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Mobility of Endogenous Ecotropic Murine Leukemia Viral Genomes Within Mouse Chromosomal DNA and Integration of a Mink Cell Focus-Forming Virus-Type Recombinant Provirus in the Germ Line

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Characterization of endogenous ecotropic Akv proviruses in DNA of low and high leukemic mouse strains revealed the presence of one to six copies of the Akv genome per haploid genome equivalent integrated in the germ line. Low leukemic strains analyzed so far contained only one complete copy of the Akv proviral DNA. The site of integration varied among strains, although genetically related strains often carried the Akv proviral gene in the same chromosomal site. The different substrains of the AKR mouse displayed the presence of variable numbers (two to six) of Akv genomes. In all substrains one Akv genome was present in an identical chromosomal site; this locus probably comprised the progenitor genome. Closely related substrains had several Akv proviral DNAs integrated in common sites. The accumulation of Akv genomes in the germ line of the AKR/FuRdA strain is likely the result of independent integration events, since backcross studies with the Akv-negative 129 strain showed random segregation of all six proviral loci. The AKR/Cnb strain carried a recombinant provirus in the germ line. This provirus resembled in structure the AKR mink cell focus-forming viruses, which are generated by somatic recombination during leukemogenesis. Therefore, the germ-line amplification of Akv proviral DNAs occurs most likely through infection of embryonic cells by circulating virus.

Most mouse strains carry multiple copies of murine leukemia viral (MLV) genomes as genetic elements in the host chromosome (15, 17, 22). These endogenous viruses have been classified according to their host range: ecotropic viruses, which can only infect mouse cells; xenotropic viruses, which are only infectious for cells of other species; and amphotropic viruses, which can infect both types of cells (7, 15, 20). These endogenous viruses are expressed in some mouse strains, whereas in other strains these genomes are repressed by host control systems (13, 16, 28). The expression of the ecotropic Akv virus is associated with the occurrence of lymphatic leukemia in various strains; a high incidence of leukemia is associated with a high level of Akv expression like in the AKR, C58, and C3H/Fg strains, and a low incidence of leukemia is seen in strains in which only occasionally Akv virus expression is observed, like BALB/c, DBA, and C3H, whereas in strains without an increased frequency of leukemia, like 129, no Akv virus is expressed (4, 9, 10, 14, 17, 21, 26). Both liquid hybridization studies and DNA analyses with the Southern transfer technique have shown that the nonleukemic strains lack the genetic information for ecotropic virus (17, 19, 26). The endogenous ecotropic viruses present in the low and high leukemic strains share a set of restriction endonuclease sites, suggesting that they have originated from a common ancestor (26). Since all of these endogenous proviruses are part of the host genome complement and are transmitted from parent to offspring according to Mendelian expectations, we were interested to see whether the characteristic integration sites of these ecotropic viruses were conserved among related mouse strains. The transposon-like structure of RNA tumor viruses and the ability to generate infectious virus and reinfect host cells would in principle allow considerable variation in the number and sites of integrations. We chose both high and low leukemic strains to perform this analysis. The high leukemic AKR strains originate from Jacob Furth (6) who started to select mice for high incidence of leukemia. C. Lynch continued inbreeding of several sublines up to 1946. Most of the now available

sublines are derived from her AKR/M line (6). Some of these lines differ in several genetic markers (1).

We analyzed 14 different substrains of the high leukemic AKR strain and various low leukemic strains to study the mobility of the Akv viral genome within the chromosomal DNA. We addressed the following questions. At what frequency do germ-line integrations of Akv occur spontaneously? What is the stability of the integrated structure and through what mechanisms are germ-line integrations established, through infection of embryonic cells by circulating virions, as suggested by the results of Rowe and Kozak (23), or through intracellular pathways?

MATERIALS AND METHODS

Virus. The AKR virus was isolated and propagated as described previously (26).

