flvi-1, a Common Integration Domain of Feline Leukemia Virus in Naturally Occurring Lymphomas of a Particular Type

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A locus in feline DNA, termed *flvi-1*, which may play an important role in the natural induction of lymphomas by feline leukemia virus (FeLV) was identified. Examination of a bank of 21 naturally occurring FeLV-positive feline lymphomas revealed that FeLV proviral integration occurs at *flvi-1* in four independent tumors (19%). Independent integrations occurred within a 2.4-kilobase region of *flvi-1*, the probability of which by random chance can be estimated as 10^{-16} . Several lines of evidence, including sequence analysis of the long terminal repeat, demonstrated that proviruses integrated at *flvi-1* are exogenously acquired and are oriented in the same transcriptional direction with respect to the locus. Molecularly cloned *flvi-1* did not hybridize with probes representing several previously described proviral integration domains or with probes representing 10 oncogenes. The natural feline lymphomas examined in this study were heterogeneous with respect to tissue of origin, cell type, and number of monoclonal proviral integrations. The four tumors in which *flvi-1* is interrupted were classified as members of a phenotypic subgroup containing seven lymphomas, i.e., at least four (57%) of seven lymphomas of this type contained FeLV proviral integration at *flvi-1*. Members of this phenotypic subgroup are non-T-cell lymphomas isolated from the spleen and contain an average of three proviruses, compared with an average of eight among all of the tumors examined. The small number of proviral integrations in tumors of this subgroup suggests that an early proviral integration event into *flvi-1* can induce malignant change.

Naturally occurring retroviruses of humans, other mammals, and birds represent causative agents of malignant, degenerative, and immunologic disorders in those species. The oncogenic potential of some retroviruses, i.e., acutely transforming retroviruses, is attributed to the viral oncogene, a sequence transduced from host chromosomal DNA. Most naturally occurring oncogenic retroviruses, however, lack genetic information to which their malignant potential can be directly attributed. These viruses induce disease chronically and after prolonged latency by mechanisms which are not well understood. Retroviral insertional mutagenesis is hypothesized as a mechanism by which proviral integration in or near a gene critical in normal cell growth or differentiation might perturb its structure or regulation, thereby leading to malignant change (25, 52). Proviral integration sites relevant to this hypothesis have been identified by using the strategy of transposon tagging. 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 an analysis demonstrates that both familiar proto-oncogenes and previously unidentified sequences are targets for insertional mutagenesis. In birds, for example, B-cell lymphoma or erythroblastosis is apparently induced by chance integration of avian leukosis virus near *c-myc* (20, 38) or *c-erbB* (16), respectively. Studies with rodents have identified retroviral integrations near *c-myc* in thymic lymphomas (9, 29, 43, 47) and near *c-myb* in Abelson virus-induced plasmacytoid lymphosarcomas (44). A number of previously unknown genetic loci have also been identified as common targets for proviral integration in retrovirally induced tumors of several

kinds. A significant number of rodent leukemias or lymphomas induced by murine leukemia viruses (MuLV) bear proviruses in loci termed *mlvi-1* (*pvt-1/mis-1* [24]), *mlvi-2*, *mlvi-3*, *mlvi-4*, *pim-1*, *fis-1*, *gin-1*, *fim-1*, *fim-2* (*c-fms*), *fim-3*, *evi-1*, *evi-2*, *spi-1*, and *dsi-1* (5, 7, 12, 33, 35, 45, 46, 49, 53, 54). Induction of mammary carcinoma by mouse mammary tumor virus is associated with proviral integration in chromosomal domains termed *int-1*, *int-2*, *int-3*, *int-41*, and *int-H* (17-19, 37, 39). The functional significance of some of the genetic loci implicated as targets for insertional mutagenesis is largely inferred on the basis of the frequency with which these loci appear as sites of proviral integration in tumors. In some cases, however, direct experimental evidence indicates the oncogenic potential of commonly integrated regions (34, 50, 51). Thus, studies of retroviral integration patterns in tumor DNA have not only addressed the mechanism of oncogenesis by slowly transforming retroviruses but also identified new proto-oncogenes in the mammalian genome.

Feline leukemia viruses (FeLV) are a family of slowly transforming C-type retroviruses responsible for the induction of a spectrum of neoplastic and degenerative lymphoid diseases in domestic cats. The lymphoid malignant diseases caused by FeLV are broad in spectrum and are referred to collectively as the leukemia-lymphosarcoma complex (22). Unlike the well-studied murine retroviruses, FeLV infects a naturally outbreeding population. Such a population, in contrast to strains of laboratory animals, provides enormous genetic heterogeneity as a substrate for proviral integration. The significance of this heterogeneity is illustrated by the relative promiscuity with which FeLV is known to interact with cellular proto-oncogenes. At least five such sequences were originally identified on the basis of their transduction by FeLV (*fes*, *fms*, *fgr*, *kit*, and *TP1*), and four sequences

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identified as oncogenes in other viruses also appear in strains of FeLV (*abl*, *sis*, *ras*, and *myc*) (4).

