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Ecotropic Murine Leukemia Virus DNA Content of Normal and Lymphomatous Tissues of BXH-2 Recombinant Inbred Mice

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BXH-2 recombinant inbred mice spontaneously produce a B-tropic murine leukemia virus (MuLV) beginning early in life and have a high incidence of non-T-cell lymphomas. These traits are not characteristic of the progenitor strains (C57BL/6J and C3H/HeJ) or of 11 other BXH recombinant inbred strains. Since B-tropic virus expression may be causally related to the high incidence of lymphoma in this strain, we have analyzed the ecotropic MuLV DNA content of both normal and lymphomatous tissues of BXH-2 mice. Southern analysis and hybridization with an ecotropic MuLV DNA-specific probe showed that DNA of normal BXH-2 tissues contained both parental N-tropic MuLV proviruses but lacked endogenous B-tropic MuLV DNA sequences. In addition, none of 116 F_1 hybrid mice derived from male BXH-2 mice spontaneously produced ecotropic MuLV early in life. These results suggest that the B-tropic virus is horizontally transmitted in BXH-2 mice. Southern analysis of DNA from tumor tissues of 12 BXH-2 mice showed that amplification of ecotropic-specific DNA sequences had occurred in lymphomatous tissues of 3 mice and suggested that these tumors were monoclonal. The number of newly acquired proviruses, which appeared to be structurally nondefective and integrated at different sites, varied from one to three copies. Since lymphomatous tissues from only 3 of 12 mice examined carried additional detectable ecotropic proviruses, these results suggest that amplification of ecotropic MuLV DNA sequences is not required for maintenance of transformation in BXH-2 lymphomas.

Murine leukemia virus (MuLV)-related DNA sequences are inherited in a Mendelian manner in all inbred strains of *Mus musculus*. These endogenous MuLVs can be grouped into three distinct classes depending on host range: ecotropic MuLVs, which infect only mouse cells; xenotropic MuLVs, which infect cells of heterologous species; and amphotropic MuLVs, which infect both mouse cells and cells of certain heterologous species (7, 26). Ecotropic MuLVs are further classified as N-tropic (replicating preferentially on NIH 3T3 cells) or B-tropic (replicating preferentially on BALB 3T3 cells). N or B tropism is determined by a single autosomal dominant gene termed *Fv-1*. Mouse cells carrying an *Fv-1*^{b} or *Fv-1*^{n} allele restrict the replication of N-tropic or B-tropic MuLVs, respectively (23).

Infectious N-tropic and xenotropic MuLVs are often spontaneously expressed in murine tissues and are inducible from cells of many inbred strains in vitro (22, 23). Spontaneous expression of B-tropic viruses is usually observed only in aged *Fv-1*^{b} mice (22), and these viruses are believed to arise by recombination between N-tropic and xenotropic MuLVs (2, 5). However, a B-tropic virus has been induced from SWR/J tissue culture cells (27, 31) and embryo cultures of two *H-2* congenic C57BL/10 strains (18). Infectious B-tropic virus has also been detected in spleens of 3-month-old (leukemia prone) B10.Y congenic mice (4), but younger mice of this strain were found to be negative for infectious virus.

Among 12 BXH recombinant inbred (RI) strains derived from crossing C57BL/6J and C3H/HeJ mice, one RI strain, BXH-2, was shown to produce spontaneously an XC-positive B-tropic MuLV beginning early in life and to have a high incidence of non-T-cell lymphomas (1). Expression of B-tropic MuLV was not characteristic of BXH-2 mice at an intermediate stage of inbreeding (F_{11}), but by the F_{25} generation, these mice were virus positive. C57BL/6J and C3H/HeJ mice, the progenitors of the BXH RI strains, do not produce detectable levels of ecotropic or xenotropic MuLVs early in life, but N-tropic and xenotropic MuLVs are inducible

from cells of these mice in vitro (11, 32). Beginning at approximately 3 months of age in C3H/He ($Fv\text{-}1^n$) mice and 6 months of age in C57BL/6 ($Fv\text{-}1^b$) mice, an antiviral immune response appears which correlates with the onset of N-tropic virus expression (9). Adult mice of both parental strains also express low levels of xenotropic MuLV (16), and B-tropic viruses have been isolated from C57BL/6 mice late in life (28). Therefore, the B-tropic MuLV of BXH-2 mice may have arisen by recombination between parental N-tropic and xenotropic viruses expressed during inbreeding of BXH-2 ($Fv\text{-}1^b$) mice. As RI strains are maintained by brother-sister matings, the B-tropic MuLV genome could then be transmitted horizontally via viremic females, or could eventually integrate within the BXH-2 germ line, or both. Although acquisition of new endogenous ecotropic viral genomes is a rare event, it has been shown to occur in some highly viremic mice (14, 29). Alternatively, the B-tropic MuLV genome may have been present but not expressed early in life in one or both progenitor strains. Generation of the BXH-2 RI strain would then have created a genetic background permissive for B-tropic virus expression.