Mice. Mice or organs from the different strains were obtained as follows: BALB/c, 129, C57B1/10ScSn, and C3H/St-Z strains from the Central Animal Laboratory of the University of Nijmegen, Nijmegen; AKR/JS*, C3H/HeJ, C57B1/6J, and A/J livers from F. Jensen, Scripps Clinic, La Jolla, Calif.; AKR/FuRdA, B10Y, ACR (or AKR/FuA), and C3H/HeA mice from Netherlands Cancer Institute, Amsterdam. NZW and DBA/2 strains from the Central Institute for the Breeding of Laboratory Animals-TNO, Zeist, The Netherlands; GRS/A strain from the Radiobiological Institute-TNO, Rijswijk, The Netherlands; AKR/Cnb and AKR/Rb-k strains from Centre d'Etude de l'Energie Nucleaire, Mol, Belgium; AKR/J and AKR/Cu strains from the Weizmann Institute of Science, Rehovot, Israel; AKR/A Bom and AKR/H Bom strains from the Breeding and Research Center, GL Bomholtgaard Ltd., Ry, Denmark; AKR/JS DNA and organs from R. Jaenisch, Heinrich Pette-Institute, Hamburg, Federal Republic of Germany; AKR/Tl strain from H. Pogosianz, All Union Cancer Research Centre, Moscow, USSR; AKR/Sn organs from Health Research, Inc., Roswall Park Memorial Institute, New York, N.Y.; AKR/NH strain from Zentral Institut für Versuchstiere, Hannover, Federal Republic of Germany; AKR/N strain from National Cancer Institute of Health, Bethesda, Md.; and AKR/Rho strain from IFFA CREDO, l'Arbresle, France.

Preparation and characterization of the cDNA probe. An AKR-MLV specific ^{32}P-labeled cDNA probe was prepared with the endogenous polymerase reaction. The AKR-MLV cDNA sequences that cross-hybridize with other mouse viruses were removed by hybridization to an excess of 129 mouse DNA followed by hydroxyapatite chromatography exactly as described by Berns and Jaenisch (2). The remaining single-stranded fraction was tested for specificity by hybridizing with DNAs of different strains (129, NIH Swiss) which do not contain the Akv provirus and by hybridization with BALB/c DNA and NIH Swiss Akv-2 congenic DNA, which contain one copy of AKR-MLV (19). All four *Bam*HI restriction endonuclease fragments of a cloned AKR-MLV integrated virus (clone 623) hybridized with this probe, indicating that this probe contains sequences complementary to the different regions of the AKR-MLV genome.

Analysis of DNA. Isolation of DNA from mouse livers, restriction endonuclease digestion gel electrophoresis, transfer to nitrocellulose filters, and hybridization were carried out as described previously (19).

RESULTS

Determination of Akv-specific sequences. To detect exclusively Akv sequences, a cDNA probe was prepared as described previously and outlined above (19). The resulting Akv cDNA probe did not cross-hybridize to non-Akv endogenous proviruses present in mouse DNA. Using this Akv-specific probe, we have analyzed the genomic organization and integration sites of Akv proviral DNA in a number of inbred mouse strains. To characterize the integration sites we used restriction endonuclease *Eco*RI, which does not cleave the Akv proviral DNA. Therefore, the size of the generated fragment is determined by the position of *Eco*RI sites in adjacent host DNA. The presence of Akv sequences in identically sized *Eco*RI fragments among different strains would therefore strongly suggest that they are integrated in the same chromosomal site. Liver DNAs from a series of laboratory mouse strains were analyzed for the presence of Akv-containing *Eco*RI DNA fragments. The results of such an analysis performed on low leukemic and nonleukemic strains is shown in Fig. 1. It appeared that different strains with low virus titers carried one copy of the ecotropic MLV genome (Fig. 1B, lanes 1 through 10), whereas in virus-negative strains no endogenous Akv sequences were detectable (Fig. 1B, lanes 11 through 13). In various strains the Akv sequences were present in *Eco*RI fragments of the same size, whereas other strains carried Akv sequences on differently sized fragments, e.g., a fragment of 20 kilobase pairs (kbp) was detected in the ACR, C3H/HeA, C3H/HeJ, A/J, and BALB/c strains of mice (Fig. 1B, lanes 1, 2, 3, 5, and 6), a 31-kbp fragment was found in the C57BL/6J, C57BL/10Sn, and B10Y strains of mice (Fig. 1B, lanes 4, 9, and 10), whereas the NZW and DBA/2 strains of mice had differently sized *Eco*RI fragments of 24 kbp (Fig. 1B, lane 7) and 16 kbp (Fig. 1B, lane 8), respectively. The differences in proviral integration sites were further substantiated by *Bam*HI digestion of DNA from these inbred strains. This analysis showed different virus-cell junction fragments (data not shown). The coincidence of the Akv-containing *Eco*RI fragments among strains is in full agreement with the genealogy of the mouse strains (Fig. 1A). The Akv genome detected in a 20-kbp fragment was apparently conserved in the segregation of BALB/c, A/J, and C3H/He strains from the Bagg strain. DNA from mice of the C3H/St-Z strain did not show hybridization with the AKR-MLV cDNA. Either the C3H

