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Molecular Analysis of Several Classes of Endogenous Feline Leukemia Virus Elements

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Five recombinant DNA clones of endogenous feline leukemia virus-related DNA sequences were isolated by screening a lambda phage genomic library of cat placental DNA with a probe specific to the *gag-pol* region of infectious feline leukemia virus. The clones containing retroviral long terminal repeat-like sequences demonstrated the existence of different size classes of endogenous elements in the cat genome, including those of nearly full length in which the *gag* region is heterogeneous but all of *pol* and most of *env* are highly conserved. Other size classes included elements with major deletions in *gag* or *pol*. A genomic DNA analysis suggested that the majority of endogenous elements were close to full length in size and that the highly truncated sequences which we described previously (Soe et al., J. Virol. 46:829–840, 1983) represented only a subset of the elements present. A restriction analysis of genomic DNA suggested a high degree of conservation in *pol* and the 5′ portion of *env* among the various endogenous sequences present in the cat genome. We also found by using DNA transfection that while all of the endogenous clones were noninfectious, there was differential expression of the elements which we examined. These findings correlate with the subgenomic expression of endogenous feline leukemia virus sequences in cat placental tissue.

Feline leukemia virus (FeLV) is a replication-competent retrovirus which is transmitted by horizontal infection and naturally causes many leukemias, lymphomas, and blastopenic diseases of domestic cats (9, 12, 15). Three subgroups of FeLV (subgroups A, B, and C) can be distinguished on the basis of type-specific neutralization and interference assays and are determined by the viral *env* gene product (36, 37).

The cellular DNA of normal uninfected cats contains sequences that are partially homologous to the genome of exogenous FeLV (1, 2, 28). These endogenous sequences are present in multiple copies dispersed throughout the cat genome and are expressed as subgenomic transcripts in a tissue-specific manner (3, 24, 25, 26), but they are not inducible as infectious viral particles (2, 28). We previously described the structure of a subset of these endogenous sequences isolated from a recombinant cat genomic library (41). These long terminal repeat (LTR)-flanked sequences were approximately 4 kilobase pairs (kb) long (significantly shorter than the cloned infectious FeLV isolates) and contained 3.3- to 3.6-kb deletions in the *gag-pol* region and 0.7- to 1.0-kb deletions in the *env* regions. The conservation of flanking DNA restriction sites among these endogenous retroviral sequences suggests that this family of truncated elements may have evolved as the result of DNA amplification involving both retroviral and flanking sequences.

The presence of additional, longer FeLV-related endogenous sequences was suggested by the results of a genomic DNA analysis with *Eco*RI, which does not cleave any of the known FeLV-related endogenous sequences internally. We reported the existence of higher copy numbers of these higher-molecular-weight bands (41), in agreement with the previous findings of Koshy et al. (18) and Mullins et al. (21). Furthermore, it has been reported that the major dissimilarity between endogenous FeLV-related sequences and exogenous FeLV genomes resides in the unique 3′ (U3) region of the LTRs (4).

A comparison of the nucleotide sequence of the *env* gene of the Gardner-Arnstein strain of FeLV subgroup B (GA-FeLV-B) with the sequences of several previously described murine retroviruses revealed a striking resemblance between the gp70 sequences of FeLV subgroup B and Moloney murine leukemia virus-derived mink cell focus-forming viruses (8). This region of homology is located in the portion of the mink cell focus-forming virus which is derived via recombination from an endogenous murine virus (13). These findings suggest that endogenous *env* sequences may recombine in an analogous manner with exogenous FeLV to form FeLV *env* gene recombinants having potential biological significance. Finally, it has also been hypothesized that activation of the endogenous FeLV-related *env* gene by recombination with exogenous virus gives rise to polymorphic tumor-specific antigens (feline oncornavirus-associated cell membrane antigens) on the surfaces of feline lymphosarcoma cells (40).

These findings reflect the significance of defining the extent of polymorphism and defectiveness among the endogenous FeLV-related sequences in cat DNA. To determine the role of these endogenous retroviral sequences in tissue-specific gene expression and in the generation of *env* recombinants with exogenous FeLV, we analyzed the genetic structures of other subsets of endogenous elements isolated from cat genomes. We demonstrated the existence of several classes of endogenous FeLV sequences in which there is a high degree of conservation of *pol* (when retained) and part of *env*, but significant divergence or defectiveness of *gag* and 3′ *env*-related sequences. DNA transfection studies with NIH 3T3 mouse fibroblast cells showed differential expression of these elements and that transcription from truncated

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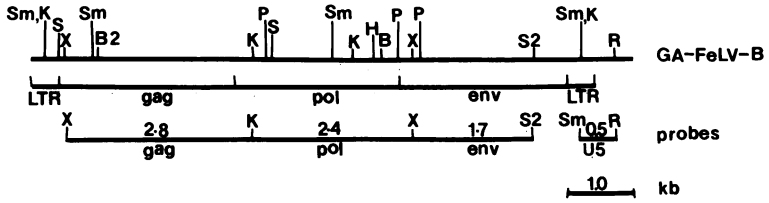

FIG. 1. Hybridization probes for the subgenomic regions of infectious GA-FeLV-B. The restriction map of infectious GA-FeLV-B is from reference 21. The estimated positions of *gag*, *pol*, *env*, and LTRs on the 8.7-kb genome were based upon homology to the data obtained with Moloney murine leukemia virus (11). The hybridization probes for the various subgenomic regions were derived from the DNA fragments indicated. Abbreviations: S2, *Sac*II; Sm, *Sma*I; K, *Kpn*I; S, *Sst*I; X, *Xho*I; B2, *Bgl*II; P, *Pst*I; H, *Hin*dIII; B, *Bam*HI; R, *Eco*RI.

classes was pronounced compared with nearly full-length counterparts. We also demonstrated that the endogenous clones are not infectious in the permissive cell system which we examined.

MATERIALS AND METHODS

Cell and tissues. Placental tissues from cats maintained in an FeLV-free colony were kindly provided by E. Hoover, Colorado State University, Fort Collins. Fresh placental tissues from house cats were obtained from local private-practice veterinarians after spay procedures. Tissue DNA was extracted by the method of Koshy et al. (18).

NIH 3T3 mouse cells were provided by G. M. Cooper, Harvard Medical School, Boston, Mass. HT1080 human fibrosarcoma cells are routinely maintained in our laboratory. NIH 3T3 cells were grown in Dulbecco modified Eagle minimal essential medium (GIBCO Laboratories) supplemented with 5% newborn calf serum. HT1080 cells were grown in minimal essential medium alpha $^+$ (GIBCO) supplemented with 10% fetal calf serum. Both cell lines were grown at 37°C in an atmosphere containing 5% CO_2 in air, and cells were subcultured by using 0.05% trypsin.

