JOURNAL OF VIROLOGY, May 1984, p. 432–438
0022-538X/84/050432-07$02.00/0

Vol. 50, No. 2

Generation of AKR Mink Cell Focus-Forming Viruses: a Conserved Single-Copy Xenotrope-Like Provirus Provides Recombinant Long Terminal Repeat Sequences

WIM QUINT, WILBERT BOELENS, PETER VAN WEZENBEEK, THEO CUYPERS, ELS ROBANUS MAANDAG, GERARD SELTEN, AND ANTON BERNS*

Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands

Received 31 October 1983/Accepted 27 January 1984

AKV and AKR mink cell focus-forming virus-specific probes from the envelope and long terminal repeat (LTR) regions were prepared for study of the structure of recombinant proviruses in tumor tissues of AKR mice. The results showed that (i) all somatically acquired proviruses possessed, besides a recombinant gp70 gene, an altered U3 LTR; (ii) in a substantial portion of the somatically acquired AKR mink cell focus-forming proviruses, the LTR comprised sequences derived from the same xenotropic-like provirus; (iii) this U3 LTR donating parental provirus (Xeno-dL) was present only once per genome equivalent in several mouse strains; (iv) in the strains containing the Xeno-dL provirus, the provirus was present in the same chromosomal site; (v) restriction analysis of the Xeno-dL revealed that the mink cell focus-forming gp70 sequences were derived from a parental provirus, different from Xeno-dL. Therefore, at least two non-ecotropic parents participate in the generation of leukemogenic AKR mink cell focus-forming viruses: a xenotropic-like virus, Xeno-dL, donating U3 LTR sequences, and another xenotropic-like virus or viruses providing gp70 sequences.

Inbred laboratory mice carry various types of murine leukemia viruses (MuLVs) as part of their genome complement (9, 21, 22, 32). These viruses can be classified according to their host ranges. The ecotropic MuLVs can infect and replicate only in mouse cells, and xenotropic MuLVs only propagate in cells of other species (21, 33). Endogenous ecotropic viruses are found both in high-leukemic strains, such as AKR, C58, and C3H/Fg, and in low-leukemic strains, such as BALB/c, DBA, and C3H (4, 18, 30, 31, 37). The various sublines of the AKR strain each harbor, besides the Akv1 locus common to all AKR strains, several AKV genomes in unique chromosomal locations (4, 31, 37). The expression of these ecotropic endogenous viruses shows a causal relationship with the development of thymic leukemias later in life (39). Several stages can be discerned in the life-span of these high-leukemic strains. From shortly after birth, these animals express high titers of the ecotropic virus (33). Before the onset of leukemia, a marked amplification of MuLV antigen expression is observed in the thymus (19). This increased antigen production coincides with the expression of endogenous xenotropic-like sequences (19). From this time on, mink cell focus-forming (MCF) viruses can often be detected in thymuses of these animals (14). Unlike AKV viruses and the class II MCF viruses (24), most of the class I MCF viruses do accelerate leukemia when injected into newborn AKR mice (10, 28). It has been suggested that these MCF viruses are generated by multiple recombination events in which at least three endogenous viruses are involved (11, 38). Comparison of the genomic structure of AKV virus with the leukemogenic MCF viruses shows that the differences are found predominantly in the 3′ half of the genome: within the 3′ portion of the p15E gene, the U3 region of the long terminal repeat (LTR), and the 5′ region of the gp70 gene (7, 16, 20, 24). In this report, we describe a more detailed analysis of the structure of AKR MCF proviruses integrated in leukemic tissues. We provide evidence that a significant portion of the leukemogenic AKR MCF viruses are generated by at least two recombinational events: (i) acquisition of heterologous U3 sequences derived from a unique, single-copy xenotropic-like parent, and (ii) acquisition of xenotropic-like gp70 sequences from a parent, differing from the U3 donor.

(This work was performed as part of the Ph.D. study of Wim Quint at the University of Nijmegen, Nijmegen, The Netherlands.)

* Corresponding author.

MATERIALS AND METHODS

Viruses and preparation of cDNA. AKR virus and Moloney MuLV (Mo-MuLV) (clone 1A) were propagated and isolated as described previously (40). Alpha-^{32}P labeled AKR and Mo-MuLV cDNA was prepared by the endogenous polymerase reaction, and the sequences that cross-reacted with heterologous endogenous viruses were removed as described previously (1, 30).

Mice. AKR/JS, AKR/J, AKR/FuRdA, BALB/c, DBA, C57BL/10ScSn, and 129 mice were obtained as described previously (31).

DNA analysis. Isolation of DNA, restriction endonuclease digestions, gel electrophoresis, electroelution, transfer to nitrocellulose, and hybridizations were carried out as described earlier (30, 40).

Molecular cloning of AKV probes. A *Pvu*II-*Eco*RI fragment from clone AKR-623 (courtesy of D. R. Lowy; see Fig. 1) was isolated from an agarose gel by electroelution and digested with restriction endonuclease *Sau*3A. The resulting fragments were ligated into the *Bam*HI site of pBR322. From the Amp^r Tet^s colonies that reacted with AKR, MuLV, or AKV cDNA but not with Mo-MuLV cDNA, plasmid DNA was isolated. These DNAs were nick translated as described previously (39) and tested for their specificity by blot hybridization to 129, BALB/c, and AKR mouse DNA.

The inserts of these plasmids were localized on the genomic map by hybridization to Southern blots, which

contained a number of restriction digests of the recombinant phage AKR-623 (23).

