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Genetics

Role for the 3′ end of the genome in determining disease specificity of Friend and Moloney murine leukemia viruses

(*in vitro* recombinants/erythroleukemia/T-cell lymphoma)

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ABSTRACT **To probe the genetic basis of disease specificity of nondefective murine type C viruses, we are constructing recombinants *in vitro* between molecular clones of Friend murine leukemia virus (Fr-MuLV) and Moloney murine leukemia virus (Mo-MuLV). Fr-MuLV induces erythroleukemias when injected into newborn NFS mice, whereas Mo-MuLV almost invariably induces T-cell lymphomas. We find that a recombinant whose genome is derived primarily from Fr-MuLV but which has 621 nucleotides of Mo-MuLV information at its 3′ end induces almost exclusively thymic lymphomas. The sequences derived from Mo-MuLV include 99 nucleotides encoding the carboxyl terminus of Prp15E, the origin of DNA +-strand synthesis, all of the U3 region, and 36 nucleotides of the R portion of the long terminal repeat. When the segment of Mo-MuLV was removed and replaced with the comparable segment from Fr-MuLV, the virus was again erythroblastosis-inducing. These results, in conjunction with studies from other laboratories [Laimins, L. A., Khoury, G., Gorman, C., Howard, B. & Gruss, P. (1982) *Proc. Natl. Acad. Sci. USA* 79, 6453–6457], suggest that transcriptional signals in U3 may determine tissue tropism and hence influence disease specificity ("targeting") of murine leukemia viruses.**

Nondefective, oncogenic murine type C viruses that possess highly related genomes can differ substantially in their disease-inducing phenotypes. Differences between viral isolates include the latent period of disease induction and the type of leukemia or lymphoma induced. Furthermore, these phenotypes can vary for a particular virus depending on the genetic constitution of the host. Two murine leukemia viruses (MuLVs) whose disease-inducing phenotypes have been studied extensively are Moloney MuLV (Mo-MuLV) and the nondefective component of the Friend virus complex, sometimes called Friend helper virus or Fr-MuLV. When injected into newborn NFS mice, Mo-MuLV induces T-cell lymphomas, while Fr-MuLV induces erythroleukemias. The latent period for the two viruses is similar, ≈1.5–3.5 months, with Fr-MuLV-induced disease appearing somewhat earlier than Mo-MuLV-induced lymphomas (unpublished data).

Recently we obtained evidence suggesting that an *in vitro* host-range property of certain MuLVs is determined by the U3 portion of the viral long terminal repeat (LTR) (unpublished data). U3, a 4- to 500-nucleotide-long sequence that is located near the 3′ end of MuLV genomic RNA and at both ends of proviral DNA (see Fig. 1), contains transcriptional signals, a "TATA" sequence, and a directly repeated sequence of about 100 nucleotides that is an analogue of the simian virus 40 enhancer sequence (ref. 1; see ref. 2 for review). The possibility that a host-range property of MuLVs may reside in U3 led us to ask whether exchanging the U3 regions of Fr-MuLV and Mo-MuLV might alter the disease specificity of the viruses. Here we report that when a 621-nucleotide-long fragment of the Mo-MuLV genome encompassing U3 replaced the corresponding region in the Fr-MuLV genome, the resulting recombinant virus induced almost exclusively T-cell lymphomas. The reciprocal recombinant has not yet been studied.

Previous studies by Scolnick and his collaborators (3) localized the genes required for the erythroleukemia-inducing phenotype of Fr-MuLV to a 4.1-kilobase (kb) segment encompassing the *env* gene, the 3′ LTR, and an amino-terminal portion of *gag*.

MATERIALS AND METHODS

Cells and Viruses. NIH 3T3 (4) and BALB/3T3 (5) cells were used for all transfection (6) and plaque-forming studies (7). Infectious DNA clones of ecotropic Mo-MuLV and ecotropic nondefective Fr-MuLV clone 57 were obtained from S. Goff (8) and A. Oliff (9), respectively. Mo-MuLV and Fr-MuLV are cloned into pBR322 in permuted form at the *Hin*dIII and *Eco*RI sites, respectively.

