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Structure and Function of the Long Terminal Repeats of Feline Leukemia Viruses Derived from Naturally Occurring Acute Myeloid Leukemias in Cats

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Long terminal repeats of feline leukemia viruses cloned from feline acute myeloid leukemias frequently contained direct repeats of 40 to 74 bp in the upstream region of the enhancer (URE). The repetitive URE conferred an enhancer function upon gene expression in myeloid cells, suggesting its association with tumorigenic potential in myeloid cells.

Feline leukemia virus (FeLV) is associated with a variety of neoplastic diseases, including lymphoma and acute myeloid leukemia (AML) in domestic cats (8, 13, 19, 23).

The U3 region of the retroviral long terminal repeat (LTR) contains the transcriptional promoter and enhancer elements necessary for gene expression (2). FeLV proviruses isolated from thymic lymphomas appear to contain duplications of the enhancer sequences (7, 10, 11); however, LTRs of FeLVs derived from nonneoplastic diseases or weakly pathogenic strains contain only a single copy of the enhancer (5, 21). In addition to these enhancer elements, a downstream enhancer element of the FeLV LTR was shown to contribute to transcription in a cell type-specific manner (1). The present study was carried out to characterize the structure and function of the FeLV LTRs derived from naturally occurring AML in cats.

Nucleotide sequences of the U3 region. Specimens of tumor cells were obtained from eight cats with AML (myeloblastic leukemia, two cats; monocytic leukemia, 1 cat; erythroleukemia, 5 cats) and four cats with thymic lymphoma. High-molecular-weight cellular DNAs of tumor cells were extracted from spleen, bone marrow, thymus, and pleural fluid. For analysis of the FeLV LTR structure, PCR was employed for amplification of the full-length U3 region in the 3′ LTR. The sequence of the 5′ primer was specific to a sequence between *env* and the 3′ LTR of exogenous FeLV (21): Fe-1S, 5′-GAGAGCTCAATA CGAT(TC)CGGACCGACCATG-3′ (nucleotides [nt] 1968 to 1989 in FeLV A/Glasgow-1 [21]; the *Sac*I linker is underlined). The 3′ primer was synthesized based on the sequence downstream of the capping site of the FeLV LTR: Fe-1R, 5′-GGT ACCCGGGGCGGTCAAGTCTCGGCAAAG-3′ (nt 539 to 510 in strain pJ7E2 [11]; the *Kpn*I site, conserved among various FeLV strains, is underlined). The sequence of the 3′ primer was common to most of exogenous and endogenous FeLV isolates. PCR products were directly cloned into a cloning vector (TA cloning kit; Invitrogen, San Diego, Calif.) and sequenced by the dideoxy chain termination method.

Twenty-two FeLV LTR clones from eight cats with AML and eight FeLV LTR clones from four cats with thymic lymphoma were sequenced (Fig. 1). In sequences from five (My1, My2, My3, My4, and My6) of 8 cats with AML, characteristic repetitive structures were found in a region, between the inverted repeat and the enhancer, which was designated the upstream region of the enhancer (URE). One unit of the direct repeat of the URE in these LTR clones from AML was 40 to 74 bp. The 5′ and 3′ termini of the URE repeat were −345 to −301 nt and −261 to −241 nt, respectively, from the presumptive RNA cap site in the FeLV/Glasgow-1 LTR (21). In clones My1-2, My1-3, and My1-5 from cat My1, a 41-bp sequence in the URE (nt −317 to −261) was duplicated or triplicated. In clones My1-1 and My1-4, a 47-bp sequence in the URE (nt −317 to −255) was duplicated and quadruplicated, respectively. The clone My2-1 contained a duplication of a 70-bp sequence (nt −345 to −255) in the URE. In three clones from cat My3, two to four copies of a 40-bp sequence in the URE (nt −301 to −241) were repeated. The clone My4-1 had a duplication of a 74-bp sequence in the URE (nt −342 to −249). In four clones from cat My6, two to five copies of a 51- or 43-bp fragment in the URE (nt −322 to −253 or nt −319 to −257) were found. LTRs from cats My5, My7, and My8 did not have any repeated sequence in the URE.

