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Recombination between Feline Leukemia Virus Subgroup B or C and Endogenous *env* Elements Alters the In Vitro Biological Activities of the Viruses

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An important question in feline leukemia virus (FeLV) pathogenesis is whether, as in murine leukemia virus infection, homologous recombination between the infecting FeLV and the noninfectious endogenous FeLV-like proviruses serves as a significant base for the generation of proximal pathogens. To begin an analysis of this issue, several recombinant FeLVs were produced by using two different approaches: (i) the regions of the viral envelope (*env*) gene of a cloned FeLV (subgroup B virus [FeLV-B], Gardner-Arnstein strain) and those of two different endogenous proviral loci were exchanged to create specific FeLV chimeras, and (ii) vectors containing endogenous *env* and molecularly cloned infectious FeLV-C (Sarma strain) DNA sequences were coexpressed by transfection in nonfeline cells to facilitate recombination. The results of these combined approaches showed that up to three-fourths of the envelope glycoprotein (gp70), beginning from the N-terminal end, could be replaced by endogenous FeLV sequences to produce biologically active chimeric FeLVs. The in vitro replication efficiency or cell tropism of the recombinants appeared to be influenced by the amount of gp70 sequences replaced by the endogenous partner as well as by the locus of origin of the endogenous sequences. Additionally, a characteristic biological effect, aggregation of feline T-lymphoma cells (3201B cell line), was found to be specifically induced by replicating FeLV-C or FeLV-C-based recombinants. Multiple crossover sites in the gp70 protein selected under the conditions used for coexpression were identified. The results of induced coexpression were also supported by rapid generation of FeLV recombinants when FeLV-C was used to infect the feline 3201B cell line that constitutively expresses high levels of endogenous FeLV-specific mRNAs. Furthermore, a large, highly conserved open reading frame in the *pol* gene of an endogenous FeLV provirus was identified. This observation, particularly in reference to our earlier finding of extensive mutations in the *gag* gene, reveals a target area for potentially productive homologous recombination upstream of the functional endogenous *env* gene.

Multiple steps are involved in leukemogenesis caused by infection of the host by replication-competent retroviruses. Among the mammalian retroviruses, considerable information on murine leukemia virus (MuLV)-induced leukemia, which appears to produce several characteristic virus-host interactions, has been generated. The envelope protein gene (*env*) of the infectious MuLV recombines with the endogenous MuLV-related sequences inherited in the mouse genome and expressed in mouse cells to give rise to recombinant MuLVs that are capable of recognizing different sets of cell surface receptors. Such altered recognition may be critical in establishing infection in various host cells including the target cells (11, 14, 28, 37). There is also evidence for insertional mutagenesis that frequently activates proto-oncogenes like c-*myc*, *pim-1*, *pvt-1*, etc., in tumor cells (4). Additionally, hematopoietic hyperplasia, cytopathic effect on bone marrow cells, and other related effects induced directly or indirectly by MuLV infection appear to contribute to the development of leukemia (9, 20). While these observed phenomena offer opportunities for a critical analysis of the mechanism of the disease in the mouse system, there is only limited information on feline leukemia virus (FeLV) infection, which naturally and frequently results in leukemogenesis in the domestic-cat population. Analogous to the mouse model, FeLV infection predominantly causes T-cell lymphoma in cats after a prolonged latency (13, 16, 21), and some recent studies point to potential interactions between exogenous and endogenous FeLV elements (19, 25). Furthermore, evidence that indicates frequent activation of c-*myc* in feline T lymphomas by either viral transduction or insertional mutagenesis (22) has accumulated.

To understand the nature of interaction between the infecting strain of FeLV and transcriptionally active but replication-defective (5, 23, 24, 35, 36) endogenous FeLV elements of the domestic-cat genome, which may be responsible for the generation of proximal leukemogens, it is necessary to delineate the effects contributed by specific domains of the endogenous sequences. With this aim, we generated chimeric FeLVs to examine their biological activities. In vitro infectivity studies with individual or mixtures of recombinants show that up to three-fourths of the envelope surface glycoprotein (gp70), beginning from the N-terminal end, could be replaced by endogenous FeLV sequences to produce biologically active chimeric FeLVs, although the efficiency of infection of these chimeras in certain cell types appears to be influenced by the locus of origin of the endogenous component as well as by the length of the endogenous insert. Multiple crossover sites on the *env* gene selected in the recombination process have also been identified. These findings along with that of a large open

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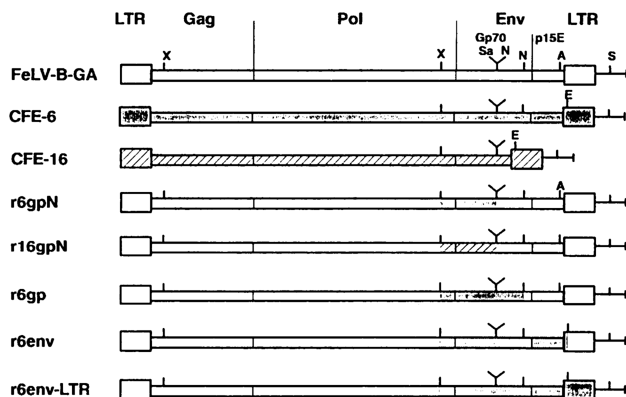

FIG. 1. In vitro construction of chimeric FeLVs. Abbreviations: X, *Xho*I; Sa, *Sau*I; N, *Nco*I; A, *Ava*II; E, *Esp*I; S, *Sal*I. The *Sau*I and 5′ *Nco*I sites are separated by six bases.

reading frame upstream of the endogenous FeLV *env* gene as potential target sites for homologous recombination are presented in this report.

MATERIALS AND METHODS

Cell lines and cloned proviruses. The feline embryo fibroblast cell line H927, mouse fibroblast cell line NIH 3T3, and mink lung cell line (ATCC CCL-64) were maintained in high-glucose Dulbecco modified Eagle medium (MEM) with 10% fetal bovine serum (FBS). HT1080 human fibrosarcoma cells were cultured in alpha MEM (αMEM) supplemented with 15% FBS. The feline T-lymphoid tumor cell line 3201B (34) was propagated in a 1:1 (vol/vol) mixture of RPMI 1640 and Leibowitz L-15 medium containing 20% FBS (30). The human B-lymphoid tumor cell line Raji and T-lymphoblastic leukemic cell line CEM were grown in RPMI 1640 with 10% FBS.

Plasmid clones pBHM1 and pFSC, representing the Gardner-Arnstein strain of FeLV subgroup B (FeLV-B-GA) and the Sarma strain of FeLV subgroup C (FeLV-C-Sarma), respectively, were kindly provided by J. Mullins. Endogenous FeLV clones pCFE-6 and pCFE-16, representing two different proviral loci (3, 36), were used in the construction of recombinant proviruses. CFE-6 is a nearly full-length endogenous proviral clone, while CFE-16 is an endogenous locus in which both *pol* and *env* genes are partially truncated. In the *env* region, CFE-16 DNA is naturally deleted in the 3′ half of the surface glycoprotein gp70 domain and all of the p15E transmembrane coding sequences (19).