Although FeLV-mediated induction of the leukemia-lymphosarcoma complex represents a valuable model for natural carcinogenesis in an outbreeding population, little is known about the molecular mechanism by which FeLV effects malignant change. FeLV contains no genetic information which encodes this capacity, and it is hypothesized that oncogenesis results from an undefined interaction between viral and host genetic information. Insertional mutagenesis of *c-myc* appears to play a role in the induction of T-cell lymphoma by FeLV, since proviral integration within or near *c-myc* is observed with a frequency as high as 40% in such tumors (14, 32). The role of insertional mutagenesis in the induction of other members of the leukemia-lymphosarcoma complex is unknown.

The aims of the present investigation were (i) to determine whether FeLV-induced tumors of cats are associated with disruption by proviral integration of distinct cellular genes and (ii) to determine whether the loci identified as targets of insertional mutagenesis by MuLV in rodents interact similarly with FeLV in malignant induction in cats. We report here the identification and characterization of *flvi-1*, the only locus, other than *c-myc*, identified in feline DNA to harbor FeLV proviral DNA in multiple, independent, naturally occurring lymphomas. The interruption of *flvi-1* by FeLV proviral integration is apparently associated with induction of lymphoma of a particular phenotype.

MATERIALS AND METHODS

Collection of naturally occurring feline lymphomas. A collection of 24 naturally occurring feline lymphomas, histopathologically characterized and provided by Murray B. Gardner and James W. Casey (27), was obtained from random-source cats which ranged in age from 10 months to 12 years. Tumors were isolated from a variety of tissues, including thymus, spleen, and various lymph node tissues, and metastatic sites, including kidney and liver tissues. Various uninvolved tissues were collected from the same animals.

Preparation and analysis of high-molecular-weight DNA. High-molecular-weight DNA was prepared by homogenizing frozen tumor or normal tissue in extraction buffer containing 10 mM Tris hydrochloride (pH 8.1), 100 mM NaCl, 200 mM EDTA, and 0.5% sodium dodecyl sulfate. Proteinase K was added to a concentration of 200 µg/ml, and the mixture was incubated at 55°C for 1 h, followed by repeated extraction with phenol and chloroform. High-molecular-weight DNA was collected by ethanol precipitation and spooling on a glass rod.

For Southern blot analyses, DNA (10 µg) was digested with restriction enzymes, electrophoresed in a 0.8% agarose gel, transferred to nitrocellulose (30), and affixed by UV irradiation for 2 min (23). Filters 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)] buffer (pH 6.8) (Calbiochem-Behring), 0.1% sodium lauroyl sarcosine, 0.01 M EDTA, 0.2 mg of denatured calf thymus DNA per ml, and 5× Denhardt solution (30). Filters were then hybridized for 18 h at 37°C in the same solution containing 2 × 10⁶ cpm of probe DNA per ml radiolabeled to a specific activity of 1 × 10⁸ to 2 × 10⁸ cpm/µg by nick translation with [α-³²P]dCTP (800 Ci/mmol; Du Pont Co.). After being washed twice in 2× SSC (30)–0.1% sodium lauroyl sarcosine at 37°C for 30 min each time and twice in

0.1× SSC–0.1% sodium lauroyl sarcosine at 50°C (45°C when heterologous, i.e., nonfeline, probes were used) for 30 min each time, filters were exposed at room temperature or at –70°C for various times to Kodak XRP-5 film with intensifying screens.

Molecular cloning and nucleotide sequence analysis. Genomic DNA libraries were prepared in bacteriophage cloning vector λEMBL3 (15). High-molecular-weight DNA was partially digested with *Mbo*I and size fractionated through a gradient of 5 to 20% potassium acetate by velocity sedimentation (30). Fragments of 15 to 20 kilobases (kb) were selected and ligated with the cloning vector. Ligated DNA was packaged in vitro by using Gigapack packaging extract (Stratagene Cloning Systems) and plated on a lawn of *Escherichia coli* P2392 (Stratagene Cloning Systems). The library (1.4 × 10⁶ PFU) was screened without amplification by in situ plaque hybridization (2) by using probes radiolabeled by nick translation to a specific activity of 10⁸ cpm/µg with [α-³²P]dCTP (800 Ci/mmol; Du Pont Co.).

