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The Feline Leukemia Virus Long Terminal Repeat Contains a Potent Genetic Determinant of T-Cell Lymphomagenicity

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Feline leukemia virus (FeLV) is an important pathogen of domestic cats. The most common type of malignancy associated with FeLV is T-cell lymphoma. SL3-3 (SL3) is a potent T-cell lymphomagenic murine leukemia virus. Transcriptional enhancer sequences within the long terminal repeats (LTRs) of SL3 and other murine retroviruses are crucial genetic determinants of the pathogenicities of these viruses. The LTR enhancer sequences of FeLV contain identical binding sites for some of the transcription factors that are known to affect the lymphomagenicity of SL3. To test whether the FeLV LTR contains a genetic determinant of lymphomagenicity, a recombinant virus that contained the U3 region of a naturally occurring FeLV isolate, LC-FeLV, linked to the remainder of the genome of SL3 was generated. When inoculated into mice, the recombinant virus induced T-cell lymphomas nearly as quickly as SL3. Moreover, the U3 sequences of LC-FeLV were found to have about half as much transcriptional activity in T lymphocytes as the corresponding sequences of SL3. This level of activity was severalfold higher than that of the LTR of weakly leukemogenic Akv virus. Thus, the FeLV LTR contains a potent genetic determinant of T-cell lymphomagenicity. Presumably, it is adapted to be recognized by transcription factors present in T cells of cats, and this yields a relatively high level of transcription that allows the enhancer to drive the requisite steps in the process of lymphomagenesis.

Feline leukemia virus (FeLV) is a significant pathogen of domestic cats that causes a range of neoplastic and degenerative disorders, including lymphomas, sarcomas, immunodeficiency, and other types of degenerative hematopoietic diseases. The most common type of malignancy induced by FeLV is a lymphoma of T-cell origin (24, 44). FeLV is related to the murine leukemia viruses (MuLVs), and, like MuLVs, often causes lymphomas by a mechanism involving insertional activation of cellular oncogenes (2, 29, 31, 32, 56). The genetic loci most frequently targeted by FeLV for insertional mutagenesis in T-cell lymphomas include c-*myc*, *pim-1*, *bmi-1*, and *fit-1*, some of which are also activated in MuLV-induced T-cell lymphomas (2, 8, 10, 19, 34, 41, 43, 47, 56–58). These similarities indicate that the molecular mechanisms involved in FeLV-mediated lymphomagenesis are at least partially analogous to those in MuLV-induced disease.

Transcriptional enhancer sequences located in the U3 region of the viral long terminal repeat (LTR) are major genetic determinants of the tumorigenic potential of MuLVs. In MuLVs, these enhancers affect the cell type specificity of viral tumorigenesis and the potency of the virus (7, 11, 13, 25, 26, 33, 50, 59). They affect multiple steps in the process of lymphomagenesis, including infection of target cells, formation and propagation of mink cell focus-forming recombinant viruses, and activation of cellular oncogenes (6, 12, 38, 45). MuLVs with tandemly repeated LTR enhancer sequences are more potent at inducing disease than otherwise identical viruses with only single copies of the tandemly repeated elements (14, 15, 23, 33).

LTR sequences may also affect the pathogenic properties of FeLV. FeLV proviruses derived from T-cell lymphomas generally contain a tandem repeat of two or three copies of the enhancer region within the LTR (14, 37). Moreover, it is interesting to note that there are some clear similarities in the transcription factor binding sites within the enhancer regions of FeLVs and MuLVs. In particular, the FeLV LTR contains an element called the enhancer core that is similar to those found in all MuLVs (16). Core elements are crucial for the pathogenicity of the potent T-cell lymphomagenic MuLVs, SL3-3 (SL3), and Moloney MuLV (Mo-MuLV) (17, 38, 50). The sequences of the core elements vary slightly among various MuLVs (16). Interestingly, FeLV shares with SL3 a distinctive core element that differs in sequence from that of weakly leukemogenic Akv virus (Akv) by a single nucleotide (26). That single-nucleotide difference has been shown to contribute significantly to the difference in lymphomagenicity between SL3 and Akv (38). Immediately upstream of the core element, there is a second transcription factor binding site that is highly conserved among MuLVs and FeLV (16). This site was originally called the LVb site in MuLVs and binds members of the Ets family of transcription factors (16, 36, 49, 53). Mutation of the LVb Ets-binding site (Ets/LVb site) significantly disrupted lymphomagenesis by Mo-MuLV (50). The same mutation in SL3 had a smaller, though statistically significant, effect on tumorigenesis (39). The presence in the FeLV LTR of sites that play important roles in lymphomagenesis by MuLVs suggested the hypothesis that the FeLV LTR contains a genetic determinant of T-cell lymphomagenicity that is similar to those of T-cell lymphomagenic MuLVs.

