Feline leukaemia virus (FeLV) is a naturally occurring retrovirus endemic in an outbreeding mammalian species, the domestic cat. The outcome of FeLV infection in nature is variable, including malignant, proliferative and degenerative diseases. Like other natural retroviruses, FeLV is not a single genomic species but is a genetically complex family of closely related viruses subject to selective pressures in the natural host (Neill et al., 1991; Overbaugh & Bangham, 2001; Rezanka et al., 1992). The LTR of FeLV is a region of remarkable genetic variation among natural isolates and LTR variants have been linked to particular disease outcome. For example, FeLV proviruses cloned directly from thymic lymphomas of T-cell origin typically contain two or three tandemly repeated enhancers in the LTR (Fulton et al., 1990; Matsumoto et al., 1992). In contrast, FeLV LTRs derived from non-neoplastic diseases or non-T-cell malignancies typically contain only a single copy of the enhancer (Jackson et al., 1996), but may contain repeated elements elsewhere in the LTR (Athas et al., 1995a; Nishigaki et al., 2002).

We previously reported the isolation of FeLV-945 from non-T-cell lymphomas classified anatomically as multicentric (Athas et al., 1995a; Levesque et al., 1990). The FeLV-945 LTR was found to contain only a single copy of the enhancer followed downstream by a 21 bp sequence triplicated in tandem. Functional studies showed that the 21 bp triplication is a modulator of LTR-driven gene expression, that it confers a replicative advantage in certain cell types and that it encodes binding sites for as-yet-unidentified nuclear proteins (Athas et al., 1995b; Prabhu et al., 1999). In the present report, FeLV LTR sequence variation and function were examined from diseased tissues of naturally infected animals in the cohort of 21 cats from which FeLV-945 was originally identified. The cohort included cats collected from essentially a single veterinary practice in Pasadena, CA, USA, over a period of 6 years (a gift from Dr Murray Gardner). Thus, the cohort represented a geographical and temporal cluster presumably exposed to a similar spectrum of horizontally transmissible FeLV. The objectives of the study were: (i) to evaluate the extent of FeLV LTR variation among naturally infected animals in the cohort; (ii) to determine whether the FeLV-945 LTR was associated uniquely with multicentric lymphoma in the cohort; and (iii) to evaluate functional attributes that may have contributed selective advantage to the predominant LTR variants.

The cohort included four cases of thymic lymphoma, 12 cases of multicentric lymphoma, one case of mast-cell leukaemia, two cases of myeloproliferative disease and two cases of anaemia. Previous studies of the cohort had shown that the thymic lymphomas were clonal tumours of T-cell origin and that the multicentric lymphomas were clonal tumours of unknown cell origin, but which did not contain clonally expanded T cells or B cells (Athas et al., 1995a). To analyse the extent of LTR variation and to determine whether the FeLV-945 LTR was associated uniquely with multicentric lymphoma, genomic DNA from diseased
tissues was amplified by PCR using primers specific for the U3 region of exogenous FeLV (Prabhu et al., 1999). Amplification products of multiple sizes were detected from most animals (data not shown) and products of each size were cloned into plasmids for automated nucleotide sequence analysis. Sequence analysis showed that thymic lymphomas in the cohort uniformly contained LTRs with enhancer duplications that varied in length from 39 to 77 bp. LTRs containing 21 bp repeat elements like that of FeLV-945 were not detected in any of the thymic tumours examined (Fig. 1). Among LTRs with duplicated enhancers, the termini of the enhancer repeat unit were variable; however, the LVb/Ets and core binding sites were conserved within the repeat unit, regardless of its length (Fig. 2a).