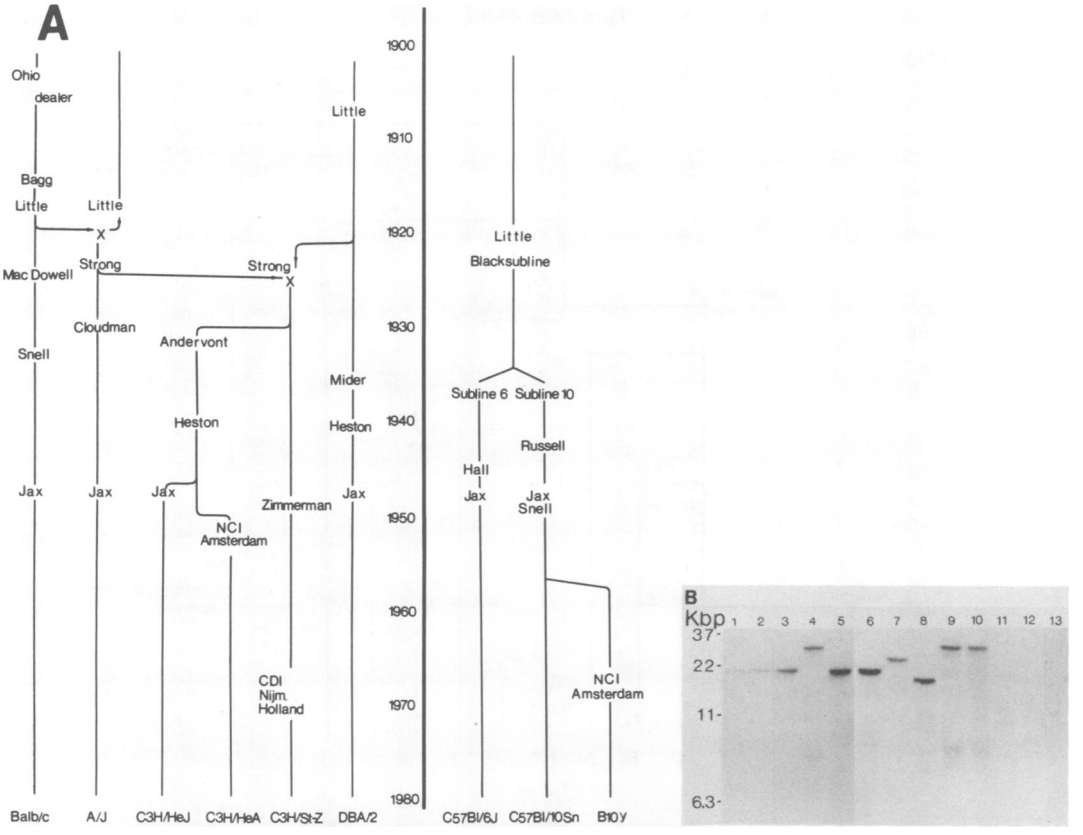

FIG. 1. Segregation of endogenous Akv proviruses during inbreeding of low-virus-titer strains. A, The genealogy of the mouse strains was derived from the Mouse News Letter; inbred strains of mice by J. Staat, (ed.), Jackson Laboratories, and are plotted against the dates for mating and segregation. B, *Eco*RI-digested DNAs were analyzed by the Southern procedure (24) and hybridized with the specific cDNA probe. *Eco*RI does not cleave the Akv viral DNA (25). Lanes 1 through 13 represent liver DNAs from the following strains: ACR (lane 1), C3H/HeA (lane 2), C3H/HeJ (lane 3), C57B1/6J (lane 4), A/J (lane 5), BALB/c (lane 6), NZW (lane 7), DBA/2 (lane 8), C57B1/10Sn (lane 9), B10Y (lane 10), GRS (lane 11), C3H/St-Z (lane 12), and 129 (lane 13). Adenovirus type 2 viral DNA and phage λ DNA digested with *Eco*RI, *Hin*dIII, and *Bam*HI were used as molecular weight markers.

strain maintained by Strong was not homozygous for this Akv locus at the time of transfer to Andervont and subsequently lost the locus during inbreeding, or the Akv locus in the C3H/St-Z has been lost. The conservation of integrated sites among strains that have been separated for nearly 60 years (BALB/c, A/J, and C3H) and 40 years (C57BL sublines) is indicative of a stable integration of the endogenous Akv genome. Furthermore, it shows a very low frequency of new integration events during the process of inbreeding of low-virus-titer strains.

Akv sequences in the AKR strains. Previously it has been shown that several sublines of the high-virus-titer AKR strain contain variable numbers of endogenous Akv genomes integrated in different chromosomal sites (19). Figure 2 shows the genealogical relation and the patterns of Akv-specific *Eco*RI fragments in liver DNA from 14 AKR sublines maintained at different laboratories. All of the sublines carry an Akv genome on a 55-kbp *Eco*RI DNA fragment. Substrains derived from the Jackson group and the Law group, which separated in 1950, have a second fragment of 12 kbp in common. In addition, each of these two groups contains a third common fragment, a 29-kbp fragment in the Law group and a 10.5-kbp fragment in the Jackson group. Besides these common integration sites, unique additional fragments can be detected in the Jackson sublines JS, JS*, and Sn, which are not seen in other related colonies. The NH and HB sublines were separated from the N colony in 1975 and contain one additional fragment. From the different endogenous genomes present in the AKR/Rho and AKR/FuRdA strains, three

