JOURNAL OF VIROLOGY, Apr. 1982, p. 165–175
0022-538X/82/040165-11$02.00/0

Vol. 42, No. 1

Continuing Germ Line Integration of AKV Proviruses During the Breeding of AKR Mice and Derivative Recombinant Inbred Strains

DAVID L. STEFFEN,[1]* BENJAMIN A. TAYLOR,[2] AND ROBERT A. WEINBERG[3]

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545[1]; The Jackson Laboratory, Bar Harbor, Maine 04609[2]; and The Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139[3]

Received 4 September 1981/Accepted 27 November 1981

The gel electrophoresis-hybridization technique of Southern was used to analyze genetically transmitted proviruses coding for the AKV strain of murine leukemia virus. We were able to identify the restriction endonuclease *Eco*RI fragments containing two previously unidentified, genetically transmitted AKV proviruses of AKR mice. Comparison of different sublines of AKR mice revealed considerable heterogeneity in their complement of germ line proviruses. This heterogeneity provides evidence that the provirus complement of AKR mice is not stable. Rather, the number of genetically transmitted proviruses increases during inbreeding. Examination of a series of sublines of the C3H strain indicated that this amplification is dependent on viremia. We estimate that, in viremic strains of mice, one new provirus becomes fixed in the germ line every 15 to 30 years.

The proviruses of the AKV strain of ecotropic murine leukemia viruses, also referred to as type 1 MLVs (1), are genetically transmitted among many laboratory strains of the mouse (3, 5). These genetically transmitted proviruses segregate in a Mendelian fashion and have, in some cases, been mapped to specific mouse chromosomes (10, 17).

The gel electrophoresis-hybridization technique of Southern (19) can be used to detect and resolve AKV proviruses integrated at different sites in the mouse genome (21). In previous work, we used this technique to associate restriction endonuclease-generated DNA fragments of AKR mouse DNA with the endogenous AKV proviruses *Akv-1* and *Akv-2* (21). One surprising result of these studies was that three sublines of AKR mice, the AKR/J, AKR/N, and AKR/Cum sublines, differed in the pattern of AKV-containing restriction endonuclease fragments. Specifically, a restriction fragment shown to carry the *Akv-2* provirus was found in the DNA of the AKR/N subline, but not in the AKR/J or AKR/Cum sublines.

Genetic crosses between AKR mice and low-virus strains of mice demonstrated two independently segregating dominant genes specifying viremia (16). Subsequently, these genes were shown to be associated with AKV proviruses (5, 21). One of these genes, designated *Akv-1*, has been mapped to chromosome 7, and is 12 map units from the *Gpi-1* locus (17). Such a gene at the *Akv-1* locus has been detected in both the AKR/N and AKR/J sublines. The other gene, *Akv-2*, was initially defined as a second, similarly behaving gene, unlinked to *Akv-1* (16). Since no linkage could be detected between this second AKV-inducing gene and known genetic markers, it is possible that the genetic location of the second gene carried by various AKR sublines could be different. Recently, one *Akv-2* gene has been localized to chromosome 16 by use of somatic cell hybridization (10). We present data here which would suggest that this mapping may only pertain to the AKR/N subline of mice. A variety of other AKV genes, each distinct from the initially defined *Akv-1* gene, are shown here to be present in other sublines of AKR mice.

In the present study we analyzed the AKV proviruses of sublines of AKR mice, of C3H mice, and of a series of AKXL recombinant inbred strains. Using these materials, we have been able to explain the polymorphism of AKV proviruses observed among sublines of AKR mice. The significance of these polymorphisms is discussed.

MATERIALS AND METHODS

Mice. The AKR/J, C57L/J, C3HeB/FeJ, and C3H/HeJ mice were obtained from The Jackson Laboratory. The NSF/N-*Akv-1* and NIH/Swiss-*AKV-2* congenic mice as well as the AKR/N mouse were a gift of W. Rowe of the National Institutes of Health. The

NIH/Swiss, C3H/An, C3H/Fg, DBA/2J, and BALB/cAn mice were obtained from the animal colony of the Massachusetts Institute of Technology Center for Cancer Research. DNA from the C3H/N mouse was a gift of J. Ihle of the Frederick Cancer Research Center. The AKR/Fu mice were a gift of W. Yang of the Oak Ridge National Laboratory. The AKR/Gs mice were a gift of L. Gross of the Veterans Administration Hospital, New York, N.Y. The AKR/Rb and the AKR/Ski mice were a gift of L. Stockert of the Sloan-Kettering Institute. These sublines were inbred by Stockert for 3 and 4 years, respectively (23). The breeding stocks for AKR/Ski derived from stocks maintained by E. Boyce at the Memorial Sloan-Kettering Institute, which were derived from the AKR/J subline in 1960 (L. Stockert, personal communication). The AKR/Rb stocks came from A. Leonard (12). The AKR/Cum mice were a gift of N. Rosenberg of Tufts University School of Medicine. The AKXL recombinant inbred strains were derived by brother-sister inbreeding beginning with the F_2 generation of the cross of AKR/J with C57L/J (25).

The L.AKR-*Akv-4* congenic strain, previously designated L.AKR-*Akv-2*, was developed from the cross of AKR/J with C57L/J, by nine serial backcrosses to the C57L/J strain, with selection for those backcross mice that expressed AKV at 4 to 7 weeks of age in either spleens or tail biopsies. The provirus present in the congenic strain was identified as *Akv-4* by the criterion that the provirus segregated independently of the *Gpi-1* locus on chromosome 7, which is closely linked to *Akv-1* (B. A. Taylor, H. G. Bedigian, and H. Meier, unpublished data). The L.AKR-*Akv-4* mice used in this study were from the sixth generation of brother-sister inbreeding and are presumed to be fixed for *Akv-4*. The change of designation of this strain is based on results presented below.

Methods. All methods used here have been previously described in detail (21).

RESULTS

Identification of DNA fragments containing viremia-inducing proviruses of AKR/J mice. The restriction endonuclease *Eco*RI does not cleave within the genome of AKV (22). *Eco*RI cleavage of mouse DNA containing AKV proviruses results in each provirus being present in a single DNA fragment. Proviruses integrated at different sites in the mouse DNA will reside in different-sized DNA fragments. Figure 1 displays DNAs from AKR/J, C57L/J, and AKXL recombinant inbred strains of mice digested with *Eco*RI, fractionated by gel electrophoresis, transferred to nitrocellulose, and hybridized to an AKV cDNA probe. This probe was prehybridized to RNA of the Moloney strain of murine leukemia virus to reduce its reactivity with non-