Molecular Cloning of Recombinant Viruses. Site-specific recombinant viruses were cloned by using both the bacteriophage λgt Wes λ-B and plasmid pBR322 as cloning vehicles. Cloning and screening of the recombinant virus-containing clones as described in the text were accomplished by using standard methods.

Transfection of Cloned Recombinant Viruses. Cloned viral DNA was transfected on NIH 3T3 or BALB/3T3 cells by using slight modifications of the calcium phosphate precipitation method (6). XC-plaque (7) and reverse-transcriptase assays were performed as described (10). Two-dimensional gel electrophoretic RNase T1 oligonucleotide patterns of the genomes of infectious viruses were prepared as described (11).

Mice. Infant (<2 days old) NFS mice were obtained from the Small Animal Production Section of the National Institutes of Health. Inoculations were by intrathymic (i.t.) or intraperitoneal (i.p.) injection (or both) of 0.02 ml of undiluted tissue culture virus stock representing $10^{4.8}$–$10^{5.3}$ plaque-forming units of virus per mouse. Animals were sacrificed when severely ill, and gross and microscopic pathologic examinations were carried out.

RESULTS

Construction and Molecular Cloning of Site-Specific Recombinants. Two restriction endonuclease sites shared by mo-

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Abbreviations: MuLV, murine leukemia virus; Fr-MuLV, Friend MuLV; Mo-MuLV, Moloney MuLV; LTR, long terminal repeat; i.p., intraperitoneal(ly); i.t., intrathymic(ally); kb, kilobase(s).

