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Recombinant Feline Leukemia Virus Genes Detected in Naturally Occurring Feline Lymphosarcomas

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Using a polymerase chain reaction strategy aimed at detecting recombinant feline leukemia virus (FeLV) genomes with 5′ *env* sequences originating from an endogenous source and 3′ *env* sequences resulting from FeLV subgroup A (FeLV-A), we detected recombinant proviruses in approximately three-fourths of naturally occurring thymic and alimentary feline lymphosarcomas (LSAs) and one-third of the multicentric LSAs from cats determined to be FeLV capsid antigen positive by immunofluorescence assay. In contrast, only 1 of 22 naturally arising FeLV-negative feline LSAs contained recombinant proviruses, and no recombinant *env* gene was detected in seven samples from normal tissues or tissues from FeLV-positive animals that died from other diseases. Four preferred structural motifs were identified in the recombinants; one is FeLV-B like (recognizing that FeLV-B itself is a product of recombination between FeLV-A and endogenous *env* genes), and three contain variable amounts of endogenous-like *env* gene before crossing over to FeLV-A-related sequences: (i) a combination of full-length and deleted *env* genes with recombination at sites in the middle of the surface glycoprotein (SU), (ii) the entire SU encoded by endogenous-like sequences, and (iii) the entire SU and approximately half of the transmembrane protein encoded by endogenous-like sequences. Additionally, three of the thymic tumors contained recombinant proviruses with mutations in the vicinity of the major neutralizing determinant for the SU protein. These molecular genetic analyses of the LSA DNAs correspond to our previous results in vitro and support the occurrence and association of viral recombinants and mutants in vivo in FeLV-induced leukemogenesis.

Feline leukemia viruses (FeLVs), naturally transmitted retroviruses, are common pathogens of the domestic cat population that frequently cause immunoproliferative, immunosuppressive, and degenerative disorders (7). Three exogenous FeLV subgroups, termed FeLV subgroup A (FeLV-A), FeLV-B, and FeLV-C, have been defined by viral interference and neutralization assays which detect genetic sequence variation in the viral surface glycoprotein (SU) gene (34, 35). FeLV-A is present in all naturally infected cats, while FeLV-B and -C occur at much lower rates and are always detected with FeLV-A. Additionally, the feline haploid genome contains approximately 15 endogenous elements highly related to FeLV, all of which are defective as a result of point or deletion mutations and therefore incapable of producing infectious viruses (38, 40). However, the long terminal repeats of these endogenous elements remain intact though negatively regulated by cellular flanking sequences (1), and the reverse transcriptase and integrase coding regions, as well as the entire *env* gene of at least one of these elements, remain open (11, 30). In fact, the endogenous *env* genes are expressed in specific embryonic as well as adult tissues (19a, 21, 24–26). It has been documented that FeLV-B is itself a recombinant between FeLV-A and endogenous elements (11, 29).

FeLV infection and development of viremia in cats are followed by a latency of 2 to 3 years before onset of neoplastic disease, the most prevalent being leukemia-lymphosarcoma complex (LSA) (7). The pathogenic mechanisms by which FeLV infection induces the development of LSAs largely remain to be elucidated, except that evidence indicating frequent activation of the c-*myc* proto-oncogene by either viral transduction or insertional mutagenesis has been obtained (19, 21). Still, less than 50% of LSAs in cats show these sorts of c-*myc* involvement (7). FeLV insertion is also detected in two other loci in feline DNA, termed *flvi-1* and *flvi-2* (12, 14). In murine leukemia virus-induced leukemia, the *env* gene has been shown to recombine with endogenous murine leukemia virus-related sequences, thus altering the cell tropism of the resultant recombinants. This recombination often precedes insertional mutagenesis of a large variety of proto-oncogenes (4, 8, 31, 41). By comparison, we have previously identified recombinant FeLVs arising under conditions of cotransfection of tissue culture cells with infectious exogenous and noninfectious endogenous proviruses (30, 37).

Epidemiological methods have been used to show association between individual exogenous FeLV subgroups (34, 35) and type of disease developed (7, 9); specifically, while FeLV-B is present in 33% of healthy infected cats, its prevalence in animals with LSA is 57%. In contrast, FeLV-A is present alone in 65% of healthy infected cats, and the prevalence of FeLV-A alone in animals developing LSA is 42%. These data were derived by isolating viruses from tumor tissues and performing interference assays to determine subgroup type by infecting feline tissue culture cells (10). The virus-infected cells were grown for many passages (more than 21 days). It has subsequently been shown that recombinants with cell tropism similar to that of FeLV-B could be generated by long-term passage of molecularly cloned FeLV-A in feline tissue culture cells (29) or by cotransfection of DNAs of FeLV-A and endogenous elements of known sequence into feline cells, followed by selection for recombinants in human cells in culture (37). In

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fact, it has been suggested that the true incidence of horizontally transmitted infections of FeLV-B in vivo may actually be lower than previously estimated, since it could be potentially generated during the in vitro assay process used to detect it (19). Similarly, the possibility exists that the pathogenic virus sequences present in tumor tissues were defective or were selected against under the assay conditions used, and therefore were not detected, in earlier experiments which involved a tedious process of virus isolation, virus propagation, and subgroup type assays.

