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Distinct Segments within the Enhancer Region Collaborate To Specify the Type of Leukemia Induced by Nondefective Friend and Moloney Viruses

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The nondefective Moloney and Friend murine leukemia viruses induce T-cell lymphomas and erythroleukemias, respectively, after being injected into newborn NFS mice. In previous studies, we showed that the distinct disease specificities of the two viruses could be switched by exchanging a small segment, about 200 nucleotides in length, encompassing their enhancer regions. This segment included the direct repeat sequence and an adjacent GC-rich region of about 20 nucleotides defined in studies of Moloney murine sarcoma virus enhancer-promoter function (L. A. Laimins, P. Gruss, R. Pozzatti, and G. Khoury, J. Virol. 49:183–189, 1984). The direct repeats of Friend and Moloney viruses are identical in a central core sequence of 32 nucleotides but have sequence differences on either side of this core as well as in their GC-rich segments. To determine whether disease specificity resides in part or in all of the direct repeat and GC-rich region, we constructed recombinants between Friend and Moloney viruses within this segment and tested them for their disease-inducing phenotypes. We found that disease specificity, in particular the ability of Friend virus sequence to confer erythroleukemogenicity on Moloney virus, is encoded throughout the region in at least three separable segments: the 5′ and 3′ halves of the direct repeat and the GC-rich segment. When just one of these segments (either both 5′ halves of the direct repeat, both 3′ halves, or just the GC-rich segment) from Friend virus was substituted into a Moloney virus genome, it conferred only a negligible or low incidence of erythroleukemia (≤5% to between 10 and 15%). Any two segments together were considerably more potent (35 to 95% erythroleukemia), with the most effective pair being the two halves of the direct repeat. Individual segments and pairs of segments were considerably more potent determinants when they were matched with a genome of the same origin. Thus, although sequences outside the enhancer region are minor determinants of disease specificity when the enhancer is derived entirely from either Friend or Moloney virus, they can play a significant role when the enhancer is of mixed origin. Some recombinant enhancers conferred a long latent period of disease induction. This was particularly striking when the 5′ halves of each copy of the direct repeat sequence were derived from Moloney virus and the 3′ halves were derived from Friend virus. The results imply that determinants lying to the 5′ and 3′ sides of a core sequence in the Friend virus enhancer cooperate to produce the high incidence and short latent period of erythroleukemogenicity characteristic of this virus. Two copies of such an enhancer are probably required for rapid disease induction (Y. Li, E. Golemis, J. W. Hartley, and N. Hopkins, J. Virol. 61:693–700, 1987).

Moloney murine leukemia virus induces T-cell lymphomas approximately 75 to 80 days after being injected into newborn NFS mice, while the replication-competent Friend murine leukemia virus induces erythroleukemias approximately 55 days after such an injection (52, 55). The disease specificity of these and other retroviruses is a complex phenomenon to which both host genes and multiple regions of the viral genome may contribute. Nonetheless, in the case of Moloney and Friend viruses, Chatis et al. (6, 7) showed that transcriptional elements in the U3 region of the long terminal repeat (LTR) are the primary determinants of the distinct disease specificities of the two viruses. More recently, our laboratories demonstrated that exchanging homologous segments of about 200 bases, which share about 85% sequence homology and encode the enhancer region of Friend or Moloney virus, was sufficient to almost completely exchange the disease specificities of the viruses (31). These 200-base segments encompassed the direct-repeat regions, shown to have enhancer activity in Moloney sarcoma virus, and a short 3′-adjacent GC-rich segment, also shown to have some function in transcription mediated by the Moloney murine sarcoma virus LTR (29). Studies from a number of laboratories have shown that the U3 region is an important determinant of disease specificity in other leukemogenic mouse retroviruses as well (11, 19, 22, 26, 49, 53). Several studies support the notion that murine leukemia virus enhancers exhibit organ and tissue tropism, and such preferential function in appropriate cell types presumably explains the role of the enhancers in determining disease specificity of murine leukemia viruses, (3–5, 12, 14, 24, 25, 46, 51, 56).

Evidence from multiple systems has shown that typical enhancers consist of an array of short-sequence motifs, each interacting with distinct *trans*-acting factors and each presumably contributing to the overall transcriptional phenotype of the element (21, 33, 43). Enhancers can function in a broad variety of cell types, as do those of simian virus 40 (43), polyomavirus (20), and, at least in cultured cells,

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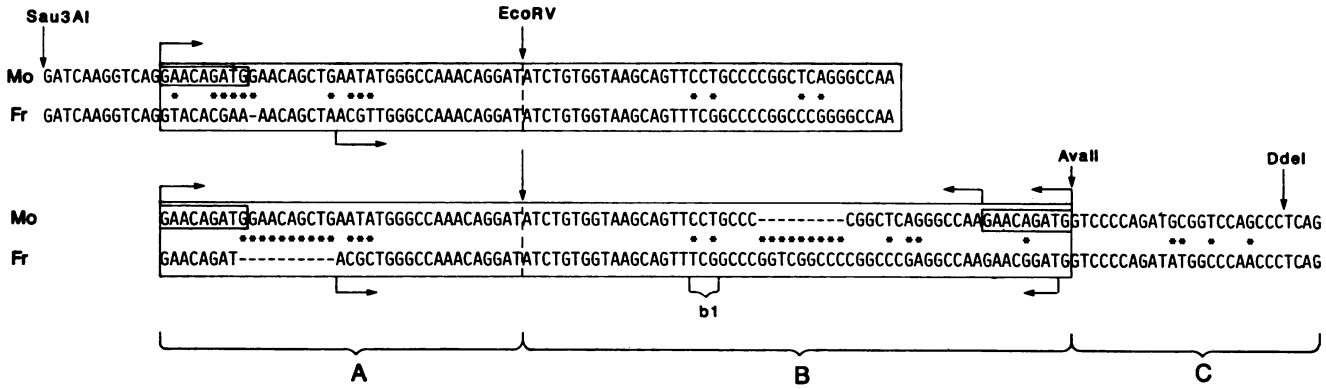

FIG. 1. Comparison of nucleotide sequences in the enhancer regions of Friend (Fr) and Moloney (Mo) viruses. Direct repeat region is boxed. Horizontal arrows indicate beginnings and ends of the repeats. Differences in sequence between the viruses are indicated by asterisks. Boundaries of the A, B, C, and b1 segments described in the text are indicated by horizontal brackets below the sequences.