Clones. GA-FeLV-B DNAs, which were cloned in Charon 4A as linear, unintegrated DNA intermediates from infected human RD cells (22, 23), were subcloned as various subgenomic regions (*gag*, *pol*, *env*, and U5). The hybridization probes used were derived from the fragments shown in Fig. 1. These clones were generously provided by J. Mullins, Harvard School of Public Health, Boston, Mass.

Snyder-Theilen FeLV subgroup B (ST-FeLV-B) DNA, which was cloned as unintegrated DNA intermediates into the *Eco*RI site of λgtWes. λB, contains deletions in LTR sequences. This clone was obtained from C. Sherr, St. Jude's Children's Research Hospital, Memphis, Tenn. (11, 39).

Endogenous FeLV clone pBCF-14 (41) consisted of the 6.0-kb *Eco*RI fragment of λCF-14 derived from the specific-pathogen free (SPF) cat placental DNA library subcloned into pBR322.

Restriction endonuclease analysis. Restriction enzymes were purchased from Amersham Corp., Bethesda Research Laboratories, Inc., or New England BioLabs, Inc., and were used according to the specifications of the suppliers. DNAs were digested with restriction enzymes, subjected to electrophoresis through 0.7% agarose gels, and transferred to nitrocellulose (Schleicher & Schuell, Inc.) as described by Southern (42). Nick translations and filter hybridizations were performed as described previously (41), except that the formamide concentration was reduced to 40% for genomic DNA blots, and hybridizations were done for 2 to 3 days with 2×10^6 dpm of ^{32}P-labeled FeLV nick-translated probe per ml for genomic DNA studies.

Isolation of recombinant phage. To isolate endogenous FeLV-related sequences, a phage library of SPF feline placental DNA partially digested with *Mbo*I was screened by using a probe homologous to the *gag-pol* region of infectious GA-FeLV-B. This library was highly amplified and contained 15- to 20-kb insertions. The procedures used for construction of the phage library and the procedures used for screening, isolation, and growth of recombinants have been described previously (41).

Transient expression assays. All cloned DNAs used for transfection were digested with *Eco*RI, separated on agarose gels, and electroeluted onto dialysis membranes as described by Yang et al. (50). Recipient NIH 3T3 cells were seeded at a density of 10^6 cells per 100-mm dish (Corning Glass Works). After incubation at 37°C overnight, the cells were treated with DNA by using a previously described modification (47) of the DEAE-dextran transfection method of McCutchan and Pagano (20). The transfection solution (2 ml per dish) consisted of 500 μg of DEAE-dextran per ml and 0.5 μg of DNA per ml in serum-free Dulbecco modified Eagle minimal essential medium. The cells were incubated initially for 1 h in an atmosphere containing 2% CO_2 in air and then for 3 h in an atmosphere containing 5% CO_2 in air. The cells were then given a 10% dimethyl sulfoxide shock for 2 min.

Cells were harvested on day 2 after transfection by scraping into phosphate-buffered saline, and they were then pelleted at 5,000 × *g* for 5 min. Total cellular RNA was extracted by using a modification (35) of the guanidine-sarcosyl procedure of Seeburg et al. (38). The final RNA precipitate was dissolved in water, the concentration was determined by spectrophotometry, and the RNA was stored as ethanol precipitates before analysis. Dot blot hybridizations were carried out as described by Thomas (48). Briefly, nitrocellulose sheets rinsed in water were soaked in 3 M NaCl–0.3 M sodium citrate. The sheets were then dried, and 1-μl portions of different dilutions of total cellular RNA were spotted onto the filters. The filters were baked at 80°C for 2 h under a vacuum, and hybridizations were carried out as described by Wahl et al. (49), using radiolabeled nick-translated hybridization probes at concentrations of 2×10^6 dpm/ml. The intensities of spots were determined by density scanning of the autoradiogram with a model GS300 scanning densitometer (Hoefer Scientific Instruments).

Infectivity assays. The transfected DNA consisted of gel-purified *Eco*RI fragments of cloned DNAs. Recipient human HT1080 cells were treated with DNA by using a previously described modification (7, 46) of the calcium phosphate coprecipitation technique of Graham and Van der Eb (10). Supernatants were first harvested on day 14 after transfec-

