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Disease Specificity of Nondefective Friend and Moloney Murine Leukemia Viruses Is Controlled by a Small Number of Nucleotides

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Moloney murine leukemia virus induces T cell lymphomas after injection into NFS mice, whereas the nondefective Friend virus induces erythroleukemias. Previous studies showed that sequences encompassing the viral transcriptional signals in U3 are the primary determinant of this phenotype in recombinants between these two viruses. To more precisely identify the sequences responsible, we constructed additional recombinants, within U3, between Friend and Moloney viruses and assayed these recombinants for their disease specificity. We found that a fragment 191 bases long that included the direct repeat (enhancer) region plus 22 nucleotides to its 3′ side from Friend virus was sufficient to convert Moloney virus to a virus that induced only erythroleukemias. A 171-base-long fragment of Moloney virus, including just the direct repeat, converted Friend virus to a virus that induced primarily lymphomas (about 85% of mice injected). We also constructed Moloney and Friend virus variants with one rather than two copies of the enhancer element. These viruses retained their disease specificity, although they exhibited a marked increase in the latent period of disease induction. Together the results suggest that 25 or fewer nucleotide differences, lying within and also just 3′ of the direct repeat, are the primary determinant of the distinct disease specificities of nondefective Friend and Moloney viruses.

Replication-competent murine leukemia viruses (MuLVs) that lack oncogenes exhibit strikingly different disease induction spectra. For example, Moloney MuLV causes T-cell lymphomas when injected into newborn NFS mice, whereas the nondefective Friend MuLV usually induces erythroleukemia in this strain (35). Previously, studies from our laboratories and others showed that sequences at the 3′ end of the viral genome are a potent determinant of disease specificity (4, 5, 19, 34). In recombinants constructed between molecular clones of Moloney and Friend viruses, sequences encompassing the transcriptional signals in the U3 region of the long terminal repeat (LTR) were the primary determinant of T-cell lymphoma induction versus erythroleukemogenicity. Thus, a recombinant virus whose genome is derived primarily from Friend but whose U3 region is derived from Moloney induces mostly T-cell lymphomas, while a virus whose U3 region is derived from Friend but whose remaining genes are derived from Moloney induces mostly erythroleukemias. The smallest fragment exchanged in these studies, 0.4 kilobase (kb), included the direct repeat that possesses the properties of a transcriptional enhancer (23, 25), as well as all promoter elements lying between the repeat and the cap site (5). These include TATA and CAT sequences, as well as a GC-rich element just 3′ of the direct repeat defined by Laimins et al. (23) in the Moloney sarcoma virus LTR.

While we had postulated that different tissue specificities of the Friend and Moloney enhancer elements could explain the results we had obtained, it is also possible that the viral promoter elements contribute to this phenotype. In any case, it was of interest to more precisely identify the sequences within U3 that determine the distinct disease specificities of Friend and Moloney viruses so that we might better understand the mechanisms that underlie this important viral phenotype. To this end we constructed and analyzed the disease-inducing properties of additional recombinants within U3 between Friend and Moloney viruses. We also studied the disease specificity of Friend and Moloney viruses from which we had deleted one of the two copies of the enhancer element.

MATERIALS AND METHODS

Cells and parental viruses. NIH 3T3 (21) and BALB/3T3 (1) cells were used for DNA transfections of virus clones. Infectivity was assayed by the XC plaque assay (32) and the reverse transcriptase assay (2). Infectious DNA clones of Moloney virus and nondefective ecotropic Friend virus clone 57 were from S. Goff (13) (Columbia University) and A. Oliff (30) (Merck, Sharp, and Dohme), respectively.

Construction of subclones recombinant in the U3 region. To construct the desired recombinants, we began with the subclones pFrCK, pMoCK, and pFrdrD (see Fig. 2A) (4). Appropriate DNA fragments (see Fig. 2) were isolated by stepwise restriction endonuclease digestion, separated by agarose gel electrophoresis, electroeluted from the gel, and concentrated by ethanol precipitation. Restriction endonucleases were from New England Biolabs and Boehringer Mannheim. DNA fragments to be ligated were ethanol precipitated together in the presence of 1 mM $MgCl_2$ and 0.3 M sodium acetate, dissolved in 10 μl of T4 ligase buffer, and incubated with T4 DNA ligase (Collaborative Research) at 16°C overnight. The ligase was then heat inactivated at 70°C for 15 min. The ligation mixture was then digested with *Cla*I and *Kpn*I, followed by heat inactivation of the enzyme for 15 min at 70°C. The resulting digestion mixture was ligated to *Cla*I-*Kpn*I-digested and phosphatase-treated pCDV-1 vector at 16°C overnight (29). This ligation mixture was used to

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transform frozen competent *Escherichia coli* DH-1 cells (17). Clones were identified by colony filter hybridization with MoCK and FrCK probes.

