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Specific Hybridization Probes Demonstrate Fewer Xenotropic Than Mink Cell Focus-Forming Murine Leukemia Virus *env*-Related Sequences in DNAs from Inbred Laboratory Mice

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We have derived hybridization probes from analogous 100-base-pair segments located within the N-terminal region of gp70 coding sequences which differentiate xenotropic from mink cell focus-forming (MCF)-related murine leukemia virus (MuLV) DNAs. The MCF probe annealed to the integrated proviruses of all six MCF MuLV isolates tested; the xenotropic probe hybridized to the DNAs of all four xenotropic proviral isolates examined. No cross-hybridization was observed, and neither probe reacted with the *env* segments of amphotropic or ecotropic MuLV DNAs. Southern blot analysis of *Hin*dIII- or *Eco*RI-digested genomic DNAs from a variety of inbred laboratory mice demonstrated the presence of more MCF- than xenotropic MuLV-related segments in every strain tested.

The genomes of inbred strains of laboratory mice contain multiple copies of murine leukemia virus (MuLV)-related sequences (13, 48, 51). The number, type, and organization of these endogenous MuLV segments have been previously examined by Southern blot hybridization employing *env*-specific DNA probes (6, 8, 10, 11, 40). Commonly used inbred mouse strains contain fewer than six ecotropic proviruses as judged by the reactivity of restricted cellular DNAs with a labeled ecotropic MuLV *env* DNA segment (10). In contrast, the nature of the multiple (~30) copies of nonecotropic endogenous MuLV proviruses remains obscure due to the failure of existing MuLV *env* probes to differentiate xenotropic- and mink cell focus-forming (MCF)-related proviral segments from one another. For example, a 455-base-pair (bp) segment (the pX_{env} probe), cloned from the *env* region of the NFS-Th-1 xenotropic provirus, hybridizes to both xenotropic and MCF *env* sequences (8).

It is now commonly thought that some nonecotropic endogenous MuLV segments have the capacity to recombine with ecotropic MuLVs to generate dual-tropic MCF viruses that can be isolated from tissues of preneoplastic and leukemic or lymphomatous mice. Detailed restriction enzyme mapping of MuLV proviruses present in murine genomic DNAs (11) and the structural analysis of multiple endogenous MuLV cloned DNAs (29, 32) have both suggested that a majority contain an MCF-related rather than a xenotropic-related *env* gene region. Since some of these nonecotropic *env* segments may be involved in the recombinational process giving rise to MCF MuLVs, the quantitation and characterization of such MCF progenitors represents an initial step in understanding MCF MuLV-mediated leukemogenesis.

In this paper we describe the identification and subcloning of analogous 100-bp segments that unequivocally differentiate xenotropic-related and MCF-related MuLV *env* genes. Although the sequences that encode host range have not been precisely mapped to within the 100-bp regions we have selected for our probes, there is a 92% or greater homology between the sequence of the MCF-specific probe and the sequences of six MCF MuLVs and four spleen focus-forming viruses (1, 2, 5, 7, 15, 24, 29, 33, 39, 53). This report demonstrates a perfect correlation between the reactivity of the MCF-specific probe with the DNAs of six MCF viral isolates and the reactivity of the xenotropic-specific probe with the DNA of four xenotropic viral isolates.

Using the newly derived probes, we have found that every inbred mouse strain tested contained endogenous MCF-related MuLV *env* segments (endogenous MCF_{env}s) which were clearly distinct from, and more numerous than, endogenous xenotropic-related MuLV *env* segments.

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MATERIALS AND METHODS

Cells, viruses, and DNAs. Mink lung cells were propagated and infected with NZB-IU-6 (19), NZB-6 (19), F/St Th-4 (42), AKR-6 (16), AKR MCF247 (21), Friend MCF (1), AKR-13 MCF (16), Akv-1 C93 MCF (17), B10.F Thy-2 (an MCF virus isolated by J.W.H. from the thymus of a 6-month-old B10.F mouse), and 1504-M L2 MCF (8) as previously described (21). Mink lung cells producing AKR-L1 ecotropic MuLV introduced by phenotypic mixing (27) were obtained from A. Ishimoto. DNA was prepared from virus-infected cells lysed in the presence of 1% Sarkosyl–100 mM NaCl–50 mM Tris (pH 7.5)–10 mM EDTA. After incubation with 50 μg of RNase A per ml for 1 h at 37°C, 50 μg of proteinase K per ml was added for 2 h at 37°C, followed by phenol and chloroform extractions, ethanol precipitation, and dialysis. Mouse liver DNAs were prepared as previously described (10, 23).

Restriction enzymes, gel electrophoresis, and DNA hybridization. Cellular DNAs cleaved by restriction enzymes were run on 0.4% agarose gels, transferred onto nitrocellulose membranes, and hybridized as previously described (23), except that duplicate nitrocellulose filters were obtained by simultaneously blotting the gel in both directions (M. D. Hoggan et al., manuscript in preparation). The membranes

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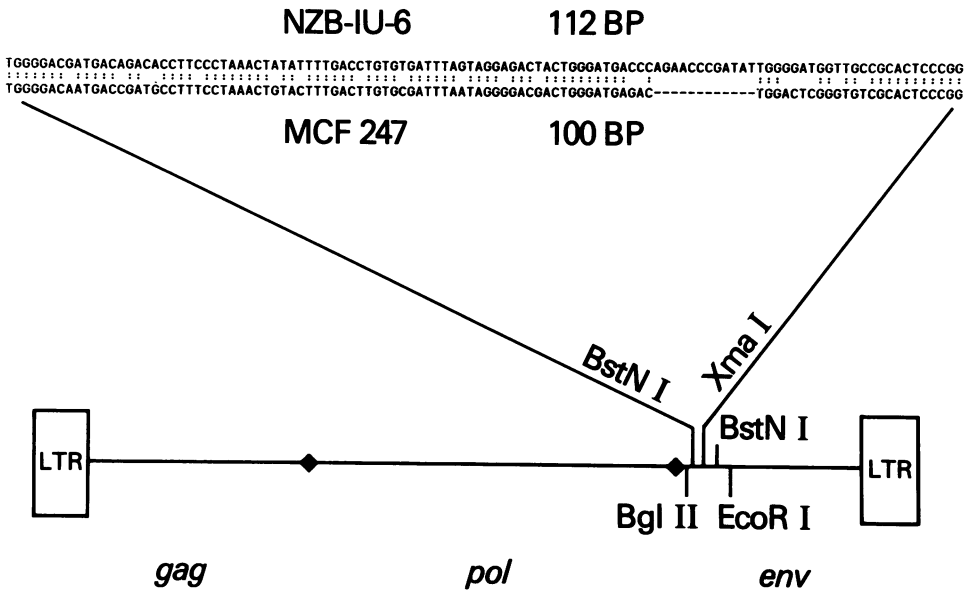

