Function of a Unique Sequence Motif in the Long Terminal Repeat of Feline Leukemia Virus Isolated from an Unusual Set of Naturally Occurring Tumors

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Feline leukemia virus (FeLV) proviruses have been characterized from naturally occurring non-B-cell, non-T-cell tumors occurring in the spleens of infected cats. These proviruses exhibit a unique sequence motif in the long terminal repeat (LTR), namely, a 21-bp tandem triplication beginning 25 bp downstream of the enhancer. The repeated finding of the triplication-containing LTR in non-B-cell, non-T-cell lymphomas of the spleen suggests that the unique LTR is an essential participant in the development of tumors of this particular phenotype. The nucleotide sequence of the triplication-containing LTR most closely resembles that of FeLV subgroup C. Studies performed to measure the ability of the triplication-containing LTR to modulate gene expression indicate that the 21-bp triplication provides transcriptional enhancer function to the LTR that contains it and that it substitutes at least in part for the duplication of the enhancer. The 21-bp triplication confers a bona fide enhancer function upon LTR-directed reporter gene expression; however, the possibility of a spacer function was not eliminated. The studies demonstrate further that the triplication-containing LTR acts preferentially in a cell-type-specific manner, i.e., it is 12-fold more active in K-562 cells than is an LTR lacking the triplication. A recombinant, infectious FeLV bearing the 21-bp triplication in U3 was constructed. Cells infected with the recombinant were shown to accumulate higher levels of viral RNA transcripts and virus particles in culture supernatants than did cells infected with the parental type. The triplication-containing LTR is implicated in the induction of tumors of a particular phenotype, perhaps through transcriptional regulation of the virus and/or adjacent cellular genes, in the appropriate target cell.

The region of the retroviral long terminal repeat (LTR) designated U3 contains the transcriptional promoter and enhancer elements necessary for viral gene expression. These regulatory sequences not only provide signals for the transcription of viral genes but also may provide signals in the appropriate target cell for the transcription of adjacent cellular proto-oncogenes. Genetic studies of murine leukemia viruses that induce T-cell lymphomas and erythroleukemias have established that sequences within the U3 region are necessary for leukemogenicity and encode determinants for tissue tropism and latency. In fact, the distinct disease specificities of certain retroviruses can be entirely switched by exchanging a segment of U3 that contains the transcriptional enhancer and immediately adjacent sequences (reviewed in reference 1). Transient-expression assays with reporter genes regulated by retroviral LTRs demonstrate a positive correlation between cell-type-specific enhancer activity and the tumorigenic spectrum of the retrovirus. For example, LTRs from viruses that induce T-cell lymphomas are more active in T cells compared with other cell types, and LTRs from erythroleukemogenic retroviruses are more active in erythroid cells (34). Thus, the relative ability of LTRs to direct transcription in a particular cell type can be considered a measure of their tumorigenic potential in that cell type (4, 5, 22, 34, 35).

The influence of the U3 in oncogenesis is mediated, at least in part, through the tissue-specific binding of nuclear transcription factors to elements of the enhancer. The enhancer consists

of a framework of multiple nuclear protein binding sites, the order and spacing of which are conserved among murine, feline, and primate type C retroviruses; however, the number of repeats of a particular element varies, as does the number of repeats of the entire enhancer (13). Generally, proviruses cloned directly from tumors contain duplications of the enhancer sequences. By comparison, tissue culture-derived proviruses, or weakly pathogenic strains, typically contain only a single copy of the enhancer (10, 12, 15, 25). Other sequence motifs in the LTR, in particular a short region immediately downstream of the enhancer, are also implicated as determinants of oncogenesis. Deletional and mutational analyses have demonstrated the region immediately downstream of the enhancer in murine leukemia viruses to contribute to leukemogenicity, latency, and tumorigenic spectrum (7, 14, 20, 23, 39).

We have identified a unique sequence motif downstream of the enhancer in the LTRs of feline leukemia virus (FeLV) integrated in an unusual set of naturally occurring feline lymphomas. The unique motif is a 21-bp tandem triplication beginning 25 bp downstream of a single copy of the enhancer. The 21-bp sequence is found in the LTRs of all FeLV strains, but its triplication has not previously been observed in the LTR of any feline or other mammalian retrovirus (18). The tumors in which the triplication-containing LTR occurs are of an unusual phenotype in that they are non-T-cell, non-B-cell lymphomas of the spleen. By comparison, the most common malignancy induced by FeLV is a T-cell lymphoma, often of thymic origin (30). The splenic lymphomas are additionally unusual in that they contain a relatively small number of clonally integrated proviruses, one of which is commonly integrated at a locus in feline DNA termed *fvi-1* (18).

In view of the repeated finding of the triplication-containing

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LTR in tumors of this unusual phenotype (18) and of the well-documented role of the LTR in determining tumorigenic spectrum, we hypothesized that the triplication-containing LTR plays a role in the induction of the tumors that contain it. As a measure of the contribution of the triplication to LTR function, we examined the ability of the triplication-containing LTR, compared with structurally distinct FeLV LTRs, to direct gene expression in a variety of cell types. We further examined biological properties of a recombinant infectious FeLV bearing the 21-bp triplication in the LTR. The results of these studies indicate that the 21-bp triplication confers enhancer activity on the LTR that contains it and that it functions preferentially in a cell-type-specific manner. On the basis of these findings, the triplication-containing LTR is implicated in the induction of tumors of a particular phenotype, perhaps through transcriptional regulation of the virus and/or adjacent cellular genes, in the appropriate target cell.