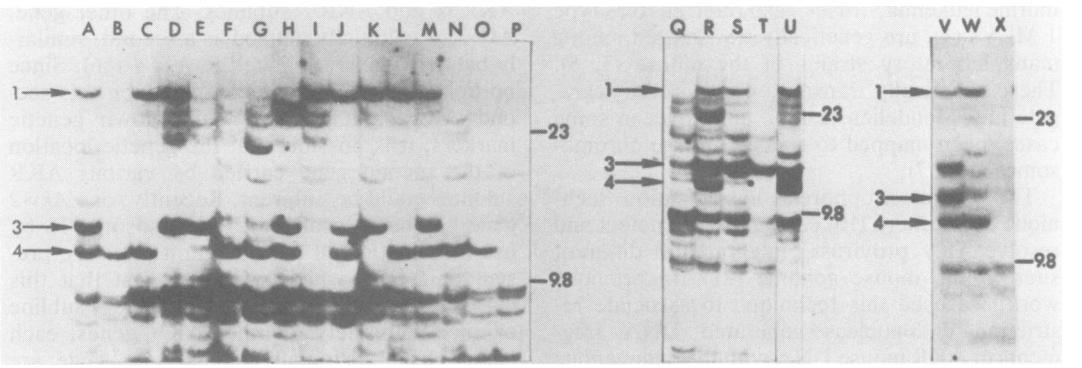

FIG. 1. *Eco*RI fragments containing AKV proviruses from DNA of AKR, AKXL, and AKV congenic mice. DNA was digested with *Eco*RI, fractionated by gel electrophoresis, transferred to nitrocellulose filters, and hybridized as previously described (21). The hybridization probe used for this experiment was AKV cDNA which was prehybridized to a 1,000-fold excess of RNA from the Moloney strain of murine leukemia virus, as previously described (21). The arrows to the left of each panel labeled 1, 3, and 4 indicate the mobilities of the *Eco*RI fragments containing the *Akv-1*, *Akv-3*, and *Akv-4* proviruses, respectively. The lines to the right of each panel represent the mobility of fragments of bacteriophage lambda DNA produced by *Hin*dIII digestion. The numbers indicate the size of these fragments in thousands of nucleotide pairs (kb). As the markers were run in a parallel lane and were run in the absence of carrier DNA, these size markers are only approximate. Because of small differences in the amounts of DNA loaded in each lane, and because of the contrast enhancement resulting from the photography used to prepare this figure for publication, the intensity of bands in different lanes varies. In particular, three bands clearly visible in lane B just below the *Akv-1* band represent non-AKV background bands that are present in other lanes as well. These represent proviral DNA fragments derived from C57L/J. The DNAs in each lane were derived from the following strains of mice: (A) AKR/J, (B) AKXL-5, (C) AKXL-7, (D) AKXL-9, (E) AKX-12, (F) AKXL-13, (G) AKXL-14, (H) AKXL-19, (I) AKXL-21, (J) AKXL-24, (K) AKXL-25, (L) AKXL-6, (M) AKXL-37, (N) AKXL-38, (O) L.AKR-*Akv-4* congenic, (P) C57L/J, (Q) AKXL-29, (R) AKXL-8, (S) AKXL-16, (T) AKXL-28, (U) AKR/J, (V) AKR/J (a different mouse than lanes A and U), (W) AKXL-17, (X) AKXL-36.

AKV endogenous proviruses present in mouse DNA (21). Although this prehybridization reduced reactivity of the probe toward non-AKV sequences, the resulting probe was not AKV specific. Rather, it hybridized both to AKV sequences and to some non-AKV murine leukemia virus sequences (21; Fig. 1–4 and 7). Consequently, we used genetic techniques throughout to identify AKV proviruses amidst the background of non-AKV endogenous murine leukemia virus sequences.

We have previously shown that the *Akv-1* provirus defined by Rowe (16) is present in AKR/J mice and is the largest viral fragment detected (21). This fragment can be seen in AKR/J DNA (Fig. 1, lane A) and is indicated by the arrow labeled 1.

Lane O of Fig. 1 displays *Eco*RI-cleaved DNA from a congenic mouse carrying a second distinct AKV provirus of strain AKR/J in the C57L/J background. C57L/J mice do not transmit any AKV proviruses (3). The pattern of *Eco*RI-digested C57L/J mouse DNA is shown in lane P. A strongly hybridizing fragment is present in the DNA of the congenic mouse (lane O) and in the DNA of the AKR/J mouse (lane A), but absent from the DNA of the C57L/J mouse (lane P). This fragment we identify as containing this second AKV provirus. We show below that this provirus is integrated at a site different from that of the proviral loci *Akv-2* (16) and *Akv-3* (7). Therefore, we propose to change the name of the second inducible AKV provirus of AKR/J mice from *Akv-2* to *Akv-4*. The DNA fragment containing this provirus is indicated by an arrow labeled 4 in Fig. 1.

The band identified as containing the *Akv-4* provirus is much darker than that containing the *Akv-1* provirus. As we have previously described (21), this is a technical artifact resulting from the high molecular weight of the *Akv-1*–containing fragment. We also note a third, intensely hybridizing fragment larger than the AKV genome indicated by an arrow labeled 3. We had previously speculated that this fragment could contain a third AKV provirus (21), the existence of which had been demonstrated by Ihle and Joseph (7). This same fragment is detected in AKR/J mouse DNA with a variety of AKV-specific probes (4, 15; W. Herr and W. Gilbert, submitted for publication), indicating that it indeed contains a third AKV provirus. We present below further evidence that this is the case.

Proof that the *Akv-2* and *Akv-4* proviruses are integrated at different sites in the mouse genome. The differences in mobilities of the *Eco*RI fragments containing the *Akv-2* and *Akv-4* proviruses could be the result of these proviruses being integrated at different sites in the mouse genome. Alternatively, the difference in DNA fragment size could result from a base change which created or abolished an *Eco*RI cleavage site in the cellular sequences adjacent to the provirus. A deletion of or an insertion into adjacent cellular sequences could also modify the size of the provirus-containing DNA fragments.

To distinguish between the alternatives described above, we compared the *Hin*dIII cleavage pattern of the AKR/N and AKR/J mice. *Hin*dIII cleaves the AKV provirus once, generating two fragments, each fused to adjacent cellular sequences (22). If the difference between the *Akv-2* and *Akv-4* proviruses were due to a restriction endonuclease polymorphism, the *Hin*dIII fragments produced by these proviruses would be the same. If the difference were due to a deletion or substitution in adjacent cellular sequences, this alteration would lie on one side of the provirus. Thus, one *Hin*dIII fragment would differ between the *Akv-2* and *Akv-4* proviruses, and one would be the same. If the differences reflected different integration sites, both *Hin*dIII fragments would differ in size.