FIG. 1. Location and nucleotide sequence of the restriction fragments used as xenotropic and MCF MuLV *env*-specific hybridization probes. The sequences of analogous *Bst*NI-*Xma*I segments of MCF247 or NZB-IU-6 proviral DNAs, which share 71% polynucleotide sequence identity, are aligned with one another. Other *Xma*I, *Bgl*II, and *Bst*NI restriction sites within the provirus are not shown (MCF247 does not contain a *Bgl*II site at 6.3 kb). The 112-bp sequence of the xenotropic probe is a subset of the 455-bp *Bgl*II-*Eco*RI pX_{env} probe of Buckler et al. (8), which fails to distinguish between MCF, xenotropic, and amphotropic MuLV sequences. LTR, Long terminal repeat.

were hybridized with the indicated radiolabeled probes and were washed (i) four times in 2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 5 min at 20°C; (ii) four times in 0.1× SSC–0.1% sodium dodecyl sulfate for 15 min at 62°C; and (iii) four times in 2× SSC for 10 min at 62°C. The 455-bp *Bgl*II-*Eco*RI *env*-specific segment of NFS-Th-1 xenotropic MuLV proviral DNA (8) was nick translated to a specific activity of 10^8 cpm/μg (38). Molecular weight markers included 7.2-, 9.8-, and 13.2-kilobase (kb) cleavage products of NFS-Th-1 xenotropic MuLV cloned in pBR322 (8), which hybridized with the xenotropic *env*-specific and pX_{env} probes but not with the MCF *env*-specific MuLV probe.

Construction of xenotropic- and MCF-specific probes. An analysis of aligned nucleotide sequences from xenotropic (45) and MCF MuLV (29) *env* genes indicated that analogous 112-bp *Bst*NI-to-*Xma*I xenotropic and 100-bp *Bst*NI-to-*Xma*I MCF MuLV DNA segments might be suitable for type-specific hybridization probes in view of their 71% homology (Fig. 1). These fragments were obtained from molecular clones of NZB-IU-6 (45) and from plasmid pMCF-1 of MCF247 (31). After digestion with *Bst*NI, fragments in the 300- to 400-bp size range were preparatively isolated from 2% agarose gels (4). The 3′ recessed ends were converted to flush ends by reaction with T4 DNA polymerase (37, 44), and the DNAs were then digested with *Xma*I. The desired 112-bp xenotropic and 100-bp MCF *Bst*NI-*Xma*I fragments were isolated by electroelution from 10% acrylamide gels and then ligated to alkaline phosphatase-treated *Hinc*II + *Xma*I-digested M13mp8 DNA. After transformation of *Escherichia coli* JM103 cells, individual clear plaques were screened for the presence of the desired 100-bp fragments by dideoxy sequencing (50).

Radiolabeled probes were prepared using modifications of the M13 dideoxy sequencing method (50). The single-stranded DNA from a 2-ml 8-h M13-infected strain JM103 cell culture was purified and suspended in 25 μl of 10 mM Tris (pH 7.5). A 5-μl sample of the DNA was annealed with 2 μl of sequencing primer (5 ng; New England Nuclear Corp.) in a total volume of 12.5 μl. After cooling, 1 μl each of 0.1 mM DTT, 1 mM dTTP, 1 mM dGTP, and ^{32}P-dATP (800 Ci/mmol) and 2 μl of ^{32}P-dCTP (400 Ci/mmol) (10 mCi/ml; Amersham Corp.) plus 2 U of Klenow fragment were added in a total volume of 19.5 μl. The mixture was incubated for 30 min at 20°C to synthesize a second strand. After the addition of 7 μl of a nonradioactive chase (1 mM deoxynucleotide triphosphates in 1× sequencing buffer [50]), incubation was continued for an additional 30 min at 20°C, after which time the reaction was terminated by incubating at 65°C for 10 min.

The MuLV *env*-specific inserts, containing short adjoining sequences from the multiple cloning site of M13mp8, were then released by simultaneous digestion with 50 U each of *Eco*RI and *Hin*dIII for 2 h at 37°C in a volume of 50 μl. After electrophoresis through a 30-cm 10% acrylamide gel, the 128-bp xenotropic or 116-bp MCF MuLV/M13mp8 bands were cut from the UV-transilluminated, ethidium bromide-stained gel, and the DNA was isolated by electroelution (37). An estimate of counts per minute per milliliter was made by Cerenkov counting in a liquid scintillation counter, and samples were boiled with salmon sperm DNA in 0.1 M NaCl–25 mM Tris (pH 8.3)–5 mM EDTA for 5 min and quenched in ice. Samples containing 100,000 cpm of the MuLV *env*-specific probe were used for hybridizations with cloned proviral DNAs; 2×10^6 to 30×10^6 cpm was incubated with membranes containing restricted cellular DNAs. Multiple in vitro labeling reactions were required to produce 30×10^6 cpm since each incubation generated approximately 5×10^6 cpm. Autoradiographic exposure of Kodak X-AR film at −70°C with intensifying screens generally required several hours for cloned DNAs or up to 2 weeks for cellular DNAs.

