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Generation of Mink Cell Focus-Forming Viruses by Friend Murine Leukemia Virus: Recombination with Specific Endogenous Proviral Sequences

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A family of recombinant mink cell focus-forming viruses (MCF) was derived by inoculation of NFS mice with a Friend murine leukemia virus, and their genomes were analyzed by RNase T_1-resistant oligonucleotide fingerprinting. The viruses were obtained from the thymuses and spleens of preleukemic and leukemic animals and were evaluated for dualtropism and oncogenicity. All these isolates induced cytopathic foci on mink cells but could be classified into two groups based on their relative infectivities for SC-1 (mouse) or mink (ATCC CCL64) cells. One group of Friend MCFs (F-MCFs) (group I) exhibited approximately equal infectivities for SC-1 and mink cells, whereas a second group (group II) infected mink cells 1,000- to 10,000-fold more efficiently than SC-1 cells. Structural analyses of the F-MCFs revealed that group I and group II viruses correlated with recombination of Friend murine leukemia virus with two distinct, but closely related, endogenous NFS proviral sequences. No correlation was found between the type of F-MCF and the tissue of origin or the disease state of the animal. Furthermore, none of the F-MCF isolates were found to be oncogenic in NFS/N or AKR/J mice. F-MCFs of both groups underwent extensive substitution of ecotropic sequences, involving much of the *gag* and *env* genes of group I F-MCFs and most of the *gag*, *pol*, and *env* genes of group II F-MCFs. All F-MCF isolates retained the 3′ terminal U3 region of Friend murine leukemia virus. Comparison of the RNAs of the F-MCFs with RNAs of MCFs derived from NFS.*Akv-1* or NFS.*Akv-2* mice indicated that the F-MCFs were derived from NFS proviral sequences which are distinct from the sequences contained in NFS.*Akv* MCF isolates. This result suggested that recombination with particular endogenous proviral sequences to generate MCFs may be highly specific for a given murine leukemia virus.

A number of ecotropic murine leukemia viruses (MuLVs) are associated with hematopoietic proliferative diseases in mice (16, 27–29, 32, 41). These include some endogenous viruses such as those expressed in strains of mice with a high incidence of leukemia (AKR and C58) (32), as well as exogenous viruses such as Friend MuLV (F-MuLV), which induces erythroleukemia (27, 41), and Moloney MuLV, which induces lymphatic leukemia after inoculation of susceptible mice (28). Both endogenous and exogenous ecotropic viruses undergo recombination with host cell retroviral sequences during the course of infection resulting in the generation of mink cell focus-forming viruses (MCFs) (14, 15, 18, 23, 41–44). These new recombinant viruses, also termed dualtropic or polytropic viruses (14, 20), can be recovered from preleukemic as well as leukemic animals and in many cases are thought to be intimately involved in the development of hematopoietic neoplasms (7, 13, 15, 18, 33, 36, 42–44). All such recombinants with ecotropic viruses recovered to date contain substituted genetic sequences in at least part of the viral envelope (*env*) gene (2, 4–6, 8, 9, 11, 12, 15, 26, 31, 37) and have acquired a widened host range of infectivity.

An extensively studied group of MCFs are those derived from AKR mice and from NFS mice congenic for endogenous ecotropic virus loci of AKR, C58, or C3HFg mice (7, 18, 26, 31, 33). Some of these virus isolates accelerate the onset of lymphomas in AKR mice or induce lymphomas in certain other strains and are classified as oncogenic, whereas other isolates appear to be nononcogenic (7). The oncogenic viruses of this group have all been isolated from the thymus, whereas most nononcogenic isolates have been derived from nonthymic tissue (7). Furthermore, all oncogenic MCFs derived from AKR mice exhibit common patterns of recombination which distinguish them from the nononcogenic isolates (26). It is not known how many distinct endogenous sequences recombine with the ecotropic parents to generate the MCFs, although it has been recently reported that at least two sequences can be distinguished in recombinants derived from the NFS genetic background (26).

Several MCFs derived from exogenous ecotropic viruses have been studied, and many have been reported to induce disease directly or after inoculation as pseudotypes into susceptible mice (13, 23, 36, 43, 44). The genomic recombination patterns of these MCF isolates appear to be dissimilar to those found in the oncogenic MCFs derived from endogenous *Akv* recombinants (3, 6, 11, 12, 37). However, groups of MCFs from exogenous viruses have not been derived in a systematic fashion which would allow the types of comparative studies that have been carried out in the endogenous ecotropic virus systems.

The endogenous murine sequences involved in the generation of MCFs are of considerable interest, and recent studies have suggested that these sequences are closely related to, but may be distinct from, xenotropic virus sequences (4, 5, 15, 31). A number of distinct endogenous sequences have been identified by genomic blotting experiments which may potentially contribute sequences to these recombinants (4, 5, 21, 22, 24, 39). Which of these closely related sequences

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recombine with ecotropic viruses to generate MCFs is not yet clear; however, in one instance a cloned endogenous proviral sequence has been identified which has an endonuclease restriction map consistent with the substitution identified in AKR MCF 247 (24).

In the present report, we derived a family of MCFs from NFS mice infected with an ecotropic F-MuLV (F-MuLV 57) (30) and examined their genomes by RNase T_1-resistant oligonucleotide fingerprinting. These studies were initiated to identify the endogenous sequences participating in the generation of these MCFs, as well as to discern the patterns of recombination. The results were then compared with the sequences and recombination patterns found in MCFs derived from NFS mice which are congenic for the ecotropic proviral genes of AKR mice (NFS.*Akv-1* and NFS.*Akv-2*) (26, 31). The data indicate that there is a high degree of specificity with regard to the particular endogenous sequences which recombine with F-MuLV 57 and reveal new recombination patterns not previously observed in MCFs.

MATERIALS AND METHODS

Cells, viruses, and mice. Friend MCF-1 (40) and F-MuLV 57 (30), a molecularly cloned NB tropic F-MuLV, were originally obtained as chronically infected Fischer rat embryo cells from E. M. Scolnick (Merck Sharp & Dohme Research Laboratories, West Point, Pa.). MCF *Akv-2*-C34, MCF *Akv-1*-C44-2, AKR MCF 247 virus, AKR-6 and NZBQ-IU-3 xenotropic viruses, and an amphotropic virus (1504A) were obtained from J. Hartley (National Institutes of Health, Bethesda, Md.). Viruses were propagated in Mv1Lu mink lung fibroblast (19) (ATCC CCL64), SC-1 (17), or *Mus dunii* cells (5). The mouse strains utilized in this study were NFS/N and AKR/J.

