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Molecular Cloning and Characterization of Endogenous Feline Leukemia Virus Sequences from a Cat Genomic Library

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Recombinant bacteriophage λ clones from a cat genomic library derived from placental DNA of a specific pathogen-free cat were screened to identify endogenous feline leukemia virus (FeLV) sequences. Restriction endonuclease mapping of four different clones indicates that there are a number of similarities among them, notably the presence of a 6.0- to 6.4-kilobase pair (kbp) *Eco*RI hybridizing fragment containing portions of sequences homologous to the *gag*, *pol*, *env*, and long terminal repeat-like elements of the infectious FeLV. The endogenous FeLV sequences isolated are approximately 4 kbp in length and are significantly shorter than the cloned infectious FeLV isolates, which are 8.5 to 8.7 kbp in length. The endogenous elements have 3.3- to 3.6-kbp deletions in the *gag-pol* region and approximately 0.7- to 1.0-kbp deletions in the *env* region. These deletions would render them incapable of encoding an infectious virus and may therefore be related to the non-inducibility of FeLV from uninfected cat cells and the subgenomic expression of these endogenous sequences in placental tissue. It appears that there is conservation in the ordering of restriction sites previously reported in the proviruses of the infectious FeLVs in sequences corresponding to the *pol* and *env* boundary as well as the region spanning the *env* gene of the endogenous clones, whereas a greater divergence occurs among restriction sites mapped to the *gag* and part of the *pol* regions of the infectious FeLV. Such deleted, FeLV-related subsets of DNA sequences could have originated either by germ-line integration of a complete ecotropic virus followed by deletion, or by integration of a preexisting, defective, deleted variant of the infectious virus.

Feline leukemia virus (FeLV) is a replication-competent type C retrovirus which is horizontally transmitted in domestic cats and has been implicated as a causative agent in naturally occurring leukemias and lymphomas of this species (12). Although the majority of cats with leukemias and lymphomas demonstrate virus-positive status, approximately one-third of these spontaneous lymphoid tumors do not contain infectious FeLV or detectable FeLV antigens (13, 15).

FeLV, like other mammalian replication competent retroviruses (4), contains three genes, *gag*, *pol*, and *env* (in the canonical order 5′ to 3′ of the genomic RNA), which code for the internal structural proteins, reverse transcriptase, and the virion envelope proteins (gp70 and p15e), respectively (4, 32). On the basis of data obtained with Moloney murine leukemia virus, it is estimated that the positions of *gag*, *pol*, and *env* genes on the 8.5-kilobase pair (kbp) DNA of Snyder-Theilen FeLV, subgroup B (ST-FeLV-B), are 1.0 to 3.2 kbp, 3.2 to 5.5 kbp, and 5.5 to 7.5 kbp, respectively, flanked by 0.49-kbp regions of long terminal repeat (LTR) (14, 30).

Cellular DNA from a normal cat is known to contain sequences partially homologous to the genome of the horizontally transmitted FeLV (1, 3, 28). These endogenous sequences are present in multiple copies in the cat genome and appear to be arranged as discrete genetic elements in nontandem fashion (18). There is evidence indicating that a portion of the infectious FeLV genome is absent in these endogenous sequences (27). This latter observation correlates with the fact that these sequences are not inducible as infectious viral particles (3). Furthermore, it has been reported that the sequences at the 3′ end of the exogenous FeLV genomes studied so far (22, 30), notably the unique 3′ (U3) region of the LTRs, are different from the endogenous FeLV-related sequences in domestic cat DNA (7).

Endogenous FeLV-related sequences are expressed as virus-specific RNA species in certain virus-negative lymphomas and in normal placen-

tal tissue in the absence of any FeLV-related *gag* protein detectability (24, 25). RNA gel analysis of the heterogeneous species of polyadenylate-selected RNA from the steady-state RNA pool of the placenta was found to contain sequences primarily homologous to the FeLV *env* region (6). To study the sequence organization and complexity of the various endogenous FeLV-related information present in cat DNA with respect to the exogenously acquired FeLV genome, and to explain the noninducibility of FeLV from uninfected cat cells and subgenomic expression of the endogenous elements, we constructed a phage library of placental DNA from a specific pathogen-free (SPF) cat and screened for FeLV-related sequences. We now describe the cloning of these endogenous sequences in a recombinant phage vector and the characterization of some of these sequences by restriction mapping and Southern blotting.

MATERIALS AND METHODS

Cat tissues. Placental tissues of cats maintained in a FeLV-free colony were kindly provided by J. Rojko and E. Hoover of the Ohio State University, Columbus, Ohio.

Preparation of placental and λJ1DNA for cloning. Tissue DNA was extracted according to the method of Koshy et al. (18). Briefly, minced tissues were homogenized and protein digested with 200 μg of proteinase K per ml in the presence of 1.0% sodium dodecyl sulfate for 3 to 5 h at 37°C. Further deproteinization of DNA with phenol-chloroform was followed by extensive dialysis against a Tris-EDTA buffer. DNA was partially cleaved with the restriction endonuclease *Mbo*I, and the fragments 17 to 22 kbp in size were isolated by rate zonal centrifugation on a sucrose gradient and checked by gel electrophoresis according to the procedure described by Maniatis et al. (20).

Lambda vector J1 was previously constructed (J. I. Mullins, unpublished data) and derived from λL47.1 (19). λJ1 was propagated on KH802 cells by the liquid culture procedure described by Blattner et al. (5). Phage and phage DNA were purified by the methods of Tiemeier et al. (34). Phage DNA was digested with *Bam*HI and separated on a sucrose gradient as described by Maniatis et al. (20).

Construction and identification of hybrid phage. Ligation of *Bam*HI-digested phage arms and size-selected placental DNA fragments was carried out according to the procedures described by Maniatis et al. (20). In vitro packaging of chimeric DNA into phage particles was done according to the procedures of Hohn and Murray (17) and Collins and Hohn (8), with modifications by Mullins et al. (22). Using this method, we obtained a packaging efficiency of 1.3×10^6 PFU per μg of inserted eucaryotic DNA. Assuming an average size of 20 kbp for the insert in the cat DNA library and a genome size of 3×10^9 base pairs, only 1 in 150,000 plaques will be expected to carry a particular single copy sequence. Thus, recombinant phage representing roughly eight genome equivalents were obtained in our cat placental DNA library.

All recombinant phage were grown on KH802 cells, and the library was amplified by subconfluent plate lysis as described by Maniatis et al. (20). The library was repeatedly screened for endogenous FeLV-related sequences, using a hybridization probe specific to the *env* region of the infectious Gardner-Arnstein FeLV, subgroup B (GA-FeLV-B). In situ plaque hybridizations were carried out as described previously (2, 19). Positive plaques were purified, and large-scale preparations of phage were obtained (5, 22). Phage DNA was purified by the methods of Tiemeier et al. (34).