RESULTS

Cloning and labeling of xenotropic- and MCF-related *env*-specific probes. The 5′ half of the ecotropic MuLV *env* gene contains a series of reciprocal insertions/deletions relative to the same region of nonecotropic MuLVs (1, 7, 24, 33, 45, 47). DNA probes from this portion of ecotropic proviral DNA fail to hybridize to xenotropic and MCF *env* genes and have been used for structural analyses of endogenous ecotropic MuLV proviruses (10). Although xenotropic and MCF MuLVs share extensive polynucleotide sequence identity in the 5′ half of their respective *env* genes (29, 45, 47, 53), an analogous 100- or 112-bp *Bst*NI-*Xma*I segment (Fig. 1) is present in each viral genome that is only 71% homologous. This portion of the *env* gene is highly conserved among different xenotropic or MCF MuLV isolates. The published *env* sequences of NFS-Th-1 (47) and NZB-IU-6 (45) xenotropic proviral DNAs are identical in this region; this portion of the MCF247 *env* gene shares 92% or more polynucleotide sequence identity with 10 other published MCF or spleen focus-forming virus MuLV *env* segments (1, 2, 5, 7, 15, 24, 29, 33, 39, 53).

Conservation of xenotropic or MCF sequences within the variable portion of the *env* gene suggested the possibility of preparing specific hybridization probes that would differentiate members of each host range type. As a first step, analogous *Bst*NI-*Xma*I *env* fragments of xenotropic (NZB-IU-6 [45]) or MCF (MCF247 [31]) MuLV proviruses were subcloned into M13mp8 as described in Materials and Methods. In initial experiments, the M13 MuLV *env* recombinants were labeled in vitro using the M13 universal probe primer procedure (25) with ^{35}S- or ^{32}P-labeled deoxynucleotide triphosphates. However, MuLV *env* hybridization probes labeled in this manner were unsuitable since excessively long exposure times were required to generate acceptable autoradiograms. An alternative method which employed the oligonucleotide sequencing primer for ^{32}P labeling of the MCF or xenotropic *env*-specific segment proved successful (Materials and Methods). The short, radiolabeled, double-stranded *env* inserts were preparatively separated from the M13 vector by simultaneous *Eco*RI and *Hin*dIII cleavage and subsequently purified by electrophoresis and electroelution.

Hybridization specificity of the cloned MCF and xenotropic *env* DNA segments. The reactivity of the cloned MCF and xenotropic *env* fragments with different classes of MuLV proviruses was initially evaluated by hybridizing them to molecular clones of ecotropic, amphotropic, MCF, and xenotropic MuLVs. The xenotropic *env*-specific probe (Fig. 2B) annealed specifically to the cloned xenotropic provirus and failed to react with the other three proviral DNAs. The MCF *env*-specific probe (Fig. 2D) exhibited a similar MuLV class specificity and only hybridized to the cloned MCF247 proviral DNA. In contrast, the pX_{env} probe, consisting of a 455-bp segment from the *env* region of NFS-Th-1 xenotropic provirus (8) inserted into pBR322 DNA, reacted strongly both with the cloned MCF and xenotropic DNAs and weakly with the amphotropic proviral DNA (Fig. 2C). The faint 16.4-kb band in the lane containing restricted ecotropic proviral DNA reflects the hybridization of pBR322 (probe) sequences with a partial cleavage product of the cloned ecotropic MuLV plasmid recombinant.

The reactivity of the xenotropic and MCF *env* probes with various MuLV proviruses was also evaluated by Southern blot analyses of DNA from virus-infected mink cells. In the experiment presented in Fig. 3, DNA was prepared from mink cells infected with MCF, xenotropic, or ecotropic MuLVs and restricted with *Pst*I, an enzyme known to cleave these MuLV proviruses both upstream and downstream from the *env* region corresponding to our probes (12). Since an acutely infected cell population is polyclonal with respect to proviral insertions, an enzyme such as *Pst*I was used to recruit common internal proviral fragments of interest into a visible band. The xenotropic-specific probe annealed to the DNAs of all four xenotropic MuLV isolates tested (Fig. 3A, lanes b to e), and the MCF-specific probe hybridized to the DNAs of all six MCF MuLV isolates tested (Fig. 3C, lanes f to k). In contrast, the pX_{env} probe hybridized with both the MCF and xenotropic MuLV DNAs (Fig. 3B, lanes b to k). The variations in intensity among the different reactive bands (Fig. 3A or C) could reflect differences in the number of proviral insertions for each virus since the same band intensity differences existed when the larger and less specific pX_{env} probe was used (Fig. 3B). The sizes of the reactive *Pst*I fragments visualized in Fig. 3 correspond to those predicted from previously published restriction maps for these MuLV proviruses (12).

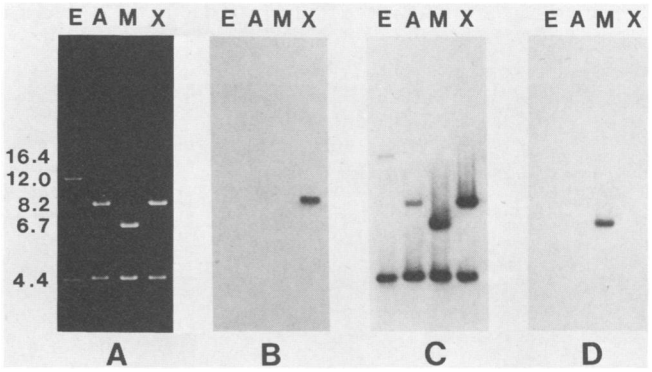

FIG. 2. Reactivity of the xenotropic-specific, pX_{env} (8), and MCF-specific hybridization probes with ecotropic, amphotropic, MCF, and xenotropic MuLV cloned DNAs. The cloned proviral DNA of AKV623 (lane E; 36), Ampho 4070A (lane A; 14), MCF247 (lane M; 31), and NZB-IU-6 xenotropic (lane X; 45) MuLVs was released from pBR322 by digestion with *Eco*RI or *Eco*RI plus *Hin*dIII and electrophoresed through 0.4% agarose gels. After the ethidium bromide-stained DNA bands were visualized under UV transillumination (A), the DNA was blotted onto nitrocellulose membranes and the filters were hybridized to the xenotropic-specific (B), pX_{env} (C), or MCF-specific (D) probes as described in Materials and Methods. The pX_{env} probe contained pBR322 sequences which hybridized to the 4.4-kb pBR322 fragment as well as to some partial digestion products (e.g., the 16.4-kb band in lane E, panel C).