To examine the prevalence of recombinant or FeLV-B genomes in feline tumors associated with FeLV infection, we designed a polymerase chain reaction (PCR) strategy for detection of FeLV *env* gene recombinants of the type that we previously identified by tissue culture experiments (37). We report here that recombinants between endogenous and exogenous *env* sequences are heterogeneous in nature and are present commonly in naturally occurring FeLV-associated feline LSAs. We also present evidence indicating that mutations exist in the vicinity of the major neutralizing epitope (5, 27) of the SU protein in some of the cases examined. Together, the results correspond closely to our previously observed findings regarding the generation of FeLV recombinants in tissue culture cells, indicate the potential genesis of recombinant and mutated FeLVs in vivo, and provide, for the first time, direct evidence, based on molecular genetic analysis, for the prevalence of FeLV-B or similar recombinant proviruses in naturally arising FeLV-induced LSAs.

MATERIALS AND METHODS

Tumor samples. Isolation of DNA from the naturally occurring LSA tissues, as well as the source of the LSAs and characterization of the affected cats as FeLV capsid antigen positive or negative on the basis of immunofluorescence assay (IFA), is described by Soe et al. (39) and Niman et al. (25). These naturally occurring LSAs included 25 from FeLV-positive and 22 from FeLV-negative tumors. An additional four LSAs from cats experimentally infected with the Rickard strain of FeLV-A were homogenized, and the DNA was extracted. Briefly, the cells in the homogenate were lysed in 1% Triton X-100–0.32 M sucrose–10 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$. Nuclei were isolated by centrifugation and incubated in 24 mM EDTA–75 mM NaCl–0.5% sodium dodecyl sulfate–0.2 mg of proteinase K per ml at 37°C overnight. Phenol and chloroform-isoamyl alcohol extractions were followed by precipitation of the DNA with sodium acetate and ethanol. DNA was spooled onto Pasteur pipettes and gently resuspended in 10 mM Tris (pH 8.0)–1 mM EDTA (pH 8.0).

PCR amplification. To detect the presence of recombinant proviruses in the tissue genomic DNA, primers that would amplify recombinants generated previously in tissue culture experiments were designed (37). The 5′ primers are specific for endogenous sequences and are oriented in the sense direction identical to the nucleotide positions indicated in the published CFE-6 sequence (11), as follows: PRB1 (600 to 619), RB53 (614 to 629), and RB56 (210 to 229). The 3′ primers, PRB2 (7878 to 7897), RB52 (7901 to 7917), and RB19 (7880 to 7900), are specific for exogenous (FeLV-A) sequences and are oriented in the antisense direction complementary to the positions indicated in the published FeLV-A (strain 61E) sequence (3).

To detect the presence of FeLV-A in the tumor samples, a strategy similar to the one outlined above was used. The 5′ primer, RB59, is specific for FeLV-A and is oriented in the sense direction identical to positions 6259 to 6278 of the published FeLV-A (strain 61E) sequence (3). The exogenous specific primers described above were used as the 3′ primer.

Additionally, to ensure that the DNA preparations were amplifiable, a conserved primer set, which would recognize and amplify the endogenous sequences present in the feline genomic DNA, was used as a positive control. This primer set was described previously as the 5′ primer pair (37).

Oligonucleotide labeling and hybridizations. Oligonucleotide probes specific for FeLV-A *env* sequences or endogenous *env* sequences were end labeled with T4 kinase and [^{32}P]ATP (17). The DNA was denatured in the dried gel and neutralized as described by Miyada and Wallace (17). Alternatively, the DNA was blotted onto GeneScreen Plus as specified by the manufacturer. The Miyada and Wallace protocol was also used for the oligonucleotide hybridizations at 42°C and washes beginning at room temperature, then at hybridization temperature, and increasing until the T_m was achieved (17). The probes used included those specific for FeLV-A *env* sequences (positions as published in reference 3; RB16 [complement of 6374 to 6404], RB17 [complement of 7310 to 7330], PRB2 [complement of 7878 to 7897] and RB19 [complement of 7880 to 7900]) and one specific for endogenous SU sequences (position as published in CFE-6 sequence in reference 11); RB13 [complement of 601 to 620]).

Cloning and sequencing. The 1.4-kb PCR products were directly cloned into a cloning vector (pCR1000 or pCRII [15]) (TA cloning kit; Invitrogen Corp.) as specified by the manufacturer. DNA for sequencing was prepared by using the Magic Minipreps DNA purification kit (Promega) according to the manufacturer's protocol. Double-stranded DNA sequencing (2) was accomplished by the dideoxy-chain termination method (33), using the M13 forward and reverse primer sites located in the polylinker region of the vector or oligonucleotides which hybridized to conserved sites internal to the insert. The sequences obtained were compared with the known *env* sequences of CFE-6 and FeLV-A (61E) (3, 11).

