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Genetic Determinants of Feline Leukemia Virus-Induced Multicentric Lymphomas

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Three discrete forms of feline leukemia virus (FeLV)-associated lymphoma have been described clinically: (1) thymic, (2) alimentary, and (3) multicentric. The most common and best-characterized lymphomas are of T-cell origin, generally occurring in the thymus. These tumors typically contain mature T-cells, involve the activation of a distinctive set of proto-oncogenes, and contain FeLV proviruses whose long terminal repeat (LTR) sequences contain tandemly repeated enhancers. Previous studies of a small group of extrathymic, multicentric lymphomas implicated a different set of genetic determinants. The present study expands those observations by examining the lineage of origin, the involvement of proto-oncogenes, and the structure of LTR and *env* gene sequences in a set of 11 natural, extrathymic lymphomas of the multicentric type. A pattern of genetic events associated with FeLV-positive multicentric lymphomas emerges from this analysis that is clearly distinct from the pattern associated with thymic lymphomas. The tumors do not contain T-cells or B-cells, as evidenced by the germ line organization of TCRβ and IgH loci. Proto-oncogenes strongly implicated in T-cell lymphomagenesis are not involved in these tumors. Rather, a distinct set of proto-oncogenes may be involved. Most striking is the repeated occurrence of an FeLV isolate whose LTR and *env* gene bear unique sequence elements.

INTRODUCTION

The feline leukemia viruses (FeLV) are a family of horizontally transmissible retroviruses of the domestic cat, associated with a wide range of malignant diseases in the natural host. The most frequently associated malignancy is a lymphoma characterized by the presence of discrete tumors and diffuse infiltration of organs by lymphoid tumor cells. Three forms of FeLV-associated lymphoma have been described clinically: (1) thymic, characterized by a rapidly progressive tumor of the anterior mediastinum; (2) alimentary, in which the tumor involves the gastrointestinal tract; and (3) multicentric, a generalized disease involving many lymphoid tissues and other organs (Crighton, 1969; Cotter, 1992). The most common and best-characterized lymphomas are of T-cell origin, generally occurring in the thymus (Neil *et al.,* 1991; Jarrett, 1992). Extensive examination of the molecular basis of these lymphomas has identified several genetic determinants. First, the T-cell origin of the tumors has been verified by the demonstration of T-cell receptor β (TCRβ) gene rearrangements (Levy *et al.,* 1988; Levesque *et al.,* 1990; Tsatsanis *et al.,* 1994). Second, the involvement of a distinctive set of proto-oncogenes, by either insertional mutagenesis or retroviral transduction, has been well documented (Levy and Lobelle-Rich, 1992; Levy *et al.,* 1993a,b; Tsujimoto *et al.,* 1993; Tsatsanis *et al.,* 1994). Finally, the FeLV proviruses cloned directly from thymic tumors are distinctive in that their long terminal repeat (LTR) sequences typically contain tandemly repeated enhancers, generally as 2 or 3 directly repeated copies (Fulton *et al.,* 1990; Matsumoto *et al.,* 1992; Rohn and Overbaugh, 1995). The results of a recent study of FeLV-induced T-cell lymphomagenesis suggested a hierarchy of genetic events involving these determinants (Tsatsanis *et al.,* 1994).

Previous studies of a small group of extrathymic lymphomas implicated a different set of genetic determinants. In particular, a group of four tumors was examined, all of which had been isolated from the spleens of diseased animals. These tumors were shown not to contain mature T-cells or B-cells, as evidenced by the germ line organization of TCRβ and immunoglobulin heavy chain (IgH) genes. A domain of feline DNA, termed *flvi*-1, was shown to be interrupted by FeLV proviral integration in the tumors, implicating *flvi*-1 as a putative proto-oncogene. The LTRs of FeLV proviruses isolated from the tumors were shown not to contain tandemly repeated enhancer sequences. Rather, they were observed to contain a unique sequence motif comprised of a 21-base-pair (bp) tandem triplication beginning 25 bp downstream of a single copy of the canonical enhancer. The repeated observation of an LTR of this unique structure in tumors of a distinctive phenotype was thought to implicate the triplication-containing LTR in the induction of tumors of

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that type (Levesque *et al.,* 1990; Athas *et al.,* 1995). The animals from which these tumors were isolated exhibited extrathymic tumors that, according to the clinical classification outlined above, would best be characterized as multicentric. In view of the distinct set of genetic determinants associated with this limited set of tumors, the objective of the present study was to examine a larger set of natural multicentric lymphomas with respect to several genetic events. First, since the feline TCRβ and IgH loci have been characterized to some extent, and probes for their somatic rearrangement have become available (Terry *et al.,* 1992, 1995; Tsatsanis *et al.,* 1994), it is possible to examine the organization of these loci in feline lymphomas with increased confidence that somatic rearrangement would be detected. The involvement of proto-oncogenes implicated in T-cell lymphomagenesis has also been examined, as has the LTR and gp70 gene structure of FeLV proviruses in tumor DNA. A pattern of genetic events associated with FeLV-positive multicentric lymphomas has emerged from this analysis that is clearly distinct from the pattern observed in FeLV-mediated T-cell lymphomagenesis.