Restriction endonuclease sites unique to the Friend or Moloney LTR (see Fig. 2) were used to confirm the structure of the recombinant subclones as follows. pModrA: the insert was isolated following *Cla*I and *Kpn*I digestion and then digested with *Bgl*I or *Xba*I (both sites were missing) and *Pst*I or *Pvu*II. The fragment sizes measured from a gel were compatible with those predicted from published sequences (22, 33). Nucleotide sequence analysis (28) was performed from the 3′ *Eco*RV site located approximately in the middle of the direct repeat, about 150 nucleotides 3′ to the *Kpn*I site. The sequence was identical to that of Moloney from the *Eco*RV to *Ava*II site and identical to that of Friend from the *Ava*II site on, except for a one-nucleotide difference in an *Eco*RII site located 49 bases 5′ of the CCAAT sequence. pFrdrD: analysis was performed on whole plasmid DNA by *Pst*I, *Pvu*II, *Bgl*I, or *Xba*I digestion. The clone lacked the *Pst*I site present in Friend and the *Pvu*II sites present in the direct repeat of Moloney, but it had the *Bgl*I site present in the second copy of the Friend direct repeat and the *Xba*I site found in the Moloney LTR. Further characterization of this clone was done by double digestion of the plasmid with *Cla*I plus *Xba*I and *Kpn*I plus *Bgl*I. Fragment sizes measured from the gel were consistent with those predicted from published sequences (22, 33). The same mapping strategy was applied to pMoP to confirm its structure, but in addition a double digestion with *Kpn*I plus *Pst*I was done. Again, digestions yielded fragment sizes consistent with published sequence data (22, 33).

Construction of recombinant viruses. The three *Cla*I-*Kpn*I inserts shown in Fig. 2B were isolated by enzyme digestion, gel electrophoresis, and ethanol precipitation. Complete recombinant viruses, designated MFdrD, FMdrA, and FMoPD, were assembled by stepwise ligation of the inserts to appropriate fragments of Friend or Moloney (as indicated in Fig. 2C), each of which had itself been molecularly cloned in pCDV1. The product of each pairwise ligation step was cloned before the next fragment was added. Fully assembled viruses were cloned in pBR322 at either the *Eco*RI or *Hin*dIII site (see Fig. 2C). The structures of the molecular clones of the fully assembled viruses were determined by restriction endonuclease mapping, and in the case of FMdrA and MFdrD, nucleotide sequencing of one clone of each (clone A10 of FMdrA and clone D1 of MFdrD) was performed from the *Cla*I site 3′ to approximately the *Sau*3AI site in U3.

Construction of viruses with one copy of the enhancer. Viruses with one copy of the enhancer rather than a direct repeat were constructed by schemes similar to that described above for the recombinant viruses. Briefly, to construct Fr(ΔRV), plasmid pFrCK (Fig. 2A; see also Fig. 1) was digested with *Eco*RV, which cuts approximately in the middle of the direct repeat in U3. It was then subjected to agarose gel electrophoresis to remove the 65-base-pair *Eco*RV-*Eco*RV fragment containing one complete but permuted copy of the enhancer, ethanol precipitated, and suspended in TE buffer. It was then recircularized with T4 DNA ligase to yield subclone FrCK(ΔRV). This clone was assembled into whole virus by stepwise ligation to subcloned fragments of Friend virus, exactly as for recombinant FMdrA described above.

To construct Mo(ΔRV), we began with a *Sau*3AI-*Kpn*I fragment of Moloney virus (see Fig. 1) cloned in the vector pUC-CAT (14) so that the *Sau*3AI end inserted into and destroyed the *Bam*HI site and the *Kpn*I end inserted into and destroyed the *Sma*I site of the polylinker. This subclone was digested with *Eco*RV to release one copy of the enhancer and then subjected to gel electrophoresis and ethanol precipitation as above. It was then recircularized with T4 DNA ligase to yield clone MoSK(ΔRV). The *Sau*3AI-*Kpn*I fragment was then isolated and, with the *Cla*I-*Sau*3AI fragment described above in the construction of MFdrD, ligated to pCDV-1 vector cut with *Cla*I and *Kpn*I to create subclone MoCK(ΔRV). The *Cla*I-*Kpn*I fragment was then isolated and ligated to the *Kpn*I-*Xho*I fragment used for assembly of MFdrD together with *Cla*I-*Xho*I-cut pCDV-1 vector to create the subclone MoCX(ΔRV). From this point, complete virus was obtained by two separate pathways. In one case, the *Cla*I-*Xho*I fragment was cut out and ligated to the subcloned 6.0-kb *Xho*I-*Cla*I fragment in the presence of pCDV-1 vector. In the other case, the full-length molecular clone of Moloney virus was digested to completion with *Cla*I and partially with *Xho*I (to avoid an *Xho*I site on the vector), and the 10.4-kb piece containing the *Xho*I-*Cla*I region on a pBR322 vector was then isolated by gel electrophoresis, ethanol precipitated, and ligated to the *Cla*I-XhoI fragment of MoCX(ΔRV) described above. In both cases, the final product was a clone of Moloney virus with a deletion of sequences lying between the *Eco*RV sites in the direct repeat in U3 (see Fig. 2C).

To confirm their structure, the Fr(ΔRV) subclones and the complete virus were digested with *Eco*RV, with *Bgl*I plus *Pst*I, and with *Cla*I plus *Kpn*I. The MoSK(ΔRV) subclone was digested with *Hin*dIII (which has a site in the pUC-CAT polylinker) plus *Sac*I and with *Eco*RV. The remaining Mo(ΔRV) subclones and the complete virus were digested with *Cla*I plus *Xba*I, with *Eco*RV, and with *Cla*I plus *Kpn*I. In addition, one complete clone of Mo(ΔRV) was sequenced by the Maxam and Gilbert (28) procedure over the region of the deletion from approximately *Ava*II to *Sau*3AI to confirm its structure.

Transfection of virus clones. After assembly into complete virus genomes, recombinants and single-enhancer virus DNAs were excised from their vectors and ligated at high concentration, and the DNA was transfected onto NIH/3T3 or BALB/3T3 cells by calcium phosphate precipitation (16). The presence of infectious virus was confirmed by XC plaque assay (32) and by the presence of reverse transcriptase activity (2) in culture fluids. Virus obtained from one recombinant, FMdrA clone A31, was further purified biologically by endpoint dilution to yield FMdrA clone A31LD virus.