Moloney virus (9), or they can have a narrowly defined cell type specificity, as do those of the immunoglobulin heavy chain (16) and light chain (40) genes, the insulin genes (54), the elastase gene (39), and many others (8, 34). Elegant studies in the simian virus 40 system (21, 38, 43, 57) have demonstrated the presence of short elements with distinct cell-type-specific enhancing activity within the 72-base-pair repeat, although the relationship of these elements and their individual specificities to the biology of simian virus 40, and in particular to its disease specificity, is unclear.

The enhancer regions of Friend and Moloney viruses are identical in sequence in a central conserved region, 32 nucleotides long, within each direct repeat but have nucleotide differences on either side of this region as well as in their GC-rich segments (28, 44). We wished to determine whether erythroleukemogenicity or T-cell lymphomagenicity could be mapped to a single short region of sequence difference within the enhancer or whether the complex phenotype of disease specificity was the product of two or more motifs or of the precise structure of the entire directly repeated sequence and its 3'-adjacent GC-rich segment. To answer this question, we carried out an analysis of the contribution of small regions within the 200-base enhancer region to the overall pathogenesis of Moloney and Friend viruses. We used a combination of cloning and mutagenesis to construct a series of viruses that would allow us to determine which parts of the Friend enhancer region, alone or in combination, were sufficient to confer erythroleukemogenicity on Moloney virus. In some cases, reciprocal constructs were made in which the identical enhancer sequence was present in both the Moloney and the Friend virus genomes in order to assess the degree to which other genomic sequences influence disease specificity.

We have characterized these Friend-Moloney enhancer-recombinant viruses for their disease-inducing phenotypes, including type of leukemia, disease incidence, and latent period after injection of newborn mice. We found that the disease specificity, and in particular the ability of the Friend virus enhancer to confer erythroleukemogenicity on Moloney virus, is determined by several regions within the enhancer. It should be noted that Ishimoto et al. (26) also found that disease specificity in the enhancer-promoter region of a Friend mink cell focus-forming virus could not be mapped to a single site, although differences in the two studies preclude a simple comparison of the results.

Consistent with the results of previous studies (6, 7, 11, 31, 37), we found that genomic sequences outside the LTR make some contribution to disease specificity and that certain combinations of enhancer sequences confer a significant increase in the latent period of disease induction. Some viruses with recombinant enhancers induced a low incidence of tumors of novel specificity rarely seen with either parental virus. It is apparent from the array of disease specificities and latent periods shown by the recombinants that variations in enhancer sequence could help to explain the wide variety of disease-inducing phenotypes seen among naturally occurring mouse retroviruses.

MATERIALS AND METHODS

Cells and parental viruses. NIH 3T3 (27) and BALB/3T3 cells (1) were used for DNA transfections of virus clones (18). Infectious DNA clones of Moloney virus (45) and nondefective Friend virus clone 57 (36) were obtained from S. Goff (Columbia University) and A. Oliff (Merck Sharp & Dohme), respectively.

Oligonucleotides. All oligonucleotides used for cloning and mutagenesis were made on an Autogen 6500 DNA synthesizer and were kindly provided by Y. Takagaki.

Mutagenesis. Site-specific mutagenesis was done on the direct repeat and GC-rich segments of the enhancer region by using the gapped-duplex method described by Lenardo et al. (30). Mutagenesis was done on *Sau*3A1-*Kpn*I subclones containing approximately 400 base pairs of viral sequence. To confirm that desired mutations had been introduced, the region of DNA extending from at least 30 bases 5' of the enhancer region through 20 bases 3' of the enhancer region was subjected to Maxam-Gilbert sequencing (32) or dideoxy sequencing (42) using the Sequenase system (50) or both. This region encompassed about 200 bases at the 5' end of the subclone, lying just 3' of *Sau*3A1. The 3' end of the subclone, proximal to the *Kpn*I site, was not sequenced.

Constructions. The sequence of the enhancer region of Friend and Moloney viruses in which we wished to make recombinants is shown in Fig. 1 (28, 44). This region consists of a direct repeat and a short 3'-adjacent GC-rich region. We designated the sequences in this region A, B, b1, and C as indicated in Fig. 1. To generate recombinants between Friend and Moloney viruses that exchanged different combinations of A, B, b1, or C, we began with subclones whose construction has been previously described (6, 7, 31). FrCK is a *Cla*I-*Kpn*I subclone of Friend virus (6). MoSK is a *Sau*3A1-*Kpn*I subclone of Moloney virus (31). Fr(ΔRV)-CAT and Mo(ΔRV)-CAT are both *Sau*3A1-*Kpn*I subclones

