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Sequences Responsible for Erythroid and Lymphoid Leukemia in the Long Terminal Repeats of Friend-Mink Cell Focus-Forming and Moloney Murine Leukemia Viruses

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Despite the high degree of homology (91%) between the nucleotide sequences of the Friend–mink cell focus-forming (MCF) and the Moloney murine leukemia virus (MuLV) genomic long terminal repeats (LTRs), the pathogenicities determined by the LTR sequences of the two viruses are quite different. Friend-MCF MuLV is an erythroid leukemia virus, and Moloney MuLV is a lymphoid leukemia virus. To map the LTR sequences responsible for the different disease specificities, we constructed nine viruses with LTRs recombinant between the Friend-MCF and Moloney MuLVs. Analysis of the leukemia induced with the recombinant viruses showed that a 195-base-pair nucleotide sequence, including a 75-base-pair nucleotide Moloney enhancer, is responsible for the tissue-specific leukemogenicity of Moloney MuLV. However, not only the enhancer but also its downstream sequences appear to be necessary. The Moloney virus enhancer and its downstream sequence exerted a dominant effect over that of the Friend-MCF virus, but the enhancer sequence alone did not. The results that three of the nine recombinant viruses induced both erythroid and lymphoid leukemias supported the hypothesis that multiple viral genetic determinants control both the ability to cause leukemia and the type of leukemia induced.

Several observations indicate that the genomic long terminal repeat (LTR) sequence determines the leukemogenicity and tissue specificity of Moloney and AKR murine leukemia virus (MuLV)-induced lymphoid leukemia by conferring tropism for the target cells (5, 9, 21). Chatis et al. (6) reported that the organ-specific pathogenicity of nondefective ecotropic Friend virus is also mainly determined by the sequence of the LTR consisting of 0.38-kilobase-pair (kbp) nucleotides. We previously reported that the erythroid nature of the leukemia induced with Friend–mink cell focus-forming (MCF) MuLV was also mainly determined by the LTR sequence (18). Despite the high degree of homology (91%) between the nucleotide sequences of Friend-MCF (1) and Moloney (28) MuLV LTRs, the pathogenicity determined by their LTR sequences is quite different. Friend-MCF MuLV is an erythroid leukemia virus, and Moloney MuLV is a lymphoid leukemia virus. The LTR sequences of retroviruses contain transcriptional enhancer elements in addition to promoter elements (CAT and TATA boxes) and transcriptional termination signals. Considerable evidence suggests that the sequences localized in a tandemly repeated region lying on the 5′ side of the CAT and TATA promoter elements in the U3 region of the LTR sequence display enhancer activity (22, 33). To clarify the role of the enhancer sequences in disease specificities, we constructed viruses with recombinant LTRs of Friend-MCF and Moloney MuLVs.

MATERIALS AND METHODS

Viruses. Infectious DNA clones (1) of Friend-MCF virus (19) consisted of two clones designated Bp-1 and Ep-2 which were subcloned into two pBR322 vectors at the *Eco*RI site, since the proviral DNA of Friend-MCF virus had two *Eco*RI sites and had been originally molecularly cloned separately into λgtWES · λB vectors at the site (Fig. 1A). An infectious DNA clone of ecotropic Moloney virus was obtained from R. A. Weinberg, Massachusetts Institute of Technology (16). It was recloned into pBR322 at the *Hin*dIII site in our laboratory and designated Molp-1. FrMCF-Mol(LTR) virus (18) is a thymic-lymphoma-inducing virus. The infectious DNA clones of FrMCF-Mol(LTR) virus also consisted of two subclones, B(m)p-3 and Ep-2. B(m)p-3 derived primarily from Bp-1 together with 621 nucleotides of Moloney virus, including the U3 region of the LTR, was constructed with Bp-1 and Molp-1 (Fig. 1A).

