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Variation in the Number of Copies and in the Genomic Organization of Ecotropic Murine Leukemia Virus Proviral Sequences in Sublines of AKR Mice

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DNAs isolated from individual mice of four AKR sublines (AKR/J, AKR/N, AKR/Cum, and AKR/Boy) were examined by hybridization of electrophoretically separated restriction enzyme fragments to a 500-base pair, ^{32}P-labeled probe specific for *env* sequences of ecotropic murine leukemia virus. Variation in the number of proviral DNA copies and in their genomic organization, as reflected by the location of restriction enzyme sites in flanking cellular sequences, was observed both between and within AKR sublines. Evidence is presented for the continual acquisition of new proviruses in the four sublines studied. The ecotropic proviral DNA copies present in the four AKR sublines can be related to their genealogy; each subline contains two or three copies of proviral DNA in common with other sublines and from one to six unique ecotropic proviruses. Overall, a new copy appears about every 12 generations of inbreeding. Some of the unique proviral DNA copies contain internal alterations, as reflected by restriction enzyme maps that differ from those of prototype ecotropic proviruses.

Two classes of endogenous murine leukemia viruses (MuLVs) with unique biological, immunological, and biochemical properties have been isolated from inbred mice (11). Ecotropic MuLVs are able to replicate only in murine cells, whereas xenotropic MuLVs can be propagated in cells from a wide variety of mammalian species but are unable to infect mouse cells. Inbred strains of mice that contain endogenous ecotropic proviruses can be further classified as "high virus" or "low virus" with respect to the spontaneous expression of their ecotropic proviral DNA. High-virus strains such as AKR or C58 begin producing infectious ecotropic MuLV shortly after birth, and nearly all animals develop leukemia by 18 months of age. In AKR mice, ecotropic proviruses have been mapped to chromosomes 7 and 16 by classical genetic techniques (7, 12). As a rule, high-virus mice contain at least three biochemically detectable copies of endogenous ecotropic proviral DNA (4, 5), and some strains may harbor as many as 12 to 15 proviruses (5). Low-virus, low-leukemia mice such as BALB/c and C57BL contain a single copy of proviral DNA and are either virus negative or produce low titers of virus in later life.

The establishment of sublines of inbred mice has permitted an evaluation of independent changes occurring in a homogeneous genetic background. For example, AKR/J and AKR/Cum were established from a common stock in 1949 and have been shown to differ from one another at several loci (1), possibly because of residual heterozygosity before subline generation. Three recent reports have demonstrated variability in the number of endogenous ecotropic MuLV proviruses in AKR sublines (6, 10, 18). In this paper we have examined the stability of ecotropic proviral DNA in four substrains of AKR mice. Unlike other mouse genes, the complement of endogenous ecotropic MuLV DNA can be amplified as a result of exogenous infection in utero with subsequent insertion of new proviral DNA copies into the germ line (13). Using an ecotropic envelope (*env*)-specific DNA probe, we have analyzed the chromosomal DNAs of four different AKR substrains by the blot hybridization method. The results obtained indicate that the number and location of ecotropic MuLV proviral copies vary not only between sublines of AKR mice but within a single AKR substrain. At least four different patterns involving five different ecotropic proviruses were identified in AKR/J mice. This type of variability was observed in an exaggerated form in the DNAs prepared from sibling AKR/Boy mice, each of which contained five to seven common,

and two unique, ecotropic proviral DNAs. The number and organization of ecotropic proviruses in a pedigreed AKR/J stock and in *Akv-1* congenic mice were also studied.

MATERIALS AND METHODS

Mice. AKR/J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Mice from the Jackson Laboratory resource stock or from the pedigreed expansion stock were used. The latter mice were maintained and bred for two additional inbred generations in our laboratory. NFS/N and AKR/N mice were obtained from the Small Animal Section, Division of Research Services, National Institutes of Health, Bethesda, Md. An AKR/Cum mouse was obtained from Cumberland View Farms, Clinton, Tenn., and two AKR/Boy mice were obtained as littermates from E. A. Boyse, Memorial Sloan-Kettering Cancer Center, New York, N.Y. The NFS.*Akv-1* (12) and NFS.*Akv-2* (7) congenic mouse colonies were maintained in our laboratory.

Mouse liver DNA isolation, restriction endonuclease digestion, and agarose gel electrophoresis. High-molecular-weight DNA was extracted as previously described (4). Restriction endonucleases were obtained from Bethesda Research Laboratories, Bethesda, Md., or from New England Biolabs, Beverly, Mass. Digestion conditions were those specified by the supplier. DNAs were digested with 3 to 4 U of enzyme per μg of DNA for 18 to 36 h. Restriction fragments were separated by electrophoresis on 0.6% agarose gels as described previously (3) and transferred to nitrocellulose membranes as described by Southern (14).

Preparation of DNA probes. The construction of a recombinant plasmid (pEc-B4) containing ecotropic MuLV *env*-specific DNA sequences has been previously described (4). Although we previously reported that some individual DNA preparations isolated from ecotropic MuLV-negative mouse strains (e.g., NZB, 129, C57LN) contained cleavage products that hybridized to the ecotropic *env* probe, we have subsequently determined that this reactivity was due to the presence of small amounts of contaminating pBR322 DNA (estimated to be three to five copies per diploid amount of cellular DNA) in the mouse DNA preparations. As a consequence of this finding, mouse liver DNAs are currently screened with ^{32}P-labeled pBR322 DNA before hybridization with probes such as pEc-B4 which contain plasmid DNA. No bands reactive with the ecotropic *env* probe have been detected in the liver DNAs of four NFS, four NZB, four 129, one C57/L, and three CBA/N mouse strains, which are negative for ecotropic virus by biological assays.

Cellular DNA segments which flank the ecotropic proviral DNA situated at the *Akv-2* locus were subcloned from recombinant λ clones derived from DNA of an NFS.*Akv-2*–Chinese hamster ovary somatic cell hybrid that contains only mouse chromosome 16 (7). To prepare recombinant phage, DNA from the hybrid cell line was digested with *Eco*RI and *Bam*HI or with *Hin*dIII, ligated to appropriately cleaved Charon 21A DNA arms, and packaged in vitro (2) before infection of *Escherichia coli* DP50 SupF (S. P. Staal, manuscript in preparation). Recombinant phages hybridizing to the MuLV cDNA (3) and containing either a 6-kilobase (kb) *Eco*RI to *Bam*HI insert or a 7-kb *Hin*dIII insert were isolated. The former contained the 5′ 1.9 kb of proviral sequences together with 4.1 kb of flanking cellular sequences, while the latter contained the 3′ 5.8 kb of proviral DNA and 1.2 kb of flanking sequences (shown in Fig. 3C). Cellular sequences which flank the 5′ (0.6-kb *Hin*dIII fragment located 1.2 kb from the 5′ end of the provirus) and 3′ (0.8-kb *Ava*I to *Hin*dIII fragment) termini were subcloned into pBR322 DNA. (The restriction map of the *Akv-2* gene region showing the location of the sites described is shown in Fig. 3C.)