Preparation of hybridization probes. We obtained ST-FeLV-B DNA in λgtWes · λB (30) as a gift from C. Sherr of the National Cancer Institute. Subgenomic probes of FeLV were derived from cloned DNAs (see Fig. 1). The *env* gene region, representing sequences encoding gp70 but not p15e, of the GA-FeLV-B was the 1.7-kbp *Xho*I to *Sac*II fragment of the plasmid clone pBHM-ɔ derived from λHF49 (22); the *gag-pol* region was the 5.2-kbp *Xho*I fragment of plasmid clone pKHM-1 derived from λHF52; the *gag*-specific region was the 5′ 2.8-kbp *Xho*I to *Kpn*I fragment of pKHM-1; the *pol*-specific region was the middle 1.5-kbp *Kpn*I fragment and 3′ 0.9-kbp *Kpn*I to *Xho*I fragment of pKHM-1; and the *U5* region of the infectious FeLV LTR was represented in the 0.55-kbp 3′ *Sma*I to *Eco*RI fragment from the λHF60 (22) clone containing half of the GA-FeLV-B LTR (38 bases R, 72 bases *U5*) plus 3′ flanking human sequences cloned into the plasmid pKHR-9 (7). The plasmid pKHR-9 was provided by A. Roach and N. Davidson. The endogenous FeLV probe used in genomic DNA studies consisted of the 6.0-kbp *Eco*RI fragment of λCF-14 derived from the SPF cat placental DNA library and subcloned into pBR322. All hybrid DNAs were digested with an excess of appropriate restriction endonucleases, indicated above, and the digestion products were electrophoretically separated on agarose gels. DNA bands were visualized by direct staining with ethidium bromide, and desired fragments were purified by electroelution onto dialysis membranes as described by Yang et al. (35). Nick-translated probes were prepared by the procedure of Mullins et al. (22), using [^{32}P]dCTP as the radiolabeled substrate, with specific activities of about 2×10^8 dpm per μg of input DNA.

Restriction endonuclease analysis. All DNAs were digested with restriction endonucleases according to the manufacturers' recommendations (Bethesda Research Laboratories or New England Biolabs). One microgram of DNA was digested for 3 h at 37°C in 30 μl of an assay buffer containing 4 U of restriction endonuclease. Digested DNA samples were mixed with 6 μl of stop reaction buffer (100 mM EDTA [pH 8.2], 25% glycerol, and 0.05% bromophenol blue), and the samples were loaded directly onto gels. Electrophoresis was conducted in 0.7% agarose gels in 40 mM Tris-acetate (pH 7.8)–5 mM sodium acetate–1 mM EDTA. Gels were run at 55 mA for 12 to 24 h. The gels were then soaked in ethidium bromide (1 μg/ml) and photographed under UV illumination. The electrophoretic standard used was *Hin*dIII digests of bacteriophage λ DNA. Transfer of DNA to nitrocellulose and filter hybridizations were carried out according to the general procedure of Southern (31), with modifications by Mullins et al. (22). All hybridizations were done for 16 to 24 h with 10^5 dpm of ^{32}P-labeled FeLV nick-translated probe per ml for restriction mapping.

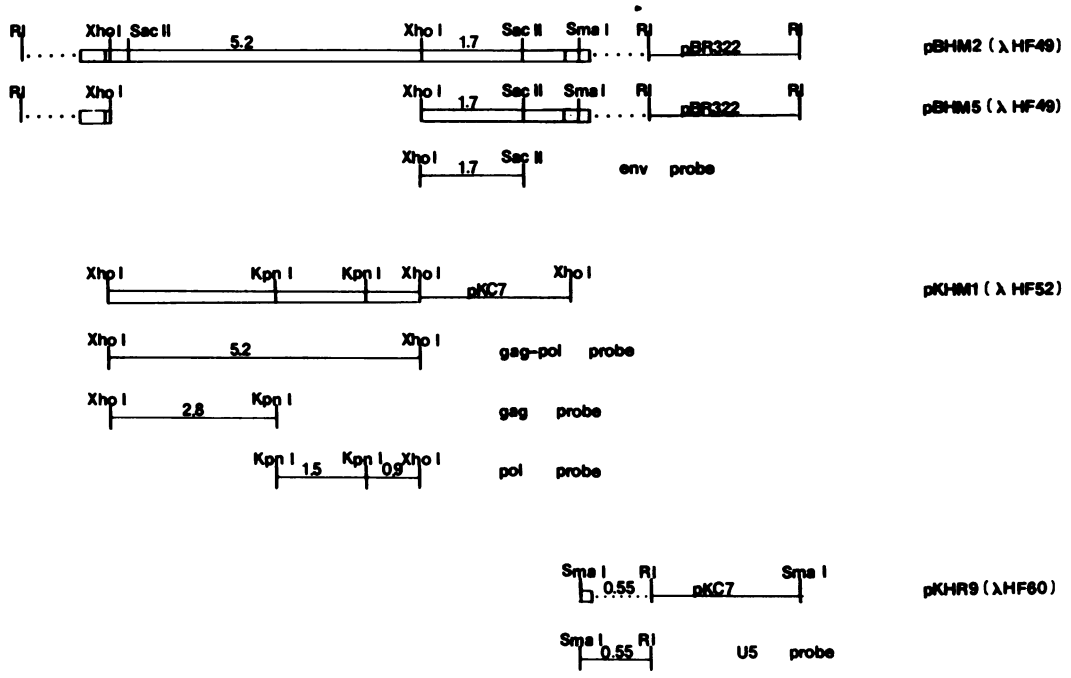

FIG. 1. Construction of FeLV *env*, *gag-pol*, and *U5*-specific hybridization probes. Open boxes represent the FeLV proviral sequence derived from the indicated λ clone. Dotted lines indicate pBR322 or pKC7 vector sequences. The subgenomic probes were obtained by gel purification of the indicated restriction fragments. Lengths are given in kilobase pairs, and RI is the *Eco*RI cleavage site.

RESULTS

Isolation and cloning of recombinant phage containing endogenous FeLV-related sequences. The modified lambda cloning vector λJ1 has a cloning capacity of approximately 8.6 to 23.8 kbp (Mullins, unpublished data). Therefore, to ensure inserts approximately 20 kbp in length, we used *Mbo*I-digested SPF placental DNA, selecting conditions for digetion which maximized the generation of fragments in this size range. The digested DNA was size-fractionated on a sucrose gradient and checked by gel electrophoresis. Fragments 17 to 22 kbp in size were pooled, dialyzed extensively against 10 mM Tris (pH 7.5)–10 mM NaCl–1 mM EDTA, and precipitated in ethanol. Size-fractionated placental DNA was ligated to the purified end fragments of *Bam*HI-digested λJ1 and packaged in vitro as described above. A total of 1.3×10^6 recombinant phage were amplified by subconfluent plate lysis, replated, and screened with a hybridization probe specific to the *env* region of the infectious GA-FeLV-B (*Xho*I-*Sac*II fragment of the plasmid pBHM-5 [22]). We obtained a total of 26 FeLV *env*-positive clones from the amplified phage preparation, denoted λCF-1 through λCF-26. We purified and analyzed the DNA from four clones by restriction mapping techniques, using subgenomic probes to the *env*, *gag-pol*, and *U5* regions of the infectious GA-FeLV-B. These four clones were selected from the 26 original isolates on the basis of their strong hybridization with the ^{32}P-labeled nick-translated FeLV *env*-specific probe. Many of the remaining clones exhibiting light and variable hybridization have not been further characterized.