Molecular cloning of AKR MCF provirus from tumor DNA. High-molecular-weight lymphoma DNA (250 μg) from AKR/JS mice was digested to completion with *Eco*RI. After gel electrophoresis in a 0.6% agarose gel, DNA was electroeluted from 2-mm gel slices (30), and the fractions, hybridizing with the AKV-specific cDNA probe, were used for cloning. Phage cloning was performed as described previously (2), except that Charon 28 was used as vector. The identity of plaques hybridizing with a total AKR cDNA probe was determined by restriction enzyme analysis and blot hybridization of mini-phage isolates. The insert of the AM35 recombinant phage selected by this procedure was subcloned in pBR322.

Cloning and labeling of MCF probes. Probes 1 and 2 (see Fig. 4) were obtained by *Bal*31 exonuclease digestion and cloning as described by Poncz et al. (29). The fragments were inserted into M13mp8 and M13mp9 (26). All probes were labeled by the primer extension reaction (34). For probe 4, a dideoxy nucleotide was included in the reaction.

Transfection. The pAM35 insert was ligated to a 15-kilobase pair (kbp) *Eco*RI fragment, which comprised the 5′ end of the Moloney MCF genome. Transfection of this DNA on mink lung fibroblasts (CCL-64) was carried out as described previously (3, 13).

RESULTS

Construction and selection of hybridization probes specific for ecotropic MuLVs. The recombinant phage AKR 623 (courtesy of D. R. Lowy, National Institutes of Health), containing an infectious AKV provirus, was used as the source for preparing ecotropic-specific hybridization probes. Various regions of the AKV genome were subcloned in pBR322 and tested for their specificity by hybridization with several mouse DNAs.

A *Pvu*II-*Eco*RI fragment mapping between coordinates 5.8 and 9.8 and containing part of the polymerase gene, the total envelope gene, and the LTR region was digested with *Sau*3A, and the resulting fragments were subcloned in the *Bam*HI site of pBR322 (Fig. 1A). (Here, and in the remainder of the paper, viral DNA sites are referred to by their distance from the 5′ end of the viral DNA.) Colonies were screened by filter hybridization, using both AKV and Mo-MuLV cDNAs as probe (30, 39). Recombinant clones that reacted with the AKV cDNA but not with the Moloney cDNA were further tested for their ecotropic specificity on Southern blots (35) containing *Eco*RI-digested DNA from different mouse strains: the 129 strain, which does not contain an endogenous ecotropic provirus, the BALB/c strain, which carries one copy of the AKV virus in its genome, and the AKR/JS, harboring four endogenous AKV genomes (31). This analysis revealed that two clones and three probes from the envelope and LTR region were ecotropic specific under the conditions used.

The positions of these probes on the genomic map are summarized in Fig. 1B. Cgp70/Np15E is located between map positions 7.2 and 7.8, corresponding to the C-terminal region of gp 70 and the N-terminal region of p15E (15). This probe overlaps the *Xba*I site. The MCp15E/U3 LTR clone, which is juxtaposed to the Cgp70/Np15E probe, extends from 7.8 to 8.5 on the genomic map, corresponding with the middle and C-terminal regions of p15E and the U3 region of the LTR (see Fig. 1B). *Pst*I digestion of the MCp15E/U3 LTR plasmid generated two fragments of 1.5 and 3.5 kbp. The 3.5-kbp fragment contained, besides pBR322 sequences, 250 base pairs of DNA corresponding to a region in the U3 LTR directly to the right of the *Pst*I site. The 1.5-kbp fragment harbored the remaining 450 nucleotides corresponding to the middle and C-terminal regions of p15E (MCp15E) and the first 30 nucleotides of the U3 region up to the *Pst*I site. Hybridization of the MCp15E with a Southern blot containing *Hin*dIII-digested DNA from BALB/c and the AKR/J strain (with one and three Akv copies, respectively) revealed one and three hybridizing fragments corresponding to the 3′ regions of the viral genomes (Fig. 2A). Since *Hin*dIII cleaves the AKV genome once (36), hybridization with the U3 LTR probe should give rise to two and six hybridizing fragments, corresponding to both 3′ and 5′ portions of the proviral genomes (Fig. 2B). The Cgp70/Np15E probe showed similar specificity for the ecotropic proviral genomes (data not shown).

As has been shown by others for the gp70 and p15E regions (8, 16), our results indicate that an ecotropic-specific U3 LTR probe can be prepared that allows the recognition of both 3′ and 5′ portions of integrated MuLV genomes.

Construction of hybridization probes for MCF proviruses. We have molecularly cloned the 3′ half of an AKR MCF provirus present in the chromosomal DNA of an AKR/JS lymphoma: the tumor DNA was digested with *Eco*RI, and the fragments reacting with an AKV-specific cDNA probe were characterized (30). Figure 3 shows the results of a blot hybridization. Lane 1 represents liver DNA, and lane 2 represents tumor DNA. Within the tumor DNA, two additional fragments of 3.5 and 20 kbp were detected. Since AKR MCFs have an *Eco*RI recognition site at position 6.9 on the genomic map, the 3.5-kbp fragment is most likely derived from the 3′ region of an AKR MCF provirus. The 3.5-kbp fragment was molecularly cloned in phage lambda (see above). Characterization of this clone with various restriction endonucleases revealed the typical structure seen in most AKR MCF viruses (see Fig. 4): the ecotropic-specific *Xba*I site at position 7.8 (6, 7), the *Sst*I site at coordinate 7.6, which is present in many MCF recombinants but absent from the ecotropic viral genome, and an *Eco*RI site at position 6.9, which is found in all MCF viruses isolated so far (7, 39). The biological integrity of this clone was further shown by ligation of this 3′ MCF clone to the 5′ part of a Moloney MCF provirus, which comprised Moloney MCF sequences up to the *Eco*RI site at position 6.9. Transfection of this DNA into mink cells resulted in the production of recombinant viruses with the expected genomic structure (data not shown).