Since B-tropic virus expression and high lymphoma incidence were traits that were acquired simultaneously during inbreeding of BXH-2 mice, it was of interest to characterize further the ecotropic MuLV DNA content of normal and lymphomatous BXH-2 tissues. In the experiments described here we have characterized these viral DNA sequences by Southern blotting and hybridization with an ecotropic MuLV DNA-specific probe.

MATERIALS AND METHODS

Mice. The BXH RI mouse strains were derived by systematic inbreeding, beginning with randomly chosen pairs of mice from the F_2 generation of the cross of C57BL/6J and C3H/HeJ inbred strains (33). C57BL/6J, C3H/HeJ, P/J, and BALB/cJ mice were obtained from Animal Resources, The Jackson Laboratory, Bar Harbor, Maine.

Cells. The cloned wild mouse embryo cell line, SC-1, was maintained as previously described (1).

Extraction of DNA. High-molecular-weight cellular DNAs were extracted from frozen mouse spleens as previously described (14). DNAs were stored at −70°C in 0.015 M NaCl–0.0015 M sodium citrate (pH 7.0).

Unintegrated BXH-2 B-tropic MuLV DNA was isolated by the Hirt fractionation procedure (8). Briefly, 300 culture dishes (100 mm) were each seeded with 10^6 SC-1 cells and maintained in Eagle minimal essential medium supplemented with 2 μg of polybrene per ml and 10% calf serum. The next day, the culture fluids were removed, and 1.0 ml of medium from SC-1 cells chronically infected with biologically cloned BXH-2 B-tropic MuLV was added to each plate. The multiplicity of infection was approximately 50. After 2 h, 9 ml of the supplemented Eagle medium was added to each dish, and the cells were fractionated by the Hirt procedure approximately 18 h later. Unintegrated B-tropic MuLV DNA was purified from the Hirt supernatant fraction as described by Lowy et al. (17).

Restriction endonuclease digestions, electrophoresis, transfers, and hybridizations. Samples of DNA were digested to completion with an excess of restriction endonuclease under reaction conditions recommended by the manufacturer (New England Biolabs, Inc., Beverly, Mass.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). DNAs (10 μg) were electrophoresed in 0.6% agarose gels and transferred to nitrocellulose filters as previously described (14). Filters were baked, prehybridized, and hybridized at 65°C with a ^{32}P-labeled (nick-translated) ecotropic MuLV DNA-specific probe (14). Nitrocellulose filters were washed (14), air-dried, and autoradiographed at −70°C using Kodak XAR-5 X-ray film and Dupont Lightning-Plus intensifying screens.

Virus assays. Expression of ecotropic MuLV in BXH-2 or BXH-2–derived hybrid mice was determined by the XC plaque assay (1) using tail biopsies or spleen cell cultures from young mice.

Esterase-1 typing. Serum samples were collected from breeding pairs of each BXH RI strain, and 20-μl aliquots were electrophoresed through 5% cyanogum-41 acrylamide slab gels in buffer containing 9 parts distilled water and 1 part EBT (107.8 g of Tris base, 9.25 g of disodium EDTA, and 55 g of boric acid). Electrophoresis was at 250 V for 2 h. Gels were stained in a mixture containing 750 mg of Fast blue BB, 0.5 ml of 14% α-naphthyl butyrate in acetone, and 150 ml of 0.025 M Na_2HPO_4–0.025 M KH_2PO_4–10^{-4} M $CaCl_2$.

RESULTS

Characterization of the endogenous ecotropic MuLV DNA sequences in BXH RI and progenitor strains of mice. We used Southern blotting and hybridization with an ecotropic MuLV DNA-specific probe to characterize the endogenous ecotropic MuLV DNA sequences in 12 BXH RI and 2 progenitor strains of mice. This hybridization probe, representing a 400-base-pair (bp) *Sma*I fragment from the *env* gene of AKR MuLV DNA which was subcloned into the *Eco*RI site of pBR322 (3), hybridizes only to ecotropic DNA sequences and not to xenotropic, amphotropic, or MCF (milk cell focus-forming) MuLV DNAs. The isolation and specificity of this 400-bp DNA fragment has been described by Chattopadhyay et al. (3), and the probe was kindly provided by D. R. Lowy (National Institutes of Health, Bethesda, Md.).

Initially, we examined the organization of ecotropic MuLV genomes in the two progenitors of the BXH RI strains. Digestion of C57BL/6J and C3H/HeJ DNA with *Pst*I, *Bam*HI, and *Kpn*I should generate single detectable fragments containing only viral sequences (25). In particular, *Pst*I only cleaves within the long terminal repeat sequences and generates an 8.2-kilobase (kb)

viral DNA fragment for each AKR-like ecotropic provirus. This was confirmed for DNAs of both progenitor strains (Fig. 1).

