Identification of DNA fragments carrying ecotropic proviruses of AKR mice

(Southern blotting/endogenous viruses/murine leukemia virus/congenic mice)

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ABSTRACT  The proviruses of the N-tropic, ecotropic virus (AKV) of AKR mice (Ak-1, Ak-2) have been studied by the Southern gel-filter transfer technique. These proviruses can be detected by cleavage of cell DNA by BamHI endonuclease, which yields characteristic subgenomic DNA fragments upon cleavage of this type of provirus. Proviruses integrated into different sites in the mouse genome can be resolved with EcoRI endonuclease, which does not cleave the AKV proviruses. Use of congenic and backcrossed mice and a radioactive DNA probe enriched for AKV sequences has allowed identification of the EcoRI fragments carrying the proviruses of the genetically defined Ak-1 and Ak-2 loci. Novel proviruses introduced by superinfection of cultured AKR cells with AKV and present in leukemic cells from AKR mice have also been identified. Comparison of substrains of AKR mice indicates some heterogeneity in their spectra of proviruses.

The AKR strain of mouse is characterized by a life-long expression of an ecotropic N-tropic murine leukemia virus, AKV, and a 70–95% incidence of leukemia (1, 2). Both of these characteristics are the result of dominant alleles present at two loci, Ak-1 and Ak-2 (3). Congenic mice have been constructed that possess either the Akv-1 or Akv-2 allele in the genetic background of the NIH/Swiss strain of mouse (4), a strain which otherwise lacks such alleles. Hybridization of DNA from such congenic mice to an AKV cDNA probe demonstrated that the Ak-1 allele segregates with and presumably represents a provirus for AKV. The allele at Ak-2 is associated with a second, apparently identical (5), provirus.

The proviruses present at Ak-1 and Ak-2 are not necessarily expressed. Embryonic AKR mice and many cell lines derived from AKR embryos do not produce virus. In vitro, expression of virus occurs at a low frequency spontaneously and at a much higher frequency upon induction with BrdUrd (6). Because AKR cells are sensitive to exogenous infection with AKV, such transient induction of virus expression leads to horizontal infection of the entire culture and subsequent continuous virus production. In vivo, expression of virus occurs from some cells in all postnatal AKR mice. The in vivo expression of virus is probably also the result of transient induction of virus by a few cells followed by horizontal infection of other cells in the animal.

The number of AKV proviruses present in virus-negative, virus-producing, and leukemic AKR cells has been estimated by liquid hybridization (7, 8). This technique allows relatively accurate quantitation of the amount of AKV sequences present in a given DNA sample. It provides no information, however, about the organization of these sequences. Recently, the Southern gel–filter transfer technique (9) has been used successfully to analyze single integrated murine leukemia virus (MuLV) proviruses in rodent cells (10). We apply this technique here to identify the Ak-1 and Ak-2 genetic loci with specific DNA fragments produced by restriction endonucleases, to identify specific restriction endonuclease fragments as markers for the presence of the AKV genome in different genetic backgrounds, and to identify newly introduced AKV and AKV-related proviruses in the DNAs of AKV-infected and leukemic AKR cells.

MATERIALS AND METHODS

Mice. AKR/N and NFS/N mice were obtained from the mouse colony at the National Institute of Health. The homozygous congenic mice containing Ak-1 and Ak-2 in an NIH/Swiss background were constructed in one of our laboratories (W.P.R.) as has been described (4). The NFS/N mice heterozygous for Ak-1 used for the segregation analysis were derived by six generations of backcrossing Ak-1 from an NIH/Swiss Ak-1 congenic into NFS/N mice. AKR/J mice used for the studies of tumor DNAs were obtained from Jackson Laboratory.

Virus and Cell Lines. Isolation of the virus coded for by the Ak-1 locus has been described (5). Akv-1 was grown in an NIH 3T3 cell line selected for its ability to grow MuLVs. These cells were infected and cloned, and a clone producing a high titer of virus was used as a source of virus. Production and purification of virus has been described (10).