Restriction endonuclease analysis of cloned endogenous FeLV DNA. A restriction endonuclease cleavage map of the four selected clones was prepared by digesting endogenous FeLV hybrid DNA with various restriction endonucleases, separating the DNA fragments by agarose gel electrophoresis, transferring the DNA fragments to nitrocellulose filters, and hybridizing the DNA to the various GA-FeLV-B subgenomic nick-translated probes. Samples of the results obtained by using each of these probes with clone λCF-14 are shown in Fig. 2; they were interpreted as follows. *Eco*RI digestion generated a single hybridizing DNA fragment of 6.0 kbp which hybridized to the nick-translated GA-FeLV-B *env*-specific probe (Fig. 2A, lane 3), *gag-pol*-specific probe (Fig. 2B, lane 3), and *U5*-specific probe (Fig. 2C, lane 3). Because the endogenous FeLV-related sequences (18, 21) have been reported not to contain internal *Eco*RI cleavage sites, this 6.0-kbp hybridizing fragment was interpreted to represent the endog-

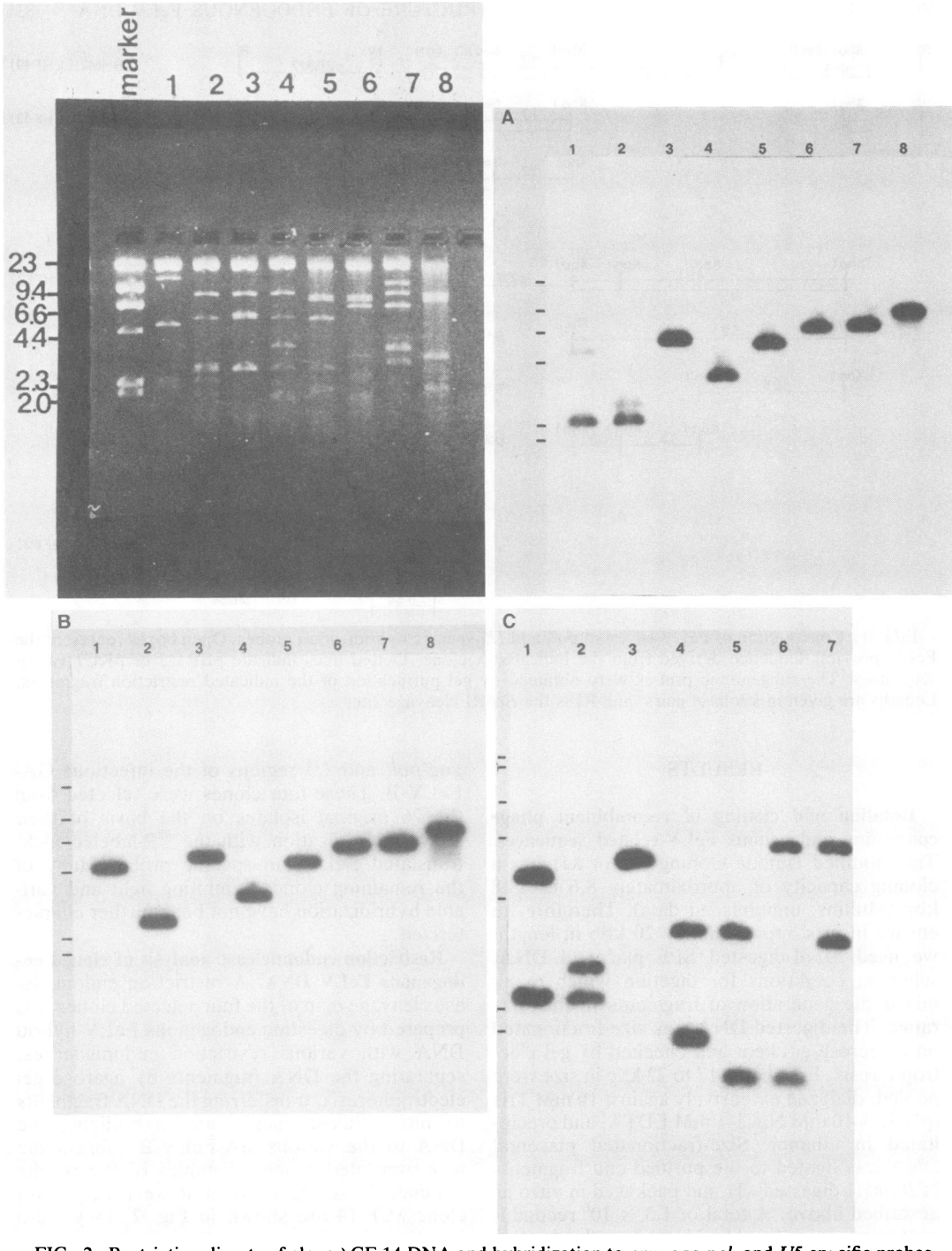

FIG. 2. Restriction digests of clone λCF-14 DNA and hybridization to *env*, *gag-pol*, and *U5*-specific probes. A preparation of λCF-14 DNA was subjected to restriction digestion, gel electrophoresis, Southern blotting, hybridization, and autoradiography as described in the text. The left panel shows the ethidium bromide staining pattern after electrophoresis on a 0.7% agarose gel. (A) Southern blot of the gel on the left probed with nick-translated ^{32}P-labeled FeLV *env*-specific DNA fragments. (B) Duplicate blot of the gel on the left which was hybridized with an FeLV *gag-pol*–specific probe. (C) Southern blot of another gel probed with ^{32}P-labeled GA-FeLV B *U5*-specific fragment. Lanes (for A, B, and C): 1, *Bam*HI; 2, *Bam*HI-*Eco*RI; 3, *Eco*RI; 4, *Eco*RI-*Hin*dIII; 6, *Bgl*II-*Hin*dIII; and 7, *Hin*dIII. For A and B: 5, *Eco*RI-*Bgl*II; 8, *Bgl*II. For C only: 5, *Eco*RI-*Hin*dIII-*Bgl*II. The far left lane of the left panel shows the positions of the fragments in a *Hin*dIII digest of wild-type λ DNA used as a molecular weight marker.

enous FeLV-related element. This 6.0-kbp *Eco*RI hybridizing fragment apparently contained sequences homologous to the major viral protein-encoding genes of the infectious FeLV as well as sequences related to the *U5* region of the LTRs. Based upon the length estimates for the infectious GA-FeLV-B (22) and ST-FeLV-B (30) genomes, this endogenous FeLV-related element appeared to contain a significant deletion of sequences.

