Insertional Mutagenesis of *flvi-2* in Tumors Induced by Infection with LC-FeLV, a *myc*-Containing Strain of Feline Leukemia Virus

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LC-FeLV is a *myc*-containing strain of feline leukemia virus (FeLV) which exhibits only partial transforming activity in vitro and in vivo. LC-FeLV infection in kittens may induce, but does not necessarily induce, thymic lymphosarcoma in viremic animals after a short latency. These observations suggest that infection with LC-FeLV is not sufficient to induce complete transformation and that another genetic event(s) is required. One possibility for such an event is that the integrating provirus acts as an insertional mutagen and thereby disrupts the structure or function of another proto-oncogene. Using a strategy of transposon tagging, this possibility was examined in eight feline T-cell lymphosarcomas, including four induced by experimental infection with LC-FeLV, three induced by natural infection with FeLV, and one FeLV-negative tumor. The analysis demonstrated one locus, termed *flvi-2*, to be structurally altered in six of the tumors examined, including three induced by LC-FeLV and three in which no activated *myc* oncogene is apparent. Inverse polymerase chain reaction was used to demonstrate the presence and transcriptional orientation of proviruses integrated at *flvi-2* in five of these tumors. The *flvi-2* locus does not hybridize to cloned probes representing 21 previously identified proto-oncogenes or common domains of retroviral integration. Thus, the data suggest that interruption of the *flvi-2* locus cooperates with the *myc* oncogene in the induction of T-cell lymphomas by LC-FeLV; indeed, the observations indicate that the insertional mutagenesis of *flvi-2* plays a role in T-cell lymphomagenesis even in the absence of feline *v-myc*.

The feline leukemia viruses (FeLV) represent a family of horizontally transmissible type C retroviruses causally associated in the domestic cat with a spectrum of lymphoid malignancies termed leukemia-lymphosarcoma complex. FeLV is a representative of the slowly transforming retroviruses, since malignant disease occurs after a prolonged viremic period (mean = 2 years), and since most natural isolates do not contain an oncogene (13, 16). Many strains of FeLV which contain oncogenes have been identified in naturally occurring tumors, however, including eight independent isolates which contain the oncogene *myc* (22, 27-29). One of the *myc*-containing strains of FeLV, termed LC-FeLV, was originally isolated as proviral DNA from a naturally occurring thymic lymphosarcoma of a domestic cat (22). The LC-FeLV provirus, 5.5 kb in length, encodes exons 2 and 3 of *c-myc* in their entirety. The sequence of *v-myc* encoded by LC-FeLV is unchanged relative to feline *c-myc* and differs only in that it is expressed as a *gag-myc* fusion protein (6, 41).

Infectious LC-FeLV, rescued by replication in the presence of FeLV as a helper, demonstrates incomplete transforming potential in early passage feline cells in vitro and in neonatal kittens in vivo (3, 21). For example, infection with LC-FeLV fails to transform early passage leukocytes from peripheral blood, spleen, or thymus from neonatal kittens. Infection of fibroblasts from feline embryos, however, gives rise to a morphologically altered population which is apparently immortalized. The cells are only partially transformed, however, since they are incapable of anchorage-independent growth and are not tumorigenic in athymic mice (3). Of animals inoculated experimentally with LC-FeLV, fewer than 50% develop malignant tumors. The tumors develop

after a relatively short latency of 4 to 6 months after inoculation, are invariably T-cell lymphomas of the thymus, and contain clonally integrated LC-FeLV. Other animals shown to be productively infected with LC-FeLV remain asymptomatic for as long as 12 months (21). These findings demonstrate that LC-FeLV may induce, but does not necessarily induce, malignant disease in infected kittens. Incomplete transforming potential is characteristic of other *myc*-containing strains of FeLV as well (11, 28).