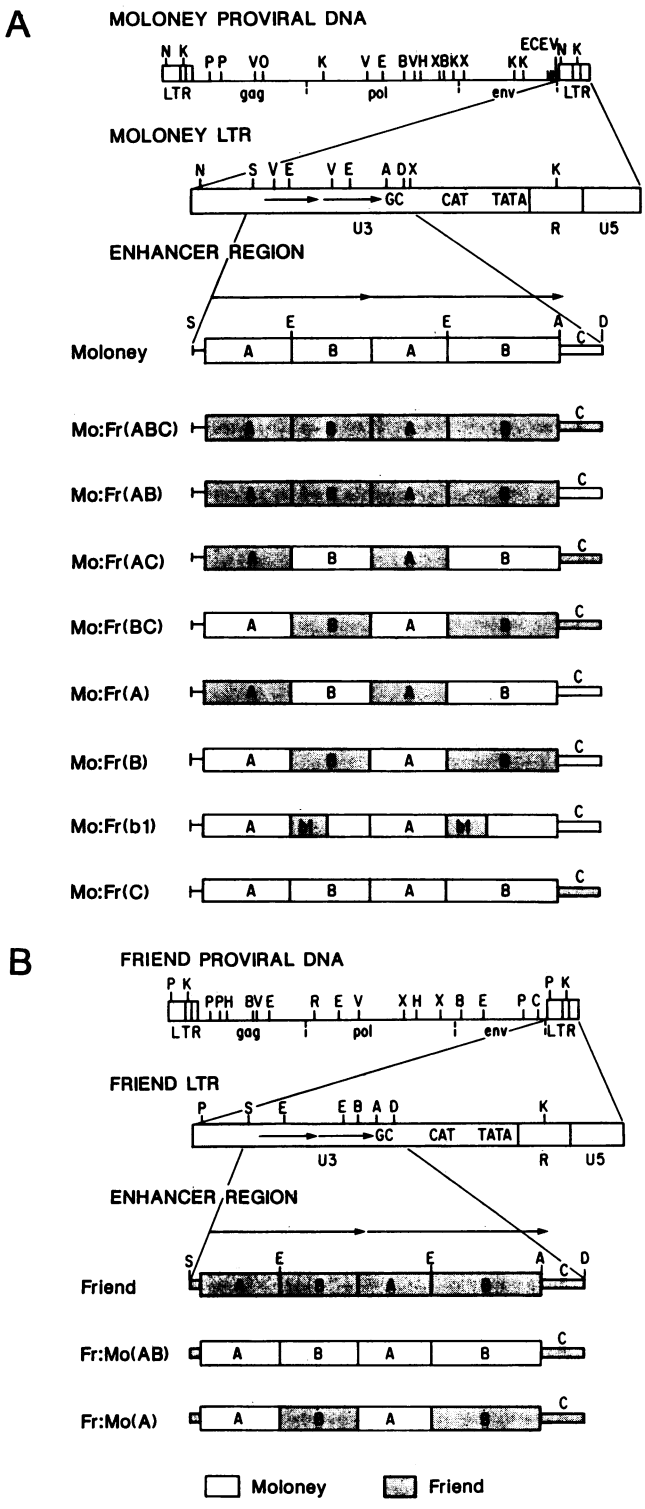

FIG. 2. Schematic representation of the enhancer regions of Friend and Moloney and recombinant viruses. (A) Diagrammatic representation of enhancer regions that were introduced into an otherwise Moloney virus genome. Sequences derived from Friend virus are shaded; those from Moloney virus are open boxes. At the top of the figure are diagrams of the Moloney proviral genome showing positions of restriction sites relevant to this study, an enlargement of the LTR with the direct repeat represented by arrows, and finally the enhancer region showing the relationship of the A, B, and b1 segments in the direct repeats and the GC-rich or C segment (see text). (B) Diagrammatic representation of enhancer regions that were introduced into an otherwise Friend virus genome. Remainder of diagram is as described for panel A. Restriction endonuclease sites: A, *Ava*II; B, *Bgl*I; C, *Cla*I; D, *Dde*; E, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; O, *Xho*I; P, *Pst*I; R, *Eco*RI; S, *Sau*3AI; V, *Pvu*II; X, *Xba*I.

in which the *Eco*RV-*Eco*RV fragment within the direct repeat has been removed from Friend and Moloney viruses, respectively (31). Mo:Fr(ABC)-CAT, previously termed MFdrD-CAT, is a *Sau*3A1-*Kpn*I subclone in which the *Sau*3A1-*Dde*I sequence is derived from Friend virus, while the *Dde*I-*Kpn*I sequence is derived from Moloney virus (Fig. 2) (31). Fr:Mo(AB)-CAT, previously termed FMdrA-CAT, is a *Sau*3A1-*Kpn*I subclone in which the enhancer sequence from *Sau*3A1-*Ava*II is derived from Moloney virus, while the sequence from *Ava*II-*Kpn*I is derived from Friend virus (Fig. 2) (31).

The enhancer compositions of all viruses used in this study are shown in Fig. 2. The strategies used to generate the complete viruses Mo:Fr(ABC) and Fr:Mo(AB) have been described previously (31). The remaining viruses were constructed as follows. Appropriate fragments extending from either *Sau*3A1-*Eco*RV, *Cla*I-*Eco*RV, or *Eco*RV-*Kpn*I from each of the subclones FrCK, MoSK, Mo RV-CAT, Fr RV-CAT, Mo:Fr(ABC)-CAT, and Fr:Mo(AB)-CAT were excised, purified on agarose gels, eluted, and religated to vectors to make a series of *Sau*3A1-*Kpn*I (SK) or *Cla*I-*Kpn*I (CK) subclones with a single copy of the direct repeat element. These initial constructs were [Mo:Fr(BC)ΔRV]SK, [Mo:Fr(A)ΔRV]SK, and [Fr:Mo(A)ΔRV]CK. To insert the second copy of the direct repeat, these constructs were cut with *Eco*RV, and synthesized, double-stranded oligonucleotides containing the desired sequence from *Eco*RV-*Eco*RV [either the 75-mer Friend(B)Moloney(A) to make Mo:Fr(BC)SK and Fr:Mo(A)CK or the 65-mer Moloney(B) Friend(A) to make Mo:Fr(A)SK] were inserted in a ligation reaction in which the oligonucleotide was in approximately 1,000:1 excess over the plasmid. These three subclones, along with the constructs MoSK and FrdrD-CAT [Mo:Fr(ABC)SK], were then used as substrates for site-specific mutagenesis in order to create the remaining constructs. Briefly, Mo:Fr(AC)SK was made by switching the C region of Mo:Fr(A)SK to the Friend virus sequence. Mo:Fr(B)SK was made by switching the C region of Mo:Fr(BC)SK to the Moloney virus sequence. Mo:Fr C was made by switching the C region of MoSK to the Friend virus sequence. Mo:Fr(b1)SK was made by switching the two nucleotides located immediately 3′ to the *Eco*RV site in each copy of the direct repeat (as indicated in Fig. 1) to the corresponding Friend virus sequence in both copies of the direct repeat in MoSK. To make Mo:Fr(AB)SK, the region from *Sau*3A1-*Bgl*I was excised from Mo:Fr(ABC)SK, the region *Bgl*I-*Kpn*I was excised from Mo:Fr(B)SK, and these purified fragments were ligated together with vector cut with *Sau*3A1 and *Kpn*I. Maxam-Gilbert sequencing was used at this stage to check that the desired enhancer sequence was present for all recombinants.

These modified *Sau*3A1-*Kpn*I or *Cla*I-*Kpn*I fragments were then excised with restriction enzymes, purified by agarose gel electrophoresis, and ligated to purified fragments corresponding to the remainder of the appropriate viral genome in several sequential subcloning steps. The end result was full-length genomic DNA, containing one LTR

with the recombinant enhancer, cloned either at *Hin*dIII (in the case of the Moloney virus-based constructs) or *Eco*RI (for the Friend virus-based constructs) into the plasmid pUC13. The structures of these recombinants were confirmed by restriction digests by using the enzymes *Eco*RV, *Pvu*II, *Bgl*I, *Cla*I, *Xho*I, and either *Nhe*I (for the Moloney virus series) or *Pst*I (for the Friend virus series). The region of the direct repeat and GC-rich segment was again checked by Maxam-Gilbert sequencing or Sequenase dideoxy sequencing or both. All results were in accordance with expected recombinant sequences.