Construction of chimeric FeLVs. The construction of five recombinant FeLVs with exchanges between FeLV-B-GA (clone pBHM1) and endogenous provirus clone CFE-6 or CFE-16 is outlined in Fig. 1. The chimeric provirus r16gpN was made by inserting the *Xho*I-*Sau*I fragment, encompassing the N-terminal half of gp70 as present in CFE-16, into the corresponding position in the FeLV-B-GA plasmid. Similarly, r6gpN was constructed with the N-terminal half of gp70 derived from CFE-6. All of the gp70, except the C-terminal 120 bases of CFE-6 isolated by *Xho*I-*Nco*I (partial) digestion, was ligated into appropriately cleaved FeLV-B-GA to generate the chimeric provirus r6gp, in which most of the gp70 sequences were of endogenous origin. In an analogous manner, the *env* region, the *Xho*I-*Esp*I fragment of CFE-6, was cloned into *Xho*I (partial)-*Ava*II-cut FeLV-B-GA to achieve r6*env*, with replacement of the entire *env* gene. The r6*env*-LTR provirus was obtained by placing the *Xho*I-*Sal*I fragment, which contained the entire *env* 3′ long terminal repeat (LTR) region of CFE-6, into *Xho*I(partial)-*Sal*I-digested FeLV-B-GA plasmid. The identity of each construct was verified by restriction mapping.

Recombination via coexpression in transfected cells. Infectious FeLV-C-Sarma proviral DNA (pFSC) was transfected into human HT1080 cells alone or in combination with noninfectious CFE-16, r6gp, r6*env*, or r6*env*-LTR by using the calcium phosphate transfection protocol as described before (12). Briefly, the DNA, purified by double CsCl density gradient centrifugation and containing either 20 μg of pFSC or 10 μg of pFSC plus 10 μg of noninfectious partner, was precipitated by $CaCl_2$ in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline under a steady stream of N_2, and the colloidal suspension was layered over the cells plated at a concentration of 5×10^5 in 10 ml of αMEM containing 15% FBS. After incubation at 37°C for 4 h, the medium was removed, and the cell layer was washed with HEPES-buffered saline and subjected to 15% glycerol shock for 2 min. Cells were then washed again and maintained in culture for 4 weeks, at which time a steady level of virus production was reached. At this point, media from individual cultures were passed through 0.22-μm-pore-size membrane filters and used as inoculum to infect fresh HT1080 cells. For infection, 3×10^5 cells were plated in a 2.5-in. (1 in. = 2.54 cm) petri dish with 5 ml of αMEM 24 h prior to the experiment. Next day, the medium was aspirated off and 5 ml of αMEM containing DEAE-dextran (18) was added. After 1 h of incubation at 37°C and following removal of the DEAE-dextran medium and washing, the cell layer was exposed for 5 min to 1.5 ml of virus-containing culture filtrate. Fresh medium (3.5 ml) was then added to each plate, and the cultures were maintained. The infected cultures that released significant levels of virus within 6 days postinfection were propagated for 4 weeks, at which time genomic DNA was prepared for characterization of the infectious virus.

Amplification of proviral DNA by PCR. To analyze the *env* gene gp70 region of the integrated proviruses, three sets of primers were synthesized. The primer sets were chosen so that together they encompassed the complete gp70 segment, beginning 61 nucleotides upstream of the *env* gene and ending at the middle of the p15E segment. Also, the primers represented the sequences highly conserved between the endogenous FeLV and pFSC genomes. The 5′ end of each primer contained restriction sites for potential use in cloning and analysis of the cloned polymerase chain reaction (PCR) products, which were expected to vary in sizes from approximately 0.5 to 0.7 kb. High-molecular-weight genomic DNA extracted from the infected cells was isolated and amplified by using 1 pmol of primer per μg of genomic DNA in a Perkin-Elmer-Cetus thermal cycler (32). After 40 cycles of amplification, the PCR products generated separately with each set of primers were analyzed by electrophoresis in 4% agarose gels (3:1 NuSieve GTG/SeaKem ME) at 50 V.

DNA sequencing. The PCR products were cloned into a 3.0-kb cloning vector (TA cloning kit, Invitrogen Corp.). Double-stranded-DNA sequencing (7) was done by the dideoxy chain termination method (33), with the M13 forward and reverse primer sites located in the polylinker region of the vector. Additionally, two internal sequencing primers (PI-1 and PI-2) were synthesized and used for sequencing the products of the mid-gp70 region.

For sequencing the *pol* gene of the endogenous FeLV

provirus, the CFE-6 DNA was used to generate seven restriction fragments from the 5.0-kb *gag-pol* region. These fragments, varying in sizes from 150 bp to 1.8 kb, were subcloned into M13mp18 and M13mp19. Deletion clones produced by the exonuclease activity of T4 DNA polymerase were isolated, and overlapping fragments were sequenced by the dideoxy chain termination procedure with the M13 universal primer (8, 33).

All sequence data were assembled on a Sun SPARC station 4.0 and analyzed by using Intelligenetics IG Suite software.

In situ gel hybridization. After electrophoresis of the PCR products on 4% agarose, the gel was dried and DNA within the gel matrix was denatured and neutralized (26). Hybridization to a specific oligonucleotide probe end-labeled with [γ-^{32}P]ATP was carried out at 42°C overnight and followed by washing with 6× SSC (0.15 M NaCl with 0.015 M sodium citrate) for 10 min at temperatures beginning at 42°C and then increasing by 5°C at a time up to the T_m. Autoradiography was performed after each washing.

Virus infectivity assays. Viral infections were achieved by priming the cells to be infected with 2 to 3 μg of Polybrene (Sigma) per ml for 1 day prior to infection (18). The cells were then washed and incubated for 4 h with virus (clarified supernatant fluids of infected cultures) at 1 ml of fluid per 10^6 cells. Fresh medium was added, and the cells were incubated for 24 h. Thereafter, the cells were washed and suspended in fresh medium and maintained in culture for up to 5 weeks for determination of infectivity. The virus released in the culture medium was regularly assayed at the time of cell passage by both measurements of particulate-associated reverse transcriptase (RT) activity (31), and the viral p27 antigen was assayed by enzyme-linked immunosorbent assay (ELISA). The VIRACHEK (Synbiotics, San Diego, Calif.) ELISA kit was used according to the supplier's specifications except that the reaction was stopped by the addition of 50 μl of 2 N sulfuric acid for reading the A_{450}. Virus production was calculated and expressed as A_{450} per 10^6 live cells or per milligram of total cell protein.

Detection of cell clumping. Clustering of the feline T-lymphoma 3201B cells following infection by FeLV-C and FeLV-C-based recombinant viruses was readily visible by light microscopy. While uninfected 3201B cells in suspension did not exhibit any significant tight aggregation at cell densities of up to 5 × 10^6/ml, the infected cells showed dramatic clumping at all cell densities. The clumps consisted of an average of 20 to 40 cells which could be disrupted to single cells only after vigorous pipetting. However, the infected cells regrouped rapidly after the clumps were disrupted and the culture was allowed to stand.