Digestion with *Eco*RI, *Pvu*II, *Xba*I, or *Hin*dIII should generate a single detectable cell-virus DNA junction fragment for each nonallelic ecotropic provirus (25). Since *Eco*RI does not cleave the DNA of most ecotropic MuLVs (25), the size of this viral DNA-containing fragment depends on where *Eco*RI cleaves in the flanking cellular DNA. *Pvu*II or *Hin*dIII digestion should generate a fragment larger than 3.0 kb (*Pvu*II) or 5.8 kb (*Hin*dIII), containing 3′ viral and flanking cellular DNA. Similarly, *Xba*I digestion should generate a fragment larger than 7.8 kb, representing a 5′ cell-virus DNA junction fragment. In all cases, digestion of C57BL/6J and C3H/HeJ DNA with *Eco*RI, *Pvu*II, *Hin*dIII, or *Xba*I produced a single fragment of molecular weight larger than the corresponding fragment of unintegrated linear AKR MuLV DNA (Fig. 1). *Eco*RI, *Pvu*II, and *Xba*I digestion produced fragments of different molecular weight in DNA of the two progenitor strains, suggesting that both strains carry single nonallelic endogenous AKR-like ecotropic proviruses. C57BL/10 (related to C57BL/6) and C3H/He mice have been shown to carry single N-tropic virus inducibility loci which have been assigned to chromosome 5, linked to phosphoglucomutase-1 (*Pgm-1*; C3H/He), and to chromosome 8, linked to esterase-1 (*Es-1*; C57BL/10) (10, 15). These results are consistent with the restriction enzyme data shown in Fig. 1.

Since digestion with *Eco*RI, *Pvu*II, and *Xba*I produced single viral DNA-containing fragments of unequal size in the two progenitor strains, we used these enzymes to characterize the ecotropic viral DNA sequences of the 12 BXH RI strains. The results obtained by *Eco*RI and *Pvu*II digestion are shown in Fig. 2 and were confirmed by *Xba*I digestion (data not shown). In addition, we have indirectly analyzed DNA of the BXH-5 RI strain. Although the strain is now extinct, a BXH-5 male had previously been outcrossed to a C57BL/6J female and the progeny were backcrossed twice to BXH-5 mice. Restriction analysis of DNA prepared from two mice of the second backcross showed that they carried no detectable ecotropic MuLV DNA sequences (Table 1), suggesting that the BXH-5 RI strain did not inherit either parental endogenous ecotropic MuLV genome.

The segregation of C3H/HeJ and C57BL/6J ecotropic proviruses in the BXH RI strains (summarized in Table 1) is consistent with two independently segregating loci. The sites of the C3H/HeJ and C57BL/6J endogenous ecotropic proviruses have been designated *C3v* and *Blv*, respectively. Two BXH RI strains inherited only *C3v*, three strains inherited only *Blv*, five strains inherited both *C3v* and *Blv*, and three strains (including BXH-5) inherited neither provirus. *C3v* segregated concordantly in 9 of 13 RI strains

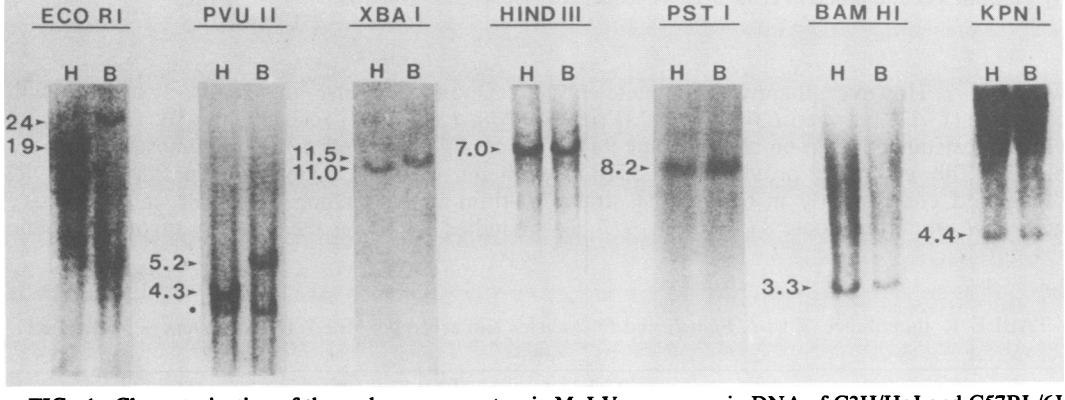

FIG. 1. Characterization of the endogenous ecotropic MuLV sequences in DNA of C3H/HeJ and C57BL/6J mice. High-molecular-weight DNAs (10 μg per lane) prepared from spleens of several C3H/HeJ (H) and C57BL/6J (B) mice were digested to completion with restriction enzymes that generate detectable cell-virus DNA junction fragments (*Eco*RI, *Pvu*II, *Xba*I, and *Hin*dIII) or internal viral DNA fragments (*Pst*I, *Bam*HI, and *Kpn*I). The molecular weight in kb of viral DNA-containing fragments, detected after electrophoresis through 0.6% agarose gels, Southern blotting, and hybridization with the ^{32}P-labeled ecotropic-specific hybridization probe, was calculated using ^{32}P-labeled *Hin*dIII-digested λ DNA electrophoresed in parallel lanes of the same gels. The band indicated by (●) does not represent hybridization of the probe to cellular DNA sequences, since the molecular weight of the fragment (3.9 kb) was unaltered by digestion with various restriction enzymes and the fragment was also detected in lanes lacking DNA but containing tracking dye. The darker smear at the top of the *Kpn*I lanes represents nonspecific hybridization of the probe to high-molecular-weight cell DNA and has not been encountered in other *Kpn*I digests of C3H/HeJ and C57BL/6J DNA.