Digestion with *Eco*RI and *Bam*HI (lane 2) produced two fragments of 2.9 and 2.3 kbp which hybridized to both the FeLV *env*-specific (Fig. 2A) and *U5*-specific (Fig. 2C) probes. Hybridization with the *gag-pol*-specific probe produced only the 2.9-kbp band (Fig. 2B). Because the sum of the two fragments, 2.9 and 2.3 kbp, was less than the total 6.0-kbp *Eco*RI fragment, *Bam*HI must have cleaved the *Eco*RI fragment twice internally, and the fragment 0.8 kbp in length, representing the balance of the *Eco*RI-*Bam*HI digestion products, does not contain endogenous FeLV-related sequences. We assumed that the endogenous FeLV element represented in clone λCF-14 contained sequences related to the viral genes *gag*, *pol*, and *env* in the canonical order 5′ to 3′ of the genomic RNA. Hence, the 2.3-kbp fragment which hybridized to both *env*-specific and *U5*-specific probes but failed to hybridize to the *gag-pol* probe represents sequences which are 3′ in orientation (with respect to viral RNA) to the 2.9-kbp fragment. Cleavage with *Bam*HI alone (lane 1) yielded two fragments, 5.2 and 2.3 kbp, which hybridized to both the *env*-specific (Fig. 2A) and the *U5*-specific (Fig. 2C) probes, but only one frgament of 5.2 kbp, which hybridized to the *gag-pol*-specific probe (Fig. 2B). The 5.2-kbp *Bam*HI fragment must represent 5′-related sequences which are subsequently cleaved by *Eco*RI to the 2.9-kbp fragment (Fig. 2B, lane 2). The 2.3-kbp *Bam*HI fragment containing *env*- and *U5*-related sequences must be the same internal 2.3-kbp fragment seen in the *Eco*RI-*Bam*HI digestion pattern. Hence, we confirmed that the 2.9- and 2.3-kbp *Eco*RI-*Bam*HI fragments are oriented 5′ and 3′, respectively, and represent contiguous sequences related to the infectious GA-FeLV-B genome. The 0.8-kbp *Eco*RI-*Bam*HI fragment which lacks sequences homologous to the infectious FeLV genome is situated at the far 3′ end of the 6.0-kbp *Eco*RI hybridizing fragment. The *Eco*RI sites are therefore contained in sequences flanking the endogenous FeLV element rather than within the LTR element itself. This is in contrast to the structure of the infectious ST-FeLV-B genome (30) in which *Eco*RI sites are present in the LTRs.

Digestion with *Eco*RI and *Hin*dIII (lane 4) yielded a fragment of about 4.0 kbp which hybridized to all three FeLV subgenomic probes. Hybridization with the *U5*-specific probe (Fig. 2C) produced an additional 2.0-kbp band. Because the 4.0- and 2.0-kbp fragments make up the total 6.0-kbp *Eco*RI band, *Hin*dIII must cleave the *Eco*RI fragment only once internally. The ability to visualize both fragments with a probe specific to the LTR-related elements suggested that these endogenous FeLV sequences are flanked by LTRs. Furthermore, sequences related to the viral structural genes of the infectious FeLV are contained in the 4.0-kbp *Eco*RI-*Hin*dIII fragment and therefore contain considerable sequence deletions. The orientation of these two fragments was determined by the *Bam*HI-*Hin*dIII digestion pattern (data not shown), in which we observed a 5.2-kbp band that hybridized to all three subgenomic probes, a 1.1-kbp band which hybridized to the *env*-specific probe, and a 1.3-kbp band which hybridized to the *U5*-specific probe. Because the 5.2-kbp fragment observed in the *Bam*HI digest alone reappeared in the *Bam*HI-*Hin*dIII double digestion, the *Hin*dIII site must be located 3′ relative to the 5′-most *Bam*HI site. Hence, the 4.0- and 2.0-kbp *Eco*RI-*Hin*dIII fragments are located 5′ and 3′, respectively, relative to the exogenous FeLV RNA genome.

*Eco*RI-*Hin*dIII-*Bgl*II digestion (Fig. 2C, lane 5) of λCF-14–cloned DNA produced two fragments 4.0 and 1.7 kbp in length which hybridized to the *U5*-specific probe. Cleavage with *Eco*RI and *Bgl*II (Fig. 2A and B, lane 5) yielded a single hybridizing fragment of 5.7 kbp. These results locate the *Bgl*II site 0.3 kbp from the 3′ *Eco*RI site and suggest that the 1.7-kbp hybridizing *Hin*dIII-*Bgl*II fragment is located at the 3′ end of the 6.0-kbp *Eco*RI fragment. In addition, there is the 0.3-kbp *Bgl*II-*Eco*RI fragment, which does not hybridize with any of our subgenomic FeLV probes. The restriction endonuclease cleavage mapping results described above are consistent with the interpretation that the nonhybridizing region of cloned λCF-14 DNA is located at the 3′ end of the 6.0-kbp *Eco*RI hybridizing fragment and to the right of the 3′ *Bam*HI cleavage site.

Using the *Eco*RI and *Bam*HI cleavage sites located above as points of reference, we used digestion with additional combinations of restriction enzymes to develop the physical map of λCF-14 cloned DNA. The data obtained by hybridization of quadruple blots were used to determine the extent of the various subgenomic regions in this clone. These results are summarized in Table 1. Briefly, the *Sst*I cleavage site located approximately 1.7 kbp from the 5′ *Eco*RI site appears to delineate the boundary between the *gag*-specific region and sequences related to the *U5* portion of the infectious FeLV LTR as the 5′ 1.0-kbp *Sst*I fragment hybridizes solely to

TABLE 1. DNA fragments from endogenous cloned FeLV λCF-14 homologous to GA-FeLV-B

Restriction enzymes	Fragment size (kbp)[a]	Labeling[b]			
		U5	Gag	Pol	Env
*Eco*RI-*Sst*I	4.3	+	+	+	+
	1.0	+	−	−	−
*Bam*HI-*Sst*I	2.3	+	−	−	+
	1.0	+	−	−	−
	1.3	−	+	+	+
*Eco*RI-*Xho*I	2.8	+	+	+	+
	1.0	−	−	−	+
	2.2	+	−	−	−
*Bam*HI-*Xho*I	2.8	+	+	+	+
	1.0	−	−	−	+
	1.4	+	−	−	−
*Eco*RI-*Kpn*I	2.3	+	+	+	+
	2.7	+	−	−	+
*Bam*HI-*Kpn*I	1.6	+	+	+	+
	0.7	−	−	−	+

[a] Sizes of fragments labeled after hybridization with various DNA probes (see Fig. 1 for map position of probes).