RESULTS

Infectivity of in vitro-constructed chimeric FeLVs. Four different chimeric FeLVs were constructed by using the frame of cloned FeLV-B-GA proviral DNA, into which increasing portions of the endogenous FeLV sequences from the clone CFE-6 were introduced. As shown in Fig. 1, the r6gpN construct contained only the N-terminal half of gp70 from the endogenous provirus, while r6gp and r6*env* had most of the gp70 and the entire gp70-p15E region, respectively, from CFE-6 DNA. The construct r6*env*-LTR was similar to r6*env* except that the donor sequences extended to include the endogenous LTR. A fifth construct, r16gpN, was analogous to r6gpN, but its endogenous region was derived from a different clone, CFE-16. Results of the in vitro

TABLE 1. Summary of infectivity of chimeric FeLV constructs in vitro

Plasmid	Infectivity for transfected cell line[a]:						
	H927	3201B	HT1080	CEM	Raji	NIH 3T3	CCL-64
FeLV-B-GA	+	−	+	+	+	−	−
r6gpN	+	−	+	+	+	−	−
r16gpN	+	−	+	+	+	−	−
r6gp	−	−	−	−	−	−	−
r6*env*	−	−	−	−	−	−	−
r6*env*-LTR	−	−	−	−	−	−	−

[a] All cell lines except lymphoid cells (3201B, CEM, and Raji) were transfected with plasmid DNA by the calcium phosphate coprecipitation method as described previously (3). For lymphoid cells, the DEAE-dextran procedure of transfection was used (12). Virus released into cell culture medium was detected by ELISA and an RT assay. Symbols: −, no virus detected in repeated transfection experiments with continued measurements up to 10 weeks posttransfection; +, good level of virus production, e.g., RT activity in terms of counts per minute of [^{3}H]dTMP incorporated per 3 ml of culture fluid per 60-min incubation at 2 to 3 weeks posttransfection ranged from 265,000 to 864,000, while pretransfection values were only 400 to 3,000.

infectivity of these chimeric FeLV constructs are summarized in Table 1. Of the five chimeric FeLVs, only two, r6gpN and r16gpN, were able to replicate in feline and human fibroblastic cells (H927 and HT1080) and in human lymphoid B- and T-cell lines (Raji and CEM). In this respect, the recombinants were similar to the parental virus (FeLV-B-GA), which also grew in the above-mentioned cell lines but like the two recombinants could not be propagated in feline T-lymphoma (3201B), mouse NIH 3T3, and mink (CCL-64) cell lines. Thus, replacement of the N-terminal half of the exogenous viral gp70 protein by the corresponding region of the endogenous sequences clearly retained infectivity. However, when the size of the exchange was extended to include most of the endogenous gp70, the entire *env*, or the *env*-LTR region, as represented by constructs r6gp, r6*env*, and r6*env*-LTR, respectively, the chimeras lost the ability to replicate in the cell lines tested.

The relative infectivity of r6gpN and r16gpN was of interest. While the abilities of these constructs to grow in fibroblastic cells were comparable and were very similar to that of the parental FeLV-B virus, they differed in their replication efficiencies in a lymphoid cell line. The recombinant r16gpN replicated well in both Raji and CEM cells, but r6gpN virus exhibited reduced growth kinetics in Raji cells. Representative data showing the relative growth activities of these viruses are shown in Fig. 2. In the substituted N-terminal gp70 region, r16gpN diverged from FeLV-B-GA at only two amino acid positions (19). The divergence in r6gpN was higher, since four amino acid substitutions could be predicted (19).

Recombination induced in cotransfected cells. The second strategy that we used to generate recombinant viruses involved expression of both exogenous-*env*- and endogenous-*env*-specific viral RNAs in the same cells so as to enhance chances of recombination by heterozygote formation. The scheme of this work using infectious FeLV-C-Sarma DNA and a noninfectious proviral DNA construct (r6gp, r6*env*, or r6*env*-LTR) is presented in Fig. 3, and the PCR strategy for analyzing the recombinants generated is shown in Fig. 4. The PCR amplification of the regions, whose fragment size varied between those of FeLV-C and endogenous elements, yielded a rapid procedure to detect recombination. For example, the first set of primers (P1 and P2) was expected to yield a 532-bp DNA product from the 5′ end of the endoge-

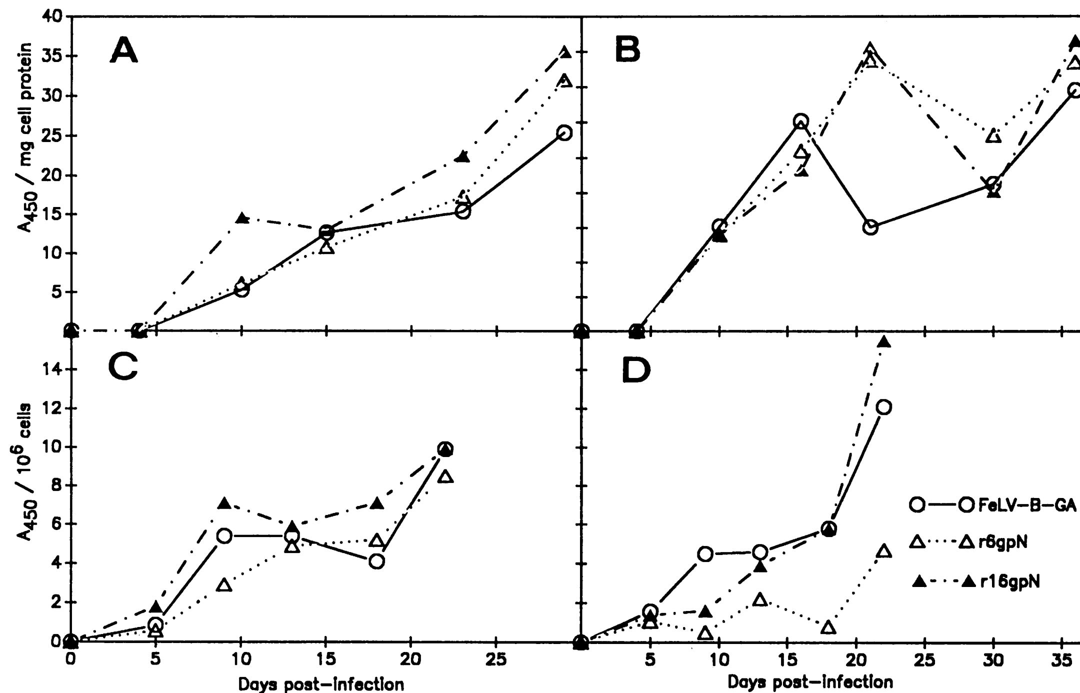

FIG. 2. Comparative replication of r6gpN and r16gpN viruses in different cell lines. Cell lines were feline embryo fibroblast H927 (A), human fibrosarcoma HT1080 (B), human T-lymphoblastic leukemic CEM (C), and human B-lymphoid tumor Raji (D).