Isolation of the AKR2B cell line has been described (11). The AKV-infected clones were obtained as follows. Supernatant fluids from a culture of AKR2B cells that had spontaneously begun virus production were passed onto a non-virus-producing culture of AKR2B. These infected cells were carried for several months and then cloned at 0.16 cells per well in microtiter wells. Clones were screened for virus production.

Isolation of DNA. Extraction of DNA from cells and animal tissues (10) has been described. A simplified procedure was used for purification of much of the DNA used here. The DNA was extracted once or twice with 1 vol of water-saturated phenol (pH 8) and once with 1 vol of 96% CHCl3/4% isoamyl alcohol (vol/vol). Isopropyl alcohol (2.5 vol) was added at 25°C, the DNA was mixed until a white clump resulted, and the clump was removed and redissolved in 10 mM Tris-HCl, pH 8/1 mM EDTA. This DNA was cleaved with restriction endonucleases as described (10) by using in each case the buffer recommended by the manufacturer of the enzyme. All restriction enzymes were obtained from New England Biolabs, except for EcoRI, which was obtained from Boehringer Mannheim. Completeness of digestion was monitored as described (10) or by adding λ DNA to each sample as a control.

Abbreviations: AKV, virus specified by the Akv-1 or Akv-2 allele of AKR mice; MuLV, murine leukemia virus; NaDodSO4, sodium dodecyl sulfate; NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate at pH 7.4.

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DNA to an aliquot of the reaction and examining the λ DNA for completeness of digestion by gel electrophoresis.

**Gel Electrophoresis of DNAs and Hybridization.** Isolation of RNA from virions has been described (12). cDNA was synthesized from purified RNA by use of avian myeloblastosis virus reverse transcriptase (unpublished data). The cDNA had a specific activity of 2 X 10^6 to 10^7 dpm/μg. This cDNA was used for Southern hybridization directly after prehybridization to Moloney MuLV RNA as follows: 70S Moloney MuLV RNA at 1000 μg/ml and cDNA at 1 μg/ml in 6X NaCl/Cit, pH 7.4 (1X is 0.15 M NaCl/0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (NaDodSO4) were hybridized for 60 min at 67°C and then used for Southern hybridization.

Gels were run and the DNA was transferred to the filters as described (13). Hybridization to the DNA on the filters was modified as follows: the filter was soaked before hybridization for 2 hr at 67°C in 6X NaCl/Cit, 10X Denhardt’s reagent (1X is 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 100 μg of boiled salmon DNA per ml, and 0.1% NaDodSO4. The filter was rinsed with 2X NaCl/Cit and dried at room temperature for several hours. Hybridizations were performed at 67°C for 16 to 24 hr. The hybridization reaction contained 6X NaCl/Cit, 10X Denhardt’s reagent, 100 μg of boiled salmon DNA per ml, 0.1% NaDodSO4, 10 μg of poly(A) per ml, and 2.5 X 10^6 dpm of AKV cDNA per ml. Prehybridized cDNA was used at twice this concentration.

After hybridization, the filter was rinsed several times briefly with 2X NaCl/Cit at room temperature and then soaked three times for 60 min and twice for 30 min at 67°C with 100–200 ml of 3X NaCl/Cit, 10X Denhardt’s reagent, 100 μg of boiled herring DNA per ml, and 0.1% NaDodSO4.

If the filter had been hybridized with cDNA prehybridized to Moloney MuLV RNA, the filter was then rinsed several times at room temperature with 2X NaCl/Cit, incubated 30–45 min in 2X NaCl/Cit, 0.5 μg of boiled RNase A per ml, and washed twice for 60 min and once for 30 min at 67°C with 100 ml of 0.3X NaCl/Cit and 0.1% NaDodSO4.

