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Detection and Cloning of Murine Leukemia Virus-Related Sequences from African Green Monkey Liver DNA

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By using low-stringency nucleic acid hybridization conditions and specific subgenomic segments of the AKR ecotropic provirus as probes, murine leukemia virus (MuLV)-related sequences were detected in African green monkey (AGM) liver DNA. The MuLV-reactive segments present in restricted AGM DNA ranged from 1.9 kilobases (kb) to greater than 10 kb in size. On the basis of this finding, a 17-kb segment was cloned from a partial *Eco*RI AGM library in λ Charon 4A which shared nearly 5 kb of homology with AKR ecotropic MuLV DNA. The MuLV-related sequences detected in restricted preparations of AGM DNA or present in the cloned monkey DNA reacted with probes mapping 2.0 to 7.0 kb from the 5′ terminus of the AKR ecotropic provirus. The AGM clone also contained repeated sequences that flanked the MuLV-related segment. Labeled, subgenomic, MuLV-reactive segments of the monkey clone hybridized to multiple restriction fragments of AGM liver DNA, indicating the presence of several copies of the MuLV-related sequences.

The chromosomal DNA of several different animal species contains the genetic information for potentially infectious retroviruses. Genetic as well as biochemical studies have shown that mouse DNA contains sequences which encode infectious ecotropic and xenotropic murine leukemia viruses (MuLV's) (8, 12, 26, 36, 41) as well as numerous copies of related sequences that may or may not be expressed in the form of viral antigens (15, 33, 44). The spontaneous or induced expression of endogenous retroviral genomes has been monitored in a variety of "virus-free" cells, including those of primate origin (42, 45). The isolation of an ever-increasing number of endogenous mammalian retroviruses has provided a variety of reagents for the analysis and comparison of virus-encoded proteins. Similarly, the availability of numerous endogenous retrovirus nucleic acid probes has permitted an examination of mammalian DNA for the presence of sequences related to a particular retrovirus. In some instances, evolutionary relationships of certain endogenous retroviruses have been deduced as well as the origin and spread of other viruses to divergent mammalian species (4-6, 45).

In this report, we have taken advantage of the great sensitivity of the blot-hybridization procedure to evaluate the conservation of MuLV-related sequences in African green monkey (AGM) DNA. Using specific subgenomic segments of AKR ecotropic MuLV DNA as probes and nonstringent hybridization conditions, we detected several discrete bands in AGM DNA which ranged in size from 1.9 kilobases (kb) to greater than 10 kb. The MuLV-related sequences present in AGM DNA reacted with subgenomic segments of AKR ecotropic MuLV DNA spanning approximately 5 kb (1.9 to 7.0 kb from the 5′ terminus) of the mouse viral genome. Based on the ability to detect MuLV-reactive sequences in monkey DNA under nonstringent hybridization conditions, we cloned a 17-kb segment from AGM DNA which appears to represent a complete endogenous retrovirus genome. Blot-hybridization experiments using specific MuLV-reactive portions of the AGM clone indicated the presence of related sequences in monkey liver DNA.

MATERIALS AND METHODS

Preparation and cleavage of DNA. High-molecular-weight AGM liver and AKR/J mouse liver DNAs were extracted and purified from fresh tissue as previously described (8). All restriction enzymes used in these experiments were obtained from New England Biolabs (Beverly, Mass.) except for *Eco*RI, which was purchased from Bethesda Research Laboratories (Rockville, Md.) and used as specified by the supplier. Completeness of digestion of cellular DNAs was monitored by adding lambda DNA to a portion of the reaction mixture and evaluating its cleavage by gel electrophoresis. Restricted DNA samples were elec-

trophoresed in 0.6, 1.0, or 1.4% horizontal agarose slab gels as described (21) and transferred to nitrocellulose membranes as outlined by Southern (40).

Preparation of specific MuLV DNA probes. Subgenomic segments of a recombinant Charon 4A phage (λAKR 623), containing an infectious AKR ecotropic provirus (28), were cloned in pBR322 as previously described (20). (i) The construction of a recombinant plasmid containing the 2.7-kb *Sal*I-*Bam*HI segment (see Fig. 1) of the AKR provirus has been previously reported (8). (ii) pBR322 recombinants containing the internal 1.9-kb *Bam*HI fragment of AKR proviral DNA (Fig. 1) and the 2.2-kb *Bam*HI fragment of λAKR 623 (pAKR5′), which contains the 5′ terminal 1.9-kb segment of the provirus as well as 0.3 kb of flanking mouse sequences, were also isolated. (iii) A plasmid recombinant consisting of the 2.4-kb *Bam*HI-*Eco*RI fragment of λAKR 623 (pAKR3′) and which contained the 3′-terminal 1.9-kb segment of the AKR provirus and 0.5 kb of flanking mouse sequences was constructed. (iv) Unintegrated supercoiled Harvey sarcoma virus DNA, containing three copies of the long terminal repeat (LTR), which was originally cloned in λgtWES·λB (9, 17) was inserted into the *Eco*RI site of pBR322 (pHaSV) and propagated in HB101.

