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Genetic Determinants of Feline Leukemia Virus-Induced Lymphoid Tumors: Patterns of Proviral Insertion and Gene Rearrangement

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The genetic basis of feline leukemia virus (FeLV)-induced lymphoma was investigated in a series of 63 lymphoid tumors and tumor cell lines of presumptive T-cell origin. These were examined for virus-induced rearrangements of the c-*myc*, *flvi-2* (*bmi-1*), *fit-1*, and *pim-1* loci, for T-cell receptor (TCR) gene rearrangements, and for the presence of *env* recombinant FeLV (FeLV-B). The *myc* locus was most frequently affected in naturally occurring lymphomas (32%; $n = 38$) either by transduction (21%) or by proviral insertion (11%). Proviral insertions were also common at *flvi-2* (24%). The two other loci were occupied in a smaller number of the naturally occurring tumors (*fit-1*, 8%; *pim-1*, 5%). Examination of the entire set of tumors showed that significant numbers were affected at two (19%) or three (5%) of the loci. Occupation of the *fit-1* locus was observed most frequently in tumors induced by FeLV-*myc* strains, while *flvi-2* insertions occurred with similar frequency in the presence or absence of obvious c-*myc* activation. These results suggest a hierarchy of mutational events in the genesis of feline T-cell lymphomas by FeLV and implicate insertion at *fit-1* as a late progression step. The strongest links observed were with T-cell development, as monitored by rearrangement status of the TCR β-chain gene, which was positively associated with activation of *myc* ($P < 0.001$), and with proviral insertion at *flvi-2* ($P = 0.02$). This analysis also revealed a genetically distinct subset of thymic lymphomas with unrearranged TCR β-chain genes in which the known target loci were involved very infrequently. The presence of *env* recombinant FeLV (FeLV-B) showed a negative correlation with proviral insertion at *fit-1*, possibly due to the rapid onset of these tumors. These results shed further light on the multistep process of FeLV leukemogenesis and the relationships between lymphoid cell maturation and susceptibility to FeLV transformation.

Feline leukemia virus (FeLV) is a widespread, naturally occurring pathogen of the domestic cat population. The virus is associated with a range of neoplastic diseases in the field, the most common form of which is lymphosarcoma of T-cell origin. Early studies of these tumors showed that the c-*myc* gene was frequently affected by FeLV insertion or transduction (23, 31, 33). The c-*myc* gene encodes a nuclear phosphoprotein belonging to a family of transcription factors which have similar basic helix-loop-helix domains and related DNA-binding motifs (2, 6, 27). The expression of c-*myc* is a feature of proliferating cells of many lineages, and the loss of normal control of its expression predisposes to tumors of correspondingly diverse tissue origin, including a high proportion of lymphoid neoplasms (27).

The significant causal role of *myc* in feline thymic lymphosarcomas was demonstrated by the capacity of the *myc*-transducing viruses to reproduce the disease very rapidly (12 to 14 weeks) after inoculation into neonatal cats (22, 34). However, further examination revealed that even the short-latency tumors induced by FeLV-*myc* recombinant viruses were of clonal origin and had phenotypic features which could not be reproduced by infection of T cells in vitro (34). These observations led us and others to screen such tumors for genes which could collaborate with *myc* in the leukemogenic process.

By examining the sites of proviral insertion in tumors induced by FeLV-*myc* viruses, two loci of common insertion were identified. The first of these loci was *flvi-2* (24), which subsequently proved to be the feline homolog of *bmi-1* (25), a *myc*-collaborating gene previously identified as a common proviral insertion site in B-cell lymphomas of *myc* transgenic mice (Eμ-*myc*) infected with Moloney murine leukemia virus (MuLV) (49). The *bmi-1* gene product is a representative of a novel nuclear protein family with characteristic helix-turn and zinc finger motifs (47). Mice overexpressing *bmi-1* are also predisposed to lymphomas, while cross-breeding with Eμ-*myc* mice showed a synergistic effect, with tumors developing with higher frequency and after a shorter latent period (12). The second common FeLV insertion locus to be identified in FeLV-*myc* tumors was *fit-1* (45). This locus was mapped to feline chromosome B2, but its coding potential is as yet unknown. The feline homolog of another known *myc*-collaborating gene (*pim-1*) also maps to chromosome B2, but RNA analysis showed no evidence of long-range activation of *pim-1* in tumors with insertions at *fit-1* (45). In the present study, a new feline *pim-1* probe was generated and used to show that *pim-1* is itself a common insertion locus for FeLV which is occupied in a small number of field case and FeLV-*myc*-induced lymphomas.