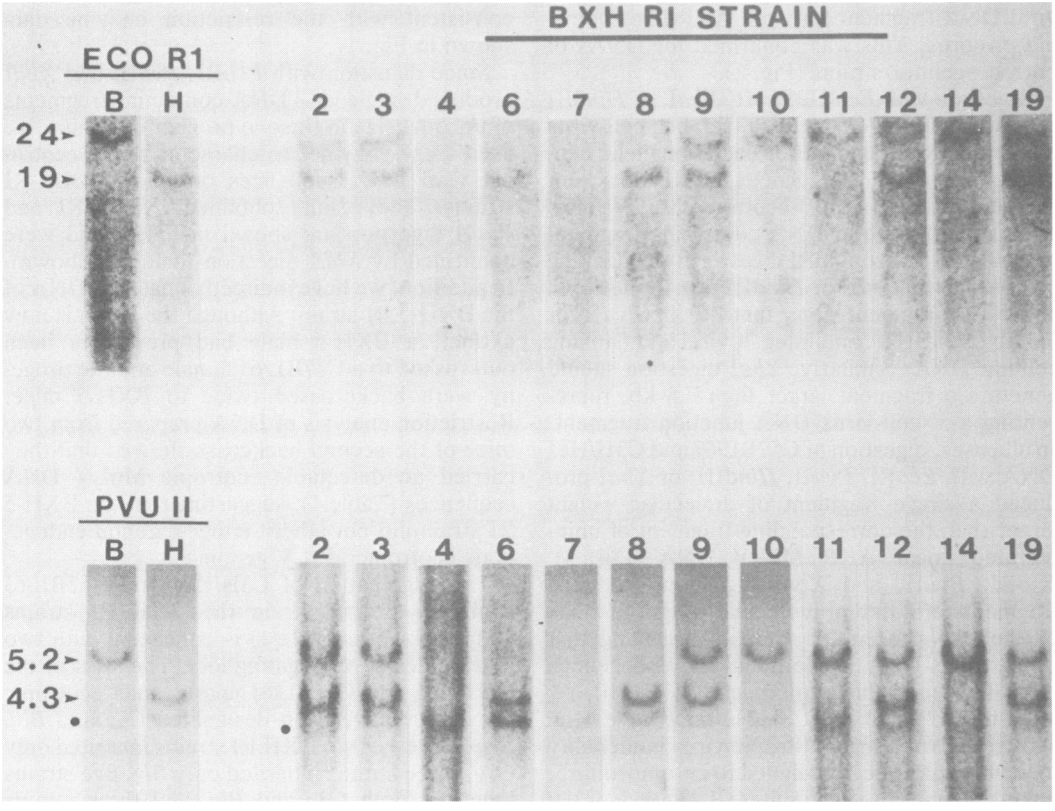

FIG. 2. Characterization of the endogenous ecotropic MuLV sequences in DNA of 12 BXH RI strains. DNAs of 12 BXH RI strains (2 to 4, 6 to 12, 14, and 19) were digested to completion with *Eco*RI or *Pvu*II and analyzed as described in the legend to Fig. 1. The results of the analysis of DNAs of the progenitor strains (B, C57BL/6J; H, C3H/HeJ) presented in Fig. 1 are shown again for reference. The bands indicated by (●) do not represent hybridization of the probe to cellular DNA sequences (see legend to Fig. 1).

with *Pgm-1*. However, there was complete concordance (13/13) between *C3v* and *Fv-2* (Friend virus-2 resistance locus) on chromosome 9 (Table 1). The ecotropic provirus of C57BL/6J segregated concordantly in 6 of 13 RI strains with *Es-1* on chromosome 8 (Table 1).

Due to the loose linkage of *C3v* and *Blv* with *Pgm-1* and *Es-1*, respectively (10, 15), we were not able to confirm the chromosomal assignments of these endogenous proviruses by RI strain analysis alone. However, in preliminary studies involving the progeny of other genetic

TABLE 1. Inheritance of *Es-1*, *Pgm-1*, and *Fv-2* alleles and ecotropic MuLV DNA sequences in BXH RI strains

Locus[a]	Alleles inherited by BXH RI strain[b]:												
	2	3	4	5	6	7	8	9	10	11	12	14	19
Blv	B	B	H	H	H	H	H	B	B	B	B	B	B
Es-1	B	H	H	B	B	B	B	B	B	B	H	B	H
Pgm-1	H	H	H	B	B	B	H	H	B	B	B	H	H
C3v	H	H	B	B	H	B	H	H	B	B	H	B	H
Fv-2	H	H	B	B	H	B	H	H	B	B	H	B	H

[a] BXH RI strains 2 through 12, 14, and 19 were classified with respect to esterase-1 (*Es-1*) as described in the text; phosphoglucomutase-1 (*Pgm-1*) (6); and Friend virus-2 restriction (*Fv-2*; F. Lilly, personal communication). The ecotropic virus loci of C57BL/6J (*Blv*) and C3H/HeJ (*C3v*) were assigned from the data in Fig. 2.