MATERIALS AND METHODS

Tumors, cell lines, and viruses. The naturally occurring FeLV-positive feline lymphomas examined in this study are non-B-cell, non-T-cell tumors of the spleen. These tumors were previously shown to contain FeLV proviral integrations within a 2.4-kb region of feline DNA termed *flvi-1* (18). A series of naturally occurring, FeLV-induced T-cell lymphomas of the thymus (19) was also examined. The FEA cell line, a continuous line of feline embryonic fibroblasts, was obtained from Jennifer Rojko, The Ohio State University, and was grown in Dulbecco modified Eagle culture medium with 10% fetal calf serum (FCS). The human Jurkat T-cell line was obtained from Fayth Yoshimura, University of Washington, and was grown in RPMI culture medium with 15% FCS. Other cell lines, including the human malignant hematopoietic progenitor cell line K-562 (ATCC CCL 243) and the canine osteogenic sarcoma cell line D-17 (ATCC CCL 183), were obtained from the American Type Culture Collection. These cell lines were grown in RPMI culture medium with 15% FCS or in Eagle minimal essential culture medium with 10% FCS, respectively. Molecularly cloned, infectious proviral DNAs of FeLV used in the study included FeLV-B/Gardner-Arnstein (3) and FeLV-A/61E (11).

PCR amplification of FeLV LTRs. Primer pair LTR13 and LTR14 were designed to yield products of 301 bp from FeLV LTRs containing the 21-bp triplication and 259 bp from LTRs lacking the triplication. LTR13 (5'-AAA GACCCCTACCCAAAACCT-3') spans nucleotides 3 to 25 and LTR14 (5'-TGAGGCGGAAGGTCGGACTC-3') spans nucleotides 303 to 284 of a triplication-containing FeLV LTR (18). PCR mixtures contained 1 µg of tumor DNA, 400 ng of each primer, and a 0.2 mM concentration of each deoxyribonucleotide triphosphate in a reaction buffer of 50 mM KCl, 10 mM Tris (pH 8.4), and 1.5 mM MgCl₂ in a total volume of 100 µl. An initial denaturation step at 98°C for 5 min was followed by annealing at 57°C for 5 min and extension at 72°C for 1 min; this was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min, with a final extension for 15 min.

PCR products were examined by agarose gel electrophoresis and Southern blotting. The presence of the triplication-containing LTR among the amplification products was verified by hybridization to an oligomer, termed LTR8 (5'-AGGCCATGCCAGCAGTTTCAAGGCCACTG-3'), which spans nucleotides 203 to 233 in a triplication-containing LTR (18). LTR8 spans the repeat junction of the triplication and recognizes only the triplication-containing LTR under the following conditions: Southern blots were prehybridized at 37°C for 2 h in a solution containing 50% formamide, 1.0 M NaCl, 0.05 M PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.8; Calbiochem-Behring], 0.1% sodium lauryl sarcosine, 0.01 M EDTA, 0.2 mg of denatured calf thymus DNA per ml, and 5× Denhardt solution (32). Filters were then hybridized for 15 h at 37°C in the same solution containing LTR8 (2 × 10⁶ cpm/ml) radiolabeled with polynucleotide kinase and [³²P]ATP (3,000 Ci/mmol; E. I. DuPont de Nemours & Co., Inc.). After two washes in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (32)-0.1% sodium lauryl sarcosine at room temperature for 10 min (each wash) and twice in the same solution at 42°C, filters were exposed at -70°C for various periods to Kodak XRP-5 film, with intensifying screens.

Construction and assay of LTR-CAT plasmids. A 1.88-kb *HindIII-SmaI* fragment containing a portion of *env* and the entire U3 region of the LTR was isolated from cloned proviral DNA of FeLV-A/61E (11) and of FeLV-945 (previously termed λ13 [18]). An analogous 2.0-kb fragment was isolated from cloned proviral DNA of LC-FeLV (19) with the enzymes *HindIII* and *SmaI*. The ends of each restriction fragment were filled with the Klenow fragment of DNA polymerase (32). Each fragment was then cloned into *SalI*-digested, blunt-ended pCAT-Basic plasmid (Promega). The pCAT-Basic plasmid contains the chloramphenicol acetyltransferase (CAT) reporter gene but lacks eukaryotic promoter and enhancer elements. Two independent clones of each LTR fragment were isolated.

A 1.53-kb fragment was also isolated from cloned proviral DNA of FeLV-A/61E with the *HincII* sites at nucleotides 6666 and 8192 (11). The *HincII* site at nucleotide 8192 occurs in the U3 region of the LTR and separates enhancer sequences from the promoter (18); thus, the fragment contains the enhancer sequences located in the U3 region of the LTR but lacks the promoter. FeLV-945 and LC-FeLV lack the *HincII* site at nucleotide 6666. Analogous 1.7-kb fragments were isolated with the *HincII* site in the U3 region of the LTR and a *PstI* site contributed by the plasmid vector. Each fragment was cloned into the pCAT-Promoter plasmid (Promega), an enhancerless reporter plasmid in which expression of CAT is driven by the simian virus 40 (SV40) promoter. Fragments were cloned at a blunt-ended *SalI* site downstream of the CAT reporter gene. Two independent clones representing each fragment (FeLV-A/61E, FeLV-945, and LC-FeLV) were isolated and were termed Δp61E, Δp945, and ΔpLC-FeLV, respectively.