Subgroup B FeLVs are generated from the common, horizontally transmissible, subgroup A form by recombination with endogenous proviral elements from which they acquire the 5′ portion of *env* (21, 42). This process is reminiscent of the generation of murine mink cell focus-forming (MCF) viruses

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from ecotropic MuLV (20). Furthermore, the endogenously derived *env* gene components of FeLV-B and MCF viruses show weak amino acid sequence homology (5). It has therefore been considered that FeLV-B might play a role as a proximal leukemogen in cats analogous to the role of MCF viruses in mice (39). Moreover, epidemiological surveys have shown that cats with lymphomas or leukemias are more likely to carry FeLV-B than are asymptomatic FeLV-infected animals (16). A recent study showed a very high prevalence of FeLV-B in tumor-bearing cats (39), but since a PCR method was used, this would not readily distinguish between FeLV-B as an element in tumor DNA or as a minor contaminant arising from tumor infiltrates or adjacent tissues. In the present study, we reexamined this issue by blot hybridization of tumor DNA, using a subgroup B-specific probe (42).

The principal aim of this study was to draw together and integrate diverse observations from a number of independent studies on the genetics of FeLV-induced lymphomas. With a similar aim, a recent study examined 21 thymic and multicentric lymphosarcomas for insertions at c-*myc*, *flvi-2*, and N-*myc* (26). In the present study, we have examined a larger series ($n = 63$) for alterations of four insertion loci and tested these for association with each other, with a standard marker of T-cell maturation (rearrangement of T-cell receptor β chain [TCRβ]), and with the presence of *env* gene recombinant FeLV-B. We find evidence for the involvement of all four host genetic loci in naturally occurring lymphomas and a very strong association between the differentiation state of the T-cell tumors and the activation of two of the cellular gene targets.

MATERIALS AND METHODS

Selection of tumor cases. A large number of tumors and cell lines have been analyzed previously by one or two of the following criteria: activation of c-*myc* (23, 31, 33, 34, 44), occupation of the *flvi-2* locus (24, 26), and occupation of the *fit-1* locus (45). To this collection of tumors, a further series of 18 field case lymphomas was added. Although FeLV infection is associated with a wide range of malignant diseases (11, 15), the present analysis was restricted to the most common form, lymphoma of presumptive T-cell origin (thymic or multicentric), and to those cases in which the integrated FeLV genome was present.

Probes. The c-*myc* locus was analyzed by using the P1 probe, a *Pst*I-*Eco*RI fragment from FeLV-CT4 v-*myc* comprising mainly exon 3 sequences of c-*myc* (33). The *fit-1* locus was analyzed with *fit-1* probe C, a 600-bp *Nco*I fragment derived from the major cluster of proviral insertions (unpublished data and references 45). The *flvi-2* probe (probe F) was a *Pst*I-*Eco*RI fragment (see Fig. 2) (24). TCRβ gene rearrangements were detected by using a 390-bp *Bgl*II fragment derived from the Cβ domain of v-*tcr* (9, 44).

The *pim-1* probes were generated from feline genomic DNA by using the following primers: 5′-CGAGTGTA(CT)AG(TC)CCTCCAGAGTGG-3′ and 5′-CTGAAGAGAC(AC)(GC)T(TC)TGCCTGAAG-3′ to amplify feline *pim-1* exon 5; and 5′-CGTCATTAGACTTCTGGAC-3′ and 5′-TCAAAGTCCGTGTAGACTG-3′ for murine *pim-1* exon 4. In the presence of 1.5 mM $MgCl_2$, genomic DNA at 10 ng/μl, and primers at 10 μM, 30 cycles of denaturation (1 min, 94°C), annealing (1 min, 57°C) and extension (1 min, 72°C) were carried out. The 172-bp feline exon 5 and 299-bp murine exon 4 *pim-1* products were subcloned into the plasmid vector pCRII.

Southern blot hybridization. DNA preparation, restriction enzyme digestion, agarose gel electrophoresis, and Southern blot hybridization analysis were all carried out as described previously (33). Blots were washed at high stringency (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.5% sodium dodecyl sulfate, 60°C) apart from those hybridized with *fit-1* probe C, for which 0.5× SSC was substituted.

Statistical analysis. Pairwise comparisons between genetic parameters were analyzed by the χ^2 test using Yates correction. Where smaller numbers were involved (expected numbers of <5 in any χ^2 cell), Fisher's exact test was used to calculate probability.

TABLE 1. FeLV-associated thymic and multicentric lymphosarcomas analyzed for proviral insertions, gene rearrangement, and *env* recombinant FeLV

Origin of tumor	*n*	No. (%) with:					
		myc insertion or transduction	insertion at:			Integrated FeLV-B	Rearranged TCRβ
			fit-1	*flvi-2*	*pim-1*		
Natural	38	12 (32)	3 (8)	9 (24)	2 (5)	24 (63)	22 (58)
Experimental FeLV-*myc*	17	14 (82[a])	6 (35)	4 (24)	1 (6)	2 (12[b])	17 (100)
Experimental FeLV	8	4 (50)	0 (0)	1 (13)	0 (0)	3 (38)	4 (50)

[a] FeLV-*myc*-negative tumors induced by FeLV-T17 complex. Includes one de novo rearrangement of c-*myc*.
[b] Inoculum included FeLV-B in both positive tumors.