Enumeration of xenotropic and MCF *env*-related sequences in cellular DNAs. The number of endogenous xenotropic- and MCF-related proviral insertions in mouse genomic DNAs was determined by using the xenotropic- and MCF-specific probes. A single conserved *Eco*RI site exists downstream from the region of the probe within *env*, at approximately 6.7 kb (Fig. 1), in all mapped xenotropic MuLVs and endogenous MCF_{env}s (12, 32, 46). *Eco*RI cleavage at this site and at an *Eco*RI site in the 5′ flanking cellular sequences would generate fragments of different sizes for each integrated xenotropic and endogenous MCF_{env} MuLV sequence. In the first group of experiments, therefore, DNAs prepared from different inbred mouse strains were digested with *Eco*RI, electrophoresed through 0.4% agarose slab gels, and hybridized to the *env*-specific probes after transfer to nitrocellulose membranes. The mouse chromosomal DNAs con-

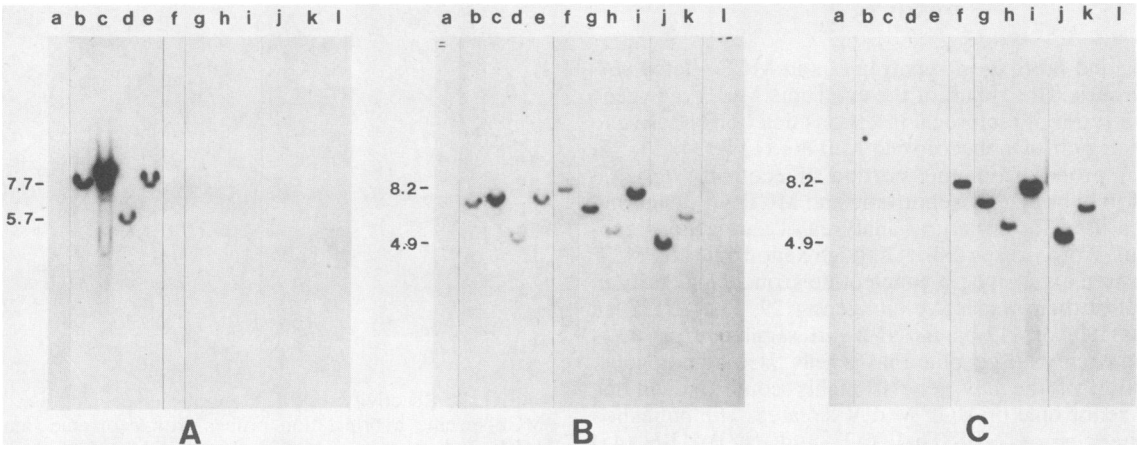

FIG. 3. Detection of various xenotropic and MCF proviral DNAs in mink cells infected with MCF or xenotropic MuLVs. DNA from mink lung fibroblasts (CCL64) (lane a), infected with xenotropic isolates NZB-IU-6 (lane b), NZB-6 (lane c), AKR-6 (lane d), and F/St Th-4 (lane e); MCF isolates AKR MCF247 (lane f), Friend MCF (lane g), AKR-13 (lane h), Akv-1 C93 (lane i), B10.F Thy-2 (lane j), and 1504-M L2 (lane k); or ecotropic AKR-L1 (lane l), was digested with *Pst*I. Restricted DNAs were transferred to nitrocellulose membranes from agarose gels and then hybridized to the xenotropic-specific (A), pX_{env} (B), or MCF-specific (C) probes as described in Materials and Methods. Panels B and C were bidirectional blots of the same gel.

tained many more copies of endogenous MCF_{env} (Fig. 4C) than xenotropic (Fig. 4A) MuLV *env*-related sequences. The number and pattern of endogenous MCF_{env}s was relatively constant among the nine mice examined, whereas xenotropic *env*-reactive segments varied greatly from strain to strain. The same mouse DNAs were analyzed with the *env*-specific probes after digestion with *Hin*dIII, an enzyme that does not cleave xenotropic MuLV proviruses (12) or endogenous MCF_{env}s (32, 46) 3′ to the *Bst*N-*Xma*I *env* segments (Fig. 5). Endogenous xenotropic MuLV proviral