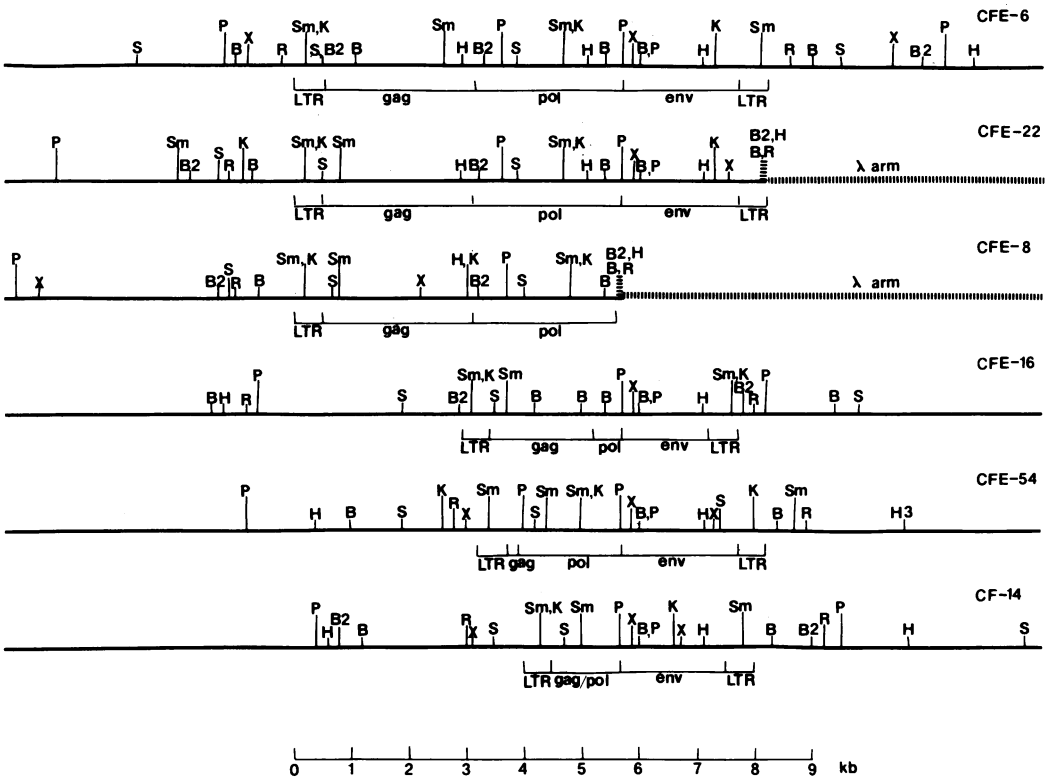

FIG. 2. Restriction map comparison of endogenous FeLV DNA sequences from lambda DNA clones. Restriction sites were mapped after digestion with the enzymes indicated above the lines or with either *Eco*RI or *Bam*HI, and the fragments were analyzed by gel electrophoresis and Southern transfer techniques. The orientation of the restriction maps is from 5′ to 3′ with respect to viral RNA. Each map shows the approximate locations of subgenomic regions which were assigned on the basis of hybridization by using the GA-FeLV-B *gag*-, *pol*-, *env*-, and U5-specific probes described in the text. Lengths are indicated (in kilobase pairs). Abbreviations: B, *Bam*HI; B2, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; S, *Sst*I; Sm, *Sma*I; X, *Xho*I.

tion, and reverse transcriptase assays were carried out as previously described (34).

RESULTS

Cloning of recombinant phage containing endogenous FeLV-related sequences. Screening of a genomic library derived from SPF cat placental DNA by using a GA-FeLV-B *env* probe resulted in the isolation of several highly deleted copies of endogenous FeLV-related sequences (41). These clones demonstrated significant deletions in the *gag-pol* region but only a small deletion in *env*. The results of genomic studies suggested the presence of additional endogenous elements larger than the truncated elements, which were not detected in the initial screening. To select for these latter clones, we screened the library with a hybridization probe specific for the *gag-pol* region of infectious GA-FeLV-B (a 5.2-kb *Xho*I fragment of plasmid pKHM1) (41). We obtained 54 FeLV *gag-pol*-positive clones, which were designated lambda clones CFE-1 through CFE-54, and extensively characterized 5 distinct clones by restriction analysis.

Structure of the endogenous FeLV-related sequences. Restriction endonuclease cleavage maps of the five selected clones were prepared by digesting cloned DNAs with various restriction enzymes singly or in combination with *Eco*RI or *Bam*HI. The fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to subgenomic probes specific to the *gag*, *pol*, *env*, and U5 regions of infectious GA-FeLV-B (Fig. 1). The restriction maps constructed for the five lambda DNA clones are shown in Fig. 2.

Endogenous FeLV-related sequences were present in several size classes, which differed as a result of the loss of large segments of specific viral protein-encoding genes. The nearly full-length isolates, as judged by the distance between U5-hybridizing fragments and the positions of analogous *Sma*I and *Kpn*I sites located in the R region of the exogenous FeLV LTR, were approximately 8.2 kb long and were contained in 8.8-kb *Eco*RI-hybridizing fragments (clones CFE-6 and CFE-22). Another clone (clone CFE-8) possessed only the 5′ one-half of the genome artifactually truncated by the cloning procedure and correspondingly contained only 5′ LTR-related sequences, *gag*, and *pol*. Clones CFE-6, CFE-8, and CFE-22 represented the most complete endogenous proviral elements, and we estimated the positions of *gag*, *pol*, and *env* genes to be at 1.0 to 3.2, 3.2 to 5.7, and 5.7 to 7.7 kb, respectively. These values closely corresponded to the values for a full complement of *gag*, *pol*, and *env* regions which have been described for infectious ST-FeLV-B (11, 39).

Another size class of endogenous elements was represented in clone CFE-16. Clone CFE-16 was also located in an 8.8-kb *Eco*RI-hybridizing fragment but contained a significant deletion in the *pol* region. The proviral element was 4.8 kb long and hybridized to a *pol*-specific probe in a region spanning only 0.5 kb. This *pol* deletion accounted for the major length difference between clone CFE-16 and clones CFE-6 and CFE-22, although variations in the ordering of

restriction sites in the *gag* gene were also noted. The structure of this clone suggested that there may be considerable heterogeneity in FeLV structure within *Eco*RI fragments similar in size.

A third size class of endogenous FeLV sequences was represented in clone CFE-54. This isolate possessed a 5.0-kb proviral element contained in a 6.1-kb *Eco*RI fragment. A 0.5-kb deletion in *pol* and a 2.3-kb deletion in *gag* accounted for the entire length difference between this clone and the higher-molecular-weight isolates.

A comparison of the five clones described above indicated that there was considerable conservation of sequences spanning the *pol* gene and most of the *env* gene. With the exception of clone CFE-16, which contained a large deletion of *pol*, all of the clones demonstrated a conserved ordering of restriction sites from the *Pst*I site on the 5′ border of *pol* to the *Hin*dIII site on the 3′ end of *env*. In contrast, there was greater variation in the restriction maps of the *gag* region among the five isolates. When clone CFE-6 was used as the prototype, clones CFE-16 and CFE-22 differed at the *Bam*HI, *Bgl*II, and *Sma*I sites, and clone CFE-16 had a 0.4-kb deletion; clone CFE-8 differed at *Bam*HI, *Bgl*II, *Sma*I, and *Xho*I sites; and clone CFE-54 contained a deletion of almost the entire *gag* region. Similarly, the 3′ end of *env* was heterogeneous with respect to the size of the deletion and the presence or absence of *Kpn*I and *Xho*I restriction sites. These results suggested that the major heterogeneity among the endogenous FeLV elements was due to the loss of some restriction sites and to larger sequence differences in the regions corresponding to *gag* and the 3′ ends of *env*-related genes.