**RESULTS**

Use of Endonucleases BamHI and EcoRI in Analysis of AKV Proviruses. As the first step in our analysis of the AKV proviruses of AKR mice, we determined the sites within the AKV genome at which BamHI cleaves. The sites of cleavage by this enzyme are shown in Fig. 1. Data supporting this map will be reported elsewhere.

Because BamHI cleaves the AKV genome at three sites, cleavage of an AKV provirus integrated into the genome of a mouse cell will result in two viral fragments linked to the adjacent cellular sequences and two internal fragments containing only viral sequences. The sizes of these two internal fragments will be invariant and will not depend on the sequences into which the provirus happens to be integrated. These two internal fragments can be used to screen for the presence or absence of an AKV provirus in a given sample of DNA.

DNA samples were screened for the presence of AKV proviruses by cleaving the DNA with BamHI and detecting the resulting fragments by the Southern procedure. When DNA from an AKR/N mouse was analyzed, the complex pattern of Fig. 2, lane b was seen. The large number of fragments hybridizing to the AKV probe represent diverse, endogenous, genetically acquired sequences with homology to the AKV probe used for detection. These endogenous sequences are observed in many strains of mice, some of which lack AKV-type proviruses (14). Among the fragments seen in Fig. 2, lane b, two of them (termed B and C) were the same size as BamHI fragments resulting from digestion of the unintegrated AKV genome (Fig. 2, lane a). We tentatively conclude that these result from the provirus at Akv-1 and Akv-2 in the AKR/N mouse DNA.

The NIH-Swiss strain of mice does not contain an AKV provirus (14). Analysis of BamHI-cleaved NIH/Swiss DNA again reveals many fragments with homology to the AKV probe (Fig. 2, lane d) but none of the size of the AKV internal fragments B and C. If DNA from a clone of AKV-infected NIH/Swiss mouse cells was analyzed (lane c), the B and C fragments

![Fig. 1. Sites on the AKV genome cleaved by BamHI. Arrows indicate sites on the genome cleaved by BamHI. Numbers above the line indicate the sizes (in kilobases) of the fragments. The 5' and 3' orientation of the map is defined with respect to the viral RNA. The fragments are labeled A–D as indicated below the line. Fragments A, B, and D are coincidentally the same size and thus migrate the same distance during gel electrophoresis (Fig. 2).](https://example.com/fig1.png)

![Fig. 2. Identification of AKV specific BamHI fragments in mouse DNAs. BamHI-digested DNAs were analyzed by the Southern procedure. The gel was run for 16 hr at 1.2 V/cm. The molecular sizes indicated to the left of the figure in kilobases were determined with HindIII-digested λ DNA. The BamHI B and C fragments (see Fig. 1) are shown to the right of the figure. Lane a, unintegrated AKV linear DNA; lane b, AKR2B DNA; lane c, AKV-infected NIH 3T3 cell DNA; lane d, NIH/Swiss mouse DNA. All lanes were hybridized with unselected AKV cDNA.](https://example.com/fig2.png)
were observed. Thus, the B and C fragments represent reliable markers for the presence of the AKV genome amid the complex, endogenous background of NIH/Swiss mouse DNA.

Unlike *BamHI*, *EcoRI* does not cleave AKV proviruses to produce fragments of characteristic size. Rather, because *EcoRI* does not cleave the AKV genome (data not shown), cleavage of DNAs containing AKV proviruses with *EcoRI* will result in intact AKV proviruses flanked on either side by cellular sequences. The size of the DNA fragment containing an AKV provirus will depend on the distance between the viral sequences and the nearest *EcoRI* site in the cellular DNA. Consequently, proviruses integrated into different sites in the mouse cell genome will in general reside in different sized *EcoRI* fragments and therefore can be resolved by gel electrophoresis. All *EcoRI* fragments containing a provirus must be larger than 8800 base pairs (8.8 kilobases), the size of the AKV provirus.

