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A 3′ End Fragment Encompassing the Transcriptional Enhancers of Nondefective Friend Virus Confers Erythroleukemogenicity on Moloney Leukemia Virus

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Nondefective Friend helper murine leukemia virus (Fr-MuLV) induces primarily erythroleukemias in NFS mice, whereas Moloney murine leukemia virus (Mo-MuLV) induces T cell lymphomas. Using molecular clones of these two viruses, we constructed a recombinant in which a 0.62-kilobase fragment encompassing the U3 region at the 3′ end of the Fr-MuLV genome replaced the corresponding region of Mo-MuLV. The recombinant virus obtained by transfection of this clone, whose genome is derived primarily from Mo-MuLV, induces almost exclusively erythroleukemias in NFS mice. This and the previous result of Chatis et al. (Proc. Natl. Acad. Sci. U.S.A. 80:4408–4411), showing that the reciprocal recombinant whose genome is primarily derived from Fr-MuLV induces almost exclusively lymphomas, argue that a strong determinant of the distinct disease specificities of Fr-MuLV and Mo-MuLV lies in this 3′ end 0.62-kilobase fragment which contains the putative virus enhancers. To more precisely define this determinant, we have begun to construct recombinants in which smaller 3′ end fragments of the Fr-MuLV and Mo-MuLV genomes are exchanged. Analysis of the first such recombinant showed that Fr-MuLV can be converted to a lymphoma-inducing virus in NFS mice by substitution of a 0.38-kilobase fragment encompassing the virus enhancers in U3 with the corresponding region of the Mo-MuLV genome.

Moloney murine leukemia virus (Mo-MuLV) induces T cell lymphomas after injection into newborn NFS mice, whereas the nondefective Friend helper murine leukemia virus (Fr-MuLV) induces almost exclusively erythroleukemias in this strain (37, 44, 45). To identify virus genes that determine this difference in disease targeting, we constructed recombinants between molecular clones of nondefective Fr-MuLV and Mo-MuLV. Chatis et al. (2) have previously reported that, when a 0.62-kilobase (kb) fragment near the 3′ end of the Fr-MuLV genome is replaced with the corresponding fragment of Mo-MuLV, the resulting recombinant, designated FM, induced primarily lymphomas of the T cell type. This result suggests that determinants of disease specificity reside in the 3′ end of the MuLV genome (see also reference 6). However, because Fr-MuLV occasionally induces T cell lymphomas in older NFS mice and induces only lymphomas and myelogenous leukemias, but not erythroleukemia, in C57BL or DBA/2 mice (43–45, 47, 48), it seemed possible that, in the FM recombinant, the 3′ end 0.62-kb fragment of Mo-MuLV might serve to enhance a latent ability of Fr-MuLV to induce T cell lymphomas rather than confer this ability. Therefore, it was of great interest to study the disease specificity of the reciprocal recombinant, namely one whose genome was derived primarily from Mo-MuLV but whose 3′ end was derived from Fr-MuLV, since, to our knowledge, cloned isolates of Mo-MuLV do not have the ability to induce erythroleukemias in any mouse strain. Here we report that this recombinant, designated MF, induces primarily erythroleukemias in NFS mice, a result that strongly suggests that a region of the Fr-MuLV genome encompassing the U3 portion of the long terminal repeat (LTR), and hence the putative virus enhancers (15, 24, 28, 51), encodes a strong determinant of erythroleukemogenesis.

We constructed additional recombinants between Fr-MuLV and Mo-MuLV to further define the 3′ end sequences that determine their different disease specificities. Consistent with the hypothesis that the specificity determinant is the virus enhancer, we found that a recombinant with 0.38 kb encompassing the Mo-MuLV enhancer but with the rest of its genome derived from Fr-MuLV induces almost exclusively lymphomas in NFS mice.

MATERIALS AND METHODS

Cells and parental viruses. NIH/3T3 cells (21) were used for DNA transfections (16) of virus clones. Infectivity was assayed by XC plaque assays (41) and the presence of reverse transcriptase activity (1) in supernatant fluids of transfected cells. Sc-1 cells (19) were used for two cycles of endpoint purification of MF viruses (described below). Virus stocks were titrated by XC plaque assays in Sc-1 cells; titers were expressed in PFU. Infectious DNA clones of Mo-MuLV (13) and nondefective Fr-MuLV clone 57 (38) were obtained from S. Goff (Columbia University) and A. Oliff (Memorial Sloan-Kettering Cancer Center), respectively. The Mo-MuLV and Fr-MuLV genomes are cloned into pBR322 in permuted form at their unique *Hin*dIII and *Eco*RI sites, respectively.