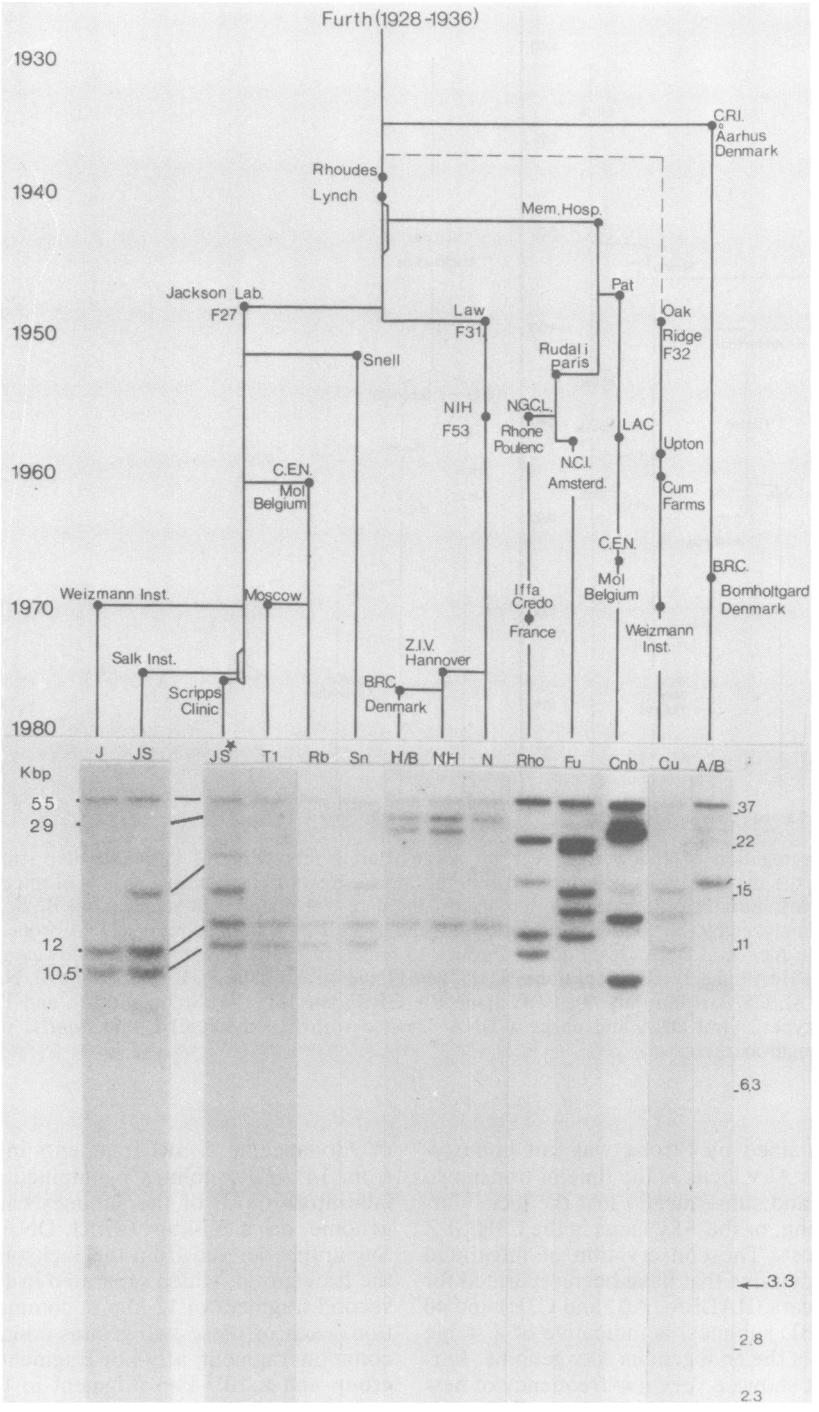

FIG. 2. Identification and segregation of endogenous Akv proviruses in 14 substrains of AKR mice. The upper part represents the genealogical tree of the AKR substrains which was obtained from the Mouse News Letter and plotted against the recorded time for segregation. The dashed line indicates the most likely historical pathway of the AKR/Cu strain. The lower part of the figure represents the Akv genotype for each substrain, which was determined by Southern blotting analysis of *Eco*RI-digested liver DNA. The molecular sizes of some individual Akv-containing fragments are indicated at the left. The molecular weight markers at the right are as described in the legend to Fig. 1.

bands are located at the same position, whereas the others are unique for each substrain. In general, the integration patterns show that strains which have been separated in the 1930's and 1940's have only the Akv genome present in the 55-kbp *Eco*RI DNA fragment in common; later-diverged strains possess several Akv genomes integrated in the same chromosomal site. The position of Akv proviruses in the different chromosomal loci is in complete agreement with the segregation pattern of the various sublines and illustrates the stability of proviral integrations in these strains.

Segregation of endogenous Akv genomes from the AKR/FuRdA strain. To study the characteristics of individual Akv loci, the AKR/FuRdA strain, which contains six endogenous genomes, was backcrossed with the ecotropic virus-negative 129 strain. We were interested to see whether the six proviral loci were genetically linked or segregated independently. Furthermore, it allows analysis of the biological activity of the individual proviral loci. From 29 mice, obtained after backcrossing 129 with (AKR/FuRdAx129) F1 males and females, liver DNA was isolated and digested with *Eco*RI. The segregation patterns of these 29 mice show random segregation of the six proviral loci (Fig. 3). Among the 29 mice analyzed, 6 mice with each one of the six loci were found. Therefore, the Akv genomes are integrated in distant unlinked positions in the AKR genome and most likely are the result of independent integration events in the germ line rather than gene duplication or tandem integrations.

