Somatically Acquired Recombinant Murine Leukemia Proviruses in Thymic Leukemias of AKR/J Mice

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We have probed the structure and arrangement of murine leukemia virus genomes in eight spontaneous AKR thymic leukemias by Southern hybridization with one ecotropic *pol* and four ecotropic *env* probes. These probes revealed many (in 2 cases over 15) somatically acquired proviruses that had undergone complex patterns of recombination. The large majority were not deleted and were structurally analogous to the oncogenic mink cell focus-inducing murine leukemia viruses isolated from AKR tumors in that the amino-terminal p15E-coding region derived from ecotropic AKR murine leukemia virus sequences, whereas certain gp70-coding sequences were nonecotropic. Nevertheless, we observed a few proviruses which did not appear to be gp70 recombinants; however, these proviruses were in general clearly recombinant within the p15E-coding sequences. Although the proviral recombination patterns were quite variable, in general the large majority of recombinant proviruses within each tumor appeared structurally identical, indicating that they originate from a common parent. Each tumor contained a unique pattern of provirus integrations; densitometer tracings of the Southern hybridizations indicated that many of the integrated proviruses were present at one copy per cell, suggesting that the tumors derive from a single cell which contained multiple integrated copies of a unique recombinant virus structurally similar to the mink cell focus-inducing viruses.

The AKR mouse strain is noted for a high incidence of thymic leukemias at 6 to 9 months of age and a complex pattern of murine leukemia virus (MuLV) expression. Two major types of MuLV have been isolated from the germline of AKR mice: ecotropic MuLV, which replicate preferentially in murine cells, and xenotropic MuLV, which grow in cells from a variety of heterologous species (24). The host range is defined by the viral envelope (*env*) gene (2, 20), which codes for the gp70 and p15E peptides.

Neonatal AKR mice produce high titers of the AKR ecotropic MuLV, AKV, but not xenotropic MuLV, presumably because of the favorable environment for reinfection and replication (47). Ecotropic viremia and the high incidence of leukemia in AKR mice are genetically related. Mice of the nonleukemic NIH/Swiss strain, which have acquired either of two independently segregating loci, *Akv-1* and *Akv-2*, by mating with AKR mice, become viremic and contract leukemia, albeit with a much longer latency period (44, 45). However, unlike viral extracts from preleukemic and leukemic thymuses, AKV virus is unable to induce leukemia in healthy

mice (21, 33). Various leukemogenic MuLV have been isolated from preleukemic AKR thymuses and AKR thymoma cell lines.

Preleukemic AKR thymuses express MuLV that have both xenotropic and ecotropic host ranges (15). These dual-tropic viruses are distinguished from amphotropic viruses by the characteristic foci they produce when they infect mink lung cells and are, therefore, termed mink cell focus-inducing (MCF) viruses (15). Unlike AKV, AKR MCF virus isolates may accelerate the onset of leukemia if injected into young AKR mice (9, 36). MCF viruses result from recombination between endogenous ecotropic and non-ecotropic MuLV, principally within the *env* and long terminal repeat sequences (6-8, 11, 25, 43).

A second class of viruses capable of accelerating leukemia in AKR mice has been isolated from cell lines derived from AKR thymomas. Unlike the MCF viruses, these "spontaneous leukemia" viruses have an exclusively ecotropic host range (34). RNase T₁ oligonucleotide maps of two spontaneous leukemia viruses show that the gp70 sequences are not recombinant, but they do appear to have significant differences from AKV near the 3' terminus (39).

Somatically acquired proviruses (5, 49), which

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have MCF virus-like recombinant structures (6, 40), appear in the DNA of leukemic AKR thymuses. We have analyzed the structure of these new proviruses with five short AKV-derived hybridization probes that map within or near the *env* gene. These probes generate simple germline hybridization patterns and show that the leukemic cells have acquired many proviruses with complex recombinant structures. Although the provirus recombination patterns are highly variable, most tumors contain multiple integrations of structurally identical proviruses.

MATERIALS AND METHODS

Molecular subcloning of AKV hybridization probes.

We constructed a series of DNA hybridization probes specific for discrete regions of the AKV *pol* and *env* genes by individually subcloning short AKV-derived restriction fragments into the plasmid vector pBR322. Figure 1 shows the AKV sequences contained within each of the seven subclones, called pAKV3-9 (Fig. 1A), and the individual subclone restriction site maps (Fig. 1B). We used the nucleotide sequence of the 3' half of the AKV genome (17a) to determine the subcloning strategies. Each of the subcloned restriction fragments derives from one of three plasmids (pAKV1-2, -6, and -9), shown at the bottom of Fig. 1A, which were constructed in determining the 3' AKV nucleotide sequence. These plasmids contain AKV sequences extending from a randomly inserted *EcoRI* linker to the 3' long terminal repeat *PstI* site within the large *EcoRI-PstI* fragment of pBR322.

Each AKV fragment to be subcloned was purified by polyacrylamide gel electrophoresis and eluted as described below before ligation with pBR322 plasmid DNA. The gel-purified DNAs were ligated by incubation with 200 U of T4 DNA ligase (New England Biolabs) in 50 μ l of 50 mM Tris-hydrochloride (pH 7.6)–10 mM $MgCl_2$ –10 mM dithiothreitol–1 mM ATP–50 μ g of bovine serum albumin per ml overnight at 15°C. Ligated samples were phenol extracted in 200 μ l of 0.3 M sodium acetate–5 mM EDTA–50 μ g of tRNA carrier per ml, ethanol precipitated, and suspended in 10 μ l of water. Transformations of HB101 cells were done by heat shock (90 s, 42°C) in 100 mM $CaCl_2$, and transformed cells were selected as single colonies on 2YT (30) plates supplemented with 50 μ g of ampicillin per ml. Positive subclones were verified and the insert orientation was identified by nucleotide sequence analysis (28).