Statistical analysis. The GraphPAD InStat program was used to determine the *P* values for the chi-square analyses. Fisher's exact test was performed to determine the independence of the following: (i) the occurrence of recombinant proviral sequences in FeLV-positive tumors to that in normal tissues or tissues from other diseases occurring in FeLV-infected animals and (ii) the occurrence of recombinants between FeLV-positive and -negative LSAs. To test the correlation of the occurrence of recombinants between categories of LSAs from FeLV-positive cats, a chi-square analysis using the Yates correction factor was done.

RESULTS

Detection of proviral *env* genes in tumor tissues. The PCR strategy used to detect recombinant or FeLV-A *env* genes in the genomic DNA extracted from feline LSAs is illustrated in Fig. 1. This strategy was chosen on the basis of previous results determining the structure of recombinants generated in tissue culture experiments. To detect recombinant proviral *env* genes, a 5′ primer specific for endogenous sequences (PRB1, RB53, or RB56) was paired with a 3′ primer specific for FeLV-A sequences (PRB2, RB52, or RB19). Additionally, FeLV-A proviral *env* genes were detected with primers specific for that sequence, the 3′ primer being the same as those used to detect recombinants (PRB2, RB52, or RB19).

Results of hybridization following PCR amplification are

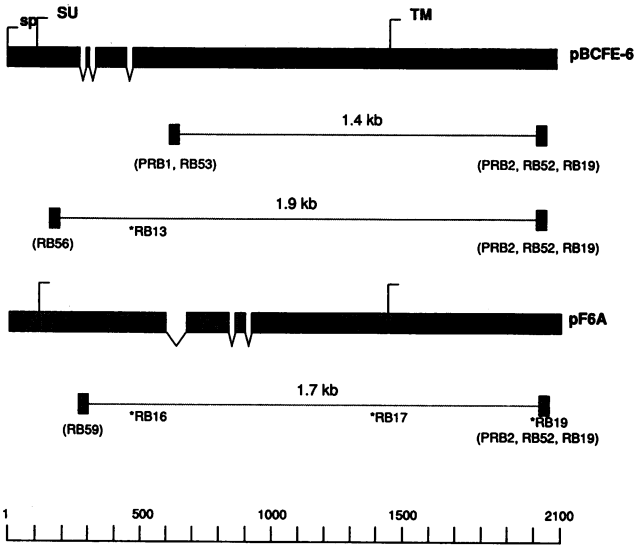

FIG. 1. PCR strategy used to detect recombinants and FeLV-A in tumor samples. The SU and TM sequences of pBCFE-6, a clone of an endogenous provirus (40), and pF6A, a clone of an FeLV-A (strain 61E) provirus (3), are illustrated. The positions of PCR primers, which are coded by the solid and hatched boxes to denote specificity, the positions of the hybridization probes used (labeled with asterisks), and the sizes of products expected are also indicated. The scale at the bottom reflects the nucleotide position numbering for the endogenous sequence as published in reference (11). sp, signal peptide.