Comparison with the highly truncated endogenous FeLV sequences. We previously detected the presence of highly incomplete endogenous sequences by screening the cat genomic library with an *env*-specific probe (41). Four distinct clones were characterized and found to demonstrate a high degree of homogeneity among the restriction sites mapped within the 6.0-kb *Eco*RI fragment. A comparison between these highly truncated elements and the five clones described above (Fig. 2) indicated that, as previously deduced, the truncated elements (e.g., clone CF-14) contained extensive deletions in *gag-pol* and a smaller deletion in *env* compared with the nearly full-length endogenous sequences. The ordering of the restriction sites mapped to the boundary between *pol* and *env* (*Bam*HI, *Pst*I, and *Xho*I) and the *env* gene (*Hin*dIII and *Kpn*I) corresponded well with the maps of clones CFE-6 and CFE-22. The *Sst*I and *Sma*I sites at the 5′ end of the truncated proviral element also appeared to correspond to the 5′-most end of the *gag* gene in clones CFE-16, CFE-22, and CFE-8. These structural analyses were consistent with the hypothesis that the *gag-pol* deletions of clone CF-14 were the result of a single large deletion extending from approximately 2.0 to 5.6 kb from the 5′ end of the proviral element of clone CFE-22, for example. This 3.6-kb deletion would have accounted for the entire length difference between the *gag-pol* regions of the truncated class of endogenous sequences and the nearly full-length sequences. Hence, within the limits of restriction mapping, the large degree of relatedness between clones CF-14 and CFE-22 suggested that the truncated elements were derived from the nearly full-length endogenous sequences.

We previously observed (41) the conservation of restriction sites mapped to the immediate flanking cat cellular sequences contained within the *Eco*RI fragment of the highly truncated endogenous elements. In the five clones isolated which were less defective in structure, we noted unique restriction sites corresponding to flanking cellular sequences. This divergence in flanking sequences confirmed their identification as discrete genetic loci.

Comparison of endogenous FeLV elements with infectious FeLV. The restriction maps of the five endogenous FeLV clones were compared with the structure of two natural infectious FeLV subgroup B isolates, GA-FeLV-B (23) and ST-FeLV-B (39). The two exogenous viruses demonstrated greater conservation in the *pol* gene and at the boundary between *pol* and *env*. Similarly, we observed conservation in the ordering of restriction sites at the *pol-env* boundary between the endogenous elements and both exogenous isolates. At a position approximately 6.0 kb from the 5′ end of the infectious FeLV genome, we identified internal *Bam*HI and *Xho*I cleavage sites and two internal *Pst*I sites separated by approximately 0.4 kb. The same pattern of restriction sites was observed in two nearly full-length endogenous FeLV clones (clones CFE-6 and CFE-22) and at an analogous position approximately 6.0 kb from the 5′ end of the endogenous genome. Similarly, this pattern of cleavage sites was present in each of the other three endogenous clones at positions corresponding roughly to the boundary between *pol* and *env*. The conservation in *pol* was evidenced by the maintenance of restriction sites from 5′ to 3′ consisting of *Pst*I sites and closely paired *Kpn*I and *Sma*I sites about 1.0 kb downstream, followed by *Hin*dIII and *Bam*HI sites separated by 0.3 kb. The same pattern was observed in both exogenous isolates and all of the endogenous isolates except clone CFE-54, which exhibited heterogeneity in *pol*. Hence, sequences shared among exogenous FeLV isolates were conserved among the endogenous clones as well.

The *Sma*I and *Sst*I restriction sites at the 5′ end of *gag* in most of the endogenous clones corresponded with similar sites at an analogous position in GA-FeLV-B. Similarly, the *Hin*dIII and *Kpn*I sites in the middle of *env* were observed in many of the endogenous FeLV isolates and in infectious ST-FeLV-B. This analysis suggested that the basic structure of the full-length endogenous FeLV sequences was similar to a mosaic of 5′ GA-FeLV-B and 3′ ST-FeLV-B sequences. It should be noted, however, that there was a higher degree of relatedness among the nearly full-length endogenous clones than between the two cloned infectious proviral DNAs. Additional heterogeneity was observed in most of the *gag* and 3′ *env* sequences of the endogenous FeLV corresponding to the regions of divergence from the exogenous FeLV. In terms of LTR structure, the endogenous FeLV elements were more closely related to GA-FeLV-B (23), as shown by the absence of *Eco*RI sites. However, the paucity of mapped restriction sites in this region limited the analysis to this general comparison at this time.

Analysis of genomic DNA for endogenous FeLV sequences. To determine the relative proportion of a particular class of endogenous elements among the multiple endogenous loci present in normal cat DNA, we digested total cat placental DNA with an excess of various restriction enzymes and analyzed the Southern blots with subgenomic FeLV probes. The results of this analysis are shown in Fig. 3 through 5.

The results of hybridization of cat placental DNA to the *pol*-specific probe confirmed the high degree of conservation of the *pol* region among the endogenous FeLV elements. Cleavage with *Bam*HI (Fig. 3, lane 1) yielded several major hybridizing fragments. The 0.6-kb *Bam*HI band corresponded to the boundary between *pol* and *env* and was conserved among the nearly full-length endogenous clones, as well as *pol* deletion copy clone CFE-16. Except for clone CFE-6, which contained a single *Bam*HI site 0.7 kb from the

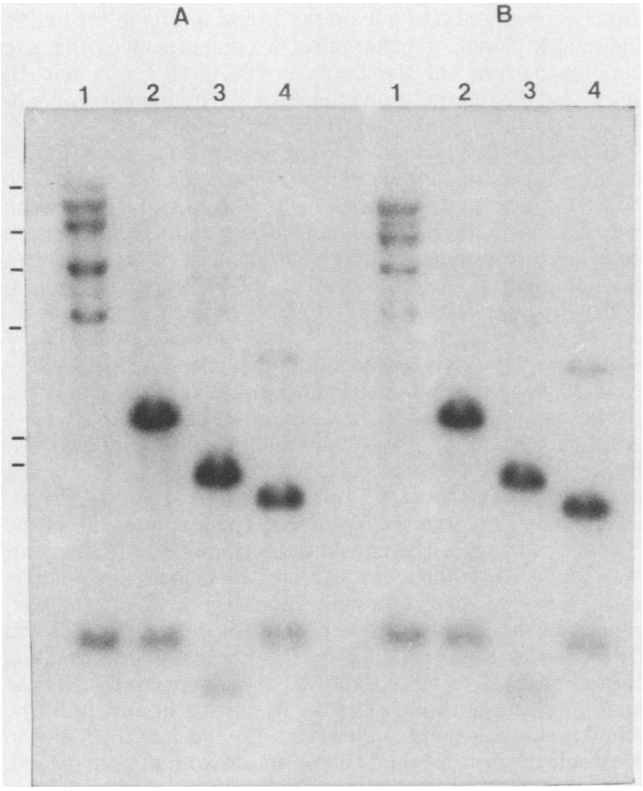

FIG. 3. Genomic analysis of uninfected cat placental DNA for endogenous FeLV *pol*-specific sequences. SPF (A) and household (B) cat DNAs were subjected to restriction digestion, gel electrophoresis, and Southern blotting as described in the text. Lane 1, *Bam*HI; lane 2, *Bam*HI-*Hin*dIII; lane 3, *Bam*HI-*Pst*I; lane 4, *Bam*HI-*Sst*I. On the left the positions of the fragments in a *Hin*dIII digest of wild-type lambda DNA used as a molecular weight marker (23.0, 9.4, 6.6, 4.4, 2.3, and 2.0 kb, from top to bottom) are shown.