[b] B and H were used to denote the alleles inherited from C57BL/6J and C3H/HeJ, respectively.

crosses we have shown that the viral DNA sequences identified in DNA of the progenitor strains represent the previously identified N-tropic virus inducibility loci and confirmed their chromosomal locations (data not shown). The complete concordance of the segregation of *C3v* and *Fv-2* on chromosome 9 is therefore unexpected. The probability of this occurring by chance is $(1/2)^{13}$ or 0.00012. This type of segregation could occur if during inbreeding strong selection occurred favoring the progenitor combinations over the recombinant types. However, the reason for this aberrant segregation remains to be elucidated.

The restriction enzyme data shown in Fig. 2 indicate that the BXH-2 RI strain carries both parental N-tropic proviruses. To determine whether B-tropic sequences are also present in BXH-2 DNA, it was first necessary to verify that the hybridization probe used in these experiments recognizes BXH-2 B-tropic MuLV DNA. Unintegrated viral DNA was prepared from SC-1 cells exogenously infected with biologically cloned BXH-2-derived B-tropic virus and then analyzed either as undigested DNA (Fig. 3, lane 1) or after digestion with *Eco*RI, *Hin*dIII, *Kpn*I, or *Bam*HI (Fig. 3, lanes 2 through 5, respectively). In all cases, one fragment was detected after hybridization with the ecotropic-specific probe (Fig. 3A). The size of the fragment was identical to that predicted from the restriction enzyme map of unintegrated AKR MuLV DNA (25). After hybridization with the ecotropic-specific probe, the filter was erased by boiling in distilled water and then rehybridized with a probe representing the entire AKR MuLV genome. Again, in all cases, fragments of molecular weights similar to those predicted from the map of AKR MuLV DNA were identified (Fig. 3B). The faint 8.8-kb doublet detected after *Hin*dIII digestion (Fig. 3B, lane 3) probably represents a small amount of closed circular viral DNA (containing one or two terminal repeats) that was present in this Hirt supernatant DNA. Due to their low abundance in this DNA preparation and their inefficient transfer during Southern blotting, these molecules were only detected after linearization by *Hin*dIII digestion. These results demonstrate that the BXH-2 B-tropic MuLV is structurally similar to the N-tropic AKR provirus and is detectable with the ecotropic-specific probe.

Collectively, these studies indicate that the B-tropic virus of BXH-2 mice is not integrated into the germ line of the BXH-2 mice used in these experiments. This result was confirmed by analysis of DNA from mice of several other litters of current generations of BXH-2 mice (data not shown). Nevertheless, most BXH-2 mice that we have recently assayed spontaneously expressed high titers of XC-positive B-tropic MuLV, presumably as a consequence of horizontal (maternal) MuLV transmission.

Expression of B-tropic MuLV in tissues of BXH-2-derived F_1 and backcross mice. To further demonstrate that the B-tropic virus is not an endogenous virus in BXH-2 mice, virus-positive BXH-2 males from current generations of inbreeding (F_{40} to F_{42}) were crossed to female mice carrying the *Fv-1*^{b} allele (C57BL/6J, P/J, and BALB/cJ). Tissues (tail or spleen) from the progeny were then analyzed, by the XC plaque assay, for B-tropic virus expression. To date, none (0/116) of the progeny tested spontaneously produced B-tropic MuLV.

These results are in contrast to those obtained at an earlier generation (F_{29}) of BXH-2 inbreeding. Approximately 50% (9/16) of the progeny generated from crossing a BXH-2 male to C57BL/6J females produced ecotropic MuLV, suggesting that the BXH-2 male was heterozygous for an endogenous B-tropic MuLV genome. This conclusion is supported by the data presented in Table 2. Of six progeny analyzed from the cross of a C57BL/6J female and an F_{29} BXH-2 male, two were virus positive. These results are consistent with the F_{29} BXH-2 male carrying heterozygously an endogenous B-tropic virus genome. From this cross, a virus-

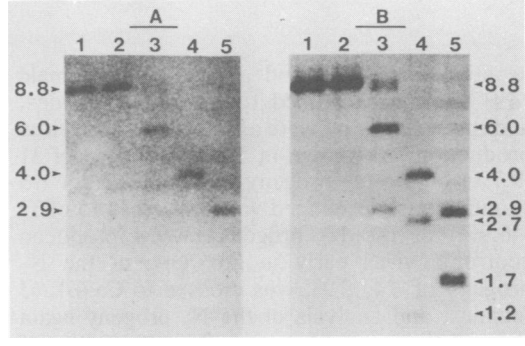

FIG. 3. Restriction enzyme analysis of unintegrated BXH-2-derived B-tropic MuLV DNA. Unintegrated viral DNA was prepared from SC-1 cells exogenously infected with biologically cloned BXH-2 B-tropic MuLV. Hirt supernatant DNA (50 μg per lane) was electrophoresed through a 0.6% agarose gel as undigested DNA (lane 1) or after digestion with *Eco*RI (lane 2), *Hin*dIII (lane 3), *Kpn*I (lane 4), or *Bam*HI (lane 5). DNAs were transferred to nitrocellulose and hybridized to the ecotropic-specific probe (A) and subsequently to a DNA probe representative of the entire AKR MuLV genome (B). The AKR genomic probe (clone 623) had been subcloned into the *Hin*dIII and *Eco*RI sites of pBR322 (D. R. Lowy, personal communication) and was kindly provided by D. R. Lowy. The molecular weight of the viral DNA fragments (in kb) was calculated by using ^{32}P-labeled *Hin*dIII-digested λ DNA electrophoresed in a parallel lane of the same gel.