Figure 4B shows the location of the fragments which were subcloned in phage M13 and used as hybridization probes. *Eco*RI-digested liver DNA from the AKR/JS mouse strain was hybridized with the gp70 MCF probes 1 and 2 and the p15E MCF probe 3. These probes reacted with approximately 20 different *Eco*RI DNA fragments (data not shown). Fragments of similar molecular weights were recognized by both the gp70 and p15E probes. No hybridization was observed with the AKV proviruses, suggesting that these hybridization probes most likely annealed to the numerous xenotropic proviral sequences, which are detectable in many mouse strains (5, 17, 19).

However, the LTR MCF probe 4, which was derived from the U3 region of our AKR MCF clone, reacted with only two *Eco*RI fragments of 6.7 and 9.1 kbp in both the BALB/c and AKR/JS mouse strains (Fig. 5, lanes 1 and 2). Hybridization of this probe with *Hin*dIII-digested liver DNA from the AKR/JS strain showed exclusive annealing with a single restriction fragment of 22 kbp (Fig. 5, lane 3). These results

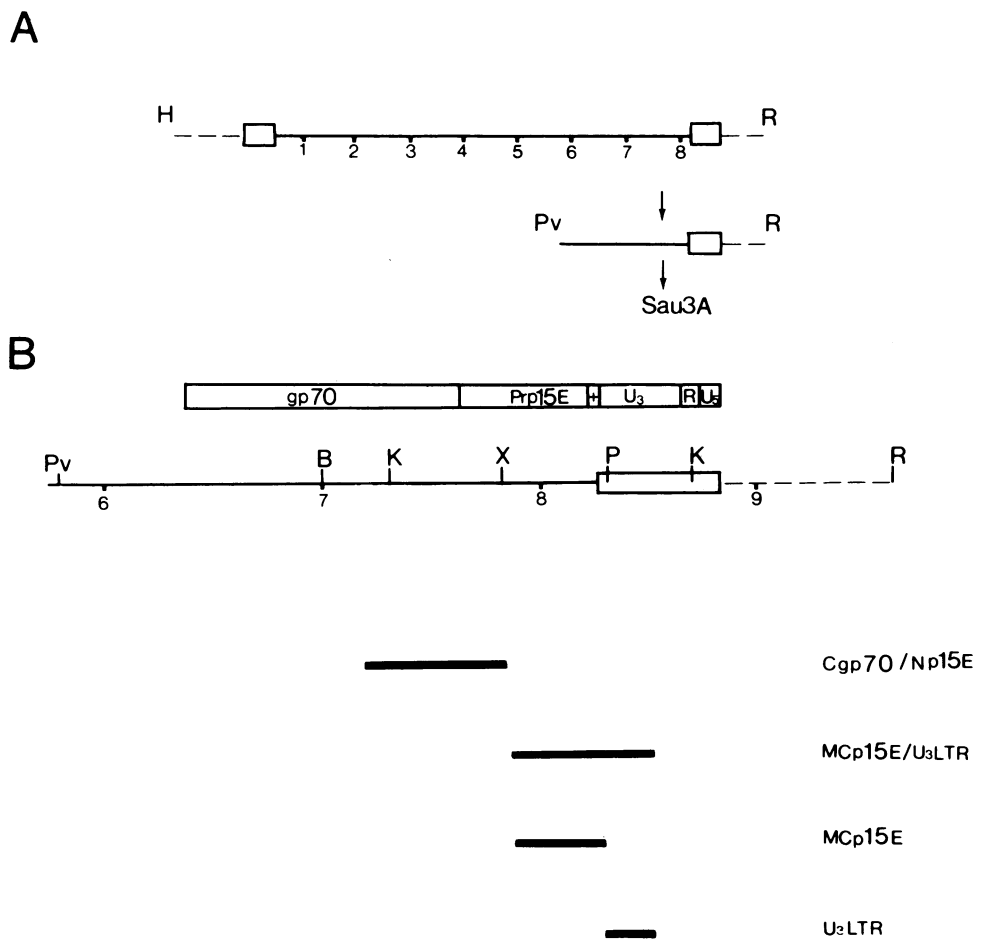

FIG. 1. Structure of AKV provirus and location of the AKV-specific probes. (A) Structure of phage AKR-623 (23), and position of the *Pvu*II-*Eco*RI fragment used for *Sau*3A digestion and cloning. The dashed line represents the flanking cellular sequence. (B) The upper box gives the location of the envelope gene and the LTR region. The positions of some restriction sites are indicated (in kbp from the 5′ end of the viral genome). The positions of the AKV subclones are shown. Abbreviations: Pv, *Pvu*II; B, *Bam*HI; K, *Kpn*I; X, *Xba*I; P, *Pst*I; R, *Eco*RI.

suggest that the MCF LTR probe recognizes a proviral genome that is present only once in the genome of BALB/c and AKR mice. We have designated this virus Xeno-dL (donating LTR xenotropic virus).