5′ end of the *gag* gene, all of the endogenous isolates contained the *Bam*HI site in 5′ flanking cellular sequences. The highly truncated class of endogenous elements (clone CF-14), *gag* deletion copy clone CFE-54, and nearly full-length clone CFE-6 containing the additional internal *Bam*HI site were all predicted to generate a 4.6-kb fragment. The intensity of this band was consistent with a representation of approximately one-fourth of the elements present in the cat genome. Nearly full-length endogenous isolates CFE-22 and CFE-8 generated a 6.2-kb band which was also roughly one-fourth of the endogenous elements, and three higher-molecular-weight bands appeared to account for the remaining endogenous elements. This finding confirmed that 5′ flanking regions were heterogeneous for the different loci.

The results of double digestion of genomic DNA with *Bam*HI and *Hin*dIII (Fig. 3, lane 2) emphasized the strong conservation of the *pol* region. The major 0.6-kb *Bam*HI fragment remained unchanged, and, in addition, since *Hin*dIII cleaved the boundary between *gag* and *pol*, a prominent 2.6-kb band was observed, which together with the 0.6-kb *Bam*HI fragment spanned the entire *pol* gene. The presence of this 2.6-kb *Hin*dIII-*Bam*HI fragment suggested that the majority of the endogenous elements in the cat genome contained the full complement of the *pol* gene, as predicted from the structure of nearly full-length clones CFE-6, CFE-8, and CFE-22. Faint bands (visible in the original autoradiogram) at 5.0 and 5.5 kb corresponding to the predicted sizes of deleted endogenous sequences suggested that these elements were present at low copy numbers.

Similarly, double digestion with *Bam*HI and *Pst*I (Fig. 3, lane 3) generated the predicted 1.8- and 0.3-kb fragments, and *Bam*HI-*Sst*I digestion (lane 4) yielded the major 1.5- and 0.6-kb fragments. These fragments corresponded to the sizes expected from the nearly full-length endogenous isolates as well as *gag* deletion element clone CFE-54. This finding confirmed restriction site conservation in *pol*, as both *Pst*I and *Sst*I sites were internal to the *pol* gene, and the contention that the majority of the sequences present were complete in the *pol* gene. Faint higher-molecular-weight bands corresponding to the fragments predicted from the highly truncated class of endogenous elements were clearly observed on autoradiograms exposed for a longer period of time, confirming their presence at lower copy numbers. The exact copy numbers of these deleted elements were difficult to assess since their diminished intensities reflected both low numbers and smaller areas of homology to *pol*. The faint band at 3.6 kb in the *Bam*HI-*Sst*I digestion pattern represented sequences spanning the 5′-most portion of the *pol* gene and most of the *gag* gene of the nearly full-length endogenous clones, and its presence suggested partial conservation in *gag* structure as well.

A comparison of the hybridization patterns of two cat DNAs (Fig. 3A and B) demonstrated that there was minimal heterogeneity among individual cats with respect to the *pol* homologous region. Hence, these findings and the findings

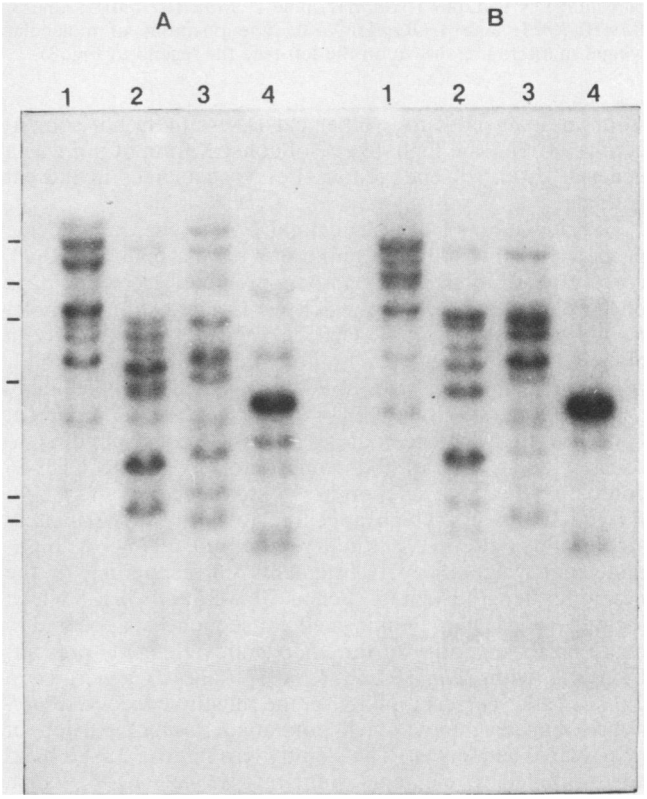

FIG. 4. Restriction analysis of genomic DNA for FeLV *gag*-specific sequences. (A) SPF cat DNA. (B) Household cat DNA. Lane 1, *Bam*HI; lane 2, *Bam*HI-*Hin*dIII; lane 3, *Bam*HI-*Pst*I; lane 4, *Bam*HI-*Sst*I. The positions of molecular weight markers are shown on the left (see the legend to Fig. 3).