To identify the *Hin*dIII fragments deriving from the *Akv-2* and *Akv-4* proviruses, we used congenic mice (Fig. 2). In each case, the *Hin*dIII patterns were compared to identify two fragments which were absent in the background strain (NIH/Swiss or C57L/J, lanes A and N, respectively), present in the congenic strains (*Akv-2*, lane C, and *Akv-4*, lane G), and present in the AKR strain (AKR/N, lanes D and E, AKR/J, lane F). We associated two *Hin*dIII fragments each with the *Akv-2* and *Akv-4* proviruses. These fragments are indicated by arrows to the left of each panel, labeled 2 and 4, respectively. Both of the *Akv-4* *Hin*dIII fragments are present in AKR/J DNA (lane F) but not in AKR/N DNA (lane E). Of the two *Akv-2* *Hin*dIII fragments generated from AKR/N DNA (lane E), the smaller one is clearly absent from AKR/J DNA, whereas the larger one appears to be present (lane F). In fact, the fragment observed in the DNA of this AKR/J mouse derives from an AKV provirus present in some but not all individuals of the AKR/J strain and only coincidentally comigrates with the fragment deriving from the *Akv-2* provirus. This provirus was recently introduced into the germ line of AKR/J mice, has not yet become fixed, and is unrelated to *Akv-2* (Herr and Gilbert, manuscript submitted).

Since the two *Hin*dIII fragments containing the *Akv-2* provirus differ in size from both of the two *Hin*dIII fragments containing the *Akv-4* provirus, we conclude that these proviruses are present at different sites in the mouse genome and constitute separate and distinct proviruses.

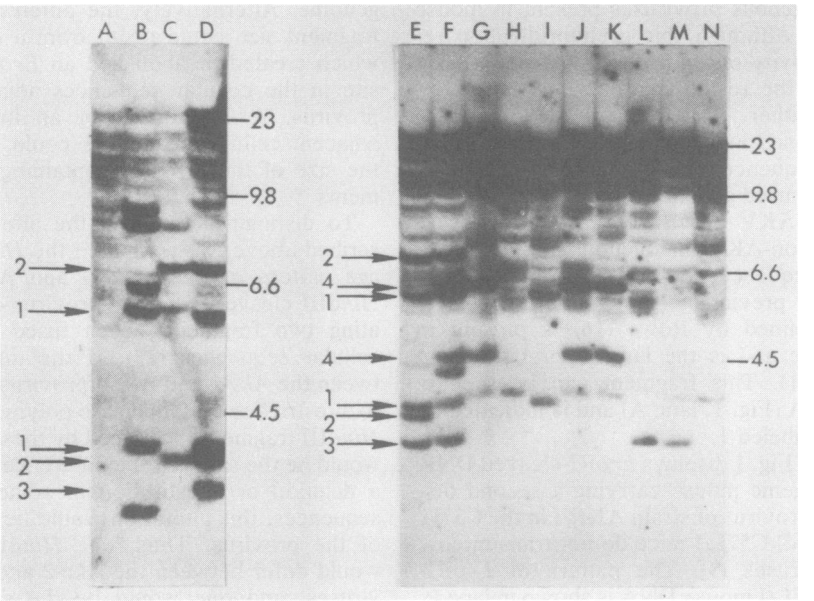

FIG. 2. *Hin*dIII fragments containing halves of AKV proviruses from DNA of AKR, AKXL, and AKV congenic mice. DNA was digested with *Hin*dIII. The arrows to the left of each panel indicate the mobilities of the fragments deriving from the *Akv-1*, *Akv-2*, *Akv-3*, and *Akv-4* proviruses. Otherwise, the experiment was done as described in the legend to Fig. 1. The DNAs in each lane were derived from the following mouse strains: (A) NIH/Swiss, (B) NSF/N-*Akv-1*, (C) NIH/Swiss-*Akv-2*, (D) AKR/N, (E) AKR/N, (F) AKR/J, (G) L.AKR-*Akv-4*, (H) AKXL-6, (I) AKXL-21, (J) AKXL-5, (K) AKXL-13, (L) AKXL-12, (M) AKXL-28, (N) C57L/J. The cleavage pattern of fragments detected by our probe in NIH/Swiss and NFS/N mouse DNAs are indistinguishable. (NSF/N is derived by inbreeding NIH/Swiss.) Thus, lane A represents the control for both lane B and lane C.

Identification of a third AKV provirus in AKR/J mouse DNA. In Fig. 1, lanes B through N, Q through T, and V and W display EcoRI-cleaved DNA from 19 AKXL recombinant inbred strains. The AKV proviruses of the AKR progenitor can be seen to segregate among them. Specifically, lane I displays AKXL-21, a strain transmitting only *Akv-1*, lanes B, F, and N display the strains AKXL-5, AKXL-13, and AKXL-38, which transmit only *Akv-2*, and lanes E and T display the strains AKXL-12 and AKXL-28, which transmit only the fragment that we hypothesized to carry *Akv-3*. We were able to use these latter two strains to demonstrate that this fragment in fact carries a third AKV provirus.

Restriction endonucleases which cleave two or more times within the AKV genome can be used to identify AKV proviruses amidst the background of other, cross-hybridizing sequences present in mouse DNA (22). One such enzyme is *Pst*I. If DNA from a laboratory strain of mouse which genetically transmits an AKV provirus is digested with *Pst*I, an 8.2-kilobase (kb) fragment results. Strains which lack genetically transmitted AKV proviruses also lack this fragment (4, 22; unpublished data). Thus, we could use *Pst*I to determine whether those AKXL strains which lack *Akv-1* and *Akv-4* proviruses, but which transmit the DNA fragment that we hypothesize to contain the *Akv-3* provirus, in fact transmit an AKV provirus.

Lanes A and B in Figure 3 display the pattern of DNA from the AKR/J and C57L/J strains digested with *Pst*I. DNA from both of these strains gave rise to a number of common hybridizing fragments. Only the DNA from the AKR/J strain, however, gave rise to the 8.2-kb AKV-specific fragment. The arrow to the left of the panel indicates the mobility of this AKV-specific DNA fragment. The *Pst*I digestion pattern of DNA from selected AKXL strains is displayed in the second panel of Fig. 3. Lane C displays AKR/J DNA as a marker for the 8.2-kb fragment, whose position is indicated by an arrow. Lane D displays DNA from the AKXL-29 strain which lacks the *Akv-1*, *Akv-3*, and *Akv-4* fragments. It does not yield a detectable 8.2-kb fragment. Lanes E and F display *Pst*I-digested DNA from the strains AKXL-21 and AKXL-5 which transmit only *Akv-1* or *Akv-4*, respectively. They both display the 8.2-kb DNA fragment, as expected. Lanes G and H display DNA from the strains AKXL-12 and AKXL-28 which puta-

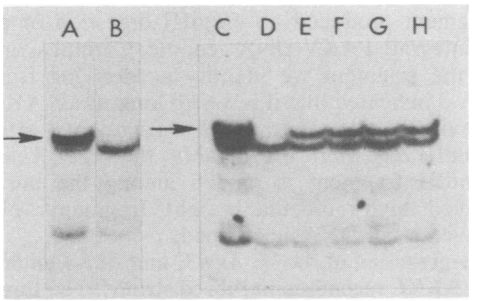

FIG. 3. *Pst*I fragments containing the center fragment of the AKV proviruses from AKR and AKXL mice. DNA was digested with *Pst*I. The arrows to the left of each panel indicate the mobility of the *Pst*I fragment diagnostic for AKV (22). Otherwise, the experiment is as described in the legend to Fig. 1. The DNAs in each lane were derived from the following mouse strains: (A) AKR/J, (B) C57L/J, (C) AKR/J, (D) AKXL-29, (E) AKXL-21, (F) AKXL-5, (G) AKXL-12, (H) AKXL-28.

tively transmit the *Akv-3* provirus. Both display an 8.2-kb DNA fragment, showing that these strains indeed transmit an AKV provirus. This result was confirmed by analogous analyses with *Bam*HI and *Kpn*I, which also generate AKV-specific DNA fragments (data not shown). We conclude that AKR/J mice do transmit a third AKV provirus. These data, taken with previously published analyses using AKV-specific probes (4, 15; Herr and Gilbert, manuscript submitted) indicate that this provirus is contained in the DNA fragment we label as *Akv-3*.