RESULTS

The c-*myc* locus. The c-*myc* locus is frequently affected in feline T-cell tumors by viral transduction (31, 33), proviral insertion (7, 26, 29, 33), or gene rearrangement (7). In addition to those cases examined in previous studies, a further 17 naturally occurring thymic or multicentric lymphosarcomas were screened, yielding two further *myc* transductions and two cases of proviral insertion at the locus. As can be seen from Table 1, the overall frequency of c-*myc* locus involvement in the naturally occurring tumors was 32%, with transduction more common than proviral insertion (eight cases compared with four). A slightly higher frequency was recorded in tumors induced experimentally by FeLV lacking transduced *myc* (50%), but in these cases, no new transductions of *myc* were observed. Not surprisingly, the highest frequency of *myc* gene involvement (82%) was seen in tumors induced by FeLV isolates carrying a transduced *myc* gene (T3, F422, T17, and LC isolates). The input FeLV-*myc* virus was detected in all of these tumors apart from those induced by FeLV-T17, in which case half of the tumors lacked detectable v-*myc* sequences and one showed instead a new proviral insertion at the locus (44).

Proviral insertions at the *fit-1* locus. Occupation of the *fit-1* locus has been found previously in tumors induced experimentally by the T3 and F422 FeLV-*myc* isolates. We examined a further 47 tumors, of which 5 showed insertions at *fit-1* locus, including three naturally occurring cases of thymic lymphosarcoma. Three of the newly identified *fit-1* rearrangements are shown in the Southern blots in Fig. 1A, in which *Sst*I digests of tumor DNA (lanes 1 to h) and control tissue (lane C) were probed with a 600-bp *Nco*I fragment (*fit-1* probe C). Four cases mapped previously are also shown here to assist cross-reference for those tumors carrying multiple rearrangements. In each case, it was possible to align a U3-hybridizing band with the rearranged *fit-1* allele, and in most cases, PCR amplification of a host-virus junction was also carried out, confirming the location and orientation of each of the inserted proviruses (not shown). In some cases (lanes a, e, g, and h), the

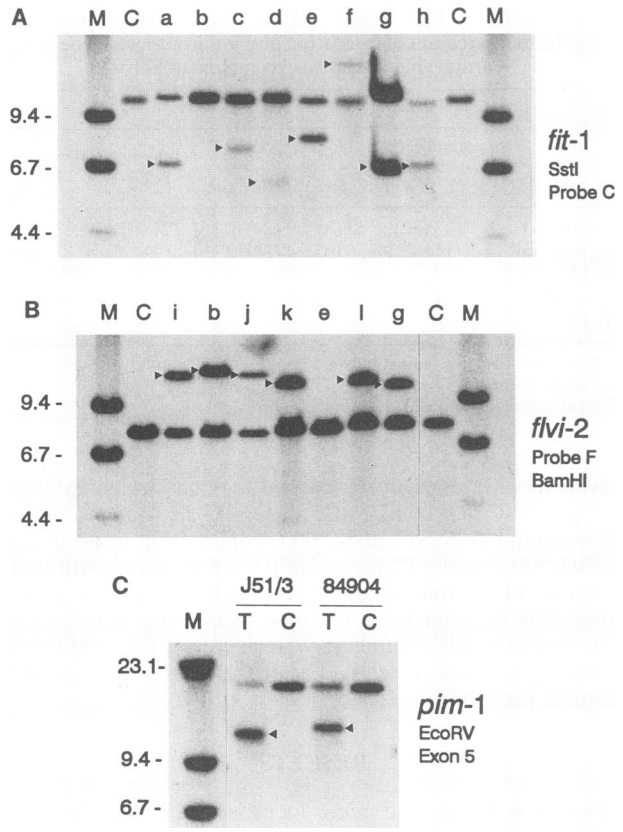

FIG. 1. Rearrangements at three common proviral insertion loci in feline T-cell lymphosarcomas and tumor cell lines. Tumor and control (lanes C) tissue DNAs were digested with restriction enzymes as show, separated on 0.8% agarose gels, and hybridized with probes as specified. Molecular size markers were end-labeled λ*Hin*dIII restriction fragments. (A) Southern blot hybridization of *Sst*I-digested DNA with *fit-1* probe C (see Fig. 2). (B) Southern blot hybridization of *Bam*HI-digested DNA with *flvi-2* probe F (see Fig. 2). Lanes: M, size markers (indicated in kilobases; a, T3C5; b, T3T (cell line); c, F42289-2; d, J51-3T; e, T21T; f, F422-4T; g, F422-1T; h, F422-2T; i, T10T; j, T11T; k, T19T; l, T23T. (C) Southern blot hybridization of *Eco*RV-digested DNA from tumor and control tissues of cases 84904 and J51-3 with a feline *pim-1* exon 5 probe (see Fig. 2).

rearranged allele was strongly represented, suggesting its occupation in all or most of the tumor cells. In contrast, in some cases (lanes c, d, and f), the rearrangement was present in only a proportion of the cells. The newly mapped insertions at *fit-1* are shown in Fig. 2 underneath the restriction map of the locus alongside the tumor identification code, with those mapped previously shown above the line. Extensive analysis of the locus around the major insertion cluster has not as yet revealed a transcription unit affected by these insertions.