TABLE 2. Spontaneous ecotropic MuLV expression in hybrid BXH-2 mice

Cross	Family[a]	Ecotropic virus production[b] (no. positive/no. tested)	% Virus positive
F_1 cross			
C57BL/6J × BXH-2 (F_{29})	1 (♂3, ♂4)	2/6	33.3
N_2 cross			
C57BL/6J × (C57BL/6J × BXH-2 [F_{29}])♂3	1	0/6	
	2	0/4	
	3	0/7	9.7
	4	0/2	
	5	0/7	
	6	3/5	
C57BL/6J × (C57BL/6J × BXH-2 [F_{29}])♂4	1	0/2	
	2	2/3	
	3 (♂93)	1/2	
	4 (♂124)	0/2	46.9
	5	3/7	
	6	1/2	
	7	3/4	
	8	3/5	
	9 (♂299, ♂300)	2/5	
N_3 cross			
C57BL/6J × N_2 ♂93	1	0/4	
	2	3/7	42.9
	3	3/3	

[a] In several cases, many families were produced by crossing a single male to several different females, and certain progeny of some families have been designated.

[b] Expression of ecotropic MuLV was determined by the XC plaque assay using tail biopsies from 5- to 10-week-old mice.

negative male (♂3) and a virus-positive male (♂4) were backcrossed to C57BL/6J females, and the N_2 progeny were again assayed for virus production. As shown in Table 2, only 3 of 31 (9.7%) of the N_2 progeny descended from ♂3 spontaneously produced virus, whereas 15 of 32 (46.9%) of the N_2 progeny of ♂4 produced ecotropic virus early in life. One of the N_2 progeny of ♂4, ♂93, was crossed to C57BL/6J females, and analysis of the N_3 progeny again showed that approximately one-half (42.8%) of these mice expressed the B-tropic MuLV genome. These results suggest that ♂4 was also heterozygous for an endogenous B-tropic provirus which was inherited in a Mendelian fashion.

Three of the N_2 progeny of ♂4 (♂124, ♂299, ♂300) were available for restriction enzyme analysis. The results obtained by *Pvu*II digestion are shown in Fig. 4. As expected, all three mice carried the C57BL/6J endogenous N-tropic provirus (5.2-kb fragment). Mouse 124 also carried (and was presumably heterozygous for) the C3H/HeJ N-tropic MuLV genome (4.3-kb fragment). In addition, ♂124 and ♂299 appeared to be heterozygous (determined by relative intensity of DNA fragments in the same lane) for an additional endogenous ecotropic MuLV genome corresponding to a 6.6-kb *Pvu*II fragment. Since retroviruses are thought to integrate randomly within mouse chromosomes and since both ♂124 and ♂299 carry an additional provirus with similar flanking cellular DNA sequences, it is likely that the viral DNA sequences characterized by the 6.6-kb *Pvu*II fragment represent an endogenous B-tropic provirus. In addition, digestion of ♂124 and ♂299 DNA with *Pst*I produced only an 8.2-kb viral DNA fragment (data not shown), suggesting that the additional ecotropic provirus is structurally nondefective.

As expected, ♂299 spontaneously produced ecotropic MuLV early in life, and ♂300 was virus negative. However, ♂124 was also negative for spontaneous virus production. Since ♂124 is now dead, we were not able to reassay ♂124 to determine whether this mouse was virus negative or produced only low titers of ecotropic MuLV that were not detected in the XC plaque assay.

In view of the results summarized in Table 2 and Fig. 4, we conclude that the B-tropic MuLV genome was present as an endogenous virus in some BXH-2 mice at an earlier stage of inbreeding but has been subsequently lost from the germ line of this strain.

Characterization of endogenous ecotropic MuLV DNA sequences in tumor tissues of BXH-2

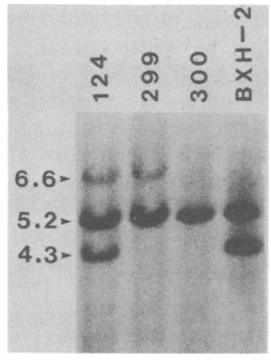

FIG. 4. Characterization of the endogenous ecotropic MuLV sequences in DNA of BXH-2-derived hybrid mice. DNAs were prepared from three progeny (♂124, ♂299, and ♂300) of the N_2 cross C57BL/6K × (C57BL/6J × BXH-2 [F_{29}])♂4 (Table 2) and from a normal BXH-2 mouse (F_{42}). After digestion with *Pvu*II, DNAs were analyzed as described in the legend to Fig. 1.

