbp from 14-9 DNA (lanes 5 and 6). We attribute this difference in size of the smaller fragment to the presence of an additional *Sma*I site near the right end of 14-9 DNA (Fig. 3). We interpret our inability to detect by hybridization the expected 0.4-kbp *Sma*I fragment at the right end of 621 DNA and the expected 0.4-kbp *Sma*I fragments at the right end of 14-9 DNA as due to their small size and limited homology with our AKR MuLV [^{32}P]cDNA probes. Consistent with this interpretation are the pronounced differences in re-

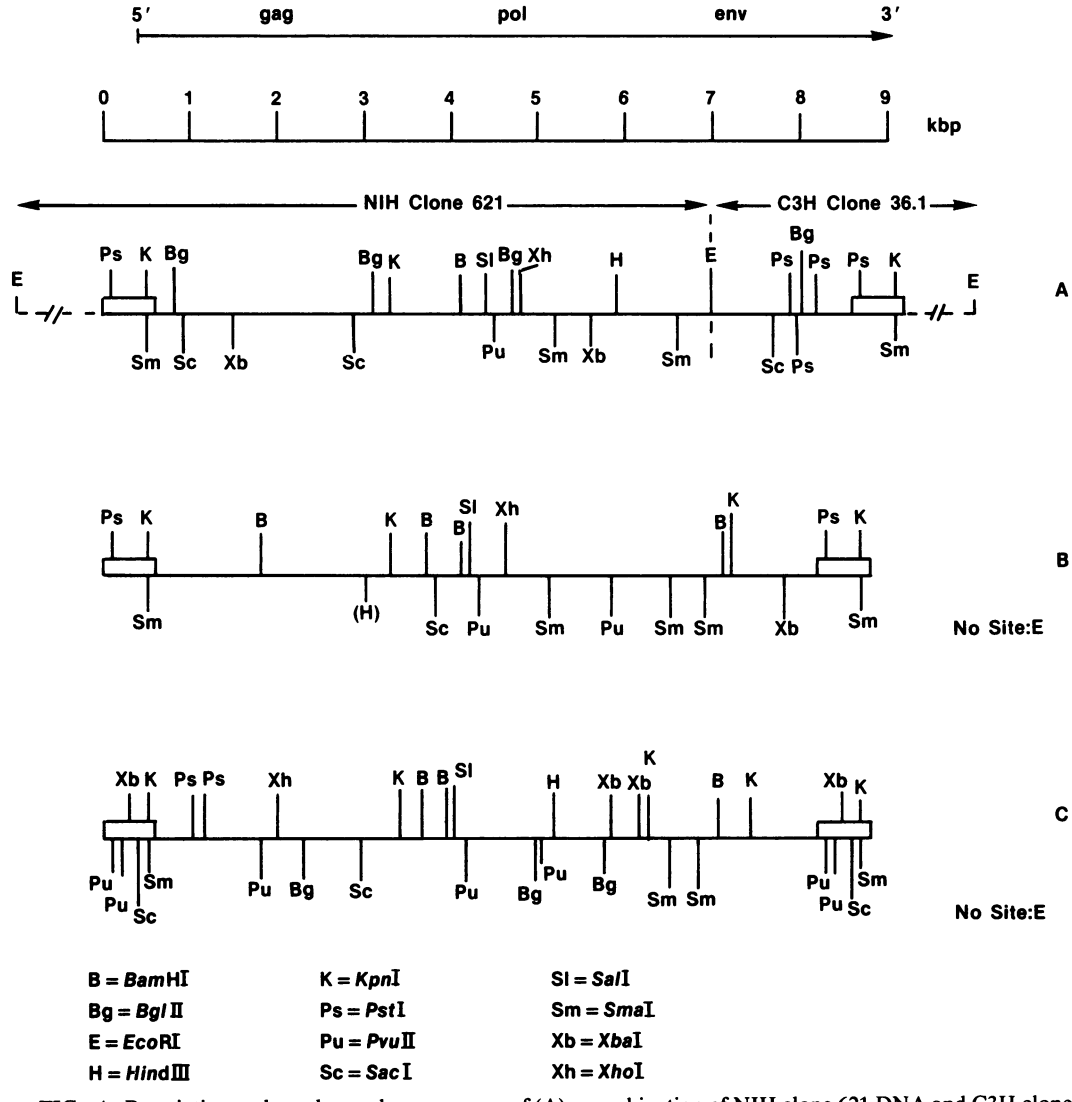


FIG. 4. Restriction endonuclease cleavage maps of (A) a combination of NIH clone 621 DNA and C3H clone 36.1 DNA, (B) cloned AKR-type MuLV DNA (NIH clone 623) (23, 24), and (C) M-MuLV cDNA (14). The boxed regions indicate viral LTR or cellular LTR-like DNA sequences. The interrupted dashed line represents varied lengths of non-MuLV-related flanking mouse DNA sequences.

striction enzyme cleavage patterns between NIH clone 623 (23) and C3H clone 14-9 DNAs starting approximately 5.5 kbp from the end of the left LTR sequence (Fig. 4).