We constructed pAKV3 by isolating the short 237-base-pair (bp) AKV *ClaI* fragment. In plasmid DNA isolated from *dam*⁺ *Escherichia coli* strains these *ClaI* sites are resistant to *ClaI* digestion because of overlapping methylated *MboI* sites. Therefore, we transformed the *dam*⁺ *E. coli* strain RB404 (obtained from R. Brent) with pAKV1-9, prepared plasmid DNA from 6 ml of saturated culture by the boiling method (19), isolated the 237-bp AKV *ClaI* fragment, and ligated it to *ClaI*-digested pBR322 plasmid DNA. pAKV3 was identified by colony hybridization (14) with a total AKV cDNA probe prepared as described elsewhere (18a).

pAKV4, pAKV8, and pAKV9 are derived from pAKV1-6. pAKV9 contains the 776-bp AKV *BglII*

fragment within the *BamHI* site of pBR322. This construction destroys both the *BglII* and *BamHI* recognition sites, but the 776-bp AKV fragment can be excised intact by *MboI* digestion. The AKV sequences within pAKV1-6 terminate at an inserted *EcoRI* site within the N-terminal region of the gp70 sequences. The region spanning from this site to the gp70 *BamHI* (pAKV4) and *BglII* (pAKV8) sites was subcloned by ligation to the large *BamHI-EcoRI* fragment of pBR322 (generously provided by J. Brosius). These three AKV subclones were identified by screening for Amp^r Tet^s colonies, followed by restriction site analysis of five candidate colonies for each subclone.

pAKV1-2 was used to construct the three p15E subclones, pAKV5, 6, and 7. pAKV5 contains the N-terminal region of p15E spanning from the inserted *EcoRI* site of pAKV1-2 to the *BglII* site (Fig. 1A). This 167-bp fragment was subcloned as described above for pAKV8. The pAKV6 and pAKV7 inserts were blunt end ligated to flush-ended molecules: the large *EcoRI-HindIII* fragment of pBR322 and *EcoRI*-cleaved pBR322, respectively. These ligations recreated the *EcoRI* and *HindIII* sites (Fig. 1B). To make the molecules flush ended, they were treated with the large fragment of polymerase I (New England Biolabs) in 50 mM NaCl, 10 mM Tris (pH 7.6), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μ M each dATP, dGTP, dCTP, and TTP overnight at 15°C. pAKV6 contains the 265-bp AKV *BalI-MboII* fragment spanning the C-terminal region of p15E, whereas pAKV7 contains the 228-bp AKV *BalI* p15E fragment. pAKV6 and pAKV7 were identified as described above for pAKV3.

Mice, DNA isolation, and restriction enzyme digests. High-molecular-weight DNA was isolated from brain and thymus tissue of eight (no. I through VIII) grossly leukemic AKR/J mice (Jackson Laboratories, Bar Harbor, Maine) by a modification of the Blin and Stafford (3) procedure. All but one of the mice were 6 to 9 months old (animal VII was 13 months old) and had thymuses approximately 10- to 20-fold larger than normal. The tissues were rapidly frozen in liquid N₂, ground to a fine powder with a mortar and pestle, and lysed in 0.5 M EDTA (pH 8)–0.1% Sarkosyl–100 μ g of proteinase K (Boehringer Mannheim) per ml by incubation for 4 to 12 h at 50°C while set on a rocker. The DNA was extracted three times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and dialyzed extensively against 10 mM Tris-hydrochloride (pH 8)–10 mM NaCl–1 mM EDTA. Contaminating RNA was digested with 100 μ g of RNase A (Sigma Chemical Co.) per ml at 37°C for 3 h. The DNA solution was made 0.1 M EDTA, further extracted twice with phenol, and extensively dialyzed against 10 mM Tris-hydrochloride (pH 8)–1 mM EDTA.

The DNA samples were digested with restriction enzymes as suggested by the manufacturer (New England Biolabs). We monitored the *EcoRI*, *PstI*, and *HindIII* digestions by incubating a portion of the digest in parallel with end-labeled, *HinfI*-digested pBR322 plasmid DNA. The largest pBR322 *HinfI* fragment (1,631 bp) is cleaved by each of these enzymes (52), and digestion of this fragment was detected by polyacrylamide gel electrophoresis and autoradiography. The digested DNA samples were ethanol precipitated and suspended in 50 to 100 μ l of water.

Gel electrophoresis and Southern hybridization. DNA samples (10 μ g) in 5% Ficoll 70, 25 mM EDTA,

and 0.01% bromphenol blue and xylene cyanol dyes were electrophoresed through horizontal submerged 0.6 or 0.7% agarose gels (0.5 to 0.7 cm thick) in 50 mM Tris-acetate (pH 8.0)–20 mM sodium acetate–1 mM EDTA (17) at 1 V/cm for approximately 24 h until the bromphenol blue dye marker had migrated 15 cm. Molecular weight markers were as described previous-

ly (18). To prepare the DNA for transfer to nitrocellulose filters (BA85; Schleicher & Schuell Co.), the gels were treated twice for 10 min with 0.25 N HCl (55), twice for 25 min with 0.5 N NaOH–1 M NaCl, and once for 45 to 60 min with 1 M Tris-hydrochloride (pH 7.4)–3 M NaCl. The DNA was transferred essentially as described by Southern (48) with 20× SSC (1× SSC

FIG. 1. AKV subclone constructions. (A) The *pol* and *env* open reading frames are shown above a restriction site map of the 3'-terminal region of AKV (17a). The restriction sites bounding each subcloned AKV fragment are shown. Bracketed *EcoRI* restriction sites represent the inserted *EcoRI* linkers from the pAKV1-2, -6, and -9 subclones shown at the bottom. (B) Restriction site maps of each AKV subclone are shown. The large pBR322 *HincII* fragment is unaltered in each subclone and is not shown. The orientation of each insert is shown by the arrows with respect to the 5' to 3' direction of the AKV viral RNA, and the size of each insert is given in base pairs. The plasmids which have been used as ecotropic virus-specific hybridization probes are identified by the coding region from which they derive. The Cgp70 probe is the internal *KpnI*-*BalI* fragment of pAKV9.

is 0.15 M NaCl–0.015 M sodium citrate)–1 M NH_4 acetate for approximately 6 h. After the transfer, the filters were washed briefly in 1 M ammonium acetate, air dried, and baked in vacuo at 80°C for 2 h.

The filters were prehybridized for 1 to 3 h in $10\times$ Denhardt solution ($1\times$ Denhardt solution is 0.02% bovine serum albumin–0.02% polyvinylpyrrolidone–0.02% Ficoll 400 [10]), $5\times$ SET ($1\times$ SET is 0.15 M NaCl–0.03 M Tris-hydrochloride [pH 8.0]–2 mM EDTA [26]), and 500 mg of heat-denatured salmon sperm DNA per ml at 65°C. The filters were hybridized with 0.5×10^6 to 1×10^6 cpm/ml of heat-denatured, nick-translated probe (see below) in $5\times$ Denhardt solution–10% dextran sulfate (55)– $5\times$ SET–250 μg of heat-denatured salmon sperm DNA per ml for 24 to 48 h at 65°C. Excess probe was washed off the filter with 100 to 200 ml of $5\times$ Denhardt solution– $2\times$ SET–100 μg of heat-denatured salmon sperm DNA per ml for 30 to 60 min at 65°C followed by a wash with $2\times$ SET–0.5% sodium dodecyl sulfate for 30 to 60 min at 65°C with shaking. Nonspecifically hybridized probe was removed by shaking the filters in 0.1 $\times$ SET–0.1% sodium dodecyl sulfate for 30 min at 65°C. After air drying, the filters were autoradiographed at –70°C with a Du Pont Cronex Lightning Plus screen (22, 53) or at room temperature without screen enhancement. To reprobe, the filters were treated with 20 mM NaOH for 30 min at room temperature before the prehybridization step.