Molecular cloning of recombinant viruses. Recombinant viruses were cloned by using pBR322 as a cloning vector. Cloning and screening of the recombinant virus containing clones as described below were accomplished by standard methods. The schemes used to generate the fragments for the MF and FMdrR recombinants are shown in Fig. 3 and 5. The parental clones were digested with the enzymes indi-

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cated by procedures suggested by the manufacturers (Bethesda Research Laboratories, Inc., New England Biolabs; and Boehringer Manheim Biochemicals) and separated on 0.5 to 1.5% agarose gels and electroeluted. The fragments were ligated to cleaved pBR322 and used to transform *Escherichia coli* DH-1 (18). The resulting colonies were screened with the appropriate ^{32}P-labeled, 0.62-kb *Cla*I-*Kpn*I fragment as a probe (17). To generate the subclones shown in Fig. 5, we isolated the 0.62-kb *Cla*I-*Kpn*I fragment of Mo-MuLV (2) and that of Fr-MuLV (see Fig. 3), and both were cloned in pCDV-1 (33) by the procedures outlined above.

RNase T1 fingerprints. Two-dimensional gel electrophoretic RNase T1 fingerprints of ^{32}P-labeled virus genomes and analysis of large T1-resistant oligonucleotides by digestion with pancreatic RNase were performed as described previously (10). The secondary digestion products of T1-resistant oligonucleotides were analyzed visually but were not quantitated.

Mice and tumor induction. Newborn (<2-day-old) NFS mice, supplied by the Small Animal Production Section of the National Institutes of Health, were inoculated by injection of 0.02 ml of undiluted tissue culture-grown virus, representing $10^{4.0}$ to $10^{4.4}$ PFU for Mo-MuLV and the MF recombinants and $10^{4.9}$ PFU for Fr-MuLV and the FMdrR recombinants. Mice received viruses either both intrathymically and intraperitoneally or intraperitoneally only and were sacrificed by ether or CO_2 anesthesia when disease was far advanced. Animals were autopsied and examined for gross evidence of erythroleukemia or lymphoma, and many animals, including all for which diagnosis was not obvious, were sampled for hematocrit testing and microscopic examination of blood smears or of spleen imprints or histological preparations or both. Diagnosis of erythroleukemia or lymphoma was based on the criteria outlined in Table 1. Myelogenous leukemia was diagnosed histologically when the splenic red pulp was enlarged and contained almost entirely immature myeloid cells compressing the white pulp. Similar myeloid cells usually infiltrated the hepatic portal areas and the medullae of lymph nodes. Mice with myelogenous leukemia had anemia, enlarged evenly colored spleens, hepatomegaly, and moderately enlarged lymph nodes.

RESULTS

Preparation of recombinants. Two restriction endonuclease sites, *Cla*I and *Kpn*I, shared by molecular clones of Mo-MuLV and Fr-MuLV allowed us to replace the 3' end region of Mo-MuLV encoding the C terminus of Prp15E, all of U3, and part of the R region of the LTR with the corresponding region of Fr-MuLV to generate recombinants designated MF (2, 13, 34, 38). The location of the *Cla*I and *Kpn*I sites on the virus genome and the sequences encoded between them in Fr-MuLV (22, 23) and Mo-MuLV (42) are shown in Fig. 1 and 2. The scheme used to construct the MF recombinants is shown in Fig. 3 (see also Materials and Methods). Two independent clones of MF, designated MF-3 and MF-15, yielded infectious virus after transfection into N1H/3T3 cells. The structure of the molecular clones was confirmed by restriction endonuclease analysis with *Kpn*I (see Fig. 3); *Xba*I, which cuts the 0.62-kb *Cla*I-*Kpn*I fragment of Mo-MuLV but not that of Fr-MuLV; and Sau3A, which cuts the 0.62-kb fragment of Fr-MuLV twice but that of Mo-MuLV once. RNase T1 fingerprints of the genomes of viruses obtained by transfection of MF-3 and MF-15 clones confirmed their genomic structure (Fig. 4). T1 fingerprints of MF viruses look very much like those of their Mo-MuLV parent (11); however, two large, well-separated RNase T1-resistant oligonucleotides (M20 and M24) encoded by the relevant 3' end *Cla*I-*Kpn*I fragment of Mo-MuLV were absent from MF virus genomes, whereas two oligonucleotides (F7 and F9) encoded by this region of Fr-MuLV (9) were present (see Fig. 2 and 4). Because even a very low level of contamination of MF recombinants by Fr-MuLV could lead to spurious results in these studies, MF-3 and MF-15 viruses obtained by transfection of the corresponding molecular clones were further purified biologically by end-point dilution. The identity of the resulting viruses, designated MF-3(LD) and MF-15(LD), was confirmed by RNase T1 fingerprinting (data not shown).