RNase T_1 fingerprint analysis. FMdrA (clones A10, A31, and A31LD), MFdrD (clone D1), and Fr(ΔRV) were analyzed by RNase T_1 fingerprinting as descibed previously (10, 11).

Mice, tumor induction, and classification of disease. Newborn (<2-day-old) NFS mice, supplied by the Small Animal Production Section of the National Institutes of Health, were inoculated with 0.02 ml of tissue culture-grown virus, representing from 10^2 to 10^5 PFU per mouse, as described in Results. Mice received viruses intraperitoneally or intraperitoneally and intrathymically and were sacrificed by CO_2 anesthesia when disease was far advanced. Animals were autopsied and examined for gross evidence of erythroleukemia or lymphoma, and for most microscopic examination of blood smears or of spleen imprints or histological preparations or both was done. Diagnosis of erythroleukemia, lymphoma, or myelogenous leukemia was based on gross pathology and histology as described previously (5).

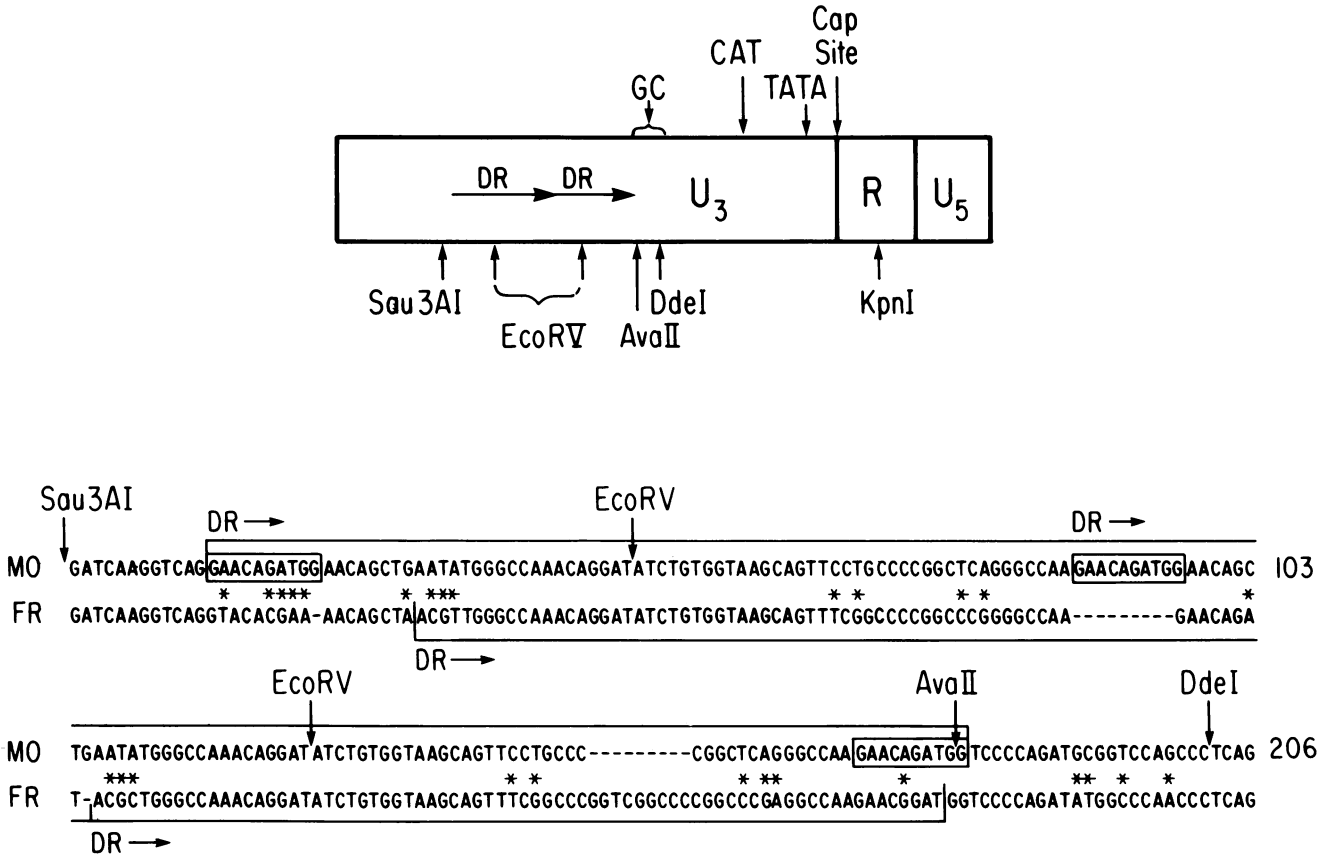

FIG. 1. (Top) Schematic representation of the LTRs of MuLVs. U3 (the sequence unique to the 3′ end of viral RNA) is about 450 bases long and includes the tandem direct repeat (DR) with enhancer activity (arrows) and TATA, CAT, and GC-rich sequences believed to be important components of the transcriptional promoter. R (the sequence repeated at both ends of the viral RNA genome) is 68 bases long and begins with the cap site. U5 (the sequence unique to the 5′ end of viral RNA) is 77 bases long. Restriction endonuclease sites shown define regions exchanged in recombinants or, in the case of the *Eco*RV sites, the region deleted to make viruses with a single copy of the enhancer. (Bottom) Comparison of nucleotide sequences (23, 33) of the direct repeats and most of the adjacent GC-rich region of Moloney (Mo) and Friend (Fr) viruses: differences are indicated by asterisks. Brackets surround the direct repeats (DR) that possess enhancer activity. To introduce the Friend virus direct repeat into Moloney, the 191-base-long *Sau*3AI-*Dde*I fragment was used. To introduce the Moloney virus direct repeat into Friend, the 171-base-long *Sau*3AI-*Ava*II fragment was used. To construct viruses with a single copy of the enhancer instead of a direct repeat, the sequences between the two *Eco*RV sites were deleted.