Isolation of MCFs. To generate MCFs, NFS/N mice less than 24 h old were injected intraperitoneally with ca. 10^4 to 10^5 focus-forming units of F-MuLV 57 as measured by the XC assay (35). Three to six weeks after injection, individual mice were sacrificed and autopsied, and their thymuses and spleens were removed aseptically. Dispersed cells (10^7 and 10^6) from each organ were overlayed on SC-1 and mink cells, which were plated at 2×10^5 cells in 60-mm tissue culture dishes the previous day as described elsewhere (7, 18). The medium was changed on days 1 and 4 after infection, and when confluent at 5 to 7 days after infection, the mink cells were scored under a dissecting microscope for the presence of MCF foci (direct mink cultures). The SC-1 cells were UV-irradiated on day 3 postinfection and overlayed with 2×10^5 mink cells and scored for MCF foci at confluency (UV mink cultures). Cultures were then passed two to four times and the viruses harvested. These virus stocks were subsequently titrated in direct mink and UV mink cultures, and those cultures infected near the limiting dilution were passed several times and the virus harvested. Each virus isolate in these analyses was cloned by three or more cycles of infection near the limiting dilution and was evaluated for homogeneity on the basis of RNase T_1-oligonucleotide fingerprinting.

Fluorescent focus assay. SC-1 or mink cells (2×10^5 cells) were plated into 60-mm tissue culture dishes containing a glass cover slip (11 by 22 mm). On the following day, the cultures were treated for 1 h at 37°C with tissue culture medium containing 20 μg of DEAE-dextran per ml and, subsequently, infected with serial dilutions of virus in regular medium. Five days later, the cover slips were removed, rinsed with phosphate-buffered saline, fixed in cold acetone for 10 min, and air dried. They were then stained for MuLV antigens with fluorescein-conjugated goat anti-Moloney MuLV antisera (Biological Carcinogenesis Branch, National Cancer Institute, Bethesda, Md.) and rhodamine counter stain, as described previously (17). The entire cover slip was scanned at ×40 magnification on a Leitz Orthoplan incident light fluorescent microscope, and the number of foci of fluorescent cells was recorded.

Leukemogenicity assays. AKR/J and NFS/N mice (1 to 3 days of age) were injected intraperitoneally with 10^3 to $10^{3.8}$ mink cell focus-forming units of each Friend MCF (F-MCF) isolate, and the mice were examined weekly for signs of illness or enlarged spleens or lymph nodes. Any mouse which exhibited clinical evidence of disease or survived for 1 year was sacrificed and autopsied.

RNase T_1-oligonucleotide fingerprinting. *M. dunii* cells were infected with each MCF isolate, and confluently infected cultures were passed into 150-cm^2 tissue culture flasks which were coated with "biocarriers" (Bio-Rad Laboratories, Richmond, Calif.) by the instructions of the manufacturer. The cells were allowed to grow to confluency and then labeled with 10 to 50 mCi of ^{32}P-labeled H_3PO_4 per flask. The labeling of cultures, harvesting of virus, and purification of viral RNA have been previously described (10). RNase T_1-oligonucleotide fingerprinting was accomplished by a two-dimensional, electrophoresis-homochromatography technique, as described earlier (10), with the following modification. After the electrophoretic separation of oligonucleotides on cellulose acetate strips, the oligonucleotides were transferred to a DEAE-cellulose thin-layer plate (20 by 40 cm; Analtech, Newark, Del.) at the bottom of its long dimension. Before chromatography, a paper wick (two thicknesses of Whatman 3MM filter paper) was clamped to the top of the plate. The chromatographic dimension of the fingerprint was developed until the wicks were saturated with the chromatographic medium (3 to 5 h). Individual T_1-oligonucleotides were analyzed for their RNase A-resistant oligonucleotides on DEAE paper (DE 81; Whatman) as previously described (10), with the following modifications to accommodate the recent alteration of the product by the manufacturer. The samples were applied along a line drawn 8 cm from the short edge of a sheet of the DEAE paper (43 by 56 cm). To avoid severe distortion, samples were not applied closer than 6 cm to the long edge of the paper. Electrophoresis was performed at 1,000 V (ca. 90 mA) for 5 h, with the temperature maintained at 25°C, at which time the xylene cyanol tracker dye migrated about 13 cm. The order of migration distances of the RNase A digestion products of the T_1-oligonucleotides was U > G > C > AC > AU > AG > AAC > AAU > AAG > AAAC > AAAU > AAAG > A_nX. At higher voltages, AC and AU were poorly resolved, even when electrophoresis was carried out at lower temperatures.

RESULTS

Source of F-MCFs and their biological properties. The viruses isolated in this study were derived from the spleens or thymuses of NFS litter mates which were inoculated at birth with F-MuLV 57. Mice were sacrificed at 3 to 6 weeks of age and were evaluated for erythroleukemia by gross examination at autopsy, and their thymus or spleen cells were cocultivated with mink and SC-1 cells for virus isolation as detailed above. The 3-week-old mice showed no obvious splenomegaly, whereas most mice sacrificed at 6 weeks of age exhibited grossly enlarged spleens and livers typical of F-MuLV-induced erythroleukemia (41). All MCF isolates were infectious for mink and mouse (SC-1) cells and

TABLE 1. Properties of F-MCF viruses from NFS mice

Virus designation[a]	Tissue of origin	Age at sacrifice (wk)	Disease state of animal	Infectivity[b] FA titer of the following cells (log_{10}): Mink	SC-1	Mink/SC-1 ratio (log_{10})	Leukemogenicity of the following mouse strains (no. leukemic/no. inoculated)[c] NFS/N	AKR/J
368-2T	Thymus	3	Normal	5.8	2.0	3/4	0/6	0/6
368-2S	Spleen	3	Normal	3.9	2.8	1/2	0/10	0/7
368-3T	Thymus	4	Normal	4.1	3.5	0/1	0/8	0/8
368-5T	Thymus	6	Splenomegaly	3.7	1.9	1/2	0/6	0/5
368-5S	Spleen	6	Splenomegaly	5.9	2.3	3/4	0/6	0/6
368-6T	Thymus	6	Normal	4.8	0.9	3/4	0/5	0/4
368-7T	Thymus	6	Splenomegaly	3.9	0.5	3/4	0/5	0/5
368-7S	Spleen	6	Splenomegaly	4.3	0.8	3/4	0/9	0/8

[a] The virus designation reflects the animal number (368-2, 368-3, 368-5, 368-6, and 368-7, respectively) and tissue of origin (T, thymus; S, spleen).