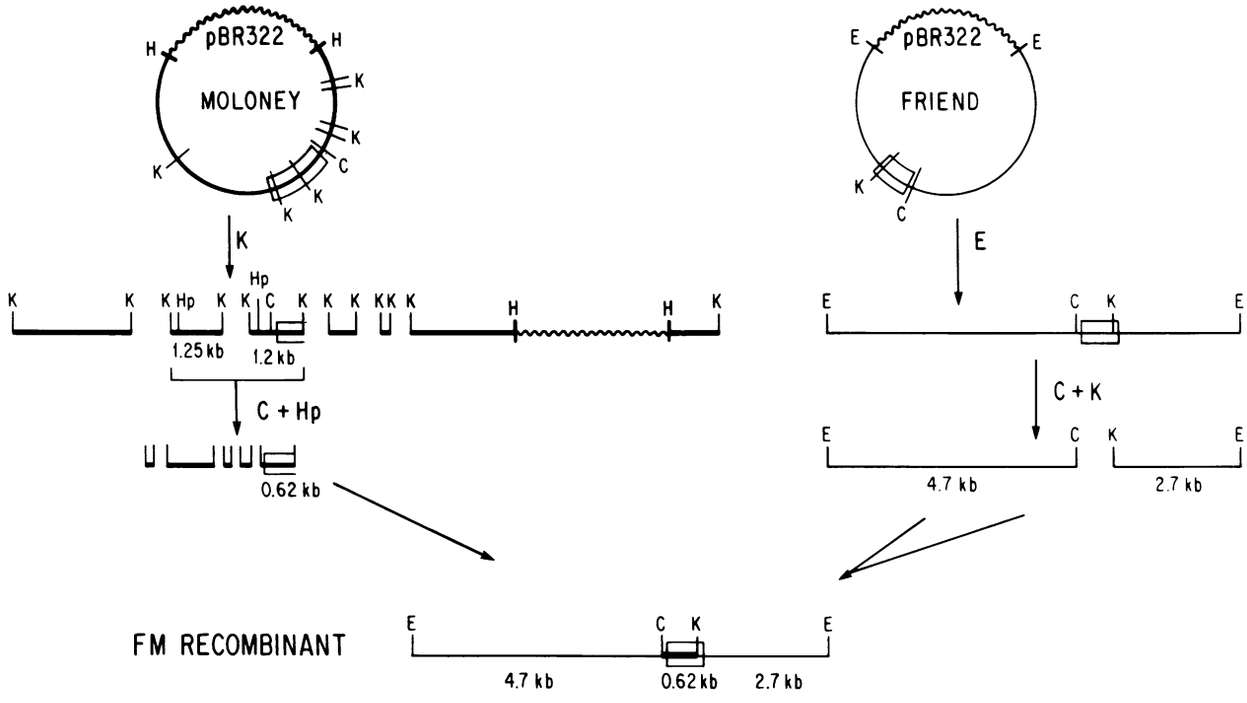

FIG. 1. Schematic diagram for the molecular cloning of the FM recombinant. Mo-MuLV and Fr-MuLV parents were cloned in pBR322 at the unique *Hin*dIII (H) and *Eco*RI (E) sites, respectively. The Mo-MuLV clone was cut with *Kpn* I (K) to generate six fragments. The desired 1.2-kb fragment comigrated with a 1.25-kb fragment. These fragments were isolated together and cut with *Cla* I (C) and *Hpa* I (Hp) to generate five fragments. The 0.62-kb C–K fragment clearly separated from the other fragments and was isolated. The Fr-MuLV clone was cut with *Eco*RI (E) to generate the full-length Fr-MuLV insert in a permuted form. This insert was then isolated and cut with *Cla* I (C) and *Kpn* I (K) to generate three fragments. The 4.7-kb E–C fragment, the 2.7-kb K–E fragment, and the 0.62-kb Mo-MuLV C–K fragment were ligated to λ phage *Eco*RI arms and packaged *in vitro*. The phage plaques were screened by using a ^{32}P-labeled 0.62-kb C–K DNA fragment as probe.

lecular clones of Fr-MuLV and Mo-MuLV allowed us to investigate the role of the 3′ end of the viral genome in determining disease specificity (refs. 3 and 12; unpublished data). A single *Cla* I site is located ≈8.05 kb from the 5′ end of viral DNAs within sequences encoding the Prp15E portion of *env*, while a common *Kpn* I site lies in the R region of the proviral LTRs (Fig. 1). The 0.62-kb DNA fragment lying between these two sites encodes the carboxyl terminal 33 amino acids of Prp15E, the last 17 of which are proteolytically removed in generating mature virion p15E (14, 15), 39 nucleotides believed to encode the origin of DNA +-strand synthesis (14, 15), all of U3, and 36 nucleotides of R. R encodes a short direct repeat, ≈70 nucleotides long in MuLVs, located at the termini of genomic RNA (1).

To introduce the 3′ terminus of Mo-MuLV into Fr-MuLV, the DNA clone of Mo-MuLV was digested with restriction endonucleases to produce the 0.621-kb *Cla* I–*Kpn* I fragment, while the DNA clone of Fr-MuLV was digested to yield a 4.7-kb *Eco*RI–*Cla* I fragment and a 2.7-kb *Kpn* I–*Eco*RI fragment (see Fig. 1). These fragments were eluted from the gel and ligated to form recombinant clone FM as shown in Figs. 1 and 2. FM was cloned in a permuted form by using *Eco*RI λ phage arms. The structure of FM recombinant clones could be confirmed by restriction endonuclease digestion because Mo-MuLV contains an *Xba* I site in its U3 region, whereas Fr-MuLV does not. FM clones possess one copy of the LTR.