Characterization of the Xeno-dL proviral genome. Southern blots containing AKR/JS DNA cleaved with a variety of restriction endonucleases were hybridized with the MCF LTR probe (data not shown), and a physical map of the proviral locus was constructed. Figure 6A shows the restriction map of Xeno-dL with adjacent cellular sequences. Comparison of this structure with the physical maps of known xenotropic MuLVs (7) showed a close relationship with the AKR-6 and BALB/2 xenotropic viruses. Some restriction sites, which were shown to be present within the genomes of AKR-6 and BALB/2 xenotropic virus (7), could not be determined in the Xeno-dL genome with the MCF LTR probe (Fig. 6B). Because of the close resemblance with these known xenotropic viruses, it is justifiable to conclude that the sequences present in the MCF LTR probe are derived from a provirus that is similar or identical to the AKR-6 or BALB/2 xenotropic virus.

To determine whether the same endogenous virus was present in other laboratory mouse strains as well, liver DNA from various strains was analyzed by Southern blot analysis. Mice of the AKR, BALB/c, DBA, and C57BL/10 strains all contained a single proviral copy of Xeno-dL, present in the same chromosomal site, as indicated by hybridization to identically sized fragments. The 129 mouse strain lacked Xeno-dL sequences. The presence of the same proviral structure in the same chromosomal location in different strains indicates that the Xeno-dL proviral genome was present in these strains before they diverged at least 80 years ago (27).

Structure of somatically acquired MCF proviruses. It has been shown previously that leukemogenesis in AKR mice is accompanied by amplification of AKV sequences in DNA of tumor tissues (1, 9, 16, 30). The structure of these somatically acquired copies closely resembled the structure of MCF proviruses. Unlike the ecotropic MuLV, they all contained an *Eco*RI site at map position 6.9 and a *Bam*HI site at map position 6.2. We have analyzed these recombinant proviruses with the different probes described above. DNA was obtained from lymphomas of the AKR/FuRdA strain, which contains six endogenous AKV genomes (30).

The ecotropic MCp15E probe shows the presence of these six endogenous proviruses in normal liver DNA upon digestion with *Eco*RI (Fig. 7A, samples 5 and 22). Within tumor DNA from the AKR/FuRdA strain, many additional fragments were recognized, representing 3′ portions of recombinant proviruses (Fig. 7A, samples 3, 4, 7, 8, 10, and 23). The

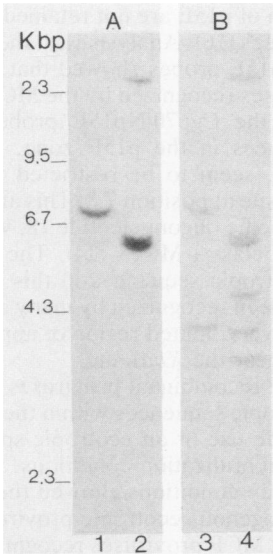

FIG. 2. Characterization of the AKV-specific MCp15E and LTR probe. *Hin*dIII-digested liver DNAs from BALB/c (lanes 1 and 3) and AKR/J (lanes 2 and 4) mice, hybridized with the MCp15E probe (A) and the U3 LTR probe (B). *Hin*dIII cleaves the endogenous AKV proviral genome once. Phage lambda, digested with *Hin*dIII, was used as molecular weight marker.

ecotropic MCp15E probe was able to recognize considerably more MCF-like proviruses than was the ecotropic Cgp70/Np15E probe, suggesting a preference for ecotropic sequences in the MCp15E region of the MCF genome. This observation is in good agreement with the previously described structural heterogeneity among MCF viruses (16).

Analysis of the same DNA samples was also performed with the ecotropic-specific LTR probe under hybridization conditions of high and low stringency. Under low-stringency conditions, tumors 4, 8, and 23 revealed the presence of somatically acquired proviruses with this probe. In tumors 3, 7, and 10, no additional proviruses were seen (Fig. 7B), although they were detected by the MCp15E probe. When the stringency of hybridization was raised, the additional fragments in tumors 4, 8, and 23 were no longer seen, although the AKV endogenous sequences hybridized normally (see Fig. 7C). The reduced efficiency of hybridization of these MCF proviruses with the ecotropic LTR probe is best explained by the presence of only part of the AKV U3 LTR sequences in the U3 regions of these MCF proviruses. The results suggest that all integrated MCF proviruses in tumor tissues have acquired non-ecotropic U3 sequences.

To obtain more information concerning the origin of the LTR sequences in MCF proviruses, we analyzed the same DNAs with the MCF LTR probe under high-stringency hybridization conditions. Tumors 3, 7, and 10 exhibit, besides the two endogenous *Eco*RI fragments of 6.8 and 9.1 kbp, a number of additional fragments (Fig. 7D). Some of these fragments also hybridized with the ecotropic MCp15E probe (compare Fig. 7A) and therefore constitute the 3′ end of recombinant genomes. The fragments detectable with the MCF LTR probe, but not those detectable with MCp15E, most likely represent 5′ ends of MCF proviruses. The somatically acquired proviral genomes within tumors 4 and 8 that reacted with the ecotropic LTR probe under low-stringency conditions were not recognized by the MCF LTR probe.