Recombinant plasmid DNAs were labeled with ^{32}P by the nick translation procedure (9) and had specific activities of 6×10^7 to 13×10^7 cpm/μg of DNA.

Blot hybridization. Assays with the ecotropic MuLV-specific probe (pEc-B4) were carried out as previously described (3). To reduce the reactivity of repetitive sequences present in the cloned segments that flank the *Akv-2* locus, hybridization conditions and washes were made more stringent. The salt concentrations in prehybridization and hybridization buffers were reduced to 0.33 M, and all incubations were carried out at 68°C. After hybridization for 18 to 24 h, the nitrocellulose membranes were washed six times for 10 min at 68°C in 0.3 M NaCl–0.03 M sodium citrate–0.2% Ficoll–0.2% polyvinylpyrrolidone–0.2% bovine serum albumin–0.1% sodium dodecyl sulfate. This was followed by three 15-min washes at 68°C in 15 mM NaCl–1.5 mM sodium citrate–0.1% sodium dodecyl sulfate. Finally, the membranes were washed three to six times for 10 min at 68°C in 0.3 M NaCl–0.03 M sodium citrate. After drying, the membranes were exposed to Kodak AR film as described previously (3).

RESULTS

Number and organization of endogenous ecotropic MuLV proviruses in four substrains of AKR mice. Previous studies (4, 6, 10, 18) have demonstrated variability in the number of ecotropic MuLV sequences present in the DNA of AKR mice. These differences were investigated in greater detail by determining the number and sizes of restriction fragments which hybridized to cloned ecotropic MuLV envelope (*env*) sequences present in pEc-B4 DNA (4). Liver DNAs were prepared from 11 AKR/J mice acquired at various times over a period of 1 year, from 2 AKR/N mice, from a single AKR/Cum mouse, and from 2 AKR/Boy mice. The restriction enzymes (Fig. 1G) used were: (i) *Eco*RI, which does not cleave ecotropic proviral DNA sequences (15); (ii) *Xba*I, which cuts ecotropic proviruses a single time (16) to the right of the cloned ecotropic *env* sequences (mapping between 6.5 and 7.0 kb) and therefore generates a restriction fragment containing flanking cellular DNA sequences that abut the 5′ terminus of the proviral DNA; (iii) *Hin*dIII and *Sac*I, each of which cleaves proviral sequences once (at 3.0 and 3.6 kb, respectively) (16) and defines cellular sequences that are located 3′ to proviral genes; (iv) *Pst*I, which cuts the ecotropic provirus once in each long terminal repeat, producing

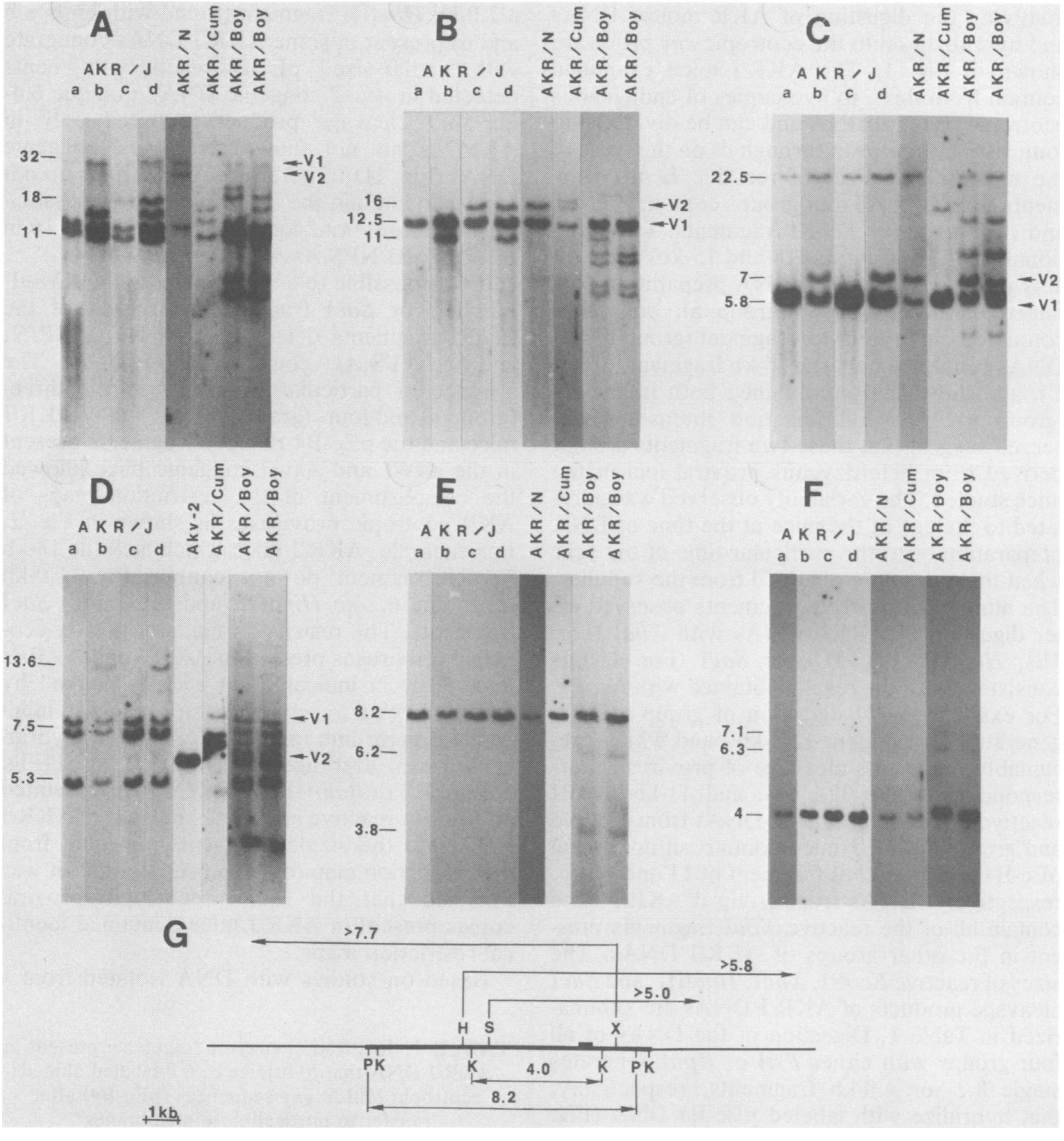

FIG. 1. (A to F) Autoradiograms of filter blot hybridization analyses of restriction enzyme-digested AKR liver DNAs. Enzyme digestion, electrophoresis on 0.6% agarose gels, transfer to nitrocellulose membranes, and hybridization to ^{32}P-labeled pEc-B4 were as described in the text. (A) *Eco*RI-digested DNAs, (B) *Xba*I-digested DNAs, (C) *Hin*dIII-digested DNAs, (D) *Sac*I-digested DNAs, (E) *Pst*I-digested DNAs, and (F) *Kpn*I-digested DNAs. Fragment sizes in kb are indicated to the left of each panel, while the position of fragments containing *Akv-1* and *Akv-2* proviral sequences (v1 and v2) are shown to the right of panels A to D. The 32-kb *Eco*RI *Akv-1* fragment (A) transfers with poor efficiency, resulting in a very low intensity on the autoradiogram. Very long exposures demonstrate the presence of this *Eco*RI fragment in all AKR DNAs studied. NFS.*Akv-2* congenic DNA was substituted for AKR/N DNA in (D) (*Sac*I digestion) to demonstrate the position of the unique *Akv-2* fragment. (G) Restriction map of an endogenous *Akv* proviral copy with the positions of the *Pst*I (P), *Hin*dIII (H), *Kpn*I (K), *Sac*I (S), and *Xba*I (X) sites indicated. The sizes, in kb, of restriction fragments that would be expected to hybridize to pEc-B4 (thick portion of the proviral sequence at the left of the third *Kpn*I site) are indicated.