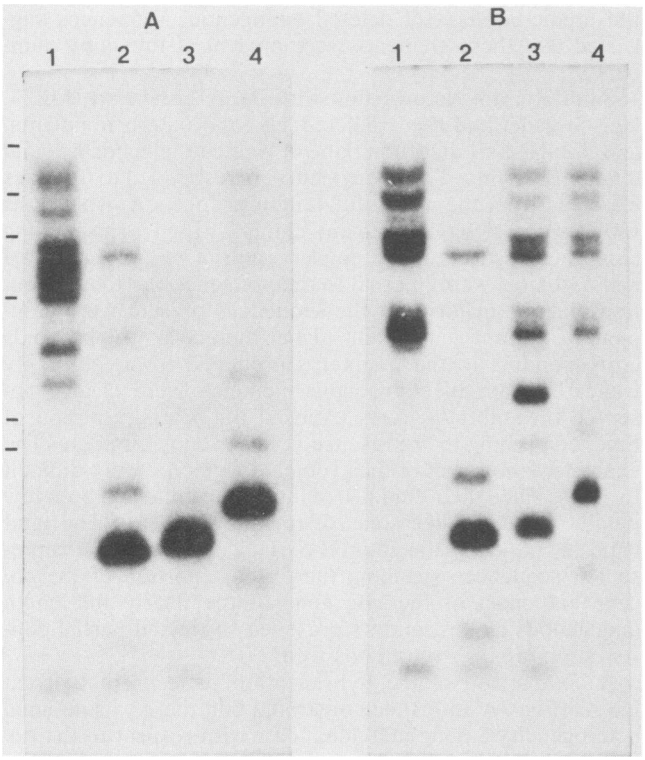

FIG. 5. Genomic hybridization of cat DNA to an FeLV *env*-specific probe. (A) SPF cat placental DNA. (B) Household cat placental DNA. Lane 1, *Bam*HI; lane 2, *Bam*HI-*Hin*dIII; lane 3, *Bam*HI-*Kpn*I; lane 4, *Bam*HI-*Xho*I. The positions of molecular weight markers are shown on the left (see the legend to Fig. 3).

from an analysis of four other cat DNAs (data not shown) confirmed that the high degree of conservation of *pol* was a general feature of endogenous FeLV sequences in the cat genome.

Hybridization of cat placental DNA to the *gag*-specific probe (Fig. 4) resulted in numerous bands, many of which corresponded in size to fragments predicted from the maps of the endogenous clones which we isolated. For example, *Bam*HI digestion (Fig. 4, lane 1) cleaved *gag* internally in clone CFE-6 to produce a 4.6-kb band on the genomic blot. A fragment similar in size would be expected from the highly truncated class (clone CF-14), and hence this fragment appeared to be present at an intensity corresponding to roughly one-fourth of the copies present. Clone CFE-16 containing the large deletion in *pol* produced 0.8- and 5.6-kb *Bam*HI fragments which appeared to be at approximately single-copy intensity. Finally, the clustering of high-molecular-weight *Bam*HI fragments corresponding to the nearly full-length isolates, such as CFE-22 and CFE-8 which located *Bam*HI to 5′ flanking cellular sequences, appeared to make up the majority of the endogenous elements present. Cleavage with *Bam*HI and *Hin*dIII (lane 2) generated a 2.6-kb band corresponding to the highly conserved *pol*-hybridizing fragment which contained a small portion of *gag*-related sequences. The slightly less intense 2.2-kb band corresponded to elements similar to clone CFE-6, which contained the internal *Bam*HI site. Digestion with *Bam*HI and *Pst*I (lane 3) generated numerous bands corresponding to heterogeneous *Pst*I sites in 5′ flanking sequences. Finally, digestion with *Bam*HI and *Sst*I (lane 4) yielded an intense 3.8-kb band. This fragment resulted from cleavage of the largely conserved *Sst*I site on the 5′ end of *gag* in the nearly full-length clones. Furthermore, a comparison of the *gag* banding patterns of the two cat DNAs (Fig. 4A and B) suggested that greater polymorphism existed among individual cats in this region than in the *pol* region.

Genomic blots hybridized to the *env*-specific probe (Fig. 5) demonstrated the high degree of conservation of the 0.6-kb *Bam*HI fragment corresponding to the boundary between *pol* and *env*. Other higher-molecular-weight bands (Fig 5, lane 1) corresponded to fragments generated from heterogeneous *Bam*HI sites in 3′ flanking DNA sequences. Digestion with *Bam*HI and *Hin*dIII (lane 2) illustrated the conserved *env* region, as *Hin*dIII cleaved the endogenous FeLV sequences internally to generate the characteristic 1.2-kb band. Digestion with *Bam*HI and *Kpn*I (lane 3) generated the 1.3-kb band predicted for endogenous FeLV clones CFE-6 and CFE-22 and the 0.8-kb band predicted for clone CF-14. In Fig. 5B, lane 3, there is also a 2.1-kb band corresponding to the fragment expected from clones CFE-16 and CFE-54, which lacked the internal *Kpn*I site. The low intensity of the 2.1-kb band suggested that, like the clone CFE-16 element, the clone CFE-54 element also probably occurs as a single copy. Finally, digestion with *Bam*HI and *Xho*I (lane 4) generated the 1.5-kb band expected for clones CFE-22 and CFE-54 and minor 3.1-, 2.7-, 1.0-kb bands representing clones CFE-16, CFE-6, and CF-14, respectively. These findings suggested that while the 5′ portion of *env* up to the *Hin*dIII site was highly conserved, greater heterogeneity in 3′ structure was observed. As observed with the *gag* probe, the *env* region banding pattern demonstrated more variation among individual cats than the *pol* pattern (see Fig. 5A and B).

Transient expression assay of transfected exogenous and endogenous FeLV DNAs. We showed previously by an RNA gel analysis (3) that the predominent FeLV-related polyadenylated RNA transcript from the steady-state RNA pool of uninfected cat placentas was homologous to the *env*-specific region. The inability to detect genomic length endogenous FeLV RNA transcripts in cat placentas suggested that there is differential expression of these elements. To determine the relative levels of expression of the various cloned endogenous isolates, we carried out DNA-mediated transfection studies.

NIH 3T3 cells were chosen as recipient cells for transfection because they do not spontaneously produce ecotropic or xenotropic endogenous murine leukemia virus (45) and because they are good recipients of exogenous DNA. Furthermore, the infectious FeLV LTR is known to function in NIH 3T3 cells (5). In transfection assays of FeLV DNAs, donor DNAs isolated from recombinant phage or plasmids propagated in bacteria were used. These DNAs consisted of *Eco*RI-digested and gel-eluted fragments of cloned isolates in which a minimum amount of flanking cellular sequences remained. We inoculated duplicate cultures with 1.0-μg portions of a given donor DNA. The transient expression level of virus-related transcripts in DNA-treated recipient cell cultures was determined by a dot blot analysis of the total cellular RNA extracted from the cultures on day 2 after transfection. The dot blots consisted of serial dilutions of RNA spotted in 2-, 1-, 0.5-, and 0.1-μg amounts. Exogenous FeLV RNA isolated from human RD cells chronically infected with ecotropic FeLV (27) was spotted as an additional positive control for hybridization.