Transfection of virus clones. After assembly into complete virus genomes, recombinant virus DNAs were excised from their vectors and ligated to form closed circles or concatamers. This DNA was then transfected onto either NIH 3T3 or BALB/3T3 cells by calcium phosphate precipitation (18). The presence of infectious virus was confirmed 1 week later by the XC plaque assay (41). At 2 weeks posttransfection, culture fluids were assayed for reverse transcriptase activity (2). Virus supernatants used for injection of mice were harvested after 2 to 3 weeks. Viruses for injection were obtained from transfection of two independent molecular clones of each construct.

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 10^3 to $10^{4.5}$ PFU per mouse. Mice received viruses intraperitoneally or intraperitoneally and intrathymically and were sacrificed by CO_2 anesthesia when diseased. Animals were autopsied and examined for gross evidence of erythroleukemia or lymphoma. For most mice, diagnosis included microscopic examination of blood smears, spleen imprints, and/or histological preparations. Diagnosis of erythroleukemia, lymphoma, or myelogenous leukemia was based on gross pathology and histology as described previously (6).

Confirmation of virus genome structure. Viruses were purified biologically by limiting dilution starting with each of the recombinant virus supernatants. These biologically cloned virus stocks were also injected into newborn NFS mice, and the resulting disease and latency were noted and compared with those induced by uncloned stocks. As an additional control, genome structure of injected viruses was determined by either RNase T1 fingerprinting [for Mo:Fr(ABC) and Fr:Mo(AB)] (13) or by restriction enzyme analysis and Southern blotting (47) of Hirt supernatants (for all other viruses) (23). Viral DNA in Hirt supernatants was analyzed by restriction endonuclease digestion with the enzymes *Bgl*I, *Pvu*II, and *Eco*RV. This analysis was performed for each recombinant virus by using the initial uncloned stocks obtained from transfection and used for injection. It was also performed on biologically cloned viruses used for injection. Hirt supernatants were also prepared from cells that had been infected with virus reisolated from tumors as follows. Mo:Fr(A) was isolated from one mouse with erythroleukemia and one mouse with erythroleukemia and lymphatic lymphoma. Mo:Fr(AC) was isolated from one mouse with erythroleukemia and one mouse with lymphoblastic lymphoma. Mo:Fr(b1) was isolated from one mouse with erythroleukemia. Analysis of these supernatants by Southern blotting indicated that viral stocks were free of contamination with parental Moloney or Friend virus.

RESULTS

(i) Construction of viruses with recombinant enhancers. The design and construction of enhancers with fragments derived from both Friend and Moloney viruses was dictated by a comparison of the sequences of the two viruses in this region of the genome (28, 44). An alignment of the sequences of the Friend and Moloney enhancer regions is shown in Fig. 1. The Moloney direct repeat has a 9-66-9-66-9 structure, with the three 9-base elements corresponding to the consensus sequence of the glucocorticoid response element (10). The two 66-base sequences are identical. The so-called GC-rich segment of Moloney virus, lying just 3′ of the last glucocorticoid response element, shares all but 4 bases with the corresponding Friend virus segment. The Friend virus direct repeat is less uniform than that of Moloney virus, following roughly a 65-65 repeat structure. There are several base changes between the two copies of the 65-base sequence, and there is a 9-base insertion in the second copy.

Nucleotide differences between the direct repeats of Friend and Moloney viruses cluster on either side of a central conserved region of identity. We wished to determine the input of each of the regions of sequence diversity to disease specificity. In order to perturb the natural enhancer structure as little as possible, we chose to construct recombinants within the Friend and Moloney virus enhancers by using the *Eco*RV site located in the center of the conserved region (Fig. 1). We have designated the region from the 5′ end of each repeat to the *Eco*RV site A and the region from *Eco*RV through the 3′ end of each Moloney virus repeat (extending to *Ava*II in the second copy) B. The GC-rich segment between *Ava*II and *Dde*I is designated C. Thus the region under study has the structure (AB) (AB) C. Using the schemes detailed in Materials and Methods, we constructed recombinant enhancers between Friend and Moloney virus in which both A segments or both B segments or just the C segment were derived from one virus and the remainder of the genome was derived from the other. Other recombinants received two sequence elements from one virus (A + B, A + C, or B + C) and the remainder of their genome from the other. We also constructed one recombinant in which the B segment was further dissected so that only the region we designate b1 from Friend virus was introduced into both copies of the Moloney virus direct repeat (Fig. 1). This involved changing only 2 bases in the b1 region of the Moloney direct repeat.

Most recombinant enhancers were made in a Moloney virus genomic background; however, some were also made in a Friend virus background, in order to assess the contribution of other genomic sequences to disease specificity. The recombinant enhancers we constructed are shown diagramatically in Fig. 2. Figure 2a shows those enhancers that were introduced into the Moloney virus genome, while Fig. 2b shows those introduced into the Friend virus background. Names of recombinant viruses give the source of the genomic background first and then the name of the virus donating small enhancer segments followed by the name of the segment(s). For example, Mo:Fr(C) is a Moloney virus whose GC-rich region has been replaced with the corresponding sequence from Friend virus.

It is important to emphasize that in each construct that involved exchanges within the direct repeat, the changes were made to both copies of the repeat sequence. For example, Mo:Fr(A) consists of a Moloney virus genome whose direct repeat has the structure (Friend A-Moloney B) (Friend A-Moloney B). While it would have been simpler

TABLE 1. Types of leukemia induced by Moloney, Friend, and recombinant viruses

Virus	No. positive[a]/no. inoculated	No. of diagnosed tumors						% of diagnosed tumors[b]		
		Lymphoma	Erythro-leukemia	Myelogenous leukemia	Megakaryocytic leukemia	Lymphoma + erythroleukemia	Other	Lymphoma	Erythro-leukemia	Other
Moloney	44/44	44						100		
Friend	61/61		59						100	
Mo:Fr(ABC)	63/63		63						100	
Mo:Fr(AB)	31/31	2	28			1		9	91	
Mo:Fr(AC)	64/64	34	21	1		7	1[c]	57	40	3
Mo:Fr(BC)	79/81	42	23	4	1	5	3[d]	57	34	9
Mo:Fr(A)	71/71	59	6	1	1	2	1[e]	85	11	4
Mo:Fr(B)	42/50	40	1				1[f]	95	2	2
Mo:Fr(b1)	32/32	25	2		2	1	2[g]	77	11	12
Mo:Fr(C)	35/38	33	1				1[h]	92	3	5
Fr:Mo(S-K)	28/28	26		1		1		93	3	3
Fr:Mo(AB)	72/75	57	7	2	3	1		82	11	7
Fr:Mo(A)	37/41	4	22	6	2		1[i]	11	63	26