Restriction enzymes and digestion. DNA was digested with 2 U of enzyme per μg of DNA under the buffer conditions specified by the manufacturer. For more than one cleavage, a restriction enzyme-cleaved DNA sample was ethanol precipitated, and the pellet was washed in 70% ethanol, air dried, and suspended in the reaction buffer of the second enzyme. Digested DNA was analyzed by electrophoresis at 30 V on 0.7% agarose horizontal slab gels. The standard size marker mixture consisted of 23.7- to 0.10-kbp DNA fragments and was prepared from the product of *Hin*dIII-cleaved λDNA.

Construction of DNA recombinants in vitro. For subcloning, samples (~10 μg) of various subclones were cleaved with restriction endonucleases. The digestion conditions used were those recommended by the suppliers (Takara Shuzo Co. Ltd., Kyoto, Japan, and Toyobo Co. Ltd., Osaka, Japan). The desired fragments were separated with a 0.7% low-melting-point agarose gel (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) under the conditions specified by the supplier. These fragments were then ligated to cleaved pBR322 with T4 DNA ligase at 12°C for 20 h in 10 μl of a solution containing 50 mM Tris hydrochloride (pH 7.5), 10 nM $MgCl_2$, 20 mM dithiothreitol, and 1 mM ATP and

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were used to transform *Escherichia coli* HB101. Colonies were screened by the alkalysis rapid isolation method (2). Positive clones were grown in mass culture. The construction of the various recombinant clones with the chimeric LTRs was confirmed by excising the insert with the appropriate restriction endonucleases, separating it by 0.7 to 1.2% agarose or 5 to 15% polyacrylamide gel electrophoresis at each step of construction to determine whether the inserted fragment was the right one. The various restriction endonucleases used to confirm the construction are shown in Fig. 2. For example, to check the fragments M5 and F5 (Fig. 1B), which are the same length and differ by only five point mutations (Fig. 2), the *Sac*I site (−20 with respect to the cap site within the F5 segment) was used to compare the length, since Moloney virus does not have the site; M5 gave rise to 67-bp *Sac*I-*Kpn*I fragment, and F5 gave rise to a 54-bp *Sac*I-*Kpn*I fragment distinguishable by 10% polyacrylamide gel electrophoresis.