[b] +, Detectable hybridization; −, undetectable hybridization.

the *U5*-specific probe (Table 1; Fig. 3). The *gag* and *pol*-related sequences are confined to the middle 1.3-kbp *Sst*I-*Bam*HI fragment, although additional hybridization of this fragment to the *env*-specific probe indicates that the *env* gene-related sequences extend into this fragment as well. Based upon earlier results by Sherr et al. (30) on the size estimates for the various regions of the ST-FeLV-B genome, we concluded that these endogenous sequences are deficient in some portion of the *gag-pol* region. Sherr et al. (30) estimated the *gag-pol* region to be approximately 4.5 kbp, whereas our restriction maps indicated the same region to be less than 1.3 kbp in length. This finding represents a significant deletion of sequences in this region and appears to account for a major difference in length between the infectious FeLV genomes and the endogenous FeLV-related sequences described in these clones.

The extent of the *env*-specific region of the endogenous FeLV element is illustrated in the *Xho*I cleavage pattern. Cleavage of λCF-14 DNA with *Eco*RI and *Xho*I (see Table 1) yielded a 2.8-kbp fragment which hybridized to all four subgenomic FeLV probes, a 1.0-kbp fragment which hybridized to only the *env*-specific probe, and a 2.2-kbp fragment which hybridized to the *U5*-specific probe. Since double digestion with *Xho*I and *Bam*HI did not alter the 2.8-kbp fragment, this fragment must be located within the 2.9-kbp *Bam*HI fragment described earlier. The 1.0-kbp *Xho*I fragment which hybridizes to the *env*-specific probe strongly suggests that the *Xho*I cleavage site located approximately 3.8 kbp from the 5′ *Eco*RI site represents the boundary between the *env* gene region and sequences related to the LTR-like elements of the endogenous FeLV DNA. Furthermore, the 1.4-kbp *Bam*HI-*Xho*I fragment (Table 1) seen with the *U5*-specific probe confirms that LTR-related sequences flank the portion of the endogenous FeLV clone containing elements homologous to portions of the viral genes *gag*, *pol*, and *env*. Cleavage with *Bam*HI and *Kpn*I confirms that the *env*-related sequences extend through the region 5′ of the second *Xho*I site as the 0.9-kbp fragment, which from earlier experiments was determined to be generated from the 5′ *Bam*HI cleavage site and the *Kpn*I site located 3.6 kbp from the 5′ *Eco*RI site, hybridized only to the *env*-specific probe. Hence, restriction endonuclease cleavage mapping results are consistent with the interpretation that the *env*-related sequences of the clone λCF-14 are located from approximately 2.7 to 3.8 kbp from the 5′ *Eco*RI cleavage site. Based upon the length estimates of the *env* gene of the infectious ST-FeLV-B (30), which was determined to be approximately 2.0 kbp, these results indicate a deletion of *env*-related sequences in our endogenous clone. However, it should be noted that the hybridization probe used to map the *env* gene consists of sequences spanning the gp70 but not the p15e protein-encoding region of the *env* gene. The size estimate for the *env*-related sequences in our endogenous clone may, therefore, represent a minimum estimation of its total length.

Comparison of endogenous FeLV sequences among different loci in cat DNA. A comparison of the restriction maps constructed for the four λ DNA clones obtained from the cat genomic library is seen in Fig. 3. There was a high degree of homogeneity among the cloned sequences within the *Eco*RI fragment, as illustrated by conservation of several restriction sites. For example, all four clones contained an *Sst*I restriction site approximately 1.8 kbp from the 5′ *Eco*RI site, two internal *Bam*HI cleavage sites, an internal *Hin*dIII site located approximately 4 kbp from the 5′ *Eco*RI site, and two internal *Pst*I sites located near the middle of the *Eco*RI fragment separated by approximately 0.4 kbp. Two internal *Sma*I cleavage sites appeared at positions approximately 2 and 4.9 kbp from the 5′ *Eco*RI site, and two internal *Xho*I cleavage sites and a single *Kpn*I site were mapped near the internal *Hin*dIII cleavage site in all clones. There appeared to be a conservation of *Sma*I and *Kpn*I cleavage sites within the 5′ LTR-related elements, although this homogeneity in structure was less apparent in the 3′ LTR-related sequences of the endogenous FeLV clones studied. The use of various subgenomic probes to the GA-FeLV-B indicated that all