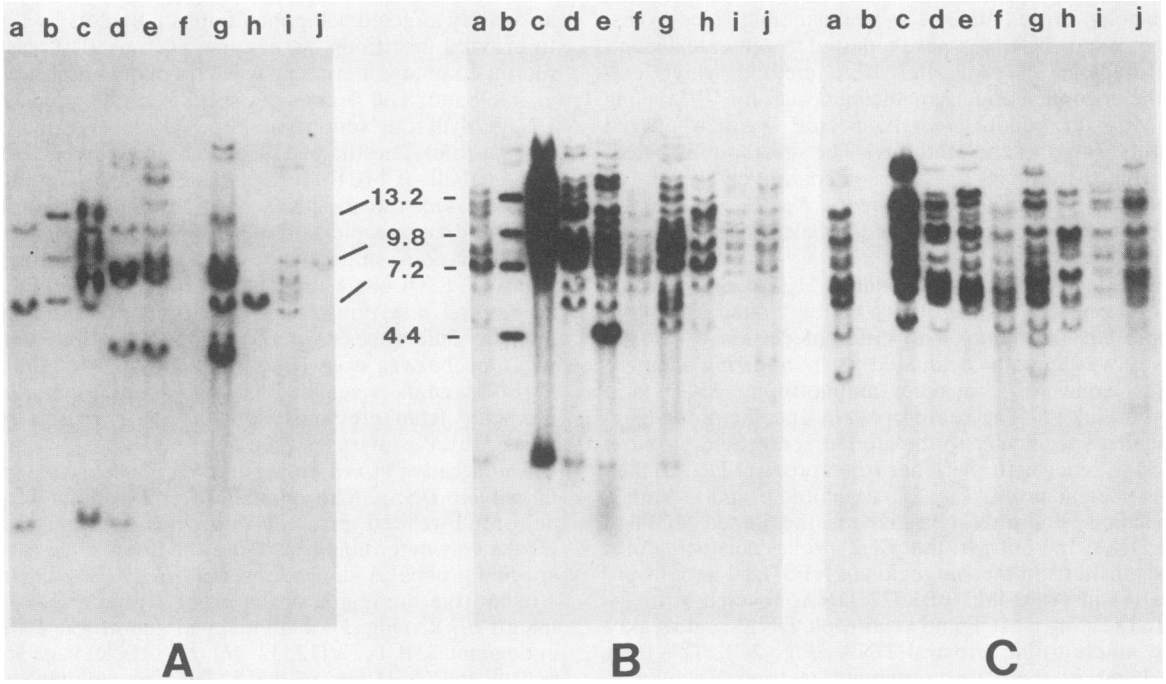

FIG. 4. Identification and enumeration of xenotropic or endogenous MCF_{env} proviral DNA segments in cellular DNAs of different laboratory mouse strains. CBA/N (lane a), C3H/FgLw (lane c), AKR/N (lane d), NZB/N (lane e), NFS (lane f), C57L/J (lane g), 129/J (lane h), BALB/cπ (lane i), and SWR/J (lane j) DNAs were cleaved with *Eco*RI, electrophoresed through 0.4% agarose gels, transferred to nitrocellulose membranes, and hybridized to the xenotropic-specific (A), pX_{env} (B), or MCF-specific (C) probes as described in Materials and Methods. Lanes b contain "internal" molecular weight markers consisting of the NFS-Th-1 cloned proviral DNA (8.8 kb of MuLV + 4.4 kb of pBR322 [8]) digested with *Xba*I, *Xba*I plus *Xho*I, or *Bst*EII. The prominent 4.4-kb band in NZB/N mouse DNA (panel B, lane e) which hybridized to the pX_{env} probe is the result of pBR322 contamination of this DNA sample. Panels A and C were bidirectional blots of the same gel.

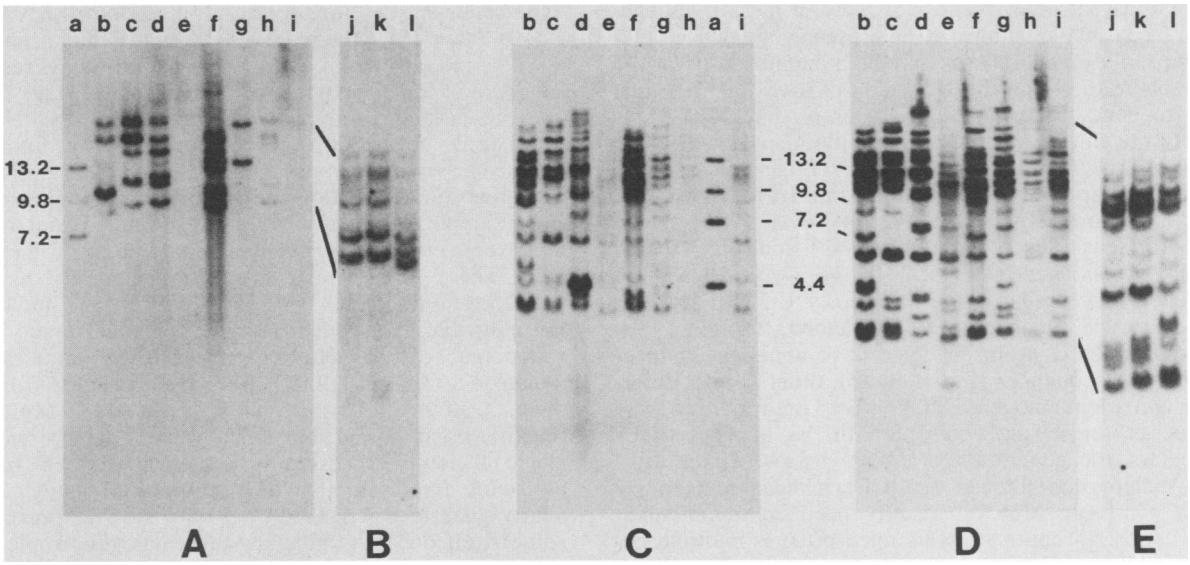

FIG. 5. Identification and enumeration of xenotropic or endogenous MCF_{env} proviral fragments in *Hin*dIII-cleaved mouse DNAs. C3H/FgLw (lane b), AKR/N (lane c), NZB/N (lane d), NFS (lane e), C57L/J (lane f), 129/J (lane g), BALB/cπ (lane h), SWR/J (lane i), F/St (lane j), C57BL/6J (lane k), and CBA/J (lane l) DNAs were cleaved by *Hin*dIII, electrophoresed through 0.4% agarose gels, transferred to nitrocellulose membranes, and hybridized to the xenotropic-specific (A and B), pX_{env} (C), and MCF-specific (D and E) probes as described in Materials and Methods. Lane a contains the "internal" molecular weight markers described in Fig. 4. The 4.4-kb band in NZB/N (panel C, lane d) DNA which hybridized to the pX_{env} probe is the result of pBR322 contamination. Panels A and D were bidirectional blots of the same gel. Panel E was first hybridized (28) to the MCF-specific probe and then was stripped (26) and rehybridized to the xenotropic-specific probe to produce the autoradiogram shown in panel B.

segments would therefore be cleaved in 5′ and 3′ flanking cellular sequences, whereas endogenous MCF_{env}s would be cleaved 5′ to the *env* region and in the 3′ flanking cellular sequence. As was observed after *Eco*RI digestion, mouse DNAs contained far fewer xenotropic-related *env* segments than endogenous MCF_{env}s (Fig. 5). The numbers of endogenous xenotropic-related proviruses present in different inbred mouse strains, deduced from the hybridization experiments involving *Eco*RI- and *Hin*dIII-restricted DNAs, are presented in Table 1. All of the mouse DNAs examined contained 20 to 30 MCF-related *Eco*RI or *Hin*dIII segments.

