Analysis of the Disease Potential of a Recombinant Retrovirus Containing Friend Murine Leukemia Virus Sequences and a Unique Long Terminal Repeat from Feline Leukemia Virus

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We have molecularly cloned a feline leukemia virus (FeLV) (clone 33) from a domestic cat with acute myeloid leukemia (AML). The long terminal repeat (LTR) of this virus, like the LTRs present in FeLV proviruses from other cats with AML, contains an unusual structure in its U3 region upstream of the enhancer (URE) consisting of three tandem direct repeats of 47 bp. To test the disease potential and specificity of this unique FeLV LTR, we replaced the U3 region of the LTR of the erythroleukemia-inducing Friend murine leukemia virus (F-MuLV) with that of FeLV clone 33. When the resulting virus, F33V, was injected into newborn mice, almost all of the mice eventually developed hematopoietic malignancies, with a significant percentage being in the myeloid lineage. This is in contrast to mice injected with an F-MuLV recombinant containing the U3 region of another FeLV that lacks repetitive URE sequences, none of which developed myeloid malignancies. Examination of tumor proviruses from F33V-infected mice failed to detect any changes in FeLV U3 sequences other than that in the URE. Like F-MuLV-infected mice, those infected with the F-MuLV/FeLV recombinants were able to generate and replicate mink cell focus-inducing viruses. Our studies are consistent with the idea that the presence of repetitive sequences upstream of the enhancer in the LTR of FeLV may favor the activation of this promoter in myeloid cells and contribute to the development of malignancies in this hematopoietic lineage.

Nonacute retroviruses lack oncogenes and induce disease, usually lymphoma or leukemia, after a long latency. Insertional mutagenesis, resulting in activation of cellular genes by the inserted viral long terminal repeat (LTR), is considered to be one of the most tenable models to explain tumorigenesis by these retroviruses (12). Studies using chimeras of the genomes of erythroleukemia- and lymphoid leukemia-inducing murine leukemia viruses have shown that the viral LTR is an important genetic determinant of the phenotype of disease induced by nonacute mouse retroviruses (2–5, 10, 11, 13, 14). The role of the viral LTR in determining the disease phenotype of other nonacute retroviruses is less clear. Feline leukemia virus (FeLV) is a nonacute retrovirus that is associated with a variety of neoplastic diseases in domestic cats, including lymphoma and acute myeloid leukemia (AML) (22). FeLV proviruses isolated from naturally occurring thymic lymphomas in domestic cats usually contain tandemly duplicated enhancer sequences in the U3 region of the LTR, while the LTRs derived from weakly pathogenic or non-neoplasia-inducing strains of FeLV contain a single copy of the LTR enhancer (6, 7, 16, 19, 23, 29). The FeLV LTRs from cats with AML (including myeloid and erythroid leukemias) were recently shown to contain a single copy of the U3 enhancer region but frequently contained tandem direct repeats of the upstream region of the enhancer (URE) (19). In order to determine the role of the FeLV LTR in disease specificity, we molecularly cloned an infectious FeLV provirus from one of these cats with AML and analyzed the disease potential of its LTR.

Molecular cloning of FeLV clone 33 and its sequence analysis. High-molecular-weight cellular DNA from the spleen of a cat with AML (19) was isolated. The DNA was digested with EcoRI and ligated to EcoRI-digested EMBL-4 (Stratagene, La Jolla, Calif.), and a DNA library was constructed. Screening of the library with an FeLV LTR U3 probe derived from the pJ7E2 FeLV provirus (17) detected about 30 positive clones out of a total of 500,000 recombinant phages. Seven positive clones were randomly chosen, and after several rounds of plaque purification, phage DNA was extracted. The insert fragment of the recombinant phage was excised by EcoRI digestion and subcloned in the EcoRI site of the plasmid vector pUC18.

To test for infectious FeLV, plasmid DNAs were transfected by using Lipofectamine (Life Technologies, Gaithersburg, Md.) in feline kidney (CRFK) cells. After transfected CRFK cells were passaged several times, the supernatant was measured for reverse transcriptase activity. One clone, designated FeLV clone 33, was found to be biologically active.