[b] Representative titers of MCFs on mink and SC-1 cells and the mink to SC-1 ratio of infectivity. Number of fluorescent foci per 11- by 22-mm area of a 60-mm dish per 0.2 ml of virus suspension. FA titer, Fluorescent antibody titer.

[c] Newborn mice were inoculated and observed for development of disease for a period of 1 year for NFS mice and 6 months for AKR mice.

induced typical cytopathic foci on mink lung fibroblast cells.

Virus designations, tissues of origin, disease state of the animals, and ages at sacrifice are presented in Table 1. Also presented in Table 1 are the titers of the isolates scored by fluorescent focus assays on SC-1 and mink cells. It is seen that, based on their relative infectivities for mouse cells compared with mink cells, the new MCFs appeared to fall into two classes. MCF isolates 368-2S, 368-3T, and 368-5T exhibited nearly equal titers on mink and SC-1 cells, whereas the remaining MCFs (isolates 368-2T, 368-5S, 368-6T, 368-7T, and 368-7S) exhibited a much higher infectivity for mink than for SC-1 cells. In control experiments, two other MCFs (AKR MCF 247 and F-MCF-1) and an amphotropic virus (1504A) exhibited approximately equal titers on mink and SC-1 cells, whereas ecotropic F-MuLV 57 and two xenotropic viruses (AKR 6 and NZBQ-IU-3) did not exhibit dual infectivities within the limits of detection in our assays (data not shown). There was no correlation between the differential mink to SC-1 infectivities of the viruses and the disease states, ages, or tissues of origin. However, as shown below, the relative titers on mink and SC-1 cells correlated with the virus structures. Studies on the oncogenicity of these viruses after neonatal inoculation of NFS and AKR mice did not reveal evidence of oncogenicity for any isolate (Table 1). In contrast, NFS neonatal mice inoculated with F-MuLV 57 almost uniformly exhibited splenomegaly detectable by palpation at 4 to 6 weeks and rarely survived beyond 3 to 4 months of age.

T_1-oligonucleotides of F-MCFs. To compare the genetic structure of the F-MCFs, we analyzed each F-MCF by T_1-oligonucleotide fingerprinting and mapping procedures. Figures 1 and 2 show the fingerprints of seven MCF isolates as well as a fingerprint of F-MuLV 57. Each numbered oligonucleotide in each virus was further identified by digestion with RNase A, as described above, to determine the shared and unique oligonucleotides of each isolate (data not shown).

The fingerprints are displayed in two groups which correspond to their infectivities on mink and SC-1 cells as described above. Group I MCFs (Fig. 1B through D) exhibited comparable infectivities on both mink and SC-1 cells, and group II MCFs (Fig. 2A through D) are viruses that were more infectious for mink cells. It is apparent from the fingerprints that a large proportion of the F-MuLV 57 oligonucleotides were substituted in many of the isolates. In the different MCFs, 43 large oligonucleotides not present in F-MuLV 57 were identified.

Close similarities were seen among the viruses of each group. Indeed, viruses 368-5T and 368-2S of group I (Fig. 1B and C) and 368-6T and 368-5S of group II (Fig. 2A and B) were indistinguishable by T_1-oligonucleotides, even though these viruses were derived from different animals. MCF 368-7S, whose fingerprint is not shown, was indistinguishable from the group II MCF 368-7T (Fig. 2C) derived from the same animal. One group I MCF (368-3T) differed from the remaining viruses of both groups in that many more F-MuLV 57 oligonucleotides were conserved, indicating less extensive substitutions.

Recombination patterns of F-MCFs. The order of T_1-oligonucleotides of F-MuLV 57 has been previously mapped (11). Thus, the identification of residual F-MuLV 57 oligonucleotides in the recombinant viruses defined in some detail the regions of recombination between F-MuLV 57 and the endogenous genetic sequences. Moreover, the precise positions of oligonucleotides residing in the *env* gene and the 3′ terminal sequences of F-MuLV 57 were determined by identification of these oligonucleotides in the nucleotide sequence of F-MuLV 57 (25; W. Koch and R. Friedrich, personal communication). The patterns of recombination observed in the F-MCFs are depicted in the bar diagrams in Fig. 3. Also included for comparison is a bar diagram representing the genome of F-MCF-1 (11). It can be seen that sequences in addition to *env* sequences were substituted in all of the new MCF isolates, and quite similar patterns of substitution were observed. Indeed, in all but one of the isolates (368-3T), sequences in the 5′ *gag* region of the genome were substituted by endogenous NFS sequences, and in the five group II isolates (368-2T, 368-5S, 368-6T, 368-7T, and 368-7S), extensive regions of the *gag* and *pol* genes were of NFS origin. Clearly, the prevalent patterns of substitution differ from previously reported MCFs derived