nous *env*, while the product size should be larger (568 bp) if the DNA was copied from FeLV-C DNA. Conversely, the second primer set (P3 and P4) should yield a larger fragment from the endogenous clone (746 bp) relative to the FeLV-C mid-gp70 region (641 bp). The results of cotransfection of

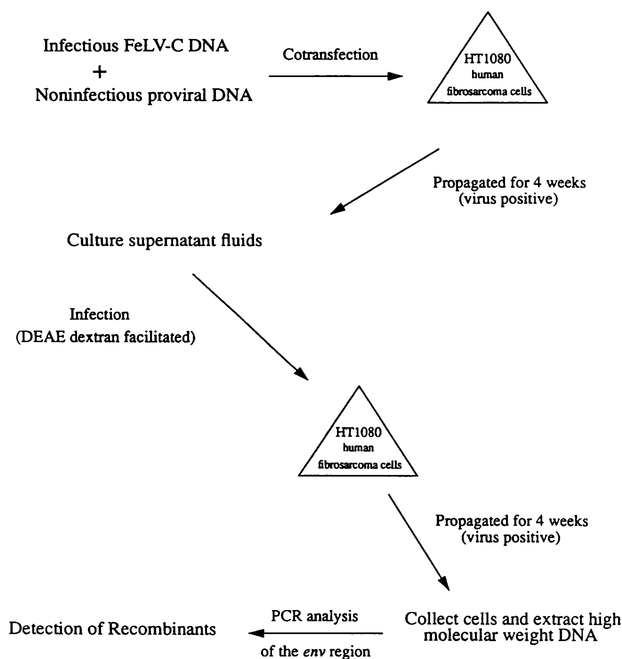

FIG. 3. Schematic representation of coexpression used to generate recombinant FeLVs.

FeLV-C DNA with constructs containing various lengths of the endogenous *env*-LTR region showed that recombinants were generated with a high frequency. As illustrated in Fig. 5, primer set 1 produced two bands (panel A), one corresponding to the size of the FeLV-C fragment (band C) and the other similar to the band for the endogenous *env* region (band E). Analysis of the mid-gp70 region with primer set 2 revealed multiple bands (Fig. 5C and D), of which the fastest-migrating FeLV-C-specific band was the lowest in intensity. One prominent band corresponded to the expected migration of a fragment similar to endogenous *env* (band E). Of the additional two bands, one migrated at an intermediate rate (band E^-) and the other migrated slower than the endogenous product (band E^+). When annealed to an oligonucleotide probe specific for the endogenous CFE-6 and located adjacent but 3′ to the P3 primer sequence, each one of bands E^-, E, and E^+ hybridized, indicating specificity of the derived PCR products and acquisition of endogenous *env*-specific domains (Fig. 5D). Figure 5B shows mainly a single band made by using primer set 3. Since the sizes of the products from the end gp70-p15E region were estimated to vary by only 3 bp, a resolution between bands of endogenous and exogenous origin, if produced together, was not expected to be manifest in 4% agarose gels. The slower-migrating minor band in lane 3 (panel B) appeared to be an artifact, since it could not be seen in repeat experiments.

Clearly, the products generated with primer sets 1 and 2 were most revealing of the rapid generation of the recombinant FeLVs containing endogenous *env* sequences. Additionally, these products had several notable aspects. First, recombinants apparently propagated in the HT1080 cells more efficiently than the parental FeLV-C virus, since the FeLV-C-specific band was only poorly visible in the gel analyses relative to the intensity of the other new bands.

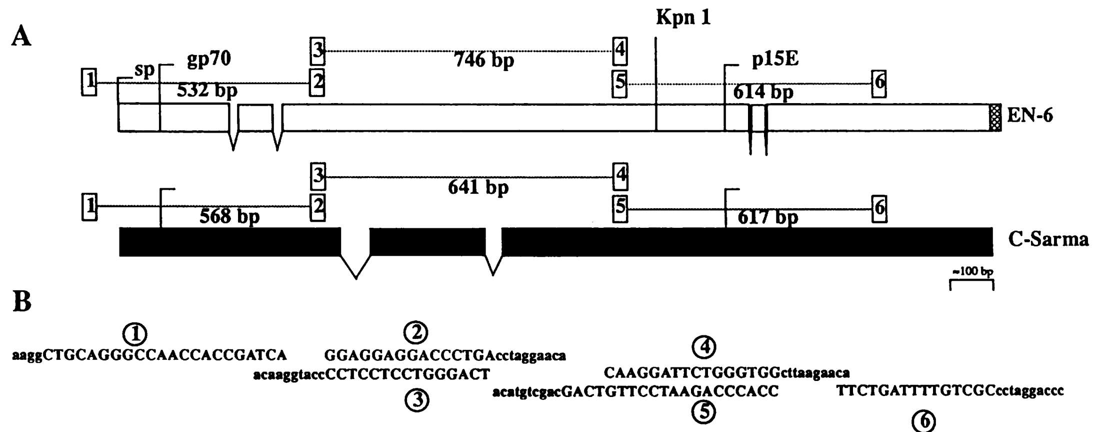

FIG. 4. Strategy used for generating PCR products. (A) Schematics of *env* gene coding sequences for endogenous clone CFE-6 (EN-6) and FeLV-C-Sarma (C-Sarma) are shown. The hatched area at the C-terminal part of EN-6 represents the extra coding sequences in the endogenous clone. Numbers in boxes denote the primers used. The lengths of the expected products including the primer sequences are shown under the dotted lines connecting each set of primers. (B) Nucleotide sequences of the primers synthesized, with lowercase letters indicating the added restriction sites.

Second, analysis of the primer set 2 products revealed multiple but discrete classes of recombinants which were capable of growing in HT1080 cells. Furthermore, while band E^- of the mid-gp70 region was prominent in crosses of FeLV-C and r6gp, crosses with r6*env* or r6*env*-LTR under otherwise identical conditions produced all three bands (E^-, E, and E^+), although E^- was only faintly visible in the r6*env*-LTR cross. Since retroviral recombination is a result of virions containing different RNA molecules (15), the primary sequence of the endogenous sequences placed in the noninfectious recombination partner probably influenced the frequency of recombinations at specific sites. This factor, along with preferential replication of certain recombinants over others formed, might have contributed to the observed effect of the endogenous-insert size variation. No recombination was detected when FeLV-C DNA was coexpressed with the CFE-16 plasmid, since additional PCR products other than the exogenous viral C band were not visible in the gel analysis (Fig. 5). Although the CFE-16 LTR was strongly active in driving transcription (3), its *env* gene is deleted from the mid-gp70 region to the end of p15E transmembrane protein (19). The functional inability of CFE-16 might be related to this large deletion, which prevented the generation of viable recombinant viruses containing domains of the *env* gene retained in this endogenous proviral clone.