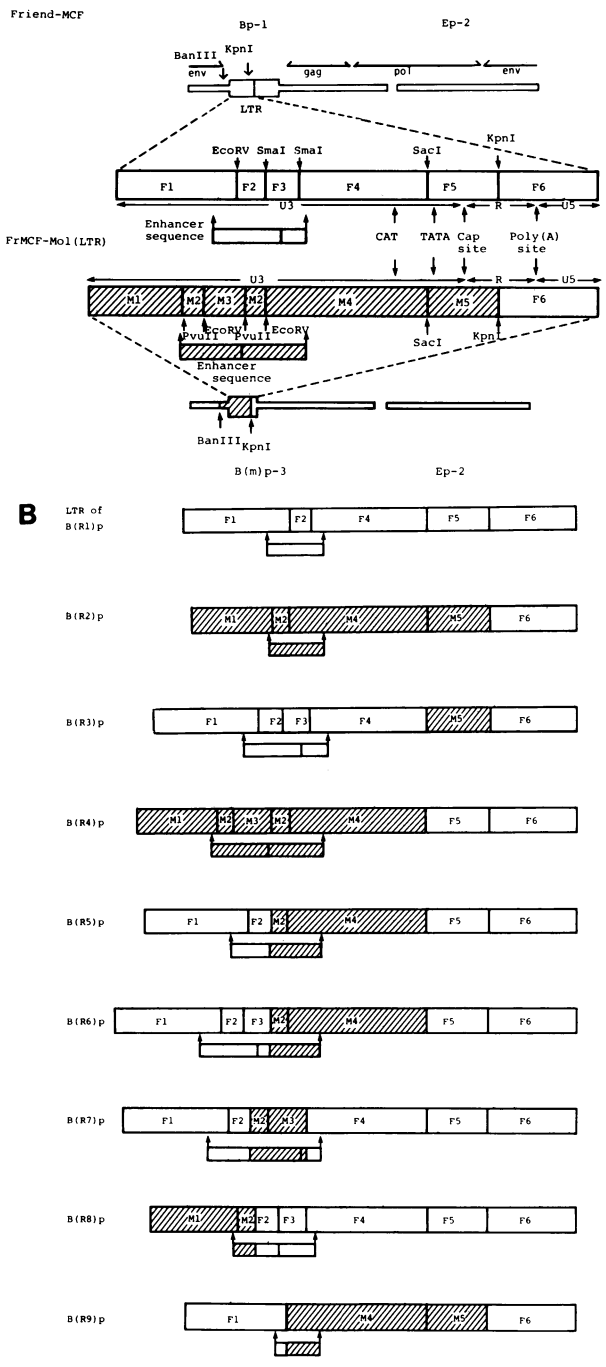

FIG. 1. (A) DNA clones of Friend-MCF (1) and FrMCF-Mol(LTR) (18) viruses and schematic representation of the configuration of the LTRs. Infectious DNA clones of Friend-MCF virus were subcloned into pBR322 at the *Eco*RI site and designated Ep-2 and Bp-1 (1). Infectious DNA clones of FrMCF-Mol(LTR) virus also consist of two viral inserts of Ep-2 and B(m)p3, whose viral insert is derived primarily from Bp-1 together with 621 nucleotides of Moloney virus at its 3′ end including the U3 region of the LTR. In LTRs, the tandem repeats implicated as enhancer elements are shown as the enhancer sequence. CAT and TATA promoter elements, and cap and poly(A) sites are indicated. Each LTR is subdivided into six or seven segments. The LTR of FrMCF-Mol(LTR) virus has sequences derived fom Moloney virus except segment F6, which is derived from the Friend-MCF virus. ▭, Sequences derived from Friend-MCF virus; ▨, sequences derived from the Moloney virus. (B) Schematic representation of recombinant LTRs of Friend-MCF and FrMCF-Mol(LTR) viruses. Nine clones with altered LTRs were constructed between Bp-1 and B(m)p-3 clones by using five restriction endonuclease sites and designated B(R1)p, B(R2)p, B(R3)p, B(R4)p, B(R5)p, B(R6)p, B(R7)p, B(R8)p, and B(R9)p. The segments derived from Friend-MCF or Moloney virus are shown in each LTR.

Transfection. Viral inserts were cleaved from subclones with the appropriate restriction endonuclease and separated with a 0.7% low-melting-point agarose gel. Viral DNAs separated from pBR322 were ligated with T4 DNA ligase. Ligation was confirmed by ethidium bromide staining of the gels; most of the DNAs were converted into circular or linear dimers and several unknown forms of higher molecular weight. The religated DNA was then transfected into NIH 3T3 or SC-1 cells in 6-cm-diameter plates by a modification (34) of the original calcium phosphate precipitation method (11).

Cells and virus assay. SC-1 (12), NIH 3T3 (30), mink lung ATCC CCL-64 (15), and S+L mink cells (27) were grown in Dulbecco-Vogt modified Eagle minimal essential medium supplemented with a 5% heat-inactivated fetal calf serum. Dual-tropic MCF virus assays were performed by the focus-forming assay in mink S+L or lung cells (13).

Mice. NFS mice are an inbred strain from an NIH Swiss mouse originally supplied by the animal production section. A continuous single line was maintained in our laboratory by sibling mating. The recovered MCF viruses (0.2 ml) (10^4 S+L focus-forming units per mouse) harvested from mink cells were inoculated intraperitoneally into newborn NFS mice.