MATERIALS AND METHODS

Feline lymphomas. Naturally occurring FeLV-positive feline tumors examined in this study are of the multicentric type and have been histopathologically classified as lymphomas. These tumors were previously examined for FeLV proviral integration in the c-*myc* locus (Levy *et al.,* 1984) and in *flvi*-1 (Levesque *et al.,* 1990).

Probes. The TCRβ locus was examined using pFeCβ, a 390-bp fragment derived from the Cβ domain of v-*tcr* (Terry *et al.,* 1992). The IgH locus was examined using pFeCμ4, a 417-bp fragment amplified by PCR from the feline IgH locus (Terry *et al.,* 1995). The *flvi*-2 locus was examined using probe D, a 1.1-kb *Eco*RI fragment cloned from feline *flvi*-2 (Levy and Lobelle-Rich, 1992). The *pim*-1 locus was examined using pFePim-1, a 172-bp PCR product amplified from exon 5 of feline *pim*-1 (Tsatsanis *et al.,* 1994). The *fit*-1 locus was examined using a 600-bp PCR product amplified from feline *fit*-1 (Tsujimoto *et al.,* 1993).

Southern blot analysis. High-molecular-weight genomic DNA was prepared from tumor tissues, and Southern blots were prepared, as previously described (Levesque *et al.,* 1990; Levy and Lobelle-Rich, 1992). Filters were hybridized for 15 hr in a solution containing 6× SSC, 5× Denhardt solution, 0.5% sodium dodecyl sulfate, 100 mg of denatured DNA per milliliter, and 0.05 *M* PIPES (pH 6.8) at 68° to probes radiolabeled by random priming and added to the hybridization solution at 2×10^6 cpm/ml. After two washes in 2× SSC–0.1% sodium lauroyl sarcosine at 37° for 30 min each, and two washes in 0.1× SSC–0.1% sodium lauroyl sarcosine at 55°, filters were exposed at −70° for various periods of time to Fuji RX film, with intensifying screens.

PCR amplification of FeLV LTRs and env gene sequences. Primer pair LTR13 and LTR14 were used in PCR reactions as previously described (Athas *et al.,* 1995) to amplify a portion of the LTR from integrated FeLV proviruses in tumor DNA. The primers were designed to yield products of 301 bp from FeLV LTRs containing the 21-bp triplication and 259 bp from LTRs lacking the triplication. Primer pair env1 and LTR14 were used to amplify a 2.1-kb fragment of FeLV proviral DNA. The env1 primer (5′-GGAATGGCCAATCCTAG-3′) begins at nucleotide 6073 (of FeLV-A/61E) in a region of the gp70 gene that is highly conserved among FeLV isolates (Donahue *et al.,* 1988). PCR reaction mixtures contained 1 μg of tumor DNA, 60 pmol of each primer, and a 0.2 m*M* concentration of each deoxyribonucleotide triphosphate, in a reaction buffer of 50 m*M* KCl, 10 m*M* Tris, pH 8.4, and 1.5 m*M* $MgCl_2$ in a total volume of 100 μl. An initial denaturation step at 98° for 5 min was followed by annealing at 57° for 5 min and extension at 72° for 1 min. This was followed by 35 cycles of denaturation at 94° for 30 sec, annealing at 57° for 30 sec, and extension at 72° for 1 min with a final extension for 15 min. Amplification products were examined by agarose gel electrophoresis and cloned into the pGEM-T plasmid vector (Promega Corp.). Nucleotide sequence analysis was performed using dideoxy chain termination reactions with Sequenase as described by the manufacturer (Amersham Life Sciences).