***Akv-1* and *Akv-3* are present at the same site in the genomes of AKR/J and AKR/N mice.** We demonstrated above that the *Akv-2* provirus of AKR/N mice and the *Akv-4* provirus of AKR/J mice, which are present in different-sized *Eco*RI fragments, represent proviruses integrated at different sites in the mouse genome. Comparison of the *Eco*RI cleavage pattern of AKR/J and AKR/N mice reveals that both display *Eco*RI fragments of the size identified with the *Akv-1* and *Akv-3* proviruses (21; Fig. 4). It is possible that these comigrations could be coincidental: that in fact these do not represent proviruses integrated at the same site in the mouse genome. To provide a second criterion for identity, we compared the *Hin*dIII cleavage patterns of these proviruses.

Comparison of the *Hin*dIII cleavage patterns of AKR/N and AKR/J mouse DNAs is shown in Fig. 2. The *Hin*dIII fragments corresponding to the *Akv-1* proviruses were identified by using an NSF/N *Akv-1* congenic mouse. This analysis was complicated by the fact that this congenic mouse has acquired extra endogenous AKV proviruses via germ line reinsertion during its inbreeding (18, 21). Thus, *Eco*RI digestion (21) and *Hin*dIII digestion (Fig. 2, lane B) produced a number of fragments absent from NIH/Swiss mouse DNA. Only one *Eco*RI fragment (21) and two *Hin*dIII fragments corresponded to fragments present in AKR/N mouse DNA (lane D), identifying these fragments as having derived from the *Akv-1* provirus. These fragments are indicated by arrows to the left of both panels of Fig. 2, labeled 1. Comparison of DNAs from an AKR/N mouse (lane E) and from an AKR/J mouse (lane F) revealed that the *Akv-1 Hin*dIII fragments are present in both, providing further evidence that the *Akv-1* proviruses of these sublines are present at the same site in the

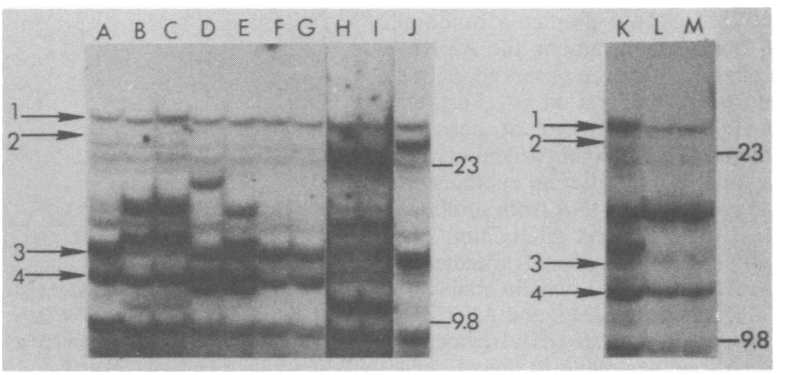

FIG. 4. *Eco*RI fragments containing AKV proviruses from DNA of different sublines of AKR mice. The experiment is as described in the legend to Fig. 1 except that the arrow to the left of the figure labeled 2 was included to indicate the mobility of the DNA fragment carrying the *Akv-2* provirus. The DNAs in each lane were derived from the following mice: (A) AKR/J, (B) AKR/Cum, (C) AKR/Cum, (D) AKR/Ski, (E) AKR/Ski, (F) AKR/Rb, (G) AKR/Rb, (H) AKR/Gs, (I) AKR/Gs, (J) AKR/N, (K) AKR/Cum, (L) AKR/Fu, (M) AKR/Fu. Where the same subline is indicated more than once, different individuals were the donors of the DNA, except that the same individual donated the DNA shown in lanes B and K.

genome. This is not unexpected, since the *Akv-1* provirus of both of these sublines has been genetically mapped to the same site on chromosome 7. Quint et al. used a similar strategy to reach the same conclusion about *Akv-1* proviruses present in the AKR sublines that they studied (15).

Since a congenic line for the *Akv-3* provirus was not available, we used an alternative strategy for the identification of the *Hin*dIII fragments containing *Akv-3* proviruses. *Hin*dIII-digested DNAs from AKXL strains carrying the *Akv-1*, *-3*, or *-4* proviruses in isolation were compared. Lane N displays C57L/J DNA. Comparison of this pattern with that of the AKR/J DNA reveals no obvious similarities, except that both display a number of poorly resolved, high-molecular-weight bands. Lane H displays DNA from AKXL-6, a strain transmitting no AKV proviruses. Again, no obvious similarity to the AKR/J pattern was observed. Lane I displays DNA from AKXL-21, a strain transmitting *Akv-1*. Comparison of this pattern with that of AKR/J reveals two prominent common bands—those previously associated with the *Akv-1* provirus. Similarly, examination of lanes J and K, which display AKXL-5 and AKXL-13, strains transmitting an *Akv-4* provirus, reveals the two *Akv-4* fragments. Lanes L and M display AKXL-12 and AKXL-28, strains transmitting an *Akv-3* provirus. Here, one fragment, indicated by an arrow labeled 3, is seen in common with AKR/J. This fragment is absent from the other AKXL strains examined (lanes H, I, J, and K), which lack *Akv-3*. This is precisely the distribution expected if this fragment were derived from the *Akv-3* provirus. An alternative explanation, that this fragment derived from a provirus other than *Akv-1*, *Akv-3*, or *Akv-4*, present in AKR/J but absent from C57L/J, would predict a random distribution of this fragment among the AKXL strains. The probability that the observed distribution derived by chance is 1 in 64. Further evidence that this *Hin*dIII fragment derived from *Akv-3* was provided by comparison of the *Hin*dIII cleavage pattern of different sublines of AKR mice (data not shown). DNA from sublines which lack *Akv-3*, AKR/Gs and AKR/Cum (21; Fig. 4), failed to produce this *Hin*dIII fragment, whereas DNA from sublines which transmit *Akv-3*, AKR/J, AKR/N, AKR/Ski, and AKR/Rb (21; Fig. 4), produced this *Hin*dIII fragment. These facts taken together make it almost certain that this *Hin*dIII fragment derived from *Akv-3*. This being the case, we conclude that the *Akv-3* provirus is at the same site in the genomes of AKR/J, AKR/N, AKR/Rb, and AKR/Ski mice.