It is noteworthy that the tumorigenic potential of LC-FeLV, as well as of other *myc*-containing isolates of FeLV, is restricted to the induction of T-cell lymphoma of the thymus, the same tumor from which such isolates were originally derived (21, 30); indeed, the acquisition of *myc* by FeLV does not expand its tumorigenic spectrum, since FeLV infection is associated with induction of the same neoplasm after a long latency (13, 16). The *v-myc* oncogene expressed by LC-FeLV is implicated in tumorigenesis because of (i) the clonality of tumors with respect to LC-FeLV proviruses and (ii) the effect of LC-FeLV on latency before the appearance of tumors. Its partial transforming activity in vitro and in vivo, however, suggests that infection with LC-FeLV is not sufficient to induce complete malignant transformation and that another genetic event(s) is required. One possibility for such an event is addressed by the hypothesis of retroviral insertional mutagenesis. According to this hypothesis, proviral integration in or near a gene critical to normal cell growth or differentiation might perturb its structure or regulation, thereby leading to malignant change (45). Insertional mutagenesis appears to be the sole mechanism, for example, by which the *myc* oncogene is activated by murine leukemia viruses (MuLV) in the induction of thymic lymphoma (9, 23, 35, 40). Insertional mutagenesis of the loci termed *mlvi-1* (= *pvt-1/mis-1* [18]), *mlvi-2*, *mlvi-3*, *mlvi-4*, *pim-1*, *fis-1*, *gin-1*, *fim-1*, *fim-2* (= *c-fms*),

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fim-3, *evi-1*, *evi-2*, *spi-1*, and *dsi-1* (5, 7, 10, 24, 26, 34, 37, 38, 43, 46, 47) occurs in a significant proportion of lymphomas and leukemias induced in rodents by infection with MuLV. Insertional mutagenesis has also been proposed as a cooperating event in leukemogenesis mediated by an oncogene-containing retrovirus, specifically, in the induction of pre-B-cell lymphoma by Abelson MuLV. In that case, a domain of murine DNA termed *Ahi-1* is commonly interrupted by helper MuLV integration (15, 31, 32). In the situation under examination here, it is possible that the helper FeLV component of the inoculum, or LC-FeLV itself, acts as an insertional mutagen. Specifically, the insertional mutagenesis of a particular domain of feline DNA may cooperate with the *myc* oncogene in tumor induction. An event of that kind may be detected by using a strategy of transposon tagging as we have employed previously (20). In this approach, host DNA sequences flanking somatically acquired proviruses are used as probes to identify regions of chromosomal DNA interrupted commonly in independent tumors. Such domains serve as targets of insertional mutagenesis and are thereby implicated functionally in tumor induction.

MATERIALS AND METHODS

Natural and experimentally induced feline lymphosarcomas. A collection of eight naturally occurring or experimentally induced feline lymphosarcomas were examined in this study. Three thymic tumors (J5-1, 981-2, 981-3) experimentally induced by infection with LC-FeLV (21) were examined. Four FeLV-positive (1110, 931, 1037, 1100) and one FeLV-negative (1433) naturally occurring tumors, histopathologically characterized and provided by Murray B. Gardner and James W. Casey (22), were also examined. Various uninvolved tissues were collected from the same animals.

Preparation and analysis of high-molecular-weight DNA. High-molecular-weight DNA was prepared from frozen tumor or normal tissue as previously described (20). For Southern blot analyses, DNA (10 µg) was digested with restriction enzymes, electrophoresed in a 0.8% agarose gel, transferred to nylon filters (Nytran; Schleicher & Schuell), and affixed by UV irradiation (17). Hybridization and washing conditions were as previously described (20), except that probes were radiolabeled with the Klenow fragment of *Escherichia coli* DNA polymerase I by using random oligonucleotide primers (33).

Preparation and screening of recombinant library. A recombinant library of the genomic DNA of tumor J5-1 was prepared in the bacteriophage cloning vector λEMBL3 (14) as previously described (20). The library (1.5×10^6 PFU) was screened without amplification by in situ plaque hybridization (1) with probes radiolabeled by random priming. Recombinants selected from the library were purified by repeated rounds of in situ plaque hybridization.

PCR amplification of proviral integration sites. Inverse polymerase chain reaction (PCR) amplification was performed as described by Silver and Keerikatte (36) to amplify the sequences at the 5' host-virus junction of proviral integrations. The template for inverse PCR was prepared from the genomic DNA of each tumor with the restriction enzymes *RsaI* and *EcoRV* as described in the text. Thirty cycles of amplification were performed, using AmpliTaq polymerase (Perkin Elmer Cetus) and a Bioscyler thermal cycler (Bios Corporation). Amplification was performed at a denaturation temperature of 94°C for 20 s, an annealing temperature of 55°C for 1 min, and an extension temperature

of 72°C for 2 min. For slot-blot analysis of PCR products, a portion of each reaction mixture was denatured in 0.1 N NaOH for 20 min at room temperature, neutralized in 10 volumes of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and spotted immediately on a Nytran filter by using a Bio-Dot SF apparatus (Bio-Rad).