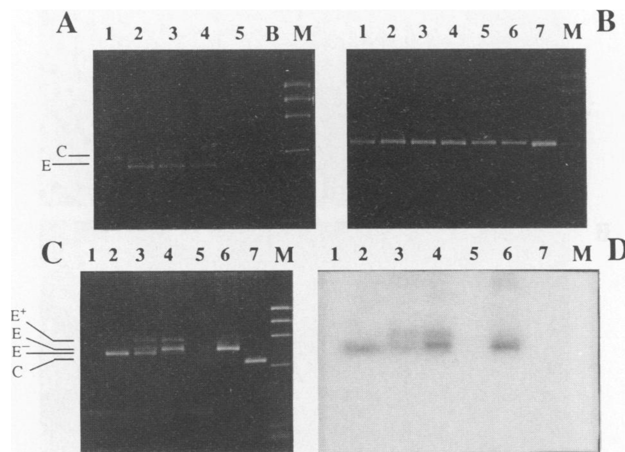

FIG. 5. Analysis of PCR products by electrophoresis. Products derived from primer sets 1 (A), 3 (B), and 2 (C) were electrophoresed on 4% agarose gels, and the dried gel of panel C was hybridized to an endogenous FeLV-specific synthetic oligonucleotide (D). The oligonucleotide represented a 21-mer sequence (nucleotide positions 587 to 607) published elsewhere (19). Lanes: 1, FeLV-C-Sarma; 2, FeLV-C × r6gp; 3, FeLV-C × r6*env*; 4, FeLV-C × r6*env*-LTR; 5, FeLV-C × pCFE-16; 6, CFE-6 plasmid DNA control; 7, FeLV-C plasmid DNA control; M, φX174-*Hae*III-cut marker; B, distilled-water blank for PCR.

In vitro infectivity of FeLV-C and the recombinant mixtures generated in human HT1080 cells was determined in the three lymphoid cell lines 3201B, CEM, and Raji. In all of these cell lines, the viruses replicated with high efficiency (Table 2). PCR analysis of the infected 3201B cells at consecutive passage levels up to 23 days postinfection revealed that the pattern of the PCR products did not change significantly in the course of propagation. As seen with the primer set 2, all three bands (E^-, E, and E^+) were retained throughout the period (Fig. 6). It should, however, be noted that at least part, if not most, of the materials in the E-band area were probably contributed by amplification at some of the inherited endogenous genomic loci. In contrast to human HT1080 cells, the human T-cell line (CEM) did not permit replication of all three mid-gp70 classes (E^-, E, and E^+) of recombinants. PCR analysis of the DNA from infected CEM cells revealed a strong E^- band, while the E band was not detected at all (data not shown). The delayed appearance of a minor E^+ band was, however, visible at 23 days postinfection. This might be related to slow replication in CEM cells of one or more of the virus populations present in the E^+ class. However, it should be noted that the PCR reactions in this work were not performed to be quantitative. Therefore, the relative amounts of the different *env* gene recombinants in the infected cells, while could be indicative of differences in the replication ability of the recombinants, did not prove that any such differences existed.

During the infectivity study, feline 3201B T-lymphoma cells exhibited a striking property of clumping. While the uninfected cells remained primarily as single cells in the

TABLE 2. Infectivity of FeLV-C and recombinant viruses in lymphoid cells

Infectious isolate[a]	Virus released in culture medium (ELISA $A_{450}/10^6$ cells)[b]					
	3201B		CEM		Raji	
	6 days p.i.	14 days p.i.	6 days p.i.	14 days p.i.	6 days p.i.	14 days p.i.
FeLV-C-Sarma	9.5	9.5	1.0	4.5	4.0	9.0
FeLV-C × r6gp	9.0	10.0	1.0	7.5	2.0	10.0
FeLV-C × r6*env*	11.0	14.0	1.0	10.0	5.0	9.0
FeLV-C × r6*env*-LTR	8.0	14.0	1.5	7.0	6.0	10.0

[a] All virus isolates were propagated in HT1080 cells prior to infection for the production of inoculum.
[b] ELISA to detect the presence of p27 core protein in the cell culture fluid was conducted by using the VIRACHEK FeLV test kit per kit protocol. Cell counts were determined on a hemacytometer with 0.2% trypan blue dye exclusion. p.i., postinfection.

suspension culture, infection with either FeLV-C or the derived recombinants prompted aggregations of, on average, 20 to 40 cells, which adhered to each other with considerable tenacity. The clumps could be disrupted mechanically, such as by pipetting the cell suspension in and out several times. However, reclumping would occur as soon as the cells were allowed to settle. Representative photomicrographs of the clumped cells and uninfected cells are shown in Fig. 7. One apparent consequence of clumping was the noticeable growth retardation of these cells infected by either FeLV-C or mixtures of the FeLV-C-derived recombinants under the culture conditions used (Fig. 8). The degree of inhibition of the growth rate, however, was not very striking; it varied only from approximately two- to eightfold after 24 days in culture relative to inhibition of uninfected 3201B cells. The clustering effect of FeLV infection could not be meaningfully compared with that caused by either FeLV-B or FeLV-A infection, since FeLV-B could not be grown and FeLV-A grew only very poorly in these 3201B cells. However, the specificity with which FeLV-C and related recombinants produced the aggregation was apparent when a comparison was made with recombinants generated from 3201B cells by introducing FeLV-A DNA into these 3201B cells by electroporation (our unpublished results). These FeLV-A-derived recombinants, not yet fully characterized, replicated in 3201B cells to levels similar to those of the FeLV-C viruses but did not induce the characteristic clustering.

Biologically selected sites of recombination. The PCR products generated from the genomic DNA of HT1080 cells infected with FeLV-C × r6gp, FeLV-C × r6*env*, or FeLV-C × r6*env*-LTR were cloned into the TA cloning vector (Invitrogen). Several clones were isolated and sequenced to determine the crossover points. A summary of the results obtained is presented in Table 3. First, we isolated and characterized the E band from the following clones amplified by primer set 1: two from FeLV-C × r6gp, two from FeLV-C × r6*env*, and two from FeLV-C × r6*env*-LTR crosses. DNA sequencing indicated that the products in each case originated from the endogenous CFE-6 DNA. This is shown diagramatically in Fig. 9 by emphasizing the areas of sequence divergence between FeLV-C and the CFE-6 DNAs in the region concerned. Similarly, cloning of PCR products from primer set 2 yielded multiple clones, some of which were characterized.