Cleavage of 621 and 14-9 DNAs with *Eco*RI and *Xba*I yielded 4.0- and 1.6-kbp hybridizing fragments from 621 DNA and 4.0- and 1.2-kbp hybridizing fragments from 14-9 DNA (Fig. 3, lanes 7 and 8). This difference is due to the presence of an additional *Xba*I site in 14-9 DNA located as shown in Fig. 3 because digestion of 14-9 DNA with *Xba*I alone fails to generate the 1.2-kbp hybridizing fragment (data not shown). Digestion of these DNAs with *Eco*RI and *Xho*I showed that both DNAs yielded a 2.3-kbp hybridizing fragment and that the other hybridizing fragment was about 10 kbp for 621 DNA compared with 9.1 kbp for 14-9 DNA (lanes 9 and 10). This indicates that the distance from the *Xho*I site to the nearest leftward *Eco*RI site is different for 621 and 14-9 DNAs, presumably because the flanking nonhybridizing mouse DNA sequences are different. Cleavage with *Eco*RI and *Bgl*II yielded 2.3- and 1.6-kbp hybridizing fragments from 621 DNA and 2.3-, 1.6-, and 1.2-kbp hybridizing fragments from 14-9 DNA (lanes 11 and 12).

In summary, these comparisons indicate that

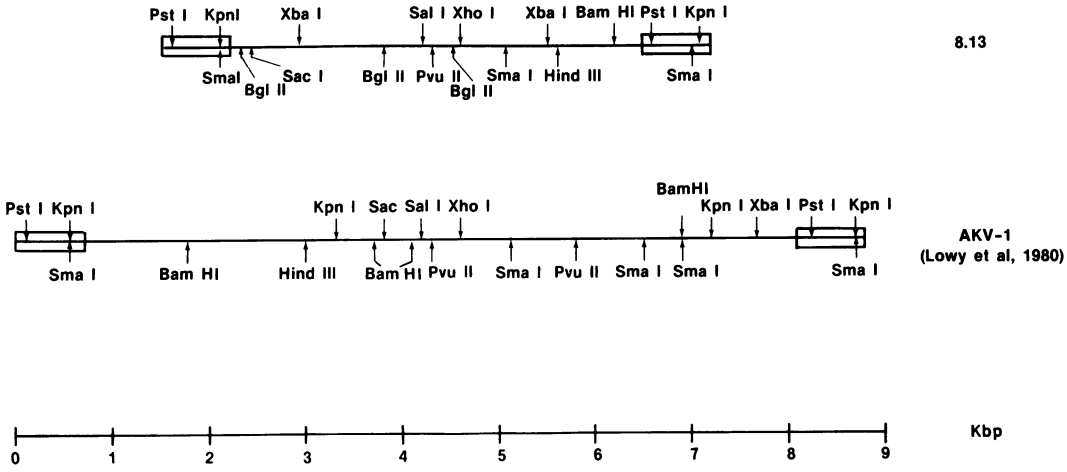


FIG. 5. Comparison of the restriction maps of MuLV-related DNA sequences within C3H clone 8.13 and Akv-1, NIH clone 623 (23). Sites were obtained by restricting DNA with single and multiple enzymes and analyzing fragments by agarose gel electrophoresis and Southern transfer techniques. Fragment sizes of cloned DNA were obtained by comparison with fragments of *Hin*dIII- and *Eco*RI-restricted λ DNA (21.9, 5.0, 4.2, 3.5, 1.95, 1.6, 1.3, 0.95, 0.85, and 0.59 kbp) which were electrophoresed into the same gel (see text).

the MuLV-related region of clone 14-9 DNA differs from that of clone 621 DNA in that (i) 14-9 has additional internal *Pst*I, *Sma*I, *Xba*I, and *Bgl*II cleavage sites, and (ii) the flanking mouse DNA segment is different.

Characterization of C3H clones 36.1 and 34.2. In addition to C3H clone 14-9, which contains approximately 7 kbp of MuLV-related DNA sequences, C3H DNA clones that have approximately 2 kbp of MuLV-related DNA sequences were also isolated. Two examples of such clones (34.2 and 36.1) have identical restriction endonuclease maps for the MuLV-related sequence, but differ in the flanking mouse cellular DNA sequence. This suggests that these MuLV-related DNA sequences are present in more than one chromosomal location within mouse genomic DNA.