RESULTS

TCRβ and IgH gene rearrangements in multicentric lymphomas. Rearrangement of the TCRβ gene, a defining event in the differentiation of the major $\alpha\beta$ T-cell lineage, is characteristic of FeLV-positive feline lymphomas of the thymus and reflects their origin in the T-cell lineage. A recent study demonstrated clonal rearrangement of the TCRβ gene in 68% of presumptive T-cell lymphomas examined (Tsatsanis *et al.,* 1994). Organization of the TCRβ locus in the DNA of multicentric lymphomas in the present study was examined by Southern blot analysis of tumor DNA digested with *Eco*RI or with *Hin*cII and hybridized to a feline Cβ-specific probe (Terry *et al.,* 1992). As described previously, clonal rearrangements of the feline TCRβ locus can be readily visualized under these conditions (Tsatsanis *et al.,* 1994). Southern blot analysis demonstrated the TCRβ locus to be in germ line configuration in all 11 multicentric lymphomas examined (representative examples shown in Fig. 1), although rearrangement was clearly evident in the DNA of 3 thymic lymphomas examined for comparison (Fig. 1, lanes a–c).

Rearrangement of the IgH locus may be considered a marker of B-cell lineage, particularly when the TCRβ locus occurs in germ line configuration (Kirsch and Kuehl, 1994). FeLV-associated B-cell lymphomas are relatively rare, although they are generally extrathymic and often

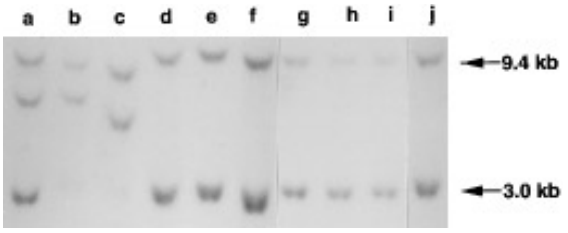

FIG. 1. Southern blot analysis of the TCRβ locus in DNA from FeLV-positive lymphomas of cats 981-3 (lane a), 1110 (lane b), 931 (lane c), 1043 (lane d), 1046 (lane e), 1112 (lane f), 945 (lane g), 934 (lane h), 1345 (lane i), and from normal feline spleen (lane j). DNA samples examined were obtained from thymic lymphomas (lanes a–c) or from extrathymic, multicentric lymphomas (lanes d–i). DNA samples were digested with *Hinc*II, and resulting Southern blots were hybridized to a radiolabeled probe representing the Cβ domain of feline TCRβ (Terry *et al.,* 1992). The 9.4- and 3.0-kb fragments definitive of germ line organization of TCRβ are indicated. In three thymic lymphomas, clonal rearrangement of TCRβ is evident (lanes a–c). In all multicentric lymphomas, the locus is in germ line configuration (lanes d–i).

associated with the alimentary tract (Cotter, 1992). Organization of the IgH locus in the DNA of multicentric lymphomas in the present study was examined by Southern blot analysis of tumor DNA digested with *Eco*RI or with *Hin*dIII, and hybridized to a feline Cμ-specific probe. Clonal rearrangements of the feline IgH locus can be readily visualized under these conditions (Terry *et al.,* 1995). Southern blot analysis demonstrated the IgH locus to be in germ line configuration in all 11 multicentric lymphomas examined (representative examples shown in Fig. 2), although an FeLV-negative multicentric tumor examined for comparison was observed to contain a rearrangement (Fig. 2, lane e).