LTR-CAT constructs were used in transient-transfection assays in which DNA was introduced into cultured cells by electroporation with the Cell-Porator Electroporation System 1 (GIBCO-BRL). FEA and K-562 cells were suspended in serum-free Dulbecco modified Eagle medium at a density of 3 × 10⁶ to 5 × 10⁶/ml, and 10 µg of LTR-CAT plasmid DNA was added to 1 ml of cells. Jurkat T cells were suspended at a density of 10⁷/ml in the same medium, and 50 µg of LTR-CAT plasmid DNA was added to 1 ml of cells. Electroporation was carried out at room temperature in 0.4-cm-diameter electroporation chambers at 1,180 µF, 300 V, and low resistance for FEA cells; at 800 µF, 300 V, and low resistance for K-562 cells; and at 1,180 µF, 250 V, and low resistance for Jurkat cells. Following electroporation, cells were resuspended in complete culture medium with 15% serum and were harvested 48 h later by three freeze-thaw cycles. CAT activity was quantified in cell homogenates by the solvent partition method (27, 32). Cell extracts (30 µl) were incubated with a solution of 0.2 µCi of [³H]acetyl coenzyme A (CoA) (4 Ci/mmol; final acetyl-CoA concentration, 280 nM; Amersham) chloramphenicol (1 mM), and a water-immiscible scintillation fluor (Econofluor; DuPont) for 24 h (FEA and K-562 cells) or 72 h (Jurkat cells). Incorporation of radiolabel into chloramphenicol was determined by liquid scintillation counting at periodic intervals. Parallel reactions lacking transfected CAT reporter plasmid were included to determine background signal, and the background thus obtained was subtracted from each datum point. For each transfection, data obtained at time points within the linear range of enzyme activity were pooled for a determination of mean counts per minute of ³H-acetylated chloramphenicol per hour. To normalize data for variations in transfection efficiency, LTR-CAT constructs were cotransfected at a 5:1 ratio with a reference plasmid, CMV-β-galactosidase (21). β-Galactosidase activity was quantified by a colorimetric assay (32).

Virus rescue assays. FeLV proviral DNAs λ13 and λ17, cloned from feline tumor 945 (18), were introduced into FEA and D17 cells as calcium phosphate precipitates (32), as were genomic DNAs prepared from feline tumors 945 and 934 (18). A total of 20 µg of cloned proviral DNAs, or 40 µg of genomic DNA, was introduced into 5 × 10⁶ cells. As positive controls, infectious molecular clones (40 µg) of FeLV-B/Gardner-Arnstein (3) and FeLV-A/61E (11) were introduced into parallel cultures. Productive infection was monitored by an enzyme-linked immunosorbent assay (ELISA; Synbiotics, Inc.) for the detection of the feline *gag* antigen in culture supernatants.

Construction of 61g945L, an infectious, triplication-containing FeLV. The recombinant virus 61g945L was constructed by substituting the triplication-containing LTR from FeLV-945 (18) into an infectious clone of FeLV-A/61E (11). An *EcoRV-BamHI* fragment was isolated from the 3' end of FeLV-945, with a unique *EcoRV* site within the single enhancer element of the LTR (18) and a *BamHI* site downstream in host flanking DNA. By a series of stepwise ligations, the fragment was substituted for the analogous fragment of FeLV-A/61E at the *EcoRV* site. Proviral DNAs of 61g945L and FeLV-A/61E were introduced into FEA cells as calcium phosphate precipitates (32). Productive infection was monitored by an ELISA (Synbiotics, Inc.) for the detection of the feline *gag* antigen in culture supernatants. To verify the structure of the recombinant LTR, reverse transcription (RT)-PCR was used to amplify the LTR from the total cellular RNA of infected cells. Samples of total cellular RNA (20 µg) from uninfected FEA cells and from cells infected with 61g945L and with FeLV-A/61E were reverse transcribed with Superscript (GIBCO-BRL) and random primers (3 µg) for 10 min at room temperature, at 42°C for 1 h, and then at 95°C for 10 min. Products of reverse transcription were then amplified by PCR with primers LTR9 and LTR10. LTR9 (5'-TGAAAGACCCCTACCC-3') spans nucleotides 1 to 17 and LTR10 (5'-ATAGCAGAAAGCGCGCA-3') spans nucleotides 357 to 339 of a triplication-containing FeLV LTR (18). Secondary amplification was then conducted with nested primers LTR11 and LTR12. LTR11 (5'-AAGG AAGTTAGAGGC-3') spans nucleotides 96 to 110 and LTR12 (5'-AATGAGG CGGAAGGTC-3') spans nucleotides 305 to 290 of a triplication-containing FeLV LTR (18). Amplification reactions were conducted as described above, except that the annealing temperature was 50°C. Amplification products were examined by Southern blot analysis with a probe for the U3 region of exogenous FeLV (6) and were directly sequenced with the *fmol* DNA sequencing kit (Promega).

Biological assays of 61g945L. Proviral DNAs (40 µg) of 61g945L and FeLV-A/61E were introduced by electroporation into 4 × 10⁷ FEA cells, and the cells were subsequently replaced into complete culture medium for 8 days. At 2, 4, 6, and 8 days after electroporation, culture supernatants were harvested for mea-