The structure of the proviral MCFs detected in tumor DNA is reminiscent of the structure of the class I MCF viruses (24, 25). The gp70 region, like the MCp15E and U3 regions of this class I virus, is not of ecotropic origin. The changes in the gp70 region of the somatically acquired recombinants include the acquisition of a characteristic *Bam*HI site at map position 6.2 (30, 39). However, the Xeno-dL provirus involved in the generation of the MCF proviruses in tumors 3, 7, and 10 does not contain a *Bam*HI site at this position (data not shown). Similarly, a *Kpn*I site present in the Xeno-dL provirus at map coordinate 7.3 is frequently

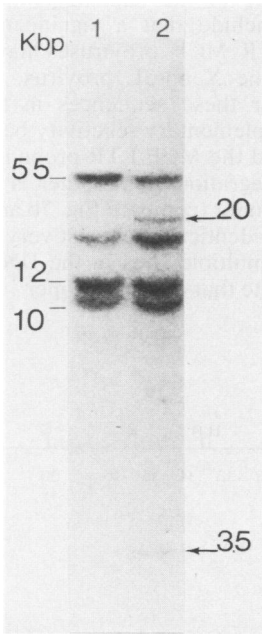

FIG. 3. Detection of somatically acquired AKV sequences within an AKR/JS tumor. *Eco*RI-digested tumor DNA (lane 2) and normal liver DNA (lane 1) were hybridized with an AKV-specific cDNA probe (30). The molecular weights of the fragments carrying the additional AKV sequences are indicated at right.

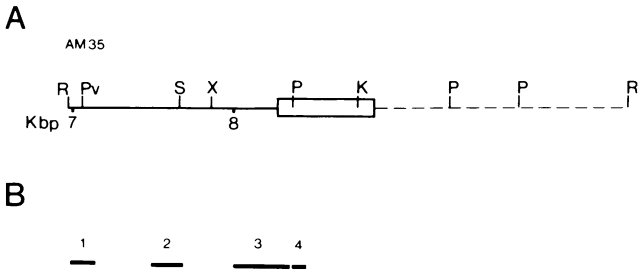

FIG. 4. Structure of the 3′ and of the AKR MCF AM35 clone and locations of the MCF probes. (A) Physical map of the 3.5-kbp insert of recombinant AM35. Flanking mouse sequences are indicated by the dashed line. The open box represents the LTR. The viral part of the provirus is indicated by the solid line. Abbreviations: R, *Eco*RI; Pv, *Pvu*II; S, *Sst*I; X, *Xba*I, P, *Pst*I; K, *Kpn*I. (B) Positions of the MCF probes on the genomic map of AKR MCF. The fragments were subcloned in M13. Numbers 1 and 2 represent gp70 probes: probe 1 corresponds to positions 6.9 to 7.05 on the genomic map of AM35, and probe 2 is located between coordinates 7.4 and 7.6. The p15E probe (number 3) comprises a *Pst*I-*Taq*I fragment of 313 nucleotides, positioned between 8.0 and 8.3. The MCF LTR probe (number 4) corresponds to a *Pst*I-*Taq*I fragment between map positions 8.30 and 8.45.

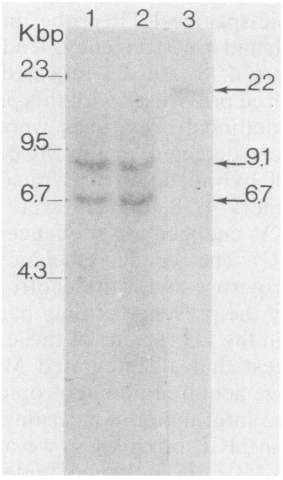

FIG. 5. Characterization of the AKR MCF LTR probe. *Eco*RI (lanes 1 and 2) and *Hin*dIII (lane 3)-digested DNAs were blot hybridized with the MCF LTR probe. Lane 1, BALB/c liver DNA; lanes 2 and 3, AKR/JS liver DNA. The molecular weights of the hybridizing fragments are indicated at right. Molecular weight markers are shown at left.

absent from MCF proviruses, which have acquired an *Sst*I site at position 7.6 (compare Fig. 4 and 6) (7, 39).

Therefore, the generation of the MCF viruses involves recombinant events in which often at least two non-ecotropic parents participate: one xenotropic-like virus, Xeno-dL, is donating U3, and possibly p15E, to many of the somatically acquired MCF proviruses, whereas (an)other xenotropic-like parent(s) provides the gp70 sequences.

DISCUSSION

Previously we have provided evidence, using a specific cDNA probe, for the presence of many somatically acquired proviruses in AKR lymphomas (30). Nearly all of these proviruses exhibited MCF structures with non-ecotropic restriction sites within the gp70 gene and ecotropic-specific restriction sites near the 3′ terminus (16, 30, 39). Here we present a more detailed analysis of these proviruses with ecotropic- and MCF-specific probes, derived from the gp70, p15E, and LTR regions. In agreement with the data of others (16), our results show the presence of two separated regions (gp70 and LTR) with non-ecotropic sequences within AKR MCF proviruses found in tumor tissues. In earlier studies, it has been shown that the ecotropic sequences comprising the C-terminal region of p15E are not retained in the majority of the MCF proviruses (16). Analysis with the ecotropic Cgp70/Np15E and MCp15E probes showed that only a fraction of the MCF proviruses recognized by the MCp15E probe could be detected by the Cgp70/Np15E probe. Therefore, the ecotropic sequences in the p15E zone, common to most MCF proviruses, seem to be restricted to a small region around the *Xba*I site at position 7.8. This area also comprises the ecotropic-specific oligonucleotide 18, which is present in all leukemogenic class I MCFs (24). The data suggest that the need for ecotropic sequences in this region, a requirement which has been recognized by many authors (6, 16, 24), is restricted to a very limited region of approximately 100 to 200 nucleotides near the *Xba*I site.