[a] Number sacrificed with severe disease or found dead. Deaths without gross or histopathological diagnosis represented 1.8% of total.
[b] Percentages were calculated as the number of a given type of leukemia divided by the sum of all leukemias upon injection of virus. For this purpose, mice diagnosed as having two distinct types of leukemias were scored twice.
[c] Erythroleukemia plus myelogenous leukemia.
[d] Erythroleukemia plus myelogenous leukemia (1) and lymphoma plus megakaryocytic leukemia (2).
[e] Lymphoma plus histiocytic sarcoma.
[f] Lymphoma plus megakaryocytic leukemia.
[g] Lymphoma plus megakaryocytic leukemia (1) and erythroleukemia plus myelogenous leukemia (1).
[h] Myelogenous leukemia plus megakaryocytic leukemia.
[i] Histiocytic sarcoma.

technically to generate recombinants with just one copy of the direct repeat sequence, previous experiments had shown that removal of one copy of the direct repeat from our clones of Moloney and Friend viruses results in viruses with a very long latent period of disease induction, a situation we wished to avoid in the present study (31). Recombinants that received Friend A or Friend B segments received the two slightly different sequences seen in the two copies of the direct repeat of the parental Friend virus.

(ii) Disease specificity of viruses with recombinant enhancers. Table 1 shows the frequency of each type of leukemia induced by viruses with recombinant enhancers. First we consider data for viruses whose genomes are derived almost entirely from Moloney virus but contain some portion of their enhancer region from Friend virus. As shown before, introduction of the entire enhancer region from Friend virus [virus Mo:Fr(ABC) in Table 1] almost completely alters the disease specificity of Moloney virus (31). Strikingly, recombinants which substitute Friend sequences into two of the three regions (A, B, or C) also demonstrated a significant alteration in disease specificity. Mo:Fr(AB), which substitutes the entire direct repeat from Friend virus but leaves the Moloney virus GC-rich segment intact, induced greater than 90% erythroleukemia. Mo:Fr(AC) and Mo:Fr(BC) each have half of the Friend virus direct repeat sequence combined with the Friend virus GC-rich region in a Moloney virus background. These two viruses induced approximately 34 to 40% erythroleukemia, 57% lymphoma, and, especially in the case of Mo:Fr(BC), a number of leukemias involving other hematopoietic lineages.

The fact that the intact direct repeat from Friend virus conferred a ≥90% incidence of erythroleukemia on Moloney virus might lead one to suppose that the GC-rich region of Friend virus (segment C) plays little role in disease specificity. In this case, the substantial incidence of erythroleukemias and other nonlymphoid tumors induced by viruses with one half of their direct repeat plus their GC-rich region from Friend virus [Mo:Fr(AC) and Mo:Fr(BC)] would be due primarily to the presence of the A or B segments of the direct repeat, respectively, and not to the GC-rich segment. Analysis of the disease specificity of recombinants with just A, B, or C sequences derived from Friend virus tested this hypothesis. As shown in Table 1, Mo:Fr(C) did indeed induce a negligible incidence of erythroleukemia (1 of 38 mice). However, in contradiction to the hypothesis that segment C is inconsequential as a determinant of erythroleukemia, when just the 5′ halves (A regions) or just the 3′ halves (B regions) of the Friend virus direct repeat were present in a Moloney virus genome, they were considerably less potent determinants of erythroleukemogenicity than they were when the Friend virus GC-rich region was also present. Thus, Mo:Fr(A) induced 11% erythroleukemias and 4% other nonlymphoid tumors instead of the 43% erythroleukemias and other nonlymphoid tumors seen with Mo:Fr(AC). Even more dramatic, Mo:Fr(B) induced only 1 erythroleukemia among 42 tumors versus 43% nonlymphoid tumors seen with Mo:Fr(BC).

A somewhat surprising result was obtained with the virus Mo:Fr(b1). Although this virus contained fewer Friend virus sequences from the B region than did Mo:Fr(B), it induced a higher incidence of nonlymphoid tumors (7 of 32).

Table 1 also shows the disease specificity of the few recombinants constructed with segments of the Moloney virus enhancer in a Friend virus genomic background. As previously reported, viruses with the entire direct repeat and GC-rich region from Moloney virus induced 93% or more T-cell lymphomas, although it should be noted that the virus analyzed in previous studies which contained the Moloney virus A, B, and C segments in the Friend virus background included the entire *Sau*3A1-*Kpn*I fragment of Moloney virus (7). The virus now designated Fr:Mo(AB), with just its direct repeat derived from Moloney virus, induced 82% T-cell disease (31). Thus, the Moloney virus direct repeat, like the Friend virus direct repeat, contains most of the determinants

of disease specificity. Fr:Mo(A), the reciprocal recombinant to Mo:Fr(A), induced 11% T-cell lymphomas. Thus, the 5′ halves of the Moloney virus direct repeat were about as potent as those of Friend virus in determining disease specificity when they were removed from their normal context and placed in the alien genome.

(iii) Influence of sequences outside the enhancer on disease specificity. The previous finding that an intact enhancer region from either Friend or Moloney virus almost completely determines disease specificity in recombinants between these two viruses argues that other genomic sequences play a minor part in this phenotype (6, 7, 31). However, the data in Table 1 suggest that the genomic context of a recombinant enhancer can influence disease specificity significantly and indicate that the degree to which it does so may depend on the integrity of the enhancer.