a single characteristic 8.2-kb fragment (16) that will hybridize to labeled pEc-B4 DNA; and (v) *Kpn*I, which cleaves the *Akv* provirus at 0.5, 3.3, 7.3, and 8.7 kb (8), with the 4.0-kb fragment (located between the *Kpn*I sites at 3.3 and 7.3 kb) containing sequences that react with the pEc-B4 DNA probe.

The results of blot hybridization experiments

involving the digestion of AKR mouse DNAs and hybridization to the ecotropic *env* probe are shown in Fig. 1. The AKR/J mice examined contain from three to five copies of endogenous ecotropic proviral DNA and can be divided into four distinct groups (a through d) on the basis of the number and sizes of reactive *Eco*RI fragments (Fig. 1A). All four groups contain 32-, 13-, and 11-kb reactive *Eco*RI fragments. Two additional *Eco*RI fragments (18 and 15 kb) are variably present: one AKR/J DNA preparation contained neither fragment (group a), one DNA contained only the 15-kb fragment (group b), six DNAs contained only the 18-kb fragment (group C), and three DNAs contained both fragments (group d). The hybridization intensities observed suggest that these two fragments are not derived from heterozygous proviral loci in the mice studied. The variability observed was unrelated to the age of the mice at the time of DNA preparation or to the particular time of the year when the mice were obtained from the supplier. The number of reactive fragments observed after digestion of AKR/J DNAs with *Xba*I (Fig. 1B), *Hin*dIII (Fig. 1C), or *Sac*I (Fig. 1D) is consistent with the results obtained with *Eco*RI. For example, *Xba*I digestion of group a DNA generates a prominent 12.5-kb band which presumably represents cleavage of proviruses corresponding to the 32-, 13-, and 11-kb *Eco*RI reactive digestion products. DNAs from group b and group c AKR/J mice contain an additional pEc-B4-reactive *Xba*I fragment of 11 and 16 kb, respectively. DNAs from group d AKR/J mice contain all of the reactive *Xba*I fragments present in the other groups of AKR/J DNAs. The sizes of reactive *Eco*RI, *Xba*I, *Hin*dIII, and *Sac*I cleavage products of AKR/J DNAs are summarized in Table 1. Digestion of the DNAs of all four groups with either *Pst*I or *Kpn*I generates single 8.2- or 4.0-kb fragments, respectively, that hybridize with labeled pEc-B4 DNA (Fig. 1E and F).

AKR/N mice contain three *Eco*RI fragments that react with labeled pEc-B4 DNA. Besides the 32- and 13-kb *Eco*RI bands which are shared with AKR/J animals, AKR/N mice contain a unique 25-kb fragment (Fig. 1A). The 32-kb *Eco*RI fragment present in both AKR/J and AKR/N animals comigrates with a single fragment present in NFS.*Akv-1* congenic mouse DNA that hybridizes with the ecotropic *env* probe (data not shown). Similar *Akv-1*-specific *Xba*I, *Hin*dIII, and *Sac*I fragments are also present in AKR/J and AKR/N DNAs. The 25-kb *Eco*RI fragment detected in AKR/N DNA comigrates with a fragment in NFS.*Akv-2* congenic mice that anneals to the ecotropic MuLV *env* probe (data not shown). Although a 16-kb *Xba*I fragment (found with groups c and d, Fig. 1) and a 7.0-kb *Hin*dIII fragment (found with groups b and d) present in some AKR/J DNAs comigrate with similar-sized pEc-B4-reactive fragments detected in *Akv-2* congenic DNA, a unique 6.4-kb *Sac*I cleavage product is found only in AKR/N (data not shown) and *Akv-2* congenic DNAs (Fig. 1D). Therefore, AKR/J mice do not appear to contain the ecotropic provirus associated with the *Akv-2* locus on chromosome 16 in AKR/N and NFS.*Akv-2* congenic mice.

It was possible to associate a particular *Xba*I, *Hin*dIII, or *Sac*I fragment with each of the *Eco*RI fragments detected in AKR/J, AKR/N, and two NFS.*Akv* congenic mouse DNAs. The absence of particular fragments in the three- (group a) and four- (groups b and c) copy AKR/J mice and the pEc-B4-reactive fragments present in the *Akv-1* and *Akv-2* congenic mice allowed the establishment of the restriction maps of AKR ecotropic proviruses as shown in Fig. 2. For example, AKR/J mice which lack the 18-kb *Eco*RI fragment do not contain the 16.0-kb *Xba*I, the 6.2-kb *Hin*dIII, and the 7.2-kb *Sac*I fragments. The restriction maps of the six ecotropic proviruses present in AKR/J and AKR/N mice (Fig. 2) indicate that each is flanked by different cellular sequences and suggest independent germ line insertions. Since the hybridization seen after digestion with *Pst*I, *Kpn*I (Fig. 1D and E), or *Bam*HI (data not shown) recruited all pEc-B4-reactive sequences present in AKR/J DNA into the single fragments expected from the restriction map of cloned AKV_{623} (8), it was assumed that the MuLV ecotropic proviral copies present in AKR/J mice contained identical restriction maps.

Based on studies with DNA isolated from a

TABLE 1. Restriction enzyme fragments present in AKR/J DNA that hybridize to ^{32}P-labeled cloned ecotropic MuLV *env* sequences (pEc-B4) after transfer to nitrocellulose membranes[a]

Restriction enzyme	Fragment sizes in kb
*Eco*RI	32, 18,[b] 15,[b] 13, 11
*Xba*I	16,[b] 12.5,[c] 11[b]
*Hin*dIII	22.5, 7.0,[b] 6.2,[b,d] 6.0,[d] 5.8[d]
*Sac*I	13.6,[b] 8.3, 7.5,[e] 7.2,[b,e] 5.3

[a]AKR/J DNAs were digested with restriction enzymes, separated on 0.6% agarose gels, transferred to nitrocellulose membranes, and hybridized to cloned ecotropic MuLV *env* proviral sequences (pEc-B4) as described in the text.

[b]Variably present.

[c]An unresolved triplet of nearly identical-sized fragments.

[d]A poorly resolved triplet of similar-sized fragments.

[e]A poorly resolved doublet of similar-sized fragments.