The sequences of the UREs of these FeLV LTRs derived from cats with AML were more similar to that of clone FeLV/pJ7E2 (11) isolated in Japan than to that of FeLV-A/Glasgow-1 (21) isolated in Great Britain. Furthermore, all of these repeated URE sequences had a relatively uniform deletion, corresponding to nt −295 to −275 in FeLV-A/Glasgow-1 (21). In contrast to the sequences of LTRs from cats with AML, the LTRs derived from four cats with thymic lymphoma did not have any direct repeat in the URE. Clones Th4-1, Th4-2, and Th3-1, derived from cats with thymic lymphoma, each had a deletion from nt −295 to −275, as seen in the UREs of the LTRs derived from cats with AML.

Direct repeats of 55 to 105 bp were found in the enhancer region of FeLV LTRs derived from all four cats with thymic lymphoma. The 5′ and 3′ termini of the direct repeats corresponded to nt −251 to −235 and nt −181 to −147, respectively. In most of these LTR clones from thymic lymphomas, leukemia virus factor b (LVb), simian virus 40 (SV40) core, NF1, the binding motif in feline leukemia virus (FLV-1), the CAT box, and the Goldberg-Hogness box were relatively well conserved. None of the 22 LTR clones from cats with AML contained the direct repeat in the enhancer region that is frequently detected in the LTRs from thymic lymphoma. A

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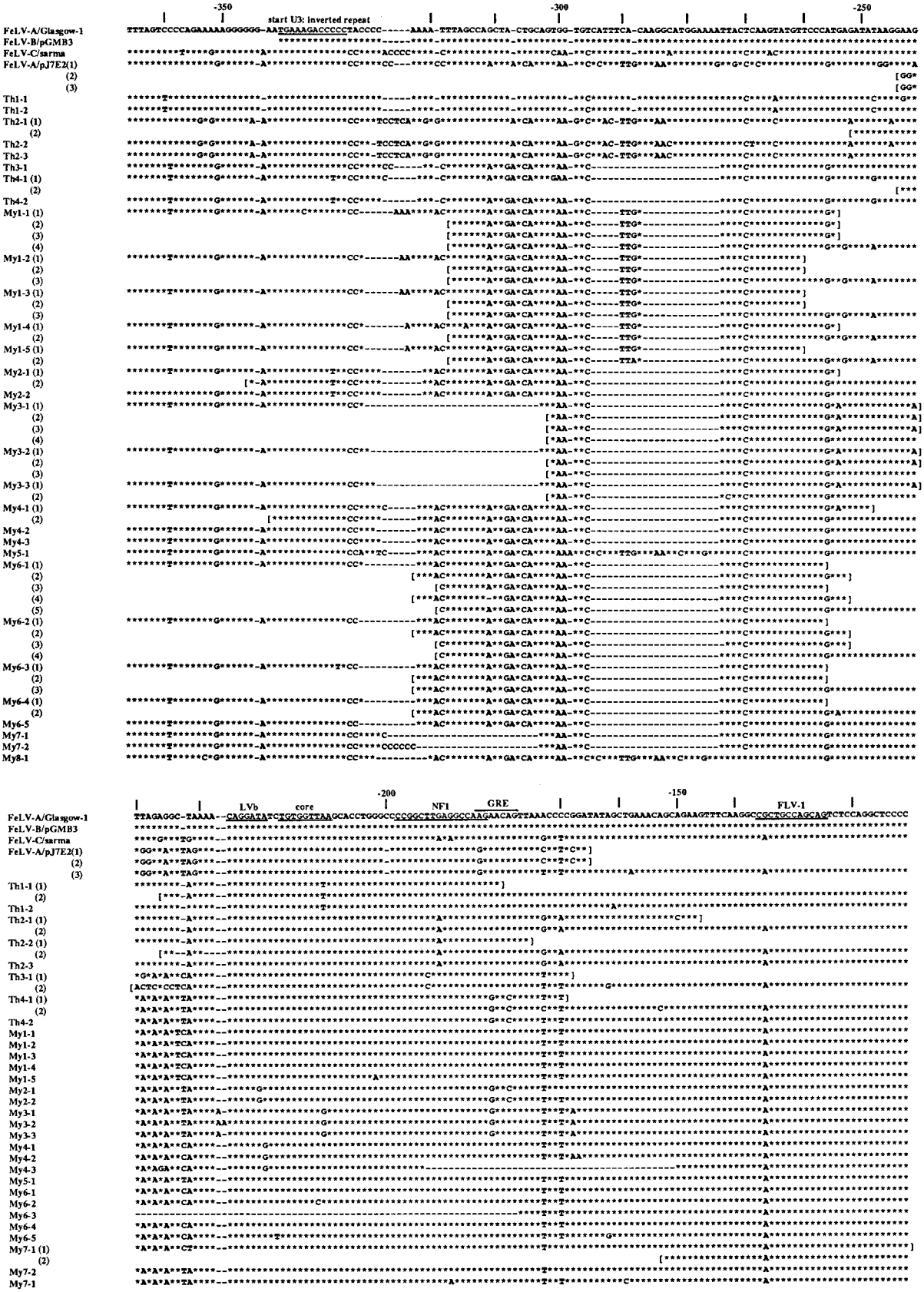