Preparation of radiolabeled hybridization probes. DNA fragments for nick translation were isolated by polyacrylamide gel electrophoresis (6% polyacrylamide, 50 mM Tris-borate [pH 8.3], 1 mM EDTA) and elution as described by Maxam and Gilbert (27), after restriction enzyme digestion as follows: the *pol* probe was the short *EcoRI*–*Bam*HI fragment of pAKV3 (Fig. 1B), the gp70 probe was the short *EcoRI*–*Sall* fragment of pAKV4, the Np15E probe was the short *EcoRI*–*Sall* fragment of pAKV5, and the Cp15E probe was the short *EcoRI*–*Bam*HI fragment of pAKV6. The Cgp70 probe was isolated by polyacrylamide gel purification of the short *EcoRI*–*Bal*I fragment of pAKV9 followed by a similar purification of the *Kpn*I–*Bal*I fragment.

The radioactively labeled probes were prepared by nick translation (41). Samples (20 to 100 ng) of purified fragment were incubated with 12 U of polymerase I (New England Biolabs) in 50 mM NaCl, 10 mM Tris (pH 7.6), 10 mM MgCl_2 , 1 mM dithiothreitol, 4 μM each [α - ^{32}P]dATP, -TTP, -dCTP, and -dGTP, 5 ng of DNase I (Worthington Diagnostics) per ml, and 500 μg of bovine serum albumin per ml for 2 to 3 h at 15°C. The polymerase I was inactivated in 10 mM EDTA at 65°C for 10 min, and unincorporated nucleotides were removed by Sephadex G50 chromatography. The labeled DNA had a specific activity of 5×10^8 to 2×10^9 cpm/ μg .

RESULTS

We used the hybridization probes shown in Fig. 2 to study the structure and arrangement of somatically acquired proviruses in eight (no. I through VIII) spontaneous AKR/J thymic leukemias. We described elsewhere (18a) the isolation of radiolabeled, single-stranded AKV cDNA restriction fragments that map within or near the

corresponding nonecotropic *env* sequences of an MCF virus (Fig. 2A). We used these restriction fragments as hybridization probes in our initial studies. To facilitate the production of hybridization probes we constructed recombinant plasmids bearing these AKV cDNA sequences (Fig. 2A). The plasmid probes derive from a short region of *pol* (*pol*), the central (gp70) and C-terminal (Cgp70) regions of the gp70-coding sequence, and the N-terminal (Np15E) and C-terminal (Cp15E) regions of the p15E-coding sequence. Figure 2B shows the location of these probes with respect to restriction site maps of AKV and the prototype MCF virus, strain 247.

Identification of germ-line proviruses. To identify somatically acquired proviruses in AKR/J leukemias we had first to identify those proviruses that were already present in the germ line of each individual. This was particularly important with AKR/J mice, because even though they have been highly inbred their germ-line patterns are variable (4, 18, 31). To determine individual germ-line patterns we analyzed the DNA isolated from the brain tissue of each diseased animal. Unlike the leukemic thymus, the brain is refractory to viral infection and shows no increase of ecotropic proviral sequences in leukemic animals (as detected by liquid hybridization [1]).

Table 1 gives the sizes of the *EcoRI*, *Hind*III, and *Pst*I restriction fragments generated by each of the six (labeled A through F) germ-line proviruses we have observed in the eight leukemic animals described here. The brain DNA hybridization patterns (not shown) showed they each had three (A through C) constant ecotropic proviruses. Two proviruses (D and E) which have been shown to be scattered throughout the Jackson Laboratories AKR mouse colony (4, 18, 31) appeared in some of these mice. Provirus D was present in animals I through III and VI through VIII, whereas provirus E appeared in animals II and VIII. A previously unidentified germ-line provirus (F) was present in animal VI.

AKR/J thymomas carry multiple recombinant proviruses: *env* structure. Figure 3 shows that the DNA from leukemic AKR/J thymomas contains many somatically acquired proviruses with complex, MCF virus-like, recombinant structures. We show four Southern blot hybridizations (48) of *EcoRI*-digested, thymoma DNA samples (Fig. 3, lanes 1 through 8) hybridized to the four ecotropic *env* probes (Fig. 2): gp70 (Fig. 3A), Cgp70 (Fig. 3B), Np15E (Fig. 3C), and Cp15E (Fig. 3D). As an undiseased tissue control we show the DNA isolated from the brain of diseased animal I (Fig. 3, lanes B). The individual brain DNA samples showed that the 50-, 12-, and 11-kilobase (kb) fragments present in each lane and the variable 14-kb and 17-kb fragments

FIG. 2. Location of ecotropic virus-specific hybridization probes (hatched boxes) and restriction site maps of AKV and MCF 247. (A) The location of *Hae*III cDNA fragments (a) identified previously (18a) and subcloned AKV probes (b) is shown with respect to the *pol* and *env* (*gp70*, *p15E*, and *R*) coding sequences determined from the nucleotide sequence of the 3' half of the AKV genome (17a). (B) Restriction site maps of AKV (50) and MCF 247 (7) are shown. The restriction sites are as follows: *Hind*, *Hind*III; *Pst*, *Pst*I; *R*I, *Eco*RI; *Xba*, *Xba*I. The positions of the subcloned probes are shown as in (A).

are the germ-line proviruses (Table 1, data not shown). The 16-kb *Eco*RI fragment in lanes 6 (see Fig. 3A, lane 6) is the new germ-line ecotropic provirus (F) we identified (Table 1) in animal VI.

The Np15E probe hybridizes to many new proviral fragments (Fig. 3C, lanes 1 through 8) that are not present in DNA isolated from the brain (compare lanes B and 1 in Fig. 3C) and most of which do not hybridize to the ecotropic *gp70* probe (Fig. 3A, lanes 1 through 8). The Np15E hybridization shows the complete repertoire of somatically acquired proviruses we detect in AKR thymomas indicating a strong selection for these ecotropic sequences.