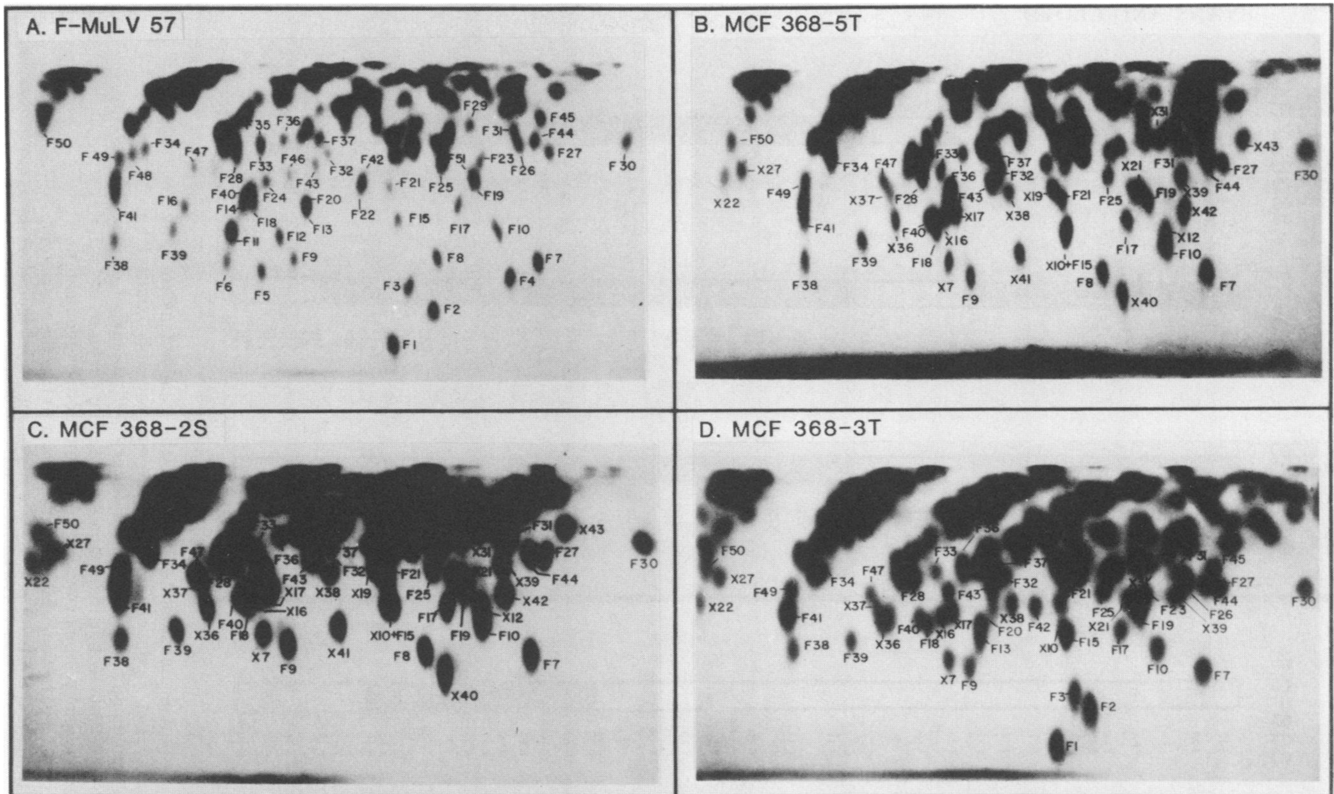

FIG. 1. RNase T_1-resistant oligonucleotide fingerprints of F-MuLV 57 and group I F-MCFs. The 70S [^{32}P]RNA ($\sim10^6$ cpm) of each virus was digested with RNase T_1 and fingerprinted as described in the text. Electrophoresis was from left to right, and homochromatography was from bottom to top. T_1-resistant oligonucleotides prefixed by an F correspond to oligonucleotides identified in F-MuLV 57 (A). Oligonucleotides prefixed by an X correspond to oligonucleotides not found in F-MuLV 57 but identified in recombinant F-MCFs (B through D).

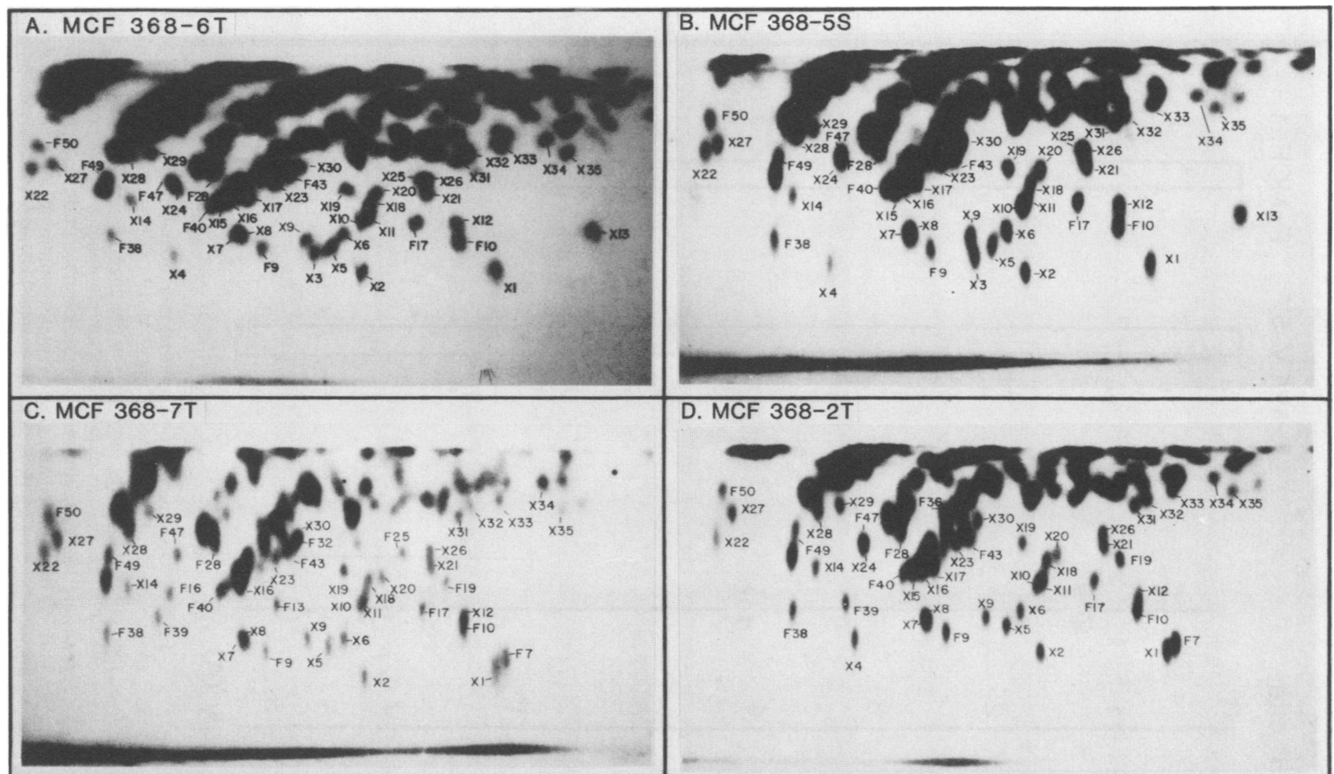

FIG. 2. RNase T_1-resistant oligonucleotides of group II F-MCFs. The viruses were fingerprinted as described in Fig. 1. Oligonucleotides of group II F-MCFs (A through D) shared with F-MuLV 57 are prefixed by an F and those identified in F-MCFs but not in F-MuLV 57 are prefixed by an X.