To exchange sequences within the LTR between Fr-MuLV and Mo-MuLV, we used the shared *Sau*3A sites located just 5' of the viral direct repeats in U3 (see Fig. 2) and the same *Kpn*I site in R that was used to construct the MF recombinants described above. The scheme for obtaining this 0.38-kb *Sau*3A-*Kpn*I fragment from Mo-MuLV and

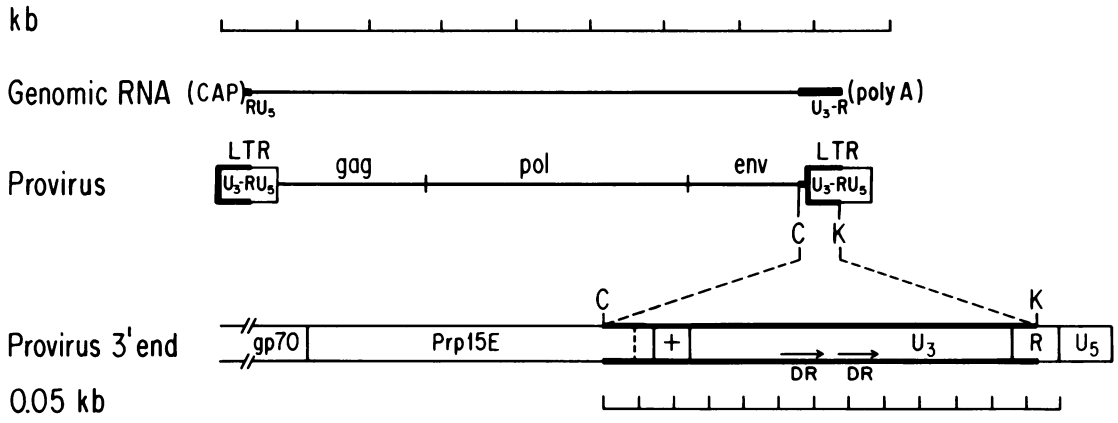

FIG. 1. Diagram showing the genomic location of the Mo-MuLV 3' end *Cla*I-*Kpn*I fragment replaced by the corresponding Fr-MuLV sequences in MF recombinants.