FIG. 1. Comparison of nucleotide sequences of LTRs of various FeLV isolates Sequences shown include FeLV-A/pJ7E2 (11), FeLV-A/Glasgow-1 (21), FeLV-B/GM-1 (23), FeLV-C/Sarma (15), and FeLV LTRs isolated from AML and thymic lymphoma in this study. The nucleotides different from those of FeLV-A/Glasgow-1 are shown. Dashes indicate gaps.

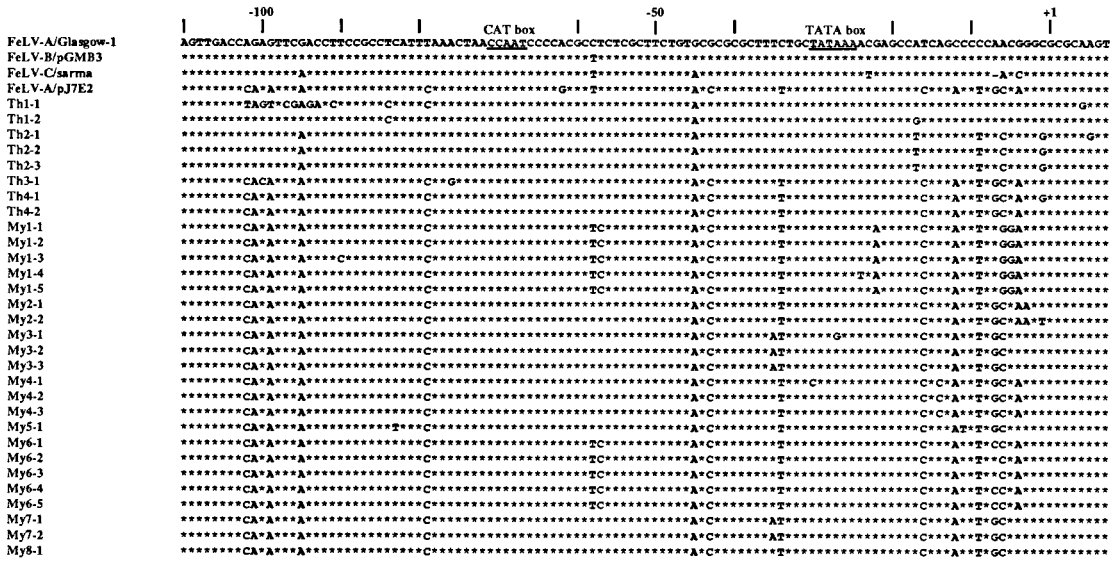

FIG. 1—*Continued.*

notable characteristic of these LTR clones derived from cats with AML was frequent nucleotide substitution in the enhancer elements. Two clones from cat My2 had a transition (CAG GATA→CAGGAGA) in the LVb binding motif and two transitions (AGAACAGT→AGGACCGT) in the glucocorticoid response element (GRE) binding motif (transitions are underlined). Three clones from cat My3 had a transition (AGAACA GT→AGGACAGT) in the GRE binding motif. In addition, clone My3-1 had a mutation in the TATA box (TATAA A→TATGAA). In clone My4-3, a 43-bp fragment including the NF1 and GRE binding motifs was deleted. Three clones, My4-1, My4-2, and My4-4, had a transition (CAGGATA→C AGGATG) in the LVb binding motif. Clone My6-2 had a T-to-C transition (TGTGGTTAA→TGTGGTCAA) in the SV40 core binding motif, corresponding to the determinant for leukemogenicity, tissue-specific transcription, and induction of latent period of disease in murine AKV (3, 12). Clone My6-3 had a deletion of a 64-bp sequence encompassing the LVb, SV40 core, NF1, and GRE binding motifs, which have been shown to influence disease specificity and induction of the latent period in Moloney murine leukemia virus (20).

In clone My7-1, a direct repeat of the URE was not observed; however, a 42-bp sequence around the FLV-1 site was duplicated in the downstream region of the enhancer (from nt −152 to −111).