DNA sequencing revealed that all of the primer set 2 products contained CFE-6 DNA sequences at the 5′ ends and variable amounts of FeLV-C DNA sequences at the 3′ ends. The crossover sites varied from product to product,

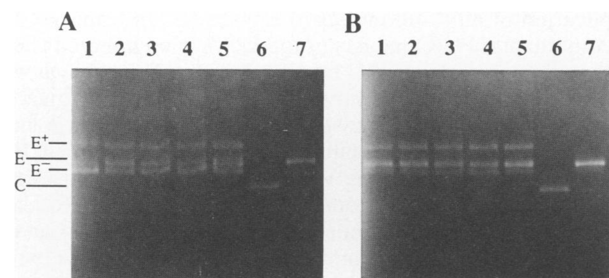

FIG. 6. Propagation of recombinant virus classes in 3201B cells. DNA was amplified by using primer set 2 from cells infected with viruses derived from FeLV-C × r6*env* (A) or FeLV-C × r6*env*-LTR (B) crosses at 6, 10, 14, 18, and 23 days postinfection (lanes 1 through 5, respectively). Lanes 6 and 7 show relative migration of the products from FeLV-C and CFE-6 plasmids, respectively.

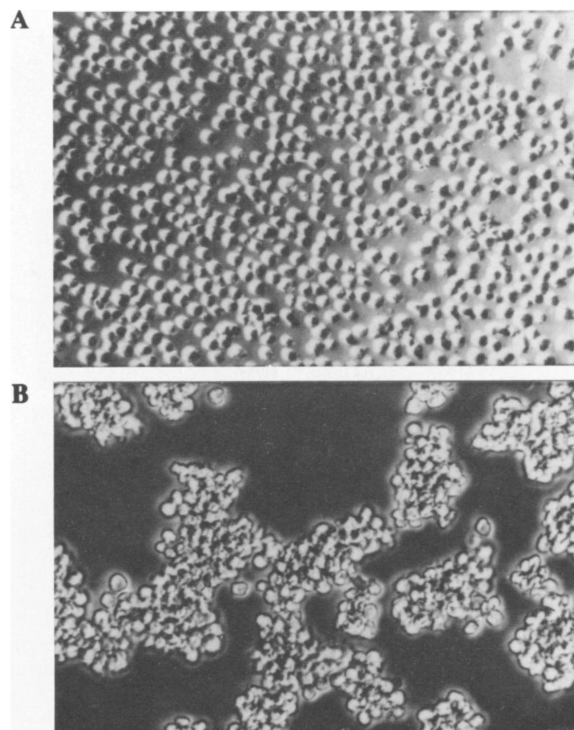

FIG. 7. Cell clumping induced by FeLV-C-related virus infections. Feline T-lymphoma 3201B cells, uninfected (A) or infected with FeLV-C-Sarma (B), are presented at ×20 magnification. The characteristic aggregation shown in panel B was also seen with all FeLV-C-based recombinants beginning 4 to 6 days postinfection.

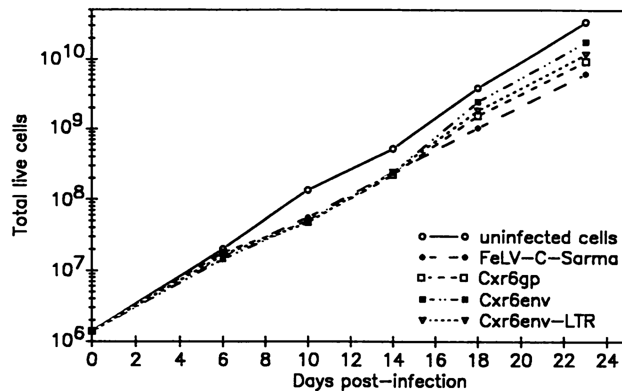

FIG. 8. Growth inhibition of 3201B cells by FeLV-C-related virus infection. Total live cells were counted by trypan blue exclusion method.

and, as illustrated in Fig. 9, under the selection conditions imposed, four regions of recombination could be identified. These four sites are marked 1 to 4 in Fig. 9, with the length of endogenous sequences increasing from 5' to 3'. In the two cloned products which had originated from a FeLV-C × r6gp cross, the recombination point (site 1) was found to be located between nucleotide positions 634 and 647, position 1 being the *Pst*I site upstream of the *env* gene of FeLV-C. Since the FeLV-C × r6gp cross yielded only E^--type products in the mid-gp70 region and since it contained only one of the two major insertions of the endogenous gp70 relative to FeLV-C, these sequences appeared to represent the E^- band. Similarly, five other clones, three derived from the FeLV-C × r6*env* cross and two derived from the FeLV-C × r6*env*-LTR cross, showed recombination points which could reside between nucleotides 670 and 760 (site 2). The location of this site could not be identified more accurately, since the homology between the recombinant partners was 100% in that domain. As stated above, these

TABLE 3. Clones of PCR products analyzed

Source of recombinant	Primer set used for amplification	No. of plasmid clones sequenced	Sequence origin (crossover sites)
FeLV-C × r6gp	1	2	CFE-6
	2	2	CFE-6/FeLV-C (site 1)
	3	2	FeLV-C
FeLV-C × r6*env*	1	2	CFE-6
	2	8	CFE-6/FeLV-C (site 2 in three clones, site 3 in three clones, site 4 in two clones)
	3	2	FeLV-C
FeLV-C × r6*env*-LTR	1	2	CFE-6
	2	7	CFE-6/FeLV-C (site 2 in two clones, site 3 in four clones, remaining clone deleted upstream of site 4)
	3	2	FeLV-C

recombinants were probably also of the E^- class. Crossover sites 3 and 4 were recognized from the analysis of the FeLV-C × 6*env* or FeLV-C × 6*env*-LTR products. Site 3 could be pinpointed to nucleotide 951 in 7 of the 10 clones analyzed. Of the remaining three clones, one was deleted at sequences 3' to nucleotide position 1007 and the other two were complete. In each of these three cases, the sequence was identical to that of the endogenous provirus CFE-6. On the basis of these results, crossover site 4 was tentatively assigned to the sequence of homology in the vicinity of the primer 5. Since recombinants involving site 3 or 4 contained equal amounts of gp70 sequences, the migration of the PCR products should be similar to band E. However, none of the cloned DNAs showed any new insertion in the recombinant mid-gp70 region to indicate a high-molecular-weight product that could correspond to the migration character of the E^+ band. PCR priming from the plasmid clones yielded only band E, suggesting that no E^+ clones were isolated (data not shown). Therefore, it appeared that the four general regions identified in this work represented some but not all of the various *env* gene recombinations. Moreover, while the recombination points were identical in multiple clones, it remained to be determined if they represented clones derived from the same or independent crossover events. Considering these limitations, it was difficult to infer if certain "hot spots" for recombination might occur within the FeLV gp70 gene sequences.

Analysis of the DNA sequences of the products of the last primer set showed complete homology to FeLV-C. All in all, six cloned DNAs were sequenced in this region, and each yielded the same sequence. Consistent with the origin of this part from FeLV-C, restriction analysis of the PCR products of all three different types of crosses suggested similarity to FeLV-C sequences. This analysis was based on the occurrence of a *Kpn*I site on the endogenous-related fragment but not on FeLV-C DNA in that area (Fig. 4 and 9). Accordingly, while the PCR band from CFE-6 plasmid could be cleaved in the middle by *Kpn*I, the products of the recombinants were completely resistant to this digestion (data not shown).