A *Hin*dIII fragment of an AKV provirus would have to be larger than 3 kb, the smaller fragment produced by *Hin*dIII digestion of an unintegrated AKV DNA genome. Careful sizing of the fragment we identify as deriving from *Akv-3* indicated that it is 3.6 kb long. Each AKV provirus will generate two fragments after *Hin*dIII digestion. Presumably, the other *Akv-3* *Hin*dIII fragment is hidden among the unresolved high-molecular-weight fragments observed in all DNAs examined.

Segregation of *Akv-1*, *Akv-3*, and *Akv-4* among the AKXL recombinant inbred strains. The three AKV provirus genes of AKR/J exhibited normal segregation among 19 AKXL recombinant inbred strains (Table 1). Approximately one-half of the strains inherited each of the three proviruses as expected for random fixation of autosomal genes. Furthermore, each provirus segregated independently of the others. Since the AKXL recombinant inbred strains have been typed with respect to numerous other genetic markers (24), it was possible to evaluate potential linkage

TABLE 1. Comparison of the distribution of *Akv-1*, *Akv-3*, and *Akv-4* among the recombinant inbred strains with the ability of these strains to produce virus, either spontaneously or after induction by iododeoxyuridine[a]

Strain	Virus	*Akv-1*	*Akv-3*	*Akv-4*
AKXL-5	+	−	−	+
AKXL-6	+	+	−	+
AKXL-7	+	+	−	+
AKXL-8	+	+	+	+
AKXL-9	+	+	+	−
AKXL-12	−	−	+	−
AKXL-13	+	−	−	+
AKXL-14	+	−	−	+
AKXL-16	+	+	+	−
AKXL-17	+	+	−	+
AKXL-19	+	−	+	+
AKXL-21	+	+	−	−
AKXL-24	+	+	+	+
AKXL-25	+	+	+	−
AKXL-28	−	−	+	−
AKXL-29	−	−	−	−
AKXL-36	−	−	−	−
AKXL-37	+	+	+	+
AKXL-38	+	−	−	+

[a] Distribution of the proviruses among the recombinant inbred strains was also examined for linkage to a set of mapped genetic markers. Among the markers tested were *In*, *Pep-3*, *ald*, *Ltw-4*, *Mls*, *Akp-1*, and *Ly-9* on chromosome 1; *Hc*, *H-3*, and *Svp* on chromosome 2; *Car-2* on chromosome 3; *Lyb-2*, *Mup-1*, *b*, *Gpd-1*, and *Akp-2* on chromosome 4; *Gus* on chromosome 5; *Ly-23* on chromosome 6; *Coh*, *c*, and *Hbb* on chromosome 7; *Es-1* on chromosome 8; *Lap-1*, *Thy-1*, *Ltw-3*, *Fv-2*, and *Bgs* on chromosome 9; *Hds* on chromosome 10; *Hba*, *Glk*, and *Es-3* on chromosome 11; *Igh-1* and *Pre-1* on chromosome 12; *Es-10* on chromosome 14; and *H-2K*, *H-2D*, *Pgk-2*, and *Thy-2* on chromosome 17. No significant linkages were observed.

relationships between the proviruses and these other markers. *Akv-1* has previously been mapped to chromosome 7, and there is no marker available in the AKXL strains which would add any more precision to its location. Regrettably, no significant linkage was detected between either *Akv-3* or *Akv-4* and any of the other markers which we tested. We can, however, conclude that there is no close linkage between the proviruses and this set of markers. These negative linkage results are summarized in the footnote to Table 1.

As additional genetic markers are discovered and applied to the AKXL recombinant inbred strains, the linkages of *Akv-3* and *Akv-4* may become apparent.

Association of *Akv-1* and *Akv-4* with viremia. The AKXL strains have been characterized with respect to their ability to express AKV, either spontaneously or after iododeoxyuridine induction (Taylor, Bedigian, and Meier, unpublished data). These data are summarized in Table 1. The one strain, AKXL-21, which transmitted only *Akv-1* was virus positive. Similarly, all four strains which transmitted only *Akv-4* (AKXL-5, AKXL-13, AKXL-14, and AKXL-38) were virus positive. In contrast, the two strains which transmitted *Akv-3* in the absence of *Akv-1* or *Akv-4*, AKXL-12 and AKXL-28, were both virus negative. These results suggest that *Akv-3*, unlike *Akv-1* or *Akv-4*, does not readily give rise to detectable viremia. This result is consistent with the work which originally suggested the existence of a third AKV provirus in AKR mice, since some mice transmitting AKV proviral sequences and displaying an immune response against AKV antigens failed to exhibit viremia (7).

Germ line integration of new AKV proviruses during inbreeding of the recombinant inbred strains and of AKR sublines. Every hybridizing *Eco*RI fragment in the AKXL strains would be expected to have derived either from the AKR/J or the C57L/J parent. Such is not the case. The strains AKXL-8, AKXL-14, and AKXL-19, whose *Eco*RI-cleaved DNAs are displayed in lanes G, H, and R of Fig. 1, each have intensely hybridizing *Eco*RI fragments which are not represented in either parent. The most likely explanation for these additional fragments is that they represent AKV proviruses which have become integrated at novel sites in the genome of these strains during their derivation.

If novel germ line integrations have occurred during the development of the AKXL strains, it would be expected that similar integrations would have occurred during inbreeding of the AKR strain itself. To determine whether this has occurred, we compared the *Eco*RI cleavage pattern of a series of sublines of AKR (Fig. 4).

It is immediately apparent from Fig. 4 that the pattern of hybridizing fragments varies considerably from subline to subline. Similar subline variability has been previously reported for a different set of AKR sublines (15). We have associated four sizes of *Eco*RI fragments with AKV proviruses, *Akv-1*, *Akv-2*, *Akv-3*, and *Akv-4*. Of these, only the *Akv-1* fragment is present in all of the AKR sublines (Fig. 4; reference 15). The others are present in one or more of the sublines, but absent from others. In addition, many of the sublines display other intensely hybridizing fragments, which almost certainly represent additional AKV proviruses.

The pattern of AKV proviruses present in the AKR sublines can be rationalized in terms of their genealogy (Fig. 5). Mice with the more recent common ancestors have the most similar pattern of proviruses. Further, the entire pattern is consistent with sequential addition of proviruses during inbreeding of the sublines. The points at which we hypothesize that proviruses were integrated are indicated by arrows. The *Akv-3* and *Akv-4* proviruses are an exception to this generalization. The AKR/Fu, AKR/N (AKR/Lw), and AKR/J sublines all diverged one from another in 1949 (13). The AKR/J subline transmits the *Akv-3* and *Akv-4* proviruses; the AKR/N subline transmits the *Akv-3*, but not the *Akv-4*, provirus; and the AKR/Fu subline transmits the *Akv-4*, but not the *Akv-3*, provirus. It would seem that at the time of the N/J/Fu divergence the *Akv-3* and *Akv-4* proviruses had become integrated in the germ line of the common ancestor colony, but were still segregating.