For nucleotide sequence analysis, restriction fragments of interest were inserted into bacteriophage cloning vector M13mp18 or M13mp19 (31). Single-stranded recombinant bacteriophage DNA was prepared, and dideoxynucleotide sequence analysis (41) was performed by using Sequenase (U.S. Biochemical) in the presence of [α-³⁵S]dATP (1,350 Ci/mmol; Du Pont Co.). Sequencing products were separated on 0.4-mm denaturing 6% polyacrylamide gels for various times. Gels were fixed, dried, and exposed to Kodak XRP-5 film at room temperature.

RESULTS

Integration patterns of FeLV proviruses in naturally occurring feline lymphomas. Implicit in the hypothesis of insertional mutagenesis as a mechanism of retroviral transformation is the monoclonality or oligoclonality of the tumor with respect to proviral integration sites. To examine the organization of exogenously acquired FeLV proviruses in the DNA of naturally occurring tumors, a bank of 21 FeLV-positive and 3 FeLV-negative histopathologically characterized, natural feline lymphomas and uninvolved tissues from the same animals was studied. The tumors were isolated from random-source animals which ranged in age from 10 months to 12 years and were identified from a variety of tissues including thymus, spleen, various lymph node tissues, and metastatic sites, including kidney and liver tissues. Thus, the collection of naturally occurring lymphomas represents a heterogeneous group characteristic of the spectrum of disease naturally induced by FeLV strains (21, 22). To study the organization of horizontally acquired FeLV proviruses in tumor DNA, Southern blot analysis was performed by using as probe a molecular clone of the U3 region of the long terminal repeat (LTR) of FeLV-B/Gardner Arnstein (27). This region has been demonstrated to be only weakly related to endogenous FeLV sequences in the feline genome (8). A typical FeLV provirus contains recognition sites for the restriction enzyme *Kpn*I at three internal sites and within the R region of the LTR (48; Fig. 1a). Thus, when hybridized to a Southern blot of *Kpn*I-digested tumor DNAs, the U3 probe recognizes two fragments from each provirus: (i) an internal fragment identical among proviruses of the same strain and (ii) a 5' host-virus junction fragment whose length depends on the position of the *Kpn*I site in the adjacent host DNA. If the tumor mass is monoclonal 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,

FIG. 1. (a) Partial restriction enzyme site map of integrated proviral DNA of FeLV-A/Glasgow (48). □, LTR; K, *KpnI*. (b) Southern blot analysis of DNAs from naturally occurring feline lymphomas of cats 1037 (lane A), 1100 (lane B), 1112 (lane C), 922 (lane D), 934 (lane E), 945 (lane F), and 1049 (lane G). Included in the analysis were thymic lymphosarcomas (lanes A and B), a lymphoma isolated from a mediastinal lymph node (lane C), and splenic lymphomas (lanes D to G). DNA samples (10 µg) were digested with *KpnI*, electrophoresed in an agarose gel, and transferred to nitrocellulose (30). 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 (27). *, Restriction fragments likely to represent the internal proviral *KpnI* fragment related to the U3 probe (see text). In lane E, the internal proviral *KpnI* fragment is not evident. Molecular sizes were determined by comparison with the migration of *HindIII*-digested lambda DNA, *TaqI*-digested pUC18 DNA, and *BglI*-digested pUC18 DNA electrophoresed in parallel. (c) Southern blot analysis of DNAs from tumor 945, a naturally occurring feline lymphosarcoma of the spleen (lane 1), an uninvolved kidney of the same animal (lane 2), and a kidney from an uninfected cat (lane 3). DNA samples (10 µg) were digested with *KpnI*, electrophoresed in an agarose gel, and transferred to nitrocellulose (30). 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 (27). Indicated are host-virus junction fragments in tumor 945 DNA of 13.0 and 0.84 kb and the internal proviral fragment of 5.3 kb (see text). Molecular sizes were determined by comparison with the migration of *HindIII*-digested lambda DNA, *TaqI*-digested pUC18 DNA, and *BglI*-digested pUC18 DNA electrophoresed in parallel.

then the host-virus junction fragments appear as discrete bands in Southern blot analysis and can be enumerated. Such an analysis demonstrated that the 21 FeLV-positive lymphomas contained monoclonally or oligoclonally integrated proviruses. The number of exogenous proviruses per cell ranged from 1 to 20 (average, 8) (portion of the data in Fig. 1b). Since this analysis demonstrates an internal proviral *KpnI* fragment, as well as host-virus junction fragments, the various sizes of internal fragments observed may be considered to represent the variety of strains of FeLV involved in induction of these tumors.

