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Lack of AKR Ecotropic Provirus Amplification in AKR Leukemic Thymuses

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A DNA fragment from the 3' region of a molecularly cloned AKR ecotropic provirus was identified to be specific for the AKR ecotropic murine leukemia virus (MuLV). This selected DNA fragment was used to analyze the integrated MuLV proviruses in normal and leukemic tissue DNAs of AKR mice. In comparison with a DNA fragment from the 5' region of the cloned AKR genome or one representing the entire genome, this selected probe hybridized to only a few MuLV proviruses. By comparing transformed and nontransformed tissue DNAs, it appeared that no amplification of proviral sequences related to the AKR ecotropic MuLV had occurred in thymomas of AKR mice during the development of leukemia in these animals. Analysis of the AKR ecotropic MuLV proviruses revealed a significant degree of polymorphism for these sequences among individuals in the AKR/J strain of mouse.

The high-leukemic AKR mouse strain possesses two dominant genetic loci, *Akv-1* and *Akv-2*, which are responsible for the expression of an N-ecotropic murine leukemia virus (MuLV) (15, 16). In addition to the ecotropic MuLV (referred to as AKR MuLV throughout this report) which is produced throughout the lifetime of AKR animals, a second class of MuLV's has been detected mainly in preleukemic and leukemic tissues. This class of MuLV, referred to as mink cell focus-inducing (MCF) virus, was first characterized by serological analysis to have properties of both ecotropic and xenotropic MuLV's (8). Evidence from a number of different studies has further shown that MCF MuLV's are recombinants between AKR MuLV and xenotropic viral sequences in the envelope region of their genomes (5, 7, 14).

Liquid hybridization studies using an AKR MuLV-selected cDNA probe revealed that AKR-specific viral sequences were amplified in the DNA of target tissues, such as thymus and spleen of leukemic animals (1). These results appeared to be supported by subsequent analyses using either AKR sequence-enriched (20) or nonenriched (3) probes and the Southern transfer technique to study the topology of the integrated AKR proviruses. From the latter studies, it appeared that a few of the amplified proviral sequences corresponded to altered forms of the AKR genome since they were subgenomic in size, suggesting that either rearrangements, deletions, or recombination of the original genome had occurred.

Identification of the integrated AKR proviral DNAs has been difficult because of the presence in the mouse genome of a large number of sequences sharing homology with AKR MuLV. This report identifies a DNA fragment which is specific for the AKR MuLV. By analyzing the DNA from target and nontarget tissues for transformation of normal and leukemic animals, we show that the number of AKR MuLV-related proviruses did not increase in transformed thymuses.

MATERIALS AND METHODS

Source of AKR-MuLV cloned DNA. A molecular clone of an integrated provirus of AKR MuLV (clone 623; 11) was generously provided by Douglas Lowy, Laboratory of Tumor Virus Genetics, National Institutes of Health, Bethesda, Md.

Source of MCF-13 MuLV. Virus from a cloned NIH/3T3 cell line productively infected with MCF-13 MuLV (provided by G. Baldwin) was used. The original AKR MCF-13 MuLV was isolated and cloned into mink lung cells by J. W. Hartley and W. P. Rowe.

Preparation of DNA. Tissue DNA was prepared by first isolating nuclei by douncing minced tissue in reticulocyte standard buffer (1.5 mM $MgCl_2$, 10 mM NaCl, and 10 mM Tris-hydrochloride, pH 7.4). Nuclei were lysed in sodium dodecyl sulfate (SDS) buffer (100 mM NaCl, 10 mM EDTA, and 10 mM Tris-hydrochloride, pH 7.5) containing 1% SDS and 0.4 mg of proteinase K per ml and incubated at 37°C for 2 h. DNA was then phenol and chloroform extracted before dialyzing against TE buffer (10 mM Tris-hydrochloride, pH 7.4, and 5 mM EDTA) overnight. It was then treated with 0.05 mg of RNase A per ml at 37°C for 1 h followed by proteinase K digestion after addition of

0.5% SDS. Phenol and chloroform extractions were followed by dialysis against 0.3 N NaCl in TE buffer and finally against TE buffer alone.