All of the MCF recombinant proviruses we have analyzed gained non-ecotropic sequences within the LTR region. This was shown by the use of an ecotropic-specific LTR probe under stringent hybridization conditions. Hybridization under high-stringency conditions allowed the exclusive recognition of the endogenous ecotropic proviruses (see Fig. 7c), indicating that all MCF proviruses recognized by the ecotropic MCp15E probe carried an altered LTR. The recombinant proviruses that could still hybridize with the ecotropic LTR probe under low-stringency conditions (Fig. 7b) did not hybridize with the MCF LTR probe, whereas the MCF proviruses that did react with the specific MCF LTR probe did not hybridize with the ecotropic-specific LTR probe (compare Fig. 7b and d). This complementary reactivity is most likely due to the different recombination positions in the MCF LTRs. Depending on the amount of ecotropic sequences left in the U3 LTR of the MCF genome, the provirus can be recognized by the ecotropic-specific LTR probe or by the MCF-specific LTR probe. In those cases in which the MCF proviruses are not recognized by the MCF-specific LTR probe, the origin of non-ecotropic sequences is still uncertain. However, from our hybridization analyses and sequence comparisons of the MCF LTR probe with the published sequence of the leukemogenic AKR MCF 247 (20), we can conclude that a significant portion of the leukemogenic AKR MCF proviruses have acquired LTR sequences from the Xeno-dL provirus. This suggests an important role for these sequences in the leukemogenic process. The complementary reactivity between the ecotropic LTR probe and the MCF LTR probe is not restricted to single proviral integrations, but rather is seen in all proviruses within one tumor (compare Fig. 7b and d). Most likely, MCF viruses with identical or at least very similar structures are integrated in multiple sites in the DNA of target cells. One could speculate that once a "proper" MCF is generated

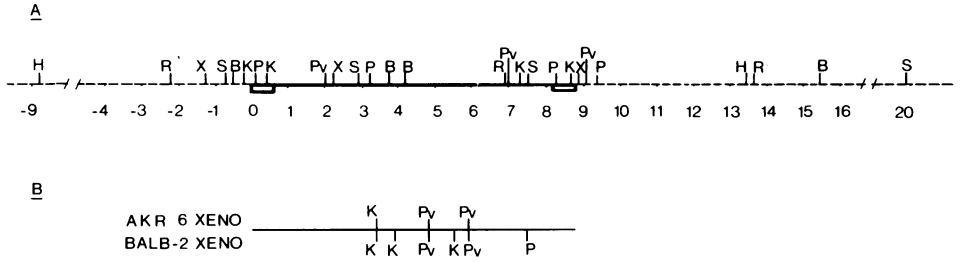

FIG. 6. Physical map of the Xeno-dL proviral genome with flanking cellular sequences. The restriction endonuclease map (A) was constructed by cleaving liver DNA from an AKR/JS mouse with various restriction endonucleases, followed by blot hybridization to the ^{32}P-labeled MCF LTR probe. This map was indistinguishable from the map of AKR-6 and BALB/2 xenotropic virus (7), except that the positions of the restriction sites shown in (B) could not be determined by using the MCF LTR probe. Restriction endonucleases are abbreviated as described in the legends to Fig. 1 and 4. H, *Hin*dIII.