Lymphomagenicity of an FeLV-MuLV recombinant virus. To test this hypothesis, a recombinant virus in which the U3 region of SL3 (26, 42) was replaced with that of an isolate of FeLV derived from LC-FeLV, a naturally occurring isolate of FeLV derived from a T-cell lymphoma was engineered (4, 30, 51). LC-FeLV was chosen because the U3 region of its LTR contains a 50-bp tandem duplication of sequences that includes the enhancer core element and the Ets/LVb site (14). Previous work with MuLVs showed that the presence of a tandem repeat of the enhancer region was important for the maximal

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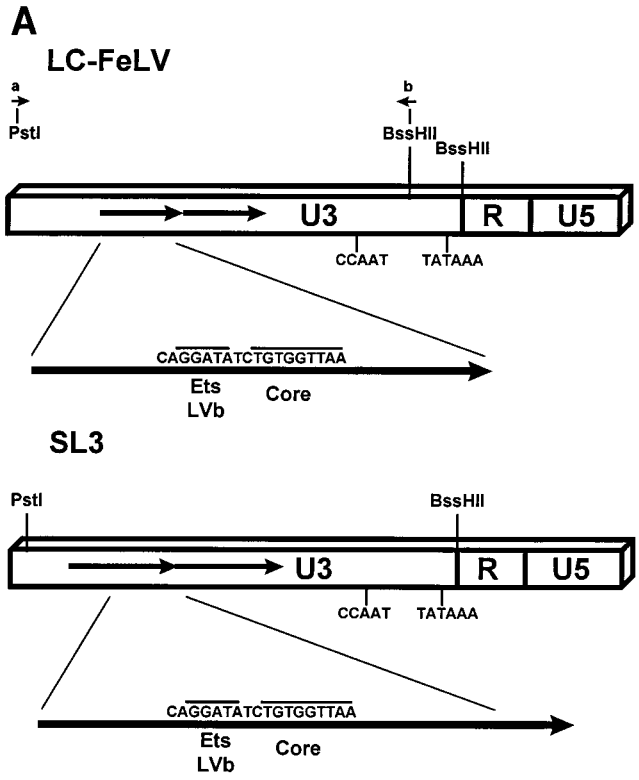


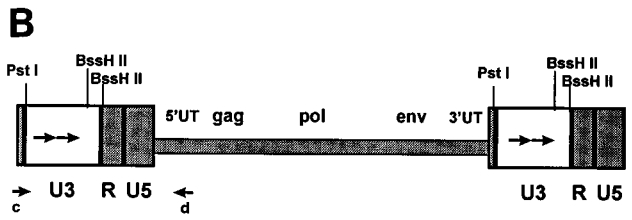
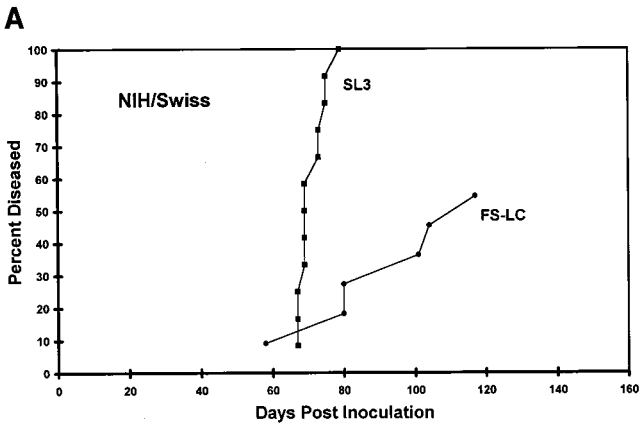