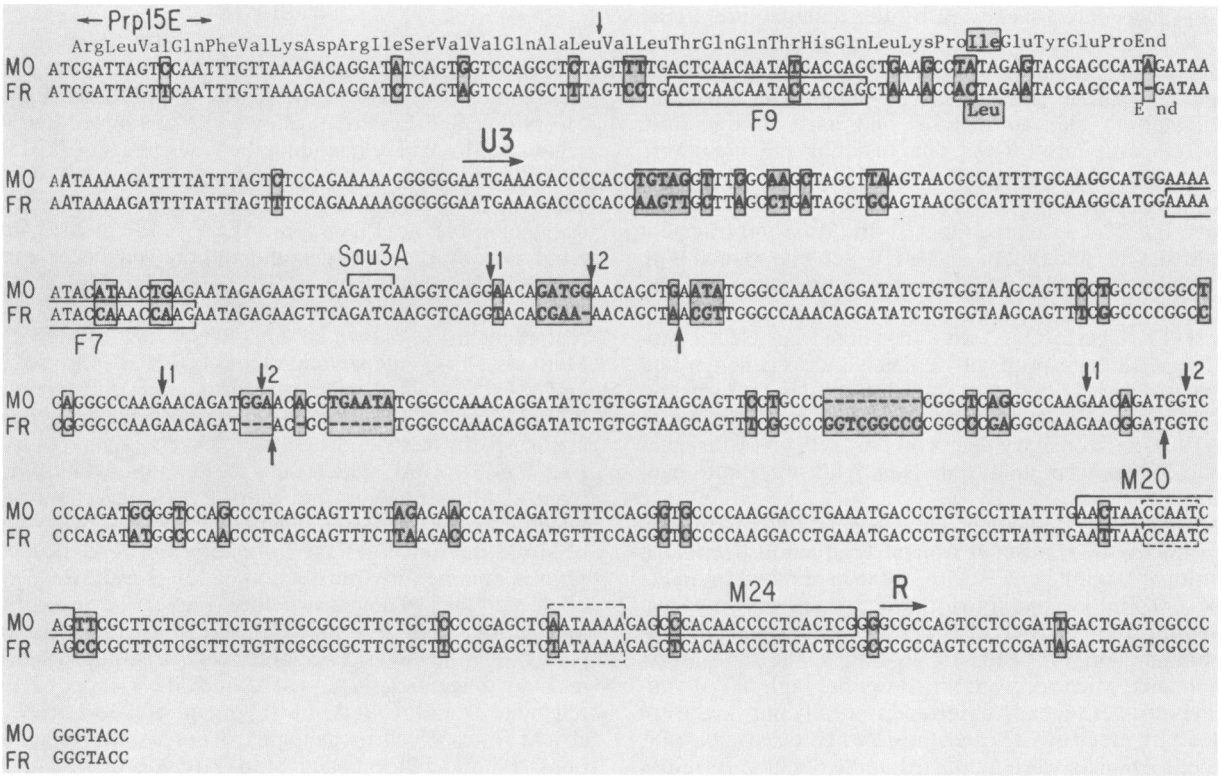

FIG. 2. Comparison of nucleotide sequences of Mo-MuLV and Fr-MuLV in the *Cla*I-*Kpn*I fragment used in this study. Mo-MuLV and Fr-MuLV sequences are from Shinnick et al. (42) and Koch et al. (22, 23), respectively. The fragment encompasses the 3′ end of the Prp15E gene, whose amino acids are indicated; all of U3; and a portion of R. Gray boxes enclose nucleotide differences between Fr-MuLV and Mo-MuLV. The arrow above the top line indicates the putative site of proteolytic cleavage within Prp15E to generate mature p15E. F9 and F7 and M20 and M24 are the Fr-MuLV- and Mo-MuLV-specific large RNase T1-resistant oligonucleotides, respectively, that were well separated on our RNase T1 fingerprints and thus diagnostic of the parental origin of the *Cla*I-*Kpn*I fragment in MF recombinants. Arrows above and below the third and fourth lines mark beginnings and ends of direct repeat sequences that probably encode the Mo-MuLV and Fr-MuLV enhancer elements. Note that a Mo-MuLV direct repeat can be drawn in two ways (marked 1, 2 in the figure), owing to the presence of three copies of a 10-base-long sequence that flanks the repeats.

TABLE 1. Criteria for classifying disease induced by parental and recombinant viruses

Determinant	Criteria for classifying disease as:	
	Erythroleukemia	Lymphoma[a]
Anemia	Severe (HCT, <30)[b]	Less marked (HCT, >30)
Gross pathology	Hepatosplenomegaly, normal or atrophied thymus, normal or minimally enlarged lymph nodes	Enlarged thymus or lymph nodes or both, splenomegaly, occasional hepatomegaly
Wright-Giemsa stain[c]	Erythroblasts: deeply basophilic cytoplasm, more condensed chromatin, variable numbers of normoblasts	Lymphoblasts: less basophilic cytoplasm, less condensed chromatin, morphologically uniform population
Histology		
Spleen	Blast infiltration of expanded red pulp, compressed white pulp	Blast infiltration of expanded white pulp
Liver	Blast infiltration within sinusoids	Occasional blast infiltration of periportal areas
Thymus	Normal	Diffuse infiltration with lymphoblasts, destroying architecture of organ
Lymph nodes	Often enlarged germinal centers, architecture preserved; very rare infiltration with erythroblasts	Diffuse infiltration with lymphoblasts, destroying architecture of organ

[a] Thymic or nonthymic lymphoblastic lymphoma of T cell type.
[b] HCT, Hematocrit value. For MF recombinants, the median value was 21 for 38 mice; for FMdr recombinants, the median was 34 for 15 mice.
[c] Performed on blood smears or splenic imprints.