Proviral insertional mutagenesis of proto-oncogenes in multicentric lymphomas. The insertional mutagenesis of host proto-oncogenes by proviral integration has been implicated in FeLV-mediated tumorigenesis. Interruption of the c-*myc, flvi*-2 (*bmi*-1), and *pim*-1 loci by FeLV proviruses has been linked to the induction of thymic tumors of T-cell lineage, as has interruption of the putative proto-oncogene, *fit*-1 (Levy and Lobelle-Rich, 1992; Levy *et al.,* 1993a,b; Tsujimoto *et al.,* 1993; Tsatsanis *et al.,* 1994). Involvement of those loci in FeLV-positive multicentric lymphomas was examined in the present study for comparison. Although involvement of the c-*myc* locus is evident in 67% of FeLV-positive lymphomas of T-cell origin (Levy *et al.,* 1993b; Tsatsanis *et al.,* 1994), previous analysis of the multicentric tumors considered in this study demonstrated the c-*myc* locus to be in germ line configuration (Levy *et al.,* 1984). The *flvi*-2 (*bmi*-1) locus is interrupted by FeLV proviral integration in 25–30% of FeLV-positive lymphomas of T-cell origin, as reported in two recent studies (Levy *et al.,* 1993a; Tsatsanis *et al.,* 1994). Organization of the *flvi*-2 locus in the present study was examined after digestion of tumor DNAs with the restriction enzyme *Pst*I or *Bam*HI, and hybridization to a 1.1-kb *Eco*RI restriction fragment of feline *flvi*-2 designated as fragment D (Levy and Lobelle-Rich, 1992). This analysis allows examination of approximately 9 kilobases (kb) of feline *flvi*-2, including the previously described domain of frequent FeLV integration (Levy and Lobelle-Rich, 1992; Tsatsanis *et al.,* 1994). Results demonstrated the *flvi*-2 locus to be in germ line configuration in all 11 multicentric lymphomas examined (data not shown). The feline *pim*-1 and *fit*-1 loci have been implicated as targets of insertional mutagenesis in smaller proportions of FeLV-positive lymphomas of T-cell origin (2 and 19%, respectively; Tsatsanis *et al.,* 1994). Organization of the *pim*-1 locus in the present study was examined after digestion of tumor DNAs with the restriction enzyme *Eco*RV and hybridization to a feline *pim*-1 exon 5 probe. This analysis allows examination of approximately 25 kb of feline *pim*-1, including the sites of integration previously described in feline thymic lymphomas (Tsatsanis *et al.,* 1994). Organization of the *fit*-1 locus was examined after digestion of tumor DNAs with *Eco*RI, *Pst*I, or *Bam*HI, and hybridization to a feline *fit*-1 probe. This analysis allows examination of approximately 26 kb of feline *fit*-1, including the major cluster of FeLV proviral integration (Tsujimoto *et al.,* 1993; Tsatsanis *et al.,* 1994). Results demonstrated both *pim*-1 and *fit*-1 loci to be in germ line configuration in all 11 multicentric lymphomas examined (data not shown).

LTRs of FeLV proviruses integrated in multicentric lymphomas. Previous studies have demonstrated the presence of an FeLV LTR of distinctive structure in four multicentric lymphomas from the collection examined in the present study. The unique LTR, not reported in any other strain of FeLV or in any other mammalian retrovirus, exhibits a 21-bp tandem triplication beginning 25 bp downstream of a single copy of the canonical enhancer. Previous studies demonstrated that the 21-bp triplication contributes enhancer function to the LTR that contains it, and that it functions preferentially in a primitive, multipotential hematopoietic cell line. Those studies associated the triplication-containing LTR uniquely with multi-

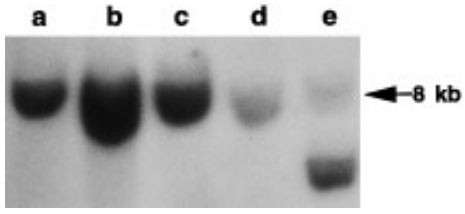

FIG. 2. Southern blot analysis of the IgH locus in DNA from normal kitten thymus (lane a), from FeLV-positive, multicentric lymphomas of cats 925 (lane b), 945 (lane c), and 1043 (lane d), and from an FeLV-negative multicentric lymphoma 1316 (lane e). DNA samples were digested with *Hin*dIII, and the resulting Southern blot was hybridized to a radiolabeled probe representing the Cμ domain of feline IgH (Terry *et al.,* 1995). The 8.0-kb fragment definitive of germ line organization of IgH is indicated. In FeLV-positive multicentric lymphomas, the locus is in germ line configuration (lanes b–d). By comparison, clonal rearrangement of IgH is evident in an FeLV-negative tumor (lane e).

centric tumors, since it was not identified in five thymic lymphomas examined (Athas *et al.,* 1995). For the present study, LTRs from seven additional multicentric lymphomas were amplified by PCR (Athas *et al.,* 1995). Amplification products were cloned into plasmid vectors, and the sequence of each was determined. LTRs amplified from four of the tumors (925, 1046, 1043, 1112) were shown to contain the 21-bp triplication. Taken together with previous studies, the triplication-containing LTR has been isolated from eight multicentric lymphomas. The sequence and position of the 21-bp triplication is identical in the LTRs from all of those tumors, with the exception of a single base change in the LTR amplified from tumor 925 (Figs. 3 and 4). By comparison, three tumors (1011, 1325, and 1345) were shown to contain LTRs exhibiting a single copy of the canonical enhancer and a single copy of the 21-bp sequence (Fig. 3). Thus, the triplication-containing LTR is isolated from most, but not all, multicentric lymphomas in this collection (8 of 11; 73%).