RESULTS

Preparation of recombinant virus. To construct the recombinant LTRs between the Friend-MCF and Moloney MuLV LTRs, we used DNA of the FrMCF-Mol(LTR) virus instead of the original Moloney virus since FrMCF-Mol(LTR) and Moloney viruses are both lymphoid leukemia viruses (18). The cleavage sites for the restriction endonucleases *Eco*RV, *Sac*I, and *Kpn*I common to Friend-MCF and Moloney MuLV LTRs were used to construct the in vitro recombinant viruses. The *Eco*RV and *Sac*I sites are located in the U3 region, and the *Kpn*I site is located in the R region of the LTR. In addition to these common sites, two *Sma*I sites in

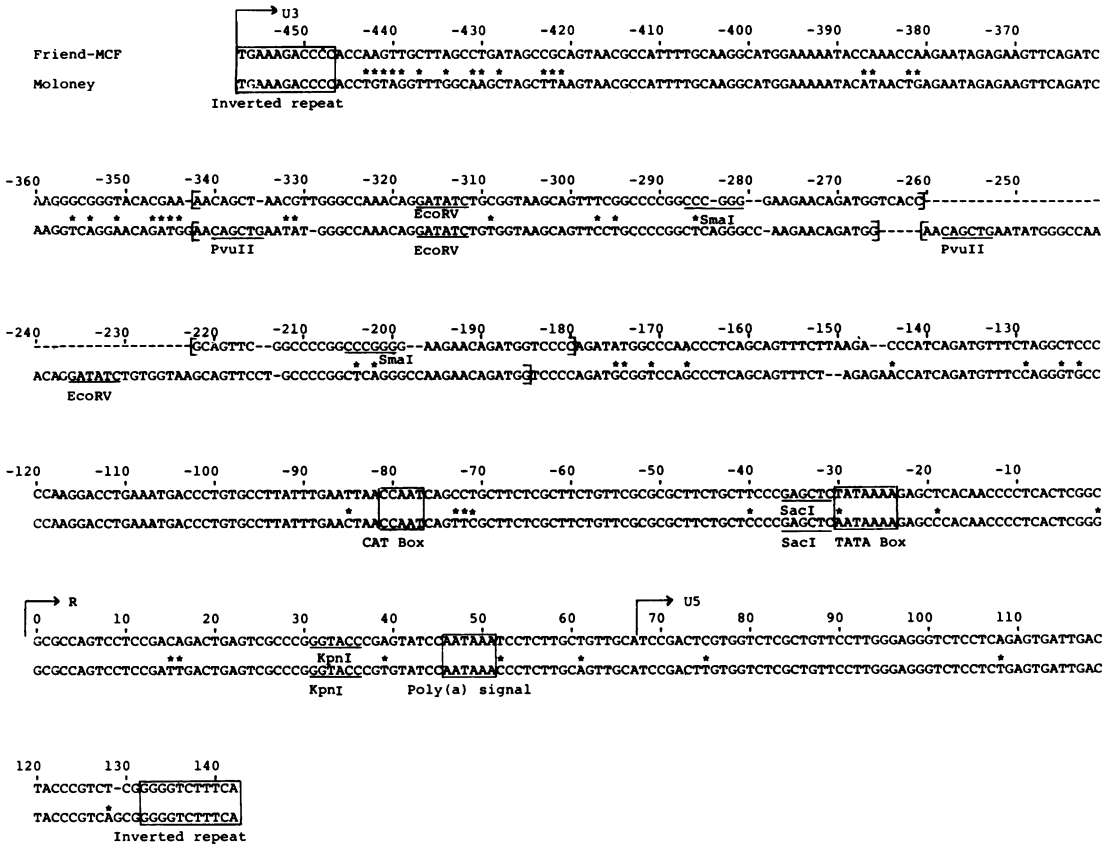

FIG. 2. Nucleotide sequence of the Friend-MCF (MCF-FrNx strain) LTR region compared with those of the Moloney LTR (1, 28). Nucleotides different from those of Friend-MCF LTR are indicated by asterisks. The major structural features of this region, i.e., the inverted repeats, the CAT and TATA boxes, and the poly(A) signal, are indicated. The tandemly repeated sequences in which enhancer elements are included are enclosed in brackets. The restriction endonucleases used for the construction of recombinant LTRs, *Sac*I, *Kpn*I, *Sma*I, *Eco*RV, and *Pvu*II, are indicated. Friend-MCF virus has one more *Sma*I site at +30 and *Sac*I site at −20 (with respect to the RNA cap site) within its LTR. Moloney virus also has one *Sma*I site at +30.