FF recombinants, in which the 0.62-kb *Cla* I–*Kpn* I fragment of Fr-MuLV was introduced back into an FM recombi-

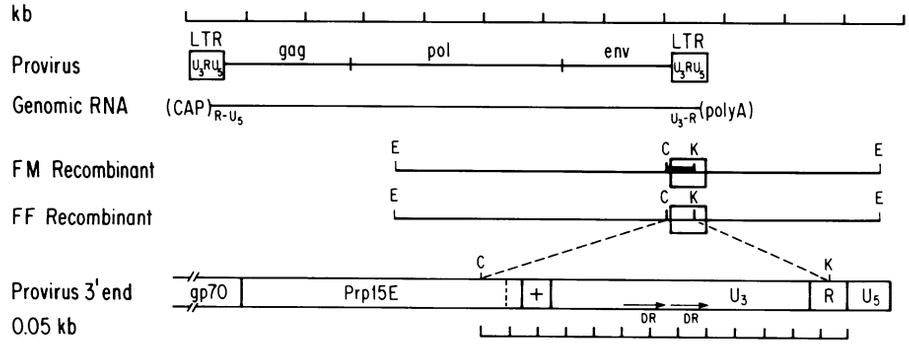

FIG. 2. Genomic structure of FM and FF recombinant viruses. The structure of integrated proviral DNA is shown at the top, with the viral genome below it, followed by the structures of the FM and FF recombinants at the DNA level. ——, Sequences derived from Fr-MuLV; ▬, sequences derived from Mo-MuLV. The relative positions of the restriction enzyme sites used to generate the recombinants are shown: E, *Eco*RI; C, *Cla* I; and K, *Kpn* I. The last line shows an expansion of the 0.62-kb *Cla* I–*Kpn* I fragment, which was replaced in the recombinants. This region consists of 99 nucleotides encoding the carboxyl-terminal end of Prp15e, the origin of DNA +-strand synthesis, U3 (containing two copies of the direct repeat sequences *Dr Dr*), and 36 nucleotides of the R portion of the LTR.

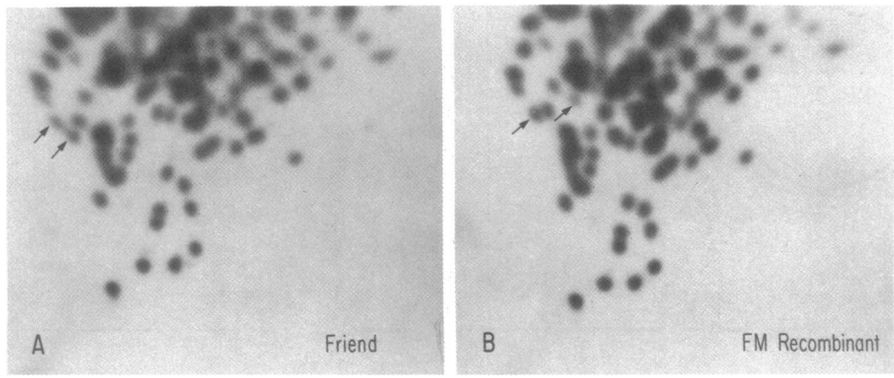

FIG. 3. Two dimensional-gel electrophoretic oligonucleotide patterns of RNase T1-resistant products from ^{32}P-labeled 70S RNA of Fr-MuLV (*A*) and FM recombinant virus (*B*). Arrows in *A* and *B* indicate oligonucleotides that are unique to Fr-MuLV and the FM recombinant virus, respectively.

nant to regenerate Fr-MuLV (see Figs. 1 and 2), were constructed in essentially the same way as FM recombinants.

Infectivity of FM and FF Recombinant Clones. Inserts from the FM or FF DNA clones were isolated from gels, ligated, and transfected onto either NIH 3T3 or BALB/3T3 cells. Infectious clones were identified by XC-plaque formation of the transfected cells and by detection of reverse-transcriptase activity in culture fluids 7 days after transfection.

Two-dimensional gel electrophoretic oligonucleotide patterns of RNase T1-resistant products were prepared of infectious viral isolates. Fr-MuLV and FM recombinants could be distinguished by four well-resolved large RNase T1-resistant oligonucleotides (Fig. 3). As expected, oligonucleotide patterns of recombinant FF viral genomes looked like those of Fr-MuLV.