PCR amplification yielded multiple products from two of the tumors examined in the present study. Tumors 1043 and 1112 were shown to contain not only the triplication-containing LTR, but also a single-enhancer LTR lacking the triplication (Figs. 3 and 4). Tumor 1112 was shown to contain a third LTR species that lacks the triplication, but contains a partial duplication of enhancer. Specifically, the LTR (indicated as 1112.1 in Fig. 4) contains an imperfect repeat of 28-bp including the core enhancer and a partial NF1 binding site. This is the only amplification product examined among the 11 multicentric lymphomas that exhibits duplication of the canonical enhancer. By contrast, FeLV LTRs derived from thymic lymphomas typically contain 2 or 3 tandem direct repeats of the enhancer (Fulton *et al.,* 1990; Matsumoto *et al.,* 1992; Rohn and Overbaugh, 1995).

gp70 gene sequence from FeLV proviruses bearing the triplication-containing LTR. Previous studies indicated that FeLV proviruses bearing the unique LTR belonged to subtype A (FeLV-A), inasmuch as molecularly cloned proviral DNA hybridized to a subtype-specific probe that distinguishes subtypes A and B (Levesque *et al.,* 1990). Sequence analysis of the triplication-containing LTR, however, suggested a relationship to subtype C FeLV (FeLV-C; Athas *et al.,* 1995). This possibility bears on the mechanism of pathogenesis mediated by the virus, since FeLV-A and FeLV-C exhibit quite different pathogenic potentials and induce diseases involving distinct cell populations (Neil *et al.,* 1991). FeLV subgroup classification is defined experimentally on the basis of viral interference and neutralization properties (Sarma and Log, 1971, 1973). Differences between FeLV-A and FeLV-C with respect to these properties have been attributed to differences in the sequence of the amino terminus of gp70 envelope protein (Neil *et al.,* 1991). In fact, a region of the gp70 gene that encodes the first three of five variable regions has been shown to determine the subgroup classification for FeLV-A and FeLV-C (Rigby *et al.,* 1992; Kristal *et al.,* 1993). Thus, in order to evaluate the subgroup classification of triplication-containing FeLV, the sequence of the gp70 gene encoding variable regions 1–3 was determined. Two FeLV proviruses bearing the triplication-containing LTR were examined. First, a provirus was examined that had been previously cloned from a genomic library of tumor 945 DNA. This provirus was integrated in tumor DNA at the *flvi*-1 locus (Levesque *et al.,* 1990). Second, a 2.1-kb fragment of proviral DNA containing a portion of the *env* gene and the LTR was amplified by PCR from tumor 934 DNA. Because the amplification product represents viral sequences only, the integration site of this provirus is not known. Nucleotide sequence analysis of both proviral DNAs verified the presence of the triplication-containing LTR (data not shown). Sequence analysis of the gp70 genes revealed them to be most closely related to FeLV-A/Glasgow. A comparison of the predicted translations of gp70 gene sequences, across variable regions 1–3, from prototypical FeLV of subtypes A, B, and C, as well as from triplication-containing FeLV proviruses is shown in Fig. 6. It can be seen that the gp70 proteins encoded by triplication-containing FeLV from two multicentric lymphomas are nearly, but not entirely, identical to each other and are most closely homologous to that encoded by FeLV-A/Glasgow.

DISCUSSION

Genetic analysis of a set of 11 natural, extrathymic FeLV-positive lymphomas of multicentric type reveals a characteristic pattern of molecular determinants. First, an examination of the TCRβ and IgH loci in tumor DNA demonstrates their germ line organization (Figs. 1 and 2). Thus, although the tumors were classified histopathologically as lymphoid, their lineage and differentiation state are unknown. By comparison, other studies have shown that 67–100% of FeLV-positive thymic lymphomas contain mature T-cells, as evidenced by somatic rearrangement of TCRβ (Levy and Lobelle-Rich, 1992; Tsatsanis *et al.,* 1994). The pattern of proto-oncogene involvement in thymic lymphomas of T-cell origin is also distinctive, and includes the retroviral transduction or insertional mutagenesis of c-*myc, flvi*-2 (*bmi*-1), *pim*-1, or *fit*-1 with varying frequencies as high as 67% (Levy and Lobelle-Rich, 1992; Levy *et al.,* 1993a,b; Tsujimoto *et al.,* 1993; Tsatsanis *et al.,* 1994). In fact, 24% of the FeLV-positive T-cell lymphomas examined in a recent study contained interruptions of more than one of those loci within a single tumor, an indication of their cooperation in T-cell lymphomagenesis (Tsatsanis *et al.,* 1994). By contrast, none of the multicentric lymphomas examined in the present study exhibited interruption of any of those loci (data not shown). These findings are consistent with the hypothesis that somatic rearrangement of TCRβ, acti-