RESULTS

Disease specificity of recombinants. We wished to construct recombinant viruses in which just the promoter or just the enhancer regions of Friend and Moloney were exchanged. The U3 regions of the two viruses are about 80% homologous and contain several moderately convenient restriction endonuclease sites that can be used for this purpose. The viral enhancers, which are present as a short direct repeat, are located about 180 nucleotides upstream from the cap site (Fig. 1). In Moloney virus the enhancer region has a 10-65-10-65-10 base repeat structure (33). The corresponding region of Friend virus has extensive homology to Moloney but its direct repeat is shorter. In contrast to Moloney, the Friend virus repeat is imperfect, with the second copy containing a 9-base insertion relative to the first.

To exchange direct repeat and promoter regions of the two viruses, we first made recombinants within 0.63-kb subclones that included the entire U3 region of the viral genomes and were flanked by a convenient *Cla*I site (located in sequences encoding the carboxy terminus of Prp15E) and the common *Kpn*I site in R (Fig. 1) (4). To introduce the Moloney virus direct repeat into Friend we used the 171-base *Sau*3AI-*Ava*II fragment whose sequence is shown in Fig. 1. To replace the Moloney virus direct repeat with that of Friend we used the 191-base-pair *Sau*3AI-*Dde*I fragment whose sequence is shown in Fig. 1. (The presence of additional *Ava*II restriction sites in the U3 region of Moloney made it too difficult to construct a precisely reciprocal Friend direct-repeat-only recombinant containing just the *Sau*3A-*Ava*II fragment of Friend virus in the Moloney genome.) To make a Friend virus in which most of the promoter region was derived from Moloney, we used the Moloney virus sequences between the *Dde*I and *Kpn*I sites shown in Fig. 1. The schemes used to construct the recombinant *Cla*I-*Kpn*I subclones, which could then be used to assemble the desired viruses, are shown in Fig. 2A and B.

The genome structures of the fully assembled recombinant viruses are shown in Fig. 2C. The three recombinants we constructed were designated MFdrD (primarily Moloney virus but with the Friend direct repeat), FMdrA (Friend virus but with the Moloney direct repeat), and FMoPD (Friend with most of its promoter region from Moloney). The procedures for generating these viruses and the restriction