	EcoRV			NF1			GRE		
	core enhancer								
FeLV-A/61E	GATATC	TGTG	GTTAAGCACC	TGGGCCCCGG	CTTGAGGCCA	AGAACAGTTA	AAACCCCGGAT	ATAGCTGAAA	
FeLV-A/Glasgow									
LC-FeLV									.G.....
FeLV-B/GM1									
FeLV-C/Sarma									.G..T.....
945									.G.....
934									.G..T.....
922									.G..T.....
1142									.G..T.....
922trip-									.G..T.....
1142trip-									.G..T.....
	1			2			3		
FeLV-A/61E	CAGCAGAAGT	TTCAAGGCCG	CTGCCAGCAG	-----	-----	-----	-----	-----	-----
FeLV-A/Glasgow				-----	-----	-----	-----	-----	-----
LC-FeLV				-----	-----	-----	-----	-----	-----
FeLV-B/GM1				-----	-----	-----	-----	-----	-----
FeLV-C/Sarma				-----	-----	-----	-----	-----	-----
945				TTTCAAGGCC	ACTGCCAGCA	GTTTCAAGGC	CACTGCCAGC	CACTGCCAGC	CACTGCCAGC
934				TTTCAAGGCC	ACTGCCAGCA	GTTTCAAGGC	CACTGCCAGC	CACTGCCAGC	CACTGCCAGC
922				TTTCAAGGCC	ACTGCCAGCA	GTTTCAAGGC	CACTGCCAGC	CACTGCCAGC	CACTGCCAGC
1142				TTTCAAGGCC	ACTGCCAGCA	GTTTCAAGGC	CACTGCCAGC	CACTGCCAGC	CACTGCCAGC
922trip-				-----	-----	-----	-----	-----	-----
1142trip-				-----	-----	-----	-----	-----	-----
	HincII								
FeLV-A/61E	---TCTCCAGG	CTCCCCAGTT	GAC						
FeLV-A/Glasgow									
LC-FeLV									
FeLV-B/GM1									
FeLV-C/Sarma									
945	AG.....								
934	AG.....								
922	AG.....								
1142	AG.....								
922trip-									
1142trip-									

FIG. 1. Nucleotide sequences of the LTRs of various FeLV isolates, shown between the conserved *EcoRV* and *HincII* sites at positions 117 and 232, respectively, in the LTR of FeLV-A/61E (11). Sequences shown include FeLV-A/61E (11), FeLV-A/Glasgow (36), LC-FeLV (12), FeLV-B/GM1 (37), and FeLV-C/Sarma (29). Also shown are the sequences of triplication-containing FeLV LTRs isolated from splenic lymphomas of animals 945, 934 (18), 922, and 1142. LTRs lacking the triplication from animals 922 (922trip-) and 1142 (1142trip-) are also shown. The sequences are compared with that of FeLV-A/61E, and only the differences in sequence are shown. Dashes are included to optimize the alignment because of the insertions in the triplication-containing LTRs relative to the other sequences. Nuclear protein binding sites in the FeLV enhancer are indicated (core enhancer, NF1, and GRE [12]). LC-FeLV contains a duplication of the enhancer (12), only the second copy of which is shown. LTRs from 945, 934, 922, and 1142 contain a 21-bp sequence which is repeated three times in tandem.

surement of reverse transcriptase activity. Reverse transcriptase activity in culture supernatants was measured essentially as described by Klement and Nicolson (17).

Total cellular RNA was also isolated at 2, 4, 6, and 8 days after electroporation, with guanidinium isothiocyanate (8). For Northern (RNA) blot analysis, 5 µg of total cellular RNA was denatured in a formamide-formaldehyde solution, electrophoresed on an agarose gel, and transferred to nylon filters as previously described (32). Filters were hybridized for 15 h in a solution containing 6× SSC, 5× Denhardt solution, 0.5% sodium dodecyl sulfate (SDS), 100 mg of denatured DNA per ml, and 0.05 M PIPES (pH 6.8) at 68°C to an FeLV U3 probe (6) radiolabeled by random priming and added to the hybridization solution at 2 × 10⁶ cpm/ml. After two washes in 2× SSC (32)–0.1% sodium lauroyl sarcosine at 37°C for 30 min (each wash) and two washes in 0.1× SSC–0.1% sodium lauroyl sarcosine at 55°C, filters were exposed at –70°C for various periods to Kodak XRP-5 film, with intensifying screens. Filters were subsequently hybridized to a probe for β-actin (9) to demonstrate uniform loading of the gel.

RESULTS

The triplication-containing LTR occurs in non-B-cell, non-T-cell lymphomas of the spleen. The triplication-containing LTR was originally identified in proviruses inserted at the *flvi-1* locus in feline tumors 934 and 945, both of which are non-B-cell, non-T-cell lymphomas of the spleen. Feline tumors 922 and 1142 had been identified as lymphomas of the same phenotype, but the LTRs in those tumors were not previously

examined (18). To determine if tumors 922 and 1142 contain proviruses bearing the triplication in the LTR, a portion of the U3 region was amplified from the DNA by PCR. Amplification was performed with primers LTR13 and LTR14, predicted to yield a product of 301 bp from FeLV LTRs containing the 21-bp triplication and 259 bp from LTRs lacking the triplication. Southern blots of amplification products were hybridized to LTR8, an oligonucleotide which crosses the repeat junction of the triplication and hybridizes specifically to the triplication-containing LTR. The results demonstrated the predicted 301-bp product, indicating the presence of the triplication-containing LTR in both tumor 922 and tumor 1142. As negative controls, DNA samples from five FeLV-positive thymic lymphomas lacking insertions at *flvi-1* were similarly amplified and did not yield a product recognized by the LTR8 probe (data not shown). Nucleotide sequence analysis of the 301-bp amplification products confirmed the presence of the 21-bp tandem triplication and demonstrated that the triplication is identical in proviruses from all tumors examined (Fig. 1). The triplication-containing LTRs share distinctive sequence hallmarks, including a change at position 11 of the 21-bp element. Two of the triplication-containing LTRs also contain a change within the core enhancer element. When compared with FeLV

FIG. 2. Diagram of the LTRs of FeLV-A/61E (11), FeLV-945 (18), and LC-FeLV (11, 19), illustrating the number of repeats of the enhancer, the 21-bp sequence, and the positioning of these elements relative to the promoter (Pro).

isolates representing each natural subgroup, it is observed that the triplication-containing LTRs most closely resemble the LTR of FeLV subgroup C (Fig. 1).