Analysis of AKV proviruses transmitted in the C3H strain of mice. Addition of AKV proviruses at new sites in the genome of AKR mice could result either from infection of germ line cells by virions, or by direct transposition of existing proviruses. If infectious virus were required for insertion of new proviruses, only viremic mice ought to acquire new proviruses over time. To test this prediction, we compared the AKV proviruses in the DNA from different sublines of C3H mice. The set of C3H sublines is especially suitable for comparison with the AKR sublines for several reasons. (i) The C3H sublines have been separated one from another for about as long as the AKR sublines (Fig. 5 and 6). (ii) They transmit genetically an AKV provirus which is expressed early in the life of the animal (8). (iii) The C3H strain is permissive for AKV replication. (iv) Unlike the AKR strain, however, mice of the C3H strain do not develop viremia. Rather, they mount a successful immune response against the virus (8).

The genetically transmitted AKV provirus of C3H mice can be identified because it is allelic with the genetically transmitted AKV provirus

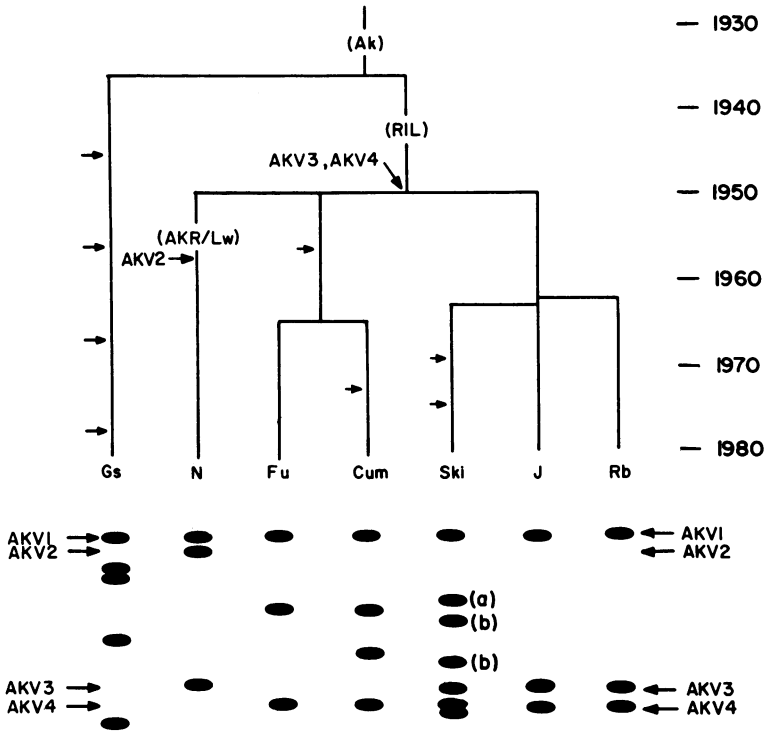

FIG. 5. Genealogy of the AKR sublines. The genealogy of the AKR sublines studied here is diagrammed in the upper portion of this figure (12–14). To the right of the figure is a time scale in calendar years. Beneath each lineage is the name of each subline, e.g., AKR/Gs, AKR/N, etc. Interrupting some lineages are parentheses containing names of lineages which have now been revised. Points in the genealogies at which we believe provirus integration to have occurred are indicated by arrows. The positions of these arrows are not, however, meant to indicate the precise time at which integration occurred. The proviruses named 1, 2, 3, and 4 are defined in the text. The remaining proviruses were named by adding to the name of each subline a number. Two AKR/Ski mice that we examined had different patterns of AKV proviruses. The proviruses which were idiosyncratic are labeled either a or b. At the bottom of the figure is a schematic drawing of the mobility of the proviruses in gel electrophoresis. To either side of the figure the mobility of each provirus is indicated by arrows.

of BALB/c mice (8). Comparison of the pattern of DNA fragments generated by *Eco*RI digestion of DNA from the C3H sublines (Fig. 7, lanes C through G) and from BALB/c (lane B) reveals a common fragment which we identify as the allelic AKV provirus. The C3H strain is derived from a cross between the dba and Bagg albino stocks from which the DBA and BALB/c strains were derived by further inbreeding. The one intensely hybridizing *Eco*RI fragment of DBA DNA is slightly smaller than the BALB/c and C3H AKV fragment (lane G). Thus, C3H mice appear to have derived their AKV provirus from their Bagg albino rather than their dba ancestor.

Comparison of four of the C3H sublines reveals that they transmit only the one, ancestral AKV provirus (Fig. 7, lanes B–E). One of the sublines, C3H/Fg, has apparently acquired multiple AKV proviruses (lane F; 3, 4). The C3H/Fg subline is also unique in that it is the only C3H subline which is viremic. These results show that amplification of provirus copy number is associated with viremia.

DISCUSSION

Different sublines of AKR mice are extremely heterogeneous with respect to the number and chromosomal integration sites of AKV proviruses (Fig. 4; reference 15). Residual heterozygosity of the AKR strain present at the time the sublines diverged one from another could explain, at most, a small fraction of the observed heterogeneity. In particular, the differences between the AKR/Fu and AKR/Cum sublines and the AKR/J and AKR/Ski sublines would have to result from heterozygosity that persisted through more than 30 years of inbreeding, an event of exceedingly low probability (Fig. 5).

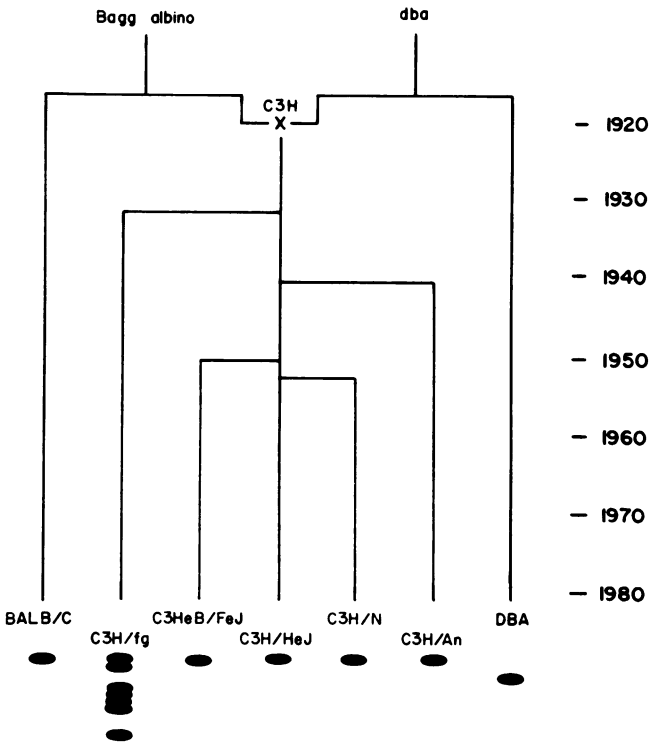

FIG. 6. Genealogy of the C3H sublines. The arrangement is essentially the same as that described in the legend to Fig. 5. The C3H strain was derived from the F_1 progeny of the cross of Bagg albino and dba stocks, as shown (20). BALB/c, DBA, and the C3H sublines were subsequently derived (2, 20). Only two AKV proviruses are named in this scheme, the proviruses transmitted in the DBA and BALB/c strains.