The restriction map of the MuLV-related sequences of C3H clone 36.1 is presented in Fig. 3. The map, which was developed initially from Southern analysis (27) of fragments of lambda C3H clone 36.1 DNA hybridizing to AKR MuLV [^{32}P]cDNA, was confirmed by mapping an *Eco*RI-*Bam*HI fragment of clone 36.1 subcloned in pBR322 by the technique of Smith and Birnstiel (28) (data not shown). C3H clone 36.1 and 34.2 DNAs have restriction sites for *Pst*I, *Kpn*I, and *Sma*I in an arrangement characteristic of the LTR sequence of AKR-type MuLVs (24, 26).

When NIH Swiss DNA is digested with *Pst*I, a major MuLV-related fragment of approximately 7.6 kbp, which is cleaved by *Eco*RI to a fragment of approximately 7 kbp, is generated (8, 29). A similar 7-kbp fragment was generated by digestion of NIH clone 621 DNA with *Eco*RI and *Pst*I (Fig. 2). Since we believe that clone 621 represents the 5′ portion of an MuLV-related provirus and that clone 36.1 represents a 3′ region of a similar provirus, we can ask what *Pst*I fragments would be generated if such sequences were joined at their *Eco*RI sites. If a 621-like sequence were joined to a 36.1-like sequence, a *Pst*I fragment of about 7.6 kbp, similar to the major *Pst*I fragment found in mouse genomic DNA, would be generated. This suggests that sequences like those in clone 621 are contiguous with sequences like those in clone 36.1 in mouse genomic DNA. Consistent with this interpretation is our observation that nick-translated C3H clone 36.1 DNA, when used as a probe, hybridized with a 7.6-kbp *Pst*I fragment from NIH Swiss spleen and thymus DNA (data not shown). Thus, DNA sequences like C3H clone 36.1 may be physically linked to DNA sequences like NIH clone 621 in C3H/Hen mouse genomic DNA.

Characterization of clone 8.13 DNA. Clone 8.13 was found to contain an *Eco*RI-resistant sequence of 7.6 kbp which hybridized to an AKR-specific cDNA. A restriction endonuclease map of clone 8.13 DNA was generated to compare it with the restriction maps of other MuLV DNAs. Figure 5 shows the sites in 8.13 DNA recognized by 10 different restriction endonucleases. Three enzymes recognize only one site: *Xho*I, *Sal*I, and *Bam*HI (*Bst*I). Each of these was then used to orient other enzymes recognizing multiple sites. *Pvu*II, *Pst*I, *Kpn*I, and *Hin*dIII recognize two sites, whereas *Sma*I, *Xba*I, and *Bgl*II recognize three. The MuLV-