Proviral insertions at *flvi-2*. *flvi-2*, the feline homolog of *bmi-1*, was previously shown to be occupied in 13 independent cases of thymic or multicentric lymphoma (24, 26). We examined a further 52 tumors and found a total of seven new examples of insertion which were mapped and tested for proviral orientation at the locus. Figure 1B shows the results of Southern blot hybridization analysis of six of the new cases which were mapped, alongside control tissues (lane C) and tumors without rearrangement at the locus (lane e). Again, the rearranged alleles coincided with U3-hybridizing fragments (not shown) and generated restriction maps consistent with proviral insertion at the site. The intensity of the rearranged alleles shown in Fig. 1B indicates that the rearrangement was carried by most or all of the cells in the tumor mass. Thus, in tumor F422-1T (Fig. 1A and B, lanes g), the major tumor cell population contains insertions at both *fit-1* and *flvi-2* as well as the integrated FTT v-*myc* gene.

Figure 2 shows the locations of the *flvi-2* insertions mapped previously (above the line) alongside the new examples (below the line). In common with previous findings (24, 26), the predominant observation was of insertion downstream and in the same orientation as the transcription unit, although there did appear to be a slight shift toward the gene in the major cluster of insertions. Whether this difference is significant or merely the result of mapping with a different *flvi-2* fragment (probe F places most of the insertions 3′ to the adjacent *Hin*dIII site) remains to be established.

As seen in Table 1, the overall frequencies of *flvi-2* involvement are similar in the natural and experimentally induced tumors and were also observed in a tumor (T10T; Fig. 2) induced by FeLV-Rickard, a highly oncogenic strain which was selected by in vivo passage of thymic tumor extracts (38) but does not contain a v-*myc* gene (10, 33).

Proviral insertions at the *pim-1* gene. The *pim-1* gene encodes a cytoplasmic protein kinase and is well established as an oncogene which can collaborate with c-*myc* in T-cell neoplasia (4, 50). In common with the *fit-1* locus, the feline *pim-1* gene is located on chromosome B2. To clarify the relationship between the two loci, we examined the prevalence and pattern of FeLV insertion at *pim-1*. Although murine *pim-1* probes hybridize with feline DNA, the signal is generally weak and requires low-stringency washing conditions. To generate a feline *pim-1* probe, primers were designed on the basis of conservation of the murine and human *pim-1* genes and used to amplify a 172-bp probe from exon 5 which shares 93.6% homology with human *pim-1* and 90.1% with the murine sequence. With this probe, it was possible to detect rearrangements of the locus sensitively under high-stringency hybridization conditions. Figure 1C shows two tumors carrying rearrangements at the *pim-1* locus detected by digestion of DNA from tumor (lane T) and control (lane C) tissue with *Eco*RV. As can be seen from the blots, the rearranged allele is at least as intense as or more intense than its normal counterpart (J51-3), suggesting that either amplification of the rearranged locus or loss of the normal allele had occurred. Interestingly, tumor J51-3 also had a rearrangement at *fit-1* (Fig. 1A, lane d). However, the low representation of the rearranged *fit-1* allele in this case suggests that this was present in a subset of the tumor cells which carried both FeLV v-*myc* and a rearranged *pim-1* gene.

Attempts to amplify feline *pim-1* exon 4 by the same approach were unsuccessful, but a murine exon 4 probe was amplified (see Materials and Methods) and used to orient the feline gene map. It was therefore possible to place the three proviral insertions detected on this outline map (Fig. 2). The orientation of the inserted elements has not yet been determined, but assuming that the architecture of the gene locus is also conserved, these correspond to insertions at the 5′ end (84904 and J51-3) and 3′ end (C2) of the transcription unit.

Concerted gene rearrangements in feline T-cell tumors. The number of tumors in which one or more of the four loci was involved is shown in the two Venn diagrams in Fig. 3. Figure 3A includes the naturally occurring tumors and those induced by inoculation of FeLV isolates without transduced *myc*, while the 17 tumors induced by inoculation of FeLV-*myc* isolates are depicted separately in Fig. 3B. It is clear from these diagrams that multiple insertion or gene activation events can be de-