The map of the restriction enzyme sites of FeLV clone 33 is shown in Fig. 1A, and the nucleotide sequence of its LTR is shown in Fig. 1B. The LTR contains three tandem direct repeats of 47 bp in the URE and a single copy of the enhancer. The URE of FeLV clone 33 also contains a 20-bp deletion compared with the URE of other FeLVs. The enhancer region of the LTR of FeLV clone 33 contains binding sites for the transcription factors LVb, CORE, NF1, GRE, and FLV-1, as previously reported for the LTR of FeLV/Glasgow-1 (7). We also determined the nucleotide sequence of the env region of
this provirus, and compared with previously reported FeLV sequences, it is most closely homologous to that of FeLV/Glasgow-1 (29). The percentages of identity in the amino acid sequence encoded by the env gene of FeLV clone 33 compared with FeLV/Glasgow-1 and FeLV-C/Sarma (23) are 88.38 and 85.38%, respectively, with the differences being within the gp70 coding region.

**Tumor induction by recombinant viruses F33V and FGLV.** To examine the influence on tumorigenesis of the tandem direct repeats in the URE from the LTR of FeLV clone 33, we constructed a recombinant virus, termed F33V, by replacing the U3 region of the LTR of Friend murine leukemia virus (F-MuLV) clone 57, which contains a tandemly duplicated direct repeat of its 75-bp enhancer (20), with U3 LTR sequences from FeLV clone 33. For comparison, we also constructed another recombinant virus, termed FGLV, in which the U3 region of the F-MuLV LTR was replaced by U3 LTR sequences from a molecular clone of the Glasgow-1 strain of FeLV, a low-virulence strain that contains a single copy of the enhancer but no tandem direct repeats in the URE. To con-
struct F33V and FGLV, the U3 region between the ClaI and KpnI restriction sites in both LTRs of F-MuLV clone 57 (15) was replaced by the analogous ClaI-KpnI fragments of the LTRs of either FeLV clone 33 or Glasgow-1 (29) (Fig. 1C). Once it was confirmed that both recombinant viruses could replicate in NIH 3T3 cells, a focal infectivity assay (18) was used to determine viral titers, and similar amounts of virus (5 × 10^4 focus-forming units [FFU] of FGLV and 14 × 10^4 FFU of F33V) were injected into newborn NIH Swiss mice (19 mice each). Ten newborn NIH Swiss mice were injected with F-MuLV (10 × 10^4 FFU) for comparison. The mice were routinely monitored for evidence of disease, and moribund mice were sacrificed. Figure 2 shows mortality (disease induction) plots for F33V and FGLV recombinant viruses compared with wild-type F-MuLV. As previously described (15, 20), all mice (10 of 10) inoculated with wild-type F-MuLV developed erythroleukemia within 14 weeks (average latency, 7 weeks). Although 84% (16 of 19) of mice inoculated with the F33V recombinant virus and 79% (15 of 19) of mice inoculated with the FGLV recombinant virus developed hematopoietic diseases, the latency periods were significantly increased, with the latency for F33V-induced tumors ranging from 13 to 67 weeks (average, 43 weeks) and that for FGLV-induced tumors ranging from 22 to 62 weeks (average, 43 weeks). Tumors induced by the recombinant viruses F33V and FGLV were diagnosed by gross pathological and histopathological examinations as well as by examination of peripheral blood smears, and the results are summarized in Table 1. In contrast to the disease induced in mice by F-MuLV, which induced erythroleukemia 100% of the time, few mice injected with the recombinant viruses developed erythroleukemia (2 of 16 for F33V and 2 of 15 for FGLV). Rather, the majority of tumors induced by F33V and FGLV were lymphomas: 9 of 16 (63%) of the F33V-induced tumors (3 of which were associated with thymic enlargement) and 13 of 15 (87%) of the FGLV-induced tumors (2 of which were thymic lymphomas and 1 of which was a lymphoid hyperplasia). Although both F33V and FGLV induced a high percentage of lymphomas, the two recombinant viruses differed in their ability to induce myeloid leukemia. Thirty-one percent of the tumors induced by F33V were classified as myeloid malignancies, with one showing evidence of both lymphoma and myeloid leukemia, while none of the mice infected with FGLV developed myeloid disease. Based on Fisher’s exact probability test, the difference between these two viruses in inducing myeloid leukemia was significant (P = 0.043).