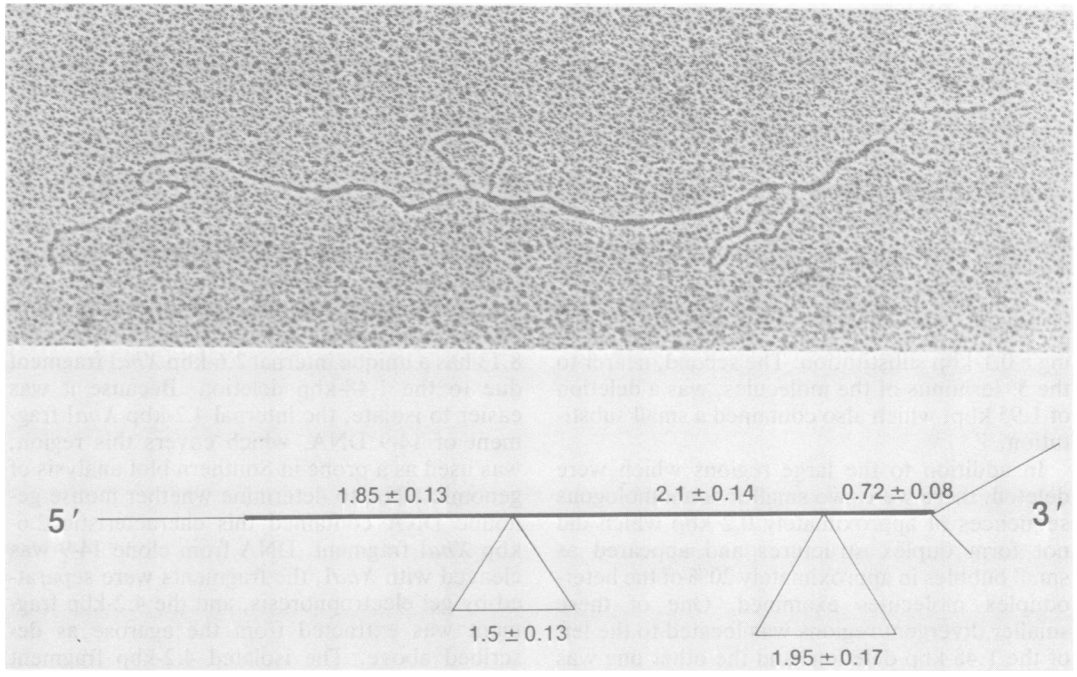


FIG. 6. Heteroduplex analysis of C3H clone 8.13 and NIH clone 623 (23) DNAs digested with *Eco*RI and annealed in the presence of formamide as described in the text and heteroduplex molecules photographed under the electron microscope. The diagram below the photomicrograph shows the approximate sizes in kbp of specific regions of the duplex molecules.

related DNA fragment contains no site for *Pvu*I. Recognition sites for *Pst*I, *Sma*I, and *Kpn*I are present at both ends of the virus-specific sequences in an arrangement suggesting an LTR structure of 0.65 kbp similar to that observed for AKv-1 (23).

When the restriction endonuclease map of clone 8.13 was compared to the map generated for NIH clone 623, an Akv-1 clone (21), three regions of similarity were observed. Approximately 1 kbp encompassing the *Sal*I and *Xho*I sites in the center of the molecules was conserved. In addition, two regions of approximately 0.65 kbp at both the 3′ and 5′ termini of the molecules containing the *Pst*I, *Sma*I, and *Kpn*I sites were conserved. The remaining region was divergent from clone 623 by restriction endonuclease analysis and consisted of two large segments. The first comprised all of the region to the left of the *Sal*I site extending to the left (5′) LTR. The second divergent region began to the right of the *Xho*I site and extended up to the right (3′) LTR. A minor difference in the LTRs of the 8.13 clone was also present. The 5′ LTR appeared identical to the LTRs of clone 623; however, the position of the *Sma*I site of the 3′ LTR of clone 8.13 was slightly but reproducibly different from the position of the *Sma*I site in the 5′ LTR of the same molecule, as well as both LTRs of clone 623 (Fig. 5).

There are two possible explanations for these differences. First, when the lengths of the virus-specific sequences were compared in clones 8.13 and 623, it was apparent that the clone 8.13 sequence lacked approximately 3.2 kbp which were present in clone 623 DNA. Thus, the differences in restriction endonuclease sites between the two cloned DNAs could be explained by a deletion of sequences from 8.13 DNA which are present in 623 DNA. Additionally, differences in restrictions sites might be because clones 623 and 8.13, although related, represent different classes of MuLV proviral DNA sequences as is the case between clone 623 and C3H clones 14-9, 621, and 36.1.

The first possibility was confirmed by heteroduplex analysis. DNA from clones 8.13 and 623 was cut with *Eco*RI, denatured, and renatured in the presence of formamide. Heteroduplex molecules were formed between the two cloned DNAs and observed under the electron microscope (Fig. 6). As shown schematically in Fig. 6, there were two major regions of nonhomology between 8.13 and 623 DNAs. The first region of nonhomology, near the 5′ terminus of the molecules, consisted of a 1.48-kbp deletion contain-

TABLE 2. DNA fragments common to clones 8.13, 14-9, 621, and 623

Restriction enzymes	Common DNA fragments (kbp) from:			
	8.13	14-9	621	623
*Sal*I-*Xho*I	0.42	0.42	0.42	0.42
*Sal*I-*Sma*I	0.9	0.9	0.9	0.9
*Sal*I-*Bam*HI	2.0	2.0	2.0	
*Kpn*I-*Xba*I	0.9	0.9	0.9	
*Kpn*I-*Xba*I		2.0	2.0	
*Xho*I-*Xba*I		3.3	3.3	

ing a 0.1-kbp substitution. The second, nearer to the 3′ terminus of the molecules, was a deletion of 1.95 kbp, which also contained a small substitution.

In addition to the large regions which were deleted, there were two smaller nonhomologous sequences of approximately 0.2 kbp which did not form duplex structures and appeared as small bubbles in approximately 20% of the heteroduplex molecules examined. One of these smaller divergent regions was located to the left of the 1.48-kbp deletion, and the other one was located to the right of the 1.95-kbp deletion (data not shown). These results show that C3H clone 8.13 DNA shares extensive sequence homology with NIH clone 623 DNA, and that the gross differences between these two DNAs are regions which are deleted from clone 8.13.