The Cgp70 and Cp15E probes identify the structural heterogeneity among the somatically acquired proviruses. None of the new proviruses in tumor I hybridize to the Cgp70 probe (Fig. 3B, lane 1), whereas, except for a 5.3-kb fragment in thymoma IV (Fig. 3C, lane 4), all of the somatically acquired proviruses in thymomas II through VIII do (Fig. 3B, lanes 2 through 8). Therefore, in general, the somatically acquired proviruses contain hybrid ecotropic-non-ecotropic *gp70* sequences because most do not hybridize to the *gp70* probe (Fig. 3A). Figure 3D shows that the proviruses in thymomas V and VII contain ecotropic C-terminal *p15E* sequences (lanes 5 and 7) whereas, except for two

proviruses in tumor IV (5.3 and 18 kb), the somatically acquired proviruses in the other tumors are missing these sequences (lanes 1, 2, 3, 4, 6, and 8).

The *gp70* probe hybridizes to a few somatically acquired proviruses (for examples, see Fig. 3A, lanes 4 and 8) which may be proviruses with entirely ecotropic *gp70* sequences. Nevertheless, in most cases these proviruses are clearly *env* recombinants because they do not hybridize to the Cp15E probe (compare lanes 8 in Fig. 3A and D).

Somatically acquired proviral *pol* structure. The *Eco*RI ecotropic *pol* probe hybridization patterns (not shown) are very different from the *env* probe patterns shown in Fig. 3. This is because MCF viruses acquire by recombination an *Eco*RI site within the *gp70*-coding sequences between the *pol* and *env* probes (Fig. 2). Therefore, to analyze the *pol* structure of the somatically acquired proviruses we digested the tumor DNAs with *Hind*III. Figure 4 shows the resulting *pol* (Fig. 4A), *gp70* (Fig. 4B), and Np15E (Fig. 4C) Southern blot hybridization patterns. Lanes a, b, and c contain control brain DNA samples from three of the diseased animals (IV, VI, and II, respectively), which display the germ-line ecotropic proviral 3' *Hind*III junction fragments described in Table 1.

As in the *Eco*RI digests (Fig. 3), the *gp70* probe hybridizes to a few thymoma-specific proviruses in the tumor DNAs (Fig. 4B, lanes 1 through 8), and the Np15E probe displays many new recombinant proviral *Hind*III fragments (Fig. 4C, lanes 1 through 8). Except for one Np15E band in both tumors I and II, the new proviruses in tumors I through IV and VIII (Fig. 4A, lanes 1 through 4 and 8, respectively) all hybridize to the ecotropic *pol* probe. In contrast, no new ecotropic *pol* probe sequences appear in tumors VI and VII (Fig. 4A, lanes 6 and 7, respectively; compare tumor VI, lane 6, with the

TABLE 1. Sizes of AKR/J germ-line ecotropic provirus restriction fragments that hybridize to the five ecotropic hybridization probes

Endonuclease	Size (kb) of restriction fragment from germ-line ecotropic provirus ^a					
	A	B	C	D	E	F
<i>Eco</i> RI	50	12	11	17	14	16
<i>Hind</i> III	5.9	26	6.2	5.9	7.0	6.5
<i>Pst</i> I	8.2	8.2	8.2	8.2	8.2	8.2

^a The three constant AKR/J ecotropic proviruses A, B, and C are the AKV-1, AKV-3 and AKV-4 alleles, respectively, described by Steffen et al. (51). The variable ecotropic proviruses D and E have been described previously (4, 18, 31), and provirus F is described in this report.

FIG. 3. Identification of somatically acquired provirus *env* structure. Samples of *EcoRI*-digested DNAs were electrophoresed through 0.6% agarose gels and hybridized by Southern blotting to the (A) gp70, (B) Cgp70, (C) Np15E, and (D) Cp15E probes as described in the text. The gp70 hybridization was a reprobing of the Cgp70 filter. Lanes 1 through 8 are DNAs from thymomas I through VIII, respectively, and lane B is the brain control DNA from mouse I. Fragment sizes are given in kilobases.

brain control, lane b), suggesting that all of the new proviruses in these two tumors are *pol* recombinants. Tumor V contains two populations of recombinant proviruses because only two of the new Np15E *HindIII* bands (Fig. 4C, lane 5) appear in the *pol* hybridization (Fig. 4A, lane 5).

An unexpected result from the Np15E hybridization in Fig. 4C is that many of the new *HindIII* fragments in tumor VI (lane 6) are shorter than 5.8 kb. Because the AKV *HindIII* site lies 5.8 kb from the 3' terminus and xenotropic viruses isolated from AKR mice do not contain any *HindIII* sites (7), we expected all of

FIG. 4. Somatically acquired provirus *pol* structure. *Hind*III-digested DNAs were electrophoresed through 0.7% gels and hybridized by Southern blotting to the (A) *pol*, (B) *gp70*, and (C) *Np15E* probes as described in the text. The *gp70* hybridization (B) was a reprob of the *pol* filter. Lanes 1 through 8 are the thymoma DNAs from mice I through VIII, respectively. The brain control samples are from mice IV (lane a), VI (lane b), and II (lane c). The sizes of fragments are shown in kilobases.

the recombinant proviruses to lie on 3' *Hind*III fragments larger than 5.8 kb. Further analysis of the recombinants in tumor VI (data not shown) showed that they have acquired a new *Hind*III site that maps near the *pol* probe sequences, and that this site is present in a subset of germ-line noncotropic proviruses which also contain a *Pst*I site 4.8 kb from the 5' terminus.

Structural integrity of recombinant proviruses. Lack of proviral hybridization to an ecotropic virus-specific probe could be due to either replacement of ecotropic sequences by noncotropic sequences through recombination or deletion of the ecotropic sequences. The germ-line noncotropic hybridization pattern is so complex (6) that we could not detect new proviruses in most tumors with a noncotropic hybridization probe (unpublished results). Therefore, to study the structural integrity of the somatically acquired proviruses, we analyzed the tumor DNAs by digestion with *Pst*I alone and in combination with *Eco*RI. Fig. 5 shows examples of the *pol* and *Np15E* hybridization patterns.