Characterization of the LTR sequences in tumors induced by recombinant viruses. Proviruses isolated from FeLV-induced tumors often show changes that are associated with a new tumor phenotype (16, 19). To determine if the LTR sequences in the proviruses isolated from F33V- or FGLV-induced tumors had undergone changes, LTRs were amplified from tumor DNA using specific PCR primers derived from FeLV or F-MuLV sequences, and the nucleotide sequences were determined. A summary of the data obtained is shown in Table 2. Considerable variation in LTR structure was detected in all F33V-induced tumors examined, including lymphomas and myeloid leukemias. In contrast, variation of LTR structure was not observed in any of the FGLV-induced tumors examined. Nucleotide sequencing revealed that tumors from mice inoculated with F33V, which contains an LTR with three direct tandem repeats of the URE, contained proviruses with variable numbers of direct tandem repeats in the URE (one to four copies).

A change of nucleotide sequence (A to G or G to A) at the start or end of repeated URE sequences was observed in two clones from F33V-induced tumors (nucleotide position 15 or 62). Interestingly, a 47-bp URE unit was precisely deleted or inserted in F33V-induced tumors. Proviruses from tumors induced in mice inoculated with FGLV, which contains only one copy of the URE, showed no changes in the URE. No changes were detected within the enhancer region of the LTRs of proviruses from either F33V- or FGLV-induced tumors.

TABLE 1. Disease induction in mice injected with recombinant viruses F33V and FGLV

<table>
<thead>
<tr>
<th>Virus</th>
<th>Disease diagnosis</th>
<th>No. of mice positive/total no. (%)</th>
<th>Latency (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F33V</td>
<td>Lymphoma</td>
<td>10/16** (62.5)</td>
<td>102–468</td>
</tr>
<tr>
<td></td>
<td>Erythroleukemia</td>
<td>2/16 (12.5)</td>
<td>89–386</td>
</tr>
<tr>
<td></td>
<td>Myeloid leukemia</td>
<td>5/16* (31.3)</td>
<td>214–402</td>
</tr>
<tr>
<td>FGLV</td>
<td>Lymphoma</td>
<td>13/15 (86.7)</td>
<td>153–431</td>
</tr>
<tr>
<td></td>
<td>Erythroleukemia</td>
<td>2/15 (13.3)</td>
<td>288–290</td>
</tr>
<tr>
<td></td>
<td>Myeloid leukemia</td>
<td>0/15 (0)</td>
<td></td>
</tr>
</tbody>
</table>

* NIH Swiss mice received intraperitoneal injections as newborns with 0.1 ml of F33V or FGLV and then were routinely observed for disease development. When moribund, they were sacrificed and their tissues (blood, spleen, liver, lymph nodes, and thymus) were examined for pathological changes by gross and histologic analyses.

** One mouse had both lymphoma and myeloid leukemia.

* Myeloid leukemia (n = 3), granulocytic leukemia (n = 1), and histiocytic sarcoma (n = 1).

† Lymphoma (n = 12) and lymphoid hyperplasia (n = 1).