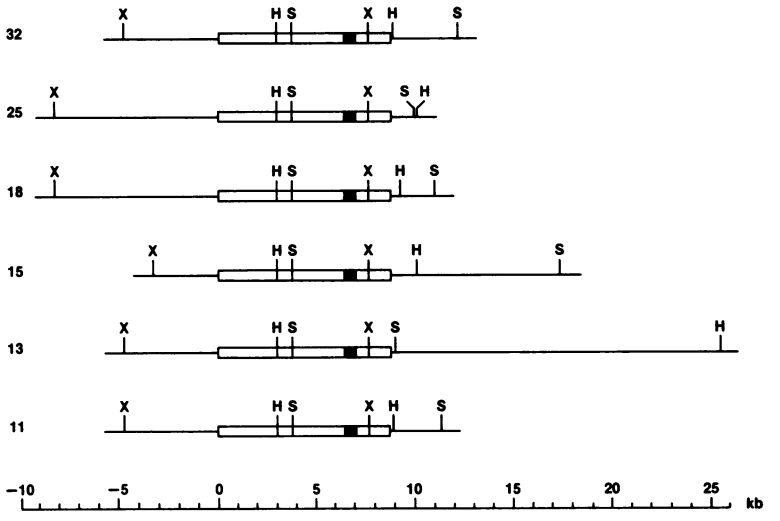

FIG. 2. *Xba*I, *Hin*dIII, and *Sac*I restriction maps of endogenous ecotropic MuLV copies and flanking cellular DNA from AKR/J and AKR/N mice. Numbers to the left of each line indicate the size, in kb, of *Eco*RI restriction fragments that contain the proviral copy shown. The 32-kb (*Akv-1*) and 13-kb *Eco*RI copies are present in both AKR/J and AKR/N DNAs. The 25-kb (*Akv-2*) *Eco*RI copy is unique to AKR/N DNAs, while the remaining copies (18-, 15-, and 11-kb *Eco*RI copies) are unique to AKR/J DNAs. The scale at the bottom of the figure is in kb from the 5′ end of the proviral copies. X, *Xba*I; H, *Hin*dIII; S, *Sac*I. The position of the restriction sites in flanking cellular DNA was determined from the fragment sizes shown in Fig. 1.

single AKR/Cum mouse, four copies of ecotropic proviral DNA sequences were observed (Fig. 1). The 32-kb *Eco*RI *Akv-1* provirus previously detected in AKR/J and AKR/N DNAs was observed only after long exposure of the hybridized nitrocellulose membrane because of poor transfer of high-molecular-weight DNAs. Other pEc-B4-reactive fragments were the 11-kb *Eco*RI fragment present in AKR/J DNA and two additional *Eco*RI fragments (14 and 12.5 kb) absent in AKR/J and AKR/N DNAs. *Xba*I, *Hin*dIII, and *Sac*I digestion of AKR/Cum DNA confirms the identity of fragments shared with AKR/J and AKR/N DNAs (Fig. 1). Mapping of *Xba*I, *Hin*dIII, and *Sac*I sites in cellular DNA flanking the unique AKR/Cum ecotropic proviruses has not yet been completed.

AKR/Boy DNAs contain seven to nine restriction fragments that hybridize to pEc-B4 DNA (Fig. 1). The 32-, 13-, and 11-kb *Eco*RI bands found in AKR/J DNA are also detected in both AKR/Boy DNAs. The appropriate *Xba*I, *Hin*dIII, and *Sac*I restriction fragments confirm the identity of these three proviral DNAs (Fig. 1). Both of the AKR/Boy liver DNAs examined contain 10.5- and 18.2-kb pEc-B4-reactive *Eco*RI digestion products, but one DNA contains additional *Eco*RI fragments of 18.5 and 13.8 kb, while the other DNA contains a unique 14.5-kb *Eco*RI fragment. This result was quite unexpected since the two AKR/Boy mice were siblings. Thus, some of the unique ecotropic proviral DNAs detected in AKR/Boy mice may represent recent germ line reinsertions present as single (heterozygous) copies in mouse genomic DNA.

Ecotropic envelope sequences present in the AKR mouse DNAs could be organized differently than those associated with proviruses of known infectious ecotropic MuLVs. Hybridization of pEc-B4 DNA to *Pst*I and *Kpn*I fragments other than the characteristic 8.2- and 4.0-kb cleavage products, respectively, would indicate such a difference. Both AKR/Boy DNAs contain at least two ecotropic proviral DNAs of this type. *Eco*RI fragments of 7.1 and 6.3 kb are clearly too small to be derived from an 8.8-kb ecotropic provirus containing no *Eco*RI restriction sites. Reactive 7.0- and 6.5-kb *Xba*I fragments, 5.6- and 4.8-kb *Hin*dIII fragments, and a 5.0-kb *Sac*I fragment are also smaller than those expected from an infectious ecotropic provirus (Fig. 1G). In addition to the 8.2-kb *Pst*I and 4.0-kb *Kpn*I cleavage products characteristic of ecotropic proviral DNA, both AKR/Boy DNAs contained other fragments that hybridized to pEc-B4. As shown in Fig. 1E and F, 6.2- and 3.8-kb *Pst*I fragments and 7.1- and 6.3-kb *Kpn*I fragments were detected in the AKR/Boy DNAs. All of the proviral DNA sequences present in AKR/J, AKR/N, and AKR/Cum mouse DNAs that hybridize with labeled pEc-B4 DNA

are recruited into single fragments of the expected size after digestion with either *Pst*I or *Kpn*I (Fig. 1E and F).

AKR/J mice do not contain the *Akv-2* locus. The data presented in Fig. 1 and 2, as well as a previous report (15), suggest that the *Akv-2* locus is absent in AKR/J mice. However, the results of some blot hybridization experiments involving restricted AKR/J mouse DNAs were ambiguous in completely ruling out the possibility that one of the ecotropic loci detected in AKR/J mice might have been derived from an *Akv-2* locus that had been altered by deletions or insertions or both in the flanking cellular sequences. For example, some AKR/J mice (groups c and d, Fig. 1) contain a 16.0-kb *Xba*I cleavage product that comigrates with the *Xba*I fragment characteristic of the *Akv-2* provirus. Furthermore, a 7.0-kb *Hin*dIII fragment present in group b and d AKR/J mice (Fig. 1) is also found in *Akv-2* congenic mice. On the other hand, none of the reactive *Eco*RI or *Sac*I fragments present in AKR/J mice comigrate with the unique 25-kb *Eco*RI or 6.4-kb *Sac*I fragment characteristic of the *Akv-2* locus in AKR/N mice.