Transcriptional activity of LTR. To test transcriptional activity of the viral LTRs, we performed transient-expression assays using the choramphenicol acetyltransferase (CAT) reporter gene. The *Sac*I-*Kpn*I fragments containing the full-length U3 region (nt 1968 to 2417 in FeLV-A/Glasgow-1 [21]) of FeLV LTRs were inserted upstream of the CAT gene in the pHdCAT plasmid (18), which contains a reporter gene but lacks eukaryotic promoter and enhancer elements, and was then introduced into a human T-lymphoblastoid cell line (Jurkat) (17) and a human acute myelogenous leukemia cell line (THP-1) (22). DNA transfection was carried out essentially as described by Ciccarone et al. (4). CAT activity of transfectants after incubation for 48 h was quantified by the solvent partition method (14). For each transfection experiment, data obtained at time points 1 to 3 h after incubation were pooled for a determination of the increase of counts per minute of [^{3}H]acetyl-chloramphenicol per hour. Control cells transfected with pHdCAT plasmid were used to determine the background level. The pSV-β-galactosidase plasmid was used to monitor the efficiency of transfection.

The transcriptional activities of various FeLV LTRs in Jurkat are shown in Fig. 2A. The FeLV LTR derived from thymic lymphoma, FeLV/pJ7E2 (three enhancer repeats), was the most transcriptionally active of the LTRs examined. Transcriptional activities of Th1-1 (two enhancer repeats) and Th1-2 (one enhancer repeat), derived from thymic lymphoma, and that of low-pathogenic strain FeLV/Glasgow-1 were lower than that of pJ7E2. Transcriptional activities of FeLV LTRs derived from five cats with AML (cats My1, My2, My3, My4, and My6) were significantly lower than those of LTR clones pJ7E2, Th1-1, and Th1-2 derived from thymic lymphomas. The LTR clones My6-1, My7-1, and My7-2 showed relatively high transcriptional activities, but these values were still lower than that of pJ7E2. In clone My3-1 with a mutation in the TATA box, the transcriptional activity was remarkably low. These results indicate that many of the FeLV LTRs derived from AML showed relatively low transcriptional activities compared with those of the LTRs from thymic lymphoma in Jurkat cells.

The transcriptional activities of various FeLV LTRs in a human myelogenous leukemia cell line, THP-1, are shown in Fig. 2B. Transcriptional activities of LTRs derived from AML in the THP-1 cells were relatively higher than those in Jurkat cells in comparison with the transcriptional activities of LTR clones from thymic lymphoma (Th1-1 and Th1-2), although the pJ7E2 with three enhancer repeats still showed a high transcriptional activity. Especially, the transcriptional activities of clones My2-1 (127%), My6-1 (130%), My6-2 (145%), and My6-4 (133%) were higher than that of pJ7E2. In addition, transcriptional activities were relatively dependent on the number of URE repeats. Transcriptional activities of My1-1 (four URE repeats), My1-3 (three URE repeats), and My1-5 (two URE repeats) were 91, 78, and 67%, respectively, of that of the pJ7E2 LTR. Moreover, LTR clones with URE repeats such as My2-1 (two URE repeats) and My6-2 (four URE repeats) showed higher transcriptional activities than their counterparts, clones My2-2 and My6-5, without the URE repeat. The My6-3 clone showed a distinct transcriptional activity