‡ Erythroleukemia (n = 1) and erythroid hyperplasia (n = 1).
TABLE 2. Copy number of URE in the proviral LTRs from F33V- and FGLV-induced tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Copy no. of URE</th>
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</thead>
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<tr>
<td>F33V-1</td>
<td>2, 3</td>
</tr>
<tr>
<td>F33V-3</td>
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<tr>
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<tr>
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<tr>
<td>F33V-9</td>
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<tr>
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<tr>
<td>F33V-14</td>
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<tr>
<td>F33V-15</td>
<td>1, 2, 3, 4</td>
</tr>
<tr>
<td>FGLV-2</td>
<td>1</td>
</tr>
<tr>
<td>FGLV-3</td>
<td>1</td>
</tr>
<tr>
<td>FGLV-4</td>
<td>1</td>
</tr>
<tr>
<td>FGLV-5</td>
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<td>1</td>
</tr>
<tr>
<td>FGLV-10</td>
<td>1</td>
</tr>
</tbody>
</table>

*LTRs were amplified by PCR with the following primers (see Fig. 1C): a, 5'-CAGATAAAGCAATACGATCCAGAC-3'; b, 5'-CAATACGATCCAGACCAACCATG-3'; c, 5'-GGGGGTCAAGTCTCAGCAAAGACTTG-3'; and d, (F-MuLV LTR specific) 5'-CAGCAAGAGCTTTATTGGGAATACGG-3'. PCR products were cloned into the vector pCR-Blunt II-TOPO (Invitrogen, Carlsbad, Calif.). The copy number of the enhancer was 1 in all cases.

MCF virus induction in F33V- and FGLV-induced tumors. Erythroleukemia induced by F-MuLV is strongly associated with recombination between the input virus and endogenous retroviral sequences, resulting in the generation of mink cell focus-inducing (MCF) viruses (9, 24). Similar viruses resulting from recombination between FeLV and endogenous retroviral sequences have also been detected in naturally occurring feline lymphomas (26). To determine if MCF viruses were generated in mice inoculated with F33V or FGLV, we carried out Western blotting on splenic extracts from diseased mice using a monoclonal antibody, 7C10 (30), that detects MCF viral envelope glycoproteins but not the envelope glycoproteins of either F33V or FGLV. As shown in Fig. 3, we could detect MCF viral envelope protein in all F33V- and FGLV-induced tumors examined, but not in the spleen from an uninfected mouse (normal spleen). Interestingly, a larger number of FGLV-induced tumors expressed low levels of MCF viral envelope protein compared with F33V-induced tumors. The level of expression of MCF viral envelope protein did not correlate with tumor phenotype or disease latency.

In summary, we have isolated an infectious FeLV provirus (clone 33) from a cat with AML. Out of seven clones obtained, only one was infectious, consistent with previous observations that naturally occurring tumors of cats usually contain multiple copies of defective FeLV proviruses. Compared with other FeLVs, FeLV clone 33 showed subtle differences in its env gene as well as an unusual LTR containing three tandem direct repeats of a URE. To determine if the unique LTR of FeLV clone 33 influenced disease specificity, we generated an infectious recombinant MuLV virus (F33V) by replacing the U3 region of the F-MuLV LTR with that from FeLV clone 33. The specificity of disease induced in mice by this virus was then compared with that induced by a recombinant murine leukemia virus (FGLV) containing the LTR from FeLV/Glasgow-1, which does not contain repetitive URE sequences. Our results

![Image](http://jvi.asm.org/)

FIG. 3. Detection of MCF viral envelope proteins in tumors induced by F33V and FGLV. Western blot analysis was carried out on cell lysates (70 μg) from the spleens of diseased F33V- and FGLV-infected mice using the monoclonal antibody 7C10, which specifically recognizes MCF viral envelope proteins. An extract from the spleen of an uninfected NIH Swiss mouse was used as a negative control, while an extract from HCD-57 cells, an erythroleukemia cell line derived from an F-MuLV-infected mouse, was used as a positive control. The number below each sample represents the level of MCF viral envelope protein relative to the level in HCD-57 cells (set at 100).
demonstrate that both of these FeLV LTRs altered the disease specificity and latency of F-MuLV. F-MuLV induces erythroleukemia in virtually 100% of susceptible neonatal mice after a short latency period. The presence of an LTR from either FeLV clone 33 or FeLV/Glasgow-1 changed the tumorigenic spectrum of F-MuLV-induced disease from erythroid to primarily lymphoid, with 63% of F33V-infected mice and 87% of FGLV-infected mice developing lymphomas. Although both recombinant viruses could efficiently induce lymphoid disease, there was a significant difference between the two viruses in their ability to induce myeloid leukemia. None of the mice infected with FGLV developed myeloid leukemia, in contrast to a third of those injected with F33V. Thus, the presence of the FeLV clone 33 LTR on an F-MuLV background significantly increases the chances of mice developing myeloid malignancies. The latency of tumor induction following F33V and FGLV injection is an average of six times longer than that associated with F-MuLV-induced erythroleukemia (an average of 43 weeks for F33V and FGLV versus 7 weeks for F-MuLV). This is most likely due to the presence of a single enhancer in the FeLV LTRs compared with two copies in F-MuLV. Single-enhancer mutants of MuLVs have been shown to induce tumors with a prolonged latency (14).