The germ-line ecotropic proviruses lie on an 8.2-kb *Pst*I fragment (Fig. 5, lanes 1 and 3; Fig. 2) that is resistant to *Eco*RI digestion (Fig. 5, lanes 2 and 4). (The *pol* probe cross-hybridizes slightly to 7.6-, 4.5-, 2.9-, and 2.4-kb noncotropic proviral *Pst*I fragments [Fig. 5, lane 1] that can be differentiated from ecotropic sequences

because they are sensitive to *Eco*RI digestion [Fig. 5, lane 2].) All of the new proviruses in tumor I (as well as in tumors III, VI, and VII [data not shown]) appear to lie on an 8.2-kb *Pst*I fragment that comigrates with the germ-line *Pst*I fragment (Fig. 5, lanes 5 and 7). The recombinant proviruses are differentiated from the germ-line proviruses by *Eco*RI digestion because of the characteristic *Eco*RI site within the noncotropic *gp70* sequences (Fig. 2). The recombinant proviral *pol* probe sequences lie on a 6.8-kb, 5' *Pst*I-*Eco*RI fragment (Fig. 5, lane 6), and the *Np15E* sequences lie on a 1.4-kb, 3' *env* *Eco*RI-*Pst*I fragment (Fig. 5, lane 8). We conclude that these new proviruses are not deleted.

Nevertheless, in three of the tumors we detected some proviruses that may be deleted. In both tumors II and V we observed one recombinant *Pst*I fragment that was larger than 8.2 kb, whereas in tumors II and VIII we detected recombinant *Pst*I fragments that were smaller than 8.2 kb. The patterns generated by tumor II are shown in Fig. 5 (lanes 9 through 12). The combination of *Pst*I and the *Np15E* probe (Fig. 5, lane 11) shows three abnormal fragments of 14, 6.8, and 5.4 kb, which all disappeared upon digestion with *Eco*RI (Fig. 5, lane 12), indicating that 3' *env* sequences are not deleted (only the 1.4-kb, 3' *Eco*RI-*Pst*I fragment appears). The largest fragment must contain mutated se-

FIG. 5. Structural integrity of somatically acquired thymoma-specific proviruses. *PstI*- and *PstI-EcoRI*-digested DNAs were electrophoresed through 0.7% agarose gels and hybridized by Southern blotting to the (A) *pol* and (B) Np15E ecotropic probes as described in the text. Odd-numbered lanes are *PstI*-digested DNAs, and even-numbered lanes are *PstI-EcoRI* double digestions. The DNAs were isolated from the thymus of a healthy 3-month-old AKR mouse (lanes 1 through 4), thymoma I (lanes 5 through 8), thymoma II (lanes 9 through 12), and thymoma IV (lanes 13 through 16). The sizes of fragments discussed in the text are identified in kilobases.

quences to the 5' side of the *EcoRI* site, whereas the smaller fragments may be either deleted or have acquired unusual *PstI* sites. The *pol* probe hybridized to the 14- and 6.8-kb *PstI* fragments, but not the 5.4-kb fragment (Fig. 5, lane 9), suggesting that the *pol* probe sequences may have been deleted in the latter provirus and that the provirus in tumor II lacking ecotropic *pol* sequences (Fig. 4A, lane 2; see also Fig. 6) does not represent a different recombinant structure. *EcoRI* digestion (Fig. 5, lane 10) shortens these two fragments, and another aberrant structure appears, producing three fragments of 11, 5.5, and 5.4 kb, further indicating that the abnormal structures lie on the 5' side of the *EcoRI* site.

Unlike the other tumors, *PstI* digestion of tumor IV DNA results in a major 5.3-kb proviral fragment (Fig. 5, lanes 13 and 15) that contains the 1.4-kb, 3' *env EcoRI-PstI* fragment (Fig. 5, lane 16) and a 3.8-kb, 5' *pol PstI-EcoRI* fragment (Fig. 5, lane 14). Yet digestion with other restriction enzymes (e.g., *PvuII*) showed that these proviruses are not deleted (data not shown). Consistent with a recombinant *PstI* site, these proviruses have lost (data not shown) the AKV *HindIII* site that maps near this new *PstI* site. Chattopadhyay et al. (7) mapped two MCF viruses isolated from AKR mice, which also have these characteristics.

Somatically acquired proviral structure. Figure

6 shows, schematically, the *pol* and *env* structure of somatically acquired proviruses in each of the eight tumors as determined from the hybridization analyses shown in Fig. 3 and 4, and *pol-EcoRI*, *Cgp70-HindIII*, and *Cp15E-HindIII* hybridization patterns (data not shown). Only proviruses which by the intensity of the bands appear to be present in all or a majority of the tumor cells are shown (see below). A solid line indicates ecotropic sequences as defined by hybridization to the ecotropic virus-specific probe shown above, whereas a dotted line indicates lack of hybridization and therefore non-ecotropic sequences. (In a few isolated cases [e.g., the provirus lacking ecotropic *pol* sequences in tumor II], lack of hybridization may be due to deletions.) The transition points are placed arbitrarily halfway between each probe. The approximate number of new proviruses in each tumor is indicated to the left of each structure.

All of the recombinant proviruses have retained the N-terminal ecotropic p15E sequences, whereas most have lost the ecotropic gp70 probe sequences. The *pol*, *Cgp70*, and *Cp15E* ecotropic probe sequences may be either present or absent in the different structures, indicating that the ecotropic or non-ecotropic nature of these sequences is not crucial. Most of these proviruses (for example, in tumors I

FIG. 6. Structure of somatically acquired proviruses in eight spontaneous AKR leukemias. The structures of new proviruses in tumors I through VIII (1 through 8 in the figure) were identified from the Southern blot analyses shown in Fig. 3 and 4 and from *pol*-*EcoRI*, *Cgp70*-*HindIII*, and *Cp15E*-*HindIII* patterns (not shown). Only those proviruses present at approximately one copy per cell (see text) are shown. Solid lines indicate ecotropic sequences, and dotted lines indicate missing ecotropic sequences. The break points between ecotropic and nonecotropic sequences are arbitrarily placed midway between each ecotropic probe shown at the top of the figure. In a few cases the dotted regions may be due to deletion of ecotropic sequences and not replacement by nonecotropic sequences (see text). The left extremity of each structure is shown as ecotropic sequence only because the 5' regions of MCF viruses are in general derived from the ecotropic parent virus. The approximate number of somatically acquired proviruses exhibiting the structures shown is noted to the left of each structure.

through IV) show two separate regions of recombination (*gp70* and *Cp15E*) that must have involved multiple recombination events. The new *pol* gene *PstI* site in the somatically acquired proviruses of tumor IV indicates that these proviruses have at least three separate nonecotropic regions.