To determine the class to which MuLV proviral DNA clone 8.13 belongs, the following experiment was performed. DNAs from clones 623, 8.13, 14-9, and 621 were digested with the appropriate pairs of enzymes and compared by agarose gel electrophoresis. If the clones contained identical enzyme sites, then the fragments generated should migrate identically. Table 2 presents a summary of the results of this experiment.

As expected, all of the clones contained the internal 0.42-kbp fragment between *Sal*I and *Xho*I. A larger fragment of 0.9 kbp, resulting from digestion by *Sal*I and *Sma*I, which contains the *Sal*I-*Xho*I fragment was also conserved among all four of the clones. However, there was divergence in the position of restriction endonuclease sites outside this region defined by *Sal*I, *Sma*I, and *Xho*I. In particular, the 2.0-kbp fragment resulting from cleavage by *Sal*I and *Bam*HI was found only in clones 8.13, 14-9, and 621. Similarly, two fragments, one of 2.0 kbp generated by the combination of *Kpn*I and *Xba*I and one of 3.3 kbp generated by *Xho*I and *Xba*I, were found only in clones 14-9 and 621 DNAs. These 2.0- and 3.3-kbp fragments are probably absent from C3H clone 8.13 DNA because of the 1.5-kbp deletion in the 5′ region (Fig. 5). An *Xba*I site present on the 5′ side of the deletion in clone 8.13 was conserved in 14-9 and 621, but was absent from 623. We, therefore, conclude that C3H clone 8.13 is more closely related to C3H clone 14-9 and NIH clone 621 than to NIH clone 623.

Demonstration of deleted internal DNA fragments like clone 8.13 within murine DNA. Although it was possible to demonstrate that clone 8.13 was representative of a class of cloned sequences from the murine genome, it was also important to determine whether these sequences exist in the murine genome as cloned. To examine this, we took advantage of the fact that clone 8.13 has a unique internal 2.6-kbp *Xba*I fragment due to the 1.48-kbp deletion. Because it was easier to isolate, the internal 4.2-kbp *Xba*I fragment of 14-9 DNA, which covers this region, was used as a probe in Southern blot analysis of genomic DNA to determine whether mouse genomic DNA contained this characteristic 2.6-kbp *Xba*I fragment. DNA from clone 14-9 was cleaved with *Xba*I, the fragments were separated by gel electrophoresis, and the 4.2-kbp fragment was extracted from the agarose as described above. The isolated 4.2-kbp fragment was then radioactively labeled by nick translation and used as a probe for homologous sequences in *Xba*I digestions of DNA from NIH Swiss, C57BL, C3H, BALB/c, and AKR mice.

Figure 7 shows the results obtained when the 4.2-kbp probe was hybridized to the cellular DNA. The specificity of the probe was demonstrated by the single hybridizing 2.6-kbp band in the lane containing 8.13 DNA. The 4.2-kbp fragment of clone 14-9 DNA was also clearly observed. The labeled higher-molecular-weight fragment seen with clone 14-9 DNA was a result of a minor partial restriction endonuclease cleavage. All of the cellular DNAs contained the 4.2-kbp fragment internal to clone 14-9. The relative intensity of hybridization of the cellular 4.2-kbp band suggests that these sequences are from a highly reiterated provirus class. A smaller fragment of 2.6 kbp internal to clone 8.13 DNA was found also in all of the cellular DNAs; however, the hybridization was much lighter, suggesting that the deleted provirus represented by clone 8.13 is present at a much lower frequency in murine DNA than the provirus represented by clone 14-9. Alternatively, fragments of about 2.6 kbp could be the result of another conserved internal *Xba*I site in an endogenous MuLV provirus that is nondeleted. Additional hybridizing bands smaller than 4.2 kbp were found in *Xba*I digests of all of the cellular DNAs examined. These smaller-molecular-weight fragments may be due to other proviruses with various deletions in the *Xba*I fragment. The fragments larger than 4.2 kbp were found primarily in strains having endogenous ecotropic viruses and probably rep-