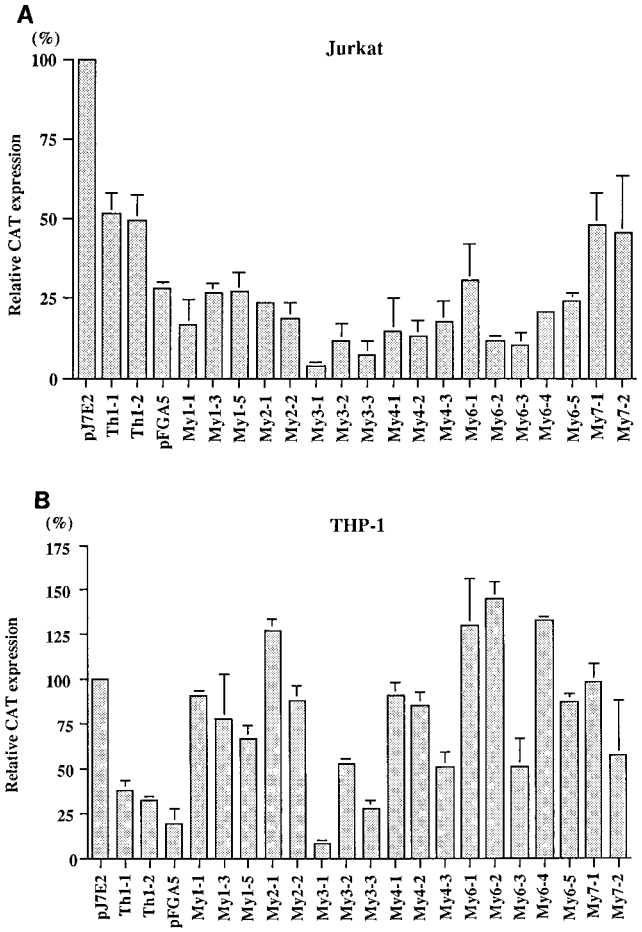

FIG. 2. Relative CAT gene expression directed by FeLV LTRs. The LTR-CAT plasmids were transfected into Jurkat (A) and THP-1 (B) cells. Results are expressed as values relative to the CAT activity of the FeLV/pJ7E2 LTR-CAT. Experiments were independently performed at least three times. The averages and standard deviations (error bars) are presented.

in cell line THP-1, even though this clone had a large deletion of the enhancer elements, including LVb, SV40 core, NF1, and GRE.

Enhancer function of URE. To examine the enhancer function of the URE and its repetitive structure, CAT reporter plasmids were constructed. *Sac*I-*Eco*RV fragments which contained URE (nt 1968 to 2159 in FeLV-A/Glasgow-1 [21]) but not promoter and enhancer sequences of the FeLV LTR were cut out from the LTRs of FeLV/pJ7E2 and clones My1-1, My1-3, My3-1, My6-1, My6-2, My6-4, and My7-2 from AML. These URE fragments were then inserted upstream of the pSVoCAT-promoter plasmid (9), an enhancerless reporter plasmid in which the expression of CAT reporter gene is driven by the SV40 promoter. CAT activity of the constructed plasmids was represented as a relative activity compared with that of the SV40 promoter plasmid (pSVoCAT). In THP-1 cells, enhancer activities of the URE fragments from three cats with AML (My1, My3, and My6) were 3.6- to 13.1-fold higher than that of pSVoCAT (Fig. 3B). In contrast, the enhancer activity of the URE fragments of pJ7E2 derived from thymic lymphoma was as low as that of pSVoCAT. Enhancer activities of the URE clones My6-1 (five URE repeats), My6-2 (four URE repeats), and My6-4 (two URE repeats) from cat My6 were found to be dependent on the number of URE repeats, indicating the positive enhancer function of the URE repeat. The enhancer activity of the My7-2 URE without the repetitive structure was lower than those of other URE clones from AML containing two to five repeats in THP-1 cells. These enhancer activities of URE were not observed in Jurkat cells (Fig. 3C).

In the present study, FeLV provirus LTRs derived from cats with AML exhibited mainly four structural properties. First, the LTRs derived from AML frequently contained two to five repeats of the URE sequence between the inverted repeat and the enhancer element. Second, the FeLV LTRs from AML contained only a single copy of the enhancer element, in contrast to the thymic lymphoma-derived FeLV proviruses, which have repeated enhancer elements (7, 10, 16). Third, LTRs derived from AML frequently contained mutations and dele-