Development of a Probe Selective for AKV. Analysis of *EcoRI*-cleaved AKR/N mouse DNA by the Southern procedure described above revealed many hybridizing DNA fragments (Fig. 3, lane a). Most of these fragments do not represent AKV proviruses, but rather other endogenous sequences with some homology to the AKV probe. We were able to use a minor variation of a technique developed by Lee Bachelor of the Salk Institute (personal communication) to remove most of the sequences from the AKV probe that hybridize to these non-AKV endogenous sequences.

We annealed the AKV cDNA probe to a 1000-fold excess of Moloney MuLV RNA before incubating the probe with the Southern filter containing mouse DNA. The sequences in the probe with homology to the MuLV RNA become unavailable for hybridization to DNA on the filter. Hybridization of *EcoRI*-cleaved AKR/N mouse DNA with this preselected probe (Fig. 3, lane c) revealed a much simpler pattern of DNA fragments than was observed with the unselected probe (lane a). Some of the fragments detected must still represent sequences other than AKV proviruses, as they are smaller in size than the intact AKV genome. There are, in fact, only three fragments large enough (>8.8 kilobases) to contain a complete AKV genome. Two of these must represent the AKV proviruses at the *Akv-1* and *Akv-2* loci.

Use of Congenic Mice to Identify *Akv-1* and *Akv-2*. We used congenic NIH/Swiss mice carrying *Akv-1* and *Akv-2* to identify the *EcoRI* fragments on which these alleles reside. The congenic mice contain either the *Akv-1* or the *Akv-2* allele amid the genetic background of NIH/Swiss mice. The respective proviruses were identified as *EcoRI* fragments absent from NIH/Swiss mouse DNA, but present both in the congenic mouse DNA and the AKR/N mouse DNA. Comparison of the DNAs of these mice is shown in Fig. 3. DNA from the NIH/Swiss *Akv-2* congenic mouse is displayed in lane b. This DNA exhibited two fragments (one at 23 and one at 9.8 kilobases) that were absent from the NIH/Swiss DNA (lane e) and were large enough to contain a complete AKV provirus. Only one of these was present in the DNA of the AKR/N strain of mouse. This fragment must contain the sequence of the *Akv-2* allele. The fragment absent from the AKR/N and NIH/Swiss DNAs but present in the congenic could represent either variability in the NIH/Swiss background or integration of a new provirus during construction of the congenic line.

DNA from the NIH/Swiss *Akv-1* congenic (lane d) contained three fragments that were absent from the NIH/Swiss DNA and that were large enough to contain a complete AKV provirus. Only one of these was present in DNA from the AKR mouse and, therefore, we identify this fragment as containing *Akv-1*. Because this fragment comigrated with a group of very high molecular weight fragments, its size was impossible to deter-

![Fig. 3. Identification of DNA fragments associated with *Akv-1* and *Akv-2* in *EcoRI*-digested mouse DNAs. *EcoRI*-digested DNAs were analyzed by the Southern procedure. The gel was run for 40 hr at 1.2 V/cm. The molecular sizes indicated to the left (in kilobases) were determined with *HindIII*-cleaved λ DNA. The mobilities of the fragments associated with *Akv-1* and *Akv-2* and the mobility estimated for an intact, unintegrated AKV genome (8.8 kilobases) are shown to the right. Lane a was hybridized with unselected AKV cDNA; the remaining lanes were hybridized with AKV cDNA prehybridized to Moloney MuLV RNA. DNAs were extracted from mouse livers. Lane a, AKR/N DNA; lane b, NIH/Swiss *Akv-2* congenic DNA; lane c, AKR/N DNA; lane d, NIH/Swiss *Akv-1* congenic DNA; lane e, NIH/Swiss DNA (a mouse obtained from the Massachusetts Institute of Technology Center for Cancer Research Animal Facility).](image-url)

mine here and its intensity was frequently low due to technical factors in electrophoresis and transfer.