The amplification of LTRs from all tumors examined yielded a product not identified by the LTR8 probe but which did hybridize in Southern blot analysis to a probe representing the U3 region of FeLV LTR. In the case of tumors 922 and 1142, this product was approximately 259 bp in size (data not shown). The nucleotide sequences of the 259-bp products from tumors 922 and 1142 demonstrated that they lack the 21-bp triplication. The LTRs otherwise contain the sequence hallmarks of the triplication-containing LTR, including the change at position 11 of the 21-bp sequence (Fig. 1).

The triplication-containing LTR acts in a manner similar to that of an LTR containing duplicated enhancers. Studies were performed to determine the effect of the 21-bp triplication on reporter gene expression modulated by the LTR. Three naturally occurring, structurally distinct FeLV LTRs were examined for their abilities to direct reporter gene expression: (i) the triplication-containing LTR from FeLV-945, a provirus integrated at *flvi-1* from tumor 945 (18); (ii) an LTR from LC-FeLV, a thymic lymphoma-derived provirus (19); and (iii) an LTR from FeLV-A/61E, a weakly pathogenic isolate of FeLV (11, 28). As represented in Fig. 2, the LTR of LC-FeLV exhibits a tandem duplication of the enhancer and only a single copy of the 21-bp sequence. The LTR of FeLV-A/61E exhibits

a single copy of the enhancer and a single copy of the 21-bp sequence. The LTRs of FeLV-945, FeLV-A/61E, and LC-FeLV are 96% identical (other than the presence or absence of repeat structures).

Recombinant constructs containing the U3 region of each LTR were prepared in a CAT reporter plasmid lacking a eukaryotic promoter or enhancer. LTR-CAT constructs were introduced by electroporation into two cell lines: FEA, a feline embryo fibroblast line, and Jurkat, a human malignant T-cell line. Cells were harvested 48 h later, and CAT activity was quantified in cell homogenates by the solvent partition method (27). Studies with FEA demonstrated that the triplication-containing LTR is 2.6-fold more active than the LTR of FeLV-A/61E, an LTR which also contains a single enhancer but lacks the triplication. The LC-FeLV LTR, which contains a duplicated enhancer but lacks the triplication, demonstrates activity which is statistically indistinguishable from that of the triplication-containing LTR (Fig. 3A). Thus, compared with the LTR of FeLV-A/61E, LTR-directed gene expression in fibroblasts is significantly higher when directed by an LTR containing a duplicated enhancer or a single enhancer with the 21-bp triplication. The activity of the triplication-containing LTR is also statistically indistinguishable from that of LC-FeLV in Jurkat T cells (Fig. 3B), a cell type in which the duplicated enhancer is thought to exert maximal influence (1). Taken together, these data suggest that the 21-bp triplication provides tran-

FIG. 3. CAT activity as a measure of expression of the CAT gene directed by FeLV LTRs. LTRs examined were from FeLV-A/61E (61E), LC-FeLV, and FeLV-945 (945). Restriction fragments containing the FeLV U3 were cloned into the pCAT-Basic plasmid (Promega), a vector lacking eukaryotic promoter and enhancer sequences. LTR-CAT plasmid DNA and CMV- β gal, a reference plasmid encoding β -galactosidase (21), were cotransfected in a 5:1 molar ratio into FEA feline embryonic fibroblasts (A) or Jurkat human T cells (B). After 48 h, cell lysates were assayed for CAT activity by the solvent partition method (27). Data obtained from time points within the linear range were normalized to β -galactosidase activity to correct for transfection efficiency and were pooled to determine mean counts per minute of [3 H]acetyl-CoA incorporated into chloramphenicol per hour. Illustrated are the pooled results of at least five replicates, with independent constructs of each LTR-CAT plasmid. Data were analyzed statistically by Student's *t* test, and statistical significance was assigned to $P \leq 0.05$. Asterisks indicate that data from LC-FeLV and from FeLV-945 are statistically indistinguishable from each other but are statistically distinct from data from FeLV-A/61E.

FIG. 4. CAT activity as a measure of expression of the CAT gene directed by FeLV LTRs in K-562 cells. The experiment was performed as described in the legend to Fig. 3. The asterisk indicates that data from FeLV-945 are statistically distinguishable from data from LC-FeLV and from FeLV-A/61E.

scriptional enhancer function to the LTR that contains it and that it substitutes at least in part for the duplication of enhancer.

The triplication-containing LTR modulates gene expression preferentially in a multipotential hematopoietic progenitor cell line. The activity of LTR-CAT reporter plasmids was also examined with line K-562, a human leukemia cell line which has been characterized as a primitive, multipotential, hematopoietic progenitor (24). Since the K-562 line represents a cell type similar to the target cell for pathogenesis mediated by members of the FeLV-C subgroup (16), it may be representative of the cell type targeted for malignant transformation in the spleen tissue of animals infected with the triplication-containing isolate of FeLV. In the K-562 line, the triplication-containing LTR is 12-fold more active than that of FeLV-A/61E and 4-fold more active than that of LC-FeLV (Fig. 4). In addition, the triplication-containing LTR is more active in K-562 than in other cells examined. For example, the level of activity of the triplication-containing LTR in K-562 cells (mean cpm of ³H-acetylated chloramphenicol per h = 2,707) is 4.2-fold higher than its activity in FEA cells (mean cpm of ³H-acetylated chloramphenicol per h = 640), taking into account differences in the efficiencies of transfection between the cell types. These data indicate that the triplication not only provides enhancer function but does so preferentially in a cell-type-specific manner.