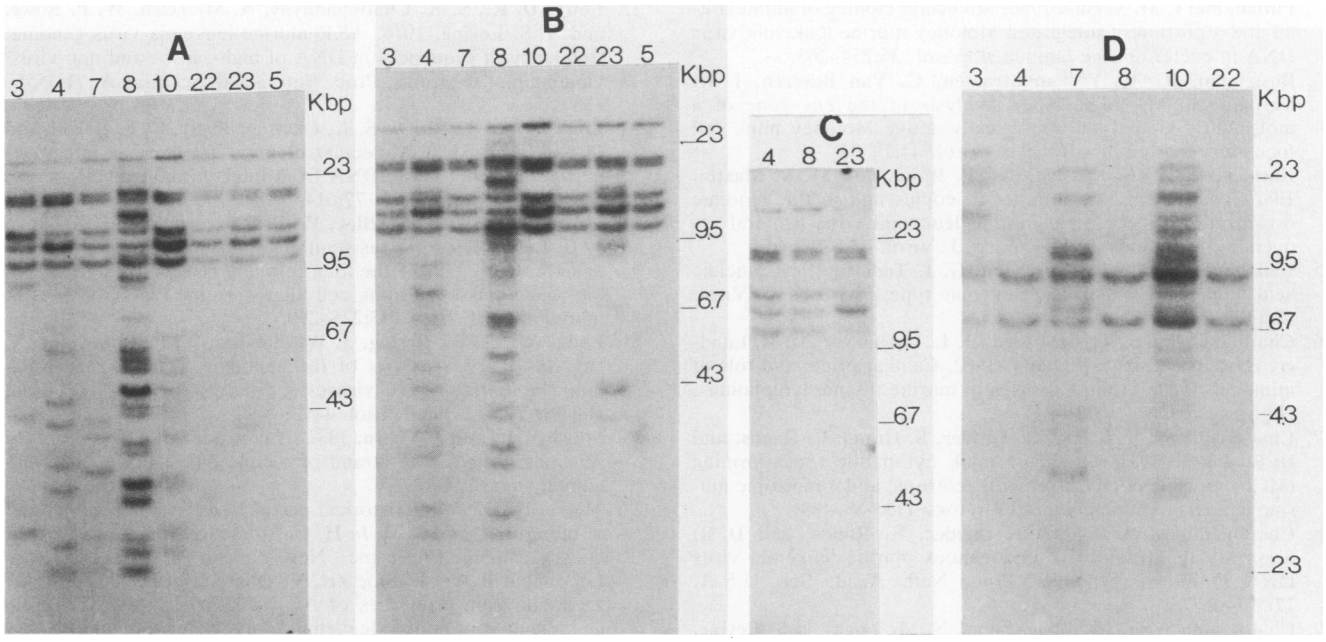

FIG. 7. Detection of somatically acquired proviruses by the AKV and MCF-specific probes. DNA (10 μg) from AKR/FuRdA mice was digested with *Eco*RI and blot hybridized. Numbers 3, 4, 7, 8, 10, and 23 represent thymoma DNAs from different mice. Numbers 5 and 22 represent control liver DNA. Molecular weight markers are as described in the legend to Fig. 2. (A) Hybridization to AKV-specific MCp15E probe. (B) Hybridization to AKV-specific U3 LTR probe under low-stringency conditions (0.1× SSC plus 0.1% sodium dodecyl sulfate, 15 min at 62°C [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). (C) Hybridization of thymoma DNAs 4, 8, and 23 to AKV-specific U3 LTR probe under high-stringency conditions (0.1× SSC plus 0.1% sodium dodecyl sulfate, 15 min at 65°C). (D) Hybridization of thymoma DNAs 3, 4, 7, 8, and 10 and control liver DNA 22 to MCF LTR probe.

it will proliferate and become responsible for most of the integration events detected in the outgrown tumor.

Hybridization of the MCF LTR probe to normal mouse DNAs revealed that the MCF LTR sequence was derived from a xenotropic-like provirus which was present only once in the same chromosomal site within the BALB/c, C57BL/10, AKR, and DBA strains but was absent from the 129 mouse strain. The presence of the Xeno-dL virus in an identical chromosomal site in such unrelated strains as BALB/c and C57BL indicates that this Xeno-dL virus became integrated in the germ line more than 80 years ago.

Restriction analysis of the proviral MCF recombinants in tumor DNA showed that the xenotropic-related gp70 in the MCFs could not be derived from the Xeno-dL virus. The MCF recombinant proviruses we analyzed (30, 39) all acquired a *Bam*HI restriction site at map position 6.2, whereas many did not contain a *Kpn*I site at position 7.3. In contrast, the Xeno-dL provirus contained a *Kpn*I site at position 7.3 and lacked the *Bam*HI site at 6.2. If the gp70 region is derived from a single parent, the presence of a *Bam*HI site at position 6.2, an *Eco*RI site at 6.9, an *Sst*I site at 7.6, and the absence of a *Kpn*I site at 7.3 place this virus in a distinct subclass of xenotropic-like sequences which have not been identified in infectious isolates. Therefore, the generation of a significant portion of the leukemogenic AKR MCF viruses involves the recombination with at least two xenotropic-like proviruses: (i) the Xeno-dL provirus, donating part of the LTR, and (ii) other xenotropic-like sequences providing gp70 genetic information. These results confirm and extend suggestions by others who documented the independent expression of the non-AKV gp70 and p15E-U3 sequences (11, 38).

The retention within MCF viruses of ecotropic p15E sequences and gathering of U3 sequences from a specific xenotropic locus corroborate the notion that, besides the need for an altered gp70, the U3 region is also required for the leukemogenicity of the MCF virus. The Xeno-dL provirus might provide LTR sequences that change the tropism of the virus, allowing it to replicate in the thymus. Such a role has recently been assigned to LTR sequences (12). The absence of the Xeno-dL locus in the 129 strain might contribute to the high resistance of this strain to AKV-induced leukemogenesis. This is in contrast to the high susceptibility of this strain for Mo-MuLV-induced leukemogenesis (39). In this respect, it is interesting to note that the leukemogenic Moloney MCF has retained Moloney-specific LTR and p15E sequences (3; unpublished data). Apparently the LTR (and p15E) sequences in Mo-MuLV can fulfill the requirements for the leukemogenic potential of the Moloney MCF. The construction of a series of hybrid viruses will eventually establish the functional requirements for the different regions involved in the generation of leukemogenic MCF recombinant viruses.

ACKNOWLEDGMENTS

We are grateful to Hans Bloemendal, in whose laboratory these investigations were carried out. We also thank Marcel Mannens for his contribution.

This study was supported by the Foundation for Medical Research, which is subsidized by the Netherlands Organization for the Advancement of Pure Research.

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. **Berns, A., M. H. T. Lai, R. A. Bosselman, M. A. McKennett, L. T. Bacheler, H. Fan, E. C. Robanus Maandag, H. van der**