The observed differences between the AKR sublines can be wholly explained by sequential addition of proviruses, as is diagrammed in Fig. 5. According to this model, the original Ak strain derived by Jacob Furth in 1928 had only one fixed, genetically transmitted AKV provirus, *Akv-1*. During subsequent inbreeding, the different sublines of the AKR strain integrated and fixed from two to five additional proviruses.

Germ line integration of new AKV proviruses has been observed by others as well. Rowe and Kozak showed the addition of new proviruses in congenic lines carrying *Akv-1* in an NFS/N background (18). Integration of additional AKV proviruses has been carefully documented in the AKR/J subline by Herr and Gilbert (manuscript submitted). We have also observed a recent germ line integration of AKV proviruses among AKR/J mice (Fig. 1, lane V).

In addition to germ line infection, two other sources of provirus instability could be imagined: loss of proviruses and movement of proviruses from one site in the genome to another. No evidence presented to date indicates that proviruses are capable of direct transposition. Loss of proviruses, by contrast, would not be unexpected. Proviruses could be lost by simple deletion, including deletion occurring by legitimate recombination at the redundant ends. Such deletions appear to have occurred among endogenous avian retroviruses (6).

Neither provirus movement nor provirus loss is supported by the variability of AKV proviruses that we observed among AKR sublines. To explain this variability by provirus loss would require that blocks of several proviruses would have to be coordinately lost from independent lines—an event of exceedingly low probability. During evolution, some proviruses are probably lost, but such a mechanism does not appear to be the major cause of the heterogeneity that we observed among AKR sublines.

The consequences of provirus movement are more complex than addition or loss of proviruses. In the case of a homozygous *Akv* locus, provirus movement would generate two heterzygous loci; the original untransposed provirus and the second provirus, transposed to a new site. Either of these proviruses could be lost or fixed by further inbreeding. Thus, in some cases, the original provirus ought to be lost. In the case of the AKR sublines, this would result in the same

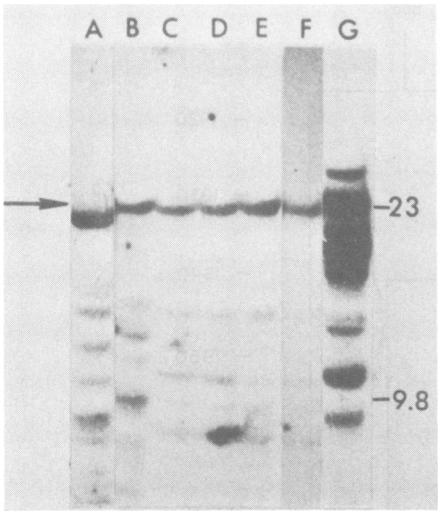

FIG. 7. *Eco*RI fragments containing AKV proviruses from DNA of BALB/c, DBA, and sublines of C3H mice. The experiment is as described in the legend to Fig. 1 except that the arrow to the left of the figure indicates the mobility of the endogenous AKV provirus of C3H mice. The DNAs in each lane were derived from the following mouse strains: (A) DBA/2J, (B) BALB/cAn, (C) C3H/An, (D) C3HeB/FeJ, (E) C3H/HeJ, (F) C3H/N, (G) C3H/Fg.

provirus being present in distantly related lines, while being absent in some lines closely related to either of them. This was not observed.

The mechanism we propose for insertion of new proviruses is infection of germ line cells by extracellular virus. Support for this model comes from comparison of AKV provirus stability in C3H and AKR sublines. The AKV provirus in C3H mice is expressed early in the life of the animal, as it is in the AKR mouse, but unlike the AKR mouse, the C3H mouse mounts a successful immune response to the virus and does not become viremic (8). If direct transposition of proviruses were responsible for the heterogeneity of AKV proviruses in AKR mice, infectious virus would have no obvious role in the process. Such a model would predict equal heterogeneity in AKR and C3H sublines. In fact, the only C3H subline showing any heterogeneity was the C3H/Fg subline, which, unlike other C3H sublines, is viremic. Thus, germ line insertions of new AKV proviruses are associated with viremia, implicating infection of germ line cells with virions as the mechanism of this insertion.

The role of viremia in germ line reinsertions of AKV proviruses was also indicated by the work of Rowe and Kozak (18), who found that germ line reinsertion occurred only if the AKV provirus was acquired from the mother. This result can be most easily interpreted in terms of a required role for infectious virus in reinsertion.

Germ line infection has been previously documented as a source of genetically transmitted proviruses. In a series of publications, Todaro and his colleagues have documented several cases of interspecies transmission of retroviruses (reviewed in reference 26). In most cases, the virus is genetically transmitted in the recipient species. Germ line infection is the only conceivable explanation for these observations. Germ line infection has also been reproduced in the laboratory. Jaenisch (9) used infection to introduce the provirus of Moloney murine leukemia virus into the germ line of BALB/c mice.

AKV proviruses have become fixed at 14 different loci in seven sublines of AKR mice during 45 years of inbreeding. Thus, one new provirus became fixed about every 15 years during inbreeding of the AKR sublines. Among the recombinant inbred strains which were viremic, three new proviruses were acquired. This would suggest the fixation of one new provirus per line per 33 years. These two rates are probably not significantly different.

It is likely that different AKV proviruses induce different levels of viremia which, in turn, could affect the rate at which subsequent germ line infections occur. Also, we suggest that different breeding programs could result in differing rates of provirus fixation. In support of such a notion, Rowe and Kozak (18) noted that novel provirus insertions were observed in the germ line only when the provirus was acquired from the mother. Thus, the rate of fixation of new proviruses may depend on a variety of factors.

The rate of acquisition of new proviruses that we observed is quite rapid when considered on the time scale of evolution. If such incrementation of provirus copy number occurred in nature, one might expect a large number of proviruses in the genome of mice. In fact, the murine leukemia virus provirus-like sequences of mice are present in 10 to 15 copies per genome (3), and the type A particle genes (M432 virus-like) are present in hundreds of copies per genome (11). These numbers, although large, fall short of the number of proviruses expected if they were being acquired at a rate of one every few decades. We suggest that some process must eventually limit the acquisition of proviruses.

Amplification of provirus copy number followed by segregation in an outbred population will lead to increased representation of the provirus in that population in the absence of any selection, an obvious advantage for the virus. Since this spread is mediated by proviruses continually integrating in new sites in the mouse genome, considerable heterogeneity will result.