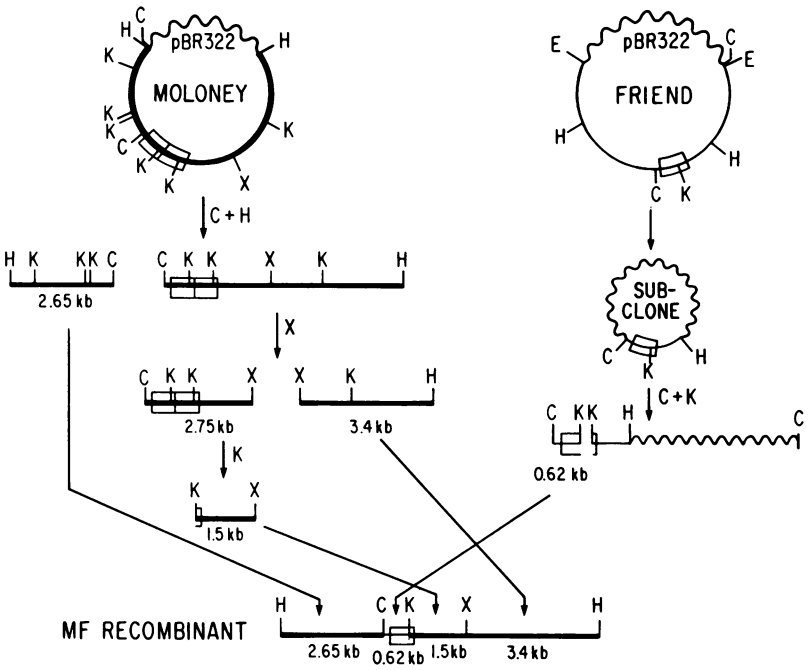

FIG. 3. Schematic diagram of the construction of the MF recombinant. Mo-MuLV and Fr-MuLV parents were cloned in pBR322 at their unique *Hin*dIII and *Eco*RI sites, respectively. We prepared a 1.4-kb *Hin*dIII-*Cla*I subclone of Fr-MuLV in pBR322. The Mo-MuLV clone and the Fr-MuLV subclone were digested with the indicated enzymes (H, *Hin*dIII; E, *Eco*RI; C, *Cla*I; K, KpnI; X, *Xho*I) to generate four fragments that were isolated from gels and ligated with pBR322 to generate the MF recombinant. Heavy lines represent Mo-MuLV DNA, thin lines represent Fr-MuLV DNA, and wavy lines represent pBR322 DNA.

inserting it into the Fr-MuLV genome to generate recombinants designated FMdrR is shown in Fig. 5. Two independent clones of FMdrR, FMdrR-1 and FMdrR-2, were prepared. RNase T1 fingerprints confirmed the structure of the FMdrR genome (data not shown). This recombinant possesses Mo-MuLV-specific T1 oligonucleotides M20 and M24 (see Fig. 2), but otherwise its T1 fingerprint resembles that of Fr-MuLV and incluces T1 oligonucleotides F7 and F9.

MF recombinants induce primarily erythroleukemia in newborn NFS mice. Newborn NFS mice were injected with MF recombinant viruses [MF-3, MF-15, MF-3(LD), and MF-15(LD)] and with Fr-MuLV and Mo-MuLV obtained by transfection of the parental clones used to construct MF recombinants. All viruses induced disease within 1½ to 4 months (Fig. 6). Criteria used for the diagnosis of erythroid or lymphoid disease are summarized in Table 1. As expected, Mo-MuLV induced T cell lymphomas, primarily thymomas, whereas Fr-MuLV induced exclusively erythroleukemias (Table 2).