Isolation of exogenous FeLV proviruses and host flanking

sequences from a single lymphoma. The analysis of proviral integration patterns described above demonstrated that no particular host-virus junction fragment was shared among the tumors examined; thus, no specific site of integration was used consistently by FeLV proviral DNA (Fig. 1b). This does not preclude the possibility that a particular domain of cellular DNA is targeted consistently for proviral integration in tumors. Examination of this possibility requires molecular cloning of all of the horizontally 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. To pursue this strategy, a tumor, 945, which was shown by Southern blot analysis to contain only two horizontally acquired, monoclonally integrated proviruses per cell, was selected. Host-virus junction fragments of 13 and 0.84 kb represent those integration events (Fig. 1c). Other fragments in tumor 945 DNA which hybridized to the probe with various intensities represent either (i) the 5.3-kb internal proviral fragment or (ii) endogenous FeLV sequences, as determined by comparison with the pattern of hybridization to the same probe in DNA from uninvolved tissues of cat 945 and other animals (Fig. 1c).

A recombinant library of the DNA from tumor 945 was prepared in bacteriophage cloning vector λEMBL3 (15) and screened (2) by using as the probe the U3 region of the LTR of FeLV-B/Gardner-Arnstein (27). Eleven recombinants were isolated from the library. Restriction enzyme site mapping demonstrated that each of these recombinants contains an FeLV provirus and represents one of two different integration events. Restriction enzyme site mapping of a representative of each type, λ17 and λ13, demonstrated that the clones contain identical full-length proviruses 8.9 kb long (Fig. 2). Restriction site maps of the proviruses resemble that of the minimally pathogenic FeLV strain FeLV-A/Glasgow (48). Hybridization of λ13 DNA to a *HincII*-*HindIII* fragment specific for the envelope of subtype A FeLV (48) identified the provirus as a member of the subtype A group (data not shown).

Four lines of evidence indicate that λ17 and λ13 represent both of the horizontally acquired proviruses and the only horizontally acquired proviruses in tumor 945. (i) The internal, U3-containing *KpnI* fragment of both proviruses is 5.3 kb long. This is the size of the internal fragment observed on Southern blot analysis of 945 tumor DNA digested with *KpnI* when hybridized to the U3 probe (Fig. 1c). (ii) Digestion of the recombinants with *HincII* and hybridization to the U3 probe demonstrated that the LTRs present in both recombinants contain a recognition site for *HincII* within U3 (data not shown). The presence of this site distinguishes all known exogenous FeLV strains from endogenous species (3). (iii) None of the 11 recombinants isolated from the library represents any integration event other than these two. (iv) In Southern blot analysis of tumor 945 DNA, restriction fragments of host DNA immediately flanking the 5' end of each provirus identified the same restriction fragments identified by the FeLV U3 probe (data not shown).

Examination of the integration sites in the DNAs of other naturally occurring tumors. The insertional mutagenesis hypothesis suggests that a region of feline DNA interrupted by proviral integration in tumor 945 can serve as an integration site for proviral DNA in other naturally occurring feline lymphomas. To examine this possibility, restriction fragments containing unique cellular sequences immediately flanking the proviruses in clones λ13 and λ17 were identified for use as probes in Southern blot analysis of other tumors in

FIG. 2. Restriction enzyme site maps of molecular clones representing horizontally acquired FeLV proviral integrations in tumor 945. The direction of transcription of proviral DNA is indicated (arrows). □, LTR; B, *Bam*HI; H, *Hind*III; K, *Kpn*I; RI, *Eco*RI; SI, *Sst*I; SII, *Sst*II; Sa, *Sal*I; S, *Sma*I. Recognition sites for *Sal*I at both ends of each insert were contributed by the cloning vector. Indicated under each map are the restriction fragments used to screen the collection of feline lymphomas for regions of common integration (see text).

the collection. Three such fragments were identified and subcloned: (i) the 0.5-kb *Hind*III-*Eco*RI fragment flanking the 5' end of the provirus in λ17, (ii) the 0.7-kb *Hind*III fragment flanking the 3' end of the provirus in λ17, and (iii) the 0.8-kb *Sma*I-*Kpn*I fragment flanking the 5' end of the provirus in λ13 (Fig. 2). Southern blots of DNAs from the FeLV-positive and FeLV-negative tumors in the collection, as well as from uninvolved tissues of the same animals, were prepared after digestion with various restriction enzymes. Fragments flanking the 5' and 3' ends of the provirus in λ17 were hybridized to blots representing the DNA samples after digestion with either *Kpn*I or *Bam*HI. The 25-kb region identified by the probes was observed to be in germ line configuration in all of the tumors examined except 945 (data not shown). Thus, the domain of proviral integration represented in λ17 is not targeted for proviral integration in the other tumors examined.