The results of the dot blot analysis are summarized in Table 1. Briefly, GA-FeLV-B DNA served as a positive control and exhibited abundant transcripts hybridizing to

TABLE 1. Levels of FeLV RNAs in NIH 3T3 cells transfected with FeLV DNA

Transfected DNA[a]	Proviral size (kb)	Relative level of FeLV RNA[b]		
		gag	*pol*	*env*
Exogenous FeLV				
GA-FeLV-B	8.8	1.00	1.00	1.00
ST-FeLV-B[c]	8.5	0.19	0.23	0.14
Endogenous FeLV				
CFE-6	8.2	0.00	0.00	0.00
CFE-22	8.2	0.08	0.00	0.00
CFE-16	4.8	1.43	1.74	2.11
CF-14	4.0	0.46	0.42	1.32
DEAE-dextran		0.00	0.00	0.00

[a] A 1.0-μg portion of the gel-purified *Eco*RI fragment of each clone (per 2×10^6 cells) was used for transfection.
[b] The autoradiographic density values for GA-FeLV-B were normalized, and the other data are presented relative to GA-FeLV-B expression. The actual density values were 1.46, 0.94, and 1.60 for GA-FeLV-B *gag*, *pol*, and *env*, respectively.
[c] ST-FeLV-B DNA contains a deletion in LTR sequences (11).

gag-, *pol*-, and *env*-related sequences; on the assumption that these GA-FeLV-B *gag*, *pol* and *env*-related transcripts were present in equal amounts, we normalized the values of 1.00 to account for variations in intensity resulting from different hybridization probes. Other transfection results were adjusted based upon these GA-FeLV-B hybridization values to allow a rough comparison among the intensities observed with the three probes. However, a quantitative analysis of the data was not possible due to variations in the extent and degree of homology between the RNAs and the probes. ST-FeLV-B DNA, which contains deletions in LTR sequences (11), demonstrated approximately fivefold-lower levels of hybridization to the three subgenomic probes than GA-FeLV-B did. Since this exogenous clone is missing the putative enhancer portion of the U3 region of the LTR, the lower level of transcription observed is consistent with its known structure (19). Among the endogenous isolates assayed, we found low or no detectable hybridization of FeLV-related sequences to RNAs isolated from cells transfected with DNAs from nearly full-length isolates CFE-6 and CFE-22. In contrast, clone CFE-16, containing a large deletion in *pol*, demonstrated abundant transcripts hybridizing to *gag*, *pol*, and *env*. The strong hybridization signal obtained with the *pol* probe reflected an elevated expression of transcripts containing the truncated *pol* gene of clone CFE-16, as well as a high degree of conservation of *pol* sequences. Elevated levels of *env*-related transcripts were also detected for highly truncated clone CF-14, whereas *gag* and *pol* expression was relatively lower. Finally, controls without DNA or without DEAE-dextran and DNA expressed no RNA that showed detectable hybridization to the three subgenomic FeLV probes. These findings suggest that there is differential expression of the endogenous elements and that transcription from the truncated classes is elevated compared with the nearly full-length classes.

Infectivity analysis of transfected cloned DNA. The endogenous FeLV-related sequences in the uninfected cat genome have been reported to be uninducible as infectious viral particles (2). To determine whether the individual cloned endogenous sequences, especially the nearly full-length elements, demonstrated this lack of infectivity, we carried out DNA-mediated transfection studies in permissive human cell line HT1080. *Eco*RI-digested and gel-purified insertions from various exogenous and endogenous cloned isolates were inoculated into HT1080 cells at a level of 0.5 μg of DNA per dish. Virus production was first measured 2 weeks after transfection by assaying for reverse transcriptase activity in the culture fluid (34).

TABLE 2. Infectivity of cloned FeLV DNA

Recombinant clone	Reverse transcriptase activity (cpm [$\times 10^4$] per ml of supernatant) at:		
	2 weeks	4 weeks	8 weeks
GA-FeLV-B	14.31	18.91	19.39
ST-FeLV-B	0.01	0.01	0.05
CFE-6	0.02	0.01	0.00
CFE-22	0.02	0.02	0.00
CFE-16	0.01	0.01	0.00
CF-14	0.00	0.01	0.00
Carrier DNA only	0.02	0.01	0.00
RD-FeLV[a]	14.47	17.83	8.34

[a] Positive controls for the polymerase assay consisted of supernatants from human RD cells infected with GA-FeLV-B.

The results of these infectivity assays are summarized in Table 2. GA-FeLV-B DNA (22)-transfected cells and the human RD cell line infected with GA-FeLV-B (27) served as positive controls. Exogenous ST-FeLV-B containing deletions in LTR sequences was noninfectious, as were all of the endogenous clones examined. In each negative case, the reverse transcriptase activities were at least 400-fold lower than those observed with GA-FeLV-B and were comparable to the background levels observed in the controls, which were inoculated with carrier DNA alone. These results confirmed the noninfectivity of the endogenous FeLV sequences tested and are consistent with the noninducibility of these sequences from uninfected cat cells.

DISCUSSION

Several classes of endogenous FeLV-related elements were isolated from a cat genomic library by screening with a probe specific for the *gag-pol* region. These classes included clones that were nearly full-length (clones CFE-6 and CFE-22), as judged by homology to the exogenous FeLV isolates. In contrast to the highly truncated class of endogenous elements isolated previously (e.g., clone CF-14) (41), which contained extensive deletions in *gag-pol* sequences and a smaller deletion in *env*, several of the new isolates possessed nearly full-length complements of sequences homologous to the three major protein-encoding genes.

Five of the clones isolated demonstrated the existence of

TABLE 3. Size classes of cloned endogenous FeLV elements

Size of *Eco*RI fragment (kb)	Genome size (kb)	Major characteristic	Clone(s)
8.8	8.2	Nearly complete[a]	CFE-6, CFE-8, CFE-22
8.8	4.8	Deletion in *pol*	CFE-16
6.1	5.0	Deletion in *gag*	CFE-54
6.0	4.0	Deletion in *gag-pol*[b]	CF-4, CF-5, CF-14,CF-19

[a] These clones are nearly full length in all internal viral genes but exhibit a large degree of *gag* gene heterogeneity.
[b] Characterization of these clones has been described previously (41).

different size classes of endogenous FeLV elements in the cat genome (Table 3). These elements included the members of the predominant group, which were nearly full-length (8.2 kb) and in which the *gag* region was heterogeneous but all of *pol* and most of *env* were highly conserved. Another size class identified was 4.8 kb long (clone CFE-16) and contained a major deletion in the *pol* gene. A third size class was 5.0 kb long (clone CFE-54) and contained the major deletion in the *gag* gene. The latter two deleted clones appeared to be present in low copy numbers, perhaps as single-copy elements, although quantitation of hybridization intensity was complicated by the extent of the deletions in the elements and the degree of homology with the probes used. In addition, the fourth size class corresponded to the highly truncated endogenous copies (clone CF-14) in which significant deletions in *gag-pol* and part of *env* were observed. These results indicate that there is considerable heterogeneity in endogenous FeLV structure and at least partially define groups of variant structures.