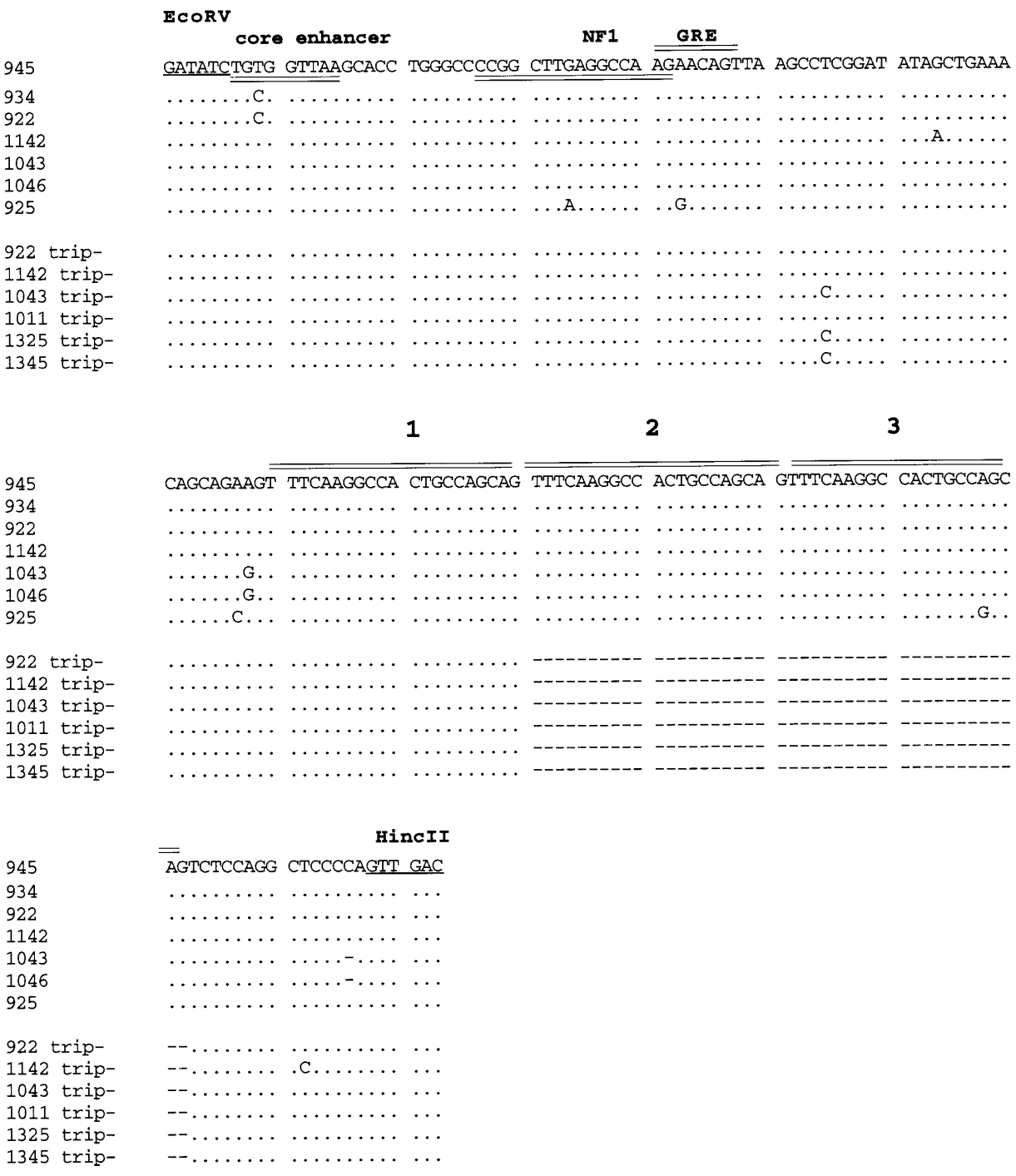

FIG. 3. Nucleotide sequence of the FeLV LTRs amplified by PCR from naturally occurring feline tumors, shown between the conserved *Eco*RV and *Hin*cII sites at positions 117 and 232, respectively, in the LTR of FeLV-A/61E (Donahue *et al.,* 1988). Sequences shown include the triplication-containing FeLV LTRs amplified from lymphomas 945, 934, 922, 1142, 1043, 1046, and 925. LTRs lacking the triplication (trip-) from animals 922, 1142, 1043, 1011, 1325, and 1345 are also shown. Sequences are compared to that of FeLV-945, and only the differences in sequence are shown. Dashes are included to optimize the alignment due to insertions in the triplication-containing LTRs relative to other sequences. Nuclear protein binding sites in the FeLV enhancer are indicated (core enhancer, NF1, GRE; Fulton *et al.,* 1990). The sequences of LTRs amplified from tumors 945, 934, 922, and 1142 have been previously reported (Athas *et al.,* 1995).