Such heterogeneity is quite apparent among laboratory mice containing AKV proviruses. Different strains of laboratory mice appear to have AKV proviruses at different loci in the mouse genome (8). Examination of the xenotropic-like proviruses leads to the same conclusion. Two wild mice captured within a few hundred yards of each other have patterns of provirus integration only somewhat similar one to another, and mice captured at independent locations (a few to several thousand miles apart) have no apparent similarity (D. L. Steffen and R. A. Weinberg, manuscript in preparation). Since integration of a provirus into the mouse genome is mutagenic, the accumulation of proviruses integrated into novel sites in the mouse genome and the resulting polymorphism of provirus integration sites might be an important source of genetic instability and polymorphism among mice.

ACKNOWLEDGMENTS

We thank W. P. Rowe, J. Ihle, W. Yang, L. Gross, L. Stockert, and N. Rosenberg for providing some of the mouse strains used in this work.

This work was supported by grant VC140 from the American Cancer Society to R.A.W., core grant CA14051 to the Massachusetts Institute of Technology Center for Cancer Research from the National Cancer Institute, and research contract N01-CP-33255 from the Division of Cancer Cause and Prevention, the National Cancer Institute, to The Jackson Laboratory. D.L.S. was supported by a fellowship from the Massachusetts Institute of Technology Weizmann Fellowship Fund. R.A.W. was a Rita Allen Scholar.

LITERATURE CITED

1. **Aaronson, S. A., and J. R. Stephenson.** 1976. Endogenous type-C RNA viruses of mammalian cells. Biochim. Biophys. Acta **458**:323–354.
2. **Bailey, D. W.** 1979. Genealogies of long-separated sublines in six major inbred mouse strains, p. 18–20. *In* P. L. Altman and D. D. Katz (ed.), Inbred and genetically defined strains of laboratory animals, Part 1, Mouse and rat. Federation of American Societies for Experimental Biology, Bethesda, Md.
3. **Chattopadhyay, S. K., D. R. Lowy, N. M. Teich, A. S. Levine, and W. P. Rowe.** 1975. Qualitative and quantitative studies of AKR-type murine leukemia virus sequences in mouse DNA. Cold Spring Harbor Symp. Quant. Biol. **39**:1085–1101.
4. **Chattopadhyay, S. K., M. R. Lander, E. Rands, and D. R. Lowy.** 1980. Structure of endogenous murine leukemia virus DNA in mouse genomes. Proc. Natl. Acad. Sci. U.S.A. **77**:5774–5778.
5. **Chattopadhyay, S. K., W. P. Rowe, N. M. Teich, and D. R. Lowy.** 1975. Definitive evidence that the murine C-type virus inducing locus *Akv-1* is viral genetic material. Proc. Natl. Acad. Sci. U.S.A. **72**:906–910.
6. **Hughes, S. H., P. K. Voght, E. Stubblefield, H. Robinson, J. M. Bishop, and H. E. Varmus.** 1979. Organization of endogenous and exogenous viral and linked nonviral sequences. Cold Spring Harbor Symp. Quant. Biol. **44**:1077–1089.
7. **Ihle, J. N., and D. R. Joseph.** 1978. Serological and virological analysis of NIH (NIH × AKR) mice: evidence for three AKR murine leukemia virus loci. Virology **87**:287–297.
8. **Ihle, J. N., and D. R. Joseph.** 1978. Genetic analysis of the endogenous C3H murine leukemia virus genome: evidence for one locus unlinked to the endogenous murine leukemia virus genome of C57BL/6 mice. Virology **87**:298–306.
9. **Jaenisch, R.** 1976. Germ line integration and Mendelian transmission of the exogenous Moloney leukemic virus. Proc. Natl. Acad. Sci. U.S.A. **73**:1260–1264.
10. **Kozak, C. A., and W. P. Rowe.** 1980. Genetic mapping of the ecotropic virus-inducing locus *Akv-2* of the AKR mouse. J. Exp. Med. **152**:1419–1423.
11. **Kuff, E. L., K. K. Leuders, and E. M. Scolnick.** 1978. Nucleotide sequence relationship between intracisternal type A particles of *Mus musculus* and an endogenous retrovirus (M432) of *Mus cervicolor*. J. Virol. **28**:66–74.
12. **Leonard, A., and Gh. Deknudt.** 1967. A new marker for chromosome studies in the mouse. Nature (London) **214**:504–505.
13. **Lynch, C. J.** 1954. The R.I.L. strain of mice: its relation to the leukemia AK strain and AKR substrains. J. Natl. Cancer Inst. **15**:161–176.
14. **Morse, H. C., III.** 1978. Introduction, p. 3–21. *In* H. C. Morse III (ed.), Origins of inbred mice. Academic Press, Inc., New York.
15. **Quint, W., W. Quax, H.v.d. Putten, and A. Berns.** 1981. Characterization of AKR murine leukemia virus sequences in AKR mouse substrains and structure of integrated recombinant genomes in tumor tissues. J. Virol. **39**:1–10.
16. **Rowe, W. P.** 1972. Studies of genetic transmission of murine leukemia virus by AKR mice. J. Exp. Med. **136**:1272–1285.
17. **Rowe, W. P., J. W. Hartley, and T. Brenner.** 1972. Genetic mapping of a murine leukemia virus-inducing locus of AKR mice. Science **178**:860–862.
18. **Rowe, W. P., and C. A. Kozak.** 1980. Germ-line reinsertions of AKR murine leukemia virus genomes in *Akv-1* congenic mice. Proc. Natl. Acad. Sci. U.S.A. **77**:4871–4874.
19. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
20. **Staats, J.** 1976. Standardized nomenclature for inbred strains of mice: sixth listing. Cancer Res. **36**:4333–4377.
21. **Steffen, D., S. Bird, W. P. Rowe, and R. A. Weinberg.** 1979. Identification of DNA fragments carrying ecotropic proviruses of AKR mice. Proc. Natl. Acad. Sci. U.S.A. **76**:4554–4558.
22. **Steffen, D. L., S. Bird, and R. A. Weinberg.** 1980. Evidence for the Asian origin of endogenous AKR-type murine leukemia proviruses. J. Virol. **35**:824–835.
23. **Stockert, E., A. B. DeLeo, P. V. O'Donnell, Y. Obata, and L. J. Old.** 1979. G(AKSL2): a new cell surface antigen of the mouse related to the dualtropic mink cell focus-inducing class of murine leukemia virus detected by naturally occurring antibody. J. Exp. Med. **149**:200–215.
24. **Taylor, B. A.** 1978. Recombinant inbred strains: gene mapping, p. 423–438. *In* H. Morse (ed.), Origins of inbred mice. Academic Press, Inc., New York.
25. **Taylor, B. A., and H. Meier.** 1976. Mapping the adrenal lipid depletion gene of the AKR/J mouse strain. Genet. Res. **26**:307–312.
26. **Todaro, G. L.** 1980. Interspecies transmission of mammalian retroviruses, p. 47–76. *In* J. R. Stephenson (ed.), Molecular biology of RNA tumor viruses. Academic Press, Inc., New York.