RESULTS

Organization of integrated FeLV proviral DNA in feline T-cell lymphomas. Examined in the present study were the malignant lymphomas of eight cats, seven of which are FeLV positive and one of which is FeLV negative. Of the FeLV-positive tumors, 1 (1110) is a naturally occurring thymic lymphoma from which the *myc*-containing strain of FeLV, LC-FeLV, was originally isolated (22). Three other FeLV-positive tumors (J5-1, 981-2, 981-3) were experimentally induced by infection of neonatal animals with LC-FeLV (21). The remaining FeLV-positive tumors (931, 1037, 1100) and the FeLV-negative tumor (1433) are naturally occurring feline lymphomas which lack LC-FeLV or any apparent rearrangement of the *c-myc* gene. Southern blot analysis of DNA from tumors 931, 1037, 1100, and 1433 demonstrates the *c-myc* gene to be in germ line configuration over a region of 16 kb (data not shown). All these tumors contain T cells as evidenced by the somatic rearrangement of the β-chain of the T-cell-receptor gene (21) (a portion of the data is not shown).

Implicit in the hypothesis of insertional mutagenesis as a cooperating event in LC-FeLV-induced transformation is the monoclonality or oligoclonality of each tumor with respect to proviral integration sites. The organization of exogenously acquired FeLV proviruses in tumor DNA was examined by Southern blot analysis using as a probe a molecular clone of the U3 region of the long terminal repeat (LTR) of FeLV-B/Gardner-Arnstein (22). This probe identifies all strains of horizontally transmissible FeLV identified to date, but is only weakly related to endogenous FeLV sequences in the feline genome (8). The probe may be used as described elsewhere (20) to identify an internal proviral fragment and the 5' host-virus junction fragments generated by *KpnI* digestion of tumor DNA. If the tumor mass is monoclonal (or oligoclonal) with respect to proviral integration sites, i.e., if the proviruses are located in integration sites which are identical in each cell in the tumor, then the host-virus junction fragments appear as discrete bands in Southern blot analysis and can be enumerated. Analysis of *KpnI*-digested tumor DNAs with the U3 probe demonstrated the FeLV-positive tumors to contain multiple, clonally integrated LC-FeLV and/or FeLV proviruses. The proviruses of LC-FeLV and FeLV may be readily distinguished in this analysis by the size of the internal proviral DNA fragments. The number of exogenous proviruses per cell was observed to range from 4 to 15 but was generally fewer in tumors induced by infection with LC-FeLV than in those lacking LC-FeLV (Fig. 1).

Molecular cloning of LC-FeLV and FeLV proviruses from tumor J5-1. The strategy of transposon tagging requires the molecular cloning of all the somatically acquired proviruses from a single lymphoma. Then, unique cellular sequences representing each integration event may be identified and used as probes to screen other tumors in the collection for proviral integration within the same region. Tumor J5-1, experimentally induced by infection with LC-FeLV, was shown by Southern blot analysis with the U3 probe to contain six proviruses which appear to be monoclonally

FIG. 1. Southern blot analysis of DNA from naturally occurring and experimentally induced lymphosarcomas of the following cats: J5-1 (lane A), 1110 (lane B), 981-2 (lane C), 981-3 (lane D), 931 (lane E), 1100 (lane F), 1037 (lane G), and 1433 (lane H). The tumors examined were isolated from the thymus (lanes A, B, C, D, and G) or from lymph nodes (lanes E, F, and H). Tumors were naturally occurring (lanes B, E, F, G, and H) or experimentally induced (lanes A, C, and D). DNA samples (10 μ g) were digested with *Kpn*I, electrophoresed in an agarose gel, and transferred to nitrocellulose. The resulting Southern blot was hybridized as described in the text to a radiolabeled probe representing the U3 region of the LTR of FeLV-B/Gardner-Arnstein (22). Indicated (arrowheads) are restriction fragments likely to represent the internal proviral *Kpn*I fragment from LC-FeLV (5.2 kb) or from FeLV (3.6 kb). Molecular sizes were determined by comparison with the migration of *Hind*III-digested lambda DNA, *Taq*I-digested pUC18 DNA, and *Bgl*I-digested pUC18 DNA electrophoresed in parallel.