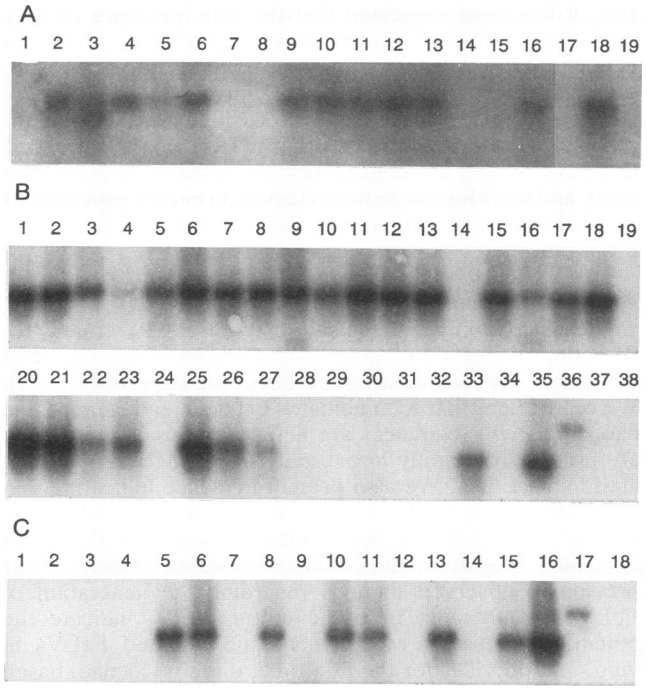

FIG. 2. Oligonucleotide hybridizations to PCR products amplified from tumor samples. (A) Recombinant proviruses amplified with RB52 and RB56 and hybridized to RB13, an endogenous specific probe. Shown are the 1.9-kb products generated by FeLV antigen-positive tumor samples P1, P2, P16, P3, P20, P4, P5, P6, P7, P8, P9, P10, P11, P21, P12, and P17 (lanes 1 to 16) as well as the controls using pF6A (an FeLV-A plasmid that does not amplify with the recombinant primer set), pBHM-1 (an FeLV-B plasmid that amplifies with recombinant primers), and H_2O (lanes 17 to 19). Note the additional deleted-size band generated from sample P16 (lane 3). (B) The 1.7-kb FeLV-A-specific products amplified from the FeLV-positive tumors with RB59 and PRB2 and hybridized to FeLV-A-specific probes RB16, RB17, and RB19. Depicted are FeLV-positive tumor samples P1, P2, P16, P3, P20, P4, P5, P6, P7, P8, P9, P10, P11, P21, P12, P17, P22, P23, P13, P24, P25, P18, P14, P15, and P19 (lanes 1 to 25); three liver samples taken from FeLV-positive animals with diseases other than LSA (feline infectious peritonitis [lane 26] and feline AIDS [lanes 27 and 28]); three normal spleen samples from FeLV-negative cats (lanes 29 to 31); two normal liver samples (lanes 32 and 34) and a normal spleen sample (lane 33) from FeLV-positive cats; and the controls using pF6A (FeLV-A plasmid), pBHM-1 (FeLV-B plasmid), genomic DNA from an uninfected H927 feline fibroblastic cell line, and H_2O (lanes 35 to 38). It should be noted that pBHM-1, as expected, did not yield the 1.7-kb product; however, an unexplained product of 3 to 4 kb was found by amplification as shown here and in panel C, lane 17. (C) Results of amplification with RB59 and PRB2 (FeLV-A specific) of FeLV-negative tumor samples and experimentally induced tumors (FeLV-A Rickard strain). Shown are FeLV-negative tumor samples N20, N11, N12, N4, and N13 (lanes 1 to 5); samples of six experimentally induced tumors (lanes 6 to 11), of which only four contained intact amplifiable DNA (lanes 6, 8, 10, and 11); normal spleen tissue from an FeLV-negative cat and an FeLV-positive cat (lanes 12 and 13); uninfected and FeLV-A-infected H927 feline fibroblastic cells (lanes 14 and 15); and controls using pF6A (FeLV-A plasmid), pBHM-1 (FeLV-B plasmid), and H_2O (lanes 16 to 18).

depicted in Fig. 2. Because primer annealing could be affected by single nucleotide variations, multiple sets of primer pairs, representing staggered locations within the region concerned, were used in various combinations, and the data presented in Tables 1 and 2 reflect the consensus of products amplified by at least one set of primers. The hybridization results shown in Fig. 2 illustrate only one primer set for each experiment illustrated, and thus products were not necessarily seen for every tumor that was determined to be positive by other primer sets. Figure 2A reveals the 1.9-kb recombinant products seen upon amplification of the FeLV-positive tumors. It was of interest that one of the FeLV-positive tumors contained not only a full-length *env* recombinant but also a deleted recombinant *env* gene approximately 300 bp shorter. (Fig. 2A, lane 3 [P16]). Figure 2B depicts the 1.7-kb products of amplification of the FeLV-positive tumors with an FeLV-A-specific primer pair. The controls shown in lanes 26 to 34 of Fig. 2B were also analyzed with recombinant-specific primer pairs, but no amplification products were detected; thus, they are not shown in Fig. 2A. Figure 2C illustrates the presence of FeLV-A sequences in FeLV-negative tumors or experimentally induced tumors described below.

Both groups of LSAs from FeLV-positive and -negative cats, as well as tissues from normal FeLV-positive or -negative animals or tissues from FeLV-positive cats that died from other diseases (in which LSAs did not develop), were thus tested for the presence of recombinant proviruses and FeLV-A. Tables 1 and 2 summarize the results of the FeLV-positive LSAs and normal or other diseased tissues, respectively. Among thymic LSAs determined to be FeLV positive, 11 of 15 contained recombinant proviruses, while 3 of 4 alimentary LSAs showed the presence of recombinants. In contrast, recombinants were detected in only two of six FeLV-positive multicentric LSAs. However, this result was not determined to be significantly different from those seen in other types of LSAs tested ($P = 0.2$).