mice. Restriction analysis of DNAs from leukemic tissues of other inbred mouse strains that also express high titers of ecotropic MuLV and show a high leukemia incidence has indicated that amplification of ecotropic proviruses often occurs in outgrown tumors (24, 34). To determine whether amplification of ecotropic viral DNA sequences occurs in lymphomatous BXH-2 tissues, we have characterized the ecotropic viral DNA content of these tissues. Nine of 12 mice examined carried only the two parental endogenous N-tropic MuLV genomes characteristic of normal BXH-2 mice (data not shown). However, three BXH-2 mice (♀530, ♀597, and ♂598) carried additional MuLV DNA sequences detectable with this probe. Analysis of DNA prepared from nodes and thymus (that were estimated at autopsy to be approximately 30% infiltrated with tumor cells) of BXH-2 ♀597 revealed an additional ecotropic provirus characterized by a 7.4-kb *Pvu*II fragment (Fig. 5). That an additional provirus can be detected by this analysis suggests that the tumor was monoclonal. This result is in agreement with recent evidence indicating that during outgrowth of the tumor, selection of cells occurs, leaving one or a few clonal descendants in the outgrown tumor (24). *Pvu*II digestion of DNA prepared from nodes and spleen of BXH-2 ♂598 also revealed an additional ecotropic MuLV genome (5.7-kb viral DNA fragment). DNA prepared from brain of ♂598, which was not infiltrated with tumor cells, did not contain the additional ecotropic provirus (Fig. 5). Digestion of DNA of lymphomatous tissues of BXH-2 ♀530 with *Pvu*II showed the presence of three additional ecotropic proviruses, corresponding to 9.3-, 7.8-, and 5.9-kb *Pvu*II fragments (Fig. 5). These fragments were not detected in brain DNA of ♀530. The 7.4-kb and 7.8-kb *Pvu*II fragments detected in DNAs of ♀597 and ♀530, respectively, were shown not to comigrate by electrophoresis in parallel lanes of the same agarose gel. The

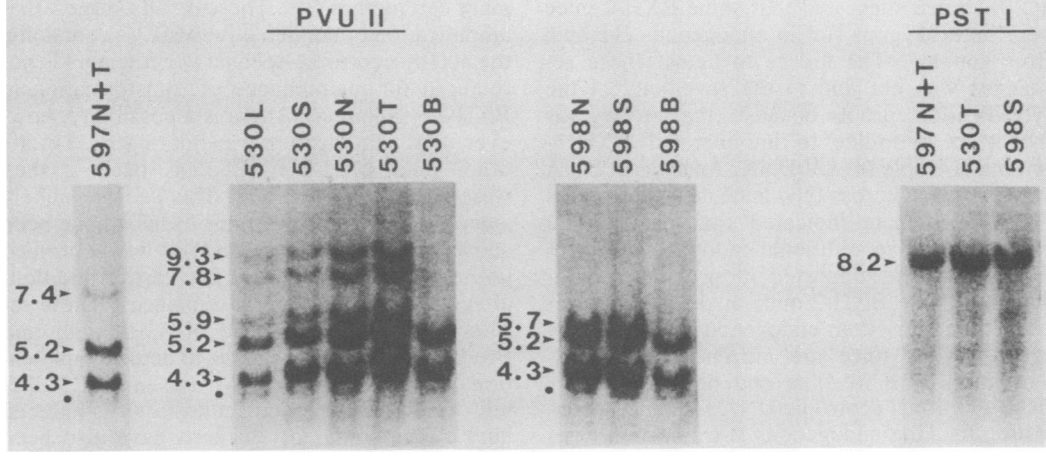

FIG. 5. Characterization of the endogenous ecotropic MuLV sequences in DNA of lymphomatous BXH-2 mice. DNAs were prepared from BXH-2 tissues (N, nodes; T, thymus; L, liver; S, spleen; and B, brain) which, in all cases except brain, were shown by histological examination to contain 30 to 95% lymphomatous cells. In the case of ♀597, tissues from both nodes and thymus were mixed before DNA extraction. DNAs were digested with *Pvu*II or *Pst*I and analyzed as described in the legend to Fig. 1. The 5.7- and 5.2-kb doublet found in *Pvu*II-digested 598N and 598S DNA is difficult to see in this figure but was easily visualized in the original autoradiograph. The bands indicated by (●) do not represent hybridization of the probe to cellular DNA sequences (see legend to Fig. 1).

monoclonal origin of these tumors is further indicated by the relative intensity of hybridization of the newly acquired viral DNAs within each tissue of ♀530 and their presence in all tumor tissues of the same BXH-2 mouse.

Digestion of DNAs from lymphomatous tissues of ♀597, ♂598, and ♀530 with *Pst*I produced only normal 8.2-kb viral DNA fragments (Fig. 5). This suggests that these recently acquired ecotropic proviruses contain long terminal repeat sequences and have not suffered large deletions or insertions in their genome. However, the presence of defective or nondefective proviruses that do not contain the 400-bp *env* sequence cannot be excluded by these experiments.