integrated (Fig. 2A). The tumor had been shown previously to contain four clonally integrated LC-FeLV proviruses per cell (21); thus, the remaining two integration events apparently represent helper FeLV proviruses. A recombinant library was prepared of the DNA from tumor J5-1 in the bacteriophage cloning vector λ EMBL3 (14) and was screened using as a probe the U3 region of the LTR of FeLV-B/Gardner-Arnstein. Recombinants were isolated from the library representing each of the six independent integration events, i.e., four LC-FeLV proviral integrations and two helper FeLV integrations (Fig. 2B). Restriction enzyme site maps of full-length FeLV proviruses in recombinants λ J₅10 and λ J₅17 are the same as the minimally pathogenic FeLV strain, FeLV-A/Glasgow (42), which served as a helper in the original inoculum used to infect cat J5-1 (21). Restriction enzyme site maps of LC-FeLV proviruses contained in recombinants λ J₅2, λ J₅14, λ J₅23, and λ J₅25 cannot be distinguished from that of the original LC-FeLV isolate (22).

Examination of integration sites in the DNA of other T-cell lymphomas. If an insertional mutagenesis event cooperates with *myc* in the induction of tumors by LC-FeLV, then at least one of the loci interrupted by proviral integration in tumor J5-1 is predicted to be interrupted by proviral integration in other tumors induced by LC-FeLV. To test this prediction, we isolated restriction fragments which represent single-copy host DNA flanking each of the six integration events in tumor J5-1 (Fig. 2B). These fragments were used as probes in Southern blot analysis to determine whether any of the six regions was interrupted by proviral integration in any other tumor in the collection. The regions harboring two helper FeLV proviruses (λ J₅10 and λ J₅17) and two LC-FeLV

proviruses (λ J₅2 and λ J₅14) were observed by examination with five restriction enzymes to be in germ line configuration in all tumors except J5-1 (data not shown). Thus, with respect to restriction enzyme sites for the enzymes *Eco*RI, *Bam*HI, *Kpn*I, *Pst*I, and *Hind*III, we observed no evidence for proviral integration in these four loci in any tumor other than J5-1.

The locus represented in recombinant λ J₅23 harbors an LC-FeLV provirus in tumor J5-1 (Fig. 2B). A 0.8-kb *Eco*RI fragment of cat DNA flanking the 5' end of the integrated provirus was used as a probe in Southern blot analysis of the DNA of other tumors. This probe identifies a 10-kb *Hind*III fragment in cat DNA. In DNA from tumor 981-3, however, the probe identifies two fragments of altered size. Thus, tumor 981-3 appears not to contain a normal allele of this locus, but contains a tumor-specific alteration of both alleles (data not shown). Tumor 981-3 is a T-cell lymphoma of the thymus, experimentally induced by infection with LC-FeLV (21). The locus represented in λ J₅23, and its tumor-specific rearrangement in T-cell lymphomas, have not yet been further studied.

The sixth integration site, represented in recombinant λ J₅25, harbors an LC-FeLV provirus in tumor J5-1 (Fig. 2B). A 1.1-kb *Eco*RI fragment of cat DNA flanking the 5' end of the integrated provirus was used as a probe in Southern blot analysis of the DNA of the other seven tumors. The probe identifies in five of the six FeLV-positive tumors a fragment which comigrates with the normal allele, as well as a fragment of a different size. Thus, one allele of the locus is tumor-specifically altered in each case (Fig. 3). The locus is not altered in the DNA of the FeLV-negative tumor from cat 1433 (data not shown). Including J5-1, the tumor from which λ J₅25 was cloned, these data demonstrate structural alteration of the locus in the DNA of six of seven FeLV-positive lymphomas examined (86%). We termed this locus *flvi-2*, an acronym for feline leukemia virus integration site 2. The *flvi-2* locus is tumor-specifically altered in three of four lymphomas induced by infection with LC-FeLV (J5-1, 1110, 981-2) and in three other T-cell lymphomas in which LC-FeLV is not involved (931, 1037, 1100).