TABLE 1. Detection of FeLV-A and recombinants in FeLV-positive tumors[a]

Tumor	LSA diagnosis	Hybridization[b]	FeLV-A[c]	Recombinant[c]
P1	Thymic	+	+	−
P2	Thymic	+	+	+
P3	Thymic	+	+	+
P4	Thymic	+	+	+
P5	Thymic	+	+	+
P6	Thymic	+	+	−
P7	Thymic	+	+	+
P8	Thymic	+	+	+
P9	Thymic	+	+	+
P10	Thymic	+	+	+
P11	Thymic	+	+	+
P12	Thymic	+	+	+
P13	Thymic	−	−	−
P14	Thymic	+	+	+
P15	Thymic	−	−	−
P16	Alimentary	+	+	+
P17	Alimentary	+	+	+
P18	Alimentary	+	+	+
P19	Alimentary	−	+	−
P20	Multicentric	−	+	+
P21	Multicentric	+	−	−
P22	Multicentric	+	+	−
P23	Multicentric	+	+	−
P24	Multicentric	+	+	+
P25	Multicentric	+	+	−

[a] Detection by IFA was positive in all cases.
[b] Presence of exogenous FeLV in the FeLV-positive tumors as detected by hybridization with the FeLV-A specific probes RB17 and PRB2 to *Eco*RI-cut genomic DNA (data not shown).
[c] Presence of proviral *env* genes as detected by PCR followed by hybridization.

However, in comparison with non-LSA tissues from FeLV-positive animals (Table 2), the presence of recombinants in LSAs was found to be significant ($P = 0.0034$). No recombinant *env* genes were found in four normal liver, spleen, or salivary gland samples from FeLV-infected cats, nor were they detected in three liver samples from animals diagnosed with feline infectious peritonitis or feline AIDS.

FeLV-A-specific *env* gene sequences were amplified in all but three of the tumors from FeLV-positive cats (samples P13, P15, and P21). Tumor P21, which contained exogenous FeLV by genomic DNA hybridization with RB17 and PRB2, amplified neither FeLV-A nor recombinant *env* sequences. It is possible that the regions to which our PCR primers would hybridize were mutated in these tumor samples or that those sequences were deleted, while in P21, at least one of the hybridization probe sites remained intact. Still, despite the use of multiple staggered PCR primers, no amplification products were detected with any of these three DNA samples.

TABLE 2. Detection of FeLV-A in FeLV-positive normal and other diseased tissues[a]

Tissue	Diagnosis[b]	FeLV-A
Liver	FIP	+
	FAIDS	+
	FAIDS	−
	Normal	−
	Normal	−
Spleen	Normal	+
Salivary gland	Normal	+

[a] IFA results were positive in all cases; no recombinant *env* gene was detected in any of the samples.
[b] FIP, feline infectious peritonitis; FAIDS, feline AIDS.

Among the FeLV-negative LSAs, only one (an alimentary LSA, tumor N11) of 22 showed the presence of recombinant proviruses, while FeLV-A sequences were not found in this tumor. Similarly, a single tumor (N13) of these 22 FeLV-negative LSAs showed the presence of FeLV-A but not recombinant *env* gene sequences. Thus, only two samples of 22 FeLV-negative LSAs contained either an FeLV-A or a recombinant provirus.

In addition to naturally occurring tumors, experimentally induced tumors were tested. Animals experimentally infected with a FeLV-Rickard preparation (FeLV-A by interference assay) that developed LSAs were studied. Four such LSAs, all shown to contain FeLV-A-specific *env* gene sequences in their DNA (Fig. 2B), lacked the presence of recombinant proviruses which could be detected by our PCR strategy (data not shown).

DNA sequences of recombinant proviral *env* genes detected in tumor tissues. The PCR products generated to detect recombinant *env* genes were cloned directly into the TA cloning vector and sequenced by priming with the M13 universal forward and reverse primers or oligonucleotides representing conserved sites internal to the insert. The portions of the sequences derived were compared with known FeLV sequences of CFE-6 and FeLV-A strain 61E (3, 11) and alterations from the known sequences were noted; the results are presented in Fig. 3, which shows the regions sequenced as representative of the sequence (CFE-6 or FeLV-A strain 61E) from which they demonstrated the fewest alterations. The sequences of some of the recombinant proviral *env* genes were similar to those of the GA and ST strains of FeLV-B (28), such as FeLV-positive tumors P4, P5, P8, P9, P10, P12, P14, P17, P18, P20, and P24 and FeLV-negative tumor N11. Since FeLV-B (strain GA), for which the complete *env* gene sequence is available (28), shares a 95% nucleotide sequence homology with positions 600 to 1100 of CFE-6 (11), this region was considered to be endogenous-like in these samples. The same region of FeLV-B strain GA, when compared with FeLV-A strain 61E sequences (3), demonstrates only 82% homology. Downstream of this region in the SU sequence and the transmembrane (TM) coding region, FeLV-B strain GA diverges to only 81% homology with the CFE-6 nucleotide sequence but shares 99% homology with FeLV-A strain 61E sequences; thus, these regions were determined to be FeLV-A related. Other sequences derived from the tumor amplification products, such as FeLV-positive tumors P2, P3, P7, P11, and P16, showed structures distinct from that of FeLV-B (GA or ST strain), as judged by the presence of endogenous-like sequences in the downstream regions of SU and/or TM or by the presence of FeLV-A-like sequences in the middle of SU. The defect detected in the deleted recombinant (tumor P16) was delineated and is also shown in Fig. 3. It was interesting that the site of crossover and deletion in this provirus in sample P16 occurred at a 9-bp sequence repeated approximately 300 bp apart. The 9-bp repeat is represented only one time in the deleted genome, resulting in a loss of 287 nucleotides and leaving the resultant gene out of its original reading frame. Thus, a stop codon occurs in the new reading frame, 11 codons from the endpoint of the deletion. This provirus, if expressed, would generate a truncated SU protein of 204 amino acids (not including the signal peptide), the final 11 amino acids being encoded by the out-of-frame sequence and thus differing from those encoded by other