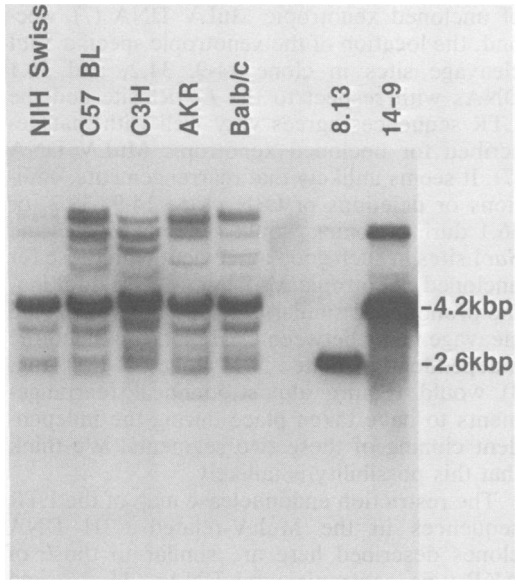


FIG. 7. Analysis of *gag-pol* region sequences within murine DNA. Cellular DNA (10 to 15 μg) from NIH Swiss, C57BL, C3H, AKR, and BALB/c mice, as well as DNA from clones 8.13 and 14-9 (0.05 to 0.1 μg), were restricted with *Xba*I and analyzed by gel electrophoresis and Southern transfer techniques as described in the text. Clone 8.13 and 14-9 specific DNA fragments were visualized by hybridization to 3×10^7 cpm of the ^{32}P-labeled 4.2-kbp *Xba*I DNA fragment from 14-9 DNA.

resent ecotropic viral sequences plus flanking cellular sequences. These results strongly suggest that the proviral viral sequences characteristic of the clone 8.13 DNA exist in the murine genome.

DISCUSSION

We cloned *Eco*RI fragments of C3H/HeN mouse DNA in a Charon 4A vector and characterized several of the isolated MuLV-related DNA sequences by restriction endonuclease cleavage. C3H clones 14-9, 34.2, 36.1, and 8.13 clearly correspond to integrated endogenous MuLV-related DNAs because (i) restriction endonuclease fragments of the cloned DNAs hybridized with AKR MuLV [^{32}P]cDNA, and (ii) their restriction endonuclease cleavage maps contained several features in common with previously published maps of unintegrated MuLV DNAs. In particular, the pattern of a *Pst*I site separated by about 0.5 kbp from coincident *Kpn*I and *Sma*I sites, which defines part of the MuLV DNA LTR sequence, has been previously observed in a wide variety of unintegrated MuLV DNAs (7, 24, 26). In addition, the pattern of single *Sal*I and *Xho*I cleavage sites separated by 0.4 kbp and located about 4.5 kbp from the 5′ end of the LTR sequence in MuLV DNAs (24, 26) was also present in C3H clone 14-9 DNA (Fig. 3). There are also features of the C3H clones which are shared with the endogenous MuLV-related sequences described by Dolberg et al. (12) in BALB/c DNA.

Although there are similarities in the maps of restriction enzyme sites between C3H clone 14-9 DNA (Fig. 3) and ecotropic MuLV DNAs (8, 14, 24, 26), C3H clone 14-9 DNA differed from ecotropic MuLV DNAs in several respects. First, unlike almost all known ecotropic MuLV DNAs, C3H clone 14-9 contains an *Eco*RI cleavage site located about 7 kbp from the left LTR. This is also a characteristic feature of NIH clone 621 (23) (Fig. 3). The sole ecotropic MuLV DNA with an internal *Eco*RI site to date, of 11 isolates tested (24), is an ecotropic MuLV from C3H/Fg cells whose DNA contains an *Eco*RI site 5 kbp from the 5′ end of the viral DNA. Second, comparing the restriction enzyme cleavage map of C3H clone 14-9 with maps for 11 independent ecotropic AKR-type MuLV isolates (24), we find that although there are 10 restriction enzyme cleavage sites in common, there are also 4 sites that are present in all AKR-type ecotropic MuLV DNAs, but not in C3H clone 14-9, and 8 sites that are present in C3H clone 14-9 DNA, but not in AKR-type ecotropic MuLV DNAs (Fig. 3 and 4). In fact, the similarities in restriction enzyme cleavage pattern are limited to the LTR sequence (0 to 0.6 kbp) and the region from 3.3 to 5.1 kbp, which may correspond to the highly conserved coding sequence for MuLV reverse transcriptase (7).