To investigate the possibility that a "covert" *Akv-2* locus was present in AKR/J DNA, cellular DNA sequences flanking both the 5′ and 3′ termini of the *Akv-2* provirus were cloned, as described above, and used as probes in Southern blot hybridization experiments. As shown in Fig. 3A (lanes b and c), both NFS.*Akv-2* and AKR/N mouse DNAs contain a single 25-kb *Eco*RI fragment that hybridizes to ^{32}P-labeled probe containing cellular DNA sequences that flank the 5′ terminus of the *Akv-2* provirus. These reactive fragments comigrate with *Eco*RI cleavage products of *Akv-2* congenic and AKR/N DNAs that hybridize with labeled pEc-B4 DNA (Fig. 3A, lanes f and g). In contrast, the cloned *Akv-2* 5′ flanking cellular sequences hybridize to 18-kb *Eco*RI fragments in both AKR/J and NFS/N mouse DNAs (Fig. 3A, lanes a and d). Both NFS.*Akv-2* and AKR/N DNAs contain a 16-kb *Xba*I fragment that hybridizes to the cloned 5′ cellular flanking sequence probe (Fig. 3B, lanes b and c). This 16-kb cleavage product comigrates with an *Xba*I fragment derived from AKR/N and NFS.*Akv-2* DNAs that hybridizes to the ecotropic *env* probe (Fig. 3B, lanes f and g). Both AKR/J DNA and NFS/N DNA contain a single 8-kb *Xba*I fragment that hybridizes with the cloned 5′ flanking cellular sequence probe (Fig. 3B, lanes a and d); this does not correspond to any of the pEc-B4-reactive bands present in AKR/J DNA (Fig. 3B, lane e).

Similar blot hybridization experiments were carried out with a 0.8-kb *Ava*I to *Hin*dIII DNA probe derived from a cloned DNA segment containing cellular sequences which flank the 3′ end of the *Akv-2* provirus. The results obtained are summarized in Table 2 and are in agreement with the experiments shown in Fig. 3A and B which indicate the absence of the *Akv-2* locus in AKR/J mice. The restriction maps of the *Akv-2* locus and the comparable segment of AKR/J and NFS/N mouse DNAs were constructed from these studies and are shown in Fig. 3C.

Stability of ecotropic proviral DNA in a pedigreed AKR/J stock. Since AKR/J commercial stock mice consist of at least four distinct subgroups with respect to the number of ecotropic MuLV proviruses that they contain, a pedigreed expansion stock breeding pair, four generations from the Jax AKR foundation stock, was obtained for further study. Individual liver DNAs were prepared from three successive inbred

TABLE 2. Restriction fragments from AKR/J, *Akv-2* congenic, AKR/N, and NFS/N mouse DNAs that hybridize to cloned sequences 5′ or 3′ to the endogenous *Akv-2* provirus[a]

Restriction enzyme	Flanking sequence probe	Fragment size in kb			
		AKR/J	NFS.*Akv-2*	AKR/N	NFS/N
*Eco*RI	5′	18	25	25	18
	3′	18	25	25	18
*Xba*I	5′	8	16	16	8
	3′	8	8	8	8
*Hin*dIII	5′	0.6	0.6	0.6	0.6
	3′	2.2	7	7	2.2
*Sac*I	5′	4	6.5	6.5	4
	3′	4	6.4	6.4	4

[a]Mouse DNAs were digested with restriction enzymes, separated on 0.6% agarose gels, transferred to nitrocellulose membranes, and hybridized to ^{32}P-labeled cloned mouse sequences derived from the 5′ or 3′ flanking region of the *Akv-2* proviral copy as described in the text.