Specific portions of the recombinant plasmids listed above were obtained by restriction enzyme digestion and two cycles of preparative agarose (0.6 to 1.4%) gel electrophoresis. MuLV subgenomic segments eluted from gel slices using 6 M sodium perchlorate and absorption-elution from glass fiber filters (13) were (i) the 0.6-kb *Kpn*I fragment from pHaSV containing a complete copy of the LTR (Fig. 1); (ii) the 1.9-kb *Pst*I-*Bam*HI fragment from pAKR5′ containing LTR and *gag* sequences (5′ probe; Fig. 1); (iii) the 0.64-kb *Bal*I-*Bam*HI fragment from pAKR5′ containing *gag* sequences (Fig. 1); (iv) the 0.80-kb *Bam*HI-*Xba*I fragment from pAKR3′ containing sequences coding for the envelope glycoprotein (Fig. 1); and (v) the 1.25-kb *Bam*HI-*Pst*I fragment from pAKR3′ containing envelope and LTR sequences (3′ probe; Fig. 1).

Recombinant plasmid DNA or DNA fragments isolated by preparative agarose gel electrophoresis were labeled by the nick translation procedure (31) and had specific activities of 6×10^6 to 13×10^6 cpm/μg.

Preparation of a generalized MuLV recombinant plasmid probe. As a by-product of the cloning of unintegrated AKR mink cell focus-forming (MCF) 247 provirus in λgtWES·λB, a noninfectious, defective clone (λMuLVgen) was obtained containing a 6.8-kb insert which hybridized to the viral sequences present in λAKR 623 DNA (A. S. Khan, R. Repaske, C. F. Garon, H. W. Chan, J. W. Hartley, W. P. Rowe, and M. A. Martin, manuscript in preparation). The proviral DNA insert present in λMuLVgen was bordered by *Eco*RI sites known to be located in the LTRs of AKR MCF 247 (34; Khan et al., manuscript in preparation; S. K. Chattopadhyay, M. R. Lander, S. Gupta, E. Rands, and D. R. Lowy, manuscript in preparation) and contained a 1.9-kb deletion mapping between 5.9 and 7.8 kb on the AKR ecotropic MuLV provirus (Fig. 1). The viral DNA contained in λMuLVgen had a restriction endonuclease cleavage map indistinguishable from AKR MuLV DNA except for the presence of *Eco*RI sites in the LTRs and the absence of restriction enzyme sites located in the deleted segment; heteroduplex analyses of the viral inserts present in λAKR 623 and λMuLVgen revealed 5.9- and 0.9-kb regions of complete homology separated by a deletion loop (Khan et al., in preparation). The proviral insert in λMuLVgen contained no detectable cellular sequences. The viral DNA present in λMuLVgen was inserted into the *Eco*RI site of pBR322 as previously described (20).

Nucleic acid hybridization. Conditions for standard blot-hybridization experiments have been previously described (21). Two nonstringent hybridization procedures were used in the experiments to be described. In the first, reaction mixtures containing ^{32}P-labeled DNA, 1.0 M NaCl, 4 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (TES) (Calbiochem), pH 7.5, 0.1% sodium dodecyl sulfate (SDS), 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, fragmented denatured salmon sperm DNA (50 μg/ml), and the indicated percentage (by volume) of formamide were incubated at 37°C for 16 to 36 h as previously outlined (18). After the annealing step, the nitrocellulose membranes incubated in 30 and 40% formamide were extensively washed in 6× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS at 58 and 65°C, respectively. In the second procedure, reaction mixtures containing ^{32}P-labeled DNA, 6× SSC, 0.1% SDS, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, and fragmented denatured salmon sperm DNA (50 μg/ml) were incubated at 55°C for 16 to 36 h. After the hybridization, the nitrocellulose filters were extensively washed in 6× SSC containing 0.1% SDS at 55°C. After drying, the nitrocellulose strips were exposed to XR-5 Xomat R (Kodak) film, using intensifying screens when necessary.

Molecular cloning of MuLV-related sequences in AGM DNA. A partial *Eco*RI AGM DNA library (30) in λ Charon 4A (48) was screened for the presence of MuLV-like sequences by carrying out the in situ plaque hybridization assay (3) in 30% formamide as described above. Positive recombinants were subcloned and amplified (10), and, in some instances, subgenomic segments were inserted into the *Bam*HI site of pBR322 as previously described (20).

RESULTS

Detection of MuLV-like sequences in AGM liver DNA. During an evaluation of ecotropic-specific MuLV sequences present in different strains of inbred mice (8), we became interested in the conservation of MuLV sequences in species other than mice. In a preliminary group of experiments, liver DNAs from AKR mice, Fischer rats, Syrian golden hamsters, and AGMs were digested with *Eco*RI and analyzed by blot-hybridization under standard conditions (22), using the generalized MuLV probe (Fig. 1). As expected, numerous bands were visualized by autoradiography in the lanes containing restricted mouse DNA; fewer, though prominent, bands were present in the rat and hamster DNA preparations, and only faint reactivity was detected with AGM DNA (data not shown).