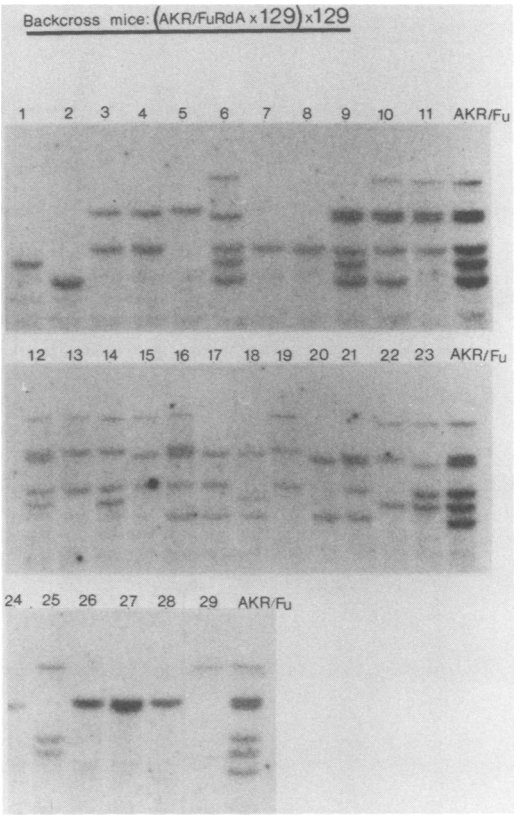

FIG. 3. Segregation of Akv proviruses in backcross of (AKR/FuRdA × 129) mice with the 129 strain. *Eco*RI-digested DNAs were analyzed by the Southern procedure. Lanes 1 through 29 represent liver DNAs from 29 mice (AKR/FuRdA × 129).

Mode of germ-line integration. The accumulation of Akv proviral loci during inbreeding in AKR substrains could be the result of two different mechanisms. Either it occurs via intracellular mechanisms, e.g., reverse transcription and subsequent reintegration, or it is the result of reinfection of (primordial) germ cells or early stage embryos. The AKR/Cnb strain allowed us to obtain more information concerning the mechanism involved in amplification of proviral loci. These mice display an *Eco*RI DNA fragment of 3.3 kbp hybridizing to the Akv-specific cDNA probe (Fig. 2). Since authentic Akv proviral DNA does not carry an *Eco*RI site, this 3.3-kbp *Eco*RI DNA fragment might comprise genetic information for part of a recombinant MLV provirus. Such recombinant proviral DNAs, however, are normally restricted to tumor tissues and are generated only later in life by somatic recombination in lymphatic tissues (8, 19, 27). The presence of such a recombinant provirus in the germ line therefore suggests infection of germ-line cells.

Liver DNA (250 μg) of AKR/Cnb mice was cleaved with *Eco*RI, and the fragments were separated on the basis of size on a 0.4% agarose gel. The 3.3-kbp *Eco*RI DNA fraction was electroeluted from gel slices and further characterized by digestion with *Xba*I and *Pst*I. After cleavage with *Pst*I and *Xba*I, one (1.5-kbp) and two (1.1- and 1.7-kbp) fragments, respectively, were found which hybridized to an Akv-specific cDNA probe (Fig. 4A). Such fragments could not be generated from AKR proviral DNA. However, fragments of 1.5 and 1.1 kbp could be obtained from 3′ parts of mink cell focus-forming virus (MCF)-type recombinant proviruses detected in all leukemic tissues of AKR mice (Fig. 4B) (3, 19); therefore, the 3.3-kbp *Eco*RI fragment represents part of a AKR-MCF recombinant genome. Since recombinant proviruses are generated by somatic recombination, the presence of such a provirus in the germ line of the AKR/Cnb strain most likely indicates that introduction of this recombinant in the germ line has been caused by infection through circulating recombinant viruses produced in lymphatic tis-