virus LTR, and two *Pvu*II sites in the tandemly repeated enhancer sequence of Moloney virus LTR were also used. *Sma*I and *Pvu*II were used to make blunt ends, and the sites cut by them were religated to each other. The LTRs of Friend-MCF and FrMCF-Mol(LTR) were divided by these restriction endonuclease sites into six and seven segments, respectively (Fig. 1A). The LTR of Friend-MCF virus was divided into segments F1 to F6. That of FrMCF-Mol(LTR) was divided into segments M1 to M5 and segment F6. The segments M1 to M5 were derived from Moloney virus, and segment F6 was derived from Friend-MCF virus.

Biological activity of the MuLVs with recombinant LTR regions. The biological activity of the viruses with deleted or recombinant LTRs was assessed by constructing a recombinant complete proviral DNA sequence, transfecting the DNA into SC-1 or NIH 3T3 cells, and bioassaying for infectious virus in vivo. All of the recovered viruses were XC-negative, NB-tropic, and dual-tropic viruses like Friend-MCF virus (1), since all genomes of the viruses with the chimeric LTR were derived primarily from Friend-MCF virus except the LTR and the sequence coding for the C terminus of p15E. The infectious viruses recovered were inoculated into newborn NFS mice to assess their pathogenicity (Fig. 3). Normal Moloney and Friend-MCF viruses have complete or incomplete tandemly repeated sequences in the U3 region. We hereafter refer to this tandemly repeated sequence as an enhancer sequence, although it is not yet clear whether the sequence actually has an enhancer activity.

Although Moloney virus has two complete copies of a 75-bp enhancer sequence and Friend-MCF virus has one complete and one incomplete copy (1, 28), many murine retroviruses have only one (3, 32), which suggests that one copy alone can determine the biological activity of a virus. To learn whether the Friend-MCF and FrMCF-Mol(LTR) viruses need more than one copy of the sequence to retain their biological activity, we first constructed LTRs with only one copy (Fig. 1B). The R1 and R2 viruses containing deleted LTRs [designated B(R1) and B(R2)], from which segments F3 and M3+M2 had been deleted, respectively, showed almost the same disease specificity as the original viruses (Fig. 3). Next, we constructed viruses containing recombinant LTRs [designated B(R3) and B(R4)], in which segments F5 and M5 had been reciprocally exchanged, to find out whether these segments were responsible for the tissue-specific pathogenicity of the viruses. Segments F5 and M5 included the TATA box and showed 93% homologous nucleotide sequences (Fig. 2). The viruses containing these recombinant B(R3) and B(R4) LTRs showed almost the same pathogenicity as the original viruses. The results obtained from the four viruses containing B(R1), B(R2), B(R3), and B(R4) LTRs suggested that the tissue specificity