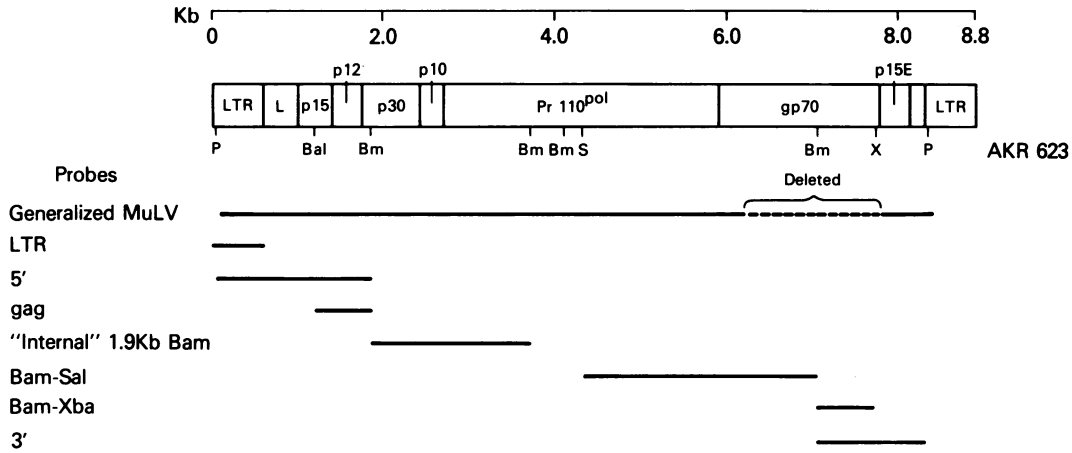

FIG. 1. *Location of various MuLV probes relative to the AKR ecotropic MuLV provirus. The map positions of virus-encoded gag (p15, p12, p30, and p10), pol, and env (gp70 and P15E) proteins or functionally important DNA segments were estimated from the sizes of the LTR, Pr75^{gag} (37), Pr110^{pol} (16, 32), and gPr90^{env} (23) and were located on the 8.8-kb map of AKR MuLV proviral DNA. Abbreviations: P, PstI; Bal, BalI; Bm, BamHI; S, SalI; X, XbaI; L, amino-terminal leader gag protein.*

Realizing that our standard conditions of hybridization might be too stringent for the detection of evolutionarily distant but related polynucleotides in AGM cells, the reaction conditions were altered (18) to permit such sequences to be identified.

A variety of radiolabeled MuLV probes were annealed to *Eco*RI-cleaved AGM DNA in reaction mixtures containing either 30 or 40% formamide (Fig. 2). Under these hybridization conditions (37°C), duplex molecules containing 30 or 24% base mismatches or less will be thermally stable (19, 29). When annealing is carried out under standard hybridization conditions (20 to 25°C below the melting temperature of the DNA) (47), molecules with 14 to 18% base mismatch or less will form stable duplexes. The generalized MuLV probe reacted with several discrete *Eco*RI restriction fragments of AGM DNA when hybridization was carried out in 30 or 40% formamide (Fig. 2A, lane 1; Fig. 2B, lane 1). The reactive AGM DNA fragments ranged from 1.9 to greater than 10 kb in size. Many of the same *Eco*RI AGM DNA fragments were detected with a subgenomic MuLV probe corresponding to the 2.7-kb *Bam*HI-*Sal*I fragment (Fig. 2A, lane 4; Fig. 2B, lane 4). Discrete *Eco*RI AGM DNA fragments were also visualized after hybridization with labeled recombinant plasmid DNA containing the internal 1.9-kb *Bam*HI fragment (Fig. 2A, lane 3; Fig. 2B, lane 3). Although the labeled *Pst-Bam* probe, complementary to the 5' 1.9-kb of AKR ecotropic MuLV provirus, reacted with restricted AGM DNA at 30% formamide (Fig. 2A, lane 2), no discrete

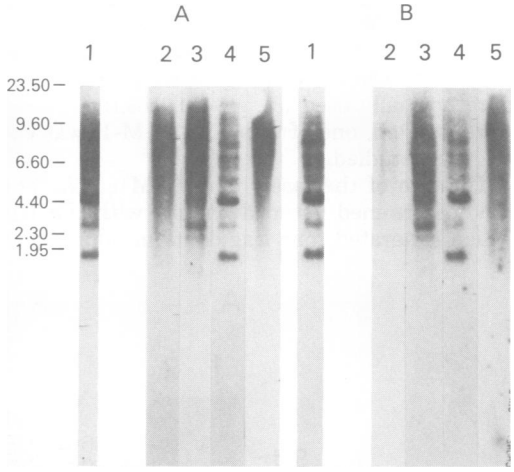

FIG. 2. *Detection of MuLV-related sequences in AGM liver DNA. Samples (5 μg) of EcoRI-digested AGM liver DNA were electrophoresed in separate lanes of a 0.6% horizontal agarose slab gel. After transfer to nitrocellulose membranes, strips corresponding to individual lanes were cut and hybridized to the indicated ^{32}P-labeled DNA probes at 30% (A) or 40% (B) formamide. Probes used (2 × 10^6 cpm/strip) were generalized MuLV (lane 1), 5' (lane 2), internal 1.9-kb Bam (lane 3), Bam-Sal (lane 4), and 3' (lane 5). Filter strips were hybridized, washed, and exposed to film as described in Materials and Methods. The markers (in kilobases) indicate the position of HindIII-cleaved lambda phage DNA.*

bands were visualized, and hybridization was virtually undetectable at 40% formamide (Fig. 2B, lane 2). Similarly, the radiolabeled 3' MuLV probe failed to resolve individual bands in the

*Eco*RI-digested AGM preparation, resulting in a smear pattern in reactions containing 30 or 40% formamide (Fig. 2A, lane 5; Fig. 2B, lane 5). In experiments using other specific 5′ (*Bal-Bam*; Fig. 1) or 3′ (*Bam-Xba*; Fig. 1) probes, discrete bands were never seen at 30% formamide, and no reactivitiy was observed at 40% formamide (data not shown). No hybridization of the generalized MuLV, *Bam-Sal*, or internal 1.9-kb *Bam* probes to salmon sperm DNA was observed under these experimental conditions, although a smear pattern was occasionally seen when annealing was carried out in the presence of 20% formamide.