The 0.8-kb *Sma*I-*Kpn*I fragment flanking the 5' end of the provirus in λ13 was hybridized to the DNA samples after digestion with *Eco*RI (portion of the data in Fig. 3). In *Eco*RI-digested DNA, the probe identified a 5.4-kb fragment representing the normal allele in all of the tissues examined. In tumor 945 DNA, an additional fragment of approximately 14 kb was observed, indicating insertion of a full-length FeLV provirus into one allele of the locus (Fig. 3, lane B). In the DNA of a splenic lymphoma from cat 934, the probe identified a fragment of 8.6 kb (Fig. 3, lane C) which was not observed in DNAs from normal tissues of the same animal (data not shown); thus, the 8.6-kb fragment represents a tumor-specific alteration of the locus in the DNA of tumor 934. Similarly, the probe identified tumor-specifically altered fragments of 8.5 and 14 kb in cats 1142 and 922, respectively, after digestion with *Eco*RI (Fig. 3, lanes D and E). Thus, four independent lymphomas were shown to contain tumor-specific alterations of the locus identified by the 0.8-kb probe. The hybridization signal representing the rearranged allele in each tumor was fainter than that representing the normal allele. This likely reflects dilution of the tumor DNA by surrounding unaffected tissue in the original samples. It may also suggest that the tumors are polyclonal with respect to the affected locus. The altered fragments in each tumor were also identified by the FeLV U3 probe (data not shown), an indication of proviral insertion in the locus. We have

termed this locus *flvi-1*, an acronym for feline leukemia virus integration site 1. Probes representing 10 retroviral oncogenes (*abl*, *fes*, *fms*, *mos*, *myb*, *myc*, *ras*, *rel*, *sis*, and *src*) and 10 previously identified common domains of retroviral integration in rodent lymphomas (*mlvi-1*, *mlvi-2*, *mlvi-3*, *mlvi-4* [49], *gin-1* [54], *pvt-1* [11], *fim-1* [46], *fim-3* [5], *pim-1* [12], and *fis-1* [45]) did not hybridize to the molecularly cloned *flvi-1* locus (data not shown).

Molecular cloning of interrupted and uninterrupted *flvi-1*. A recombinant library of tumor 934 DNA was prepared in bacteriophage cloning vector λEMBL3 (15) and screened (2) with the 0.8-kb *Sma*I-*Kpn*I fragment from λ13 (Fig. 2). Recombinants representing the interrupted and uninterrupted *flvi-1* alleles were thus isolated and distinguished on the basis of hybridization to the FeLV U3 probe (Fig. 4A and B). Restriction enzyme site mapping of a U3-hybridizing recombinant demonstrated that the altered *flvi-1* allele contains a deleted FeLV provirus 3.2 kb long. This observation is consistent with Southern blot analysis (Fig. 3, lane C), which demonstrated an anomalous 8.6-kb fragment in 934

FIG. 3. Southern blot analysis of DNAs from the normal spleen of cat 932 (lane A) and from splenic lymphomas of cats 945 (lane B), 934 (lane C), 1142 (lane D), and 922 (lane E). DNA samples (10 µg) were digested with *Eco*RI, electrophoresed in an agarose gel, and transferred to nitrocellulose (30). The resulting Southern blot was hybridized as described in the text to the 0.8-kb *Sma*I-*Kpn*I fragment flanking the 5' end of the provirus in clone λ13 (Fig. 2). 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.

FIG. 4. (A) Restriction enzyme site map of FeLV proviral integration in the *flvi-1* region in tumor 934. The direction of transcription of proviral DNA is indicated (arrow). □, LTR; B, *Bam*HI; H, *Hind*III; K, *Kpn*I; RI, *Eco*RI; SI, *Sst*I; SII, *Sst*II; Sa, *Sal*I; S, *Sma*I. (B) Restriction enzyme site map of uninterrupted *flvi-1* molecularly cloned from tumor 934 DNA. The map represents a composite of several overlapping bacteriophage recombinants selected from the library. Indicated over the map are the positions (vertical arrows) and transcriptional directions (horizontal arrows) of proviral insertions in *flvi-1* in four independent lymphomas (see text). Indicated under the map are restriction fragments used to screen DNAs from a collection of feline lymphomas for integrations at *flvi-1* (see text). B, *Bam*HI; H, *Hind*III; K, *Kpn*I; RI, *Eco*RI; SI, *Sst*I; SII, *Sst*II; Sa, *Sal*I; S, *Sma*I. (C) Restriction enzyme site maps of the points of insertion of FeLV proviruses in *flvi-1* in four independent feline lymphomas. The direction of transcription of the provirus is indicated (arrows). □, LTR; B, *Bam*HI; H, *Hind*III; K, *Kpn*I; SI, *Sst*I; SII, *Sst*II; S, *Sma*I.

tumor DNA (5.4-kb fragment from the unaltered allele plus a 3.2-kb insert). The proviruses inserted at *flvi-1* in tumors 934 and 945 were in the same transcriptional direction with respect to the locus (Fig. 4C).