Restriction endonuclease digestions. All DNAs were digested with restriction endonucleases according to the manufacturers' recommendations (Bethesda Research Laboratories or New England Biolabs). Completeness of digestion of tissue DNA was monitored by adding λ DNA to a portion of the reaction and examining the λ DNA fragments after gel electrophoresis and ethidium bromide staining.

Agarose gel electrophoresis of DNAs and hybridization. Digested DNA samples of 5 to 20 μg were electrophoresed through horizontal slab gels of varying percentages of agarose (SeaKem). Electrophoresis conditions were as reported earlier (24), using either low-salt E buffer (40 mM Tris-hydrochloride, pH 7.9, 5 mM sodium acetate, and 1 mM EDTA) or high-salt E buffer (40 mM Tris-hydrochloride, pH 8.3, 50 mM sodium acetate, and 1 mM EDTA). Transfer of DNA from agarose gels to nitrocellulose filters was based on the method developed by Southern (18) and described in a previous report (24).

Nitrocellulose filters that had DNA baked onto them were pretreated with a solution of 6× SSC, pH 7.4 (SSC = 0.15 N NaCl plus 0.15 N sodium citrate), 10× Denhardt solution (1× = 0.2% bovine serum albumin, 0.2% polyvinyl pyrrolidone, and 0.2% Ficoll), 0.1% SDS, 0.01 mg of polyadenylate per ml, and 0.1 mg of herring sperm DNA per ml for 3 h at 67°C. Hybridization was carried out under the conditions of 5× SSC, pH 7.4, 50% formamide, 20 μg of yeast RNA per ml, 20 μg of herring sperm DNA per ml, 1× Denhardt solution, and 0.02 μg of ^{32}P-labeled DNA per ml for 24 to 48 h at 41°C.

After hybridization, filters were rinsed in 2× SSC–1× Denhardt solution for 20 min at room temperature, followed by a rinse in 0.1× SSC–0.1% SDS for 1 h at 50°C and finally by 5-min rinses, each of 0.1× SSC, 0.1% SDS, and 0.1× SSC at room temperature. Filters were exposed to Kodak X-Omat X-ray film with DuPont Cronex Lightning Plus intensifying screens at −70°C for 1 to 14 days (22).

Recovery of DNA from agarose. Agarose gel slices were placed in dialysis tubing, and DNA was recovered by electroelution at 100 V for 2 h in 40 mM Tris-hydrochloride, pH 7.9, 5 mM sodium acetate, and 1 mM EDTA. After repeating once, the pooled portions were centrifuged through glass wool, extracted with *sec*-butanol and then ether, and dialyzed against 0.2 N NaCl in TE buffer and finally against TE buffer. One microgram of this DNA was nick translated to a specific activity of 1×10^8 to 2×10^8 cpm/μg by the procedure of Maniatis et al. (12).

RESULTS

Identification of a DNA fragment specific for AKR ecotropic MuLV. To analyze the integrated AKR provirus DNAs in normal and leukemic tissues, it was necessary to derive an AKR MuLV-specific probe which would not cross-hybridize with other endogenous viral sequences present in the AKR mouse genome. To do this, we relied on the information from previous studies which demonstrated that a region of nonhomology located to the envelope region of the genome existed between AKR MuLV and various recombinant MCF MuLV's (5, 14). A molecular clone of an AKR MuLV provirus (clone 623; 11), which was obtained from D. Lowy, was sequentially digested with three different restriction endonucleases, *Kpn*I, *Sma*I, and *Xba*I, which have cleavage sites in the envelope region of the AKR genome (11, 21). These three enzymes were chosen to generate small fragments of just a few hundred bases which would increase the probability of their having only sequences specific for AKR MuLV. Sequential digestion of clone 623 DNA with these three enzymes yielded the expected AKR DNA fragments as analyzed by agarose gel electrophoresis and the Southern transfer technique, using a ^{32}P-labeled 8.2-kilobase pair (kbp) *Pst*I DNA fragment representing most of the AKR genome for hybridization (21; Fig. 1, lane A). The large DNA fragments in Fig. 1 were overexposed to allow the detection of the small fragments (400 and 300 base pairs [bp]) generated from the envelope region.