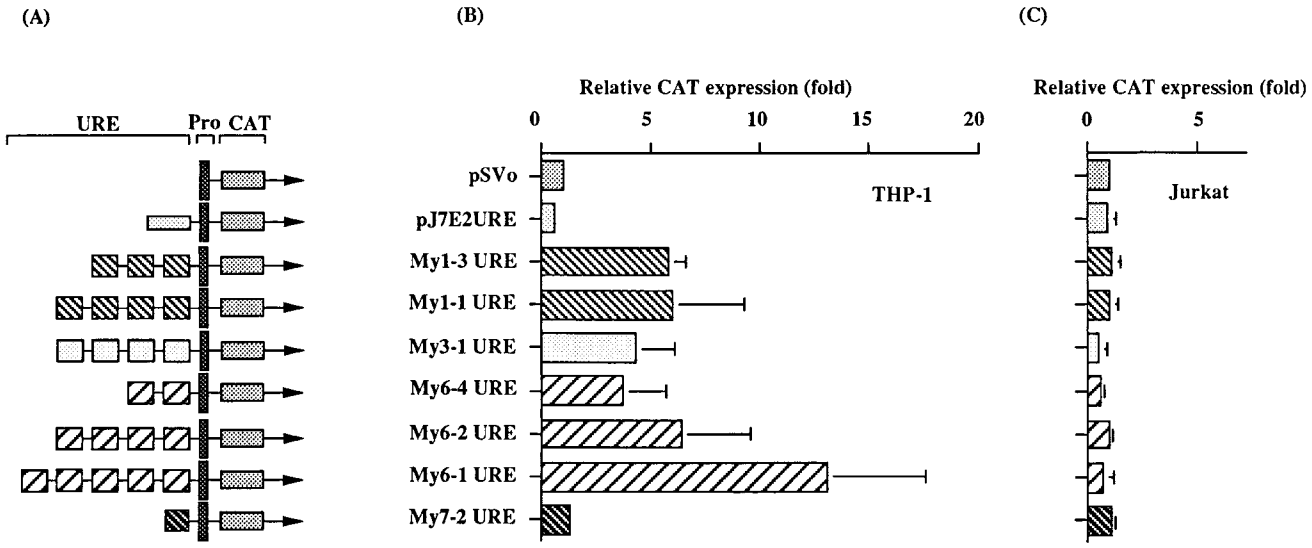

FIG. 3. Relative CAT gene expression directed by the SV40 promoters and UREs of FeLV LTRs inserted upstream of the CAT reporter gene. (A) CAT plasmid constructs were used for transfection. THP-1 (B) and Jurkat (C) cells were transfected with the pSVoCAT constructs inserted with the URE fragments of FeLV LTRs from pJ7E2, My1-3, My1-1, My3-1, My6-4, My6-2, My6-1, and My7-2. Results are expressed as values relative to the CAT activity of the pSVoCAT reporter plasmid (pSVo). Error bars, standard deviations.

tions within the enhancer elements. Fourth, an LTR derived from one cat with AML had a duplication in the downstream region of the enhancer element.

The repeated UREs from cats with AML originated from the corresponding sequences with deletions of 18- to 22-bp fragments. LTRs with or without URE repeats isolated from the same animal showed mostly identical nucleotide sequences outside the URE. Moreover, the deleted sequences in the URE and the elements of the repeated URE were very similar among a number of FeLV LTRs with URE repeats from AML. These findings provide implications for the origin of the FeLVs with direct repeats of URE. The structure of the initial inoculum in these naturally occurring leukemias is, of course, not known, but conceivably the animals were originally infected with a virus with a single URE sequence, and then LTRs with two to five copies of the URE could have arisen de novo in each infected cat, an event that may be associated with the induction of AML. A transcription factor database search indicated the presence of motifs of C/EBP, Myb, GATA, and PEA-3 binding sites (6) in the direct repeat of URE. Further experiments are required to show the direct evidence for the binding of these transcription factors. The transcriptional activities of LTRs containing URE repeats were relatively low in T cells but high in THP-1 cells. Furthermore, the enhancer activities of URE were clearly observed in the THP-1 cells but not in Jurkat cells, indicating that the enhancer function of the URE of FeLV LTR was cell type specific. It can be hypothesized that the formation of tandem direct repeats of URE accelerate the induction of AML.

In addition to this event, deletions and mutations of the protein binding motifs such as LVb, SV40 core, NF1, and GRE within the enhancer region were frequently detected in FeLV LTR clones from AML. The mutations of the enhancer elements such as the LVb, SV40 core, NF1, and GRE binding motifs were shown to influence disease specificity and the induction of the disease latent period in Moloney murine leukemia viruses (20). Furthermore, the mutation in the SV40 core binding motif found in clone My6-2 was the same as that in the nonleukemogenic AKV, corresponding to the determinant for leukemogenicity, tissue-specific transcription, and the induction of the latent period of disease in AKV (3, 12). These mutations and deletions of enhancer elements in LTRs might be related to the disease induction of AML rather than thymic lymphoma.

The present studies disclosed characteristics of the structure and function of FeLV LTRs derived from naturally occurring AML in cats. Establishing an experimental infection system using the FeLV molecular clones characteristic of those obtained from AML would help to further the understanding of molecular pathogenesis in AML.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession no.: AB003994, AB003995, AB003996, AB003997, AB003998, AB003999, AB004000, AB004001, AB004002, AB004003, AB004004, AB004005, AB004006, AB004007, AB004008, AB004009, AB004010, AB004011, AB004012, AB004013, AB004014, AB004015, AB004016, AB004017, AB004018, AB004019, AB004020, AB004021, AB004022, and AB004023.

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