An analysis of the five clones indicated that *pol* and the 5′ 1.2-kb portion of *env* up to the 3′ internal *Hin*dIII site are highly conserved. In contrast, *gag* and the 3′ end of *env* are heterogeneous in structure. When cat genomic DNA is analyzed by Southern blot techniques, using enzymes which recognize conserved sites within the various cloned isolates, a pattern of fragments corresponding to the various size classes is observed. The different bands are due to the loss of some restriction sites and to larger sequence differences among the various types of endogenous genes. We observed a high degree of conservation in *pol* and the 5′ portion of *env* among the endogenous elements present in the cat genome. In contrast, approximately one-fourth of the endogenous elements maintained limited conservation in *gag* and the 3′ end of *env*, while the majority of sequences exhibited considerable restriction site heterogeneity in these regions.

These results compare well with regions of the genome which have been conserved among endogenous and infectious FeLV isolates in which *pol* and part of *env* demonstrate a closer restriction site identity. The high degree of conservation of *pol* correlates well with results suggesting a major *pol* gene progenitor for mammalian type C viruses (6). Furthermore, these findings suggest that the homogeneity in *pol* and the 5′ region of *env* is a reflection of the general similarity in this region among the infectious FeLV isolates which originally infected the cat genome and gave rise to the endogenous elements.

Genomic DNA analysis also confirmed previous findings (41) that the majority of endogenous elements are close to full length in size. This suggests that the highly truncated elements only represent a subset of the total endogenous elements present. Also, in contrast to this truncated gene family in which we observed similar restriction maps in DNA flanking endogenous sequences, the nearly full-length isolates demonstrate unique flanking sequences. Hence, these different classes vary in their presumed genesis, as the higher-molecular-weight proviral elements do not appear to have arisen from gene amplification of DNA segments larger than those defined by the limits of the LTRs. However, we cannot rule out the role of other gene duplication events in the generation of nearly full-length endogenous elements.

The existence of different classes of retrovirus-related endogenous sequences in mammalian genomes in which one class consists of nearly full-length proviral structures and the other class is defined by truncated retroviral elements has also been described in other systems. Murine leukemia virus-related human endogenous sequences fall into two major classes, including those that are full length and those that contain deletions in *env* and some *pol* sequences (31). The truncated class is bounded by a tandem array of imperfect repeats and, like the truncated FeLV elements, contains conserved flanking DNA restriction sites, suggesting a process of DNA amplification involving both retroviral and flanking sequences (41, 44). Similarly, endogenous AKR murine leukemia virus-related endogenous sequences in the murine genome are found in several size classes, including those that are full length and those that contain 1- to 2-kb internal sequence deletions in both *pol* and *env* (16, 33). Finally, among the multiple copies of endogenous RD-114 retroviral sequences in the cat genome, a single full-length copy and numerous truncated copies containing variable deletions in *env* have been observed (30, 43).

In contrast to the generally homogeneous endogenous RD-114 reactive band pattern observed in different cats (30, 43), more extensive polymorphism exists among individual cats in sequences corresponding to the endogenous FeLV elements. This sequence polymorphism is more pronounced in regions corresponding to the *gag*- and *env*-related regions and suggests that different evolutionary pressures act upon the maintenance of these two retroviral gene families in the cat genome.

Finally, despite the existence of multiple copies of nearly full-length endogenous proviral elements in uninfected cat genomes, these FeLV-related sequences remain noninducible as infectious viral particles (2). Transfection of DNAs from these endogenous clones into permissive cells confirmed their lack of infectivity. The highly truncated class of endogenous FeLV sequences (41) contained significant deletions in internal genes that necessarily precluded their expression as infectious virus. Similarly, other deleted endogenous elements bearing defects in *gag* or *pol* alone are restricted from infectivity. In contrast, the expression of nearly full-length isolates must be limited by other means. The lack of infectivity of these latter sequences may be due to either structural defects in the internal viral genome or alterations in LTR sequences regulating gene transcription. Alternatively, the presence of negative *cis*-acting control elements in adjacent cellular sequences may interfere with transcription. However, this latter possibility is highly unlikely as most of the cloned DNAs contain only a small segment (less than 1.0 kb) of flanking sequences and, moreover, the truncated classes are transcriptionally active in transient expression analysis.

Since it is clear that many additional nearly full-length FeLV copies are present in the cat genome, our analysis was limited to the subset of sequences which have already been cloned and characterized. However, the results suggesting transcription from the highly truncated class of endogenous elements are consistent with the elevated expression of *env*-related sequences in cat placental tissue (3, 24–26). The finding that the nearly full-length endogenous elements are not efficiently transcribed is also consistent with the inability to induce infectious virus from uninfected cat cells. It is tempting to think that lack of infectivity may be the result of alterations in sequences regulating gene expression (e.g., viral LTRs). On the other hand, LTRs are known to contain enhancers which act in tissue- and species-specific fashion (17). Thus, experimental findings are limited by the recipient cell system used for transfection. Sequence analysis and studies aimed at defining the transcriptional efficiency of the endogenous LTRs will be important in elucidating the relationship among different classes of these elements.

It has been suggested that endogenous retroviruses may

confer a selective advantage on the host animal by protecting it against infectious viruses (14). For example, chickens free of functional endogenous viruses demonstrate an increased susceptibility to infection with exogenous retroviruses (32). The expression of endogenous FeLV *env*-related transcripts in cat placental tissue (3) may similarly reflect a functional role for these sequences in providing a deterrent to transplacental infection. Such a function has been postulated for the *env*-related mRNAs expressed in human term placentas (29), in which it was suggested that the presence of gp70 on the surface of trophoblastic cells might inhibit the adsorption of related infectious retroviruses. In this regard, it is interesting to note the many similarities between feline and human endogenous retroviruses, including the presence of different size classes of endogenous elements and their expression as subgenomic transcripts in placental tissue. Further studies on the structure and expression of these endogenous retroviral sequences may elucidate the biological significance for conservation of these elements in mammalian genomes.

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