To characterize the alterations of *flvi-1* in tumors 922 and 1142, Southern blot analysis of tumor DNA was performed by using the FeLV U3 probe and single-copy host DNA probes representing various parts of *flvi-1* (Fig. 4B). Restriction site mapping data generated by these analyses (Fig. 4C) demonstrated that tumor 922 contains a full-length FeLV provirus 8.9 kb long inserted at *flvi-1* in the same transcriptional direction with respect to the locus as the other insertions examined. Tumor 1142 contains a deleted FeLV provirus 3.0 kb long inserted at *flvi-1*. The transcriptional orientation of the provirus inserted at *flvi-1* in tumor 1142 was not determined because of its defective structure. Com-

TABLE 1. Classification of 21 FeLV-positive feline lymphomas into three phenotypic groups

Group	No. of tumors	Site ^a	Presence of T cells ^b	No. (range; avg) of proviruses ^c
1	4	Thymus	Yes	10–20; 15
2	7	Spleen	No	1–5; 3
3	10	Other	No	5–14; 9

^a Anatomic site from which lymphoma was isolated.

^b Determined by Southern blot analysis of lymphoma DNA hybridized to murine TCR β , a probe which detects somatic rearrangement of the TCR β gene in feline DNA (26).

^c Determined by Southern blot analysis of lymphoma DNA hybridized to the U3 region of the FeLV LTR and enumeration of host-virus junction fragments (see text).

parison of the relative positions of insertions within *flvi-1* demonstrated that the four independent integrations occurred within a domain of feline DNA 2.4 kb long (Fig. 4C).

Phenotype of lymphomas in which *flvi-1* is interrupted. The 21 FeLV-positive lymphomas examined in this study were classified phenotypically on the basis of several parameters, including the tissue of origin, tumor type, and number of integrated proviruses (Table 1). With respect to tumor type, FeLV is known to induce both T- and B-cell lymphomas (21, 22). To determine which of the lymphomas in our collection contained T cells, the organization of the gene which encodes the β chain of the T-cell receptor (TCR β) was examined. The somatic rearrangement of this gene in feline T cells may be detected by Southern blot analysis following digestion of DNA with *Eco*RI or *Sac*I and hybridization to a probe representing the constant region of murine TCR β (26). This analysis of our tumor bank revealed that only four of the FeLV-positive lymphomas contained T cells (data not shown). All of the lymphomas shown to contain T cells were thymic in origin and were shown by Southern blot analysis using the U3 probe to contain a relatively large number of proviruses per cell (range, 10 to 20; average, 15; portion of the data in Fig. 1b, lanes A and B). A second phenotypic group which contains seven lymphomas of splenic origin, none of which was demonstrated to contain T-cells, was identified. The seven splenic lymphomas were shown by Southern blot analysis to contain a relatively small number of proviruses per cell (range, 1 to 5; average, 3; portion of the data in Fig. 1b, lanes D to G). The remaining 10 tumors were isolated from sites other than thymus or spleen tissue and did not contain T cells. These tumors contained 5 to 14 proviruses per cell (average, 9; portion of the data in Fig. 1b, lane C). All of the lymphomas in which *flvi-1* was interrupted were shown to be members of the second phenotypic group as determined by the following three criteria: (i) all are splenic lymphomas, (ii) all contain a relatively low number of proviral integrations, and (iii) none contains T cells. Thus, proviral integration at *flvi-1* may be associated with induction of lymphoma of a particular type.

It is not known whether lymphomas with interrupted *flvi-1* contain B cells. Three probes known to demonstrate somatic rearrangement of immunoglobulin loci in murine B cells were used in Southern blot analysis of the collection of feline lymphomas: (i) a 2.0-kb *Bam*HI-*Eco*RI fragment of pJ₃J₄ (provided by Philip Tucker) containing the J₃ and J₄ regions of the immunoglobulin heavy-chain gene, (ii) a 0.8-kb *Xba*I-*Eco*RI fragment of p2-1 (56) containing sequences immediately 3' to the immunoglobulin heavy-chain gene J region cluster, and (iii) a 2.7-kb *Hind*III fragment of pHJ_K (28) containing the entire J region of the immunoglobulin locus. The probes identified apparently single-copy loci in cat DNA

under conditions of relatively low hybridization stringency; however, no gene rearrangement from the germ line patterns was observed after digestion of feline tumor DNAs with several restriction enzymes (data not shown). These observations may indicate that none of the tumors in the study contains mature B cells; however, since the feline immunoglobulin loci have not been cloned and characterized, it is not possible to draw this conclusion with confidence.