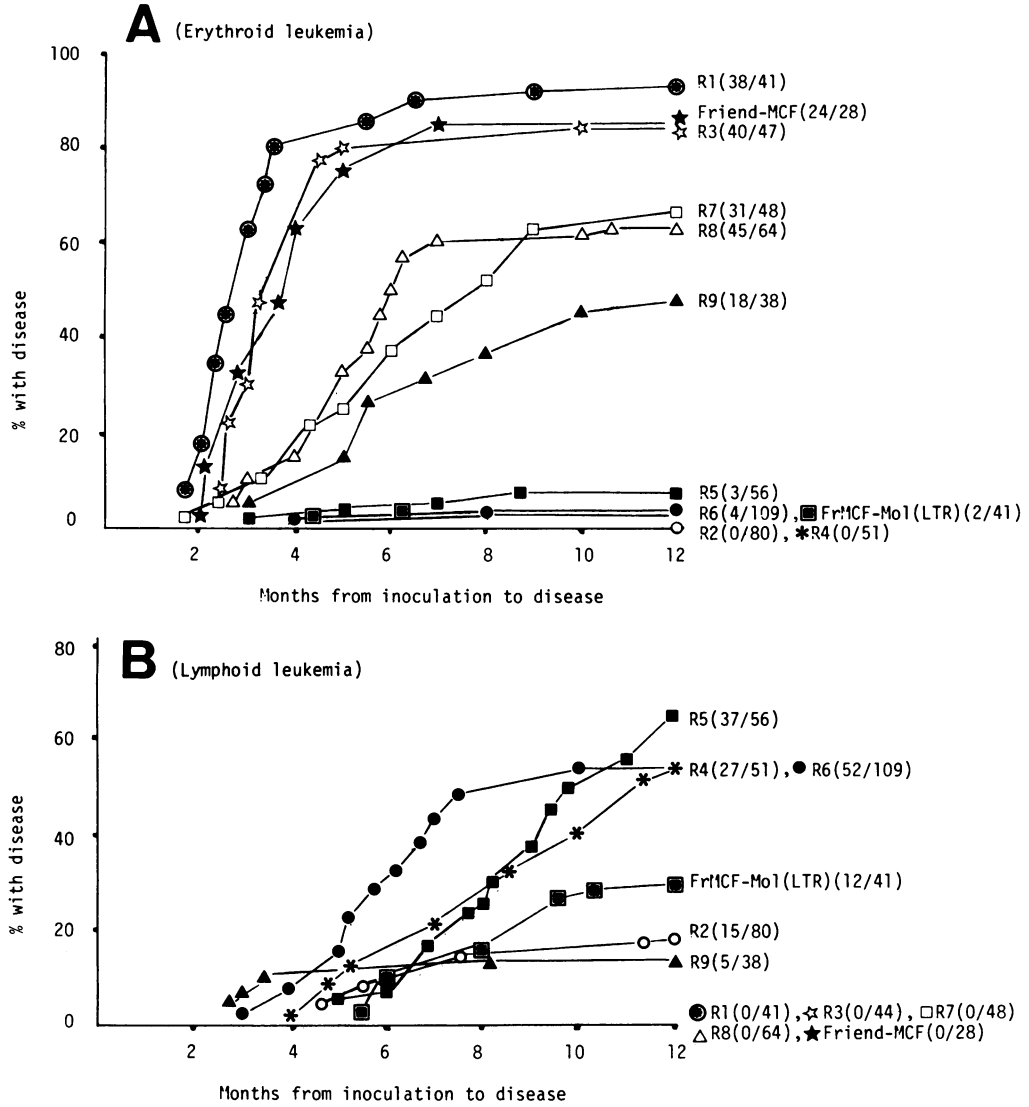

FIG. 3. Cumulative incidence of erythroid (A) and lymphoid (B) leukemia in NFS mice induced by the parental and recombinant viruses. Numbers in parentheses indicate the number of mice with leukemia/the number of mice inoculated. The infectivity of recombinant MuLV DNA with altered LTRs was tested by the calcium phosphate transfection procedure (11, 34) into NIH 3T3 and SC-1 cells. Viral inserts in B(R1)p to B(R9)p and in Ep-2 were excised by cleavage with *Eco*RI. Viral DNAs from Ep-2 and each clone with an altered LTR were ligated with T4 DNA ligase. The viruses recovered after the transfection were XC-negative, dual-tropic MCF viruses. Newborn NFS mice, usually less than 24 h old, were injected intraperitoneally with 0.1 ml of virus stock (10^4 S+L mink cell focus-forming units per mouse). Friend-MCF and viruses with altered B(R1), B(R3), B(R5), B(R6), B(R7), B(R8), and B(R9) LTRs induced erythroid leukemia. The erythroid mice showed marked hepatosplenomegaly but no enlargement of thymus or lymph nodes. Histologically, they had typical erythroid leukemia. Most of the leukemias developed between 75 and 110 days after inoculation. FrMCF-Mol(LTR) and viruses with altered B(R2), B(R4), B(R5), B(R6), and B(R9) LTRs induced lymphoid leukemia. Histologically, most of the leukemias were typical T-cell lymphomas with involvement of spleen, lymph nodes, thymus, and liver. No pathological differences were observed between lymphomas induced with Moloney virus and the recombinant viruses, except for the incidence of leukemia and the latent period.