Putten, and I. M. Verma. 1980. Molecular cloning of unintegrated and a portion of integrated Moloney murine leukemia virus DNA in bacteriophage lambda. J. Virol. **36**:254–263.
3. **Bosselman, R. A., F. van Straaten, C. Van Beveren, I. M. Verma, and M. Vogt.** 1982. Analysis of the *env* gene of a molecularly cloned and biologically active Moloney mink cell focus-forming proviral DNA. J. Virol. **44**:19–31.
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. **Callahan, R., M. M. Lieber, and G. J. Todaro.** 1975. Nucleic acid homology of murine xenotropic type C viruses. J. Virol. **15**:1378–1384.
6. **Chattopadhyay, S. K., M. 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. **Chattopadhyay, S. K., M. R. Lander, E. Rands, and D. R. Lowy.** 1980. Structure of endogenous murine leukemia virus DNA in mouse genomes. Proc. Natl. Acad. Sci. U.S.A. **77**:5774–5778.
9. **Chattopadhyay, S. K., D. R. Lowy, N. M. Teich, A. S. Levine, and W. P. Rowe.** 1974. Qualitative and quantitative studies of AKR-type murine leukemia virus sequences in mouse DNA. Cold Spring Harbor Symp. Quant. Biol. **39**:1085–1101.
10. **Cloyd, M. W., J. W. Hartley, and W. P. Rowe.** 1980. Lymphomagenicity of recombinant mink cell focus-inducing murine leukemia viruses. J. Exp. Med. **151**:542–552.
11. **Datta, S. K., C. Y. Thomas, J. A. Nicklas, and J. M. Coffin.** 1983. Thymic epithelial genotype influences the production of recombinant leukemogenic retroviruses in mice. J. Virol. **47**:33–45.
12. **Des Groseillers, L., E. Rassart, and P. Jolicoeur.** 1983. Thymotropism of murine leukemia virus is conferred by its long terminal repeat. Proc. Natl. Acad. Sci. U.S.A. **80**:4203–4207.
13. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
14. **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.
15. **Herr, W., V. Corbin, and W. Gilbert.** 1982. Nucleotide sequence of the 3′ half of AKV. Nucleic Acids Res. **10**:6931–6944.
16. **Herr, W., and W. Gilbert.** 1983. Somatically acquired recombinant murine leukemia proviruses in thymic leukemias of AKR/J mice. J. Virol. **46**:70–82.
17. **Hoggan, M. D., C. E. Buckler, J. F. Sears, H. W. Chan, W. P. Rowe, and M. A. Martin.** 1982. Internal organization of endogenous proviral DNAs of xenotropic murine leukemia viruses. J. Virol. **43**:8–17.
18. **Jenkins, N. A., N. G. Copeland, B. A. Taylor, and B. K. Lee.** 1982. Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. J. Virol. **43**:26–36.
19. **Kawashima, K., H. Ikeda, J. W. Hartley, E. Stockert, W. P. Rowe, and L. J. Old.** 1976. Changes in expression of murine leukemia virus antigens and production of xenotropic virus in the late preleukemic period in AKR mice. Proc. Natl. Acad. Sci. U.S.A. **73**:4680–4684.
20. **Kelly, M., C. A. Holland, M. L. Lung, S. K. Chattopadhyay, D. R. Lowy, and N. H. Hopkins.** 1983. Nucleotide sequence of the 3′ end of MCF 247 murine leukemia virus. J. Virol. **45**:291–298.
21. **Levy, J. A.** 1973. Xenotropic viruses: murine leukemia viruses associated with NIH Swiss, NZB, and other mouse strains. Science **182**:1151–1153.
22. **Lowy, D. R., S. K. Chattopadhyay, N. M. Teich, W. P. Rowe, and A. S. Levine.** 1974. AKR murine leukemia virus genome: frequency of sequences in DNA of high-, low-, and non-virus-yielding mouse strains. Proc. Natl. Acad. Sci. U.S.A. **71**:3555–3559.
23. **Lowy, D. R., E. Rands, S. K. Chattopadhyay, C. F. Garon, and G. L. Hager.** 1980. Molecular cloning of infectious integrated murine leukemia virus DNA from infected mouse cells. Proc. Natl. Acad. Sci. U.S.A. **77**:614–618.
24. **Lung, M. L., J. W. Hartley, W. P. Rowe, and N. H. Hopkins.** 1983. Large RNase T_1-resistant oligonucleotides encoding p15E and the U3 region of the long terminal repeat distinguish two biological classes of mink cell focus-forming type C viruses of inbred mice. J. Virol. **45**:275–290.
25. **Lung, M. L., C. Hering, J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1979. 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. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double digest restriction fragments. Gene **19**:269–276.
27. **Morse, H. G.** 1978. Historical perspective on the development of inbred mice, p. 3–23. *In* H. C. Morse (ed.), Origin of inbred mice. Academic Press, Inc., New York.
28. **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.
29. **Poncz, M., D. Solowiejczyk, M. Ballantine, E. Schwartz, and S. Surrey.** 1982. "Nonrandom" DNA sequence analysis in bacteriophage M13 by the dideoxy chain-termination method. Proc. Natl. Acad. Sci. U.S.A. **79**:4298–4302.
30. **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.
31. **Quint, W., H. van der Putten, F. Janssen, and A. Berns.** 1982. 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. J. Virol. **41**:901–908.
32. **Rowe, W. P., and J. W. Hartley.** 1978. Chromosomal location of C-type virus genomes in the mouse, p. 289–295. *In* H. C. Morse (ed.), Origin of inbred mice. Academic Press, Inc., New York.
33. **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.
34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**:5463–5467.
35. **Southern, W. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
36. **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.
37. **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 derivate recombinant inbred strains. J. Virol. **42**:165–175.
38. **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.
39. **van der Putten, H., W. Quint, J. van Raaij, E. Robanus Maandag, I. M. Verma, and A. Berns.** 1981. M-MuLV induced leukemogenesis: integration and structure of recombinant proviruses in tumors. Cell **24**:729–739.
40. **van der Putten, H., E. Terwindt, A. Berns, and R. Jaenisch.** 1979. The integration sites of endogenous and exogenous Moloney murine leukemia virus. Cell **18**:109–116.