A striking feature of the *pol*, *Cgp70*, and

Cp15E hybridization patterns (Fig. 3 and 4) is that in most cases these probes either hybridize to all or none of the new proviruses in each tumor. Because the nucleotide sequences within these regions may be either ecotropic or nonecotropic, the lack of structural heterogeneity among the new proviruses within each individual tumor suggests that the somatically acquired proviruses are each related to a common MCF virus. Tumor V is an exception in which there are two populations of recombinant proviruses in which the ecotropic *pol* sequences are either present or missing (Fig. 4 and 6).

Copy number of somatically acquired proviruses. Because the integration patterns observed in the tumor DNAs appear to be random (Fig. 3 and 4), each new fragment probably represents a single provirus integration. If one or more of these new proviruses is present once per diploid genome then the tumors probably arise from a single cell. To estimate the copy number of the somatically acquired proviruses we have scanned the films with a densitometer and compared the intensities of the germ-line bands with those of the new proviruses. Figure 7 shows the densitometer tracings of the *EcoRI*-*Np15E* hybridization patterns of tumors I and VII (from Fig. 3C, lanes 1 and 7) and the copy number of each new provirus (in tumor VII one of the new proviruses comigrates with the 11-kb germ-line provirus). All but one (the 6.3-kb fragment in tumor I) of the new proviruses in both tumors are present in equal amounts, about one copy per cell. The shorter fragments give slightly higher copy numbers; this probably reflects higher yields of these fragments because it is less likely that they will be degraded by random shearing or DNase digestion during the isolation and digestion procedures. These results indicate that the tumors are monoclonal and that the leukemia precursor cell has already acquired multiple proviruses. Nevertheless, the presence of a few proviruses at less than one copy per cell (for example, the 6.3-kb proviral *EcoRI* fragment in tumor I) suggests that proviral integrations may also occur after tumorigenesis.

DISCUSSION

In each of eight AKR thymic leukemias, we observed many somatically acquired proviruses with complex recombinant structures. All of the recombinants hybridized to an ecotropic N-terminal *p15E* (*Np15E*) hybridization probe, whereas most did not hybridize to an ecotropic *gp70* probe (*gp70*). Quint et al. (40) and Chattopadhyay et al. (6) have shown that recombinant proviruses in AKR leukemias have acquired nonecotropic restriction sites within the *gp70* sequences and retain an ecotropic *XbaI* site near the 3' terminus. The nucleotide sequence of the

FIG. 7. Densitometry tracing of the ecotropic virus-specific Np15E hybridization to *Eco*RI-digested tumor I and VII DNA (Fig. 3C, lanes 1 and 7). An unenhanced exposure was scanned by an Ortec 4310 densitometer, and the relative intensity of each band was measured by weighing the area under each peak. The sizes of the *Eco*RI fragments contained in the peaks are shown in kilobases. The weight of each peak was normalized to the 12-kb germ-line peak and doubled to indicate the number of copies per diploid genome (cell).

AKV envelope gene (17a, 23) shows that the ecotropic *Xba*I site lies within the N-terminal half of the p15E-coding sequences. Therefore, in agreement with Quint et al. (40) and Chattopadhyay et al. (6), our results indicate that a common feature of recombinant proviruses in AKR leukemias is nonectropic gp70 and ecotropic p15E *env* sequences.

Two major classes (I and II) of MCF viruses have been identified (46). Class I viruses are thymotropic, and many of these isolates accelerate leukemogenesis when inoculated into young AKR mice, whereas class II MCF viruses are nononcogenic (9, 36). Structural analyses show that both classes acquire certain nonectropic gp70 sequences, but class I viruses retain ecotropic N-terminal p15E coding sequences, whereas the class II *env* genes seem to be entirely nonectropic (7, 25). Therefore, the somatically acquired proviruses in AKR thymomas are structurally analogous to the thymotropic and oncogenic class I viruses.

The probes corresponding to the C-terminal regions of gp70 and p15E and the *pol* gene show that these sequences may be either ecotropic or nonectropic within the tumor-specific proviruses. The majority of recombinant proviruses contain two separate regions of nonectropic sequences (gp70 and Cp15E) surrounding the ecotropic Np15E sequences (Fig. 6). Probably all of these proviruses contain recombinant sequences within the 3'-terminal sequences (U3) which are duplicated in the long terminal repeat; Lung et al. (25) identified two nonectropic U3 RNase T₁ oligonucleotides within oncogenic

MCF viruses. The different structures we observe within the C-terminal region of p15E probably reflect variable recombination points between the nonectropic U3 and ecotropic N-terminal p15E sequences. Because retention of the ecotropic p15E sequences requires multiple recombinations, this further identifies the importance of the Np15E sequences in conferring a thymotropic and oncogenic phenotype.

Although the recombination patterns are highly variable, most of the tumors appear to contain multiple proviruses with identical recombinant structures. This suggests that the new proviruses are related to a single parent virus. This could be because the preleukemic cell is susceptible to infection by only a particular recombinant, or new proviruses may arise within individual cells, except that O'Donnell et al. (35) have shown that individual MCF isolates, when injected intrathymically into young AKR mice, are expressed after a few weeks on over 90% of the thymus cells. This suggests instead that the oncogenic MCF viruses arise infrequently and are subsequently amplified in the thymus.

In the gp70 probe hybridizations a few somatically acquired proviruses appear (Fig. 3A and 4B) which lack the MCF *env* *Eco*RI site. Yoshimura and Breda (56) and Chattopadhyay et al. (6) did not observe new proviruses in AKR leukemias with similar ecotropic gp70 probes. This may be because the proviruses with these ecotropic gp70 sequences are few in number and missing from some tumors.

The new proviruses which retain the ecotropic gp70 sequences in general contain nonectropic

Cp15E sequences (Fig. 6). These proviruses may be analogous to the oncogenic ecotropic spontaneous leukemia viruses isolated from AKR thymoma cell lines (34). Spontaneous leukemia viruses have AKV gp70 sequences, but contain non-AKV sequences within the C-terminal p15E and U3 regions (39). Because some of the tumors appear to lack new proviruses with the ecotropic gp70 probe sequences, the ecotropic gp70 proviruses we observe are probably not directly oncogenic. Instead, they may represent intermediates in the generation of multiply recombinant MCF viruses. Consistent with this interpretation, Thomas and Coffin (54) have shown that similar p15E recombinant MuLV appear in preleukemic AKR mice, and Famulari et al. (12) have shown that tumors induced by Gross passage A MuLV (spontaneous leukemia-type virus) always contain MuLV which have undergone further recombination within the gp70-coding sequences.