Disease Specificity Tests. Parental Fr-MuLV and recombinant viruses obtained from DNA transfections were injected i.t. or i.p., or both, into infant NFS mice. As expected, Fr-MuLV induced erythroleukemia after 1.5–2.5 months (Fig. 4); the mice developed severe anemia and, at autopsy, showed marked splenomegaly, often enlargement and mottling of the liver, and no enlargement of thymus or lymph nodes. Histology showed typical erythroleukemia. As in other mouse strains, Mo-MuLV induced T-cell lymphomas in NFS mice; about one-third of these were of nonthymic origin (unpublished data).

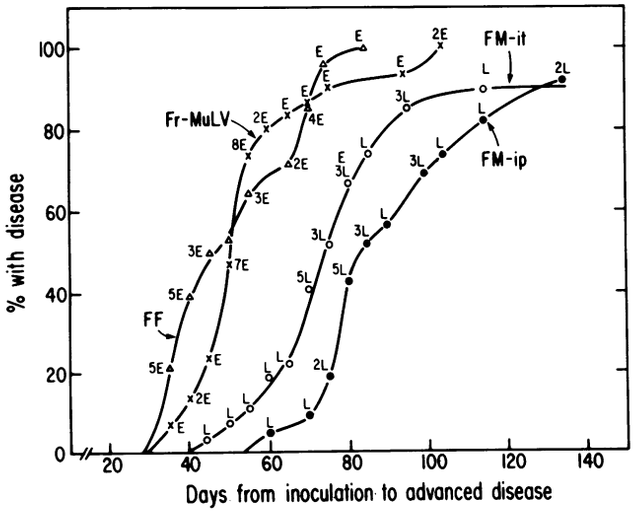

FIG. 4. Incidence and forms of tumor induced by Fr-MuLV (×) and by FM (●, ○) and FF (△) recombinant viruses. The symbols adjacent to the data points are the number of animals autopsied and their pathologic diagnoses: E, erythroblastosis; and L, lymphoma. The mice injected with Fr-MuLV and FF viruses were generally inoculated both i.t. and i.p.; the FM-it mice were inoculated either i.t. plus i.p. or i.t. only; and the FM-ip mice were inoculated i.p. only.

Strikingly, two independent isolates of recombinant FM induced T-cell lymphomas. In a total of 60 animals injected, 51 died after a latent period of 2–3.5 months, and 47 of these were autopsied; 46 had T-cell lymphomas and 1 had erythroleukemia. Of the 46 lymphomas, 36 were of thymic origin. Histologically, the tumors were typical T-cell lymphomas with involvement of spleen, lymph nodes, liver, and kidney. Fig. 4 shows the results with one recombinant, FM-I 2.

To confirm the identity of the recombinant virus that induced the T-cell lymphomas, virus was recovered from an extract of an FM-induced thymoma, and the two-dimensional gel electrophoretic oligonucleotide pattern of its RNase T1-resistant products was prepared. This autoradiogram was indistinguishable from that of injected FM virus.

To further demonstrate that it was indeed the presence of Mo-MuLV genetic information in recombinant FM that caused it to induce T-cell lymphomas, rather than an undetected secondary alteration in some part of the Fr-MuLV genome, we tested the disease specificity of recombinants designated FF (see above), derived from FM recombinants by restoring the Fr-MuLV *Cla* I–*Kpn* I segment. This recombinant should have a genome structure identical to that of Fr-MuLV. As expected, FF viruses induced erythroleukemias identical to those induced by parental Fr-MuLV (Fig. 4).

Route of inoculation did not affect the type of disease induced; that is, both i.t. and i.p. inoculation of FM virus gave thymic lymphomas, although with longer latent periods in the i.p. injected mice (Fig. 4). Conversely, i.t. injection of Fr-MuLV and FF viruses induced only erythroleukemias.