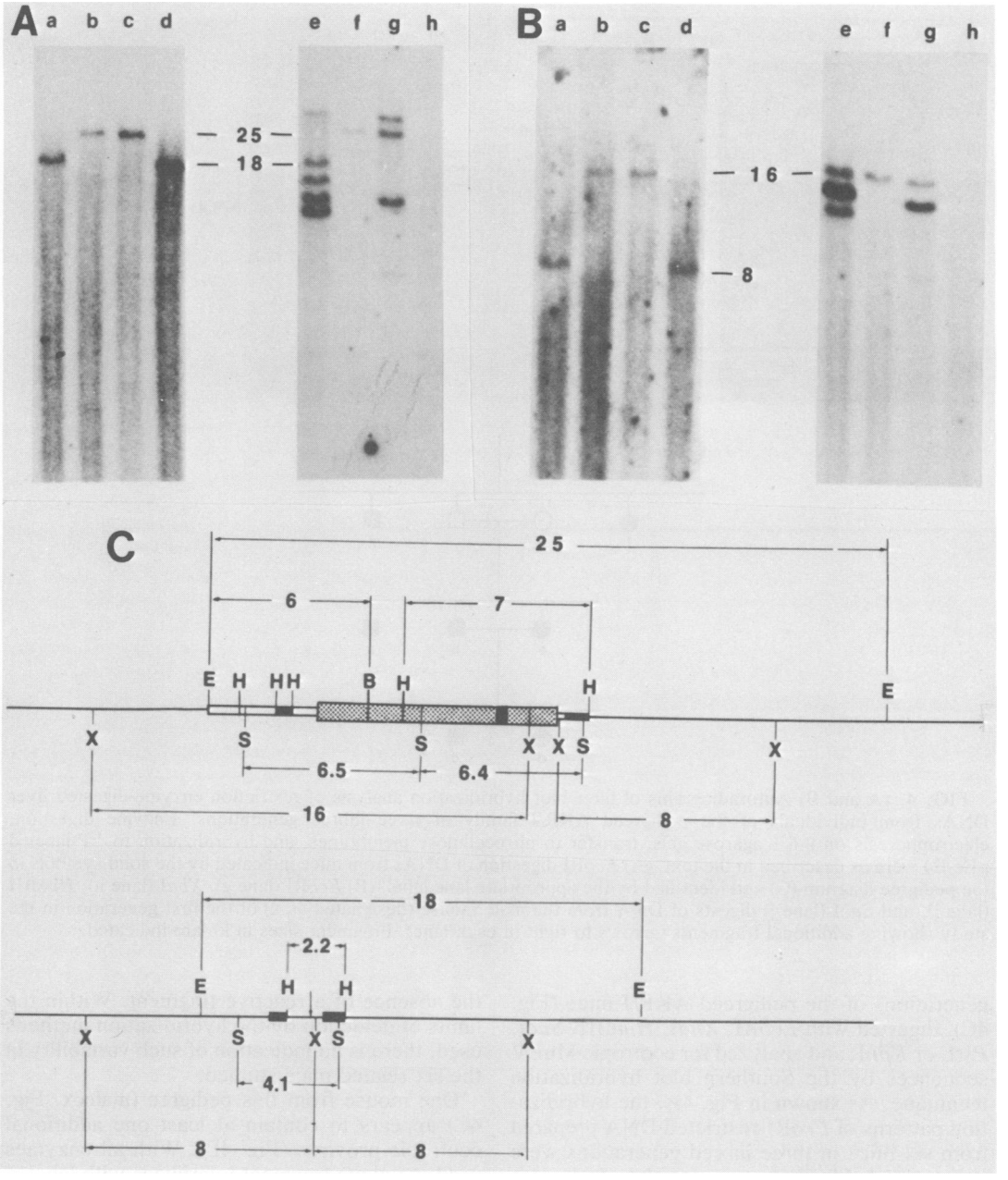

FIG. 3. (A and B) Autoradiograms of filter blot hybridization analysis of restriction enzyme-digested AKR and NFS/N.*Akv-2* DNAs. Enzyme digestion, electrophoresis on 0.6% agarose gels, transfer to nitrocellulose membranes, and hybridization to ^{32}P-labeled cloned *Akv-2* 5′ flanking sequences (lanes a to d) or pEc-B4 (lanes e to h) were as described in the text. AKR/J (lanes a and e), NFS.*Akv-2* congenic (lanes b and f), AKR/N (lanes c and g), and NFS/N (lanes d and h) DNAs were digested with *Eco*RI (A) or *Xba*I (B). The AKR/J DNA examined in lanes a and e contains five copies of endogenous ecotropic proviral DNA (group d) including the 18-kb *Eco*RI fragment which anneals to the pEc-B4 probe. The comigration of this cleavage product with a fragment hybridizing to the *Akv-2* cloned 5′ flanking DNA probe is fortuitous. (C) Restriction maps with *Xba*I (X), *Eco*RI (E), *Hin*dIII (H), and *Sac*I (S) of AKR/N and NFS.*Akv-2* DNAs (upper diagram) or of AKR/J and NFS/N DNAs (lower diagram) that contain fragments which hybridize to cloned sequences located in flanking mouse cellular DNA 5′ and 3′ to the *Akv-2* proviral copy in NFS.*Akv-2* DNA. The construction of these flanking sequence clones is described in the text. The stippled region of the upper diagram represents the inserted *Akv-2* proviral copy with the position of the pEc-B4 sequences shown by a dark band. The location of the cloned flanking sequences is indicated by darkened portions on the upper diagram. The positions of the 6-kb *Eco*RI to *Bam*HI fragment (B) and of the 7-kb *Hin*dIII fragment from NFS.*Akv-2* DNA that were cloned in λ vectors as described in the text are shown on the upper diagram. Fragment sizes are in kb.

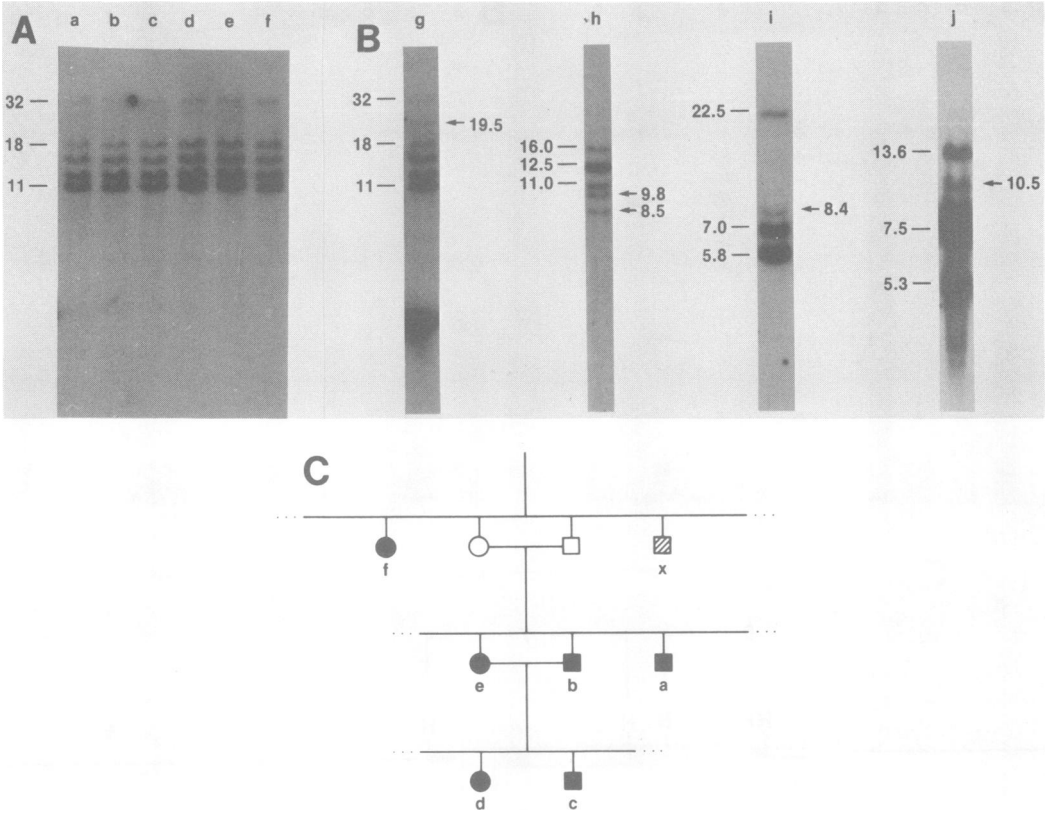

FIG. 4. (A and B) Autoradiograms of filter blot hybridization analysis of restriction enzyme-digested liver DNAs from individuals of the pedigreed AKR/J family of three inbred generations. Enzyme digestion, electrophoresis on 0.6% agarose gels, transfer to nitrocellulose membranes, and hybridization to ^{32}P-labeled pEc-B4 were as described in the text. (A) *Eco*RI digestion of DNAs from mice indicated by the solid symbols in the pedigree diagram (C) and identified by the appropriate lane label. (B) *Eco*RI (lane g), *Xba*I (lane h), *Hin*dIII (lane i), and *Sac*I (lane j) digests of DNA from the male mouse (designated as x) of the first generation in the study showing additional fragments (arrows to right of each lane). Fragment sizes in kb are indicated.

generations of the pedigreed AKR/J mice (Fig. 4C), digested with *Eco*RI, *Xba*I, *Hin*dIII, *Sac*I, *Pst*I, or *Kpn*I, and analyzed for ecotropic MuLV sequences by the Southern blot hybridization technique. As shown in Fig. 4A, the hybridization patterns of *Eco*RI-restricted DNA prepared from six mice in three inbred generations were indistinguishable from one another and were identical to those seen in AKR/J group d animals (five proviral DNA copies). In every case, digestion with the other restriction enzymes generated the expected pattern for the five ecotropic MuLV proviruses. Since the 18- and 15-kb *Eco*RI fragments are variably present in AKR/J mice (Fig. 1), it is possible that these proviral DNAs might exist in a heterozygous state in the AKR/J foundation stock breeders. Heterozygosity for any of the ecotropic proviruses present in the pedigreed AKR/J mice examined in this study would be detected by variation in the intensity of hybridization to any fragment or by the absence of a reactive fragment. Within the limits of detection by the hybridization methods used, there is no indication of such variability in the six related mice studied.

One mouse from this pedigree (male x, Fig. 4C) appears to contain at least one additional ecotropic provirus (Fig. 4B). With all enzymes used, a unique fragment hybridizes with labeled pEc-B4 DNA. In each case, the intensity of hybridization is less than that observed with the other AKR/J proviral DNA fragments, consistent with a newly acquired, heterozygous locus. Unfortunately, this mouse was not used in further breeding studies.