The MF recombinants induced primarily erythroleukemias (Tables 2 and 3), and there was no observable difference between the two independent clones of MF (MF-3 and MF-15) or their biologically cloned derivatives [MF-3(LD) and MF-15(LD)]. Of 57 mice diagnosed, 53 developed erythroleukemia, 1 developed a thymic lymphoma, and 3 developed myelogenous leukemias (Table 2). The four non-

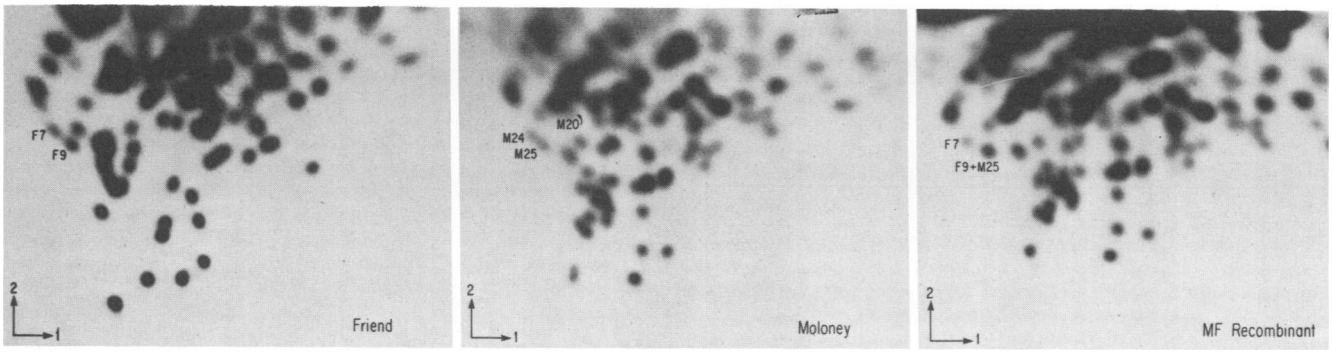

FIG. 4. Two-dimensional gel electrophoretic RNase T1 fingerprints of ^{32}P-labeled 70S RNA of Fr-MuLV, Mo-MuLV, and MF-3. Electrophoresis in the first dimension was from left to right and that in the second dimension was from bottom to top. The Fr-MuLV and Mo-MuLV T1 oligonucleotides (F7, F9, M20, and M24) used to confirm the parental (Fr-MuLV) origin of the 3' end *Cla*I-*Kpn*I segment of MF are indicated by arrows.

TABLE 2. Pathogenicity in NFS mice of Mo-MuLV, Fr-MuLV, and the MuLV recombinants MF and FMdrR

Virus[a]	No. of mice dead or diseased/no. observed	No. autopsied	No. with following diagnosis[b]:			
			E	L		M
				T	N	
Mo-MuLV	16/16	15	0	11	4	0
Fr-MuLV	55/55	50	50	0	0	0
MF-3[c]	21/21	21	20	0	0	1
MF-15[c]	36/36	36	33	1	0	2
FMdrR-1	9/9	9	1[d]	4	4	0
FMdrR-2	11/11	11	0	9	2	0

[a] Mo-MuLV and Fr-MuLV were inoculated intrathymically, intraperitoneally, or intrathymically and intraperitoneally. MF viruses were inoculated intraperitoneally or intrathymically and intraperitoneally. FMdrR viruses were inoculated intraperitoneally.

[b] Diagnoses were based on the criteria shown in Table 1. E, Erythroleukemia; L, lymphoblastic lymphoma, either thymic (T) or non-thymic (N); M, myelogenous leukemia.

[c] Since no significant difference was found in the pathogenicity of molecularly cloned MF viruses and their biologically cloned derivatives [MF-3(LD) and MF-15(LD)], the data obtained with both stocks are pooled. Although no difference was seen between independent MF clones (MF-3 and MF-15) or independent FMdrR clones (FMdrR-1 and FMdrR-2), data for independent clones is shown separately.

[d] This mouse also had microscopic evidence of lymphoblastic lymphoma.

erythroid leukemias induced by MF viruses occurred relatively early (see Fig. 6). Although the type of disease induced by MF recombinants resembled that induced by Fr-MuLV, the latent period of disease (median time, 82 days) was slightly longer than that of Fr-MuLV (median time, 50 days) and was similar to that of Mo-MuLV (median time, 78 days) (Fig. 6). The longer latent periods might be due to the slightly lower infectivity titers of the MF and Mo-MuLV stocks; this seems unlikely, however, since mice inoculated with parental Fr-MuLV diluted to contain equivalent numbers of PFU developed erythroleukemia with a median latent period of 56 days (data not shown).