FIG. 1. Sequences involved in generation of the FS-LC recombinant virus. (A) Structures of the LC-FeLV and SL3 LTRs. The arrows labeled a and b represent the primers used for PCR amplification of the LC-FeLV U3 sequences. Positions of the *Pst*I and *Bss*HII sites are shown. Arrows within the U3 regions represent the tandemly repeated enhancer units. Shown below each U3 region is the sequence of the Ets/LVb core region. Overlining indicates the sequence of each site. (B) Structure of the proviruses derived from the FS-LC recombinant genome. Positions of *Pst*I and *Bss*HII sites are shown. Arrows labeled c and d below the genome represent the primers that were used for PCR amplification of the proviral 5′ LTRs from mouse genomic DNA for reversion analysis. Transfections were performed with a clone of the *Pst*I fragment that contains most of the LTR at one end. Thus, the resulting LTRs of the subsequent proviruses are both derived from a single LTR and are identical. The arrows within the U3 regions are as defined for panel A.

pathogenicity of these viruses (14, 15, 23, 33). Most of the LC-FeLV U3 region was used to replace the corresponding sequences of SL3 in a recombinant virus termed FS-LC (Fig. 1B). Infectious virus with two identical LTRs was generated by transfection of the recombinant genome into NIH 3T3 fibroblasts as previously described (27, 38). FS-LC replicated to the same titer as SL3 in these cells as determined by XC plaque assays and by reverse transcriptase levels in the culture supernatants. Maintenance of the FeLV U3 sequences in the replicating virus was confirmed by infection of a fresh culture of NIH 3T3 cells, isolation of genomic DNA from these cells, PCR amplification of proviral 5′ LTRs from these cultures, and sequencing of the PCR product. The ability of FS-LC to induce disease was monitored following intraperitoneal injection of 10^3 XC PFU in 0.1 ml of fibroblast culture supernatant into newborn NIH/Swiss and AKR/J mice. Wild-type SL3 was also injected into control mice as a parallel control. All mice that developed tumors had enlarged thymuses, lymph nodes, and/or spleens.

All 12 NIH/Swiss mice injected with SL3 developed tumors after an average of 71 days postinjection (Fig. 2A). FS-LC was inoculated into 11 NIH/Swiss mice. Fifty-four percent (6 of 11) of these mice developed tumors between 60 and 120 days after inoculation (Fig. 2A). The mean latent period to disease in the six mice that developed tumors was 90 days.

In AKR/J mice, wild-type SL3 also induced tumors in 100% (10 of 10) of inoculated individuals (Fig. 2B). The mean latent period for disease was 78 days. In this strain, FS-LC induced tumors in 100% (9 of 9) of inoculated individuals with an average latent period of 89 days, only slightly longer than that for SL3 (Fig. 2B). One of the FS-LC mice did not evidence disease until about 140 days postinoculation (Fig. 2B). The potent tumorigenicity of FS-LC contrasts with the near lack of lymphomagenicity of a recombinant virus containing the Akv LTR linked to the remainder of the genome of SL3 or of a mutant of SL3 with a disrupted c-Myb binding site in its LTR (28, 39). The more potent tumorigenicity of FS-LC in AKR/J



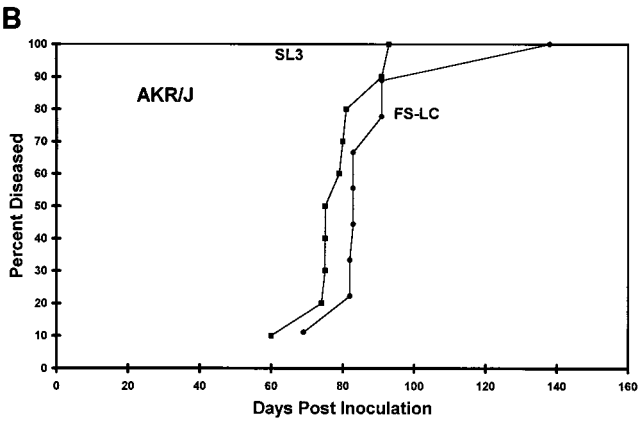


FIG. 2. Tumor induction by the FS-LC recombinant virus. (A) Time course of tumorigenesis in NIH/Swiss mice. The five surviving mice were monitored for 1 year. (B) Time course of tumorigenesis in AKR/J mice.