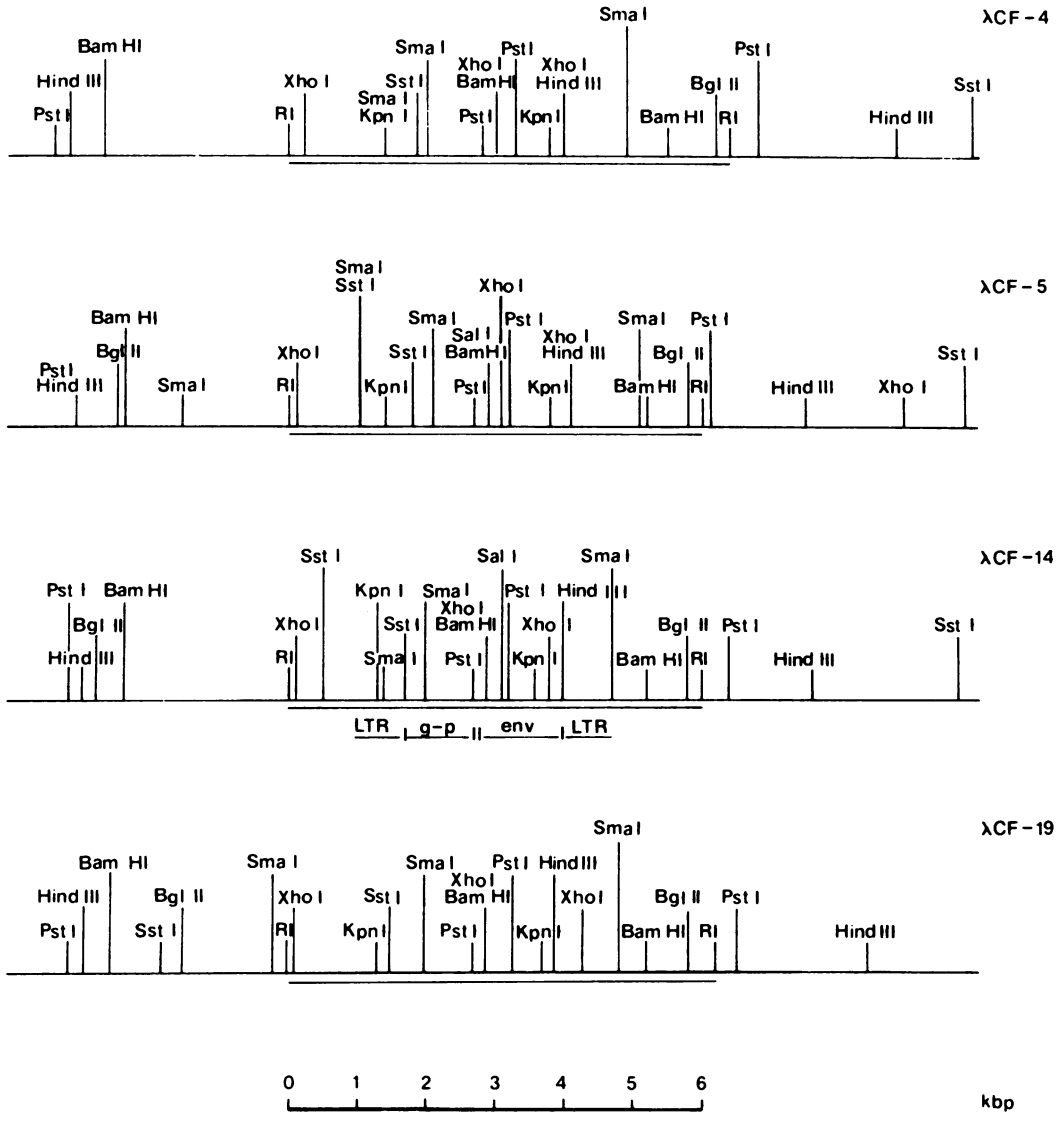

FIG. 3. Comparison of the restriction maps of endogenous FeLV DNA sequences in four λ clones. Restriction sites were mapped after digestion with the enzyme indicated alone or with either *Eco*RI or *Bam*HI, and the fragments were analyzed by gel electrophoresis and Southern transfer techniques. The orientation of the restriction maps is from 5′ to 3′ with respect to viral RNA. Double lines correspond to the *Eco*RI fragment containing the endogenous FeLV sequences; they are used to aid in orientation. Single lines indicate cat cellular flanking DNA sequences. Clone λCF-14 also shows the approximate location of subgenomic regions which were assigned according to hybridizations employing the FeLV *gag*, *pol*, *env*, and *U5*-specific probes described in the text.

clones contained an *env* (gp70) region approximately 1.0 to 1.3 kbp in length and a *gag-pol* region of approximately 0.9 to 1.2 kbp, and that they were flanked by LTR-like elements. *Eco*RI sites appeared to be present in sequences flanking the endogenous FeLV-related elements, as well as 5′ *Xho*I and 3′ *Bam*HI, *Bgl*II, and *Pst*I restriction sites. Hence, there was considerable conservation of restriction sites among the various λ clones representing some of the multiple gene loci of the endogenous FeLV sequences.

The four λ DNA clones were also distinguished as discrete genetic loci due to variations in their restriction maps. For example, although each clone appeared to contain a single *Eco*RI hybridizing fragment, this fragment varied from

6.0 to 6.4 kbp in length. An internal *Sal*I site and an *Sst*I site located 0.8 kbp from the 5′ *Eco*RI cleavage site were present in clones λCF-5 and λCF-14 but were absent from clones λCF-4 and λCF-19, and an internal *Sma*I site located 1.3 kbp from the 5′ *Eco*RI site was present in all clones but λCF-19. Although there was some clustering of restriction sites in 5′ flanking sequences, there was also a divergence present in the sequences flanking the *Eco*RI fragment, indicating different sites of occurrence of the endogenous FeLV elements in the cat genome.

Comparison of endogenous FeLV sequences with the infectious FeLV. There are several natural FeLV isolates which have been described, and restriction maps have been deduced for two subgroup B isolates. These are the ST-FeLV-B (30) and GA-FeLV-B (22). The composite map for these two infectious FeLV isolates is shown in Fig. 4. Although there is significant homology between these two isolates, they differ in overall length: ST-FeLV-B is 8.5 kbp and GA-FeLV-B is 8.7 kbp. The ST-FeLV-B LTRs contain *Eco*RI sites which are absent in the GA-FeLV-B DNA. The most significant difference observed between the endogenous FeLV sequences and the infectious FeLV was in the overall length of the virus-related elements. Although the infectious FeLV isolates varied from 8.5 to 8.7 kbp in length, the four clones of the endogenous FeLV sequences studied to date appeared to be approximately 4.0 kbp in length, including the flanking LTR sequences. The latter estimates were based upon the results of Southern blot analysis, using various subgenomic probes as well as homology to the known infectious FeLV LTR structure.

A more detailed comparison of the restriction cleavage sites present in the endogenous and exogenous FeLV revealed several important similarities (Fig. 3 and 4). For example, there was homology to the endogenous FeLV in the region located approximately 5.5 to 6 kbp from the 5′ end of the exogenous FeLV genome. In this region, we noted internal *Bam*HI and *Xho*I cleavage sites and two internal *Pst*I sites immediately flanking the *Bam*HI site and separated by about 0.4 kbp. This same pattern of restriction enzyme cleavage sites was observed in the endogenous FeLV sequences at a position approximately 3 kbp from the 5′ *Eco*RI cleavage site; it corresponds to the boundary between *gag-pol* and *env* genes as judged by estimates of the location of these regions using subgenomic FeLV hybridization probes. Furthermore, we observed a similarity in the region approximately 1.0 kbp from the 3′-internal *Pst*I site in which a *Kpn*I and *Hin*dIII site appeared to be present in the infectious ST-FeLV-B at a position approximately 7.2 kbp from the 5′ end of the RNA genome. A similar pattern was noted in the endogenous FeLV sequences at a position ap-

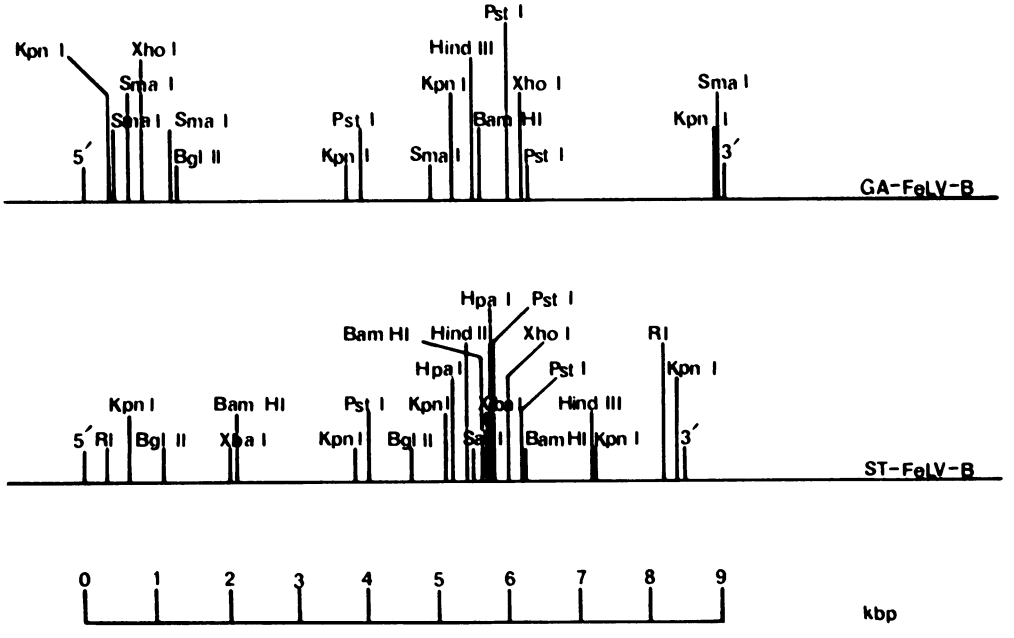

FIG. 4. Restriction maps of cloned infectious FeLV DNA. GA-FeLV-B was isolated and mapped by Mullins et al. (23), and ST-FeLV-B was cloned and mapped by Sherr et al. (30).

proximately 4.0 kbp from the 5′ *Eco*RI site; this corresponds to the region spanning the *env* gene. Hence, consistent with the finding of length estimates of the various subgenomic regions, we observed a conservation of restriction sites in the region corresponding to both the 3′ end of the putative *pol* gene and the *env* gene of the infectious FeLV genome in our endogenous FeLV clones.