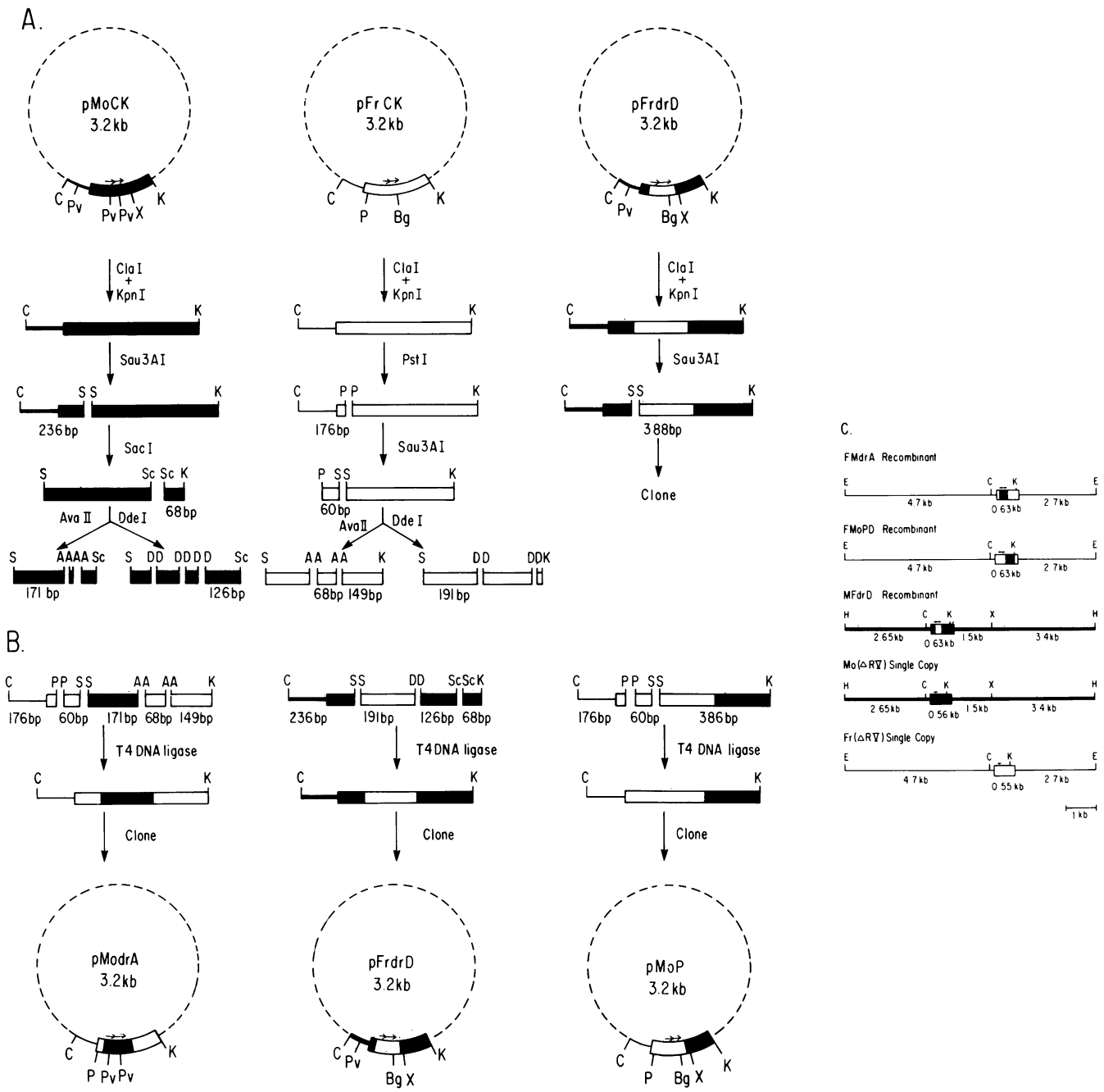

FIG. 2. Strategy for constructing recombinants between molecular clones of nondefective Friend and Moloney viruses. (A) Isolation of DNA fragments used to construct recombinant subclones in the *Cla*I-*Kpn*I regions of Moloney and Friend viruses. pMoCK is a subclone of the *Cla*I-*Kpn*I fragment isolated from the molecular clone of Moloney. pFrCK is a subclone of the corresponding fragment of Friend virus clone 57. pFrdrD is a Moloney-Friend virus recombinant in the *Cla*I-*Kpn*I region, constructed as indicated in the figure. Heavy lines represent Moloney sequences, with the solid boxed regions representing Moloney LTR sequences. Thin lines and boxes (LTR region) represent Friend virus sequences. Dashed lines represent the vector pCDV-1. The figure is drawn to scale, and sizes (in base pairs) are given for fragments that were used in constructions. (B) Schematic representation of the assembly of recombinant subclones. Restriction endonuclease sites unique to the Friend or Moloney LTRs and hence useful for confirming the structure of recombinant clones are indicated in the circular diagrams. (C) Schematic representation of the structures of molecular clones of three Friend-Moloney recombinants and the two viruses possessing one rather than two copies of the enhancer. Heavy lines indicate portions of the genome derived from Moloney, thin lines and open boxes in the LTR represent portions derived from Friend. The direct repeat (enhancer) is indicated by arrows. Abbreviations: C, *Cla*I; K, *Kpn*I; S, *Sau*3AI; Sc, *Sac*I; A, *Ava*II; D, *Dde*I; P, *Pst*I; Pv, *Pvu*II; X, *Xba*I (A and B) and *Xho*I (C); Bg, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; bp, base pairs.

TABLE 1. Type of leukemia induced by Moloney, Friend, and recombinant viruses after injection into newborn NFS mice

Virus strain and clone	Source of:		No. dead or sacrificed/no. inoculated[a]	Mean latent period (days)	Diagnosis (no. of tumors)			
	Genome	Direct repeat			Lymphoma	Erythroleukemia	Myelogenous leukemia	Megakaryocytic leukemia
Moloney			35/35	81	34			
Friend			61/61	55		59		
MFdrD	Moloney	Friend	54/57	76		54		
FMdrA[b]	Friend	Moloney	64/70	92	54	7[c]	2	3[d]
A31			18/18	90	11	5	1	1
A31LD			19/22	87	18	2[c]		
A10			12/13	123	10		1	1
A35			15/17	79	15			1[d]
FMoPD	Friend	Friend	10/10	44	1[e]	10		

[a] Mice were sacrificed when they showed evidence of advanced disease. In several cases animals died and autopsy was not possible, so the number of mice in this column can exceed the number with diagnosed disease.

[b] Data for FMdrA are a summary of data for all three molecular clones of FMdrA and for the biologically purified derivative of clone A31 designated A31LD, shown separately below.

[c] One mouse with thymic-origin lymphoma also had erythroleukemia.

[d] One mouse with nonthymic lymphoblastic lymphoma also had megakaryocytic leukemia.

[e] One mouse with erythroleukemia also had lymphoblastic lymphoma in one mediastinal lymph node.

endonuclease digestions and other methods used to confirm their structures are described in Materials and Methods. Four independent molecular clones of MFdrD, three of FMdrA, and one of FMoPD were selected for further studies. Recombinants were excised from their pBR322 vectors and ligated, and the DNA was transfected onto NIH/3T3 cells by calcium phosphate precipitation (16). The presence of infectious virus was confirmed by XC plaque assay and reverse transcriptase activity in culture fluids (2, 33). Virus obtained from one recombinant, FMdrA clone A31, was further purified biologically by endpoint dilution to yield FMdrA clone A31LD virus. FMdrA (clones A10, A31, and A31LD) and MFdrD (clone D1) viral RNAs were analyzed by RNase T_1 fingerprinting (10, 11). As expected, the T_1 fingerprints of MFdrD looked like the T_1 fingerprints of Moloney virus itself, while the fingerprints of FMdrA looked like the T_1 fingerprints of the Friend virus genome (not shown).

Viruses obtained from transfection of the molecularly cloned parental and recombinant viruses and the biologically cloned derivative of FMdrA were injected into newborn NFS mice. All were capable of inducing neoplasms of the hematopoietic system (Table 1). Strikingly, the type of disease induced by the recombinant viruses correlated strongly with the source of the direct repeat sequences. The recombinant MFdrD, whose genome was almost entirely derived from Moloney but whose direct repeat region plus 22 nucleotides 3′ of the direct repeat was derived from Friend, induced only erythroleukemias (54 mice). The recombinant FMdrA, whose genome was derived from Friend except for the Moloney direct repeat region, induced lymphoblastic lymphomas in more than 80% of injected mice. The recombinant FMoPD, whose genome (including direct repeat) was derived primarily from Friend, but whose promoter region was taken from Moloney, induced erythroleukemias in all 10 of the mice injected (although 1 of the 10 also had evidence of a lymphoblastic lymphoma).