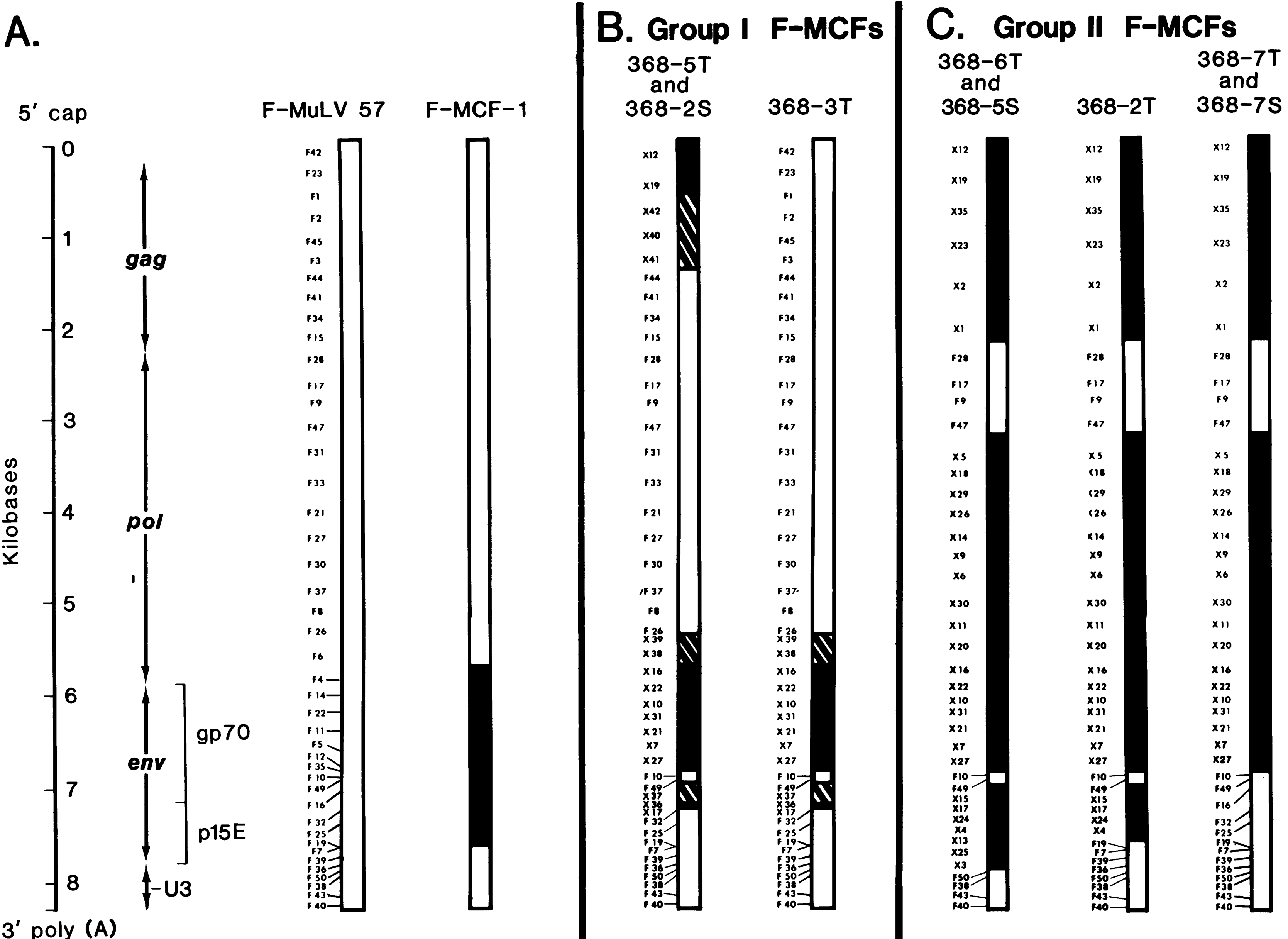

FIG. 3. Oligonucleotide maps and recombination patterns of F-MCFs. The order of T_1-resistant oligonucleotides relative to the 3' polyadenylate [poly (A)] terminus of each F-MCF and F-MuLV 57 was determined as described in the text. The order of oligonucleotides on each map was approximated by their autoradiographic intensities in the fingerprint of the smallest size class of polyadenylate-containing fragments in which they were identified. A total of 8 to 10 size classes of polyadenylate-containing fragments were fingerprinted to deduce the oligonucleotide map of each virus. The positions of MuLV genes are based on the Moloney MuLV nucleotide sequence (38). Bar diagrams indicating genomic regions containing oligonucleotides identical to F-MuLV 57 (unshaded areas) and regions containing endogenously derived oligonucleotides (black or slashed areas) are juxtaposed to the oligonucleotide maps. Lines drawn from individual oligonucleotides to discrete points on the bar diagrams indicate the precise positions of oligonucleotides identified in the F-MuLV 57 nucleotide sequence (see text). Slashed areas of the bar diagrams indicate regions of group I F-MCFs that contain endogenously derived oligonucleotides which were not found in group II F-MCFs. (A) Oligonucleotide map of F-MuLV 57 and the recombination pattern of F-MCF-1. (B) Oligonucleotide maps and recombination patterns of group I F-MCFs. (C) Oligonucleotide maps and recombination patterns of group II F-MCFs.

from endogenous or exogenous ecotropic parents. The exception is isolate 368-3T which had oligonucleotides identical to F-MuLV 57 in the 5′ half of its genome and more closely resembled F-MCF-1 in its pattern of substitution.

It is noteworthy that the elucidation of the sequence of the gp70 gene of F-MuLV 57 by Koch et al. (25) enabled us to define the borders of recombination of F-MCF-1. Examination of the residual F-MuLV 57 oligonucleotides of that virus (11) has indicated that the entire gp70 coding region is of endogenous origin (from outbred NIH Swiss mice). This indicates that dualtropism is not necessarily the result of a recombinant gp70 gene and corroborates the conclusions reached by Chattopadhyay et al. (4, 5) on the basis of restriction endonuclease maps of MCF proviral DNA and endogenous virus-related sequences.