There were notable differences in the restriction maps of the infectious and endogenous FeLV, and these divergences were seen in the *Kpn*I and *Pst*I cleavage sites located 3.8 and 4.0 kbp, respectively, from the 5′ end of the infectious FeLV genome. These two sites were not seen in any of the four endogenous FeLV clones. Similarly, the *Bgl*II site located 1.8 kbp from the 5′ end of the infectious FeLV genome and the single *Bam*HI cleavage site located 2.1 kbp from the 5′ end of the infectious ST-FeLV-B were also absent from the endogenous clones studied to date. These four restriction sites can be mapped to the *gag* and part of the *pol* regions of the infectious FeLV; they confirmed the results of restriction analyses indicating the presence of deletions in the *gag-pol* region of the endogenous FeLV sequence. Additionally, we consistently observed a *Bam*HI and *Bgl*II site within 1.0 kbp of the 3′ *Eco*RI site in all four clones analyzed. These two sites are located outside the LTR-like elements, explaining why we failed to demonstrate hybridization of sequences in this region to the various FeLV subgenomic probes.

Significantly, there appeared to be a partial conservation of LTR structure between the endogenous and the infectious FeLV sequences. *Kpn*I and *Sma*I sites can be mapped within the 5′ LTR-like elements. Although these cleavage sites are indicative of the positions within the LTRs, we do not yet have a definite boundary established for the extent of the LTRs in our endogenous clones.

Demonstration of deleted endogenous FeLV elements within cat placental DNA. To confirm that the endogenous FeLV-related sequences isolated in the cat genomic library existed in normal cat DNA, we digested total cat placental DNA with an excess of *Eco*RI and analyzed the Southern blots with FeLV *env*-specific probe as described above. Cleavage of the cat placental DNA (Fig. 5, left panel) yielded several major hybridizing fragments, including bands 6 to 7 kbp and larger in molecular weight. The presence of larger-molecular-weight bands is in agreement with earlier findings by Koshy et al. (18) and Mullins et al. (21), in which it was reported that 10 to 12 bands 8 kbp and larger were visible in cat genomic DNA digested with *Eco*RI, using cDNA probes to the infectious FeLV. We have now found, in addition, several 6- to 7-kbp bands, representative of the endogenous FeLV, which hybridized with comparable intensity to the FeLV *env*-specific probe as the other higher-molecular-weight bands. It is not clear at this time whether this difference from earlier findings is related to the specificity of the probes or to the nature of the tissues examined. However, it is noteworthy that the existence of *Eco*RI bands 6 to 7 kbp in length has also been reported in other cat tissues and cell lines (J. I. Mullins, J. W. Casey, J. B. Santon, K. B. Burck, M. O. Nicolson, and N. Davidson, manuscript in preparation). Figure 5 also shows the presence of a lower copy number of the 6- to 7-kbp bands in cat placental DNA from a house-

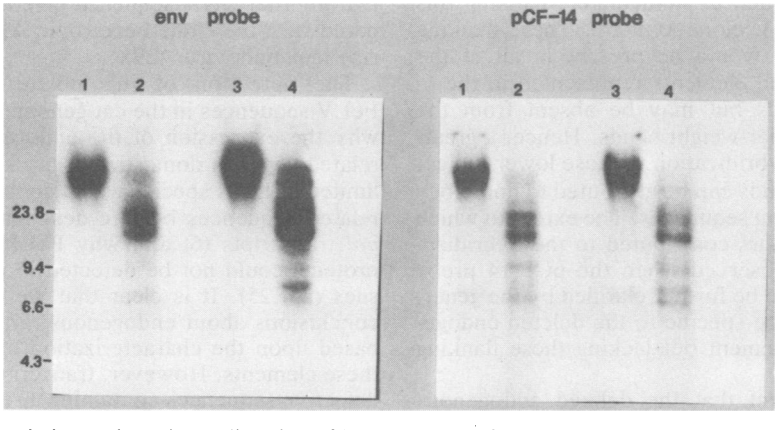

FIG. 5. Restriction endonuclease digestion of household and SPF cat placental DNA. The left panel shows the autoradiogram of a blot probed with an FeLV *env*-specific probe. The right panel represents the autoradiogram of a blot probed with pCF-14 DNA fragments. Lanes: household cat placental DNA uncut (1) and digested with *Eco*RI (2); SPF cat placental DNA uncut (3) and digested with *Eco*RI (4).

hold domestic cat (lane 2) as compared with the higher copy number observed in the DNA from a cat raised in an SPF colony (lane 4). The differences in banding patterns observed from cat to cat appear to underscore the heterogeneity in the endogenous FeLV sequences among individual cats.

The results obtained when the 6.0-kbp EcoRI fragment from λCF-14 DNA was subcloned into pBR322, used to generate a probe (pCF-14), and hybridized to *Eco*RI-digested cat cellular DNA are shown in Fig. 5, right panel. The 6.0-kbp fragment of clone λCF-14 was clearly observed to hybridize to several bands in the size range of 6 to 7 kbp as well as to other higher-molecular-weight bands. The hybridization results obtained when the *env* and pCF-14 probes were used differed in that the predominance of higher-molecular-weight bands did not appear to be as pronounced when the latter probe was used. This observation was interpreted to be the result of two factors. First, the deleted endogenous FeLV-related elements represented in the clones described share the same extent of homology to the various size classes of provirus-like elements which exist in the cat genome. This is in contrast to the *env*-specific regions defined by our gp70 hybridization probe, which apparently possess greater homology to the higher-molecular-weight *Eco*RI-digested hybridization bands. This finding suggests that the DNA bands 6 to 7 kbp in length observed in cat placental DNA are missing some portion of the *env* gene-related region specified by the hybridization probe, and it is in concordance with the results of restriction map analysis which confirms deletions mapped to the *env* gene of the endogenous FeLV clones. Second, the pCF-14 probe consists of both sequences related to the deleted endogenous FeLV element as well as the conserved sequences immediately flanking this element (Fig. 3, clone λCF-14). These flanking cat sequences would be present in all of the deleted proviral elements represented in the 6- to 7-kbp bands but may be absent from the higher-molecular-weight bands. Hence, a greater degree of hybridization to these lower-molecular-weight bands can be attributed to homology to these flanking sequences. The extent to which these two factors contributed to the hybridization pattern observed when the pCF-14 probe was used could be further clarified by the generation of a probe specific to the deleted endogenous FeLV element but lacking these flanking sequences.