DISCUSSION

We have described the construction of M13 phage recombinants containing analogous 100-bp murine retroviral *env* inserts that differentiate xenotropic from MCF MuLV-related sequences. A potential problem with such highly specific DNA probes is that they may fail to hybridize with other members of the same class or group of sequences that deviate slightly in primary structure. This does not appear to be the case with the two MuLV *env* probes we have derived. The MCV *env*-specific segment hybridized well to Friend MCF proviral DNA (Fig. 3, lane g) despite an 8% nucleotide heterogeneity (1) relative to the *Bst*NI-*Xma*I segment of MCF247. Furthermore, the MCF probe annealed efficiently to MCF proviruses originally isolated from several different mouse strains (spontaneously produced in AKR, NFS.Akv-1, and B10.F mice, or after exogenous ecotropic MuLV infection of NIH Swiss or NFS mice [Fig. 3]). Furthermore, the MCF-specific probe hybridized (data not shown) with the DNA of endogenous MuLV clone A-12 (32), whose nucleotide sequence (29) is consistent with being the progenitor of the 5′ *env* region of MCF viruses, as well as with the DNA of clones A-1, A-5, and B-34, which also appear to be related to the 5′ *env* region of MCF viruses (32). Similarly, the xenotropic *env* probe reacted with members of the α and β class (9) of xenotropic viruses (AKR-6 and NZB-6 in Fig. 3) and with both the spontaneously expressed NZB-X1 and iododeoxyuridine-induced NZB-X2 xenotropic proviruses, despite differences in the structure of their envelope glycoproteins (19; NZB-6 and NZB-IU-6 in Fig. 3).

The MCF *env* probe hybridized to 20 to 30 different bands in restricted preparations of liver DNAs representative of several inbred mouse strains (Fig. 4C and Fig. 5D and E). These endogenous MuLV segments belong to a family of noninfectious proviral DNA sequences (32) that we have designated endogenous MCF_{env}(s). Unlike the proviruses associated with infectious MCF MuLVs, the endogenous MCF_{env}s examined to date contain *gag* and *pol* regions that have unusual restriction maps (11, 32) and a long terminal repeat sequence characterized by a 190-bp insertion (30, 46). Although multiple endogenous MCF_{env}s are present in the mouse genome, to date they have not been shown to be expressed as replication-competent viruses. It seems likely

TABLE 1. Estimation of the number of xenotropic-related segments in various inbred mouse strains

Mouse strain	No. of reactive *Eco*RI segments	No. of reactive *Hin*dIII segments
C57L/J	11–14	11–15
NZB/N	9–10	8–10
AKR/N	8	8
BALB/Cπ	7	7
C3H/FgLw	6–8	5–6
CBA/N	4	4
129/J	2	2
SWR/J	1	1
NFS/N	1	1

that the apparent defectiveness of most, if not all, endogenous MCF$_{env}$s may result from the presence of deletions (32) and aberrant stop codons, or may be a manifestation of the 190-bp insertion within the U_3 region of their long terminal repeat sequences (30, 46). The apparent defectiveness is probably not due to their unusual glutamine tRNA primer binding site (18, 43, 46). Nonetheless, endogenous MCF$_{env}$s appear to donate a portion of their *pol* and *env* regions during the recombination events that give rise to MCF and spleen focus-forming viruses; to date, every MCF and spleen focus-forming virus *env* gene (1, 2, 5, 7, 15, 24, 29, 33, 39, 53) has a sequence very similar to that of the *Bst*NI-*Xma*I segment of the prototype endogenous MCF$_{env}$ clone, A-12 (29).

Although inbred strains of mice have abundant endogenous MCF$_{env}$ sequences (Fig. 4 and 5), other factors determine whether leukemogenic MCF viruses are produced. The presence of replicating ecotropic MuLVs appears to be required for the generation of MCF viruses. Thus, AKR mice, which harbor endogenous MCF$_{env}$s and spontaneously express ecotropic MuLVs, readily produce recombinant MCF viruses. In contrast, NFS mice possess multiple endogenous MCF$_{env}$s but do not spontaneously generate MCF viruses because they lack an endogenous ecotropic provirus (10). However, MCF viruses can be detected in NFS mice that are congenic for the ecotropic loci from AKR, C58, and C3H/Fg mice (12, 17) or that have been exogenously infected with ecotropic MuLVs (20). Furthermore, the particular endogenous MCF$_{env}$ segment in NFS mice which participates in the recombination event giving rise to the newly formed MCF virus appears to vary depending on whether Moloney or Friend ecotropic MuLV is inoculated (20). In this regard, the immunochemical variability noted in different MCF virus isolates (16) is compatible with a model involving different endogenous MCF$_{env}$s participating in the recombination events that yield MCF MuLVs.

Although some endogenous MCF$_{env}$s are associated with the genesis of pathogenic viruses, others may not be deleterious to their host. CBA/N and DBA/2 mice both contain a determinant (*Rmcf*) that confers resistance to exogenous MCF MuLV infections (3, 22, 49). Since mice bearing the *Rmcf* gene synthesize MCF gp70-related proteins (3) which may protect animals by binding to cell surface receptors, it is possible that an endogenous MCF$_{env}$ is constitutively being expressed, a situation analogous to the *Fv-4* ecotropic MuLV resistance determinant (26).

In contrast to MCF *env*-related fragments, whose number is relatively constant among different inbred mouse strains, the number of xenotropic *env*-related fragments varies from 1 to more than 10 copies (Table 1). This variation probably reflects the different lineages of the inbred mouse strains from the pet animal stocks of wild and oriental mice in the early 1900s (for a review, see reference 41). It is unlikely that exogenous infection with xenotropic viruses has produced additional germ line xenotropic MuLV proviruses subsequent to the establishment of inbred mouse strains, because of the inability of exogenous xenotropic MuLVs to infect mouse cells and the presence of secondary blocks to xenotropic MuLV replication (27).

No xenotropic *env*-reactive cleavage fragments were easily detected in the single restricted sample of NFS mouse DNA evaluated in Fig. 4 and 5. Our difficulty in identifying a xenotropic *env*-reactive segment in this NFS mouse DNA preparation is probably due to partial degradation of this DNA sample, in view of the absence of high-molecular-weight bands in Fig. 4 (lane f) and 5 (lane e). DNA prepared from a second NFS mouse strongly reacted with the xenotropic *env*-specific probe after digestion with *Hin*dIII or *Eco*RI (data not shown); a single band which comigrated with the reactive 22-kb *Hin*dIII and 8.9-kb *Eco*RI restriction fragment of the closely related SWR/J mouse (Fig. 4, lane j, and Fig. 5, lane i) was readily detected.