The triplication-containing LTR acts as a bona fide enhancer. Regarding the molecular mechanism by which the 21-bp triplication contributes enhancer function to the LTR, at least two possibilities were considered. One possibility is that the 21-bp triplication contributes genuine enhancer function, perhaps via the binding of nuclear transcription factors. An alternative possibility is that the 21-bp triplication functions to maintain the appropriate spacing in the LTR between the enhancer and the promoter. A spacer function might be particularly relevant in an LTR in which the enhancer is not tandemly repeated, like the triplication-containing LTR. To examine the mechanism by which the 21-bp triplication contributes enhancer function in FeLV LTR-directed gene expression, reporter plasmids in which the spacing was disrupted were constructed. A promoterless restriction fragment containing the enhancer and the 21-bp triplication was removed from the LTR of FeLV-945. Analogous fragments were removed from the LTRs of FeLV-A/61E and LC-FeLV. As represented in Fig. 5A, the fragment of each LTR containing the

enhancer and triplication (if present) was then cloned into an enhancerless plasmid in which expression of the reporter gene is driven by an SV40 promoter. LTR fragments were cloned downstream of the reporter gene, such that CAT expression was dependent on transcriptional enhancement by the inserted LTR sequence. The constructs were introduced by electroporation into K-562 cells, and CAT activity was quantified (27) in cell homogenates 48 h later. The results show that CAT activity modulated by the promoterless triplication-containing fragment ($\Delta p945$) is statistically indistinguishable from that modulated by the duplicated enhancer-containing fragment ($\Delta pLC\text{-FeLV}$) and is 3.6-fold higher than that modulated by the single-enhancer LTR ($\Delta p61E$) (Fig. 5B). These experiments do not rule out a spacer function for the 21-bp triplication but indicate that it contributes a bona fide enhancement to the LTR that contains it.

Construction of an infectious recombinant FeLV containing the 21-bp triplication in the LTR. A prediction of the findings from transient-transfection assays (Fig. 3 to 5) is that transcription and replication of a triplication-containing FeLV would be enhanced in infected cells relative to those of FeLV-A/61E by virtue of the triplication-containing LTR. A test of that prediction requires infectious FeLV particles containing the 21-bp triplication in the genome. Two full-length FeLV proviral DNAs containing the 21-bp triplication in the LTR, originally cloned from tumor 945 (18), were tested for infectivity. Intact genomic DNA from tumor 945 was similarly tested, as was genomic DNA from tumor 934 which also contains triplication-bearing FeLV proviruses (18). DNAs were tested for infectivity by stable transfection into two cell lines, FEA and D-17. As positive controls, parallel cultures were transfected with infec-

FIG. 5. (A) Diagram of a reporter gene construct in which a restriction fragment of the FeLV-945 LTR, containing the transcriptional enhancer and the 21-bp triplication, was cloned downstream of the CAT gene in an enhancerless reporter plasmid in which expression is driven by the SV40 promoter. (B) CAT activity directed by the SV40 promoter, with promoterless restriction fragments of FeLV LTRs inserted downstream of the CAT gene. Promoterless restriction fragments of FeLV LTRs examined were from FeLV-A/61E ($\Delta p61E$), LC-FeLV ($\Delta pLC\text{-FeLV}$), and FeLV-945 ($\Delta p945$). The experiments shown were performed with K-562 cells, by the methods and analysis of data described in the legend to Fig. 3. As a negative control, the assay was performed with the reporter plasmid lacking an FeLV insert (Pro). The asterisks indicate that data from $\Delta pLC\text{-FeLV}$ and from $\Delta p945$ are statistically indistinguishable from each other but are statistically distinct from the data for $\Delta p61E$ and the negative control.

FIG. 6. (A) Diagram of the 61g945L recombinant FeLV (not to scale). 61g945L contains the 5' LTR and genomic backbone of FeLV-A/61E (shaded boxes). The 3' LTR is substituted from the *EcoRV* site with the triplication-containing LTR from FeLV-945 (open boxes). Indicated within U3 are the enhancer and 21-bp triplication. Transcription of recombinant proviral DNA is predicted to yield an RNA molecule which contains the triplication within U3; thus, progeny virus is predicted to contain the triplication in both LTRs. (B) Southern blot analysis of RT-PCR products from infected and uninfected FEA cells. Total cellular RNA from uninfected cells (lane A) or from cells infected with FeLV-A/61E (lane B) or with 61g945L (lane C) were reverse transcribed and amplified by PCR as described in the text. Products were hybridized to a radiolabeled probe representing the U3 region of the FeLV LTR. Indicated are the predicted products of 209 bp, from the triplication-containing LTR, and 167 bp, from the FeLV-A/61E LTR. The nucleotide sequences of the predicted products were verified (see text).

tious molecular clones of FeLV-A/61E or FeLV-B/Gardner-Arnstein. Productive infection, as monitored by ELISA for FeLV *gag*, was demonstrated in positive control cultures at 21 days after transfection. By comparison, productive infection was not detected in cells transfected with tumor DNA or tumor-derived proviruses for as long as 6 weeks after transfection (data not shown).