Molecular cloning of uninterrupted *flvi-2* and demonstration of proviral integration. The recombinant library of tumor J5-1 DNA described above was screened with the 1.1-kb *Eco*RI fragment from λ J₅25 which flanks the 5' end of the integrated LC-FeLV provirus (Fig. 2B). Recombinants representing the uninterrupted *flvi-2* allele in tumor J5-1 were thus isolated and characterized by restriction enzyme site mapping (Fig. 4). Molecularly cloned *flvi-2* did not hybridize to probes representing 10 retroviral oncogenes (*abl*, *fes*, *fms*, *mos*, *myb*, *myc*, *ras*, *rel*, *sis*, and *src*) and 11 previously identified common domains of retroviral integration in rodent leukemias-lymphomas (*mlvi-1*, *mlvi-2*, *mlvi-3*, *mlvi-4*, *gin-1*, *pvt-1*, *fim-1*, *fim-3*, *pim-1*, *spi-1*, and *fis-1*) (data not shown).

We infer that the tumor-specific alterations of *flvi-2* as demonstrated by Southern blot analysis of tumor DNA (Fig. 3) result from the integration of FeLV provirus within the locus. Molecular cloning of the altered allele demonstrates this to be the case in tumor J5-1 (Fig. 2B). We employed a strategy of inverse PCR (36) to determine whether proviral integration has occurred at *flvi-2* in the other tumors with altered alleles. As represented in Fig. 5A, our strategy for inverse PCR depends on the presence of the 4-bp recognition site for *Rsa*I at position 374 in the FeLV LTR (42). Tumor DNA was digested with *Rsa*I to obtain 5' host-virus junction fragments whose length depends on the position of the

FIG. 2. (A) Southern blot analysis of DNA from tumor J5-1, a T-cell lymphosarcoma of the thymus experimentally induced by infection with LC-FeLV (21). DNA (10 μ g) was digested with *Kpn*I, electrophoresed in an agarose gel, and transferred to nitrocellulose. The resulting Southern blot was hybridized as described in the text to a probe representing the U3 region of the LTR of FeLV-B/Gardner-Arnstein (22). Indicated (arrowheads) are restriction fragments likely to represent the internal proviral *Kpn*I fragment from LC-FeLV (5.2 kb) or from FeLV (3.6 kb). Also indicated (squares) are 5' host-virus junction fragments in tumor J5-1 DNA. Molecular sizes were determined by comparison with the migration of *Hind*III-digested lambda DNA, *Taq*I-digested pUC18 DNA, and *Bgl*II-digested pUC18 DNA electrophoresed in parallel. (B) Restriction enzyme site maps of molecular clones representing the two horizontally acquired FeLV and four LC-FeLV proviral integrations in tumor J5-1 DNA. LC-FeLV integrations are designated by the presence of the *myc* oncogene within the provirus. The direction of transcription of proviral DNA is indicated (arrow). Symbols: open box, LTR; B, *Bam*HI; H, *Hind*III; K, *Kpn*I; R, *Eco*RI; SI, *Sst*I; Sa, *Sal*I; S, *Sma*I. Recognition sites for *Sal*I at both ends of each insert are contributed by the cloning vector. Indicated under each map are restriction fragments used to screen the collection of feline tumors for regions of common integration (see text).

proximal *Rsa*I site in flanking host DNA. These fragments were then circularized by ligation and digested with *Eco*RV, whose 6-bp recognition site occurs at position 118 in the FeLV LTR (42). PCR amplification was then performed with the indicated primers (Fig. 5A). Specifically, the primers used were 5'-CAGGGATCCGTAATTTCCATGCCTTG TG-3', complementary to positions 52 to 71 in the FeLV LTR (42), with the addition of a *Bam*HI site at the 5' end, and 5'-TAGGAATTCGTCTCCAGGCTCCCCAGTT-3', identical to positions 217 to 235 in the FeLV LTR (42), with the addition of an *Eco*RI site at the 5' end. These primers were selected because they meet three criteria. First, they anneal to appropriate positions in the LTR, with respect to the *Eco*RV and *Rsa*I sites, to allow for inverse PCR. Second, they are not significantly related to the LTR of endogenous FeLV (2). Third, the primer sequences are conserved within the LTRs of the multiple FeLV subtypes (2). Amplification of the template with these primers yields products which contain host DNA from the 5' host-virus junction fragment, flanked on both ends by LTR sequences. Thus, if rearrangement of the *fli-2* locus in multiple tumors results from