The AKR leukemias we have analyzed contain on average eight new gp70 recombinant proviruses (Fig. 6), which, in general, appear to be structurally nondefective. This contrasts with the number and structure of proviruses in avian leukosis virus-induced bursal tumors in chickens. Neel et al. (32) and Payne et al. (38) have identified between one and three new proviruses in such tumors, many of which are structurally defective; they are missing sequences from the 5' half of the proviral genome. The few aberrant proviral structures we observe may be due to deletion; these, likewise, do not seem to involve the 3' region of the genome.

The integration-promotion model for non-acute, virus-induced leukemias, suggests that the transforming virus integrates adjacent to, and abnormally promotes the expression of, a cellular gene (42). This model predicts that only certain integration events will successfully induce leukemia. Hayward et al. (16) have shown that ALV integrates next to the *myc* gene in many bursal tumors, and Neel et al. (32) have identified common integration-specific proviral restriction fragments in different tumors. The AKR tumors also display common proviral restriction fragments within different tumors; however, the large number of new proviruses prevents identification of specific integrations which may have induced these tumors. Indeed, Payne et al. (37) have shown that ALV integrations adjacent to the *myc* gene may occur in both orientations and both to the 5' or 3' end of the *myc* gene. Therefore, to determine whether MCF viruses are integrated within specific regions of the cellular genome, it will be necessary to obtain sequences flanking the proviruses to use as hybridization probes.

McGrath and Weissman (29) have proposed

that the recombinant MCF virus *env* peptides stimulate the immune system and that if a T cell that is primed to respond to the new *env* peptides is infected by this virus, it may become autostimulated and proliferate. Consistent with this, the AKR leukemias contain nondefective recombinant proviruses, and preleukemic and leukemic T cells express high levels of recombinant *env* products (13, 36). The hybridization patterns shown in Fig. 3 and 4 and the densitometer tracings in Fig. 7 indicate that many identical recombinant proviruses were already integrated into the genome of the precursor cell of these monoclonal tumors. If T cells become transformed by antigenic stimulation, either MCF virus-infected T cells are unable to respond to the recombinant viral antigen until a later stage or T cells begin proliferating after initial infection, but only one of the daughter cells, after further viral integrations, overtakes the thymus T cell population. Both of these possibilities may explain the relatively long latent period (2 months or more) in the development of thymic leukemias after initial expression of recombinant MCF viruses.

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LITERATURE CITED

1. Berns, A., and R. Jaenisch. 1976. Increase of AKR-specific sequences in tumor tissues of leukemic AKR mice. *Proc. Natl. Acad. Sci. U.S.A.* 73:2448-2452.
2. Besmer, P., and D. Baltimore. 1977. Mechanism of restriction of ecotropic and xenotropic murine leukemia viruses and formation of pseudotypes between the two viruses. *J. Virol.* 21:965-973.
3. Blin, N., and D. W. Stafford. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res.* 3:2303-2308.
4. Buckler, C. E., S. P. Staal, W. P. Rowe, and M. A. Martin. 1982. Variation in the number of copies and in the genomic organization of ecotropic murine leukemia virus proviral sequences in sublines of AKR mice. *J. Virol.* 43:629-640.
5. Canaan, E., and S. A. Aaronson. 1979. Restriction enzyme analysis of mouse cellular type C viral DNA: emergence of new viral sequences in spontaneous AKR/J lymphomas. *Proc. Natl. Acad. Sci. U.S.A.* 76:1677-1681.
6. Chattopadhyay, S. K., M. W. Cloyd, D. L. Linemeyer, M. R. Lander, E. Rands, and D. R. Lowy. 1982. Cellular origin and role of mink cell focus-forming viruses in murine thymic lymphomas. *Nature (London)* 295:25-31.
7. Chattopadhyay, S. K., M. R. Lander, S. Gupta, E. Rands, and D. R. Lowy. 1981. Origin of mink cytopathic focus-forming (MCF) viruses: comparison with ecotropic and xenotropic murine leukemia virus genomes. *Virology* 113:465-483.
8. Chien, Y., I. M. Verma, T. Y. Shih, E. M. Scolnick, and N. Davidson. 1978. Heteroduplex analysis of the sequence relations between the RNAs of mink cell focus-inducing and murine leukemia viruses. *J. Virol.* 28:352-360.
9. Cloyd, M. W., J. W. Hartley, and W. P. Rowe. 1980. Lymphomagenicity of recombinant mink cell focus-induc-