To evaluate the functional significance of enhancer duplications of variable length in thymic lymphomas, the U3 region of LTRs containing the most complete repeat unit (74 bp duplication) and the least complete repeat unit (39 bp duplication) were cloned into the firefly luciferase reporter plasmid pGL2-Basic (Promega). The LTR U3 region from FeLV-A/61E was similarly cloned for comparison. FeLV-A/61E is a horizontally transmissible virus containing only a single enhancer unit in U3 (Donahue et al., 1988). The reporter plasmids were then introduced into FEA feline embryonic fibroblasts, FC6.BM feline adherent bone-marrow-derived cells (ATCC CRL-6081), K-562 human malignant multipotential haematopoietic cells (ATCC CCL-243) and Jurkat, a human malignant T-cell line. For reporter gene assays, 2 \times 10^5 cells were deposited in quadruplicate wells of a 24-well dish. The next day, reporter plasmids (400 ng) were introduced by lipid-mediated transfection in the presence of pRL-TK (8 ng) in a 50 : 1 ratio. pRL-TK encodes Renilla luciferase and was used as an internal control for transfection efficiency. Firefly and Renilla luciferase activities were quantified 24 h later using the Dual-Luciferase reporter assay system (Promega). As shown in Fig. 2(b), the activities of LTRs with duplicated enhancers were statistically indistinguishable from FeLV-A/61E in FEA, FC6.BM and Jurkat cells. In K-562 cells, duplication of enhancer of either length conferred a modest (1-7-fold) but statistically significant transcriptional advantage. The activities of LTRs containing 74 bp or 39 bp enhancer duplications were statistically indistinguishable from each other in FC6.BM, K-562 and Jurkat cells. In FEA cells, the 39 bp duplication conferred a modest (1-7-fold) but statistically significant transcriptional advantage over the 74 bp duplication. These findings are consistent with previous reports (Nishigaki et al., 1997; Plumb et al., 1991) and indicate that duplication of the enhancer, while predominant in T-cell lymphomas, offered little transcriptional advantage. Furthermore, a longer, more complete repeat unit conferred no advantage over a shorter one.

LTRs from non-T-cell diseases frequently contained a 21 bp sequence element repeated in two, three or four tandem copies. LTRs containing 21 bp repeats were identified in 7 of 12 multicentric lymphomas and in all cases of...
myeloproliferative disease and anaemia (Fig. 1). Unlike the enhancer duplications, the termini of the 21 bp repeat units were precisely conserved (Fig. 3a). Southern blot analysis was performed to confirm that the LTR amplification products with various numbers of 21 bp repeat elements accurately represented the cohort and were not artefacts of the amplification reaction. Genomic DNA samples from three multicentric lymphomas, two cases of myeloproliferative disorder and one case of anaemia were digested with Sau3AI and KpnI to release an LTR-containing fragment varying in size from 444 to 507 bp, depending on the number of 21 bp elements in the LTR. The fragment was visualized by hybridization to a probe for the LTR U3 of exogenous FeLV (Fig. 3b). For the most part, the findings from Southern blots were consistent with results from PCR amplification. For example, Southern blot analysis of DNA from tumour 1306 (Fig. 3b, lane C) demonstrated LTR-hybridizing bands consistent with two or three copies of the 21 bp repeat, as was observed by PCR amplification. Southern analysis of tumour 945 DNA (Fig. 3b, lane E) demonstrated only an LTR containing three copies of the repeat, also consistent with PCR amplification. In some cases, LTRs identified by PCR amplification were not apparent by Southern blot analysis. For example, Southern blot analysis of DNA from cat 903 (Fig. 3b, lane D) showed a single LTR-hybridizing band consistent with four copies of the 21 bp repeat, whereas PCR demonstrated LTRs with three or four copies. The most likely explanation for this discordance is that some LTRs may be present at a frequency below the limit of detection of the Southern blot, but could be PCR amplified. Overall, the analysis confirmed the presence of FeLV LTRs containing one, two, three or four copies of the 21 bp repeat element.

Triplication of the 21 bp repeat in the FeLV-945 LTR was previously shown to confer a replicative advantage compared with an LTR containing only one copy of the 21 bp sequence (Prabhu et al., 1999). In that study, the LTRs of interest were substituted into an infectious molecular clone of FeLV subtype B and replication was examined in a human haematopoietic cell line. The present study extended those findings by comparing the replication of FeLV LTR variation and disease association
virus with one, two, three or four copies of the 21 bp sequence. The U3 region of FeLV-A/61E was substituted between PstI and HinclII restriction sites with homologous sequences from the LTRs of cats 1306 (two 21 bp repeats), 945 (three 21 bp repeats) and 903 (four 21 bp repeats).