vation of *myc,* and proviral insertion at *flvi*-2, are defining events in FeLV-mediated T-cell lymphomagenesis (Levy *et al.,* 1993b; Tsatsanis *et al.,* 1994). The lack of involvement of *myc, flvi*-2, *pim*-1, and *fit*-1 in non-T-cell non-B-cell lymphomas of the multicentric type suggests the likelihood that other as yet unidentified proto-oncogenes play a role in their induction. Apparently unique to tumors of this type is the insertional mutagenesis of *flvi*-1 by

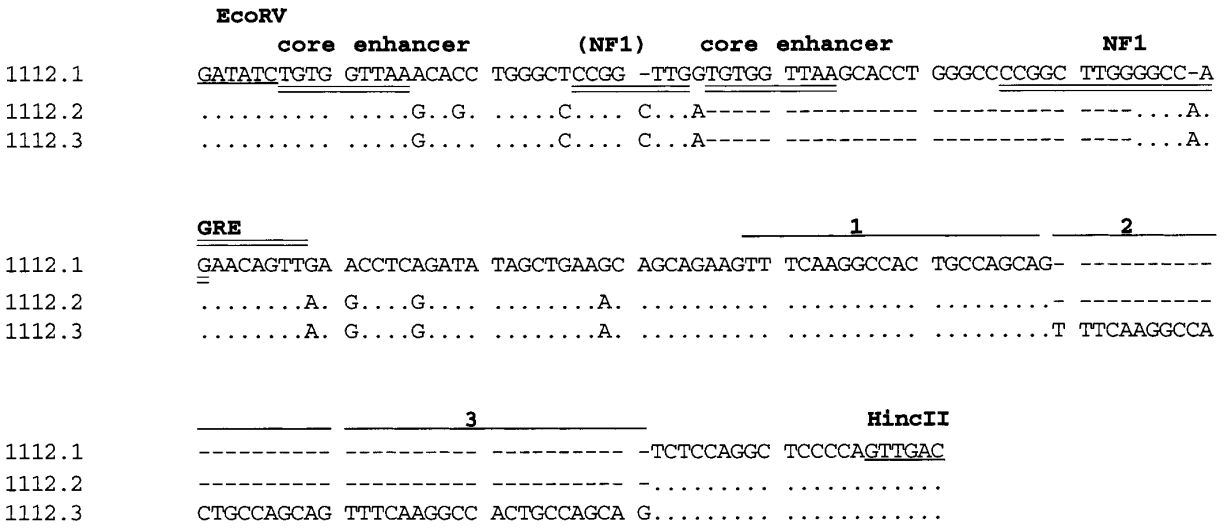

FIG. 4. Nucleotide sequence of FeLV LTRs amplified by PCR from a naturally occurring feline tumor, shown between the conserved *Eco*RV and *Hin*cII sites at positions 117 and 232, respectively, in the LTR of FeLV-A/61E (Donahue *et al.,* 1988). Three distinct LTRs that were amplified (1112.2, 1112.3) or cloned directly (1112.1; Levesque, 1990) from the DNA of tumor 1112 are shown. Sequences are compared to that of 1112.1, and only the differences in sequence are shown. Dashes are included to optimize the alignment. Nuclear protein binding sites in the FeLV enhancer are indicated (core enhancer, NF1, GRE; Fulton *et al.,* 1990). A partial NF1 site in the first enhancer repeat is also indicated (NF1).

FeLV proviral integration, as previously described in 4 of 11 (36%) of the tumors examined in this study (Levesque *et al.,* 1990). The coding capacity of the *flvi*-1 locus is not yet known.

More striking is the identification of an LTR of unique structure in 8 of 11 (73%) of non-T-cell non-B-cell lymphomas of the multicentric type (Figs. 3 and 4). The LTR is distinctive in two respects. First, it contains only a single copy of the enhancer. By comparison, FeLV proviruses derived from thymic lymphomas typically contain a tandem direct repeat of two or three copies of the enhancer (Fulton *et al.,* 1990; Matsumoto *et al.,* 1992; Rohn and Overbaugh, 1995). Second, the LTR contains a 21-bp sequence repeated in tandem, beginning 25 bp downstream of the enhancer. This unique LTR, originally described in proviruses integrated at *flvi*-1 in non-T-cell non-B-cell lymphomas of the spleen, has been shown to function preferentially in a primitive, multipotential hematopoietic cell line (Athas *et al.,* 1995). Its repeated isolation, uniquely from non-T-cell non-B-cell lymphomas of the multicentric type, strongly implicates the LTR in the induction in tumors of this phenotype.