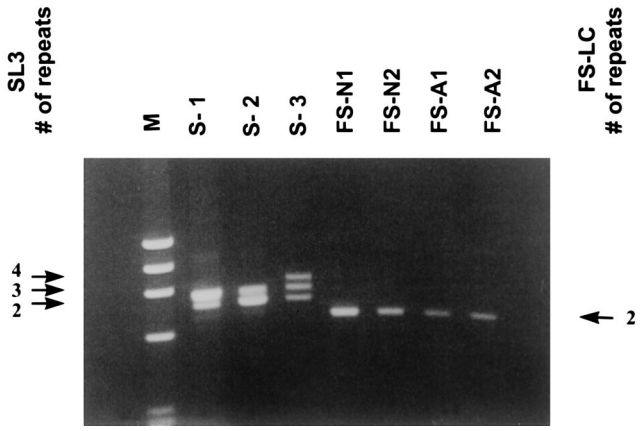


FIG. 3. PCR amplification of 5′ LTRs of proviruses in tumors. Following PCR amplification, the DNAs were resolved on an agarose gel containing ethidium bromide. Each lane contains DNA from a different tumor. Arrows on the left show the positions of the PCR products from SL3-induced tumors that contain two, three, or four enhancer repeats. The arrow on the right indicates the position of the PCR product from FS-LC-induced tumors that contains two repeat units. M indicates a marker lane with *Hae*III-digested ΦX-174 DNA. S-1, S-2, and S-3 indicate three SL3-induced lymphomas. FS-N1 and FS-N2 indicate two FS-LC tumors induced in NIH/Swiss mice. FS-A1 and FS-A2 indicate two FS-LC tumors induced in AKR/J mice.

than in NIH/Swiss mice has previously been observed with other mutants of SL3 (39). Presumably, this reflects polymorphic genetic loci within the mouse strains that affect viral pathogenicity.

FeLV LTR sequences are maintained in proviruses in tumors induced by FS-LC. Exogenous MuLVs can undergo recombination with endogenous viral sequences following infection of mice (18, 46, 52). To test whether the FeLV U3 sequences were maintained in the viruses that induced the tumors, LTRs from proviruses in tumors were amplified by PCR and sequenced. Amplification of the viral LTRs resulted in the production of a single band of 638 bp from each tumor (Fig. 3). This was the size expected for proviruses derived from the FS-LC virus. One surprising aspect of this result was that no variation in the number of enhancer repeat units was detected. Tumors induced by MuLVs with tandemly repeated enhancer sequences generally contain a mixed population of proviruses with variable numbers of the repeated units, presumably due to template misalignment events involving reverse transcriptase (5, 38). In particular, proviruses with a triplication of a repeat unit are frequently seen in tumors that occur in mice inoculated with a virus that contains two copies of the repeat unit. Possible explanations for the lack of detection of proviruses with variable numbers of repeats are that these particular sequences are somehow not prone to polymerase slippage or jumping events or that viruses with altered numbers of enhancer repeats have some selective disadvantage. The entire U3 regions from 15 of the tumors were amplified by PCR. In every case, only the FeLV U3 sequences with two enhancer repeats were present, four of which are shown in Fig. 3 along with controls from SL3-induced tumors where the variable numbers of enhancer repeats are easily seen. Individual bands from FS-LC-induced tumors were excised and directly sequenced with the CircumVent DNA sequencing system (New England Biolabs) and a minus-strand primer in the R region of the SL3 genome (5′-TGCAACAGCAAAAGGCT TTATTGGATAC-3′). No nucleotide changes were detected in any of the LTRs.

We conclude from these studies that FS-LC is a potent, tumorigenic virus that induces disease nearly as effectively as SL3 and that the LC-FeLV U3 region contains a genetic determinant of tumorigenicity.

Tumors induced by FS-LC are T-cell lymphomas. Tumors induced by SL3 are strictly T-cell lymphomas and involve enlargement of the thymus, lymph nodes, and/or spleen (17, 20, 21, 26, 40, 42). Similarly, the most common type of malignancy induced by FeLV is T-cell lymphoma (24, 44). About 80% the tumors induced by FS-LC evidenced gross enlargement of the thymus; this is equivalent to the fraction of mice that had enlarged thymuses following inoculation with SL3. Gross enlargement of the spleen and lymph nodes was also seen in every mouse. These data on pathology, particularly the enlargement of the thymuses, are consistent with the tumors being T-cell lymphomas.