To provide further evidence for our identification of the *Akv-1*-containing DNA fragment, we followed the segregation of the *Akv-1* allele in a genetic cross. A mouse with the genetic background of the NFS/N strain (an inbred strain derived from the NIH/Swiss strain) and heterozygous for *Akv-1* (*Akv-1/−*) was crossed with an NFS/N mouse lacking any *Akv* alleles (−/−). About half the progeny of this cross should inherit the *Akv-1* allele (*Akv-1/−*) while the remaining mice will lack *Akv-1* alleles (−/−). Eight suckling mice resulting from this cross were tested for IdUrd-inducible virus. Three mice were virus positive. DNA from all eight mice was cleaved with either *BamHI* or *EcoRI*. As shown in Fig. 4, the same three mice (nos. 1, 5, and 7) that were positive for virus expression also possessed an AKV provirus, as scored by generation of the B and C AKV marker fragments upon cleavage of their DNA with *BamHI*. 

![Fig. 4.](image-url)
in revealed the Akv-1 lines of AKR/N mice, the Akv-1 and Akv-2 containing the we right. indicated A EcoRI or from shown a (-) and resulting Akv (+) and resulting mobilities (Lower), 4. fragment fragment. A fragments associated with Akv-1 and Akv-2 and the mobility estimated for an intact, unintegrated AKV genome (8.8 kilobases) are shown. Novel provirus-containing fragments are located between the 9.8- and 20-kilobase markers in lanes b and c and are indicated by small arrows in lanes f-i. Lane a, AKR2B DNA; lane b, clone 1 of AKV-infected AKR2B DNA; lane c, clone 2 of AKV-infected AKR2B DNA; lane d, AKR/Cu liver DNA; lane e, AKR/J embryo DNA; lane f, AKR/J lymphoma 1 DNA; lane g, AKR/J lymphoma 2 DNA; lane h, AKR/J lymphoma 3 DNA; lane i, AKR/J lymphoma cell line clone 1 DNA; and lane j, AKR/J lymphoma cell line clone 2 DNA. All lanes were hybridized with AKV cDNA prehybridized to Moloney MuLV RNA.

Finally, we note that nos. 1, 5, and 7 were the only mice containing the EcoRI-generated DNA fragment identified in Fig. 3 as containing Akv-1, thus confirming the identification.

Although the provirus-containing fragments associated with the Akv-1 and Akv-2 alleles were identifiable in the DNA of AKR/N mice, subsequent examination of DNA from two other lines of AKR mice, AKR/Cu (Fig. 5, lane d) and AKR/J (lane e) revealed the Akv-1-associated fragment but failed to show the Akv-2-associated fragment. Noting yet other variabilities in the proviruses of these three lines of the AKR strain, we conclude that there is substantial variation between these three lines. As a consequence, our identification of the Akv-2 associated fragment is applicable only to the DNA of AKR/N mice.

Identification of Novel Proviruses in AKV-Infected and Leukemic Cells. The Akv loci in cell lines derived from AKR embryos are generally not expressed. Such cells can be infected in vitro with AKV and will thereafter express virus at high levels. Does this infection result in the introduction of new active proviruses, or alternatively, in the induction of expression of the preexisting proviruses? It has been reported (8) that, after infection of AKR embryo fibroblasts and a cell line derived from AKR embryo fibroblasts, no increase in the number of AKV proviruses was detectable by liquid hybridization. The relatively small number of EcoRI fragments detected in AKR DNA with our selected AKV probe encouraged us to re-examine this question by the analysis described above. Results of this analysis are shown in Fig. 5. Lane a contains DNA from AKR2B, a cloned line of AKR cells derived from AKR embryo fibroblasts (11). This line does not express virus. The pattern of EcoRI fragments observed is very similar to that seen when AKR/N mouse DNA was analyzed. AKR2B cells were infected with AKV, and DNA from two subsequently derived clones is displayed in lanes b and c of Fig. 5. DNA fragments are present in the infected cell lines but absent from the uninfected parent line. These fragments contain AKV proviruses integrated into new sites in the cellular genome after in vitro infection. Each clone contains several new proviruses and in each clone the proviruses are integrated in different sites. Thus, infection of AKR fibroblasts in vitro results in integration of AKV proviruses in a number of new sites in the mouse genome.
DNA from tumor cells of spontaneous leukemias in AKR mice has more AKV sequences than DNA from normal cells, as measured by liquid hybridization (8). Analysis of such DNAs with restriction endonucleases and procedures similar to those described here, except with an unselected AKV probe, has revealed novel DNA fragments in tumor DNAs (15). Using our selected AKV probe, we compared DNAs from leukemic cell DNA with embryo DNA of AKR/J mice (Fig. 5). As expected, a number of fragments were detected in the tumor DNAs that were absent from the embryo DNA (lane e). DNA from each tumor displays a unique pattern of novel fragments. These two observations taken together indicate that the tumors consist largely of the clonal descendants of one transformed cell (8). Consistent with this is the observation that two clonal cell lines derived from the same tumor have the same pattern of novel proviruses (lanes i and j). Not expected is the observation of fragments in the embryo DNA absent from the tumor DNAs. We do not understand this observation.