was determined by any one or some segments of F1, F2, F4, M1, and M2, M4. To see which of these segments was responsible for the disease specificity, we constructed recombinant viruses with LTRs designated B(R5), B(R6), B(R7), B(R8), and B(R9). B(R5) and B(R6) LTRs had Friend-MCF virus segments except segment M2+M4, and B(R7) LTR had Friend-MCF sequences except segment M2+M3. The infectious viruses containing B(R5) and B(R6) LTRs induced lymphoid leukemia 2 to 12 months after inoculation into newborn NFS mice; their latent periods were longer than those of the parent viruses (Fig. 3). Segment M2+M4 consisted of 217-bp nucleotides, which included a 70-bp almost-complete copy of the Moloney enhancer sequence (−255 to −185) and the CAT box. Although segment F1+F2 included a 57-bp incomplete Friend-MCF enhancer sequence (−342 to −285), segment F1+F2+F3 included a 78-bp complete copy of the sequence. The virus containing the B(R6) LTR, which included the complete sequence of Friend-MCF enhancer sequence and an almost-complete copy of the Moloney enhancer se-

quence, induced mainly lymphoid leukemia (52%) similar to that induced by Moloney virus and partly erythroid leukemia (4%).

To learn whether the 75-bp enhancer sequence of Moloney virus was responsible for the lymphoid leukemia inducibility, a recombinant LTR B(R7) was constructed in which an almost complete copy of the Moloney enhancer sequence, segment M2+M3, was inserted into B(R1) LTR. However, the infectious virus containing B(R7) LTR induced only erythroid leukemia. To elucidate the specific regions within the enhancer sequence responsible for the pathogenicity, recombinant B(R8) and B(R9) LTRs were constructed by cleavage at the restriction endonuclease *Eco*RV site shared by the Friend-MCF and Moloney viruses, which is located in the enhancer sequence. The recombinant viruses containing the B(R8) LTR, which consisted of a 27-bp nucleotide sequence of the 5′ end of the Moloney enhancer sequence, induced only erythroid leukemia. However, the recombinant virus with B(R9) LTR containing a 48-bp nucleotide sequence of the 3′ end of the Moloney enhancer sequence and its downstream sequence induced lymphoid leukemia (13%) in addition to erythroid leukemia (47%). The pathogenicity of the various viruses containing the deleted or recombinant LTRs suggested some distinctive features. (i) One copy of the enhancer sequences is sufficient to determine the disease specificity of Friend-MCF and Moloney viruses. (ii) Sequences upstream (−457 to −315) from the tandem repeat of Moloney virus, which show low (78%) homology, are not responsible for the specific pathogenicity of this virus. (iii) Some of the sequences within segment M2+M4 consisting of 217-bp nucleotides, or segment M4 consisting of 195-bp nucleotides, are responsible for the organ-specific pathogenicity of Moloney virus. (iv) Both the enhancer and its downstream sequences appear to be necessary for the tissue-specific leukemogenicity. The Moloney virus enhancer and its downstream sequence exerted a dominant effect over the Friend-MCF virus enhancer sequence, but the enhancer sequence alone did not. (v) The TATA box and its downstream sequence (−30 to +142) are not responsible for the organ-specific leukemogenicity. (vi) Unknown sequences, except segments F2+F3+F4+F5 (−314 to +33), are also partially responsible for the erythroid leukemia inducibility of Friend-MCF virus.

DISCUSSION

Several investigators have suggested that the mechanism of leukemogenesis by MuLV is the activation of a specific cellular gene by insertion of proviral DNA, including viral transcription regulatory elements into the host genome (14, 29, 31). Although some observations indicate that enhancer sequences determine the tissue-specific leukemogenicity of MuLV by conferring tropism for efficient viral replication in target cells and by increasing the probability of the requisite integration event (4, 7, 9), it is not yet known how the enhancer sequence confers tissue-specific efficient viral replication in the target cells.