C3H clone 14-9 and NIH clone 621 (23) both have an *Eco*RI cleavage site 7 kbp from the left LTR. We propose that they correspond to the 5′ portion of cellular DNA sequences related to xenotropic MuLVs for the following reasons. First, Chattopadhyay et al. (7) have developed restriction endonuclease cleavage maps for DNAs from 9 xenotropic and 11 mink cytopathic focus-forming viruses, and all 20 of these DNAs have an *Eco*RI cleavage site 7 kbp from the left LTR. This same result also was obtained for an additional four independently characterized MuLV xenotropic viruses in another laboratory (D. Joseph, personal communication). Second, when the xenotropic MuLV restriction maps developed by Chattopadhyay et al. (9) were compared with the ecotropic MuLV DNA restriction maps developed by Rands et al. (24), two additional "xenotropic specific" cleavage sites were identified. One of these is an *Sac*I cleavage site located 2.9 kbp from the left LTR. This cleavage site is also present in both C3H clone 14-9 and NIH clone 621 (23) DNAs (Fig. 3). Thus, the mouse genomic isolates, C3H

clone 14-9 and NIH clone 621, both contain two restriction enzyme cleavage sites that have been shown to be highly characteristic features of all of the xenotropic MuLV DNAs analyzed to date. Because of the variability in restriction endonuclease cleavage patterns among different xenotropic and MCF MuLV DNAs (7), we have limited this discussion to a comparison of those sites that have been shown to be most characteristic of xenotropic viruses.

Similarly, we propose that C3H clone 36.1 (Fig. 3) and the very similar C3H clone 34.2 correspond to the 3′ terminal portion of cellular DNA sequences related to xenotropic MuLVs for the following reasons. First, these clones contain an *Eco*RI cleavage site located about 1.9 kbp from a DNA sequence with the *Pst*I, *Kpn*I, and *Sma*I cleavage patterns characteristic of an MuLV LTR. When combined with a 5′-terminal portion of MuLV DNA containing an *Eco*RI site at 7 kbp from the left LTR (e.g., C3H clone 14-9 or NIH clone 621), C3H clones 36.1 and 34.2 produce an 8.9-kbp DNA sequence bounded by two LTR sequences comparable to full-length infectious MuLV DNAs. Second, C3H clone 36.1 contains the other xenotropic specific cleavage site identified by Chattopadhyay et al. (9), a *Sac*I cleavage site located 7.5 kbp from the left LTR (or about 0.6 kbp from the *Eco*RI site of C3H clone 36.1 as shown in Fig. 4). This *Sac*I site and the two *Pst*I sites are also present near the 3′ terminal LTR in several independently characterized MuLV xenotropic viruses (D. Joseph, personal communication). In addition, it is noteworthy that C3H clones 36.1 and 34.2 contain a *Pst*I cleavage site located such that, if combined with a 5′ terminal sequence like that of NIH clone 621, it would generate the prominent 7.6-kbp internal *Pst*I fragment previously described for mouse genomic MuLV-related DNA (8).

To investigate whether combining the DNAs from C3H clone 14-9 or NIH clone 621 with C3H clone 36.1 would yield infectious virus DNA, transfection experiments were performed. Combinations of DNA from clones 14-9 or 621 and DNA from clone 36.1 were cotransfected into cells by the calcium phosphate technique. In some experiments, the cloned DNAs were first digested with *Eco*RI and then ligated before transfection of cells. The transfected cells were assayed for infectious virus on both mouse and mink (Mv 1 Lu) cells. In no case was the production of infectious virus detected (R. Mural and A. Rein, unpublished observations).

We believe that C3H clones 14-9, 34.2, and 36.1 are unrearranged and undeleted sequences of genomic DNA. As discussed above, the distance from the internal *Eco*RI site to the LTR sequence corresponds well to that characteristic of uncloned xenotropic MuLV DNA (7). Second, the location of the xenotropic specific *Sac*I cleavage sites in clone 14-9, 34.2, and 36.1 DNAs with respect to the *Eco*RI site and the LTR sequences agrees very well with that described for uncloned xenotropic MuLV DNA (7). It seems unlikely that rearrangements, additions or deletions of C3H clone 14-9, 34.2, or 36.1 during cloning would generate *Eco*RI and *Sac*I sites in such good agreement with those for uncloned xenotropic MuLV DNA. In addition, the pronounced similarity in restriction enzyme cleavage maps between C3H clone 14-9 and the independently isolated NIH clone 621 (21) (Fig. 3) would require almost identical rearrangements to have taken place during the independent cloning of these two segments. We think that this possibility is unlikely.

The restriction endonuclease map of the LTR sequences in the MuLV-related C3H DNA clones described here are similar to those of AKR-type ecotropic viral DNAs (24, 26) and those of most xenotropic viral DNAs (7) in having a *Pst*I site located 0.1 kbp from the left end of the LTR sequence. As previously noted by Rands et al. (24), these *Pst*I-type LTR DNA sequences all differ from those previously characterized for MuLV (14, 30, 31) and Moloney sarcoma (11) and Harvey sarcoma (15) viruses which have two *Pvu*II sites and no *Pst*I site near the left end of their LTR sequences (Fig. 4). Whether the LTR sequences in C3H clones 14-9, 36.1, and 34.2 correspond to the larger or smaller variants of the MuLV LTR sequence (7, 24, 26) remains to be determined.