A number of non-AKV MuLVs, some of which are derivatives of AKV, can be isolated from leukemic AKR mice (16-20). Thus, some of the novel fragments observed in tumor DNAs may result from non-AKV proviruses. We point out that some of the novel fragments are smaller than the complete AKV genome, indicating that these fragments cannot result from the integration of an unaltered AKV genome.

DISCUSSION

We have identified DNA fragments containing the genetically acquired ecotropic proviruses corresponding to the Akv-1 and Akv-2 alleles. These alleles, and thus the associated DNA fragments, are present in AKR mice but absent from NIH/Swiss mice. By introducing the Akv-1 and Akv-2 alleles into the NIH/Swiss genetic background, we were able to correlate physically defined DNA fragments with these genetically defined loci.

Ihle and Joseph (21) have presented evidence supporting the existence of a third AKV provirus in AKR mice. Our identification of two AKV proviruses in AKR mice (Akv-1 and Akv-2) does not bear on existence of additional AKV proviruses in these mice. We note the presence in the DNA of AKR/N mice of a third EcoRI fragment (12-15 kilobases) that hybridizes strongly to our selective probe and that is large enough to contain a third AKV provirus (Fig. 3).

The Akv-1-associated fragment was identified by two independent procedures, analysis of an NIH/Swiss Akv-1 congenic mouse (Fig. 3) and segregation of Akv-1 in a cross (Fig. 4). This fragment is present in all three lines of AKR mice examined: AKR/N, AKR/J, and AKR/Cu. The Akv-2-associated fragment, identified by analysis of an NIH/Swiss Akv-2 congenic mouse (Fig. 3), is present in the AKR/N line (as well as the cell line AKR2B) but is absent from the AKR/J and AKR/Cu lines. This difference, and other differences between the bands observed in these lines, demonstrates that there is substantial genetic variability between lines of the AKR strain of mouse.

AKR cells can be infected by the AKV specified by their endogenous proviruses. Here we have shown that this infection results in the presence of additional proviruses beyond the genetically acquired AKV proviruses. These additional proviruses are difficult to resolve from the background of endogenous sequences in the AKR mouse DNA when an unselected AKV probe is used for detection (data not shown), but are clearly resolved by using our preselected AKV probe. We have identified such additional proviruses in AKR fibroblasts infected in vitro, as well as in AKR leukemic cells. Due to the diversity of virus types isolated from leukemic AKR mice, the identity of the proviruses in the leukemic cells is not clear. In fact, the subgenomic size of some of the novel fragments identified in the leukemic cells is inconsistent with their containing unaltered AKR proviruses (Fig. 5). It is likely that virus production in infected AKR cells results from transcription of these newly introduced proviruses and that cells producing virus in adult AKR mice also contain newly introduced proviruses. An unresolved question concerns the differences between the poorly expressed endogenous AKV sequences and the apparently efficiently expressed proviruses introduced via infection.

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