Generation of recombinant FeLVs in FeLV-C-infected feline T-lymphoid cells. Pursuant to our earlier observation that feline T-lymphoma tissues naturally express significant quantities of endogenous FeLV sequences (24), it was thought that results analogous to induced coexpression might also be produced when T-lymphoma cells in culture were infected with FeLV-C. Since 3201B is such a cell line which is not infected by FeLV but expresses high levels of endogenous FeLV RNA (20a), we infected these cells with cloned FeLV-C grown in human HT1080 cells and monitored the generation of recombinant FeLVs by using the PCR method with primer set 2. Figure 10 shows that the E^+ band was clearly detected at 14 and 23 days postinfection. The DNA yield from the sample at 18 days postinfection was inexplicably low. Perhaps impurities present in this DNA sample contributed to the poor quality of the resulting PCR products. The intensity of the E band was the greatest in this series of experiments. Most of the E-band materials were apparently derived from amplification of the proviral sequences conserved in the germ line of the cats, since uninfected 3201B cell DNA showed a single strong E band. At this time, we did not confirm that this band from postinfection cells represented recombinant viruses, although this possibility remains viable in relation to the results obtained in coexpression experiments.

One known primary difference between the natural and in vitro-reconstructed feline endogenous proviruses is that the

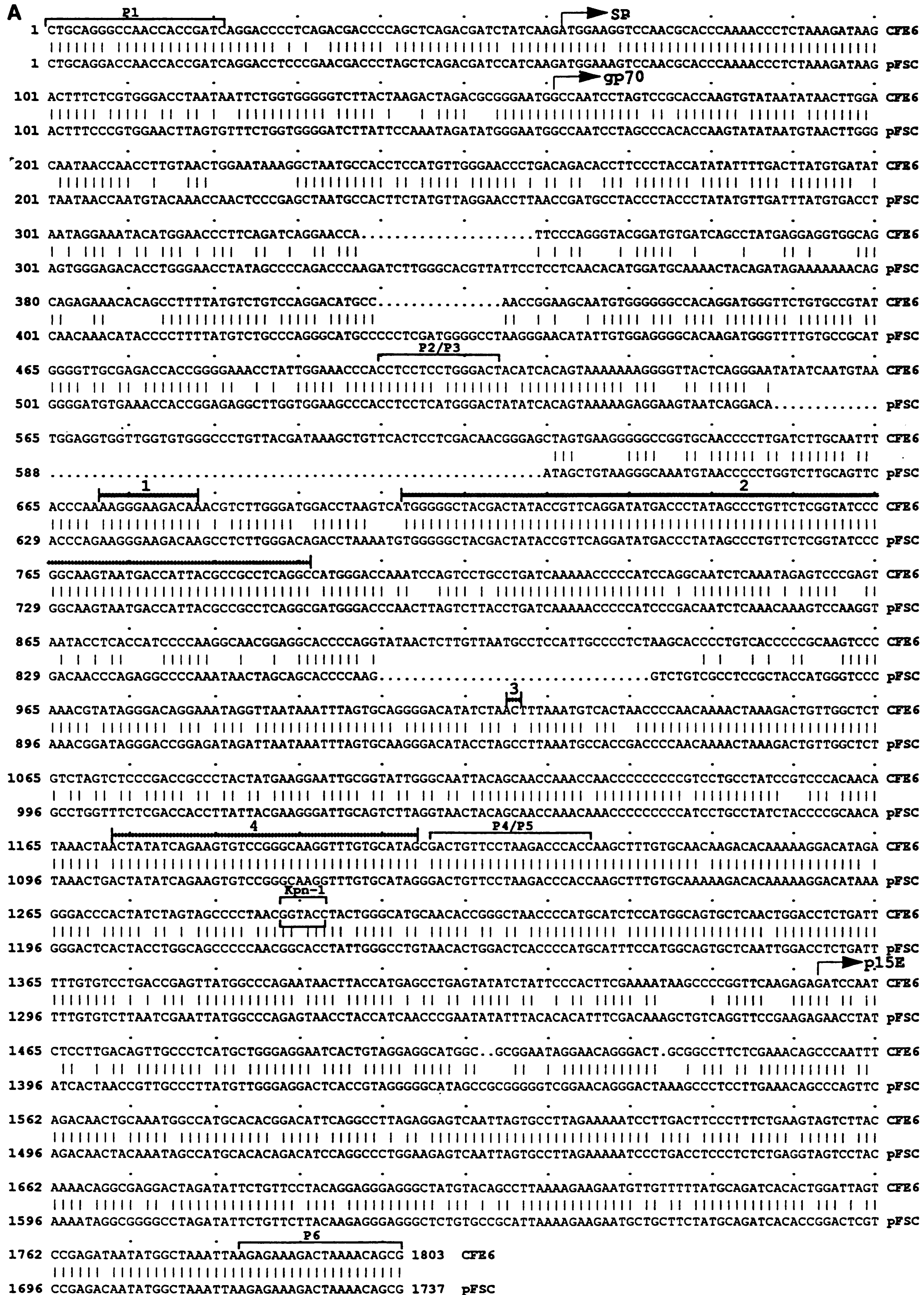

FIG. 9.

gag gene of the endogenous elements is defective because of both nonsense and missense mutations (3), while that of the constructs was not defective because the constructs contained the *gag-pol* region from infectious FeLV-B. These mutations in the *gag* region of the endogenous provirus, however, were not expected to interfere with heterozygote formation in virion RNA, since the packaging signals are conserved in the endogenous proviral sequences (3). We asked if the *pol* gene of endogenous proviruses was mutated or not. If it is not, then the recombination could naturally occur upstream of the endogenous *env* at some sites in the *pol* gene. However, if *pol* is severely defective like *gag*, then the selection of the 5′ recombination sites must be restricted to areas close to the *env* open reading frame of the endogenous elements (19). To address this important question, we sequenced the entire *pol* gene of the endogenous provirus CFE-6.

DNA sequence of CFE-6 *pol* gene. By using Sanger's dideoxy procedure and the rapid deletion method (8), the 3.5-kb region of CFE-6 DNA encompassing the *pol* gene was sequenced. The final analysis provided a 3,639-bp-long *pol* gene (Fig. 11) which exhibited 94.6% nucleotide homology to the published sequence of this gene from infectious FeLV-A (10). Within the *pol* gene, in which the three functions protease-RT-integrase (INT) are located in the order stated, both RT and INT were found to be highly conserved in the endogenous provirus, with each exhibiting an open reading frame. The amino acid homology (Fig. 12) of these two regions to the corresponding sequences of FeLV-A was >94%. The sequence of the protease gene, which is also highly conserved, however, showed an insertion of CC sequence near the 5′ end which yielded three extra termination codons (Fig. 12) in addition to the amber codon normally present at the *gag-pol* boundary in murine and feline oncoviruses. Thus, except for a short stretch at the 5′ end of the endogenous *pol* gene, most of the *pol* gene appeared to be similar to that of infectious FeLV and conceivably would be available for exchanges in recombination events with the infectious-FeLV *pol* gene. However, for the exchanges to be viable, the portions of the endogenous *pol* gene captured must be functionally compatible. This issue remained largely unexplored except that the C-terminal portion of the INT protein, which was included in the constructs of r6gpN and r16gpN, proved to be functional.