To obtain infectious FeLV particles containing the 21-bp triplication in the genome, a recombinant virus, 61g945L, was constructed by substituting the triplication-containing LTR from FeLV-945 into an infectious clone of FeLV-A/61E (Fig. 6A). 61g945L was stably introduced into FEA cells, culture supernatants of which demonstrated productive infection by 2 weeks. To verify the structure of the recombinant LTR, RT-PCR was used to amplify the LTR from total cellular RNA of infected cells. Samples of total cellular RNA from cells infected with 61g945L and with FeLV-A/61E and from uninfected FEA cells were reverse transcribed and amplified with primers LTR9 and LTR10. Secondary amplification was then conducted with nested primers LTR11 and LTR12. Southern blot analysis of the amplification products demonstrated the predicted 209-bp fragment from 61g945L-infected cells and the 167-bp fragment from FeLV-A/61E-infected cells (Fig. 6B). The nucleotide sequences of the amplification products were also verified by direct sequencing (data not shown).

Biological assays of 61g945L. The infectious recombinant 61g945L was used to test the prediction that the 21-bp triplication confers enhanced growth properties on the virus that contains it. Equal amounts of cloned proviral DNA of 61g945L and of the parental genome, FeLV-A/61E, were introduced by electroporation into parallel cultures of FEA cells. At days 2, 4, 6, and 8 after electroporation, culture supernatants were harvested for measurement of reverse transcriptase activity. Four repetitions of this experiment demonstrated that virus particles accumulate more rapidly and to higher levels in supernatants from cells infected with 61g945L than in those in-

FIG. 7. Reverse transcriptase activity present in the culture supernatants of FEA cells transfected with cloned proviral DNA of FeLV-A/61E or the FeLV recombinant 61g945L. Proviral DNAs were introduced into FEA cells by electroporation, and reverse transcriptase activity in culture supernatants was measured at 2, 4, 6, and 8 days thereafter. Shown are the results of two representative experiments using independently prepared proviral DNAs of 61g945L (●) or FeLV-A/61E (□).

FIG. 8. Northern blot analysis of total cellular RNA from FEA cells transfected with cloned proviral DNA of FeLV-A/61E or the FeLV recombinant 61g945L. Proviral DNAs were introduced into FEA cells by electroporation, and total cellular RNA was prepared at 2, 4, 6, and 8 days thereafter. Northern blots of RNA (5 μ g) from cells infected with 61g945L (lanes A) or with FeLV-A/61E (lanes B) were then hybridized to a radiolabeled probe representing the U3 region of the FeLV LTR. The blot was subsequently hybridized to a probe representing β -actin as a control to demonstrate uniform loading of the gel. Indicated are the viral RNA transcripts (vRNA), corresponding to full-length FeLV genomic RNA and subgenomic mRNA, and the β -actin transcript.

fected with FeLV-A/61E. Data from two of those experiments are shown (Fig. 7). Total cellular RNA was also isolated at days 2, 4, 6, and 8 after electroporation. Northern blot analysis with an FeLV U3 probe demonstrated that viral RNA transcripts accumulate more rapidly and to higher levels in cells infected with 61g945L (Fig. 8).

DISCUSSION

FeLV proviruses isolated from non-T-cell, non-B-cell lymphomas of the spleen exhibit an LTR which is distinctive in two respects. First, the LTR contains only a single copy of the enhancer. This is unusual, since most tumor-derived FeLV proviruses exhibit a direct repeat of two or three copies of the enhancer (12, 25, 31). Second, the LTR contains a 21-bp tandem triplication beginning 25 bp downstream of the enhancer. Triplication of the 21-bp sequence occurs uniquely in proviruses from tumors of this phenotype and has not been observed previously in any other isolate of FeLV or in any other mammalian retrovirus (18) (Fig. 1). In two of four cases, the triplication-containing provirus was shown to be integrated at *flvi-1*, a common domain of retroviral integration identified in these tumors (18). The repeated finding of the triplication-containing LTR in proviruses integrated in non-B-cell, non-T-cell lymphomas of the spleen suggests that the unique LTR is an essential participant in the development of tumors of this particular phenotype.

Examination of the sequence of the triplication-containing LTR indicates that the virus bearing it is related to FeLV subgroup C (Fig. 1). Further study is required to define the relationship between the triplication-containing virus and the FeLV-C subgroup, particularly including examination of the host range for infection and of the *env* genes for sequence hallmarks distinctive to subgroup C (26, 33). It is intriguing that FeLV-C, while not associated with malignant disease, typically induces degenerative disease involving hematopoietic progenitors of the erythroid lineage (16). The cell type of origin of feline splenic tumors has not been identified, al-

though the tumors were characterized as non-B-cell, non-T-cell (18). The relationship between the triplication-containing virus and FeLV-C suggests that the cell type targeted for transformation might indeed not be lymphoid but that it might be a hematopoietic progenitor.

Analysis of LTRs from splenic tumors also demonstrated single-enhancer FeLV proviruses lacking the triplication but containing the features of the nucleotide sequence distinctive to the triplication-containing LTR (Fig. 1). This finding has implications for the origin of the triplication-containing LTR. The structure of the initial inoculum is not known in naturally occurring tumors such as those examined; however, sequence heterogeneity is commonly observed among FeLV LTRs cloned from a single natural tumor (12, 25). It is possible that animals were originally infected with a virus whose LTR resembles that of FeLV-C and which lacks the triplication. In that case, the triplication would have arisen *de novo* in each infected animal. In fact, a recent study has demonstrated evolution of the FeLV LTR *in vivo* during lymphomagenesis (31). Alternatively, each animal may have been originally infected with a complex of viruses containing both LTR types. Evidence to date appears to support the second possibility, considering that (i) LTRs lacking and containing the triplication, isolated from the same animal, are not identical in sequence (Fig. 1), (ii) the position and sequence of the tandem triplication is precisely identical among the animals (Fig. 1), and (iii) the animals were collected from a limited geographic area and may have been exposed to the same natural isolate.