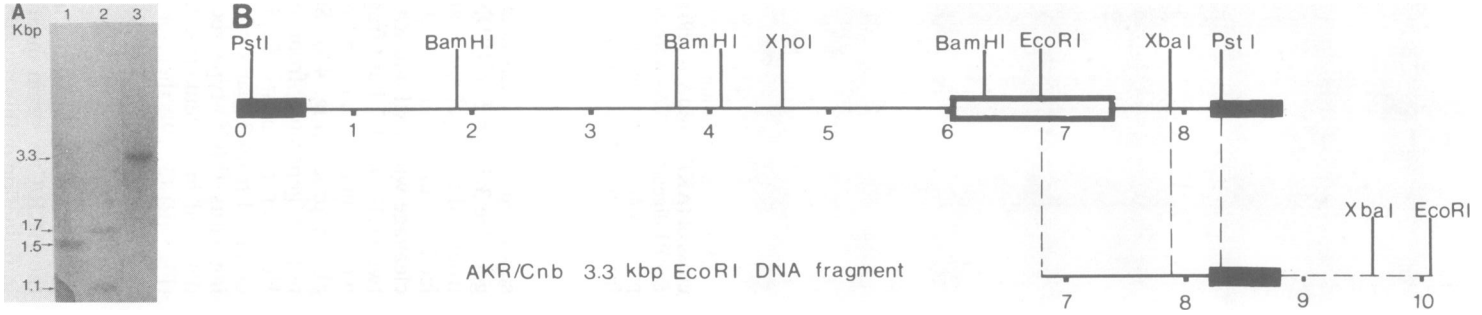

FIG. 4. Recombinant AKR-MLV sequences in AKR/Cnb mice. A, The 3.3- kbp *Eco*RI fragment from liver DNA (250 μg) of AKR/Cnb mice was isolated as described previously (19). This fragment was cleaved with *Pst*I (lane 1) and with *Xba*I (lane 2). Lane 3 represents the uncleaved 3.3-kbp *Eco*RI fragments. The molecular sizes of fragments containing the AKR-MLV sequences are indicated to the left. B, The 1.5-kbp *Pst*I fragment and the 1.1-kbp *Xba*I fragment could be mapped on the linear map of the MCF-type recombinant proviruses (19) (upper line). The positions in kbp of some restriction sites are indicated. Black boxes represent the long terminal repeat sequences. The solid frame represents the substituted sequences present in the MCF recombinant proviral genomes (19, 27). The *Eco*RI site (at 6.8) is characteristic for MCF recombinants and causes the generation of the 1.1- and 1.5-kbp fragments (19). The line at the bottom represents the structure of the 3.3-kbp *Eco*RI fragment. The solid line represents proviral sequences, and the dashed line represents the flanking cellular sequences.

sues. Whether sperm cells, oocytes, or developing embryos are the target for infection remains obscure for the moment.

DISCUSSION

We have characterized the integration sites of endogenous ecotropic genomes in DNAs from several low- and high-virus-titer strains. Previous studies have shown that cell DNA from mice with high incidence of leukemia contained multiple copies of ecotropic proviral DNA, whereas mice with low incidence of leukemia comprise in general only a single ecotropic viral genome (4). Our analyses confirm these results. The Akv genome in the low leukemic strains is found in only a few different chromosomal loci. The Akv genome in C3H/HeA, C3H/HeJ, A/J, and BALB/c strains is present in the same chromosomal site. Since these strains have been separated for nearly 60 years, this observation illustrates the stability of integrated proviral elements (Fig. 1A). The same stability is found within the C57Bl/6J and C57Bl/10Sn strains, which have been separated for 40 years and which both carry their Akv sequences in a 31-kbp *Eco*RI fragment (Fig. 1B). The presence of Akv genomes in different chromosomal loci of low leukemic strains supports the hypothesis that the endogenous Akv sequences in these strains were acquired by independent germ-line integrations (26).