Origin of proviruses integrated at *flvi-1*. The feline genome contains endogenous FeLV proviruses, 8 to 10 copies per haploid genome, which are defective and are not known to encode virus particles. They may play a role in pathogenesis, however, particularly by serving as a substrate for recombination with somatically acquired species (3, 48). Endogenous FeLV proviruses may be distinguished from exogenous species on the basis of their structure and the sequence of the LTR (3). In particular, the U3 regions of endogenous and exogenous LTRs are divergent and cross-hybridize only weakly (8); thus, isolation of proviruses integrated at *flvi-1* by using the U3 region of exogenous FeLV-B/Gardner-Arnstein as the probe indicated that these proviruses were indeed exogenously acquired. To examine the origin of proviruses integrated at *flvi-1*, two analyses were performed. (i) The proviruses molecularly cloned from tumors 945 and 934 were shown by Southern blot analysis to contain a recognition site for the enzyme *HincII* within the U3 region of the LTR (data not shown). The *HincII* site occurs in LTRs from all of the exogenous and none of the endogenous FeLV sequences examined (3). (ii) The nucleotide sequence of the 5' LTR of the provirus integrated at *flvi-1* in tumor 934 was determined (Fig. 5). This analysis demonstrated that the 934 LTR is identical, aside from insertions, to that of a horizontally transmissible strain, FeLV-A/Glasgow (48), at 508 of 526 positions (96.5%). The 934 sequence was shown to contain the hallmarks of the LTR of exogenous FeLV strains, including a single copy of the region predicted to contain transcriptional enhancer elements (32). The 934 LTR lacks hallmarks which identify endogenous FeLV LTRs (3). The 934 LTR is different from other FeLV LTR sequences reported in that it contains triplication of a 21-base-pair sequence within U3, beginning 29 base pairs downstream of the predicted enhancer (Fig. 5).

Use in feline lymphomas of common retroviral integration sites identified in other mammalian systems. At least 14 loci in mouse and rat DNAs have been shown frequently to harbor proviral insertions in retrovirally induced lymphoid tumors in those animals (5, 7, 12, 33, 35, 45, 46, 49, 53, 54). To determine whether the loci involved in MuLV-induced lymphomas also serve as common domains of FeLV proviral insertion in natural feline lymphomas, molecular clones representing *mlvi-1*, *mlvi-2*, *mlvi-3*, *mlvi-4* (49), *gin-1* (54), *pvt-1* (11), *fin-1* (46), *fin-3* (5), *pim-1* (12), and *fis-1* (45) were used as hybridization probes in Southern blot analysis of DNA from the collection of FeLV-positive feline lymphomas and normal tissues of the same animals. Probes representing *mlvi-4* and *gin-1* did not cross-hybridize to feline DNA under the conditions of moderate hybridization stringency used (data not shown). Other probes identified single- or multiple-copy restriction fragments in feline DNA under conditions of moderate hybridization stringency. No tumor-specific rearrangements of any of the corresponding loci in feline DNA were observed after digestion with either of two different restriction enzymes (data not shown). Thus, the loci whose interruption plays a role in lymphomagenesis by murine retroviruses are apparently not involved frequently in oncogenesis by feline retroviruses. A similar analysis of

FIG. 5. Nucleotide sequence of the 5' LTR of the FeLV provirus integrated at *flvi-1* in tumor 934 compared with that of FeLV-A/Glasgow (48). Only residues of FeLV-A/Glasgow which differ are indicated. Indirect repeats that demarcate the termini of the LTR are underlined. The dashes included to optimize the alignment represent insertions in the 934 LTR sequence relative to that of FeLV-A/Glasgow (48). Predicted transcriptional regulatory and signal sequences are indicated, including the 67-base-pair region predicted to contain transcriptional enhancer elements (overline) (32), CCAAT (1, 6) and TATA (6, 10) boxes, and the polyadenylation signal (AATAAA) (40). Also indicated is a *HincII* recognition site within U3 which is characteristic of exogenous but not endogenous FeLV (3). The 934 LTR contains a 21-base-pair sequence which is repeated three times in tandem (brackets).

the organization of *c-myb*, *c-sis*, and *c-abl* proto-oncogenes demonstrated no tumor-specific rearrangements of those loci in feline DNA (data not shown). As reported previously (27), a T-cell lymphoma of a single animal, cat 1110, contains a tumor-specific alteration of the *c-myc* locus. A strain of FeLV which contains *myc* as an oncogene was isolated from this lymphoma and has been described elsewhere (26, 27).