Cloning of MuLV-related sequences from AGM DNA. The detection of MuLV-like DNA in preparations of AGM liver DNA raised the possibility that such sequences could be cloned by using nonstringent hybridization techniques to screen recombinant phage. As described in Materials and Methods, Charon 4A clones, containing MuLV sequences from a partial *Eco*RI AGM library (30), were identified by using the generalized MuLV probe in reaction mixtures containing 30% formamide. Although background reactivity was markedly increased compared with the more stringent screening procedure normally used (3), several positive clones were isolated, one of which (λ-AGM-1) was extensively studied.

The size of the insert in λ-AGM-1 (17.4 kb) was determined after digestion with *Eco*RI, which generated four fragments in addition to two lambda arms. Two of the four *Eco*RI fragments (5.25 and 4.75 kb) hybridized to the generalized MuLV probe (Fig. 3A). Cleavage of λ-AGM-1 with *Kpn*I yielded seven fragments, three (11.8, 2.6, and 1.3 kb) of which reacted with the generalized MuLV probe (Fig. 3B). *Bam*HI digestion of the λ-AGM-1 clone produced 12 fragments, 3 (2.7, 1.2, and 0.9 kb) of which hybridized strongly to the generalized MuLV probe (Fig. 3C) and 1 (1.8 kb) of which reacted weakly (see Fig. 7A) with this probe. To ascertain more precisely which portions of the MuLV genome were represented in λ-AGM-1, subgenomic segments of cloned AKR ecotropic MuLV DNA (28) were nick translated and annealed to *Bam*HI-digested λ-AGM-1 DNA. The 1.9-kb internal *Bam*HI fragment of AKR ecotropic MuLV DNA (Fig. 1) hybridized to the 2.7- and 0.9-kb *Bam*HI fragments of the AGM clone (Fig. 3C). The labeled 2.7-kb *Bam*HI-*Sal*I fragment isolated from AKR MuLV DNA (Fig. 1) reacted with the 2.7- and 1.2-kb *Bam*HI cleavage products of the cloned monkey DNA (Fig. 3C). No hybridization was detected when the AGM clone was annealed to the LTR, *gag*, or *Bam-Xba* (see Fig. 1 for map coordinates of specific probes) probes derived from MuLV DNA (data not shown).

The blot-hybridization reactions presented in Fig. 3 were all carried out in 6× SSC at 55°C, and the nitrocellulose membranes were extensively washed in 6× SSC at 55°C before autoradiography. Since these annealing conditions

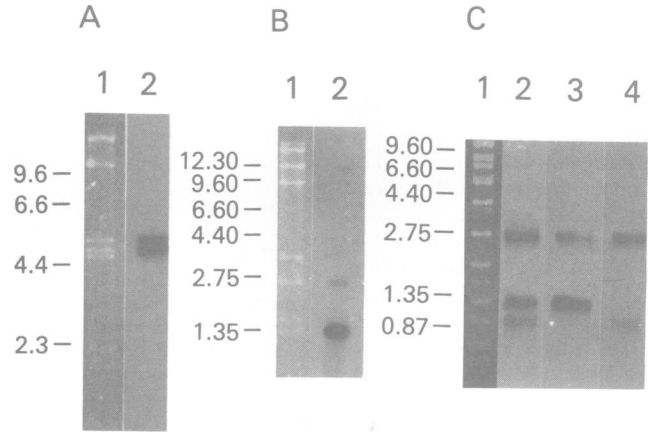

FIG. 3. *Detection of MuLV-reactive sequences in cloned monkey DNA. λ-AGM-1 DNA (0.4 μg/lane) was digested with EcoRI (A), KpnI (B), or BamHI (C) and electrophoresed in 1% (A) or 0.6% (B and C) horizontal agarose slab gels. In each panel, lane 1 shows a UV-fluorescence photograph of ethidium bromide-stained gels. The numbers indicate the size (in kilobases) of a marker mixture consisting of λ DNA cleaved with HindIII or SmaI and φX DNA cleaved with HpaII or HaeIII. The generalized MuLV probe (3 × 10⁶ cpm/strip) was used in lane 2 of each panel. In (C), the Bam-Sal probe (2 × 10⁶ cpm) was used in lane 3 and the internal 1.9-kb Bam probe (2 × 10⁶ cpm) was used in lane 4. Hybridization reactions were carried out in 6× SSC at 55°C as described in Materials and Methods.*

(T_m − 36°C) were relatively nonstringent, we were curious how stable the hybrids detected in these experiments actually were. We therefore digested λ-AGM-1 DNA with *Kpn*I and hybridized nitrocellulose membranes containing the restricted DNA with the generalized MuLV probe, the internal 1.9-kb *Bam*HI fragment of AKR MuLV DNA, and the 2.7-kb *Bam*HI-*Sal*I fragment of AKR MuLV DNA under two different annealing conditions: 6× SSC and 55°C or the very stringent procedure described by Jeffreys and Flavell (22) which involves hybridization in 3× SSC at 60°C followed by a wash in 0.1× SSC at 60°C (equivalent to T_m − 8.5°C). Bands were visualized on the membranes hybridized and washed under stringent conditions, although the autoradiograms had to be exposed nearly 10 times longer than the reactions carried out in 6× SSC at 55°C (Fig. 4). It should be noted that no annealing of the 1.9-kb *Bam*HI MuLV fragment to the 1.35-kb *Kpn*I fragment of the monkey DNA clone was detected under stringent hybridization conditions (Fig. 4, lane 6).