Certain F-MuLV 57 oligonucleotides were uniformly conserved among all the MCFs derived in this study. These included all oligonucleotides of the U3 region (F50, F38, F43, and F40), two closely positioned oligonucleotides in the 3′ half of the gp70 coding sequence (F49 and F10), and four oligonucleotides that reside in the 5′ half of the genome, perhaps near the *gag-pol* junction (F47, F9, F17, and F28).

Evidence that F-MCFs were derived from two distinct endogenous proviral sequences. Another level of analysis of the MCFs is a comparison of the endogenously derived oligonucleotides present in each isolate. The oligonucleotide maps of the MCFs and F-MuLV 57 are presented in Fig. 3. Comparison of the endogenously derived oligonucleotides revealed two subclasses of MCFs which corresponded to groups I and II (Fig. 1 and 2), respectively, based on the infectivity data presented above. All endogenously derived oligonucleotides contained in isolates of each group were found in the most extensively substituted member of that group, strongly suggesting that the two groups of F-MCFs corresponded to recombinants of F-MuLV 57 with only two closely related, but distinct, endogenous proviruses. The map regions of the endogenously derived oligonucleotides of group I MCFs which differed from those of group II MCFs are indicated in the bar diagrams (Fig. 3) by slashed areas. The recovery of the same oligonucleotides in different isolates strongly argued against point mutation after recombination as the basis of the oligonucleotide differences.

Comparison of F-MCFs with MCFs derived from NFS mice congenic for *Akv*. A preliminary comparison of the F-MCFs described here with published T_1-oligonucleotide analyses of the MCFs derived from NFS.*Akv-1* and NFS.*Akv-2* by Hopkins and co-workers (26, 31) suggested that the F-MCFs in this study may contain proviral sequences that are distinct from endogenous sequences present in the *Akv* recombinants. To directly compare the F-MCFs with *Akv* MCFs, we analyzed two MCFs derived from NFS.*Akv-1* or NFS.*Akv-2* mice (*Akv-1*-C44-2 and *Akv-2*-C34, respectively). These two isolates have been reported to result from recombination with two different endogenous sequences and have undergone substitutions which include all or nearly all of their *env* genes (26). The fingerprints of *Akv-1*-C44-2 and *Akv-2*-C34 are shown in Fig. 4, and the T_1-oligonucleotides pertinent to the comparison are numbered in the figure. It can be seen that both *Akv-1*-C44-2 and *Akv-2*-C34 shared several oligonucleotides with the F-MCFs (Fig. 1 and 2). These included oligonucleotides not found in F-MuLV 57 (oligonucleotides prefixed by X in Fig. 4), as well as oligonucleotides which were found in F-MCFs and F-MuLV 57 (F9, F10, F17, F47, and F49). Interestingly oligonucleotides F10 and F49, which were uniformly present in all F-MCF isolates, were also found in *Akv-2*-C34 but were absent from ecotropic *Akv* viral RNA (data not shown). This result indicates that these oligonucleotides are contained in some endogenous viral sequences of NFS mice and therefore raises the possibility that they may also be of endogenous origin in the F-MCFs.

Some oligonucleotides of *Akv-1*-C44-2 or *Akv-2*-C34, which were previously identified as nonecotropic (oligonucleotides 110, 112, and 114; Fig. 4) (26, 31), were not present in any of the F-MCFs (Fig. 1 and 2). Two of these (110 and 114) are located in or very near the *env* gene of the *Akv* MCFs, and one or both of these oligonucleotides are found in all MCFs derived from NFS.*Akv-1* or NFS.*Akv-2* mice (26). Since the exact positions of oligonucleotides 110 and 114 are not known, it is not clear whether the *env* gene substitution in group I F-MCFs encompassed the sequences which may contain these oligonucleotides. Thus, it is possible that some other *Akv* MCFs not directly compared here share identical sequences with group I F-MCFs. In contrast, the entire *env* gene (with the possible exception of oligonucleotides F10 and F49), as well as most of the adjacent *pol* sequences of some group II F-MCFs, were of endogenous origin (Fig. 3), and therefore, included the regions which would contain oligonucleotides 110 and 114 if they were present. The possibility that an endogenous sequence containing oligonucleotides 110 and 114 was substituted by an F-MuLV 57 sequence containing F10 and F49 appears very unlikely in that all four oligonucleotides were identified in *Akv-2*-C34. Thus, we conclude that none of the *Akv* MCFs isolated from NFS.*Akv-1* or NFS.*Akv-2* were derived from an endogenous proviral sequence identical to the sequence giving rise to group II F-MCFs.

Figure 5 shows the oligonucleotide maps of the most extensively substituted isolates of group I and group II F-MCFs (368-6T and 368-5T, respectively) and the oligonucleotides of these viruses shared with *Akv-1*-C44-2 and *Akv-2*-C34. It can be seen that both F-MCFs contained endogenously derived oligonucleotides in their *env* genes that were not found in the corresponding regions of the two *Akv* MCFs. These results substantiate the fact that group II F-MCFs were derived from an endogenous sequence which differed from the sequences involved in the generation of the *Akv* MCFs and indicated that group I MCFs also were derived from a sequence which differed from the nonecotropic sequences found in either *Akv-1*-C44-2 or *Akv-2*-C34. A direct analysis of all of the remaining *Akv* MCFs will be required to determine whether any of those isolates contain sequences corresponding to group I F-MCFs.

DISCUSSION

In this study, a family of MCFs derived from a molecularly cloned exogenous MuLV was obtained to examine the sequence heterogeneity with respect to the endogenous component(s) participating in the generation of the MCFs, as well as with respect to the particular patterns of recombination observed in the different isolates. The viruses were isolated from the thymuses and spleens of preleukemic and leukemic mice to determine whether variations in the tissue of origin or the state of disease influenced the type of virus generated. The viruses were further characterized with respect to their dualtropism and their oncogenicity to determine whether structural correlations could be defined with these parameters. NFS mice were used in these analyses because of their lack of ecotropic virus genes and their low expression of endogenous xenotropic MuLVs. In addition, the use of NFS mice allowed us to compare the structures of the MCFs generated in this study with MCFs isolated from