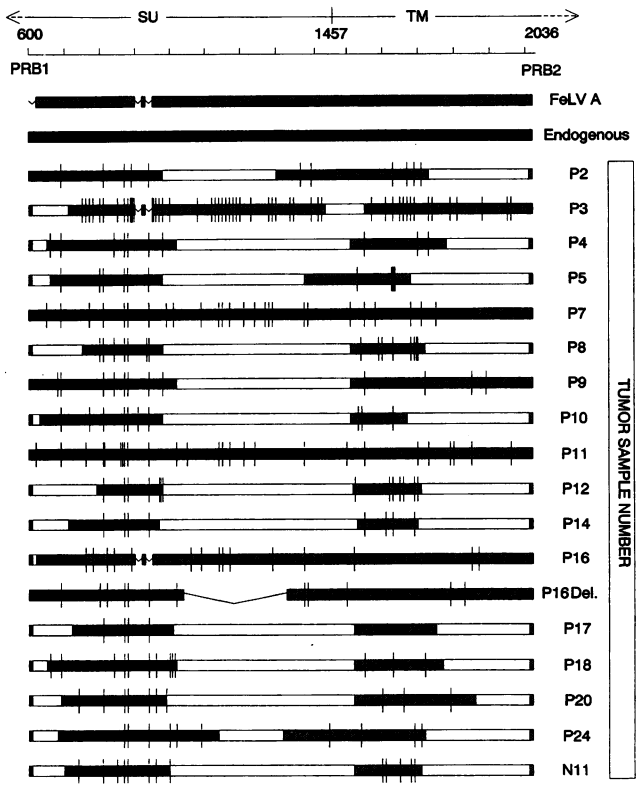

FIG. 3. Sequences of recombinant proviral PCR products amplified from tumor samples. Sequences of portions of the 1.4-kb PCR products amplified with PRB1 and PRB2 (or RB52 and RB53) are shown. The scale shown at the top delineates which portions of the SU and TM genes are illustrated, using the position numbering derived from the CFE-6 sequence (11). FeLV-A-related sequences are indicated by cross-hatching; endogenous-like sequences are depicted by solid black boxes (the white boxes designate regions not sequenced). The lines through the boxes symbolize nucleotide changes between the sequence indicated by hatching and the sequence derived for that clone. The V-shaped lines connecting boxes represent deletions relative to the endogenous sequence. The tumor sample numbers correspond to the FeLV-positive tumors described in Table 1 (P16Del. represents the deleted provirus cloned from PCR amplification of tumor sample P16), with the exception of the sequence marked N11, which is described in Table 2 (FeLV-negative tumor sample N11).

TABLE 3. Sequences in the vicinity of the pentapeptide epitope

	Sequence[a]
FeLV-A	GCA ATG GGA CCA AAC CTA GTC TTA
	Ala *Met Gly Pro Asn Leu* Val Leu
Endogenous	
CFE-6	GCc ATG GGA CCA AAt CcA GTC cTg
	Ala *Met Gly Pro Asn* **Pro** Val Leu
CFE-16	GCc ATG GGA CCA gAt CcA GTC cTg
	Ala *Met Gly Pro Asp* **Pro** Val Leu
FeLV-B	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P2	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P3	GCg ATG GGA CCA AAC CTA GTC TTA
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P4	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P5	Gtc ATG GGA CCA AAt CTA GTC cTg
	Val *Met Gly Pro Asn Leu* Val Leu
Tumor P7	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P8	GCc ATa GGA CCA AAt CTA GTC cTg
	Ala ***Ile*** *Gly Pro Asn Leu* Val Leu
Tumor P9	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P10	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P11	GCc ATG GGA CCA AAt CTA tTC cTg
	Ala *Met Gly Pro Asn Leu* **Phe** Leu
Tumor P12	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P14	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P16	GCA ATG GGA CCA AAC CTA GTC TTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P16 defective	GCc ATG GGA CCA AAt CTA GTg cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P17	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P18	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P20	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor P24	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu
Tumor N11	GCc ATG GGA CCA AAt CTA GTC cTg
	Ala *Met Gly Pro Asn Leu* Val Leu

[a] The pentapeptide epitope is shown in italics; the mutations in relation to the FeLV-A sequence are indicated by lowercase letters and boldface.

FeLV genomes. The generation of this provirus may be the result of illegitimate homologous recombination due to misalignment of the 9-bp repeat.