LTR DNA sequence of MuLV and Moloney sarcoma virus have been shown to contain possible DNA sequence signals for initiation of RNA transcription and polyadenylation (11, 31). High-level transcription of genomic DNA sequences immediately adjacent to a newly introduced avian leukosis virus LTR sequence has recently been proposed as a mechanism to explain high-level transcription of specific cellular *myc* DNA sequences (17). It will be of interest to determine whether the LTR DNA sequences contained within C3H clones 36.1 and 34.2 serve as promoters for transcription of flanking mouse DNA sequences. Assuming that they are found to lack mouse repetitive DNA sequences, flanking mouse DNA sequences from C3H clones 36.1 and 34.2 can be used to assay for RNA transcripts from this region.

Individual cloned MuLV-related cellular DNA sequences like C3H clones 14-9, 36.1, and 34.2 and NIH clone 621 (23) provide the starting materials for the construction of a variety of specific DNA sequence probes which will prove useful in the analysis of the multiple MuLV-related DNA sequences in mouse genomic

DNA. Candidates for such specific probes are the *Hin*dIII-*Bam*HI fragment from 5.6 to 6.4 kbp of C3H clone 14-9 and the *Eco*RI-*Pst*I fragment of C3H clone 36.1 which are probably located in the region corresponding to the *env* gene of MuLVs. Chan et al. (6) and Chattopadhyay et al. (8) have demonstrated the utility of this approach in the analysis of mouse genomic DNA sequences related to ecotropic viruses.

C3H clone 8.13, which represents a deleted endogenous provirus, has been shown by both restriction site mapping and heteroduplex analysis to have gross DNA sequence homology to cloned DNA from an ecotropic, AKR-type MuLV (23). The two DNA sequences are sufficiently homologous to form stable heteroduplexes in regions which are not deleted in clone 8.13. These include the 5′ LTR (0.0 to 0.6 kbp), leader (0.7 to 1.0 kbp), 5′ portion of the *gag* gene (1.1 to 2.7 kbp), *pol* (2.8 to 5.9 kbp), and 3′ LTR (8.2 to 8.8 kbp) regions. However, the presence in C3H clone 8.13 of *Xba*I, *Hin*dIII, and *Bam*HI cleavage sites at positions similar to their location in C3H clone 14-9 and NIH clone 621 (Fig. 3) indicates the similarity of C3H clone 8.13 to these xenotropic virus-related DNA sequences. Therefore, we favor the interpretation that C3H clone 8.13 is a deleted, xenotropic virus-related C3H genomic DNA segment.

Compared with AKR-type MuLV DNA (23), C3H clone 8.13 has deleted a 1.48-kbp DNA segment from the *gag-pol* region of the MuLV genome and a 1.95-kbp DNA segment from the *env* region. To our knowledge, this is the first report of a deleted MuLV-related provirus from murine genomic DNA. If such a deleted, MuLV-related DNA sequence exists in C3H mouse DNA, as opposed to being generated during cloning, digestion of C3H DNA with *Xba*I should generate a 2.6-kbp fragment that hybridizes with a *gag-pol* region probe. The results of such a hybridization experiment (Fig. 7) indicated that the expected 2.6-kbp hybridizing fragment could be detected in *Xba*I digests of C3H DNA. Similar-sized hybridizing fragments were also observed in *Xba*I digests of NIH Swiss, C57BL, AKR, and BALB/c mouse DNAs. These results strongly support our interpretation that at least one MuLV-related DNA sequence with a deletion of about 1.5 kbp like C3H clone 8.13 DNA exists in mouse genomic DNA. Such a deleted, MuLV-related DNA sequence could have originated either by germ line integration of a complete xenotropic virus-related DNA followed by deletion, or by integration of a preexisting defective, deleted variant of the xenotropic virus. Because the right end of the 1.95-kbp deletion maps within 0.2 kbp of the start of the 3′ LTR, we have not been able to use this same genomic fragment hybridization approach to analyze the origin of the 1.95-kbp deletion. It remains possible, therefore, that the 1.95-kbp deletion arose during the cloning process.

The data presented here for C3H clone 8.13 characterize it as an MuLV-related mouse genomic DNA segment which contains two internal DNA sequence deletions when compared with the DNA sequence of a cloned ecotropic AKR-type MuLV (21) (Fig. 2). To our knowledge, this is the first report of a deleted MuLV-related murine genomic DNA segment.

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