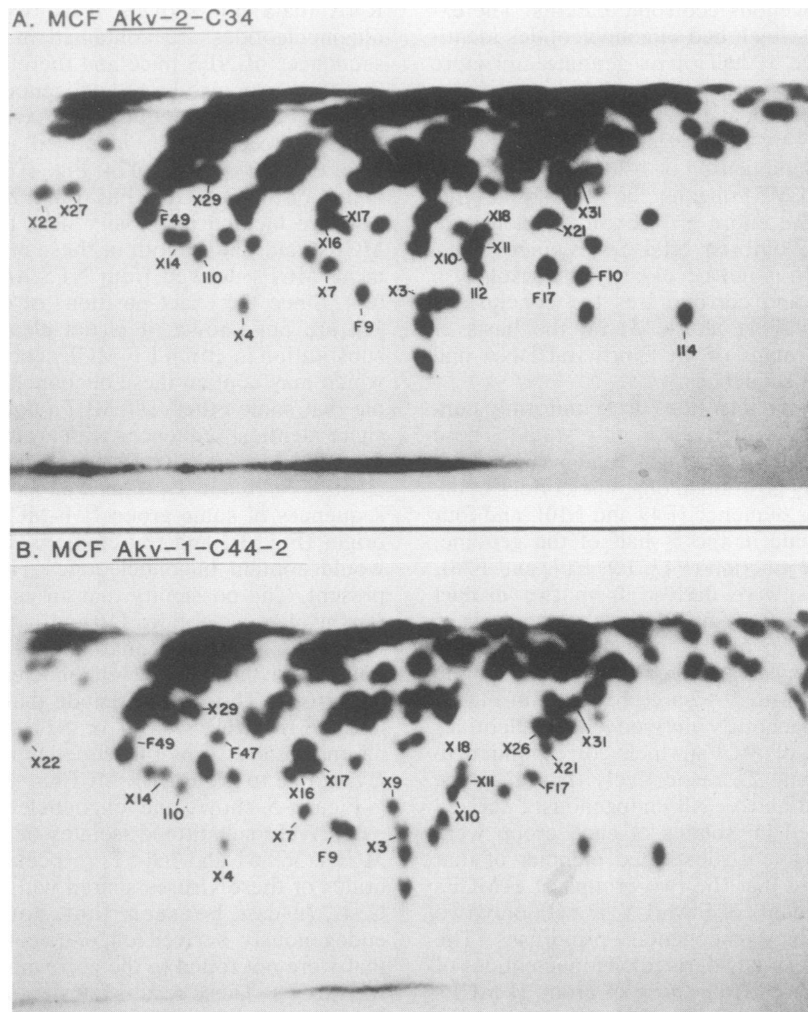

FIG. 4. RNase T_1-resistant oligonucleotide fingerprints of MCFs *Akv-2*-C34 (A) and *Akv-1*-C44-2 (B). Viral RNAs were fingerprinted as described in Fig. 1. Oligonucleotides which were shared with F-MuLV 57 are prefixed by an F. Oligonucleotides which were shared with F-MCFs but absent from F-MuLV 57 are prefixed by an X. Oligonucleotides which have been previously identified as non-*Akv* elements and were not found in any F-MCF isolate are numbered according to their original designation (see text).

mice with the same genetic background but congenic for endogenous ecotropic virus loci (26, 34).

Our isolation procedure for MCFs included passage of the viruses through SC-1 cells, and it was noted that during the isolation of certain of the F-MCFs, this passage could only be accomplished with low dilutions of the virus stock. After cloning, we quantitated the relative titers of the MCFs on mink and SC-1 cells, and, as shown above, two classes of viruses were defined which correlated with the two structural groups of F-MCFs. This result is reminiscent of the results of Rowe et al. (33), who demonstrated differential infectivities of *Akv* recombinants on SC-1 and NFS embryo cells. In that case, the infectivities also correlated with structural differences, as well as with the oncogenicity of the isolates. It is noteworthy that although most MCFs studied to date are dualtropic for both mouse and mink cells, some MCFs have been described that exhibit tropism for mink rather than tropism for mice (1). The structural basis for the differences in infectivity of the two groups of F-MCFs described in this report is unclear. There are many structural differences between the groups which could potentially alter infectivity. These include different recombination patterns, as well as sequence variations between the endogenously derived sequences.

Thus far, we have no evidence of oncogenicity for any of the F-MCF isolates we obtained, even though the NFS mice inoculated with either group I or group II isolates were followed for up to a year after inoculation. It should be noted, however, that the oncogenicity of an MCF administered exogenously may not reflect the oncogenicity of an MCF generated in vivo, particularly if the MCF is generated in the target cell. F-MCF-1 has also been reported to be nononcogenic unless pseudotyped with an amphotropic virus (36), and we are currently attempting to induce disease with our isolates by this procedure. Interestingly, MCFs isolated from NFS.*Akv-1* or NFS.*Akv-2* mice are also nononcogenic or only weakly so (7). Perhaps the particular endogenous sequences of NFS mice with which *Akv* and F-MuLV 57 recombine lack a function necessary for oncogenicity.

We found no correlation with either the tissue of origin or the disease state of the animal from which a particular type of F-MCF was isolated. Viruses of both groups were isolated