DISCUSSION

The nondefective murine leukemia viruses Mo-MuLV and Fr-MuLV induce significantly different types of neoplasms. The data presented here show that substitution of 621 nucleotides at the extreme 3′ end of the Fr-MuLV genome with the corresponding region of Mo-MuLV converts an almost exclusively erythroblastosis-inducing virus into one that induces almost exclusively thymomas in NFS mice. Although these results clearly demonstrate that sequences encompassing U3 influence disease specificity of MuLVs, it seems unlikely that this small piece of genetic information is the sole determinant of disease targeting. Many studies have suggested that disease specificity in nondefective murine and avian leukemia viruses is a multigene phenomenon, being determined by host genetic factors (16), age of injected recipient, and the genetic structure of the virus (17–19).

Studies by Scolnick and his collaborators with the Fr-MuLV–erythroleukemia system have implicated the 3′ portion of the genome (gp70, p15E, LTR) as playing a determining role in the specificity of Fr-MuLV-associated disease (3, 20, 21). Oliff *et al.* (3) molecularly cloned a subgenomic 4.1-kb fragment of Fr-MuLV that contains the gene(s) responsible for Fr-MuLV-induced erythroleukemia. This 4.1-kb DNA fragment includes the gp70 and p15E coding sequences, the origin of DNA +-strand synthesis, the LTR sequences, and 0.5 kb from the 5′ end of the genome. This result is clearly compatible with the findings we have reported here.

Linemeyer *et al.* (21) showed that mutations introduced into the gp52-encoding gene of defective Friend spleen focus-forming virus (SFFV) destroyed its disease-inducing potential, indicating that for spleen focus-forming virus, gp52 is crucial to disease induction. This result does not rule out a role for the LTR in contributing to the disease specificity of spleen focus-forming virus; furthermore, our data do not rule out a role for gp70 in Fr-MuLV-induced erythroleukemia. It seems quite possible that both genetic elements—envelope glycoprotein and the LTR—might play a role in disease induction.

One might argue that the introduction of Mo-MuLV information into Fr-MuLV does not so much confer thymic lymphomagenesis as remove a determinant of erythrotropism, allowing expression of a latent phenotype of Fr-MuLV. However, our observations argue against this interpretation and in favor of the introduction of thymotropism determinants present in Mo-MuLV. First, the time course of induction of thymic neoplasms by FM viruses and of erythroid neoplasms by Fr-MuLV in these studies overlap considerably. If Fr-MuLV possessed a potential for inducing thymic neoplasms equal to that exhibited by recombinant FM, one would expect Fr-MuLV to give rise to a mixture of thymic and erythroid neoplasms in NFS mice. Careful histologic examination of the thymuses of mice with Fr-MuLV-induced erythroleukemia showed no evidence of tumor, even in animals with the longer incubation periods. Thus, the results argue that a preference for inducing thymic neoplasm resides in part within a 621-nucleotide fragment from the 3′ end of Mo-MuLV. Because Fr-MuLV can induce T-cell lymphomas in certain strains of mice and occasionally in older NFS mice (unpublished data), it seems most likely that a latent potential of the virus has been enhanced in the FM recombinant by the introduction of Mo-MuLV information.

The 621-nucleotide fragment studied in our experiments encompasses transcriptional signals in U3, a sequence 5′ of U3 required for initiation of DNA +-strand synthesis, and sequences coding for the carboxyl terminus of Prp15E. We do not yet know which of these sequences is important in influencing disease specificity. However, it is tempting to speculate that the enhancer sequence in U3 may have tissue specificity as suggested by Laimins *et al.* (ref. 22; see also ref. 23), allowing more efficient viral expression in certain target cells, which in turn affects the disease process.

The thymotropism of the FM recombinants does not represent a qualitative change from the Fr-MuLV parent because the latter can replicate in NFS thymocytes, though less efficiently than do the FM recombinants (unpublished data). However, this does not necessarily imply that the determining effect of the 3′ genome segment on thymic lymphomagenesis represents a direct effect on efficiency of thymocyte transformation. Because both thymic lymphomagenesis and Fr-MuLV erythroleukemia may require generation of mink cell focus-forming recombinant MuLVs (24, 25), the possibility must be considered that the 3′ segment in some way determines which type(s) of mink cell focus-forming virus will be generated, which in turn is the determinant of tissue specificity.

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