To identify AKR DNA fragments lacking homology with the MCF MuLV's, a cDNA representative probe of one MCF MuLV, MCF-13, was also hybridized to the AKR DNA fragments generated by digestion with *Kpn*I, *Sma*I, and *Xba*I. The two small fragments (400 and 300 bp) from the envelope region which had hybridized to the AKR probe failed to hybridize to the MCF-13 ^{32}P-labeled cDNA probe (Fig. 1B). DNA fragments from other parts of the AKR genome hybridized to both probes (Fig. 1, lanes A and B).

The 400-bp fragment which could be generated by *Sma*I digestion alone of AKR DNA was selected for further analysis. DNA representing the 400-bp fragment was prepared by digestion of clone 623 DNA with *Sma*I, electrophoresis through a 1.4% agarose gel, and identification of the appropriate band by staining with ethidium bromide. The recovered DNA was ^{32}P labeled by nick translation (12) and used to hybridize back to *Sma*I-digested AKR clone 623 DNA to test its specificity. The 400-bp probe selectively hybridized to its corresponding AKR DNA *Sma*I fragment (Fig. 2, lane B). As an alternative probe for these studies, a second *Sma*I fragment of 4.8 kbp derived from the 5′ end of the cloned AKR provirus (11) was also purified and ^{32}P labeled by nick translation. This 5′-end fragment also hybridized to its corresponding fragment and to a lesser degree to other higher-molecular-weight fragments generated by *Sma*I digestion of clone

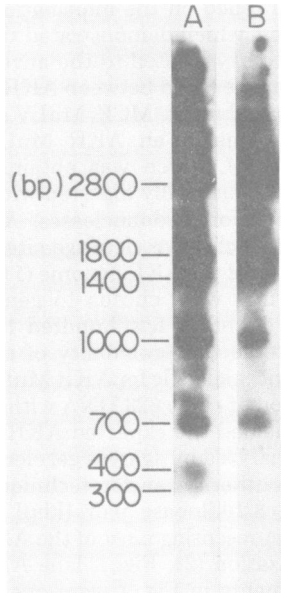

FIG. 1. *Analysis of cloned AKR-MuLV DNA digested with KpnI, SmaI, and XbaI with two different hybridization probes. Sequentially digested DNA (0.26 μg) was electrophoresed in two separate lanes through a horizontal 1.4% agarose gel in high-salt E buffer at 1.6 V/cm for 20 h and analyzed by the Southern transfer technique. (A) Hybridization with nick-translated ^{32}P-labeled 8.2-kbp PstI fragment of cloned AKR DNA; (B) hybridization with ^{32}P-labeled MCF-13 MuLV cDNA prepared by exogenous reverse transcription using deoxyoligonucleotide primers as described by Taylor et al. (23). The sizes of the DNA fragments in base pairs as determined by published restriction endonuclease maps of AKR-MuLV DNA (11, 21) are indicated to the left of the figure.*

623 DNA (Fig. 2, lane C). The AKR MuLV representative probe (8.2 kbp) also hybridized to these large fragments (Fig. 2, lane A), which probably represent λ vector arms containing the *Sma*I 5′- and 3′-end fragments generated by cleavage in the long terminal repeat regions of the AKR proviral DNA.

To test the specificities of the AKR MuLV 400-bp and 4.8-kbp probes, they were hybridized to Hirt-extracted linear DNA (8.8 kbp) of either MCF-13 or Moloney MuLV. The 400-bp fragment failed to hybridize to either linear DNAs (Fig. 3, lanes A and B); the 4.8-kbp fragment, on the other hand, hybridized to both of these DNAs (Fig. 3, lanes C and D). The smear toward the top of Fig. 3, lane C, was caused by some contamination of cellular DNA in this particular preparation of MCF-13 Hirt DNA. The four separate lanes shown in Fig. 3 (lanes A through D) contained equivalent amounts of linear DNAs electrophoresed through the same agarose gel. Hybridization conditions and autoradiography exposure times were identical for both probes.