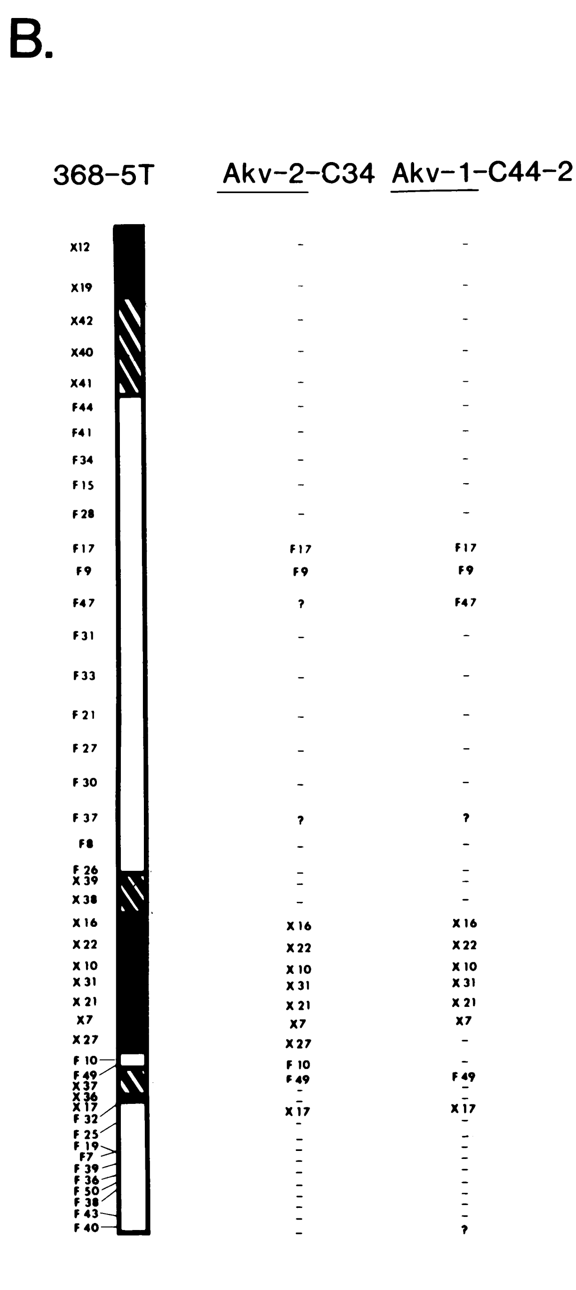

FIG. 5. F-MCF oligonucleotides shared with *Akv-2*-C34 and *Akv-1*-C44-2. (A) Map positions of group I F-MCF oligonucleotides shared with *Akv-2*-C34 and *Akv-1*-C44-2. (B) Map positions of group II F-MCF oligonucleotides shared with *Akv-2*-C34 and *Akv-1*-C44-2. The positions of MuLV genes are as shown in Fig. 3. The oligonucleotide maps and bar diagrams showing recombination patterns of the most extensively substituted isolates of group I (A) and group II (B) F-MCFs were depicted to include all of the endogenously derived oligonucleotides of F-MCFs which were identified. Oligonucleotides of each *Akv* MCF which were shared with group I or group II MCFs are listed by their prefix (oligonucleotides prefixed by an F are those identified in F-MuLV 57; those prefixed by an X are oligonucleotides not found in F-MuLV 57) and number and are juxtaposed to the respective F-MCF oligonucleotide map. Map positions of F-MCF oligonucleotides which had no counterpart in the *Akv* MCFs are designated by dashes.

from spleens and thymuses and from preleukemic as well as leukemic animals.

Although endogenous sequences similar to MCF-specific sequences have been identified in mouse genomes (4, 21, 22, 24, 39), it is still largely unknown which of these actually recombine with ecotropic viruses to give rise to MCFs or whether different ecotropic viruses preferentially recombine with particular endogenous sequences. A surprising and fortuitous finding in our analyses of F-MCFs was the high proportion of endogenous sequences present in almost all of the isolates. Such extensive recombination provided us with an unexpectedly large number of oligonucleotides as markers for assessing the origin of the endogenous sequences. A minimum of 23 endogenously derived oligonucleotides were identified and mapped in all group II MCFs (Fig. 3). This enabled us to directly compare a noncontiguous sequence of ca. 400 bases among all of the isolates of this group. From this analysis, we conclude that all group II viruses are the result of recombination of F-MuLV 57 with the same endogenous sequence. It is conceivable that two or more endogenous proviruses gave rise to the group II MCFs, but if this is the case, they must differ by less than 0.25% in their sequences. Similarly, the structures of the group I viruses were consistent with recombination of F-MuLV 57 with sequences from a second locus. It is, of course, possible that recombination of F-MuLV 57 with other proviruses occurs less frequently, but we have no evidence for this. Preliminary analyses of several additional F-MCF isolates suggest that the two groups of MCFs described here are the predominant species and that group II MCFs are isolated about three times more frequently than group I MCFs. The finding that the sequences of these F-MCFs differ from endogenous NFS sequences found in the NFS.*Akv* MCFs strongly suggests that proviral sequences, other than the two we identified, are capable of participating in the generation of MCFs in NFS mice. Thus, the particular endogenous sequences which give rise to MCFs may be specific for a given MuLV. We have not formally excluded the fact that the differences observed between F-MuLV 57 and *Akv* MCFs reflect divergence of allelic proviral sequences in NFS and NFS.*Akv-1* or NFS.*Akv-2* mice or divergence of the *Akv* MCFs subsequent to isolation; however, studies in progress suggest that Moloney MuLV may recombine with an endogenous NFS sequence to form MCFs which differ from either F-MuLV 57 or *Akv* recombinants.

The similarities of the oligonucleotide patterns of the MCFs within each group were remarkable, even though most of the MCFs appeared to be substituted in at least two discontinuous segments, suggesting multiple points of recombination. In the case of the group I F-MCFs, this could reflect recombination between F-MuLV 57 and a single endogenous provirus that occurs at very precise points in their genomes. Alternatively, this could reflect recombination of F-MuLV 57 occurring sequentially with two distinct endogenous proviruses in an equally precise manner. In this regard, evidence for sequential recombination in the generation of MCFs from mice has been reported by Thomas and Coffin (40). With group II F-MCFs, the only variations in the oligonucleotide maps were found at the junction between endogenous viral sequences and the F-MuLV 57 sequence which contained the 3′ terminus (Fig. 3). Almost all of the remaining oligonucleotides shared between F-MuLV 57 and group II F-MCFs which would suggest multiple recombination sites were identified in one or both of the *Akv* MCFs analyzed in this report. We therefore consider it quite plausible that the entire sequence encoding the *gag*, *pol*, and *env* proteins of the most extensively substituted group II MCFs is of endogenous origin. We are currently investigating this possibility by inoculating NFS mice with other F-MuLVs which lack one or more of the oligonucleotides in question and by examining the resulting recombinant viral genomes for the presence of these oligonucleotides.

Our data indicate that the MCFs were generated by recombination of F-MuLV 57 with proviruses which contain largely functional retrovirus coding sequences. However, it is not clear whether the endogenous parents correspond to replication-competent viruses. A few xenotropic MuLVs have been isolated from NFS mice, and we are in the process of determining whether they are related to either of the F-MCF groups described in this report.

Another approach to the identification of the endogenous NFS sequences giving rise to F-MCFs is, of course, to directly compare F-MCF proviral DNA with homologous NFS genomic sequences. As noted earlier, this approach has been used to identify an endogenous *env* sequence in AKR mice which has a structure consistent with the *env* gene of AKR MCF 247 (24). It is not clear, however, whether this is the only endogenous sequence which is consistent with the structure of that MCF. With the MCFs described here, a large proportion of the viral genome was of endogenous origin, which may facilitate an unambiguous identification of the endogenous parental sequences.

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