The locations of *Eco*RI, *Kpn*I, *Bam*HI, and *Hin*dIII cleavage sites in λ-AGM-1 DNA (Fig. 5) were determined in a series of double and triple digestions, using labeled cloned subgenomic *Bam*HI fragments 6 (2.7 kb), 9 (1.2 kb), and 10 (0.9 kb) of λ-AGM-1 DNA (data not shown). These three fragments reacted with the generalized MuLV probe as well as with specific subgenomic AKR ecotropic MuLV probes (see Fig. 3C). In a reciprocal experiment, labeled *Bam*HI λ-AGM-1 DNA fragments 6, 9, and 10, purified by two cycles of electrophoresis, were annealed to *Bam*HI-restricted λAKR 623 DNA (28), a clone containing a complete infectious copy of AKR ecotropic MuLV DNA. *Bam*HI λ-AGM-1 fragments 10, 6, and 9 reacted with a 1.9-kb, a 1.9-kb plus the 2.9-kb, and the 2.9-kb *Bam*HI fragments of λAKR 623 DNA, respectively (Fig. 6). Since λ-AGM-1 *Bam*HI fragments 10, 6, and 9 had been previously ordered (Fig. 5) within the monkey clone in independent experiments, the result presented in Fig. 6 indicates that a continuous 4.8-kb segment of λ-AGM-1 DNA is homologous to a 4.85-kb region of a cloned

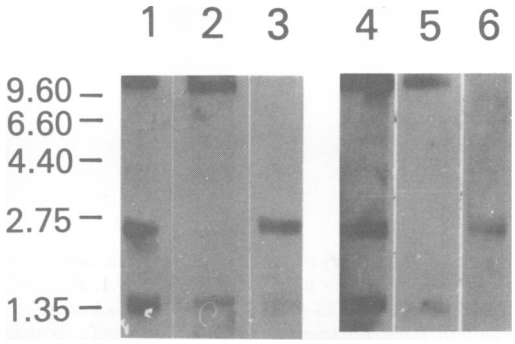

FIG. 4. *Effect of hybridization conditions on the reaction of MuLV-related sequences contained in the monkey clone. λ-AGM-1 DNA (0.3 μg/lane) was digested with KpnI, electrophoresed in a 0.6% horizontal agarose slab gel, and transferred to nitrocellulose membranes. Then 1.0-cm strips cut from the nitrocellulose sheet were hybridized and washed in 6× SSC at 55°C (lanes 1 to 3) as described in Materials and Methods or in 3× SSC with a 0.1× SSC wash at 60°C (lanes 4 to 6) as outlined by Jeffreys and Flavell (22). The generalized MuLV probe (3 × 10⁶ cpm) was used with the strips shown in lanes 1 and 4, the Bam-Sal probe (3 × 10⁶ cpm) was used with strips shown in lanes 2 and 5, and the internal 1.9-kb Bam probe (3 × 10⁶ cpm) was used with strips shown in lanes 3 and 6. The strips shown in lanes 1 to 3 were exposed for 36 h at −70°C; strips from lanes 4 to 6 were exposed for 2 weeks at −70°C. In all cases, intensifying screens were used. Size markers (in kilobases) are described in Fig. 3.*

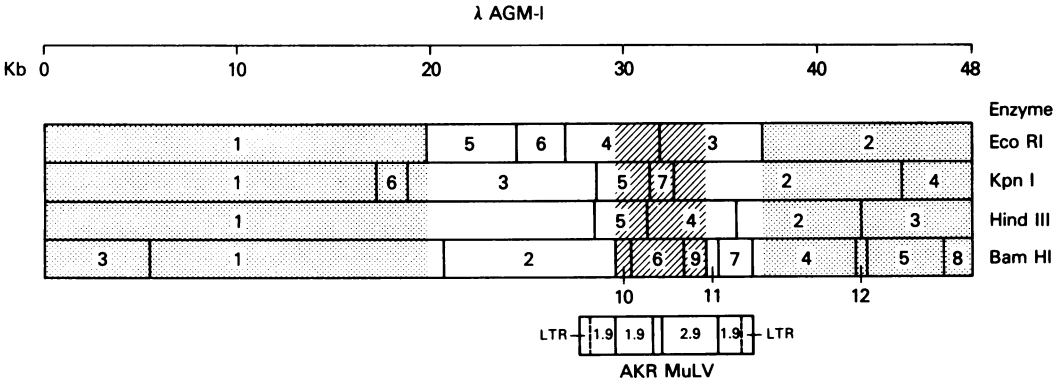

FIG. 5. *Cleavage map of λ-AGM-1 DNA. The stippled regions indicate the position of λ Charon 4A DNA; the diagonal area corresponds to segments that react with MuLV DNA probes. The 8.8-kb AKR ecotropic MuLV provirus is positioned to show best-fit regions of polynucleotide homology.*

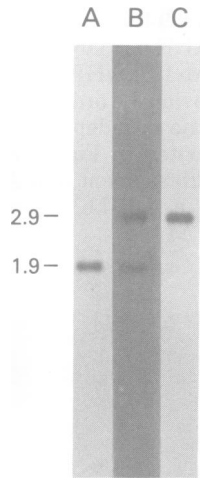

FIG. 6. *Reactivity of labeled subgenomic segments of the monkey clone with λ AKR 623 DNA. BamHI-digested λ AKR 623 (0.35 μg/lane) was electrophoresed in 0.6% horizontal agarose slab gels, transferred to nitrocellulose paper, and strips, cut from the membrane, were hybridized in 6× SSC at 55°C to ^{32}P-labeled (3×10^6 cpm/lane) BamHI fragments 10 (A), 6 (B), and 9 (C) of the monkey clone (see Fig. 5 for the map position of the three BamHI fragments of λ-AGM-1). BamHI λ-AGM-1 DNA fragments 10, 6, and 9 were purified by two cycles of agarose gel electrophoresis in 1.4% agarose gels as described in Materials and Methods and nick translated to a specific activity of 6×10^7 to 8×10^7 cpm/μg.*