Reinsertion proviral genes in *Akv-1* congenic mice. Backcross studies of some families of *Akv-1* congenic mice to the NFS/N strain indicate that additional copies of ecotropic proviral DNA have been acquired in the congenic lines (13). Three individuals from such an *Akv-1* reinsertion family were examined for the number

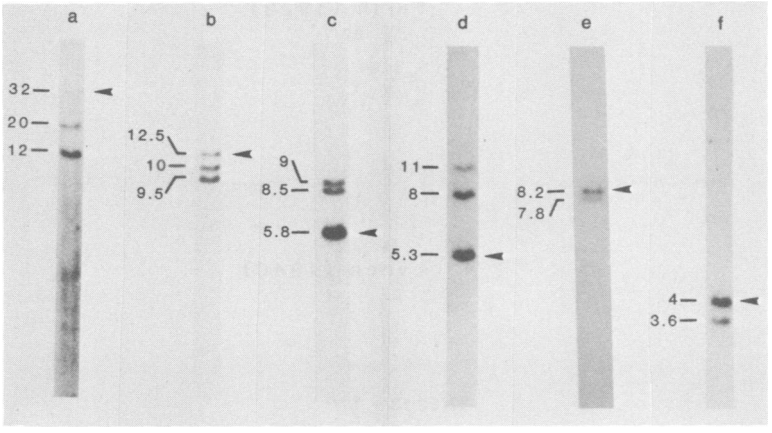

FIG. 5. Autoradiograms of filter blot hybridization analysis of restriction enzyme-digested NFS.*Akv-1* reinsertion line mouse liver DNA. Enzyme digestion, electrophoresis on 0.6% agarose gels, transfer to nitrocellulose membranes, and hybridization to ^{32}P-labeled pEc-B4 were as described in the text. DNA was digested with *Eco*RI (lane a), *Xba*I (lane b), *Hin*dIII (lane c), *Sac*I (lane d), *Pst*I (lane e), or *Kpn*I (lane f). Fragment sizes in kb are indicated to the left of each lane, and the position of the fragment containing *Akv-1* sequences is indicated by arrows to the right of each lane.

and organization of endogenous ecotropic proviruses. The results obtained with all three mouse DNA preparations were identical. Hybridization patterns observed with one of the DNAs are shown in Fig. 5. In addition to the expected *Akv-1* fragments of 32 kb with *Eco*RI, 12.5 kb with *Xba*I, 5.8 kb with *Hin*dIII, and 5.3 kb with *Sac*I (indicated by the arrows in lanes a to d, Fig. 5), two additional fragments that hybridize with pEc-B4 DNA were detected in each case. The sizes of these additional fragments were 20 and 12 kb with *Eco*RI, 11 and 8 kb with *Xba*I, 9 and 8.5 kb with *Hin*dIII, and 11 and 8 kb with *Sac*I. The sizes of these restriction fragments were compatible with the presence of complete ecotropic proviruses. However, at least one of the reinsertion copies is atypical since digestion with *Pst*I produces a 7.9-kb, rather than an 8.2-kb, fragment that hybridizes with the ecotropic *env* probe (Fig. 5, lane e). Digestion of these mouse DNAs with *Kpn*I produces an additional 3.6-kb cleavage product (Fig. 5, lane f), which is also smaller than the 4.0-kb *Kpn*I fragment characteristic of ecotropic proviral DNA.

DISCUSSION

The results presented extend the previously reported observations on the number of copies and the genomic organization of ecotropic MuLV proviral DNA in AKR mice (4–6, 10, 18). Although each of the AKR substrains examined in this study contains unique ecotropic MuLV proviral loci, some common ecotropic proviral copies are present. AKR/J, AKR/N, AKR/Cum, and AKR/Boy each contain the *Akv-1* (32-kb *Eco*RI) provirus; the 13- and 11-kb *Eco*RI copies are present in several sublines.

Heterogeneity of ecotropic proviral DNA copies was also observed within the AKR substrains. AKR/J mice can be divided into at least four distinct groups with respect to the types of endogenous ecotropic proviruses that they currently contain. Three ecotropic MuLV proviral copies (32-, 13-, and 11-kb *Eco*RI fragments) are present in the DNAs of all four groups, while two additional proviral DNAs (corresponding to the 18- and 15-kb *Eco*RI fragments) are variably present. These observations suggest that heterozygosity with respect to the two variable proviral copies exists within the AKR/J foundation stock. However, the maintenance of inbred strains contains a strong bias against heterozygosity. The acquisition of a new copy of proviral DNA by germ line reinsertion will have less than a 10% chance of remaining heterozygous after eight additional brother-sister inbred generations (about 2 years of inbreeding) and less than a 1% chance after 5 years of inbreeding. Therefore, if heterozygosity accounts for the variability of these two ecotropic MuLV proviral copies in AKR/J mice, then reinsertions must have occurred during the last 5 years. The first AKR/J DNA studied was extracted from the liver of a mouse born in July, 1979 and was found to be a five-copy (group d) AKR/J DNA, while the one DNA containing only three ecotropic MuLV copies (group a) was obtained from a mouse born in November, 1980.