A weak correlation exists between the number of xenotropic *env*-related sequences (Fig. 4 and 5) and the level of expression of infectious virus in a particular mouse strain. For example, NFS, SWR/J, and 129/J mice contain fewer xenotropic *env*-reactive segments than the other mice examined. These three strains are also categorized as "low" producers of infectious xenotropic viruses (35), and they are not inducible by iododeoxyuridine for xenotropic virus expression (34; J. W. Hartley, unpublished data). Multiple xenotropic MuLVs (19, 52) have been isolated from NZB mice, a strain harboring multiple xenotropic MuLV *env*-reactive fragments (Table 1). However, the presence of a single efficiently expressed proviral locus would be functionally more important than the presence of many silent or nearly silent loci. *Bxv-1*, a structural gene found on chromosome 1, encodes an inducible infectious xenotropic MuLV (34; Hoggan et al., in preparation). Mice of the AKR, BALB/c, C57L, and C57BL strains contain the *Bxv-1* locus, but it was not possible to identify which band(s) in Fig. 4 or 5 was associated with *Bxv-1*.

It is not known why more MCF than xenotropic *env*-related fragments exist in the DNAs of inbred mouse strains. Presumably the original integration of the progenitors of both xenotropic and endogenous MCF$_{env}$s was a relatively ancient event in the *Mus* germ line. It is possible that the retrovirus progenitor with structural features typical of endogenous MCF$_{env}$s infected mice at a much earlier time or for longer evolutionary periods than did xenotropic MuLVs. Consistent with this observation, preliminary experiments involving 14 species and subspecies of wild mice have indicated that 8 species and subspecies contain MCF *env*-related fragments, 4 subspecies contain xenotropic *env*-related segments, and 6 species and subspecies contain neither type of sequence (C. A. Kozak and R. O'Neill, Curr. Top. Microbiol. Immunol., in press).

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LITERATURE CITED

1. **Adachi, A., K. Sakai, N. Kitamura, S. Nakanishi, O. Niwa, M. Matsuyama, and A. Ishimoto.** 1984. Characterization of the *env* gene and long terminal repeat of molecularly cloned Friend mink cell focus-inducing virus DNA. J. Virol. **50**:813–821.
2. **Amanuma, H., A. Katori, M. Obata, N. Segata, and Y. Ikawa.** 1983. Complete nucleotide sequence of the gene for the specific glycoprotein (gp55) of Friend spleen focus-forming virus. Proc. Natl. Acad. Sci. USA **80**:3913–3917.
3. **Bassin, R. H., S. Ruscetti, I. Ali, D. K. Haapala, and A. Rein.** 1982. Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection. Virology **123**:139–151.
4. **Benson, S. A.** 1984. A rapid procedure for isolation of DNA fragments from agarose gels. BioTechniques **2**:66–67.
5. **Bestwick, R. K., B. A. Boswell, and D. Kabat.** 1984. Molecular cloning of biologically active Rauscher spleen focus-forming virus and the sequences of its *env* gene and long terminal repeat. J. Virol. **51**:695–705.
6. **Blatt, C., K. Mileham, M. Haas, M. N. Nesbitt, M. E. Harper,**