DISCUSSION

The endogenous ecotropic MuLV DNA content of 12 BXH RI strains and their two progenitor strains were characterized by Southern blotting and hybridization with an ecotropic MuLV DNA-specific probe. Single endogenous N-tropic proviruses were detected in DNA of C3H/HeJ and C57BL/6J mice, and they segregated as unlinked loci in the BXH RI strains. Of particular interest was the ecotropic viral DNA content of normal tissues of BXH-2 mice which were found to have inherited both parental N-tropic proviruses. However, we were unable to detect any other viral sequences that might represent an endogenous B-tropic provirus in either progenitor strain or in BXH-2 mice. This was surprising since analysis of (C57BL/6J × BXH-2 [F_{29}])F_1 mice suggested that some BXH-2 mice were heterozygous for an endogenous B-tropic virus genome. The failure to detect these sequences was not due to the specificity of the hybridization probe because the probe was shown to hybridize to unintegrated BXH-2–derived B-tropic MuLV DNA. Analysis of DNA from three backcross (N_2) mice derived from an F_{29} BXH-2 male indicated that this BXH-2 mouse carried an additional endogenous ecotropic provirus and supported our previous conclusion that some BXH-2 mice at the F_{29} stage of inbreeding carried an endogenous B-tropic provirus. BXH-2 mice are currently at the F_{42} generation of inbreeding and no longer carry these additional ecotropic DNA sequences, suggesting that the endogenous B-tropic virus was lost during subsequent inbreeding of this strain. This conclusion is supported by the lack of B-tropic virus expression in many F_1 hybrid mice derived from male BXH-2 mice at the F_{40} to F_{42} stage of inbreeding. However, BXH-2 mice are still viremic, suggesting that the B-tropic virus is horizontally transmitted.

Viremic female mice can infect their offspring at several stages of development including preimplantation or transplacental infection, or via the milk. The frequency of transplacental infection of embryos is reportedly low (13), although viral transmission via the milk is highly effective and nearly all newborn mice become infected with MuLV when foster nursed by viremic mothers (13). However, some BXH-2 embryos from the F_{40} to F_{42} generation of inbreeding also expressed high titers of B-tropic MuLV (our unpublished data). Therefore, milk transmission is apparently not the sole route of B-tropic virus infection in BXH-2 mice. The mechanism of virus transmission in BXH-2 mice is currently being investigated in more detail.

DNAs prepared from lymphomatous tissues of several BXH-2 mice were also analyzed for their ecotropic MuLV DNA content. In all cases, the two normal endogenous ecotropic proviruses of BXH-2 mice were unaltered in tumor tissues. However, amplification of ecotropic-specific MuLV DNA sequences was found in lymphomatous tissues of 3 of 12 mice examined. The number of newly acquired ecotropic proviruses varied from one to three copies, and the genomes appeared to be integrated at different sites as determined by *Pvu*II digestion. In the cases where newly acquired proviruses were found, the outgrown tumors appeared to be composed largely of clonal descendants of a single transformed cell that metastasized to various tissues. Similar results have been obtained with tumors from BALB/Mo mice (12, 35), AKR mice (24, 30), Mo-MuLV–infected mice (34), and mouse mammary tumor virus-induced mammary carcinomas (19). These results suggest that amplification of nondefective MuLVs containing the 400-bp ecotropic-specific *env* fragment is not required for maintenance of transformation in BXH-2 lymphomas. There is a possibility, however, that amplification was not observed in the other nine BXH-2 lymphomas because they were not of clonal origin. This seems unlikely since tumors in other strains of mice have been shown to be monoclonal, and this was confirmed in the three BXH-2 tumors that carried additional ecotropic MuLV DNA sequences. These results are similar to those of Yoshimura and Breda, who were also unable to detect amplification of AKR ecotropic proviruses in AKR leukemic tissues (36). Avian leukosis virus-induced bursal lymphomas of chickens have also been shown to be monoclonal in origin and do not require viral gene products for the maintenance of transformation (20, 21). In this respect, the non-T-cell lymphomas of BXH-2 mice resemble both the bursal tumors of chickens and AKR leukemias.

Several investigators have reported that outgrown murine tumors contain both authentic

ecotropic MuLV genomes and recombinant genomes (24, 34). The recombinant genomes are integrated in multiple copies in tumor tissues, are all *env* gene recombinants with a genome size similar to the parental ecotropic MuLV, and have acquired xenotropic-like sequences (similar to MCF viruses). The presence of the same recombinant proviral DNA sequences in DNAs of AKR-MuLV and Mo-MuLV induced leukemias (24, 34) suggests that the formation of these recombinant viruses may be necessary for leukemic transformation.

A dual tropic recombinant virus that induces cytopathic changes in mink lung cells and in mouse embryo fibroblasts carrying the *Fv-1^b* allele has been isolated from lymphoid cells of a lymphomatous BXH-2 mouse (1). However, these recombinant viruses would not have been detected in DNAs of BXH-2 tumors since the hybridization probe used in these studies does not recognize this class of MuLV (3, 36). We are currently analyzing DNAs of BXH-2 tumor tissues for the presence of these recombinant proviruses.

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