FeLV proviruses bearing the 21-bp triplication in the LTR were originally classified as members of FeLV subtype A, on the basis of hybridization to a probe that distinguishes FeLV of subtypes A and B (Levesque *et al.,* 1990). The possibility that triplication-containing FeLV might represent subtype C was subsequently suggested by examination of the LTR sequence (Athas *et al.,* 1995). This distinction is important because FeLV subtype classification is tightly linked to pathogenicity, disease specificity, and presumably to pathogenic mechanism (Neil *et al.,* 1991; Jarrett, 1992). The gp70 protein of FeLV-A can be readily distinguished from FeLV-B and FeLV-C by comparison of amino acid sequence, particularly at the amino terminus. Five variable regions in gp70 harbor most of the mutational changes that distinguish the FeLV subtypes (Neil *et al.,* 1991), the first three of which are known to determine the subgroup classification for FeLV-A and FeLV-C (Rigby *et al.,* 1992; Kristal *et al.,* 1993). To examine the subtype classification, and particularly to evaluate the possibility that triplication-containing FeLV may be related to subtype C, the sequence of the gp70 gene was determined across a region known to encode determinants of subtype (Rigby *et al.,* 1992; Kristal *et al.,* 1993). Examination of this region of the gp70 gene of two independent isolates of triplication-containing FeLV reveals them to be nearly identical to each other, and most closely related to FeLV-A (Fig. 5). It is noteworthy, however, that the gp70 genes of FeLV-945 and FeLV-934, while most closely related to FeLV-A/Glasgow, differ from it to an extent greater than the known FeLV-A isolates differ from each other. For example, FeLV-945 and FeLV-934 differ from FeLV-A/Glasgow at 4 of 36 residues (11%) in variable region 1, and at 2 of 9 residues (22%) in variable region 2. By comparison, three FeLV-A isolates differ across these same regions by only 5 and 0%, respectively, although they were isolated from distant geographical locations over a period of 13 years (Donahue *et al.,* 1988). It has been shown that subtle mutational changes accumulate in the gp70 genes of FeLV proviruses in infected animals, and that such changes may be responsible for biological properties such as receptor affinity, host range, growth kinetics, and pathogenic potential (Kristal *et al.,* 1993; Rohn *et al.,* 1994). Functional studies remain to be performed to evaluate

FIG. 5. Predicted coding sequences of FeLV gp70^{env}, shown between amino acids 44 and 242 of FeLV-A/Glasgow (Stewart *et al.,* 1986). Sequences obtained from triplication-containing FeLV proviruses amplified by PCR (FeLV-934) or cloned directly (FeLV-945) from tumor DNA are shown. Sequences from FeLV-A/Glasgow (Stewart *et al.,* 1986), FeLV-C/Sarma (Riedel *et al.,* 1986), and FeLV-B/Gardner-Arnstein (Elder and Mullins, 1983) are also shown. Sequences are compared to that of FeLV-A/Glasgow, and only the differences in sequence are shown. Dashes are included to optimize the alignment. Indicated are the variable regions vr1–vr3 (Neil *et al.,* 1991), and a pentapeptide sequence (MGPNL) representing the major neutralizing epitope encoded by FeLV *env* (Elder *et al.,* 1987).

the significance of the distinctive gp70 sequence of triplication-containing FeLV.

In summary, these findings delineate a set of molecular events associated with the FeLV-mediated induction of non-T-cell non-B-cell lymphomas of the multicentric type. The oncogenic mechanisms that lead to these tumors can be clearly distinguished from those leading to thymic lymphomas, and involve unique determinants encoded both by the virus and the host. By comparison to the more extensively characterized thymic lymphomas, the most distinctive features include the involvement of (i) a target cell of unknown lineage and differentiation state, histopathologically characterized as lymphoid, (ii) proto-oncogene(s) different from those implicated in thymic lymphomagenesis, and (iii) an FeLV isolate whose LTR and *env* gene bear unique sequence elements. Further characterization of the role and interaction of these features is required to dissect the pathogenic mechanisms of the FeLV-mediated induction of tumors of this type.

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