Further evidence that the tumors were of T-cell origin was obtained by examining rearrangements of the T-cell receptor (TCR) β-chain enhancer by Southern blotting (1, 22). DNA from four FS-LC-induced tumors and two SL3-induced tumors was examined. All six had clonal rearrangements of the TCR β-chain gene (Fig. 4), consistent with the tumors being of T-cell origin. All six of the tumors also showed clonal rearrangements of the immunoglobulin heavy chain D-J region, events that are frequently seen in MuLV-induced T-cell lymphomas (60). We conclude that the tumors induced by FS-LC are T-cell lymphomas.

Transcriptional activity of the LC-FeLV LTR U3 region. Transcriptional activity of MuLV LTR enhancers generally reflects the type of tumor that a particular virus causes. Thus, the enhancers of T-cell lymphomagenic SL3 and Mo-MuLV are more active in T lymphocytes than are the enhancers of erythroleukemogenic Friend MuLV or weakly B-cell lymphomagenic ecotropic viruses such as Akv (3, 35, 48, 54, 61). Since FS-LC approached SL3 in its ability to induce T-cell lymphomas, we compared the transcriptional activity of its U3 region with those of the U3 regions of SL3, Mo-MuLV, and Akv. The U3 region and part of the R region of the LTR from FS-LC was placed 5′ to the chloramphenicol acetyltransferase (CAT) reporter gene in a reporter plasmid (Fig. 5A). Transcriptional activity was measured relative to those of LTR sequences of the other viruses in otherwise identical CAT constructs by using three T-cell lymphoma lines, Jurkat, L691-6, and SL3H (Fig. 5B). Multiple trials were performed for each experiment. Averages and standard deviations were determined as previously described (38, 39, 62, 63). In each of the three cell lines, the SL3 LTR was the most active. The FeLV

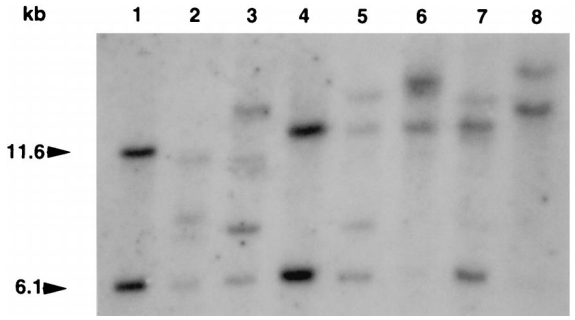


FIG. 4. Southern blot analysis of the TCR β loci in DNAs from FS-LC-induced tumors. Lanes 1 to 3 contained DNA from AKR/J mice, and lanes 4 to 8 contained DNA from NIH/Swiss mice. Lanes 1 and 4 were negative controls from uninfected mice. Lanes 2, 3, 5, and 6 were from FS-LC-induced tumors. Lanes 7 and 8 were from positive-control, SL3-induced T-cell lymphomas. Arrowheads show the known sizes of the germ line *Hpa*I fragments of AKR/J mice.