DISCUSSION

Insertional mutagenesis by retroviral integration has been proposed as a mechanism of altering the expression of proto-oncogenes, or other genes important in normal growth regulation, during malignant change. In particular, the insertional mutagenesis hypothesis offers an explanation for the malignant potential of retroviruses which contain no viral oncogene (52). FeLVs, representatives of this type of retrovirus, induce a spectrum of neoplasms of hematopoietic origin in a naturally outbreeding population, domestic cats, by mechanisms which are not known. Evidence reported here indicates that insertional mutagenesis of a locus in feline DNA, termed *flvi-1*, may play a role in the natural induction of lymphoma by FeLV.

FeLV proviral integration was demonstrated to have occurred at *flvi-1* in four independent lymphomas within a 2.4-kb region (Fig. 3 and 4). The 2.4-kb domain represents approximately 6×10^{-7} of the cat genome, if the cat genome is comparable to other mammalian genomes in size (55). Since 21 tumors were examined which contain eight proviral integrations on the average, the probability for a single

proviral insertion to have occurred in the 2.4-kb region by random chance can be estimated as $8 \times 21 \times (6 \times 10^{-7}) = 10^{-4}$. Thus, the probability that four independent proviral insertions would have occurred within this region by random chance is $(10^{-4})^4 = 10^{-16}$. The repeated finding of FeLV proviral DNA in *flvi-1* is therefore not likely to reflect random chance but indicates that integrations in the *flvi-1* region are selected in tumor induction.

FeLV is known to induce a broad spectrum of diverse hematopoietic neoplasms (21, 22), and indeed, the collection of tumors examined here is heterogeneous (Table 1). Although 4 (19%) of 21 FeLV-positive lymphomas were shown to contain integration at *flvi-1*, all 4 may be classified phenotypically within a subgroup containing only 7 of the tumors. Thus, at least four (57%) of seven lymphomas of this type contain FeLV proviral integration at *flvi-1*. All tumors in this subgroup were originally isolated from the spleen, and no tumors in the collection other than these were identified in the spleen. Tumors in the subgroup do not contain T cells, but it is not known whether they contain B cells. Probes which demonstrated somatic rearrangement of immunoglobulin loci in murine DNA failed to demonstrate rearrangement of the loci in feline DNA digested with several restriction enzymes. However, since the feline immunoglobulin loci have not been molecularly cloned and characterized, it is possible that the restriction enzyme digests used were not appropriate for demonstration of somatic rearrangement. It is interesting that tumors with integrations at *flvi-1* contain a remarkably low number of exogenously acquired proviruses (range, 1 to 5; average, 3) compared with the entire collection (range, 1 to 20; average, 8). This suggests that integration at *flvi-1* is an early event in malignant change and that the entire cascade of events involved is small.

Identification of *flvi-1* as a frequently targeted domain of integration in lymphomas of a particular type suggests that *flvi-1* provides a function in target cells which, when disrupted, confers a selective growth advantage or otherwise contributes to malignant induction. In this respect, *flvi-1* may represent a new proto-oncogene. *flvi-1* did not cross-hybridize with probes representing 10 oncogenes or with probes representing loci commonly interrupted by proviral integration in lymphomagenesis by MuLV. The proviruses integrated at *flvi-1* are oriented in the same transcriptional direction with respect to the locus (Fig. 4C). In other cases of insertional mutagenesis described in mammals, the provirus is often integrated downstream of the gene in an orientation consistent with transcriptional activation through the retroviral enhancer (9, 13, 29, 36, 42, 43, 47). By analogy, a gene sequence within *flvi-1* would be predicted to reside upstream of the region of proviral integration. It is important to determine whether *flvi-1* encodes a gene product in tumor cells, but neither intact cells nor RNAs from the original collection of feline lymphomas examined in this study are available. Analysis of the expression of *flvi-1* in various feline tissues and cell lines of hematopoietic and other origins is ongoing.

The study reported here demonstrated that loci identified as frequently targeted domains for MuLV integration in rodent lymphomas are apparently not interrupted by FeLV integration in feline lymphomas. The significance to malignant induction of insertional mutagenesis of these regions may be related to the action of particular retroviruses or to the induction of particular tumor types. In view of the likelihood, however, that these regions contain sequences which influence cell growth or proliferation, the importance

of their identification transcends their role as targets for insertional mutagenesis by retroviruses.

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