It is interesting that recombinant FMdrA, whose direct repeat came from Moloney but the remainder of whose genome was from Friend, induced a number of nonlymphoid neoplasms (12 of 64 mice, including 6 who died of erythroleukemia). In previous studies, a recombinant whose enhancer and promoter (*Sau*3A-*Kpn*I) were both derived from Moloney induced only one erythroleukemia and 19 lymphomas. Together, these results, as well as the clear-cut disease specificity of recombinant MFdrD in the present study, suggest that the GC-rich element in the promoter region may contribute to disease specificity. However, the data in Table 1 are somewhat ambiguous on this point. Of the three independent molecular clones of FMdrA (A10, A31, and A35), only one, A31, gave a substantial number of erythroleukemias. It is unlikely that the ability of clone A31 to induce erythroleukemias was due to viral contamination for two reasons. First, A31LD, the biologically purified derivative of virus obtained from clone A31, also induced some erythroleukemias, and second, virus isolated from one of the erythroleukemias induced by clone A31 virus induced lymphoblastic lymphomas in four mice that were injected. Further studies would be needed, however, to confirm that FMdrA recombinants have a less sharply defined disease specificity than viruses in which the promoter and enhancer region are both derived from the same parent.

Disease induction by viruses with one versus two copies of the enhancer element. Having established that the viral direct repeat regions contain a potent determinant of disease specificity, we wished to further identify which of the nucleotide differences between Friend and Moloney in this region are important in contributing to their distinct specificities. To this end, we wished to determine whether Moloney and Friend viruses with just one copy of the enhancer element, as opposed to a direct repeat sequence, would retain their target cell specificity.

It was possible to construct single-enhancer viruses quite easily from the molecular clones of Friend and Moloney by using the *Eco*RV sites within their direct repeats (Fig. 1). The schemes used to generate these virus clones, which are designated Mo(ΔRV) and Fr(ΔRV), and the restriction digests used to confirm their structures are described in Materials and Methods. The structures of the viral genomes are shown schematically in Fig. 2C.

Viruses obtained from transfection of one Friend and three Moloney single-enhancer virus clones were injected into newborn NFS mice. These viruses were capable of inducing leukemia. Mo(ΔRV) induced lymphoblastic lymphomas, while Fr(ΔRV) induced primarily erythroleukemias. [Several megakaryocytic and one myeloid tumor were also induced by Fr(ΔRV). These types of tumors have not been seen with the parental Friend virus in these studies

TABLE 2. Latent period and disease specificity of viruses with one or two copies of the enhancer and relationship to input titer of virus[a]

Virus	Approx. inoculum (log_{10} PFU/mouse)	No. dead or sacrificed/no. inoculated	Mean latent period (days)	Diagnosis (no. of tumors)			
				Lymphoma	Erythroleukemia	Myelogenous leukemia	Megakaryocytic leukemia
Friend	4.3	61/61	55		59		
	3.3	21/21	61		21		
	2.3	14/14	65		14		
Fr(ΔRV)	3.3	20/24	151		16	1	3[b]
Moloney	5.0	35/35	81	34			
	4.0	5/5	90	4			
Mo(ΔRV)	5.0	8/15	137	8			
	4.0	5/15	150	5			

[a] See Table 1, footnote *a*.
[b] One mouse with erythroleukemia also had megakaryocytic leukemia.

(Table 2) (4, 5). While the significance of this is unknown, conceivably it could be related to the longer latent period of disease induction by Fr(ΔRV) relative to Friend virus itself.]

Although viruses with one instead of two copies of the enhancer element essentially retained their disease specificity, they had a significantly longer latent period for disease induction (Fig. 3). When tests of Fr(ΔRV) were terminated at 218 days, the average latent period for disease induction was 151 days instead of the 60 days seen with Friend virus. In tests of Mo(ΔRV), by 162 days only 38% of the mice had contracted disease, with an average latent period of 122 days, compared with the 100% incidence and 81-day latent period found for a similar dose of Moloney virus with a direct repeat (Table 2). By 194 days, 53% of the Mo(ΔRV)-infected mice had developed lymphomas, with a mean latent period of 137 days.

The difference in latent period of single- versus two-enhancer viruses is not due to differences in the amount of virus injected, since all mice for whom data are shown in Fig. 3 received the same input dose ($10^{3.3}$ PFU per mouse for Friend viruses, 10^5 PFU per mouse for Moloney viruses). In fact, the input dose of virus had a remarkably small effect on the latent period of disease induction by Friend and probably also Moloney viruses. A 100-fold difference in input titer of Friend virus increased the mean latent period for erythroleukemia induction by only 10 days (from 55 to 65 days) (Table 2).

DISCUSSION

The results described above demonstrate that the direct repeat in U3, which in the case of Moloney has been shown to have the properties of a transcriptional enhancer, is a determinant of disease specificity in nondefective MuLVs. In at least some recombinants between Friend and Moloney viruses this element is the most potent determinant of this phenotype. The studies, as well as previous analysis of Friend-Moloney virus recombinants, indicate that this element is not the sole determinant of specificity (4, 5). Studies by Oliff and Ruscetti (31) had indicated that the viral *env* gene may contain a determinant of erythroleukemogenicity. The present studies suggest that the promoter region, in particular the GC-rich segment defined by Laimins et al. (23), may also contribute to this phenotype.