MuLV DNA. To more accurately determine the position of MuLV-reactive sequences within λ-AGM-1 DNA, the monkey clone was digested with the restriction enzymes (singly or in combination) listed in Fig. 5 and hybridized to subgenomic segments of cloned AKR MuLV DNA. Since the labeled 1.9-kb internal *Bam*HI fragment of AKR viral DNA hybridized to no cleavage product of λ-AGM-1 DNA located to the left of 28.4 kb (Fig. 5) and the labeled 2.7-kb *Bam*HI-*Sal*I fragment of AKR viral DNA annealed to no restriction fragment of λ-AGM-1 DNA to the right of 34.2 kb (Fig. 5), the regions of homology between AKR MuLV and the reactive sequences in the monkey clone were positioned as indicated in Fig. 5.

The location of the MuLV-related segment within the monkey clone would predict that sequences analogous to the 3′-terminal 1.9-kb *Bam*HI fragment of AKR MuLV DNA and its associated LTR segment might be present in *Bam*HI fragment 7 (Fig. 5) of λ-AGM-1 DNA. As mentioned above, we observed no reaction of *Bam-Xba*, 3′, or LTR MuLV probes with the monkey clone in several independent experiments, a result compatible with the analyses of restricted AGM DNA presented in Fig. 2. On a few occasions, however, overexposed autoradiograms from experiments using high-specific-activity generalized MuLV probes revealed hybridization to *Bam*HI fragment 7 (1.8 kb) of the monkey clone in addition to significantly stronger reactions with *Bam*HI fragments 6, 9, and 10 (Fig. 7A). To evaluate the possibility that the monkey clone contained LTR sequences, one of which would be located in *Bam*HI fragment 7 (based on the alignment of AKR MuLV DNA shown in Fig. 5), this *Bam*HI fragment was nick translated and hybridized to a *Bam*HI digest of the monkey DNA clone. *Bam*HI fragment 7 reacted with itself (as expected) and also annealed to the 8.8-kb *Bam*HI fragment 2 (Fig. 7A). On the other hand, *Bam*HI fragments 6, 9, and 10 of λ-AGM-1 DNA each reacted with a single homologous *Bam*HI cleavage product (Fig. 8, lanes 3, 6, and 9). If the monkey clone contains a complete copy of an endogenous retrovirus that is similar to AKR MuLV in size and organization (Fig. 5), the hybridization of *Bam*-HI fragment 7 to *Bam*HI fragment 2 suggests the presence of an additional putative LTR located near the 5′ end of MuLV-reactive sequences. The dual reactivity of *Bam*HI fragment 7 was further evaluated by hybridizing labeled *Bam*HI fragments 6, 7, 9, and 10 to an *Eco*RI-plus-*Kpn*I digest of the monkey DNA clone. Labeled *Bam*HI fragments 10, 6, and 9 hybridized to one, four, and one *Eco*RI-plus-*Kpn*I fragments, respectively (Fig. 7B), predicted from the cleavage map presented in Fig. 7C. Besides annealing to a 4.5-kb fragment as expected, the labeled *Bam*HI fragment 7 probe also hybridized to a smaller 1.4-kb cleavage product (*Eco*RI-plus-*Kpn*I fragment 7) (Fig. 7B, lane 4). These experiments point to the presence of cross-reacting sequences within the monkey clone that flank a 5-kb MuLV-reactive segment which are separated by a minimum of 6.6 and a maximum 9.8 kb. This separation is within the 8- to 9-kb range characteristic of the LTRs of MuLV and type C primate proviral DNAs.

Normal mouse DNA contains numerous copies of MuLV-related sequences which have been identified in blot-hybridization experiments using cDNA or cloned DNA probes (1, 8, 11, 41). To ascertain the number and size distribution of MuLV-reactive sequences in AGM DNA, labeled, cloned, subgenomic segments (*Bam*HI fragments 6, 9, and 10) of λ-AGM-1 DNA were hybridized to *Bam*HI-restricted monkey liver DNA (Fig. 8). In each case the most prominent reactive cleavage product of AGM DNA comigrated with the *Bam*HI fragment of the monkey clone used in the hybridization assay. Other