Studies of LTR-directed reporter gene expression in FEA and Jurkat cells indicate that the 21-bp triplication provides transcriptional enhancer function to the LTR that contains it and that it substitutes at least in part for the duplication of enhancer. In fact, gene expression directed by the triplication-containing LTR is indistinguishable from that of the LTR containing a duplicated enhancer, even in T cells (Fig. 3). This is remarkable, because T cells represent the cell type in which the duplicated enhancer is thought to exert its maximal effect (reviewed in reference 1). The triplication-containing LTR is 12-fold more active in K-562 cells than is that of FeLV-A/61E (Fig. 4) and is 4.2-fold more active in K-562 cells than it is in FEA cells. These data indicate that, with respect to the limited set of cell lines examined, the triplication provides enhancer function in a cell-type-specific manner.

The function of the triplication-containing LTR was compared in this study with those of two other naturally occurring FeLV LTRs (Fig. 2): LC-FeLV, which contains a duplication of the enhancer but lacks the triplication (12, 19), and FeLV-A/61E, which contains only a single copy of the enhancer and lacks the triplication (11). The triplication-containing LTR is 96% identical to the LTRs of LC-FeLV and FeLV-A/61E. The most prominent structural difference is the presence of the triplication, but other differences occur as well and may contribute to the function of the triplication-containing LTR. One of those nucleotide differences occurs within the triplication (Fig. 1, A versus G at position 11 of the 21-bp repeat). A systematic mutational analysis will be required to determine the contribution of each of the nucleotide sequence changes to the function of the triplication-containing LTR. The presence of the triplication also alters the spacing between the enhancer and promoter elements in the triplication-containing LTR. Indeed, the triplication may contribute to LTR-mediated gene expression by maintaining the proper spacing. A spacer function might be particularly relevant in an LTR, like the triplication-containing LTR, in which the enhancer is not tandemly repeated. Assays of reporter gene expression with constructs in which the natural spacing is disrupted indicate that the tripli-

cation does contribute bona fide enhancer function, independently of spacing (Fig. 5). The possibility of a spacer function for the triplication is not excluded by these studies, nor are the two functions mutually exclusive. For example, an element termed DEN, found downstream of the enhancer in the murine leukemia virus MCF13, contributes to LTR function by two mechanisms. DEN provides genuine transcriptional enhancer function as well as maintains the appropriate spacing in a single-enhancer LTR (39, 40).

The cell-type-specific enhancement observed for the intact triplication-containing LTR in K-562 cells (Fig. 4) is not seen in reporter gene constructs with promoterless LTR fragments (Fig. 5). It is possible that the activity of the intact LTR in K-562 cells is dependent on appropriate spacing between enhancer and promoter. It is also possible that the activity of the triplication-containing LTR in K-562 cells is dependent on interaction with the homologous promoter and is thus not apparent with the SV40 promoter used in the reporter construct. The dependence of LTR-derived enhancers on the homologous promoter for maximal activity has been previously demonstrated by others (2, 38).

The findings from assays of LTR-directed reporter gene expression (Fig. 3 and 4) predict that transcription and replication of FeLV with the 21-bp triplication would be enhanced in infected cells relative to those of FeLV lacking the triplication. A test of this prediction requires infectious FeLV containing the 21-bp triplication in U3; however, natural FeLV proviruses bearing this structure were found not to be infectious in cells of feline or canine origin. A recombinant FeLV, termed 61g945L, in which the triplication-containing LTR was substituted for the LTR of FeLV-A/61E, was constructed (Fig. 6A). When equivalent amounts of proviral DNAs of 61g945L and the parental virus, FeLV-A/61E, were introduced into parallel cultures, viral RNA transcripts and virus particles were observed to accumulate more rapidly and to higher levels in cells into which 61g945L was introduced (Fig. 7 and 8), thus supporting the predictions of our previous findings. In FEA cells, the triplication-containing LTR is approximately threefold more active than the LTR of FeLV-A/61E (Fig. 3); however, the relative accumulation of viral RNA transcripts and virus particles is greater than threefold in cells infected with 61g945L (Fig. 7 and 8). It is possible that the LTR functions differently in the context of the intact virus compared with its function in the reporter gene constructs and/or that factors other than LTR strength contribute to the accumulation.

Taken together, our findings indicate that the 21-bp triplication confers transcriptional enhancer function on the FeLV LTR that contains it and that it modulates LTR-driven gene expression in a cell-type-specific manner. It has been well documented for murine retroviruses that the ability of the LTR to direct gene expression in a cell-type-specific manner is positively correlated with tumorigenic potential (reviewed in reference 1); for example, the LTR of SL3-3 murine leukemia virus directs gene expression to the highest levels in T-cell lines, and SL3-3 is a potent inducer of T-cell lymphoma (4, 34). The triplication-containing LTR functions preferentially in K-562, a primitive, multipotential hematopoietic cell line, consistent with a role for the triplication-containing LTR in induction of the non-B-cell, non-T-cell tumors from which it was originally derived. Also consistent with such a role is the occurrence of the triplication-containing LTR uniquely in tumors of this unusual phenotype. It is our hypothesis that the triplication-containing LTR participates in the malignant process by modulating the expression of proviral genes and/or of host genes at a site of integration in the appropriate target cell in the spleen.

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