These results indicated that the 400-bp fragment was AKR MuLV specific. To further test whether this was indeed correct, we hybridized this probe to *Eco*RI-digested 129 or NZB mouse liver DNA. Neither mouse strain carries sequences related to the AKR MuLV (10), and the 400-bp probe did not hybridize to either of the liver DNAs (Fig. 3, lanes E and F). Hybridization of the same liver DNAs with the AKR *Pst*I DNA fragment (8.2 kbp) resulted in a complex pattern of bands (data not shown) similar to that observed for AKR mouse tissue DNAs as discussed below.

Analysis of AKR proviral DNAs from normal and leukemic tissues of AKR/J mice. We used the different DNA probes representing various parts of the AKR genome to analyze the proviral DNAs present in tissues

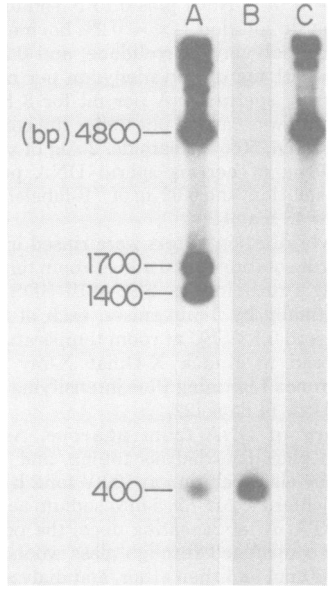

FIG. 2. *SmaI-digested cloned AKR-MuLV DNA hybridized with three different probes. SmaI-digested cloned AKR DNA (0.2 μg) was electrophoresed through a horizontal 1.4% agarose gel at 1.6 V/cm for 20 h in high-salt E buffer and analyzed by the Southern transfer technique. (A) Hybridization of SmaI-digested DNA with 8.2-kbp PstI DNA fragment representative of practically all of the AKR genome; (B) hybridization with a 400-bp SmaI fragment from the envelope region of the AKR genome; (C) hybridization with a 4.8-kbp SmaI fragment representing the 5′ end of the AKR genome. AKR DNA fragments used as hybridization probes were ^{32}P labeled by nick translation.*

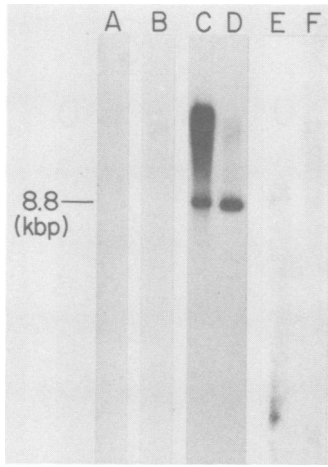

FIG. 3. *Hirt-extracted MCF-13 or Moloney MuLV linear DNAs and NZB or 129 mouse liver DNAs hybridized with two different probes. One microgram of undigested Hirt-extracted linear DNAs and 15 μg of EcoRI-digested mouse liver DNAs were electrophoresed through horizontal 0.7% agarose gels at 2.4 V/cm for 16.5 h in low-salt E buffer and transferred to nitrocellulose filters by the Southern technique for hybridization to either the 400-bp or 4.8-kbp SmaI DNA fragments ^{32}P labeled by nick translation. (A) MCF-13 MuLV linear DNA hybridized with the 400-bp fragment; (B) Moloney MuLV linear DNA hybridized with the 400-bp fragment; (C) MCF-13 MuLV linear DNA hybridized with the 4.8-kbp fragment; (D) Moloney MuLV linear DNA hybridized with the 4.8-kbp fragment; (E) NZB mouse liver EcoRI-digested DNA hybridized with the 400-bp fragment; (F) 129 mouse liver EcoRI digested DNA hybridized with the 400-bp fragment.*