In addition to a role in determining disease specificity, direct repeat elements (enhancers) have been shown to be important determinants of the ability of mouse retroviruses to induce leukemia (9, 18, 24, 27). The analysis here of viruses with one and two copies of the enhancer shows that these transcriptional elements also have a dramatic effect on the latent period of disease induction by nondefective retroviruses.

The direct repeat regions of Friend and Moloney viruses are highly homologous, having 121 of 160 nucleotides in common. They are distinct, however, in that the Moloney virus enhancer has a 10-65-10-65-10 base structure, with the 10-base element having the sequence of a glucocorticoid receptor (GRE)-binding site (Fig. 1) (7). The first of these GRE sites is absent, and the third has a nucleotide alteration in the shorter Friend direct repeat, which can be drawn as an approximately 65-base direct repeat. The direct repeat in Friend clone 57 is not perfect since the second copy has a 9-base insert relative to the first and also differs at one other nucleotide.

Both Moloney and Friend viruses with one instead of two copies of the enhancer replicate efficiently in vitro and in vivo, although the single-enhancer viruses achieve slightly lower titers than their progenitors (unpublished results). Since efficient replication plays an important role in disease induction by murine retrovirusus, it is presumably these small differences in titer that underlie the delay in disease induction by the single-enhancer viruses. Differences in input did not account for the longer latent period of the

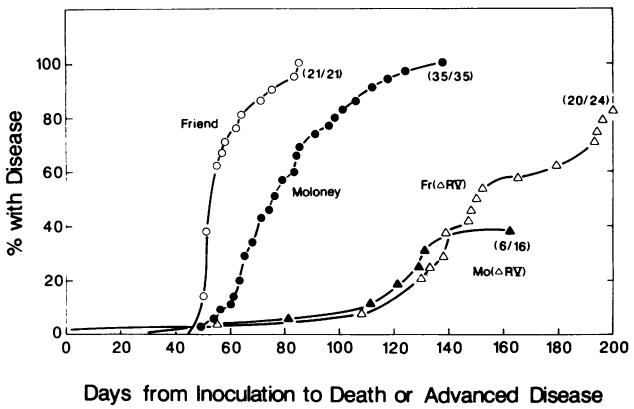

FIG. 3. Incidence of tumors induced in NFS mice by Friend, Fr(ΔRV), Moloney, and Mo(ΔRV) viruses. Numbers in parentheses indicate the number of mice deceased or with advanced disease/number injected. Data for Mo(ΔRV) viruses was collected for 162 days. Data plotted here are also included in Table 2 and correspond to data for Friend and Fr(ΔRV) viruses at an input titer of approximately $10^{3.3}$ and for Moloney and Mo(ΔRV) viruses at an input titer of approximately 10^5.

single-enhancer viruses. Indeed, a 10- to 100-fold difference in input multiplicity had only a small effect on the latent period of disease induced by Friend and possibly also Moloney viruses (Table 2). Finally, because the two copies of the Friend virus enhancer in clone 57 are not identical, we cannot be certain that the increased latent period of the Friend single-enhancer virus is due to the lack of two copies of the enhancer rather than to the particular sequence of the one copy it possesses.

Different transcriptional activity in different cell types presumably explains the different disease specificities conferred by Friend and Moloney enhancers. Moloney virus and recombinants that have the Moloney virus direct repeat replicate more efficiently in the thymus than do recombinants with the Friend virus direct repeat or Friend virus itself (unpublished results). This observation could explain why Moloney virus induces T-cell lymphomas in NFS mice while Friend virus does not. It is doubtful that this result reflects a strict T-cell tropism of the Moloney virus enhancer region, as suggested for certain other leukemogenic, thymotropic mouse retroviruses (3, 8, 36). In fact, it has been thought that Moloney virus transcriptional signals lack significant tissue specificity and are efficient in many different cell types with the possible exception of embryonal carcinoma cells (12, 15, 26). Evidence has suggested that the developmental stage of the mouse at the time of injection restricts Moloney virus replication to lymphatic cells rather than an intrinsic target tropism of the virus (6, 20). More recently, however, Cone et al. (6) reported that Moloney virus transcription is depressed in the mouse erythroleukemia cell line MEL, a result that suggests a possible explanation for why Moloney virus fails to induce erythroleukemias.

Our studies define a small number of nucleotide differences that underlie the different types of leukemia induced by the nondefective Friend and Moloney leukemia viruses. These differences lie within transcriptional signals that are thought to function by interacting with nuclear, sequence-specific DNA binding proteins. Current studies involving in vitro mutagenesis of these sites and analysis of cellular proteins that interact with them may ultimately provide a more detailed molecular description of the specificity of a retrovirus-induced disease.