Virus was recovered from the diseased, erythroleukemic spleen of one animal injected with MF-3(LD) virus. An RNase T1 fingerprint of its genome confirmed that the virus was indeed an MF recombinant.

FMdrR recombinants induce primarily lymphomas in NFS mice. With the exception of a single erythroleukemia, mice inoculated with the FMdrR recombinant viruses developed lymphoblastic lymphomas, about one third being of non-

TABLE 3. Correlation between hematocrit values and diagnosis in NFS mice inoculated with MF and FMdrR recombinant viruses

Hematocrit	No. of mice with indicated disease after inoculation with[a]:					
	MF			FMdrR		
	E	L (T)	M	E	L	
					T	N
≤14	1					
15–19	10					
20–24	8		1	1[b]		
25–29	6	1	1			
30–34					4	2
35–39					2	2
≥40					1	1

[a] Diagnoses were based on microscopic histological examination or hematological examination or both. E, Erythroleukemia; L, lymphoblastic lymphoma, thymic (T) or nonthymic (N); M, myelogenous leukemia. Data from independent clones of MF or FMdrR are pooled.

[b] This mouse also had evidnece of lymphoblastic lymphoma.

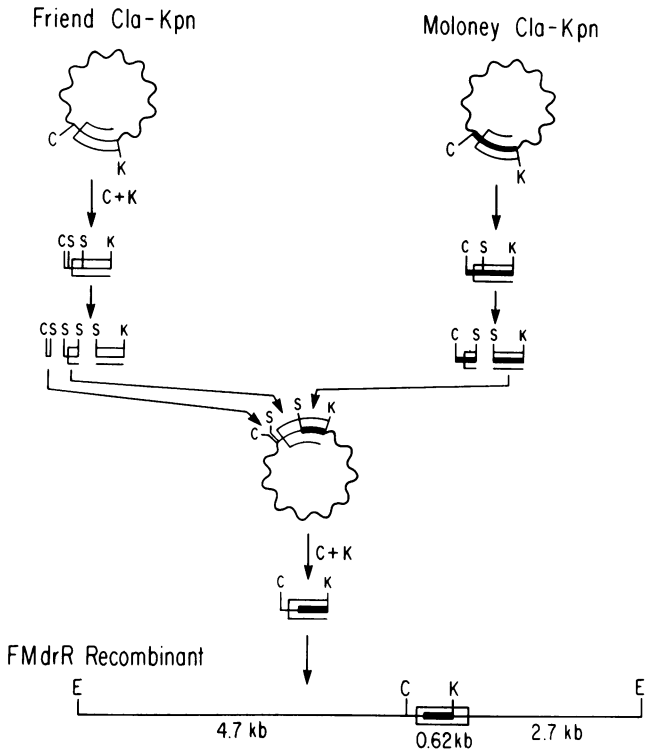

FIG. 5. Schematic diagram for the construction of the FMdrR recombinant. The 0.62-kb *Cla*I to *Kpn*I fragments of Mo-MuLV and Fr-MuLV were subcloned in the plasmid pCDV-1. These subclones were digested with the indicated enzymes (C, *Cla*I; K, *Kpn*I; S, *Sau*3A). The 4.7- and 2.7-kb fragments of the Fr-MuLV clone were isolated as described previously (2). Heavy lines represent Mo-MuLV DNA, thin lines represent Fr-MuLV DNA, and wavy lines represent pCDV-1 DNA.