FIG. 3. Southern blot analysis of DNA from normal (N) and tumor (T) tissues of cats 1110 (A), 981-2 (B), 931 (C), 1037 (D), and 1100 (E). DNA samples (10 μ g) were digested with *Bam*HI (lanes A, B, and E) or *Hind*III (lanes C and D), electrophoresed in an agarose gel, and transferred to nitrocellulose. The resulting Southern blot was hybridized as described in the text to the 1.1-kb *Eco*RI fragment indicated in the restriction map of λ J525 (Fig. 2B). Molecular sizes were determined by comparison with the migration of *Hind*III-digested lambda DNA, *Taq*I-digested pUC18 DNA, and *Bgl*II-digested pUC18 DNA electrophoresed in parallel.

FIG. 4. Restriction enzyme site map of uninterrupted *flvi-2* molecularly cloned from tumor J5-1 DNA. Indicated over the map are the positions (vertical arrows) and transcriptional directions (horizontal arrows) of proviral insertions in *flvi-2* in six independent lymphomas (see text). Indicated under the map are restriction fragments used to screen the amplification products of inverse PCR (see text and Fig. 5). The map is incomplete with respect to recognition sites for the enzyme *PstI*. A single *PstI* site is indicated between fragments E and F because it was used in the preparation of those restriction fragments. Symbols: B, *Bam*HI; H, *Hind*III; K, *Kpn*I; R, *Eco*RI; SI, *Sst*I; Sa, *Sal*I.

proviral integration, then inverse PCR as described is predicted to yield an amplified product which hybridizes to probes for both *flvi-2* and the FeLV LTR. Further, since the amplified product contains DNA from only the 5' host-virus junction fragment, the orientation of integrated proviruses may be determined by the pattern of hybridization to *flvi-2* probes.

Inverse PCR as described above was performed on DNA from tumors containing an altered allele of the *flvi-2* locus. Inverse PCR products from the amplification of each tumor DNA were examined by slot-blot analysis, using as probes the eight restriction fragments isolated from *flvi-2* DNA (indicated as A to H in Fig. 4). The amplification products were also hybridized to a probe representing the U3 region of the FeLV LTR. This analysis confirms the position and direction of the provirus integrated at *flvi-2* in the DNA of tumor J5-1 (Fig. 5B). As predicted by the restriction enzyme site map (λJ₅25; Fig. 2B), inverse PCR yields an amplification product which hybridizes to the FeLV U3 probe and to *flvi-2* probes B, C, and D, but not to probes A, E, F, G, and H. The position and direction of other proviruses integrated at *flvi-2* were determined similarly, combining data from Southern blot analysis of tumor DNAs with that from inverse PCR. Regarding tumor 931, Southern blot analysis with the *flvi-2* fragments as probes localized the rearrangement within the 0.2-kb *Hind*III-*Eco*RI fragment indicated as probe C (data not shown). Inverse PCR of tumor 931 DNA yields an amplification product which hybridizes, as predicted, to probe C, but also to probes B and D as well as to FeLV U3. These data demonstrate that the tumor-specific alteration of *flvi-2* in tumor 931 results from FeLV proviral integration within fragment C and that the transcriptional orientation of the provirus is as indicated in Fig. 4. Regarding tumors 981-2 and 1110, Southern blot analysis with the *flvi-2* fragments as probes localized rearrangements in both tumors within the 1.0-kb *Bam*HI-*Eco*RI fragment designated as probe B, but outside the 0.2-kb probe C fragment (data not shown). The pattern of hybridization to amplification products of these tumors similarly demonstrated proviral integrations within fragment B, integrated in the same transcriptional orientation as tumors J5-1 and 931 (Fig. 4 and