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LITERATURE CITED

1. **Aaronson, S. A., and G. T. Todaro.** 1968. Development of 3T3-like lines from BALB/c mouse embryo cultures: transformation susceptability to SV40. J. Cell. Physiol. **72**:141–148.
2. **Baltimore, D.** 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature (London) **226**:1209–1211.
3. **Celander, D., and W. A. Haseltine.** 1984. Tissue-specific transcription preference as a determinant of cell tropism and leukaemogenic potential of murine retroviruses. Nature (London) **312**:159–163.
4. **Chatis, P. A., C. A. Holland, J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1983. Role for the 3′ end of the genome in determining disease specificity of Friend and Moloney murine leukemia viruses. Proc. Natl. Acad. Sci. USA **80**:4408–4411.
5. **Chatis, P. A., C. A. Holland, J. E. Silver, T. N. Frederickson, N. Hopkins, and J. W. Hartley.** 1984. A 3′ end fragment encompassing the transcriptional enhancers of nondefective Friend virus confers erythroleukemogenicity on Moloney leukemia virus. J. Virol. **52**:248–254.
6. **Cone, R. D., A. Weber-Benarous, D. Baorto, and R. C. Mulligan.** 1987. Regulated expression of a complete human β-globin gene encoded by a transmissable retrovirus vector. Mol. Cell. Biol. **7**:887–897.
7. **DeFranco, D., and K. Yamamoto.** 1986. Two different factors act separately or together to specify functionally distinct activities at a single transcriptional enhancer. Mol. Cell. Biol. **6**:993–1001.
8. **DesGroseillers, L., E. Rassart, and P. Jolicoeur.** 1983. Thymotropism of murine leukemia virus is conferred by its long terminal repeat. Proc. Natl. Acad. Sci. USA **80**:4203–4207.
9. **DesGroseillers, L., R. Villemur, and P. Jolicoeur.** 1983. The high leukemogenic potential of Gross passage A murine leukemia virus maps in the region of the genome corresponding to the long terminal repeat and to the 3′ end of *env*. J. Virol. **47**:24–32.
10. **DeWachter, R., and W. Fiers.** 1972. Preparative two-dimensional polyacrylamide gel electrophoresis of P-labeled RNA. Anal. Biochem. **49**:184–197.
11. **Faller, D. V., and N. Hopkins.** 1977. RNase T_1-resistant oligonucleotides of B-tropic murine leukemia virus from BALB/c and five of its NB-tropic derivatives. J. Virol. **23**:188–195.
12. **Gautsch, J. W., and M. C. Wilson.** 1983. Delayed de novo methylation in teratocarcinoma suggests additional tissue-specific mechanisms for controlling gene expression. Nature (London) **301**:32–37.
13. **Gilboa, E., S. Goff, A. Shields, F. Yoshimura, S. Mitra, and D. Baltimore.** 1979. In vitro synthesis of a 9 kbp terminally redundant DNA carrying the infectivity of Moloney leukemia virus. Cell **16**:863–874.
14. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
15. **Gorman, C. M., P. W. J. Rigby, and D. P. Lane.** 1985. Negative regulation of viral enhancers in undifferentiated embryonic stem cells. Cell **42**:519–526.
16. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 4 DNA. Virology **52**:456–467.
17. **Hanahan, D.** 1983. Studies on transformation of Escherichia coli with plasmid. J. Mol. Biol. **166**:557–580.
18. **Holland, C. A., J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1985. At least four viral genes contribute to the leukemogenicity of murine retrovirus MCF 247 in AKR mice. J. Virol. **53**: 158–165.
19. **Ishimoto, A., A. Adachi, K. Sakai, and M. Matsuyama.** 1985. Long terminal repeat of Friend-MCF virus contains the sequence responsible for erythroid leukemia. Virology **141**:30–42.
20. **Jaenisch, R.** 1980. Retroviruses and embryogenesis: microinjection of Moloney leukemia virus into midgestation mouse embryos. Cell **19**:181–188.
21. **Jainchill, J. L., S. A. Aaronson, and G. J. Todaro.** 1969. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. J. Virol. **4**:549–553.
22. **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.
23. **Laimins, L. A., P. Gruss, R. Pozzati, and G. Khoury.** 1984. Characterization of enhancer elements in the long terminal repeat of Moloney murine sarcoma virus. J. Virol. **49**:183–189.
24. **Lenz, J., D. Celander, R. L. Crowther, R. Patarca, D. W. Perkins, and W. A. Haseltine.** 1984. Determination of the leukemogenicity of a murine retrovirus by sequences within the long terminal repeat. Nature (London) **308**:467–470.
25. **Levinson, B., G. Khoury, G. Vande Woude, and P. Gruss.** 1982. Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma virus. Nature (London) **295**:568–572.

26. **Linney, E., B. Davis, J. Overhauser, E. Chao, and H. Fan.** 1984. Non-function of a Moloney murine leukemia virus regulatory sequence in F9 embryonal carcinoma cells. Nature (London) **308**:470–472.
27. **Lung, M. C, C. Hering, J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1980. Analysis of the genomes of mink cell focus-inducing murine type-C viruses: a progress report. Cold Spring Harbor Symp. Quant. Biol. **44**:1269–1274.
28. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
29. **Okayama, H., and P. Berg.** 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. Mol. Cell. Biol. **3**:280–289.
30. **Oliff, A. I., G. L. Hagen, E. H. Chang, E. M. Scolnick, H. W. Chan, and D. R. Lowy.** 1980. Transfection of molecularly cloned Friend murine leukemia virus DNA yields a highly leukemogenic helper-independent type C virus. J. Virol. **33**:475–486.
31. **Oliff, A. I., and S. Ruscetti.** 1983. A 2.4-kilobase-pair fragment of the Friend murine leukemia virus genome contains the sequences responsible for Friend murine virus-induced erythroleukemia. J. Virol. **46**:718–254.
32. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42**:1136–1139.
33. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine virus. Nature (London) **293**:543–548.
34. **Vogt, M., C. Haggblom, S. Swift, and M. Haas.** 1985. Envelope gene and long terminal repeat determine the different biological properties of Rauscher, Friend, and Moloney mink cell focus-inducing viruses. J. Virol. **55**:184–192.
35. **Weiss, R., N. Teich, H. Varmus, and J. Coffin (ed.).** 1982. RNA tumor viruses, p. 785–998. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Yoshimura, F. K., B. Davison, and K. Chaffin.** 1985. Murine leukemia virus long terminal repeat sequences can enhance gene activity in a cell type-specific manner. Mol. Cell. Biol. **5**: 2832–2835.