Replication kinetics of recombinant FeLVs bearing one, two, three or four copies of the 21 bp element were then compared in three feline cell lines: FEA embryonic fibroblasts, FC6.BM adherent bone-marrow-derived cells and 3201 T-lymphoid cells. While relatively few cell lines of feline origin are available for study, these lines were chosen to represent tissues in which FeLV is known to replicate in vivo (Rezanka et al., 1992). Plasmid DNA encoding each recombinant provirus (250 ng) was introduced to FEA cells (2 × 10^6 per well) or FC6.BM cells (8 × 10^6 per well) plated the previous day in six-well plates. For 3201 cells, plasmid DNA encoding each recombinant FeLV was electroporated into 4 × 10^6 cells. As a confirmatory approach, 4.5 × 10^6...
3201 cells were infected with each recombinant FeLV normalized for equivalent amounts of reverse transcriptase activity. At regular intervals after transfection, electroporation or infection, culture supernatants were collected for measurement of reverse transcriptase activity (Prabhu et al., 1999). As shown in Fig. 3(c), the impact of the number of 21 bp repeat units varied with the cell type examined. In FEA cells, virus containing two, three or four 21 bp repeat units replicated to statistically significantly higher levels than did virus bearing only a single 21 bp element within the first week after transfection. By day 4, reverse transcriptase levels were 4- to 8-fold higher in cultures containing viruses with 21 bp repeats. In FC6.BM cells, the presence of three or four copies of the 21 bp repeat unit conferred a statistically significant replicative advantage evident within the first week after transfection. By day 8, virus containing three copies of the 21 bp repeat replicated to significantly higher levels than other viruses examined. Virus containing two copies of the 21 bp repeat replicated rather poorly in FC6.BM cells, to levels 6- to 12-fold lower than others at days 4 and 8 after transfection. Considering our previous demonstration that the 21 bp triplication encodes specific binding sites for nuclear proteins in cells in which it confers a replicative advantage (Prabhu et al., 1999), a possible explanation is that the 21 bp duplication does not provide the appropriate spacing and/or sequence for assembly of the relevant transcription factor complex in FC6.BM cells. In 3201 T cells exposed to virus by electroporation or infection, only recombinant virus containing three copies of the 21 bp sequence replicated efficiently. Since LTRs containing the 21 bp triplication were not found in T-cell lymphomas, it was unexpected to observe that the triplication conferred a significant replicative advantage in feline T cells.

In summary, the present report describes natural FeLV LTR variation in diseased tissues from a geographical and temporal cohort. LTRs with duplicated enhancer sequences were isolated uniformly from T-cell tumours in the cohort but were rarely identified in non-T-cell diseases (Fig. 1). In non-T-cell diseases, FeLV LTRs frequently contained tandem repeats of a 21 bp sequence downstream of the enhancer (Fig. 3a). Unlike the enhancer duplication, whose repeat length and termini were variable among animals in the cluster (Fig. 2), the 21 bp repeat unit did not vary in sequence or position in the LTR (Fig. 3a). While LTRs were identified in non-T-cell diseases that contained one, two, three or four copies of the 21 bp sequence, triplication of the 21 bp sequence was observed to confer the optimal replicative advantage (Fig. 3c). This observation is consistent with the predominance of the triplicated form in animals from the cohort (Fig. 1). The most significant replicative advantage was observed in feline T cells, an unexpected finding since the triplication-containing LTR was not detected in T-cell lymphomas. In an experimental situation, in contrast, T-cell lymphomas were readily induced in mice infected with a recombinant Moloney murine leukaemia virus into which the FeLV-945 LTR was substituted (Starkey et al., 1998); thus, the triplication-containing LTR is capable of directing the induction of T-cell lymphomas under some circumstances. Its absence in T-cell lymphomas in naturally infected cats may reflect the influence of the distinctive surface glycoprotein (SU) encoded by FeLV-945 (Athas et al., 1995a). One possibility is that the FeLV-945 SU and LTR act in concert to induce non-T-cell disease in naturally infected animals.

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References