Two pairs of viral constructs contain identical recombinant enhancers in either the Friend or Moloney virus genome (Fig. 2 and Table 1). Mo:Fr(BC) and Fr:Mo(A) both have direct repeats whose A regions are derived from Moloney virus and whose B regions come from Friend virus, and both derived their GC-rich segments from Friend virus. A virus in which this enhancer resides in a Moloney virus genome, Mo:Fr(BC), induced about 57% T-cell lymphomas, 34% erythroleukemias, and 9% myelogenous leukemias. When the same enhancer was present in a Friend virus genome, the resulting virus, Fr:Mo(A), induced only about 11% T-cell lymphomas, 63% erythroleukemias, and 26% myelogenous leukemias. These observations reveal the need for caution in interpreting the contribution of short segments of the enhancer to disease specificity in a quantitative way. In the example just given, Moloney virus A segments conferred an incidence of 57% T-cell lymphomas when they resided in the Moloney virus genome but only 11% T-cell lymphomas when they resided in the Friend virus genome.

In contrast to this example, the pair Mo:Fr(C) and Fr:Mo(AB), which both have their entire direct repeat from Moloney virus and their GC-rich region from Friend virus, reflect far less influence of outside sequences. Mo:Fr(C) produced about 92% T-cell lymphomas and 3% erythroleukemia, while Fr:Mo(AB) produced about 82% T-cell lymphomas and 11% erythroleukemia. Together these results may indicate that when the enhancer is a potent specificity determinant, the contribution of other genomic sequences is almost undetectable, and as the enhancer is weakened, the other sequences play an increasingly important role.

(iv) Latent period of disease induction by viruses with recombinant enhancers. Curves showing the latent period of disease induction for viruses with recombinant enhancers in a Moloney virus genome are presented in Fig. 3a and b along with previous data showing disease induction by Moloney virus and Moloney virus with a complete Friend virus enhancer region [Mo:Fr(ABC)]. Ignoring for the moment the type of disease induced, Moloney virus induced disease with an average latent period of 81 days, and Mo:Fr(ABC) had a similar latency, 77 days, under our conditions. The two viruses with their direct repeat from one parent and their GC-rich region from the other, Mo:Fr(C) and Mo:Fr(AB), both had slightly delayed latencies of 100 days and 111 days, respectively (Fig. 3a). Interestingly, viruses whose direct repeats were split so that the A and B sequences came from different donors fell into two groups (Fig. 3b). The two viruses whose A regions came from Friend virus and whose B regions came from Moloney virus, Mo:Fr(A) and Mo:Fr(AC), exhibited no delay in average latency to disease relative to parental Moloney virus. In sharp contrast, viruses whose A regions were derived from Moloney virus and whose B regions were derived from Friend virus exhibited a significant increase in average latent period of disease induction; Mo:Fr(B) and Mo:Fr(BC) had average latencies of 143 and 150 days, respectively. When just half of the B region of the direct repeat was derived from Friend virus and the A regions were derived from Moloney virus (i.e., virus Mo:Fr(b1), there was also a delay in the latent period of disease induction; in this case, the average was 127 days (Fig. 3a).

Disease induction as a function of time for viruses with recombinant enhancer regions in the Friend virus genomic background is plotted in Fig. 3c. Parental Friend virus induced erythroleukemia with an average latent period of 55 days. Fr:Mo(S-K), whose entire enhancer region was derived from Moloney virus, induced lymphomas with an average latent period of 73 days. Fr:Mo(AB), with its direct repeat derived from Moloney virus and its GC-rich region derived from Friend virus, exhibited a latency of 97 days, similar to the latency of the two other viruses described above whose direct repeats were derived from one donor and whose C region was derived from another [Mo:Fr(AB) and Mo:Fr(C)]. The virus Fr:Mo(A), with the same enhancer composition as Mo:Fr(BC), had a latency of 118 days, showing again the significant delay associated with this enhancer whose A regions are derived from Moloney virus and whose B regions are derived from Friend virus.

We investigated whether the latent period for disease induction was related to the type of leukemia induced. The data pertaining to this question are summarized in Table 2. For the most part, the latent period is not related to the type of leukemia induced; erythroleukemias and T-cell lymphomas are fairly evenly distributed throughout the disease induction curves. The exceptions to this pattern are Mo:Fr(b1), Mo:Fr(B), and Mo:Fr(C). In the case of these viruses, T-cell lymphomas occurred on average between 96 and 142 days after injection, whereas the few erythroleukemias and myelogenous leukemias occurred considerably later and were clustered at the end of each latency curve.

DISCUSSION

In our previous reports, we established that the primary determinant of disease specificity in Friend and Moloney murine leukemia viruses was their enhancer regions, consisting of the direct repeat plus GC-rich sequence (31). With the present results we provide a detailed analysis of the effect of shorter sequences within this region on the disease profile of these viruses. Our most striking finding is that no single segment within the enhancer region was able to program completely erythroleukemogenicity or T-cell lymphomagenicity. Rather, these phenotypes are the products of the contributions of distinct regions within the enhancer.

We divided the region of interest into sequences in the 5′ half of the direct repeat, sequences in the 3′ half of the direct repeat, and the GC-rich region. The data show clearly that all three segments of Friend virus contribute to specificity. Both halves of the Moloney virus direct repeat are important for T-cell lymphomagenesis, while a role for the Moloney virus GC-rich sequence in specifying this phenotype is implied but less conclusively demonstrated by the particular constructs studied.

Previous results showing that an intact enhancer region from either Friend or Moloney virus almost completely determines the type of leukemia induced by recombinants between these two viruses argued that sequences outside the enhancer region play only a small part in determining disease