It was previously reported that FeLV LTR sequences can substitute for MuLV LTR sequences to induce T-cell lymphomas in mice (21, 28). Our studies are consistent with the idea that the FeLV LTR favors the development of lymphomas, since the majority of the mice infected with either F33V and FGLV developed lymphoid disease. This is most likely due to the conservation in the FeLV enhancers of the LvB and CORE binding sites, which have previously been shown to be associated with lymphomagenesis (1, 27). However, mice infected with the F33V recombinant virus described in this study developed a lower incidence of lymphoma and a much higher incidence of myeloid leukemia than mice infected with the FGLV recombinant. Differences in the region of the FeLV LTR upstream of the enhancer appear to play a role in the disease phenotype induced since the only major difference in the LTRs present in F33V and FGLV is the presence in the F33V LTR of three tandem repeats of a 47-bp URE. In contrast, the enhancer regions of both viruses contain minor differences, with the known transcription binding sites being conserved. Thus, the presence of repetitive URE sequences in the LTR of FeLV clone 33 most likely is responsible for the altered tumorigenic spectrum induced by F33V. The unique LTR of F33V underwent further change during tumor induction, often resulting in addition of copies of the 47-bp URE, while no changes occurred in the URE of FGLV and no changes occurred in the enhancers of either viruses.

Although F33V and FGLV chimeric viruses induce disease in mice after a long latency compared with F-MuLV, this does not appear to be due to the failure of these chimeric MuLVs to generate MCF viruses, which play an important role in the induction of MuLV-induced diseases. We could easily detect the expression of MCF viral envelope proteins in all of the F33V- and FGLV-induced tumors examined. However, it is unclear whether the MCF viruses generated by these MuLV/FeLV chimeras are the same as those generated by F-MuLV.

The mechanism by which the unique F33V LTR shifts the disease spectrum of MuLV/FeLV LTR recombinants towards myeloid leukemia is not known. The LTR of FeLV clone 33 may specifically activate myeloid gene transcription in trans by production of a unique LTR RNA transcript. Such an LTR-generated RNA transcript was recently implicated in the activation of AP-1 by a lymphoma-inducing strain of FeLV (8). Alternatively, the unique LTR of FeLV clone 33 may contain binding sites for transcription factors expressed in myeloid cells. A transcription factor database search indicated the presence of putative DNA binding motifs for the transcription factors CCAAT/enhancer binding protein, Myb, and Stat proteins in the URE direct repeat. Stat 1 activated by gamma interferon in promonocytic cells has been shown to bind to and activate the LTR of caprine arthritis-encephalitis virus (25), and we have preliminary data using gel shift analysis that Stat 1 and Stat 3 can bind to URE sequences from FeLV clone 33. Our previous study demonstrated that repetitive URE sequences have enhancer function in myeloid cells but not T cells (19). Thus, the presence of a repetitive URE in the LTR of an FeLV may allow significant binding of myeloid transcription factors, resulting in activation of host genes at the site of viral integration in myeloid cells and favoring the development of myeloid leukemias.

**Nucleotide sequence accession number.** The FeLV clone 33 env and LTR sequences have been deposited into the GenBank nucleotide sequence database (accession number AB060732).

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**REFERENCES**