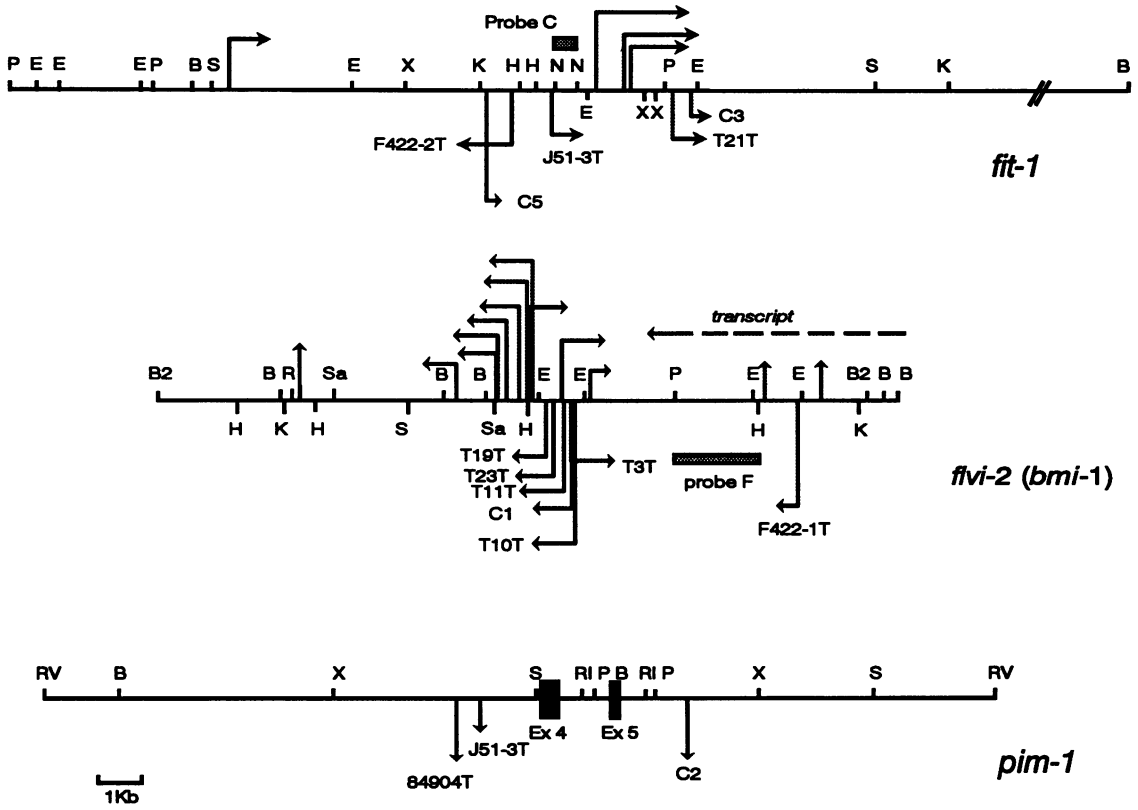

FIG. 2. Restriction maps of three common insertion loci for FeLV, showing the positions and orientations of integrated proviruses. Insertions mapped previously (24, 26, 45) are indicated as arrows above the linear restriction maps, while those newly mapped in this study are shown below the lines, along with the tumor identification code. Where orientation has not been determined, a vertical line is shown. Probes used in the Southern blot analysis in Fig. 1 (*fit-1* probe C, *flvi-2* probe F, and *pim-1* exon 5) are also illustrated. Restriction enzyme abbreviations: B, *Bam*HI; B2, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; RV, *Eco*RV; Sa, *Sal*I; S, *Sst*I; X, *Xba*I.

tected in a significant number of tumors in both groups. Not surprisingly, the number of tumors for which at least one event was scored was significantly higher in group B, in which a v-*myc* gene was introduced with the inoculum. However, the subset of group A tumors which had activated *myc* also showed a high score for secondary events, and this subset was not significantly different from that for group B, even for the number of tumors involving three hits (3 of 15 compared with 0 of 16; $P > 0.10$). This observation suggests that we have not selected for an unnatural set of gene activation events by inducing lymphomas with FeLV-*myc* isolates.

The group A tumors also included eight cases in which insertions at *flvi-2*, *fit-1*, or *pim-1* occurred in the absence of any detectable abnormality of the c-*myc* locus. Overall, only *fit-1* insertion was strongly associated with *myc* activation ($P = 0.04$). However, this association was due mainly to the high incidence of *fit-1* insertions in group B tumors. The occurrence of field case lymphoma in which the only identified genetic lesion was an insertion at *fit-1* suggests that the association is not obligatory.

TCRβ gene rearrangements in feline lymphomas. Rearrangement of the TCRβ gene is a crucial commitment point in the thymic development of the major αβ T-cell lineage (30), and the detection of a unique rearrangement of the TCRβ gene is widely used as a marker of clonality for tumors of T-cell origin. The possibility that the T-cell repertoire plays an even more significant and direct role in oncogenesis was suggested

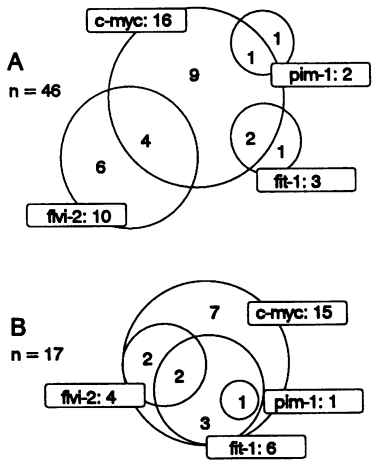

FIG. 3. Venn diagrams depicting the extent of overlap of FeLV-induced genetic alterations in 63 feline T-cell lymphomas and cell lines. These are subdivided into two groups, 46 tumors occurring spontaneously or induced experimentally by FeLV isolates lacking a transduced *myc* gene (A) and 17 tumors induced by inoculation of FeLV complexes including a transduced *myc* gene (B). For the purposes of this analysis, activation of c-*myc* by proviral insertion or transduction is assumed to be equivalent.