Because of the large number of mutations in comparison with the CFE-6 or FeLV-A strain 61E sequences (3, 11) detected in the tumor proviral sequences, the changes were examined to determine whether any led to nonsense mutations. In two cases, nonsense mutations were observed; however, other clones for those same tumor samples did not show the nonsense alterations. Therefore, we believe that with the exception of the deleted provirus from tumor P16, each tumor contains recombinant proviral *env* genes with intact open reading frames. However, since (i) the first ~600 bp of the SU coding region were not examined in our studies and (ii) not all samples were sequenced in their entirety, this observation of intact open reading frames cannot be asserted to indicate the ability of the proviruses present in the tumor samples to express functional *env* proteins.

Mutations detected in the vicinity of the pentapeptide MND. The major neutralization determinant (MND) for FeLV-A was a focus for sequence determination, given our previous results showing the presence of an endogenous sequence or mutated sequences encoding the pentapeptide, MGPNL. In three tumors, P5, P8, and P11, mutations altering the deduced amino acid sequence were detected in the vicinity of the epitope. These sequences are detailed in Table 3. Silent mutations were seen in other samples, including tumor samples P3, P16 full-length product, and P16 deleted product, indicating that this region may be a mutational hot spot as was indicated by the previous in vitro recombination results (37). The mutation seen in tumor P8, altering the methionine to isoleucine, was, in fact, identical to one identified in our in vitro-generated recombinants which were only weakly neutralized by a monoclonal antibody recognizing this epitope (37). The mutations in tumor samples P5 and

P11 involved the codons immediately adjacent to the pentapeptide epitope, sample P5 showing an alteration of the amino-terminal alanine to valine and sample P11 showing a change in the carboxy-terminal valine to phenylalanine.

DISCUSSION

The mechanisms of leukemogenesis induced by FeLVs largely remain to be clarified. In the effort to define proximal leukemogens, we examined, by molecular techniques, the FeLV proviral sequences in naturally arising feline LSAs. On the basis of previous in vitro studies in which we determined the structure of recombinant FeLVs (5′ SU sequences stemming from an endogenous source and 3′ SU sequences resulting from FeLV-A [37]), we developed a PCR amplification strategy to detect similar recombinants in genomic DNA from 25 FeLV-positive (by IFA) LSAs. Our results indicate that 11 of 15 thymic LSAs (73%), 3 of 4 alimentary LSAs (75%), and 2 of 6 multicentric LSAs (33%) contained recombinants with structure similar to that we previously defined. The differences in prevalence of recombinants between categories of LSAs were not determined to be significantly different ($P = 0.2$). In contrast, recombinant proviruses were not detected in any normal tissue or tissue from other diseases taken from FeLV-positive animals ($P = 0.0034$). FeLV recombinants also were not detected in LSAs arising in FeLV-negative animals, with the exception of 1 case in 22 ($P = 0.00002$). Similarly, only one of these tumors harbored FeLV-A DNA. This finding may suggest that animals that have no apparent current FeLV infection, as a result of either immunological clearance or lack of exposure to virus, also generally lack parental or recombinant proviruses in the LSAs which developed subsequently. These data appear to differ from the view that FeLV is also involved in many FeLV-negative LSAs (7), although a hit-and-run mechanism involving past FeLV infection cannot be ruled out. Thus, it seems likely that in animals with immunologically cleared infections or in unexposed animals, the mechanisms of leukemogenesis differ from those in persistently infected animals. Additionally, in experimentally induced lymphomas from animals infected by the Rickard strain of FeLV-A, an uncloned strain, recombinants of the types detected by this strategy do not appear to be involved. Experimentally induced tumors caused by the Rickard strain predominately activate c-*myc* by insertional mutagenesis, while less than 50% of naturally arising lymphomas are associated with c-*myc* activation, and these cases involve oncogene transduction as well as insertional mutagenesis (6, 7, 13, 16, 18–20, 22, 32). Also, in two tumors arising in experiments with cloned FeLV-A strains, the involvement of c-*myc* was not detected (19). Thus, the prevalence of pathogenic mechanisms seen in experiments with the Rickard strain does not seem to be representative of those involved with natural infections (21), and our data are consistent with this earlier observation.

With regard to the nature of the recombinants detected in the natural LSAs, our sequence data demonstrated involvement of only a small number of different sequence motifs, as illustrated in Fig. 4. The structural motifs of these recombinants, based on nucleotide sequence comparisons, include a structure similar to FeLV-B and three others which differ from FeLV-B and contain various amounts of endogenous-like *env* sequences before crossing over to FeLV-A-like sequences. We could not determine by the techniques used whether these proviruses were transmitted to the cat upon infection or were generated subsequent to viremia.