It is apparent that the deleted endogenous FeLV elements observed in our genomic phage library are present in cat DNA, but it is unclear why the 8-kbp and larger *Eco*RI hybridizing fragments were not found in the recombinant clones studied to date. One possibility is that these larger-molecular-weight bands are present in our phage library as overlapping clones which exhibit light or variable hybridization. Alternatively, these sequences may have been selectively lost during the amplification of the phage library. These and other contentions remain to be investigated.

DISCUSSION

Four distinct λ clones were isolated by screening a cat genomic library derived from SPF placental DNA with a hybridization probe specific to the *env* region of the infectious virus, GA-FeLV-B (22). Restriction endonuclease mapping indicated that there were a number of similarities among the four cloned isolates, notably the presence of a 6.0- to 6.4-kbp *Eco*RI hybridizing fragment containing portions of sequences homologous to the *gag-pol*, *env*, and LTR-like elements of the infectious FeLV. We also demonstrated that there was considerable conservation in the ordering of restriction sites previously reported to be present in the proviruses of infectious FeLVs (22, 30) in the sequences corresponding to the *pol* and *env* boundary as well as in the region spanning the *env* gene of the endogenous FeLV, whereas a greater divergence was found to occur among restriction sites mapped to the *gag* and part of the *pol* regions of the infectious FeLV. These results are consistent with a previous report (21) that sequences similar to the *pol* and *env* genes are conserved in the cat DNA and present in multiple copies. The presence of this type of defective endogenous proviral sequences containing significant deletions of internal proviral genes has also been observed in the murine system, in which it was reported that a λ clone isolated from mouse genomic DNA contained two internal DNA sequence deletions, as compared with the cloned ecotropic AKR-type murine leukemia virus (29).

The detection of incomplete endogenous FeLV sequences in the cat genome may explain why the expression of the endogenous FeLV-related genes in domestic cat placental tissue is limited to RNA species which contain *env* gene-related sequences but are deficient in *gag* and *pol* transcripts (6) and why FeLV-related *gag* proteins could not be detected in placental tissues (24, 25). It is clear that we cannot draw conclusions about endogenous viral expression based upon the characterization of a subset of these elements. However, transcription of these defective sequences containing a relatively larger representation of the *env* gene would be consistent with their observed subgenomic expression. It would be important to define the functional ability of these endogenous LTR se-

quences in cat DNA, as well as the extent of homology between the *U3* region of the endogenous LTRs and that of the infectious FeLV LTRs, where the putative transcriptional promoter is located (16, 33) to delineate the role of these deleted endogenous sequences. We suggest that the expression of FeLV-related transcripts in virus-negative lymphomas in which *gag* proteins are not detected may be, similarly, the result of the induction of these endogenous FeLV sequences deficient in *gag-pol*-related elements.

Despite the large number of endogenous FeLV-related sequences detected in cat DNA by liquid and blot hybridization analysis, these sequences remain noninducible as infectious viral particles (3). Previous studies by Okabe et al. (27), involving hybridization kinetic analyses, indicated that a portion of the FeLV genome is not endogenous in cat cells; this is consistent with our observations demonstrating deletions in the various subgenomic regions of the endogenous FeLV. Our analyses of the four different LTR-containing endogenous FeLV proviruses point to several structural features which are bound to affect their expression as infectious viral particles. For example, the endogenous FeLV sequences isolated from the cat genomic library are significantly shorter than the cloned infectious FeLV isolates, the latter of which are 8.5 to 8.7 kbp in length (22, 30), as compared with our clones, which are approximately 4 kbp in length. All of the proviruses have 3.3- to 3.6-kbp deletions in the *gag-pol* region and 0.7- to 1.0-kbp deletions in the *env* gene region (Fig. 3) and would therefore be incapable of encoding an infectious virus.

Although the proviral DNA segments present in the different endogenous clones were related to one another on the basis of similarities in several internal restriction sites, a comparison of the restriction maps of the flanking regions indicates that these sequences are present at different loci in the cat DNA (Fig. 3). The finding of endogenous FeLV sequences at a number of sites in the host genome is in agreement with the results of Koshy et al. (18), which indicated that the endogenous FeLV-related sequences consisted of multiple, discrete, nontandemly arranged genetic elements. Such deleted, FeLV-related DNA sequences could have originated either by germ line integration of a complete ecotropic virus-related DNA followed by deletion, or by integration of a preexisting, defective, deleted variant of the infectious virus. Furthermore, the degree of homogeneity observed among the various cloned isolates argues for their origin as a result of such a germ line integration of an exogenous virus at a single or limited number of chromosomal loci in a relatively recent ancestor of the cat, followed by gene duplication events and subsequent sequence divergence. This would be in contrast to an origin invoking multiple random infections by different strains of complete or deleted exogenous FeLV. This argument is based upon the presence of a larger degree of divergence among the cloned infectious proviral DNAs as compared with the divergence among the isolated endogenous clones. The gene duplication model for endogenous FeLV origin, although consistent with distribution in certain, but not all, *Felis* species (3) does not, however, strictly rule out other mechanisms by which independently integrated sequences may be conserved. For example, homogeneity between pairs of relatively closely linked endogenous sequences may be the result of gene conversion mechanisms by which similar sequences on homologous or nonhomologous chromosomes may occasionally pair up, and any mismatch in sequences between them is eliminated (11).

The homogeneity observed among the various cloned isolates also extends into the flanking cat cellular sequences. The conservation of these flanking sequences is not inconsistent with the model for endogenous FeLV origin as a result of gene amplification. The presence of units of gene amplification which are larger than the size of the functional gene has been previously noted with amplified CAD (10), DHFR (26), and *myc*-related genes (9). Similarly, it appears that the amplified units for the deleted endogenous FeLV sequences extend beyond the limits defined by the LTR-like elements.

Finally, we are able to confirm the presence of these endogenous FeLV sequences represented in the cat genomic library in normal cat DNA by Southern blot analysis. We observed multiple bands of FeLV *env*-related sequences present in the *Eco*RI digestion of cat DNA, including bands corresponding in size to our λ clones (6 to 7 kbp). The presence of higher-molecular-weight *Eco*RI hybridizing fragments, although not found in the recombinant clones studied to date, reflects the occurrence of other subsets of endogenous sequences. Additional experiments will be required to define these various types of deletions or incompleteness that represent the remnants of the original germ line infection of FeLV now present in the genomes of domestic cats.

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