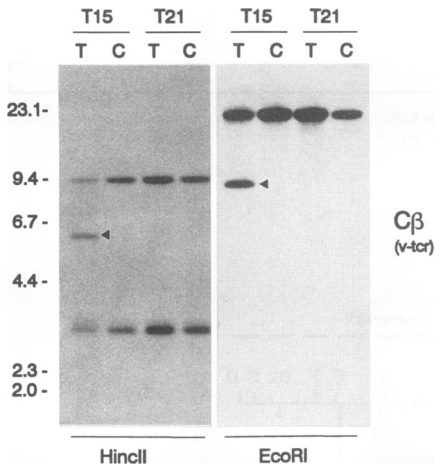

FIG. 4. Rearrangement of the TCRβ gene in feline T-cell tumors. Tumor (lanes T) and control (lanes C) tissue DNAs from naturally occurring cases of lymphosarcoma (T15 and T21) were digested with *Eco*RI or *Hinc*II, separated on 0.6% agarose gels, and hybridized with a probe derived from the Cβ domain of the transduced TCRβ gene (v-*tcr*) (9). In tumor T21, the gene is in germ line configuration, while in tumor T15, a clonal tumor cell population carrying a unique rearrangement of the Cβ1 locus has arisen. Sizes are indicated in kilobases.

by the transduction by FeLV of a full-length, functionally rearranged TCRβ gene in a naturally occurring thymic lymphosarcoma (9). In the same tumor, there was a separate population of proviruses which contained a transduced *myc* gene, suggesting the possibility of oncogenic cooperation between *myc* and an activated TCR (9, 44).

The relationship between T-cell genotype and other genetic markers was examined further in the present tumor series. The TCRβ locus was examined by Southern blot hybridization using a Cβ-specific probe (44). Digestion with *Eco*RI generates a 22-kb fragment spanning Cβ1 and Cβ2, while digestion with *Hinc*II separates these tandemly duplicated elements into 9.4-kb (Cβ1) and 3-kb (Cβ2) fragments. As shown in the examples in Fig. 4, comparison of tumor DNA with uninvolved control tissue revealed whether the genes were in germ line configuration or rearrangement had taken place. Also, the presence of a non-germ line fragment at high intensity in tumor T15 (arrowed) revealed the presence of a clonal cell population carrying a unique rearrangement.

The lineage and differentiation state of the 20 tumors with TCRβ in germ line configuration remains undetermined. Given the thymic lymphoid origin of many of these tumors, it is possible that these arise from immature cells which have not yet commenced TCR rearrangement or alternatively from a distinct non-αβ lineage such as γδ T cells (36). Clonal rearrangement of the immunoglobulin heavy-chain gene was observed in only two of these tumors (not shown).

Table 2 lists the numbers and percentages of tumors positive for each of the other parameters when the tumor panel is subdivided on the basis of TCRβ rearrangement. Inspection of these data suggests that c-*myc*, *flvi-2*, and *fit-1* are positively associated with TCRβ rearrangement. Statistical analysis shows that association is significant only for the first two of these ($P < 0.001$ and $P = 0.020$, respectively). Although a similar trend for *fit-1* appears to be emerging, with the small numbers involved, this does not attain statistical significance ($P = 0.12$).

TABLE 2. Genotypic parameters of FeLV-associated lymphomas subdivided on the basis of rearrangement of TCRβ

TCRβ (*n*)	No. (%) with:				
	c-*myc* alteration	*flvi-2* insertion	*fit-1* insertion	*pim-1* insertion	Integrated FeLV-B
Rearranged (43)	29 (67)[a]	13 (30)[b]	8 (19)	1 (2)	19 (44)
Germ line (20)	2 (10)[a]	1 (5)[b]	1 (5)	2 (10)	11 (58)

[a] Very highly significant difference ($P < 0.001$, χ^2 test with Yates correction).
[b] Significant difference ($P = 0.020$, Fisher's exact test).

Occurrence of recombinant FeLV-B proviruses in tumor DNA. Subgroup B viruses invariably derive the 5′ portion of their *env* gene from endogenous FeLV sequences by recombination (21, 35, 39, 42). Using a probe (B/S) derived from this portion of the FeLV-B genome, it is possible to screen cells for the presence of FeLV-B by blot hybridization. Digestion of feline cell DNA with *Kpn*I and probing with B/S at high stringency reveals the presence of exogenous FeLV-B as a novel hybridizing fragment which is distinguished from endogenous FeLV sequences by its unique size and its absence from uninfected cell DNA (42).