FIG. 5. (A) Partial restriction enzyme site map of integrated FeLV proviral DNA indicating the positions of recognition sites for *Rsa*I and *Eco*RV used in preparing genomic DNA templates for inverse PCR (see text). Arrows indicate the positions and directions of oligonucleotide primers used in inverse PCR. (B) Slot-blot analysis of the amplified products of inverse PCR. Amplification products were denatured in 0.1 N NaOH for 20 min at room temperature, neutralized in 10 volumes of 6× SSC, and spotted immediately onto Nytran filters. Filters were hybridized to probes A to H, representing portions of the *flvi-2* locus as shown in Fig. 4. A filter was also hybridized to a probe representing the U3 portion of the FeLV LTR (lane I). The relatively intense hybridization signal to the U3 probe reflects the multiple copies of exogenously acquired proviruses in each tumor DNA, only one of which is integrated at *flvi-2*.

5B). Southern blot analysis of DNA from tumor 1037 demonstrated the rearrangement to occur within the 1.9-kb *Eco*RI-*Pst*I fragment designated as probe E (data not shown). The pattern of hybridization to amplification products of this tumor demonstrated proviral integration within fragment E, i.e., an amplified product was demonstrated to hybridize both to probe E and to FeLV U3. The observed hybridization to probes B, C, and D, however, suggests that the provirus is integrated in a transcriptional orientation opposite that of the other proviruses examined (Fig. 5B). Thus, the technique of inverse PCR, in combination with data from Southern blot analysis, demonstrates the presence and orientation of FeLV proviral DNA integrated at *flvi-2* in five of six tumors which contain structural alterations of the *flvi-2* locus.

Southern blot analysis of tumor and uninvolved tissues of cat 1100 demonstrated a tumor-specific rearrangement of *flvi-2* within the fragment designated in Fig. 4 as probe H (data not shown). Inverse PCR analysis of tumor 1100 DNA was performed as described above to determine whether the tumor-specific alteration of *flvi-2* represents proviral integration in the locus. Inverse PCR of tumor 1100 did not demonstrate a product which hybridized to *flvi-2* probes (Fig. 5B); thus, it is not clear that proviral integration is responsible for the tumor-specific alteration of *flvi-2* in cat 1100.

DISCUSSION

The FeLV family contains the only naturally occurring mammalian retroviruses known to have transduced *myc* as an oncogene. At least eight *myc*-containing strains of FeLV have been isolated, all from natural or experimentally induced T-cell lymphosarcomas of the thymus, and each of apparently independent origin in domestic cats (22, 27–29). Feline *v-myc* as represented in one of these, termed LC-FeLV, is identical to feline *c-myc* over that portion of the coding sequence which they share, except that it is expressed as a *gag-myc* fusion product (6, 41).

The tumorigenic capacity of LC-FeLV is restricted to the induction of T-cell lymphosarcoma of the thymus, the same tumor from which it was originally isolated (21, 22). This restriction is characteristic of other *myc*-containing FeLV strains as well (30). Thus, the acquisition of *v-myc* does not expand the spectrum of tumors induced by FeLV alone, since FeLV induces the same neoplasm after long latency (mean = 2 years [13, 16]). By comparison, the broad transforming potential of the *myc*-containing avian retroviruses, including MC29 and its relatives, is remarkable and is determined at least in part by the avian *v-myc* gene itself (12, 25). The failure of feline *v-myc* to expand the tumorigenic spectrum of FeLV may reflect the close sequence relatedness between feline *v-myc* and *c-myc* in all *myc*-containing strains of FeLV (6, 28, 41). Alternatively, the narrow tumorigenic spectrum of LC-FeLV and its relatives may reflect determinants encoded in the LTR. Certainly, enhancer sequences of the LTR are major determinants of tissue tropism in MuLVs (4, 39).