removed from normal and leukemic AKR/J mice. The necessity of using an AKR-specific probe for this type of analysis is demonstrated in Fig. 4, where tissue DNAs digested with restriction endonuclease *Eco*RI, which does not cleave the AKR MuLV genome (20), were analyzed by the Southern transfer technique. Filters were hybridized with the ^{32}P-labeled 8.2-kbp *Pst*I DNA fragment representative of most of the AKR genome. Figure 4 shows *Eco*RI-digested DNAs from thymus or brain tissue of a normal (2 months old) and a leukemic mouse. The thymus from the leukemic animals had presented as a grossly enlarged thymoma. By hybridizing with this AKR MuLV representative probe, we observed similar patterns of endogenous viral sequences for DNAs from brain and thymus of the normal mouse (Fig. 4A, lanes A and B) compared with brain and thymoma of the leukemic mouse (Fig. 4A, lanes C and D). Because of the large number of endogenous viral sequences with homology to AKR MuLV present in the mouse genome, we were unable to detect any sequences that may have been amplified in the thymoma DNA compared with untransformed tissue DNAs. Additional comparisons of normal and transformed tissue DNAs yielded similar results (data not shown). Other investigators have also reported similar difficulties in detecting amplified sequences in AKR thymoma DNAs by using nonselected probes (3).

The 5′-end *Sma*I 4.8-kbp fragment was ^{32}P labeled and used to hybridize to the same tissue DNAs to determine whether any increase in specificity might be achieved by using this subgenomic fragment. The 8.2-kbp AKR representative probe was melted off the same nitrocellulose filters shown in Fig. 4A, and the same filters were rehybridized with this 5′-end probe (Fig. 4B, lanes A through D). The results from this hybridization, however, were similar to those obtained from using the AKR 8.2-kbp probe. On the other hand, when we used the 400-bp AKR MuLV-specific probe to hybridize to the same filters after melting off the previous probes from them, significantly simplified patterns were obtained for the tissue DNAs analyzed (Fig. 4C, lanes A through D).

When we compared untransformed brain with thymoma DNAs from the same leukemic animal, however, we still could not detect any extra bands in the DNA from the leukemic thymus (Fig. 4C, lanes C and D). We then analyzed other DNAs from both normal and leukemic tissues of different animals, using the 400-bp probe for hybridization. Tissues which were considered normal or untransformed were either brain and thymus from normal 2-month-old animals (Fig. 5, lanes A through C) or brain from leukemic animals (Fig. 5, lanes D through E). All of the transformed tissues examined were thymomas from animals which had developed spontaneous leukemia (Fig. 5, lanes F through K). Data presented in Fig. 5 have been taken from different agarose gels that were electrophoresed at different times throughout the course of this study. The lengths of time that samples were electrophoresed through these gels varied, but marker DNAs (*Hin*dIII and *Eco*RI fragments of λ DNA) were included with every gel. Sizes of these marker fragments are indicated to the left of the lanes. In Fig. 5 we have also included the DNAs presented in Fig. 4C (lanes A through D) to simplify the comparisons.

Table 1 summarizes the sizes of the *Eco*RI fragments generated from DNAs of both normal and leukemic tissues as shown in Fig. 5. Three common *Eco*RI fragments (26, 13.8, and 12.2 kbp) were found in all tissue DNAs tested in-