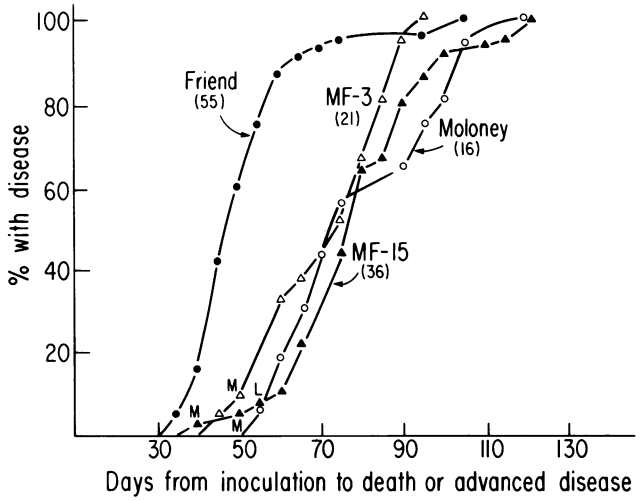

FIG. 6. Incidence of tumor induced in NFS mice by Fr-MuLV, Mo-MuLV, and two clones of MF recombinant viruses. Symbols adjacent to some MF data points represent pathological diagnoses of those rare animals that did not have erythroleukemia. L, Lymphoma; M, myelogenous leukemia. Mice received MF viruses either both intrathymically and intraperitoneally or intraperitoneally only. Numbers in parentheses indicate the number of mice injected.

thymic origin (Tables 2 and 3). The median latent period was 69 days. In the exceptional case in which erythroleukemia was diagnosed, there was also evidence of lymphoblastic lymphoma: clear-cut extensive proliferation of erythroblasts within the splenic red pulp and many erythroid precursors were observed within hepatic sinusoids, but in addition, lymphoblastic infiltration was seen in the thymus, and to a lesser extent, diffusely, within lymph nodes. The liver and spleen were not sites of lymphoblastic infiltration.

Hematocrit values were found to be predictive with a high degree of confidence of the final hematopathological diagnosis (Table 3).

DISCUSSION

The ability to switch the disease specificities of nondefective Fr-MuLV and Mo-MuLV by exchanging 0.62 or even 0.38 kb of information near the 3' ends of their genomes indicates that strong determinants of the different disease specificities of the viruses lie within this small region (Fig. 2). This region includes the virus transcriptional enhancers. There is now considerable evidence for tissue-specific enhancer function in various biological systems, and it seems likely that the 3' end determinants of different disease specificity we have identified are the virus enhancer sequences of Fr-MuLV and Mo-MuLV that lie within the direct repeat sequences in the virus U3 regions (2, 6, 14, 15, 25).

We do not feel that our studies exclude the possibility that virus genes outside the extreme 3' end of the genome may also play a role in determining the complex phenotype of MuLV disease specificity. In fact, available evidence indicates that both the ability to cause leukemia and the type of disease induced may have multiple virus genetic determinants in both murine (30, 31, 46; C. A. Holland, J. W. Hartley, W. P. Rowe, and N. Hopkins, submitted for publication) and avian (40, 49) retroviruses. It seems, however, that a single, very strong determinant of either phenotype may mask or obviate the contribution of other, weaker genes (7, 27). For example, in this study the recombinant called FMdrR, whose genome was derived primarily from Fr-MuLV but a portion of whose LTR was from Mo-MuLV, induced one erythroleukemia in addition to 19 T cell lymphomas (see also reference 2). Since presumably the Mo-MuLV sequences in FMdrR could not have contributed the ability to induce erythroleukemia, Fr-MuLV must have determinants of erythroleukemogenicity outside the 3' end *Sau*3A-*Kpn*I fragment. The Fr-MuLV *env* gene is probably such a determinant, as indicated by the studies of Oliff and colleagues (35–37).

Two mechanisms of MuLV-induced leukemogenesis have been proposed. One, by analogy to avian retroviruses (12, 20, 39), is that DNA copies of the viruses integrate adjacent to cellular oncogenes and thereby activate them, a model for which there is increasing evidence with murine retroviruses that induce T cell lymphomas (4, 5, 26, 50; Y. Li, C. A. Holland, J. W. Hartley, and N. Hopkins, Proc. Natl. Acad. Sci. U.S.A., in press). The second proposed mechanism is that certain virus envelope glycoproteins are mitogens for lymphoid or erythroid cells (8, 29, 32). The two models are not mutually exclusive. Tissue-specific enhancers could be expected to play a role in determining the type of leukemia induced by either mechanism.

The possibility that transcriptional signals possibly are the determinant of the disease specificity that we have identified receives support from the recent finding of Chen et al. (3) of tissue-specificity in LTRs of the human retrovirus HTLVII.

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