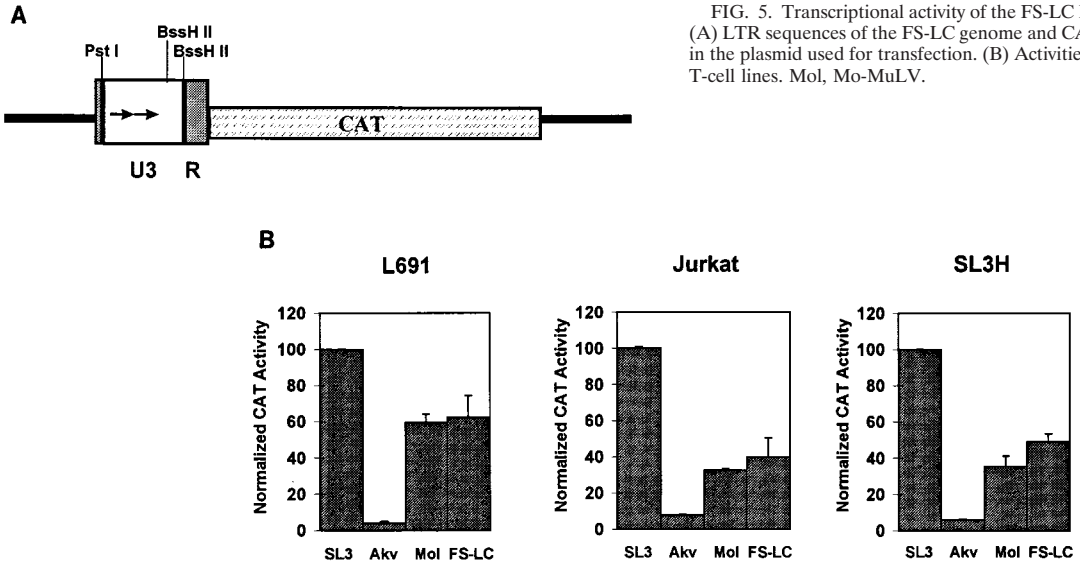


FIG. 5. Transcriptional activity of the FS-LC LTR in T-lymphocyte cell lines. (A) LTR sequences of the FS-LC genome and CAT sequences that were present in the plasmid used for transfection. (B) Activities of the viral LTRs in the three T-cell lines. Mol, Mo-MuLV.

LTR exhibited about 40 to 60% as much activity as the SL3 LTR in the three lines. This was comparable to the activity of the Mo-MuLV LTR. It was also severalfold higher than the activity of the Akv LTR in each of the cell lines tested. Thus, the level of transcriptional activity of the LC-FeLV U3 region approaches that of the SL3 U3 region in T cells.

The ability of FeLV LTR sequences to substitute for equivalent sequences in the strongly T-cell lymphomagenic MuLV SL3 shows that the FeLV LTR contains a potent genetic determinant of T-cell lymphomagenicity. LTR sequences appear to affect multiple steps in the process of T-cell lymphomagenesis, including infection of target cells, formation and propagation of mink cell focus-forming recombinant viruses, and activation of cellular oncogenes (6, 12, 38, 45). Our results imply that the FeLV LTR is capable of participating in the events that lead to lymphomagenesis in a manner similar to that of the SL3 LTR. We believe that the FeLV LTR likely functions in a similar manner in FeLV-induced T-cell lymphomas in cats.

Transcriptional activity of MuLVs in the type of cell in which a particular virus causes tumors is proportional to the tumorigenic potential of the virus (3, 35, 48, 54, 55, 61). The FeLV LTR had about half the activity of the SL3 LTR in the three lines that were tested here. The plasmids that were used here contained U3 sequences of FeLV and R region sequences of SL3. The R region sequences of the two viruses are not identical (9). They can affect the activity of the LTR (9), and the presence of the SL3 R region sequences may have decreased the activity of the FeLV LTR. Nonetheless, the activity of the FeLV construct was similar to that of Mo-MuLV and clearly greater than that of Akv. Thus, the transcriptional activity of the FeLV LTR in T cells likely reflects the lymphomagenic potential of the virus.

Enhancer sequences within the LTRs are crucial elements for determining the pathogenic potential of MuLVs. It is likely that sequences within the enhancer region of the FeLV LTR are also important for T-cell lymphomagenicity. LC-FeLV was chosen for these studies because it has a 50-bp repeated unit in its LTR that corresponds to the tandemly repeated enhancer region of SL3 and other MuLVs. The presence of duplicated enhancer sequences significantly affects the efficiency of lymphomagenicity by MuLVs and likely was important for the fairly high level of pathogenicity evidenced by FS-LC (14, 15, 23, 33).

The enhancer sequences are adapted to be recognized by host transcription factors present in the target cells for disease. Presumably, the cellular transcription factors that bind to the FeLV LTR enhancer allow the virus to perform the necessary steps in T cells for lymphomagenesis to occur. It is likely that at least a subset of the factors that bind to the FeLV enhancer are the same as those that bind to SL3. Two elements are identical in the FeLV and SL3 enhancers, the core element and the Ets/LVb site. Both of these affect lymphomagenicity by SL3 and by Mo-MuLV (17, 38, 39, 50). The core element binds transcription factor CBF (also called AML1 and PEBP2), while the Ets/LVb site can bind multiple members of the Ets family of factors (16, 36, 49, 53). Presumably, the FeLV elements are adapted to be recognized by the same factors in cats. Adjacent to the Ets/LVb site and core sequences, the FeLV and MuLV enhancer regions also share binding sites for NF1 and the glucocorticoid receptor (16). In addition to these common sites, there are other sites that are unique to a subset of the viruses, and these can vary in their importance for pathogenicity (39, 50). The precise importance of various sites in the FeLV LTR for T-cell lymphomagenicity needs to be determined empirically by mutagenesis analysis. One of the advantages of FeLV-MuLV recombinants is that they constitute a more facile system for performing these types of studies than one involving the testing of FeLV mutants in cats.

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