- ing murine leukemia viruses. *J. Exp. Med.* 151:542-552.
10. Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-646.
 11. Elder, J. H., J. W. Gautsch, F. C. Jensen, R. A. Lerner, J. W. Hartley, and W. P. Rowe. 1977. Biochemical evidence that MCF murine leukemia viruses are envelope (*env*) gene recombinants. *Proc. Natl. Acad. Sci. U.S.A.* 74:4676-4680.
 12. Famulari, N. G., C. F. Koehne, and P. V. O'Donnell. 1982. Leukemogenesis by Gross passage A murine leukemia virus: expression of viruses with recombinant *env* genes in transformed cells. *Proc. Natl. Acad. Sci. U.S.A.* 79:3872-3876.
 13. Famulari, N. G., J.-S. Tung, P. V. O'Donnell, and E. Fleissner. 1980. Murine leukemia virus *env*-gene expression in preleukemic thymocytes and leukemia cells of AKR strain mice. *Cold Spring Harbor Symp. Quant. Biol.* 44:1281-1287.
 14. Grunstein, M., and D. S. Hogness. 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. U.S.A.* 72:3961-3965.
 15. Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe. 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. *Proc. Natl. Acad. Sci. U.S.A.* 74:789-792.
 16. Hayward, W. S., B. G. Neel, and S. M. Astrin. 1981. Activation of a cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukosis. *Nature (London)* 290:475-480.
 17. Helling, R. B., H. M. Goodman, and H. W. Boyer. 1974. Analysis of endonuclease R Eco RI fragments of DNA from lambdaoid bacteriophages and other viruses by agarose-gel electrophoresis. *J. Virol.* 14:1235-1244.
 - 17a. Herr, W., V. Corbin, and W. Gilbert. 1982. Nucleotide sequence of the 3' half of AKV. *Nucleic Acids Res.* 10:6931-6944.
 18. Herr, W., and W. Gilbert. 1982. Germ-line MuLV reintegrations in AKR/J mice. *Nature (London)* 296:865-868.
 - 18a. Herr, W., O. Schwartz, and W. Gilbert. 1983. Isolation and mapping of cDNA hybridization probes specific for ecotropic and nonectropic murine leukemia proviruses. *Virology* 125:139-154.
 19. Holmes, D. S., and M. Quigley. 1981. A rapid method for the preparation of bacterial plasmids. *Anal. Biochem.* 114:193-197.
 20. Ishimoto, A., J. W. Hartley, and W. P. Rowe. 1977. Detection and quantitation of phenotypically mixed viruses: mixing of ecotropic and xenotropic murine leukemia viruses. *Virology* 81:263-269.
 21. Kaplan, H. S. 1967. On the natural history of murine leukemias: presidential address. *Cancer Res.* 27:1325-1340.
 22. Laskey, R. A., and A. D. Mills. 1977. Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized film. *FEBS Lett.* 82:314-316.
 23. Lenz, J., R. Crowther, A. Straceski, and W. Haseltine. 1982. Nucleotide sequence of the AKV *env* gene. *J. Virol.* 42:519-529.
 24. Levy, J. A. 1973. Xenotropic viruses: murine leukemia viruses associated with NIH/3T3, NZB, and other mouse strains. *Science* 182:1151-1153.
 25. Lung, M. L., C. Hering, J. W. Hartley, W. P. Rowe, and N. Hopkins. 1980. Analysis of the genomes of mink cell focus-inducing murine type C viruses: a progress report. *Cold Spring Harbor Symp. Quant. Biol.* 44:1269-1274.
 26. Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and A. Efstradiatis. 1978. The isolation of structural genes from libraries of eucaryotic DNA. *Cell* 15:687-701.
 27. Maxam, A. M., and W. Gilbert. 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. U.S.A.* 74:560-564.
 28. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499-560.
 29. McGrath, M. S., and I. L. Weissman. 1978. A receptor mediated model of viral leukemogenesis: hypothesis and experiments, p. 577-589. *In* B. Clarkson, P. A. Marks, and J. E. Till (ed.), *Differentiation of normal and neoplastic hematopoietic cells*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 30. Miller, J. H. 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 31. Moore, J. L., and H. W. Chan. 1982. Identification of ecotropic proviral sequences in high- and low-ecotropic-virus-producing mouse strains. *J. Virol.* 43:1038-1045.
 32. Neel, B. G., W. S. Hayward, H. L. Robinson, J. Fang, and S. M. Astrin. 1981. Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. *Cell* 23:323-334.
 33. Nishizuka, Y., and K. Nakakuki. 1968. Acceleration of leukemogenesis in AKR mice by grafts, cell suspensions, and cell free centrifugates of thymuses from preleukemic AKR donors. *Int. J. Cancer* 3:203.
 34. Nowinski, R. C., and E. F. Hays. 1978. Oncogenicity of AKR endogenous leukemia viruses. *J. Virol.* 27:13-18.
 35. O'Donnell, P. V., R. C. Nowinski, and E. Stockert. 1982. Amplified expression of murine leukemia virus (MuLV)-coded antigens on thymocytes and leukemia cells of AKR mice after infection by dualtropic (MCF) MuLV. *Virology* 119:450-464.
 36. O'Donnell, P. V., E. Stockert, Y. Obata, and L. J. Old. 1981. Leukemogenic properties of AKR dualtropic (MCF) viruses: amplification of murine leukemia virus-related antigens on thymocytes and acceleration of leukemia development in AKR mice. *Virology* 112:548-563.
 37. Payne, G. S., J. M. Bishop, and H. E. Varmus. 1982. Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. *Nature (London)* 295:209-214.
 38. Payne, G. S., S. A. Courtridge, L. B. Crittenden, A. M. Fadly, J. M. Bishop, and H. E. Varmus. 1981. Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of the tumor state. *Cell* 23:311-322.
 39. Pedersen, F. S., R. L. Crowther, D. Y. Tenney, A. M. Reimold, and W. A. Haseltine. 1981. Novel leukemogenic retroviruses isolated from cell line derived from spontaneous AKR tumour. *Nature (London)* 292:167-170.
 40. Quint, W., W. Quax, H. van der 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.
 41. Rigby, P. W. J., M. Diekmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237-251.
 42. Robinson, H. L., M. N. Pearson, D. W. DeSimone, P. N. Tschlis, and J. M. Coffin. 1980. Subgroup-E avian-leukosis-virus-associated disease in chickens. *Cold Spring Harbor Symp. Quant. Biol.* 44:1133-1142.
 43. Rommelaere, J., D. V. Fallar, and N. Hopkins. 1978. Characterization and mapping of RNase T₁-resistant oligonucleotides derived from the genomes of AKV and MCF murine leukemia viruses. *Proc. Natl. Acad. Sci. U.S.A.* 75:495-499.
 44. Rowe, W. P. 1972. Studies of genetic transmission of murine leukemia virus by AKR mice. *J. Exp. Med.* 136:1272-1285.
 45. Rowe, W. P. 1978. Leukemia virus genomes in the chromosomal DNA of the mouse. *Harvey Lect. Ser.* 71:173-192.
 46. Rowe, W. P., M. W. Cloyd, and J. W. Hartley. 1980. Status of the association of mink cell focus-inducing viruses with leukemogenesis. *Cold Spring Harbor Symp.*

- Quant. Biol. 44:1265-1268.
47. Rowe, W. P., and T. Pincus. 1972. Quantitative studies of naturally occurring murine leukemia virus infection of AKR mice. *J. Exp. Med.* 135:429-436.
 48. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
 49. 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.
 50. Steffen, D. L., S. Bird, and R. A. Weinberg. 1980. Evidence for the asiatic origin of endogenous AKR-type murine leukemia proviruses. *J. Virol.* 35:824-835.
 51. Steffen, D. L., B. A. Taylor, and R. A. Weinberg. 1982. Continuing germ line integration of AKV proviruses during the breeding of AKR mice and derivative recombinant inbred strains. *J. Virol.* 42:165-175.
 52. Sutcliffe, J. G. 1978. pBR322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. *Nucleic Acids Res.* 5:2721-2728.
 53. Swanstrom, R., and P. R. Shank. 1977. X-ray intensifying screens greatly enhance the detection by autoradiography of the radioactive isotopes ^{32}P and ^{125}I . *Anal. Biochem.* 86:184-192.
 54. Thomas, C. Y., and J. M. Coffin. 1982. Genetic alterations of RNA leukemia viruses associated with the development of spontaneous thymic leukemia in AKR/J mice. *J. Virol.* 43:416-426.
 55. Wahl, G. M., M. Stern, and G. R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulfate. *Proc. Natl. Acad. Sci. U.S.A.* 76:3683-3687.
 56. Yoshimura, F. K., and M. Breda. 1981. Lack of AKR ecotropic provirus amplification in AKR leukemic thymuses. *J. Virol.* 39:808-815.