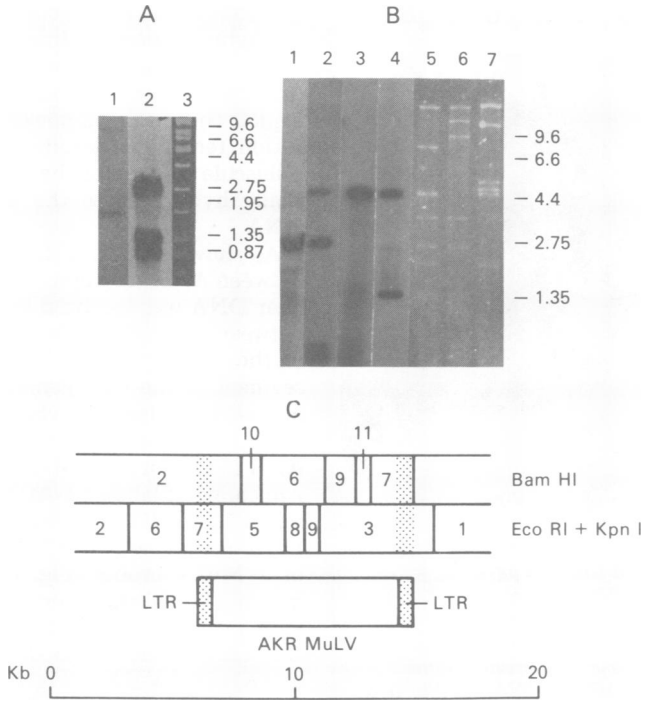

FIG. 7. *Reiteration of nucleotide sequences in the monkey clone. (A) λ-AGM-1 DNA (0.4 μg/lane) was cleaved with BamHI, electrophoresed in a 0.6% horizontal agarose slab gel, transferred to nitrocellulose membranes, and hybridized in 6× SSC at 55°C with ^{32}P-labeled (3 × 10^6 cpm) generalized MuLV probe (lane 2) or with ^{32}P-labeled (5 × 10^6 cpm) BamHI fragment 7 of λ-AGM-1 DNA (lane 1) (see Fig. 5 or panel C of this figure for the map position of this BamHI fragment). Lane 3 shows a photograph of an ethidium bromide-stained gel of BamHI-digested λ-AGM-1 DNA. (B) λ-AGM-1 DNA (0.4 μg) was cleaved with EcoRI (lane 7), KpnI (lane 6), or EcoRI plus KpnI (lanes 1 to 5) and electrophoresed in a 0.6% agarose gel. Lanes 5 to 7 show a UV-fluorescence photograph of the ethidium bromide-stained gel. The λ-AGM-1 DNA, digested with EcoRI plus KpnI, was transferred to a nitrocellulose membrane after electrophoresis, and 1.0-cm strips were hybridized under standard stringent annealing conditions to ^{32}P-labeled (4 × 10^6 cpm/strip) BamHI fragments 10 (lane 1), 6 (lane 2), 9 (lane 3), or 7 (lane 4) previously purified by two cycles of preparative agarose gel electrophoresis. The cleavage sites of MuLV-reactive BamHI and EcoRI-plus-KpnI fragments of λ-AGM-1 DNA are shown in panel C as well as the 8.8-kb AKR ecotropic MuLV provirus positioned as in Fig. 5. The stippled areas represent the location of LTR sequences in the MuLV DNA and putative LTR sequences in the monkey clone. The size markers (in kilobases) are described in Fig. 3.*

*Bam*HI fragments of AGM DNA also hybridized with the subgenomic probes, including several which were smaller than the 2.7-kb *Bam*HI fragment 6 (Fig. 8, lane 2). The MuLV-reactive subgenomic clones of λ-AGM-1 also reacted with *Bam*HI-digested AKR/J mouse DNA, but the bands observed were less discrete than those detected in AGM DNA (Fig. 8, lanes 1, 4, and 7).

DISCUSSION

Using nonstringent hybridization conditions, we have identified MuLV-related sequences in AGM DNA and have cloned and partially characterized a 17-kb segment from normal monkey liver DNA which shares nearly 5 kb of homology with the AKR ecotropic provirus. Although no infectious retroviruses have been isolated from AGM cells (46), the annealing of vervet monkey chromosomal DNA with several labeled endogenous simian type C viral DNA probes pointed to the presence of related sequences (5, 39, 45, 46). The thermal stabilities of the hybrids formed between AGM DNA and primate retroviral cDNA probes suggest that the type C virus-related sequences detected in vervet monkey DNA are more closely related to the baboon endogenous virus (5) than to the stumptail monkey endogenous virus (46).

Attempts to demonstrate polynucleotide homology between MuLV probes and primate DNAs (4) or labeled endogenous primate

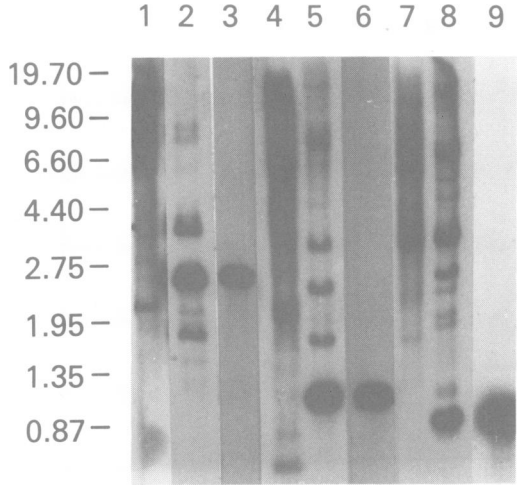

FIG. 8. *Identification of MuLV-related sequences in AGM liver DNA, using subgenomic probes derived from the monkey clone. AKR/J mouse (10 μg/lane; lanes 1, 4, and 7), AGM liver (10 μg/lane; lanes 2, 5, and 8), and λ-AGM-1 (0.3 μg/lane; lanes 3, 6, and 9) DNAs were digested with BamHI, electrophoresed through a 0.6% horizontal agarose gel, and transferred to a nitrocellulose membrane. BamHI fragments 6, 9, and 10 of λ-AGM-1 DNA (see Fig. 5) were separately inserted into the BamHI site of pBR322, yielding recombinant plasmids pAGM-B6, pAGM-B9, and pAGM-B10, respectively. Lanes 1 to 3 were hybridized to ^{32}P-labeled (12 × 10^6 cpm) pAGM-B6 DNA, lanes 4 to 6 were hybridized to ^{32}P-labeled (10 × 10^6 cpm) pAGM-B9 DNA, and lanes 7 to 9 were hybridized to ^{32}P-labeled (20 × 10^6 cpm) pAGM-B10 DNA. Hybridization to nitrocellulose strips containing AGM or λ-AGM-1 DNAs was carried out under stringent conditions. Membranes containing restricted mouse DNA were annealed and washed in 6× SSC at 55°C as described in Materials and Methods. Lane 9 was exposed for 2 h; lanes 3 and 6 were exposed for 5 h; lanes 2, 4, 5, 7, and 8 were exposed for 3 days; lane 1 was exposed for 10 days. The size markers (in kilobases) used in this experiment are described in Fig. 3.*