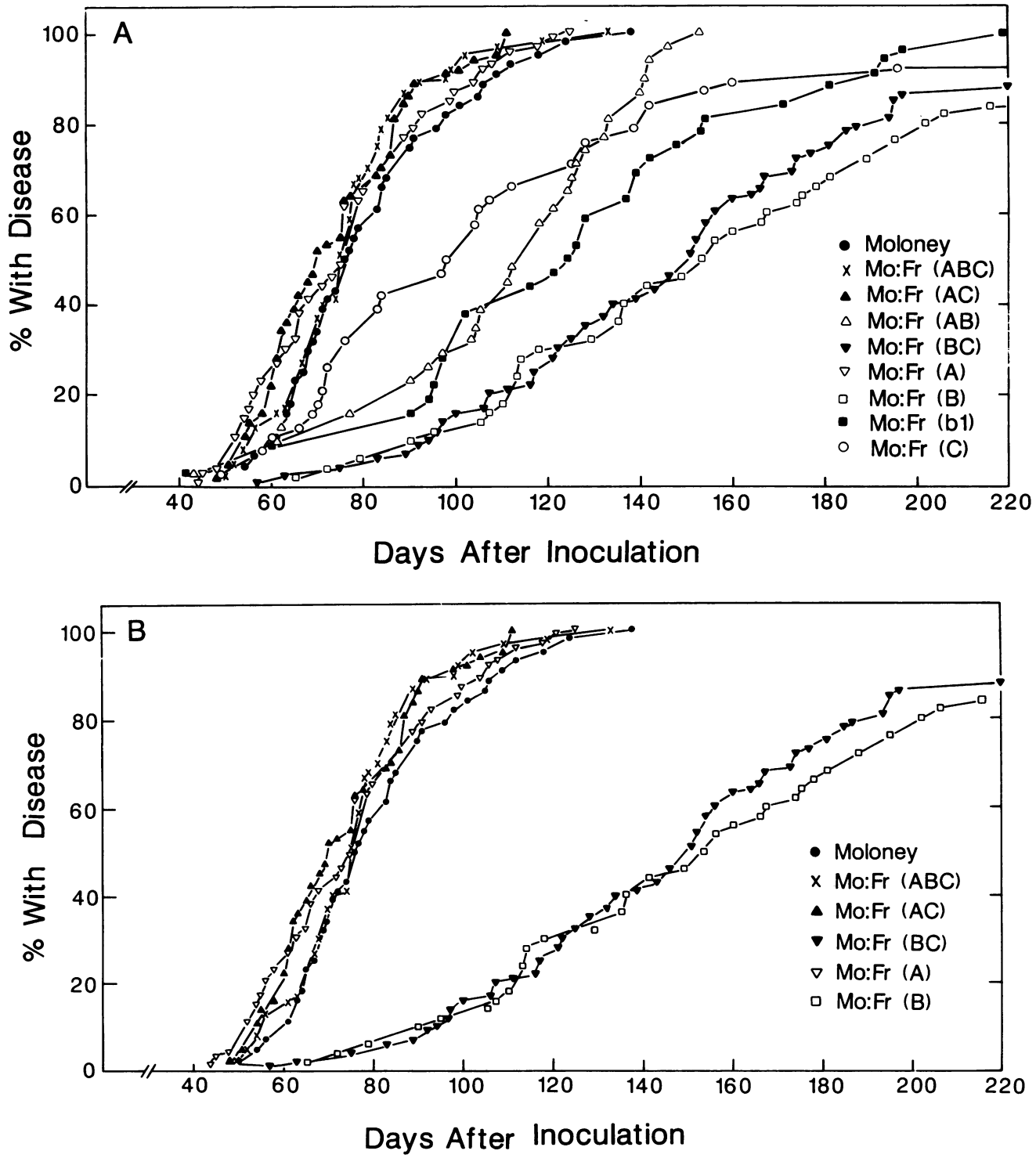

FIG. 3. Leukemia induction by Friend, Moloney, and enhancer-recombinant viruses in NFS mice. (A) Leukemia induction as a function of time following injection of newborn mice with Moloney virus or with recombinants whose genome is predominantly derived from Moloney virus. (B) Curves for six of the viruses in panel A redrawn to emphasize the differences in latent period for the two groups of recombinants whose direct repeats are half Moloney virus, half Friend virus sequences. (C) Leukemia induction as a function of time following injection of newborn mice with Friend virus or with recombinants whose genome is predominantly derived from Friend virus.

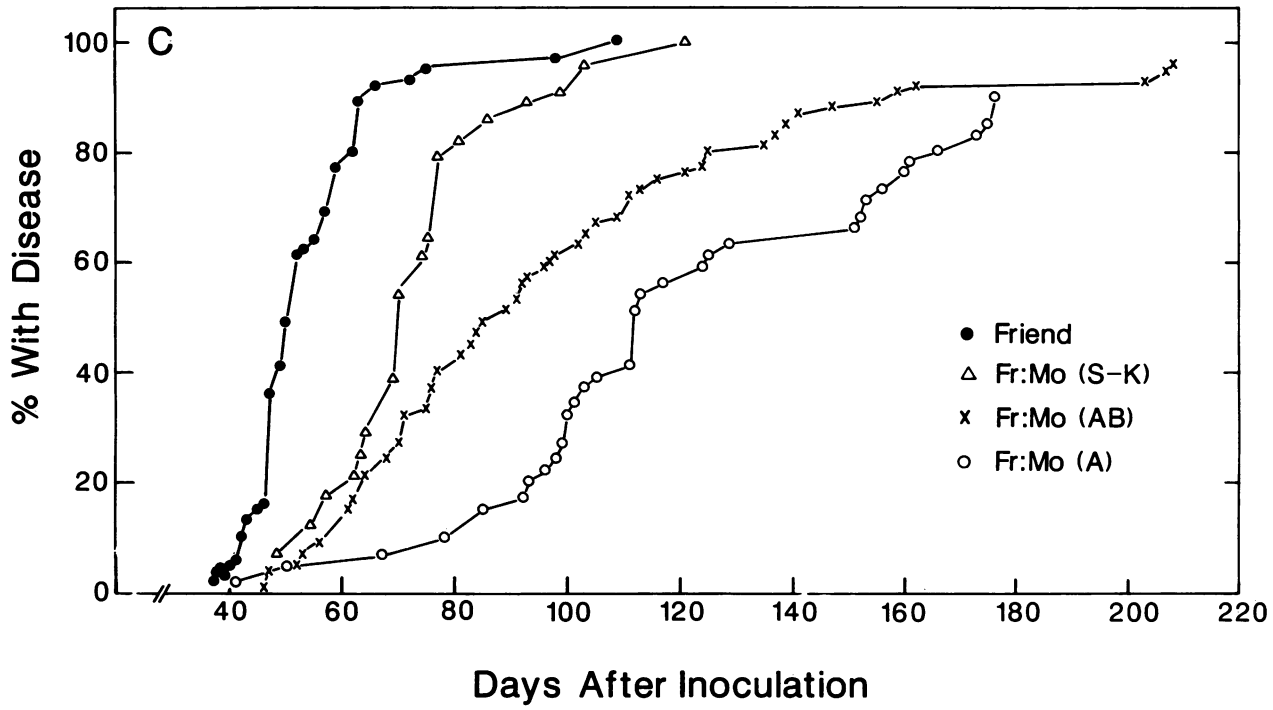

FIG. 3—*Continued.*

specificity (6, 7, 11, 31, 37). In the present studies, it was apparent that as the ability of the enhancer to determine the type of leukemia induced is weakened by mixing sequences from Friend and Moloney viruses, sequences outside this region assume a greater role in determining the outcome of an infection. Thus the true, intrinsic potency of individual enhancer segments as determinants of specificity cannot really be known but can only be compared for segments lying in the same genomic context.