Previously, we reported a difference in number and in site of integrations of Akv genomes among five substrains of the high-virus-titer AKR strain (19). Besides this variability, all of these strains contain an Akv genome in an *Eco*RI fragment of 55 kbp which is also present in nine additional AKR sublines (Fig. 2). These results clearly suggest a progenitor function of this locus as a parental gene for the other endogenous genomes. This Akv genome in a 55-kbp *Eco*RI fragment has also been found in the AKR/N, AKR/J, and AKR/Cu mouse strains by others and corresponds to the Akv-1 locus (4, 25). AKR sublines which have segragated for long periods of time (AKR/A, AKR/Cu, AKR/FuRdA, AKR/Cnb, and AKR/N) have only the Akv genome present in the 55-kbp fragment in common, whereas sublines which have segregated more recently carry several Akv genomes in identical chromosomal sites, e.g., the Jackson and Law are more related to each other (Fig. 2). This indicates that the viral genomes become immobilized in the host chromosomal DNA and behave as cellular genes.

The difference between AKR/J and AKR/N mouse strains (Fig. 2) most likely results from new integration events after the separation of these strains. However, the difference between these two sublines could also be explained by transposition of an Akv proviral genome in one of these strains after separation. Within progeny of the A/JxDBA/2 mice (Fig. 2) the DBA/2-specific locus (16-kbp *Eco*RI fragment) is not present. The absence of this Akv genome could be the result of an excision event or could be ascribed to the heterozygosity for this locus at the moment of crossing. However, the absence of this genome within the A/JxDBA/2 progeny and its presence within the DBA/2 strain could also be due to an independent germ-line integration event within the DBA/2 strain after 1925, the year in which the DBA/2 strain was crossed with the A/J strain. It is obvious that no solid evidence for an excision event can be found in any of the low leukemic strains.

The observed diversity and accumulation of Akv genomes within the sublines could be due to gene duplication, tandem integration, or reinfection of germ cells. Backcrossing the AKR/FuRdA strain with the ecotropic virus-negative 129 strain revealed independent segregation of the six loci. Therefore, the variation of Akv loci within the AKR substrains is more likely the result of independent germ-line integration events which occur on average once in 30 generations. As a consequence, integration of provirus in chromosomal DNA of germ cells has to occur with a much higher frequency, since the newly acquired genomes might be easily lost during inbreeding by the heterozygous or even mosaic character of the newly acquired locus or by being lethal in the homozygous state. The acquisition of new viral integrations is predominantly seen in strains with high virus titers and seems therefore related to the expression of the provirus. The introduction of MLV proviruses in the germ line through infection has already been reported; in vitro infection of preimplantation embryos results in the acquisition of genetically transmitted proviruses (11, 12). The observed amplification in the Akv-1 congenic mouse strain (23) and in the AKR strains most likely occurs by infection of germ-line cells. We provide evidence that, indeed, circulating virus has been involved in germ-line integration. We detected an MCF-type recombinant provirus in the germ line of the AKR/Cnb strain. Since these types of recombinants are generated only later in life in the lymphatic tissues of preleukemic and leukemic AKR mice by somatic recombination between ecotropic AKR viruses and endogenous xenotropic-like sequences (8), integration in the germ line most likely occurs by infection of primordial germ cells, oocytes, or early embryos by circulating virus. Although one could theoretically envisage the intracellular generation of MCF genomes in germ cells, this seems rather unlikely. The results of Rowe and Kozak (23), who showed that the acquisition of novel

virus-containing loci is only associated with virus-positive females, also suggest that the most likely in vivo mechanism of germ-line amplification is by the infection of oocytes or developing embryos. However, at this point we cannot exclude that postcoital infection of sperm cells occurs or that the virus is introduced during fertilization. In vitro fertilization studies in the presence of infectious virus are being performed to test the possibility of this last mode of infection.

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