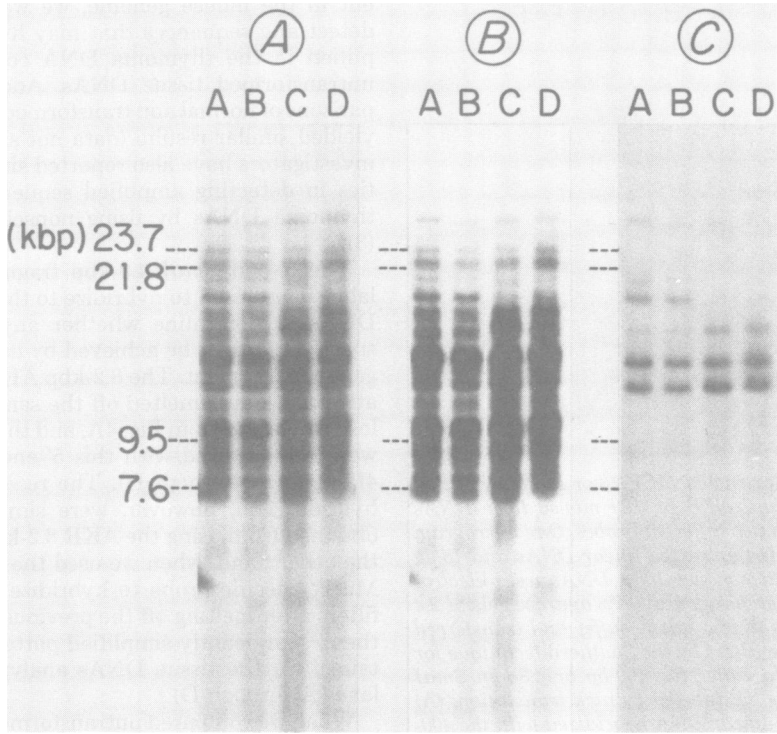

FIG. 4. *Analysis of EcoRI-digested tissue DNAs by hybridization with three different AKR MuLV probes. EcoRI-digested tissue DNAs (15 μg) were electrophoresed through a 0.7% horizontal agarose slab gel in high-salt E buffer at 26V for 46 h. After transfer to nitrocellulose filters, DNAs were hybridized with AKR DNA fragments ^{32}P labeled by nick translation. Marker DNAs (EcoRI and HindIII-digested λ DNA) were electrophoresed in an adjacent lane, and fragment sizes in kilobases are indicated to the left of the figure. The same nitrocellulose filter was hybridized, treated for melting, and rehybridized by using three different probes which were as follows: (A) the 8.2-kbp PstI fragment representing most of the AKR genome; (B) the 5'-end 4.8-kbp SmaI fragment; and (C) the 400-bp SmaI fragment. The lanes in all three sections are: (A) brain from normal mouse (N1); (B) thymus from normal mouse (N1); (C) brain from leukemic mouse (L1); (D) thymoma from leukemic mouse (L1). Notations for the animals are N (normal mouse) and L (leukemic mouse).*

cluding brain, thymus, and thymoma from normal or leukemic animals. Three other *Eco*RI fragments (20, 18.4, and 16.0 kbp) were also detected but in different combinations in these DNAs. Furthermore, Table 1 and Fig. 5 show that all *Eco*RI fragments hybridizing with the 400-bp probe that were present in leukemic tissue (Fig. 5, lanes F through K) were also seen in normal tissue DNAs (Fig. 5, lanes A through E). Extra bands besides those found in control tissue DNAs were not present in DNAs of leukemic thymuses. The multiple number of *Eco*RI fragments hybridizing to the 400-bp probe was surprising, however, since only two and possibly three genetic loci have been identified with AKR MuLV expression (9, 15, 16). Another surprising observation was that there appeared to be different patterns of the six *Eco*RI fragments cited above in tissue DNAs from individual animals, indicating a polymorphism for these sequences in this population of inbred AKR/J mice. DNAs extracted from different tissues of the same animal, however, had invariant patterns of *Eco*RI fragments (Fig. 4C, lanes A through D, and Table 1).

DISCUSSION

We have identified a 400-bp *Sma*I DNA fragment specific for the AKR MuLV genome based on the observation that it did not hybridize to either MCF-13 or Moloney MuLV Hirt-extracted linear DNAs. Nor did it hybridize to liver DNAs from a 129 or NZB mouse, two strains of mouse which lack sequences related to AKR MuLV but possess xenotropic virus-related sequences. This fragment was derived from the envelope region located between 6.7 and 7.1 kbp from the 5' end of the AKR MuLV DNA (11), which has been characterized by RNase T_1 oligonucleotide mapping and heteroduplexing