TABLE 2. Latent period of disease induction by enhancer-recombinant viruses and relationship to disease specificity

Virus	Avg latent period (days)	Avg latency for:		No. positive[a]/ no. inoculated
		Lymphoma (no.)	Erythro-leukemia (no.)	
Moloney	81	81 (44)		44/44
Friend	55		55 (59)	61/61
Mo:Fr(ABC)	77		77 (63)	63/63
Mo:Fr(AB)	111	95 (3)	110 (29)	31/31
Mo:Fr(AC)	74	71 (41)	78 (29)	64/64
Mo:Fr(BC)	150	149 (49)	148 (29)	79/81
Mo:Fr(A)	75	74 (62)	86 (8)	71/71
Mo:Fr(B)	143	142 (41)	174 (1)	42/50
Mo:Fr(b1)	127	116 (27)	172 (4)	32/32
Mo:Fr(C)	100	96 (33)	156 (1)	35/38
Fr:Mo(S-K)	73	71 (27)	74 (1)	28/28
Fr:Mo(AB)	97	87 (58)	113 (8)	72/75
Fr:Mo(A)	118	113 (4)	116 (22)	37/41

[a] Any discrepancies between totals and numbers for lymphoma and erythroleukemia are due to concomitance of these tumors, to occurrence of another category of hematopoietic neoplasm, or to deaths without definitive diagnosis.

Most of our data pertain to recombinants with primarily a Moloney virus genome and fragments of the Friend virus enhancer. For these viruses, the data show that single elements of the Friend virus enhancer region (5′ or 3′ halves of the direct repeat or the GC-rich region) are weak or, in the case of the B and C segments, negligible determinants of specificity. They also show that the 5′ and 3′ halves of the direct repeat function together to produce a potent determinant of this phenotype. The data suggest that pairs of elements (particularly the 5′ and 3′ halves of the direct repeat) function cooperatively to specify erythroleukemogenicity. Preventing a firm conclusion for other pairs is concern about a possible discrepancy between the potency of the Friend B and b1 segments as determinants of nonlymphoid tumors. A comparison of the data for Mo:Fr(B) and Mo:Fr(b1) by the Fisher exact test (one tailed) gave a value of $P = 0.03$ when the number of lymphomas was compared with that for all other hematopoietic neoplasms. Given the small number of nonlymphoma diagnoses in these groups, analysis of a larger series of animals would be required to substantiate the significance of these findings biologically.

Viewing the data for these viruses from the other point of view, namely, the potency of Moloney virus enhancer fragments at conferring T-cell lymphomagenicity on a virus whose genome is mostly derived from Moloney virus, we can see that the 5′ or 3′ halves of the direct repeat are quite potent in this situation. However, when the 5′ half of the Moloney virus direct repeats was introduced into the Friend virus genome, as with single segments of the Friend virus enhancer in a Moloney virus background, it became considerably less potent and conferred only a low incidence of T-cell lymphomagenicity.

Our results show that some combinations of enhancer sequences can have striking effects on the latent period of disease induction and can also generate new disease specificities. Many naturally occurring mouse retroviruses show

long latent periods of disease induction and induce tumors of mixed hematopoietic cell lineages (55). It seems likely that enhancer sequences are a frequent contributor to these phenotypes.

We imagine that specific enhancer sequences confer particular disease specificities by conferring preferential growth in certain cell types (35). But how? Is the inability of Moloney virus to induce erythroleukemia, for example, due to a lack of erythroid elements that allow its enhancer to function properly in appropriate target cells, or is it due to the presence of a binding site for a repressor present in these cells? Evidence obtained from transient expression assays (chloramphenicol acetyltransferase) (17) indicate that both positive and negative factors regulate Moloney virus enhancer expression in some systems (3, 22, 51). A suggestion that certain sequences present in the Friend virus enhancer may contribute in a positive way to erythroleukemogenicity comes from the fact that these sequences are found in the enhancer regions of other independent isolates of erythroleukemia-inducing mouse retroviruses but not in exclusively T-cell-leukemia-inducing viruses (E. Golemis, observation from computer alignment of published murine leukemia virus LTR sequences).

Enhancers are thought to function by interacting with specific nuclear factors, and many such proteins have now been identified. Using gel shift assays (15), Speck and Baltimore (48) identified protein-binding sites in the enhancer region of Moloney virus, and Manley, Sharp, and Hopkins (manuscript in preparation) have identified binding sites in the Friend virus direct repeat and GC-rich regions. Particularly relevant to our results, these two studies have identified factors that bind specifically to either the Friend or Moloney virus enhancers in regions identified in our studies as playing a role in disease specificity. These factors include a protein that binds to the glucocorticoid response element consensus sequence, LVa (48), NF-1, and several Friend virus-specific factors designated FVa, FVb1, and FVb2 (N. Manley et al., unpublished results). So far, no tissue-specific distribution of these proteins has been seen which could explain the disease specificity of Friend or Moloney virus; however, studies in some systems have shown that such distributions may be subtle and difficult to detect simply by gel-shift assays (S. McKnight, personal communication). Thus, given the results of our genetic analysis showing the importance of the A, B, and C regions of the Friend and Moloney virus enhancer regions to erythroleukemogenicity and T-cell lymphomagenicity, we think it possible that these protein-binding sites might in some way confer this specificity.

The experiments in this report address the question of which viral enhancer sequences contribute to determining disease specificity. Disease induction by murine leukemia viruses is a complex phenomenon comprising many separate steps. A complete understanding of this process requires analysis of each of these steps and will require a number of different approaches. These include studies of the strength and cell type specificities of different viral enhancers in vitro; examination of viral replication in infected, preleukemic mice; attempts to locate the putative target cell for leukemic transformation; and characterization of the patterns of recombinant mink cell focus-forming virus formation following infection by ecotropic viruses. Through use of the Friend-Moloney recombinant viruses described here, we will be able to use a number of these approaches to pinpoint the steps in leukemogenesis at which U3 exerts its control over disease specificity and so arrive at a detailed molecular picture of how transcriptional signals influence the complex process of disease induction by nondefective mouse retroviruses.

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