The Moloney virus 75-bp tandem repeated sequences in the LTR have been expected to function as enhancer in the same way as the Moloney sarcoma virus 72-bp tandem repeated sequences reported by Laimins et al. (20). Although no one has shown directly that the tissue-specific enhancer function resides within the MuLV tandem repeats, some investigators have found that sequences, including the repeated sequence, have an enhancer activity as detected by the CAT gene assay (4) and pathogenicity of the virus (6, 8, 21).

In the present study we attempted to identify the sequences in the LTRs responsible for lymphoid and erythroid leukemia by inserting various segments of the Moloney LTR into the Friend-MCF LTR. The downstream sequence of the tandemly repeated enhancer, in addition to the tandem repeat itself, seems to be important in determining the viral tissue-specific leukemogenicity. In particular, one of the major determinants in the induction of lymphoid leukemia seems to reside in the downstream sequence. However, data showing that three of the nine recombinant viruses induced both erythroid and lymphoid leukemia may support the possibility that multiple viral genetic determinants control the ability to cause leukemia (17, 25).

We examined here the pathogenicity of the recombinant viruses with various chimeric LTRs between Friend-MCF and Moloney viruses. However, the *gag*, *pol*, and *env* genes of all of the viruses were derived from Friend-MCF virus except the sequence coding for the C terminus of p15E. If we had constructed the recombinant viruses with the genes derived from Moloney virus in addition to Friend-MCF virus, we might have obtained some more interesting information about the role of *gag*, *pol*, and *env* genes in the leukemogenicity of the viruses. The reason why we did not construct the viruses with the genes from Moloney virus is because of the technical difficulties in constructing infectious proviral DNA, as shown in our previous study (18).

It is also unknown how the insertion of a fragment consisting of a 195-bp nucleotide sequence, including a 75-bp nucleotide Moloney MuLV enhancer, into the Friend-MCF virus changes the type of leukemia induced. Insertion of the fragment may impair the ability to produce erythroid leukemia or add the ability to produce lymphoid leukemia. Odaka et al. (24) reported that Friend virus induced the lymphoid leukemia in DDD-Fv^r mice, which are Fv-2-resistant mice (24). Induction of lymphoid leukemia in some strains of mice by Friend virus suggests that Friend virus has both an erythroid and a lymphoid leukemia-determining sequence. If so, when a particular recombinant between the Friend-MCF and Moloney viruses induces lymphoid leukemia, it could be because the introduction of the Moloney sequence has destroyed an erythroid-leukemia-determining sequence present in Friend virus rather than because the Moloney sequence conferred lymphoid disease. Even if this recombination did not destroy the sequence, it may have altered the distance between the promoter and the enhancer sequences, which is important for specific pathogenicity.

Davis et al. (7) reported that an enhancer sequence of polyomavirus exerted a dominant effect over the Moloney MuLV enhancer sequence in Moloney MuLV. However, our data showed that the enhancer sequence of Moloney MuLV required its downstream sequence to exert a dominant effect over the Friend-MCF enhancer.

Our major concern in this study is the lack of characterization of the inoculated viruses. Although we have no evidence to the contrary, we are not certain whether the virus inoculated into the mice has the same structure as the cloned DNA transfected. It has been reported that a transfecting retrovirus DNA frequently becomes rearranged and, sometimes, a virus with a genome different than the one transfected is observed due to the recombination between the transfected DNA and the endogenous viral DNA (23, 26). However, the integrity of the genomes of chimeric virus could be checked in some experiments with a specific probe and some restriction endonucleases by using unintegrated viral DNA (26) and DNA of the tumors induced by the viruses (10). Unfortunately, in our experiments neither an

appropriate probe nor restriction endonucleases have been obtained to confirm the integrity of the genome, since the fragments inserted into the Moloney MuLV LTR or into the chimeric viruses described here were derived from the similar virus.

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