and M. I. Simon. 1983. Chromosomal mapping of the mink cell focus-inducing and xenotropic *env* gene family in the mouse. Proc. Natl. Acad. Sci. USA **80**:6298–6302.
7. **Bosselman, R. A., F. 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.
8. **Buckler, C. E., M. D. Hoggan, H. W. Chan, J. F. Sears, A. S. Khan, J. L. Moore, J. W. Hartley, W. P. Rowe, and M. A. Martin.** 1982. Cloning and characterization of an envelope-specific probe from xenotropic murine leukemia proviral DNA. J. Virol. **41**:228–236.
9. **Callahan, R., M. M. Lieber, and G. J. Todaro.** 1975. Nucleic acid homology of murine xenotropic type C viruses. J. Virol. **15**:1378–1384.
10. **Chan, H. W., T. Bryan, J. L. Moore, S. P. Staal, W. P. Rowe, and M. A. Martin.** 1980. Identification of ecotropic proviral sequences in inbred mouse strains with a cloned subgenomic DNA fragment. Proc. Natl. Acad. Sci. USA **77**:5779–5783.
11. **Chattopadhyay, S. K., M. W. Cloyd, D. K. 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.
12. **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.
13. **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.
14. **Chattopadhyay, S. K., A. I. Oliff, D. L. Linemeyer, M. R. Lander, and D. R. Lowy.** 1981. Genomes of murine leukemia viruses isolated from wild mice. J. Virol. **39**:777–791.
15. **Clark, S. P., and T. W. Mak.** 1983. Complete nucleotide sequence of an infectious clone of Friend spleen focus-forming provirus: gp55 is an envelope fusion glycoprotein. Proc. Natl. Acad. Sci. USA **80**:5037–5041.
16. **Cloyd, M. W., J. W. Hartley, and W. P. Rowe.** 1979. Cell-surface antigens associated with recombinant mink cell focus-inducing murine leukemia viruses. J. Exp. Med. **149**:702–712.
17. **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.
18. **Colicelli, J., and S. P. Goff.** 1986. Isolation of a recombinant murine leukemia virus utilizing a new primer tRNA. J. Virol. **57**:37–45.
19. **Elder, J. H., J. W. Gautsch, F. C. Jensen, T. Lerner, M. Chused, H. C. Morse, III, J. W. Hartley, and W. P. Rowe.** 1980. Differential expression of two distinct xenotropic viruses in NZB mice. Clin. Immunol. Immunopathol. **15**:493–501.
20. **Evans, L. H., and M. W. Cloyd.** 1985. Friend and Moloney murine leukemia viruses specifically recombine with different endogenous retroviral sequences to generate mink cell focus-forming viruses. Proc. Natl. Acad. Sci. USA **82**:459–463.
21. **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. USA **74**:789–792.
22. **Hartley, J. W., R. A. Yetter, and H. C. Morse III.** 1983. A mouse gene on chromosome 5 that restricts infectivity of mink cell focus-forming recombinant murine leukemia viruses. J. Exp. Med. **158**:16–24.
23. **Hoggan, M. D., C. E. Buckler, J. F. Sears, W. P. Rowe, and M. A. Martin.** 1982. Organization and stability of endogenous xenotropic murine leukemia virus proviral DNA in mouse genomes. J. Virol. **45**:473–477.
24. **Holland, C. A., J. Wozney, and N. Hopkins.** 1983. Nucleotide sequence of the gp70 gene of murine retrovirus MCF 247. J. Virol. **47**:413–420.
25. **Hu, N., and J. Messing.** 1982. The making of strand-specific M13 probes. Gene **17**:271–277.
26. **Ikeda, H., F. Laigret, M. A. Martin, and R. Repaske.** 1985. Characterization of a molecularly cloned retroviral sequence associated with *Fv-4* resistance. J. Virol. **55**:768–777.
27. **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.
28. **Israel, M. A., D. F. Vanderryn, M. L. Meltzer, and M. A. Martin.** 1980. Characterization of polyoma viral DNA sequences in polyoma-induced hamster tumor cell lines. J. Biol. Chem. **225**:3798–3805.
29. **Khan, A. S.** 1984. Nucleotide sequence analysis establishes the role of endogenous murine leukemia virus DNA segments in formation of recombinant mink cell focus-forming murine leukemia viruses. J. Virol. **50**:864–871.
30. **Khan, A. S., and M. A. Martin.** 1983. Endogenous murine leukemia proviral long terminal repeats contain a unique 190-base-pair insert. Proc. Natl. Acad. Sci. USA **80**:2699–2703.
31. **Khan, A. S., R. Repaske, C. F. Garon, H. W. Chan, W. P. Rowe, and M. A. Martin.** 1982. Characterization of proviruses cloned from mink cell focus-forming virus-infected cellular DNA. J. Virol. **41**:435–448.
32. **Khan, A. S., W. P. Rowe, and M. A. Martin.** 1982. Cloning of endogenous murine leukemia virus-related sequences from chromosomal DNA of BALB/c and AKR/J mice: identification of an *env* progenitor of AKR-247 mink cell focus-forming proviral DNA. J. Virol. **44**:625–636.
33. **Koch, W., W. Zimmerman, A. Oliff, and R. Friedrich.** 1984. Molecular analysis of the envelope gene and long terminal repeat of Friend mink cell focus-inducing virus: implications for the functions of these sequences. J. Virol. **49**:828–840.
34. **Kozak, C., and W. P. Rowe.** 1980. Genetic mapping of xenotropic murine leukemia virus-inducing loci in five mouse strains. J. Exp. Med. **152**:219–228.
35. **Levy, J. A.** 1978. Xenotropic type-C viruses. Curr. Top. Microbiol. Immunol. **79**:111–214.
36. **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. USA **77**:614–618.
37. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. USA **72**:1184–1188.
39. **Mark, G. E., and U. R. Rapp.** 1984. Envelope gene sequence of two in vitro-generated mink cell focus-forming murine leukemia viruses which contain the entire gp70 sequence of the endogenous nonecotropic parent. J. Virol. **49**:530–539.
40. **Meruelo, D., A. Rossomando, M. Offer, J. Buxbaum, and A. Pellicer.** 1983. Association of endogenous viral loci with genes encoding murine histocompatibility and lymphocyte differentiation antigens. Proc. Natl. Acad. Sci. USA **80**:5032–5036.
41. **Morse, H. C., III.** 1978. Introduction, p. 3–21. *In* H. C. Morse III (ed.), Origins of inbred mice. Academic Press, Inc., New York.
42. **Morse, H. C., III, C. A. Kozak, R. A. Yetter, and J. W. Hartley.** 1982. Unique features of retrovirus expression in F/St mice. J. Virol. **43**:1–7.
43. **Nikbakht, K. N., C-Y. Ou, L. R. Boone, P. L. Glover, and W. K. Yang.** 1985. Nucleotide sequence analysis of endogenous murine leukemia virus-related proviral clones reveals primer-binding sites for glutamine tRNA. J. Virol. **54**:889–893.
44. **O'Farrell, P. H., E. Kutter, and M. Nakanishi.** 1980. A restriction map of the bacteriophage T4 genome. Mol. Gen. Genet. **179**:421–435.
45. **O'Neill, R. R., C. E. Buckler, T. S. Theodore, M. A. Martin, and R. Repaske.** 1985. Envelope and long terminal repeat sequences of a cloned infectious NZB xenotropic murine leukemia virus. J. Virol. **53**:100–106.
46. **Ou, C.-Y., L. R. Boone, and W. K. Yang.** 1983. A novel sequence segment and other nucleotide structural features in the long terminal repeat of a BALB/c mouse genomic leukemia

virus-related DNA clone. Nucleic Acids Res. **11**:5603–5620.
47. **Repaske, R., R. R. O'Neill, A. S. Khan, and M. A. Martin.** 1983. Nucleotide sequence of the *env*-specific segment of NFS-Th-1 xenotropic murine leukemia virus. J. Virol. **46**:204–211.
48. **Rowe, W. P.** 1978. Leukemia virus genomes in the chromosomal DNA of the mouse. Harvey Lect. **71**:173–192.
49. **Ruscetti, S., L. Davis, J. Feild, and A. Oliff.** 1981. Friend murine leukemia virus-induced leukemia is associated with the formation of mink cell focus-inducing viruses and is blocked in mice expressing endogenous mink cell focus-inducing xenotropic viral envelope genes. J. Exp. Med. **154**:907–920.
50. **Sanger, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
51. **Steffen, D., and R. A. Weinberg.** 1978. The integrated genome of murine leukemia virus. Cell **15**:16–24.
52. **Varnier, O. E., A. D. Hoffman, B. A. Nexo, and J. A. Levy.** 1984. Murine xenotropic type C viruses. V. Biologic and structural differences among three cloned retroviruses isolated from kidney cells and one NZB mouse. Virology **132**:79–94.
53. **Wolff, L., E. Scolnick, and S. Ruscetti.** 1983. Envelope gene of the Friend spleen focus-forming virus: deletion and insertions in 3′ gp70/p15E-encoding region have resulted in unique features in the primary structure of its protein product. Proc. Natl. Acad. Sci. USA **80**:4718–4722.