Previous studies with LC-FeLV or its *myc*-containing relatives demonstrate that infection may induce, but does not necessarily induce, thymic lymphosarcoma in the natural host. In the infected animals in which tumors occur, feline *v-myc* is directly implicated because (i) the tumors contain monoclonally integrated *myc*-containing proviruses, and (ii) the tumors occur significantly more rapidly than they do following infection with FeLV alone (21, 30). The low pathogenicity of LC-FeLV, i.e., its inability to induce tumors in some animals in whom its presence is demonstrated (21), indicates that feline *v-myc* may indeed not represent a proximal leukemogen. Rather, it may act as one of a set of cooperating events in multistep oncogenesis. We demonstrate here that a candidate for another such cooperating event is the insertional mutagenesis by proviral integration of a locus in feline DNA termed *flvi-2*. FeLV proviral integration was demonstrated to have occurred at *flvi-2* in three of four LC-FeLV-induced tumors examined (75%; tumors J5-1, 1110, and 981-2). Proviral insertions were localized within a 1.0-kb region (Fig. 4). The 1.0-kb domain represents approximately 3×10^{-7} of the cat genome, assuming the cat genome is comparable to other mammalian genomes in size (48). Considering that four LC-FeLV-induced tumors were examined which contain five proviral integrations on the average (Fig. 1), the probability for a single proviral insertion to have occurred in the 1.0-kb region by random chance can be estimated as $5 \times 4 \times (3 \times 10^{-7}) = 6 \times 10^{-6}$. Thus, the probability that three independent proviral insertions would have occurred within this region by random chance is $(6 \times 10^{-6})^3 = 6 \times 10^{-18}$. The repeated insertional mutagenesis of *flvi-2* by proviral integration is therefore not likely to reflect random chance. Rather, the findings indicate that integrations in the *flvi-2* region are selected in tumorigenesis and may cooperate with feline

v-myc in the induction of thymic lymphosarcoma in LC-FeLV-infected animals.

The function of *flvi-2* is not yet known. The proviruses integrated at *flvi-2* in LC-FeLV-induced tumors (J5-1, 981-2, 1110) are oriented in the same transcriptional direction with respect to the locus (Fig. 4). In other cases of insertional mutagenesis by mammalian retroviruses, the provirus is often integrated in an orientation consistent with transcriptional activation through the retroviral enhancer (19). A gene sequence within *flvi-2* would therefore be predicted to reside in the region of proviral integration flanking the 5' end of proviral DNA. A gene sequence in this region is actively being sought by using appropriate *flvi-2* probes in Northern (RNA) blot analysis of tumor RNA. *flvi-2* does not cross-hybridize with probes representing 10 retroviral oncogenes or 11 loci commonly interrupted by proviral integration in MuLV-induced tumors. In particular, molecularly cloned *flvi-2* does not cross-hybridize to a probe representing *pim-1*, nor is the feline *pim-1* locus tumor-specifically altered as examined by two restriction enzymes in Southern blot analysis (data not shown). This is significant because insertional mutagenesis of the murine *pim-1* locus is associated cooperatively with activation of the *myc* oncogene in the induction of T-cell lymphoma in mice (44).

The data presented here further implicate the structural alteration of *flvi-2* in three other naturally occurring FeLV-positive T-cell tumors examined (Fig. 3; tumors 931, 1037, and 1100). These tumors do not contain LC-FeLV, nor do they exhibit any alteration of the feline *c-myc* gene (data not shown). Proviral integration was demonstrated in tumor 931 to occur in the same transcriptional direction and within the same 1.0-kb fragment shown to harbor proviruses in LC-FeLV-induced tumors (Fig. 4). Proviral integration occurred within approximately 2 kb of that region in tumor 1037, although the transcriptional orientation of the provirus is opposite that of the other integration events examined (Fig. 4). In tumor 1100, a structural alteration of *flvi-2* is demonstrated by Southern blot analysis (Fig. 3), although we cannot be certain at this point that proviral integration occurred in the locus in this tumor (Fig. 5). Thus, these data indicate that interruption of *flvi-2* occurs repeatedly in natural and experimentally induced feline thymic lymphosarcomas, including those which do and those which do not involve the action of a feline *v-myc* oncogene.

Our findings implicate both feline *v-myc* and the insertional mutagenesis of *flvi-2* as members of the set of cooperating events responsible for thymic lymphomagenesis in the domestic cat. The fact that interruption of *flvi-2* occurs repeatedly, in the presence or absence of feline *v-myc*, suggests that insertion at *flvi-2* is an early and essential event in the multistep cascade. In the presence of *v-myc*, however, the latency for tumor induction is significantly reduced. An implication of this finding is that the action of *v-myc* represents a later event in the cascade and that those cells containing both *v-myc* and interrupted *flvi-2* are nearer to its completion.

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