We compared tumor and uninvolved tissues from each case and scored a positive result for FeLV-B wherever such a novel fragment was seen. Examples of these analyses are shown in Fig. 5, in which only tumor TR3 has the typical exogenous FeLV-B *env* fragment of 3.7 kb. Table 3 lists the numbers and percentages of tumors positive for each of the other parameters when the tumor panel is subdivided on the basis of detection of a FeLV-B recombinant. As can be seen, the other parameters are distributed relatively evenly between the two subsets, with the exception of insertion at *fit-1*, which is negatively associated with the presence of FeLV-B ($P = 0.001$, Fisher's exact test).

However, further insight into the possible basis of this relationship is revealed in Table 1, in which the tumors are subdivided according to the mode of their induction. It is evident that the discordance is due mainly to the rapidly arising tumors induced by FeLV-*myc* viruses which show a high

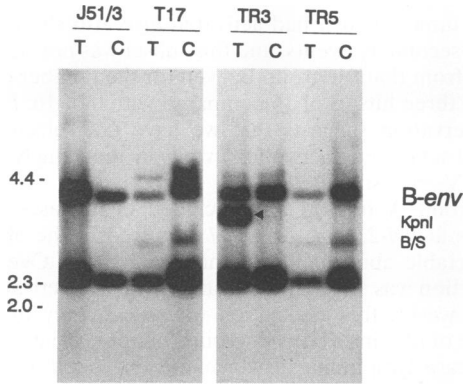

FIG. 5. Detection of FeLV-B in feline thymic tumors. Tumor (lanes T) and control (lanes C) tissue DNA samples were digested with *Kpn*I and separated by agarose gel electrophoresis prior to blotting and hybridization with a *Sau*3A probe fragment (B/S) derived from the *env* gene of FeLV-B/Gardner-Arnstein (42). The presence of a recombinant FeLV-B in tumor TR3 DNA is identified as a novel hybridizing fragment of 3.7 kb which can be distinguished from endogenous FeLV-related sequences by its absence from control somatic tissues. Sizes are indicated in kilobases.

TABLE 3. Genotypic parameters of FeLV-associated lymphomas subdivided according to detection of integrated FeLV-B

FeLV-B (*n*)	No. (%) with:				
	c-*myc* alteration	*flvi-2* insertion	*fit-1* insertion	*pim-1* insertion	Rearranged TCRβ
Present (30)	14 (47)	6 (20)	0 (0)[a]	1 (3)	19 (63)
Not detected (32)	16 (50)	8 (25)	9 (28)[a]	2 (6)	26 (81)

[a] Highly significant difference ($P = 0.001$, Fisher's exact test).

prevalence of *fit-1* insertions and a low rate of detection of FeLV-B. This trend is even more striking when two tumors induced by the LC FeLV-*myc* isolate are excluded on the basis that the inoculum already contained an FeLV-B component.

DISCUSSION

This study extends previous observations on the high frequency of c-*myc* gene activation by FeLV in naturally occurring tumors (32%). The incidence of proviral insertions at *flvi-2* in a series of 38 naturally occurring tumors was almost as high (24%). Analysis of this larger series of tumors also confirmed the lack of a strong association between these two gene activation events (26). This observation is somewhat surprising in view of the demonstrated ability of *myc* and *bmi-1* genes to collaborate in transgenic mice (12), and it would appear that both *myc* and *bmi-1* are relatively promiscuous in their ability to act in concert with other genes. In contrast, occupation of the *fit-1* locus was most commonly seen in tumors with activated *myc* and appeared in some cases to affect only minor tumor cell populations. The oncogenic potential of *fit-1* insertion may be expressed only when c-*myc* or a related gene is activated, a scenario which would occur readily in the tumors where an active *myc* oncogene was introduced with the viral inoculum. An appreciation of the functional basis of this association will require identification and characterization of the transcription unit affected by *fit-1* insertions, but it appears likely that the occupation of *fit-1* is often a late step in tumor development.

Our analysis indicates that each of the four insertion loci studied here can contribute to the neoplastic state independently of the others and that no combination of two events is obligatory. The finding that several tumors had concomitant alterations at three different loci (c-*myc* and two others) is in line with models of leukemogenesis by murine retroviruses in which a requirement for three to six hits has been postulated (49). Also, we noted a higher frequency of two or three recorded hits in the tumors induced experimentally by FeLV-*myc* viruses. It would appear that the introduction of a transduced *myc* allele with the inoculum virus not only shortens the latent period required for tumor induction (34) but increases the probability of detecting concerted gene rearrangements. This observation is analogous to studies of virus-accelerated tumors of oncogene transgenic mice in which proviral tagging has been used to show high frequency targeting of collaborating genes (49, 50). Thus, the introduction of an initiating oncogene in the germ line or in the virus inoculum reduces the number of steps required to generate a tumor and selects for the activation of only those genes which can act in concert with the initiating oncogene. This experimental approach should be of further value in the analysis of oncogene complementation in vivo and the identification of additional genes which are likely to be involved.