cDNA's and mouse chromosomal DNAs (45, 46) have, in general, been unrewarding. A low level of reactivity was reported, however, between an endogenous type C viral DNA of rhesus monkey and mouse DNA (35). More recently, low but consistent levels of hybridization were observed between M28 endogenous baboon virus cDNA and several ecotropic or xenotropic MuLV RNAs (6). In the latter experiments, DNA-RNA hybrids were permitted to form under extremely nonstringent conditions and analyzed on hydroxyapatite. Other investigators reported the annealing of size-fractionated MuLV cDNA's to several different mammalian DNA preparations consistent with a region of homology localized to a segment 50 to 500 nucleotides from the 3′ terminus of the RNA genome (24, 25). Although the hybridization conditions used in the present study were somewhat more stringent than those used by Bonner and Todaro (6), we were able to exploit the resolving power of the blot-hybridization technique despite the fact that only a miniscule fraction of the input radioactivity actually hybridized to the heterologous cellular DNA.

As shown in Fig. 2, the region of homology between AKR ecotropic viral DNA and monkey liver DNA was localized to fragments mapping between 1.9 and 7.0 kb (from the 5′ terminus) on the MuLV provirus. The conservation of this segment of the viral genome was corroborated in a set of reciprocal hybridization experiments using labeled subgenomic fragments of cloned AKR MuLV or λ-AGM-1 DNA (Fig. 3C and 6). Annealing of labeled λ-AGM-1 DNA to the terminal 1.9-kb *Bam*HI fragments of AKR MuLV DNA or the reaction of LTR, 5′, *gag*, *Bam-Xba*, or 3′ MuLV probes (Fig. 1) to AGM or cloned monkey DNA was never detected. Based on the map positions of the reactive 1.9-kb internal *Bam*HI and the 2.7-kb *Bam*HI-*Sal*I fragments of AKR MuLV DNA (Fig. 1), our results indicate the conservation of viral DNA sequences encoding reverse transcriptase and the 3′ portion of the *gag* region (viz., p30 and p10). In this regard, although antiserum to MuLV polymerase fails to inhibit type C simian viral reverse transcriptase activity (38), competition radioimmune assays indicated that murine and monkey virus polymerases share interspecies antigenic determinants (27). Similarly, the reported antigenic cross-reactivity of the p30 and p10 (2) internal viral proteins of Rauscher MuLV with analogous type C primate virus peptides would also be consistent with the region of polynucleotide sequence homology we observed.

As pointed out previously, little if any hybridization of labeled subgenomic MuLV probes, which map in the terminal 3′ or 5′ 2.0 kb of AKR MuLV, to AGM DNA (Fig. 2) or to the cloned monkey DNA was observed. Attempts to demonstrate antigenic similarities between the envelope glycoproteins of primate and nonprimate type C viruses (which partially map in the terminal 3′ 2.0 kb) have also been unsuccessful (7, 14, 43). The differences in gp70 detected immunologically and the lack of conservation of nucleotide sequences encoding this protein are certainly compatible with the role of the viral envelope in determining host range. The evolutionary divergence of the AKR MuLV LTR compared with analogous sequences in AGM DNA suggested from our data implies that they too may be species specific, regulating the expres-

sion and integration of retroviral DNA.

Recent experiments carried out in our laboratory indicate that labeled cDNA from type C baboon endogenous virus hybridizes to λ-AGM-1 *Bam*HI fragments 6, 9, and 10 as well as to fragments 2 and 7, which are thought to contain putative LTR sequences (Fig. 5 and 7; M. A. Martin, T. Bryan, S. Rasheed, and A. S. Khan, Proc. Natl. Acad. Sci. U.S.A., in press). In addition, sequences homologous to λ-AGM-1 *Bam*HI fragments 6 and 9 were found to be present in the chromosomal DNAs of higher primates (Martin et al., in press). In this regard, it should be noted that AGM chromosomal DNA contains multiple different gene segments that are related to the MuLV-like sequences present in λ-AGM-1 DNA (Fig. 8). In the present context, *Bam*HI fragments 6, 9, and 10 of the monkey clone have been defined as "internal" MuLV-reactive segments and, indeed, hybridize strongest with similar-sized *Bam*HI cleavage products of AGM liver DNA (Fig. 8). The presence of other intense reactive bands, some of which are even smaller than one of the λ-AGM-1 subclones used in this experiment (compare lanes 2 and 3, Fig. 8), is very reminiscent of the endogenous MuLV-related sequences present in mouse DNA by using defined subgenomic ecotropic (8, 11) or xenotropic proviral DNA probes (M. D. Hoggan, J. Sears, C. E. Buckler, J. W. Hartley, W. P. Rowe, H. W. Chan, and M. A. Martin, in preparation).

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