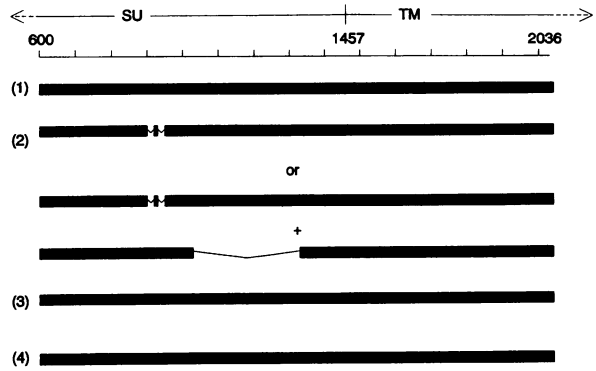

FIG. 4. Classes of *env* gene recombinants detected in tumor samples. The sequences illustrated in Fig. 3 are represented as structural motifs. The scale is as described in the legend to Fig. 3. (1) Tumors showing this structure are highly related to known FeLV-B sequences (as a result of the accumulation of mutations during the evolution of FeLV-B strains, the crossover point from endogenous to FeLV-A is not clearly delineated despite being represented here as a sharp demarcation). (2) These tumor samples containing either full-length or both full-length and deleted genomes show crossover sites, in the full-length proviruses, from endogenous to FeLV-A-related sequences upstream of the pentapeptide epitope, with second and third crossover sites further downstream in the middle of the SU coding region. Likewise, the crossover site from endogenous to FeLV-A-like sequences in the deleted genome is in the middle of SU, with the deletion occurring at the site of crossover, which corresponds to a 9-bp repeat located approximately 300 bp apart (only one copy of this 9-bp sequence is present in the deleted genome). (3) The tumor displaying this motif contains the entire SU region from endogenous-like sequences, while the TM region is from FeLV-A-related sequences. (4) Tumors demonstrating this structure include the entire SU region and approximately half of the TM region from endogenous-like sequences, before crossing over to FeLV-A-like sequences in the final half of the TM region.

However, these molecular methods confirmed the statistical occurrence of strains of FeLV-B (which are in fact recombinants between FeLV-A and endogenous proviruses), determined by virological methods in earlier work by other researchers (7, 9). As the occurrence of FeLV-B in animals developing LSAs (57%) has been observed to be much higher than that in healthy infected cats (33%), it is possible that at least a portion of these recombinants, seen in LSAs, developed by recombination with endogenous elements after initial infection with FeLV-A. In fact, a necessity for this recombinational event could help to explain the long latency seen prior to development of disease. Alternatively, this greater association with disease may reflect a higher probability for FeLV-B or similar recombinants to induce leukemia, perhaps by expanded cell tropism that increases the probability of infection of an appropriate target cell susceptible to leukemia development (7, 36). It was interesting to note that in every FeLV-positive tumor containing recombinant *env* genes, FeLV-A sequences were also detected. Possibly, the increased number of genetic events associated with infection by more than one FeLV subgroup increases the risk of appropriate targets for leukemogenesis, e.g., proto-oncogenes or tumor suppressor genes, being hit. Interference to superinfection would decrease this probability in infections with FeLV-A alone but would not eliminate the risk. Thus, the lower prevalence of FeLV-A alone in naturally arising LSAs might be explained (7, 9).

In addition to the recombinant structure detected by PCR amplification from LSA genomic DNA, sequence determination elucidated the presence of mutations in the vicinity of a pentapeptide of the SU gene known to be the MND for FeLV-A and one of the MNDs for FeLV-B and -C (5, 27). We found mutations affecting the region encompassing this pentapeptide in proviruses of three thymic LSAs. Our previous results and the work of others show that mutations in this region allow the altered viruses to escape neutralization by a monoclonal antibody directed against this pentapeptide epitope (23, 37). At a concentration of monoclonal antibody that completely neutralized infection by FeLV-A, 70% of viral yield was retained by a population of viruses containing mutations in this epitope (37). Thus, it is reasonable to believe that these mutations may allow the viruses containing them to evade the host immune response and infect the animal persistently. Persistently infected cats are known to be at higher risk for disease development than are those that can clear the viral infection (7).

The data presented in this study show that recombinant proviruses were detected in a majority of thymic and alimentary LSAs and in one-third of multicentric LSAs which developed naturally in FeLV-infected cats. In contrast, only 1 of 22 FeLV antigen-negative LSAs contained recombinants. These data, obtained by direct molecular genetic analysis of the tumor DNAs, may indicate an important role of recombinant FeLVs in leukemogenesis at thymic and alimentary sites in animals with persistent FeLV infection. This finding confirms earlier data generated by virological experiments for a higher prevalence of FeLV-B, a recombinant, in cats developing LSA than in healthy infected cats. The recombinants fall into four sequence-structure motifs containing increasing amounts of the *env* gene encoded by endogenous sources (depicted in Fig. 4). Three of the tumors contained proviruses with a mutation in the vicinity of the major neutralizing pentapeptide epitope. It will now be important to construct recombinant or mutant FeLVs mimicking the proviruses detected in the LSAs to examine the potential role of individual *env* variants in the induction of neoplasia in experimental animals.

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