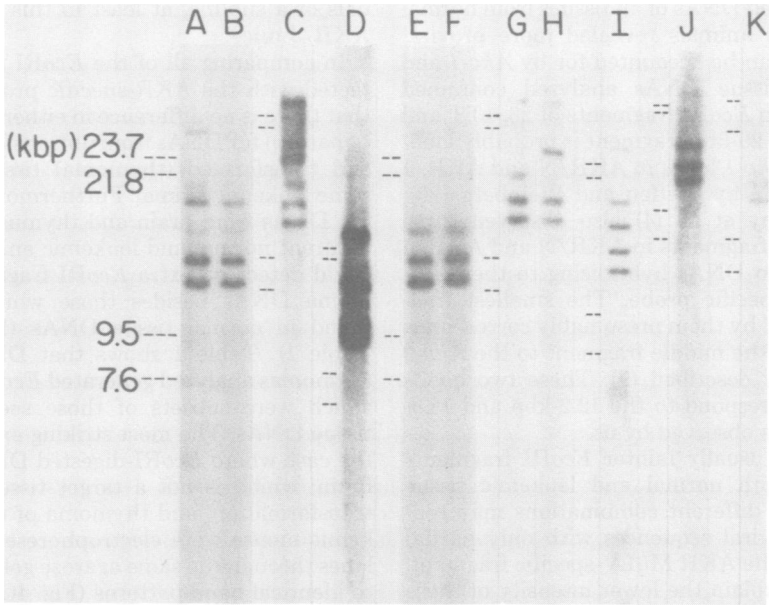

FIG. 5. *EcoRI-digested tissue DNAs analyzed by hybridization with the 400-bp AKR MuLV-specific fragment. EcoRI-digested tissue DNAs (15 μg) were electrophoresed through 0.7% horizontal agarose slab gels in either low- or high-salt E buffer at 25 to 50 V for varying lengths of time. DNAs after transfer to nitrocellulose filters were hybridized with the ^{32}P-labeled 400-bp SmaI fragment. Marker DNAs (EcoRI- and HindIII-digested λ DNA) were coelectrophoresed through every gel, and fragment sizes in kilobases are indicated to the left of the figure. Dashed lines indicate the same marker DNA fragments for different gels. (A) Brain from normal mouse (N1); (B) thymus from normal mouse (N1); (C) thymus from normal mouse (N2); (D) brain from normal mouse (N3); (E) brain from leukemic mouse (L1); (F) thymoma from leukemic mouse (L1); (G) thymoma from leukemic mouse (L2); (H) thymoma from leukemic mouse (L3); (I) thymoma from leukemic mouse (L4); (J) thymoma from leukemic mouse (L5); (K) thymoma from leukemic mouse (L6). Notations for the animals are N (normal mouse) and L (leukemic mouse). Numbers refer to individual animals.*

TABLE 1. *EcoRI-digested tissue DNA fragment sizes*

B(N1)[a]	T(N1)	T(N2)	B(N3)	B(L1)	T(L1)	T(L2)	T(L3)	T(L4)	T(L5)	T(L6)
26.0[b]	26.0	26.0	26.0	26.0	26.0	26.0	26.0	26.0	26.0	26.0
20.0	20.0	20.0								20.0
18.4	18.4	18.4	18.4			18.4	18.4	18.4		18.4
16.0	16.0	16.0	16.0	16.0	16.0	16.0		16.0	16.0	
13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8	13.8
12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2

[a] Notations used for animals and tissues from which DNAs were isolated and digested with *Eco*RI are: B, brain; T, thymus from normal animals or thymoma from leukemic animals; N1 through N3, normal AKR mouse 1 through 3; L1 through L6, leukemic AKR mouse 1 through 6. Each number refers to a single animal.

[b] Numbers refer to sizes of *Eco*RI fragments in kilobase pairs.

studies to be the major region of nonhomology between the AKR and MCF MuLV's (5, 14). Since this is also the region which has been substituted by xenotropic viral sequences in MCF MuLV's (7), the 400-bp fragment presumably lacks homology with xenotropic sequences present in MCF MuLV's and in 129 or NZB mouse liver DNAs. It is not clear what viral sequences, which also lack homology to the 400-bp fragment, are present in the Moloney MuLV envelope region. It has been shown, however, that these sequences are not homologous to NZB xenotropic viral RNA as shown by heteroduplex studies (2). While this work was in progress, Chattopadhyay et al. (4) reported that they had identified the same 400-bp fragment described here to be ecotropic type-specific sequences, which lacked homology with a number of xenotropic, amphotropic, and MCF MuLV DNAs.

Hybridization of the 400-bp AKR probe to

*Eco*RI-digested DNAs of all tissues from normal and leukemic animals revealed more proviral copies than can be accounted for by *Akv-1* and *Akv-2*. All tissue DNAs analyzed contained three common *Eco*RI fragments of 26, 13.8, and 12.2 kbp. The 26-kbp fragment is probably identical to the *Akv-1* locus in AKR/N and AKR/J mice described by Steffen and Weinberg (20). Chattopadhyay et al. (4) also observed three major *Eco*RI fragments in AKR/N and AKR/J mouse embryo DNAs hybridizing to their ecotropic type-specific probe. The smallest fragment detected by them presumably corresponds to *Akv-2* and the middle fragment to the *Akv-3* locus recently described (9). These two bands appear to correspond to the 12.2-kbp and 13.8-kbp fragments observed by us.