In lymphomas of *pim-1* transgenic mice induced by MuLV or woodchuck liver tumors induced by woodchuck hepatitis virus, viral insertional mutagenesis often activates c-*myc* or N-*myc* but never both genes (8, 48). From this and similar examples, a strong negative association between the occupation of two insertion loci in individual tumors may infer that these loci represent either functionally related genes or genes on a common growth regulatory pathway. In the tumor series examined here, an exclusive relationship was observed between *pim-1* and *flvi-2* insertions. However, overlap was noted previously between *pim-1* and *bmi-1* insertions in two virus-accelerated tumors of Eμ-*myc* transgenic mice (49), so it is likely that the apparent exclusion is due to the small number of observed *pim-1* insertions in our series rather than noncomplementing genetic functions.

Further evidence that insertions at the *fit-1* locus accomplish more than long-range activation of *pim-1* comes from the identification here of a single tumor carrying insertions at both loci. To our knowledge, occupation of both alleles of a single gene has never been observed when transcriptional activation is the relevant mechanism. In contrast, insertion at both alleles could occur when the selective advantage is loss of function, as for Friend MuLV insertions at both alleles of the p53 gene (13, 32). However, such considerations would not apply to *pim-1*, which is an activation target (50), and the lack of any instance in our tumor series in which insertions have occurred at both of the *fit-1* and *pim-1* alleles also argues against such an interpretation. It seems more likely that the two insertion sites mediate independent and possibly complementary steps in lymphoma development.

The results here reinforce and extend the evidence of a close association between the presence of an activated *myc* gene and T-cell differentiation state, as monitored by rearrangement of the TCRβ gene. Moreover, a similar relationship has emerged for *flvi-2*. We have postulated previously that a window of sensitivity to transformation might arise in the thymus, where T cells undergo TCR rearrangement and generate autoreactive cells normally destined for deletion by the induction of programmed cell death (14, 19, 40). We considered that such cells might evade negative selection and proliferate under the influence of *myc* and collaborating oncogenes (44). This model is currently difficult to test in the outbred cat, but preliminary evidence indicates that potentially autoreactive T-cell tumors arise in mice transgenic for the human c-*myc* gene targeted to the thymus (3, 41).

The frequency of detection of FeLV-B by Southern blot hybridization of tumor DNA in field cases of lymphosarcoma was similar to that seen on previous virological (16) and PCR (39) studies of tumor-bearing cats. Thus, it would appear that in most cases, there is a correspondence between the isolation of FeLV-B and its presence within the tumor cell. However, this does not always hold, since we have shown that myeloid leukemias induced by FeLV-AB/GM1 may lack the subgroup B genome even though the host is clearly infected (46). Also, we observed a marked difference in the tumors induced by FeLV-*myc* isolates in which FeLV-B was rarely detected unless it was introduced with the inoculum. It is likely that this difference also explains the negative association of FeLV-B with *fit-1* insertions which were detected mostly in the tumors induced by FeLV-*myc* isolates.

The role of subgroup B viruses in lymphoma development remains uncertain, but it is clear that they do not represent an obligatory step analogous to MCF virus generation in AKR lymphomas (43). It is possible that the generation of FeLV-B is just one of the ways in which FeLV can overcome the interference barrier created by a resident FeLV-A provirus, thereby allowing a further hit of viral integration to occur in a

developing neoplastic clone. Since there are other ways in which this barrier might be overcome (1, 37), and FeLV-B envelope proteins could conceivably help to deliver a non-FeLV-B genome by pseudotype formation (17), the frequent but not universal detection of FeLV-B in tumor DNA could thus be explained. Also, the introduction of an activated *myc* gene with the FeLV-*myc* viruses would reduce by at least one the number of insertions required to complete the transformation process, further relieving the requirement for FeLV-B. Although this seems to us the most likely explanation, it must be acknowledged that a more positive role or conversely an innocent bystander role for FeLV-B cannot be excluded. Also, the dissemination of FeLV-B in vivo may be slow (18, 46) and constrained by interference due to endogenous FeLV *env* gene expression in normal lymphoid cells (28).

In conclusion, the pattern of proviral insertion and gene activation in feline T-cell lymphomas suggests that a pool of genes is involved which is significantly larger than that currently known. In particular, the subset of thymic tumors which lack rearrangement of TCRβ genes show only a low incidence of involvement of the known target genes and are ripe for further for genetic analysis. Also, the activation in feline tumors of genes homologous to two established *myc*-collaborating genes of the mouse (*bmi-1* and *pim-1*) indicates cross-species conservation of their oncogenic functions and should serve as a further stimulus to examine their roles in human hemopoietic malignancies.

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