The genealogy of the four AKR substrains

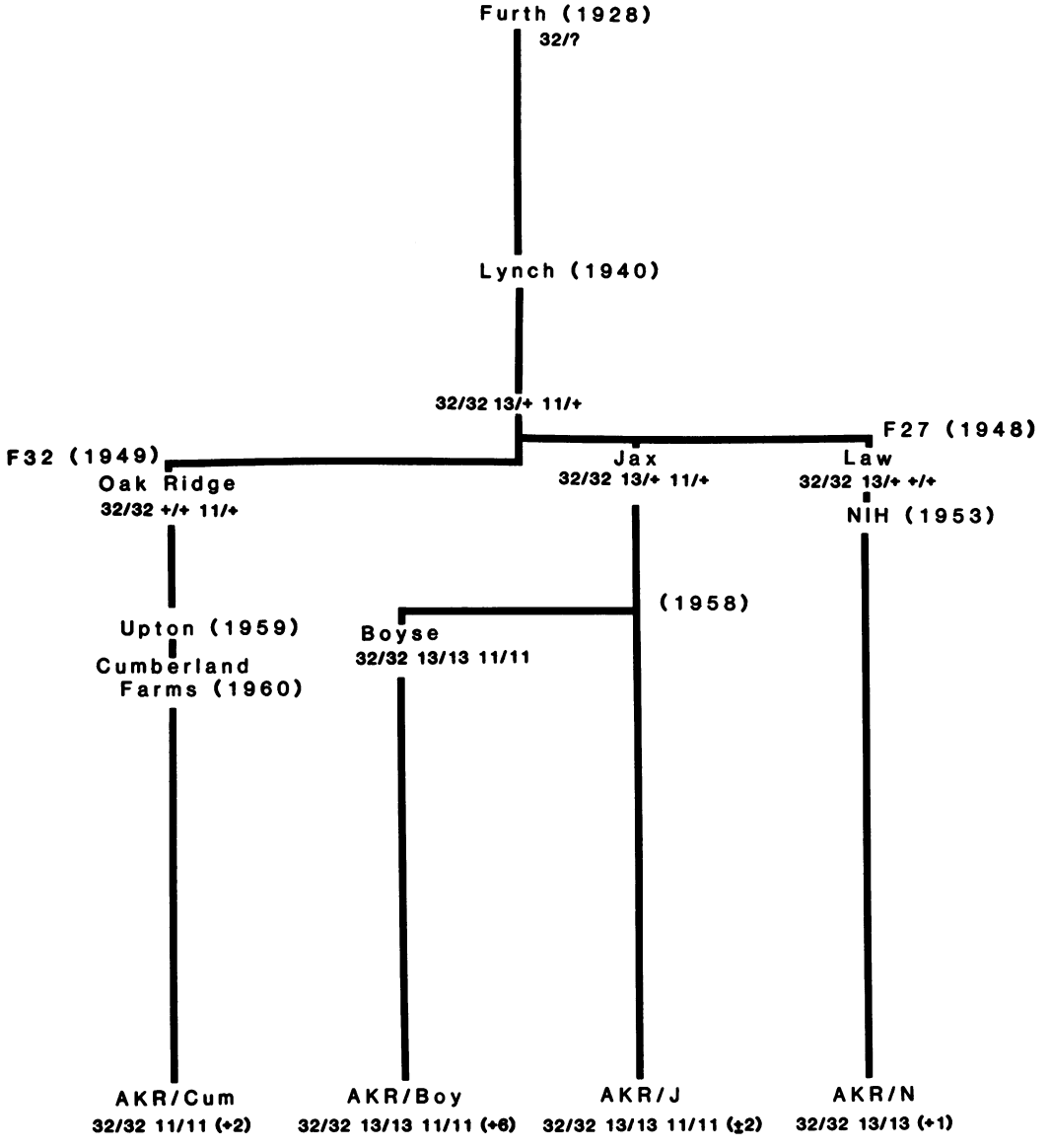

FIG. 6. Genealogy of the AKR sublines studied. Possible genotypes of ecotropic MuLV proviral copies utilize the size of the unique *Eco*RI fragment containing each proviral fragment for identification. Thus, 32/32 13/+ 11/+ indicates homozygosity for the 32-kb *Eco*RI fragment (*Akv-1*) and heterozygosity for the other two copies.

studied is shown in Fig. 6, together with possible genotypes that would explain the status of the mice used in this study. The 32-kb *Eco*RI fragment (containing *Akv-1* proviral sequences) is most likely the original ecotropic provirus that was present in the AKR line when Furth began inbreeding in the late 1920s. Before 1948, two additional ecotropic proviruses associated with the 13- and 11-kb *Eco*RI fragments were acquired by AKR mice. At that time, these two new proviral DNAs were probably present as heterozygous copies in the AKR germ line. In 1948 and 1949, three of the sublines studied by us were established. Chance segregation of the heterozygous proviral copies and subsequent inbreeding of the sublines led to the establishment of only the 13-kb *Eco*RI copy in AKR/N mice and only the 11-kb *Eco*RI copy in AKR/Cum mice. Both proviral copies became fixed in AKR/J mice. In 1958, the AKR/Boy subline was

established from the AKR/J stock and contained the three copies of ecotropic proviral DNA present in AKR/J mice at that time.

Subsequent to establishment, each subline has acquired additional ecotropic proviruses: one additional copy in AKR/N (*Akv-2*, 25-kb *Eco*RI fragment), two copies in AKR/J (18- and 15-kb *Eco*RI fragments), two copies in AKR/Cum (14- and 12.5-kb *Eco*RI fragments), and at least six additional copies in AKR/Boy. The Cumberland, Boyse, and National Institutes of Health substrains may contain even more proviral copies since only one or two individual DNAs have been examined in each case.

A germ line reinsertion establishes a heterozygous proviral locus. With further inbreeding, only 0.25 of these will by chance be fixed as homozygotes. Thus, we estimate that in the stem lines of AKR/J, AKR/N, and AKR/Cum mice, an insertion occurs about once in every 12 generations. In AKR/Boy, however, the apparent rate of adding new copies of ecotropic MuLV proviral DNA is about twice that for the other three substrains. These estimates do not mean to imply that reinsertion must be occurring at a constant rate but are derived from dividing the total number of apparent reinsertions by the total years of existence of a given subline and then dividing by 0.25, the probability of homozygous fixation of a single reinsertion. Possibly certain endogenous ecotropic loci are more functionally active than others and are more likely to lead to infection of fetuses or germ cells. While this work was in progress, findings similar to those reported here which evaluated the number and organization of ecotropic proviral DNAs in AKR mice were communicated to us (N. A. Jenkins, D. Steffen, and W. Herr, personal communication).

The variability in the number and location of endogenous ecotropic proviruses in AKR substrains is most likely due to exogenous infection of embryos in utero by virus-shedding mothers. Germ line reinsertions have not been observed in low-virus strains such as BALB/c and C57BL which have a single and stable copy of ecotropic proviral DNA (unpublished data; N. A. Jenkins, personal communication). Support for a reinsertion mechanism of ecotropic provirus amplification also comes from studies of some *Akv-1* and *Akv-2* congenic mice which contain newly acquired copies of ecotropic proviral DNA. Only those mice with virus-positive mothers contain additional ecotropic proviruses (13). Mechanisms other than germ line reinsertion secondary to infection could also produce the variability in proviral copy number observed. Transposon-like amplification of retroviral DNA sequences similar to that reported for transposable elements in procaryotes could explain the results obtained. New proviral DNA copies acquired by such a mechanism would be indistinguishable from those acquired by reinsertion following exogenous infection. Translocation of regions of the mouse genome containing an ecotropic provirus followed by fixation of the resultant heterozygous copies could also lead to the increase in the copy number of ecotropic proviral DNAs. Retention of some similar restriction sites in the cellular DNA sequences flanking such translocated proviral copies would support such a mechanism. However, none of the endogenous ecotropic proviruses detected in AKR/J or AKR/N mice seems to meet this requirement.

Although some high-virus mice such as F/St, C3H/Fg, C58/Lw, and *Mus mus molossinus* contain from 13 to 15 ecotropic MuLV proviruses (5; unpublished data), no mouse has yet been found that contains more than 20 copies of these sequences. Some mechanism must exist to keep the total number below this level. Selection based on non-viability of embryos containing more than 20 proviral copies or the rare specific excision of inserted retroviral sequences (17) could potentially limit the total number of proviral copies. As demonstrated in Fig. 3, cloned *Akv-2* flanking cellular sequences were used to detect the presence of ecotropic proviral DNA at the *Akv-2* locus in mouse chromosomal DNAs. In AKR/J and NFS/N mice, the cellular sequences surrounding the *Akv-2* locus are uninterrupted, and these mice therefore lack a detectable ecotropic provirus at this site. Since loss of endogenous MuLV DNA by specific excision might leave remnants of proviral DNA, the use of DNA probes containing sequences that flank endogenous ecotropic proviruses could demonstrate whether or not such a mechanism is operative.

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