The other, usually fainter *Eco*RI fragments present in both normal and leukemic tissue DNAs but in different combinations may represent other viral sequences with only partial homology to the AKR MuLV-specific fragment. This would explain the lower intensity of these bands compared with the legitimate *Akv* loci. Another explanation for the fainter bands may be that they represent germ line reintegrations of the AKR MuLV, a phenomenon recently described by Rowe and Kozak (17) for *Akv-1* congenic mice. The faintness of the bands would suggest either heterozygosity at these chromosomal sites or reintegration of only a fraction of the 400-bp fragment. It is unlikely that the majority of fainter bands resulted from partial digestion of the DNA since all digestions were monitored for completeness by the addition of λ DNA to a portion of the reaction mixture as described in Materials and Methods. Moreover, different tissue DNAs from the same animal had identical patterns, and it would be unlikely that different DNA preparations would be partially digested to the same extent.

We observed three basic patterns of AKR proviruses present in tissues of the individual animals used in this study. These three patterns are represented by the DNA fragments of (i) 26.0, 16.0, 13.8, 12.2, (ii) 26.0, 18.4, 13.8, 12.2, and (iii) 26.0, 20.0, 18.4, 13.8, 12.2 as seen in Table 1 and Fig. 5 for the brain of a leukemic animal (L1), the thymoma of a leukemic animal (L3), and the thymoma of a leukemic animal (L6), respectively. All other provirus patterns can be attributed to sums of two of these three basic patterns. It may be the case that other individuals in this colony of inbred mice have even fewer proviruses than those we have observed. It appears that variations in AKR ecotropic sequences exist not only in different sublines of the AKR mouse strain as reported by other investigators (4, 20) but also in different individuals of a subline, at least in this population of AKR/J mice.

In comparing all of the *Eco*RI fragments detected with the AKR-specific probe, it is clear that there is no difference in either their number or pattern for DNAs from untransformed (brain) and transformed (thymoma) tissues from the same leukemic animal. Furthermore, in comparing DNAs from brain and thymus tissues from different normal and leukemic animals, we also could detect no extra *Eco*RI fragments in thymoma DNAs besides those which were also found in normal tissue DNAs (Fig. 4 and 5; Table 1). Table 1 shows that DNAs from all thymomas analyzed generated *Eco*RI fragments which were subsets of those seen in normal tissue DNAs. The most striking example was in the case where *Eco*RI-digested DNAs from the brain, which is not a target tissue for MuLV transformation, and thymoma of the same leukemic mouse were electrophoresed in adjacent lanes through the same agarose gel and gave rise to identical band patterns (Fig. 4C, lanes C and D).

These results indicate that no amplification of at least the complete AKR MuLV genome or the region corresponding to the 400-bp probe has occurred during the development of leukemia in AKR mice. There is a possibility that the reason we did not observe any amplification was because the thymomas used in this study were not of clonal origin. This is not likely, however, since the majority of cells from similar or other tumors (3, 6, 13, 20) have been shown to be monoclonal, and by using an MCF-13 MuLV probe we have been able to detect extra bands in DNAs isolated from thymomas of animals injected with MCF-13 MuLV at birth (F. K. Yoshimura and M. Breda, unpublished data). The increased copy number which other investigators have reported possibly corresponded to parts of the 5′ region of the genome which share homology with recombinant viruses. The amplification which they observed might possibly represent the integration of these recombinant viruses such as the MCF MuLV's. Because of the difficulty in detecting these extra sequences by using either an unselected probe or one representing the 5′ region of the AKR genome (4.8-kbp *Sma*I fragment), it will be necessary to develop a complementary xenotropic specific probe(s) which will recognize various recombinant viruses for further analysis of the involvement of different MuLV's in AKR leukemogenesis.

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