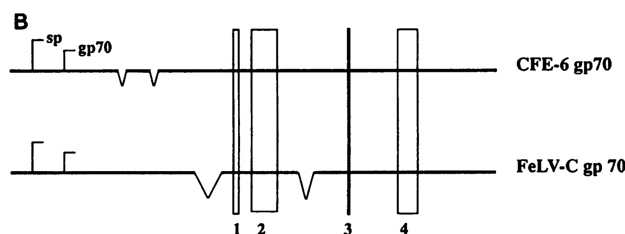

FIG. 9. Identification of recombination sites on the *env* gene. (A) The sequences presented are those of the endogenous CFE-6 provirus (19) and FeLV-C-Sarma (pFSC) (29), with numbering beginning from 61 bases upstream of the *env* gene. Dotted lines denote positions of the gaps in the sequence alignment, and P1 to P6 indicate positions of the PCR primers. The four recombination sites or regions identified are indicated by the braided lines above the sequences involved. Also marked are the start points of the signal peptide (SP), the gp70 and p15E proteins, and the *Kpn*I site present in CFE-6 but not in FeLV-C. The two internal primers, PI-1 and PI-2, used in DNA sequencing correspond to nucleotide positions 729 to 744 and 1036 to 1052, respectively, on the CFE-6 sequence. (B) Genetic maps of the endogenous CFE-6 and exogenous FeLV-C sequences from panel A are presented schematically for ease in determining the location of crossover regions on the gp70 protein. The numbers below the vertical lines denote the identified recombination regions.

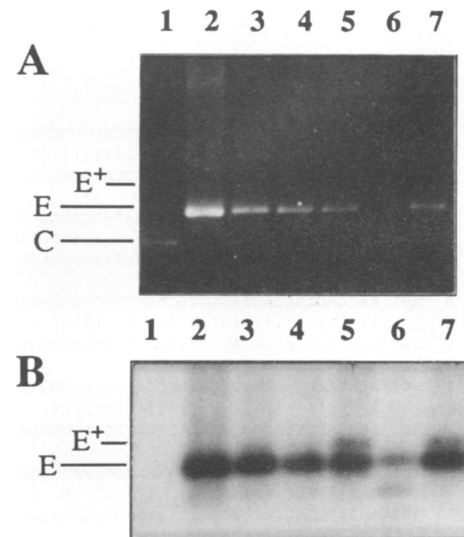

FIG. 10. Detection of recombinants in FeLV-C-infected 3201 B cells. Cellular DNA was amplified by using primer set 2 from 6, 10, 14, 18, and 23 days postinfection (lanes 3 through 7, respectively) and electrophoresed as for Fig. 5. (A) Ethidium bromide stained gel. (B) Southern blot of the same gel hybridized to an endogenous FeLV-specific oligomer (same as in Fig. 5). The PCR of DNA from uninfected 3201B cells, although not included here, was run separately at different times, and each time showed a single E band similar to that seen in lane 3 of each panel. Lanes 1 and 2, FeLV-C and CFE-6 plasmid controls, respectively.

DISCUSSION

We have generated several recombinant FeLVs by in vitro chimeric constructions or by induced recombination in transfected cells. In these recombinants, the exogenous FeLV-B or -C viral sequences in the gp70 surface glycoprotein area are variably replaced by the corresponding regions of endogenous noninfectious-FeLV proviral sequences. Individual and mixtures of recombinant viruses produced in this manner have been tested for in vitro host cell tropism, replication efficiency, and effects on cell growth and behavior. Analysis of FeLV recombinants formed by coexpression also reveals some of the points of crossover in the gp70 protein, which are selected on the basis of biological activity.

These results have uncovered several new facts which may be potentially significant in the pathogenesis of FeLV-induced diseases. The endogenous FeLV sequences which retain a completely open reading frame in the *env* gene (19) are shown here to possess the capacity to contribute up to approximately 75% of their gp70 protein from the N-terminal end to the frame of an exogenous FeLV-B or -C when viable recombinants are produced. It is of interest that the recombinants generated by substitution of the N-terminal half of the gp70 (constructs r6gpN and r16gpN), while capable of propagation in certain feline and nonfeline cell lines like their progenitor FeLV-B virus, may differ in their replication efficiencies in specific cells. Compared with r6gpN, r16gpN grows well in human Raji cells, but the replication effi-

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        PRO
A    1 CTCAACcTAGAAGATTAGGAagGTCAGGGCCAGGACCCCCCCCcccGAGCCCAGGATAACCcTAAAAgTgGGGGGGCAACCaGTGAaCcTTtCTGGTGG
B    1 CTCAACtTAGAAGATTAGGAgaGTCAGGGCCAGGACCCCCCCC   tGAGCCCAGGATAACCtTAAAAaTaGGGGGGCAACCgGTGA CtTTcCTGGTGG

A  101 AtACGGGAGCCCAGCACTCgGTAcTgACTCGACCAGAcGGACCTCTCAGTGACCGCtCAGCCCTGGTGCAAGGAGCCACaGGAAGCAAAAACTACCGaTG
B   98 AcACGGGAGCCCAGCACTCaGTAtTaACTCGACCAGAtGGACCTCTCAGTGACCGCaCAGCCCTGGTGCAAGGAGCCACgGGAAGCAAAAACTACCGgTG

A  201 GACCACCGACAGGAGGGTgCAACTGGCAACCcGTAAGGTGACTCATTCTTTTTATATGTACCTGAATGTCCCTACCCGTTATTAGGgAGAGACCTATTA
B  198 GACCACCGACAGGAGGGTaCAACTGGCAACCgGTAAGGTGACTCATTCTTTTTATATGTACCTGAATGTCCCTACCCGTTATTAGGaAGAGACCTATTA
                                                                                          RT
A  301 ACcAAACTcAAGGCtCAgATCCATTTcACCGGgGAAGGaGCTAATGTTGTTGGaCCCAtGGGcTcACCCCTACAAGTCCTcACctTgCAAcTAGAAGAAG
B  298 ACtAAACTtAAGGCcCAaATCCATTTtACCGGaGAAGGgGCTAATGTTGTTGGgCCCAgGGGtTtACCCCTACAAGTCCTtACtcTaCAAtTAGAAGAAG

A  401 AGTATCGGCTATTTGAGCCgaAAAGTgaACtgAAACAaGgtATGGACAgTTGGCTTAAAAACTTTCCCCgGGCATGGGCAGAAACAGGAGGTATGGAAT
B  398 AGTATCGGCTATTTGAGCCagAAAGTacACaaAAACAgGagATGGACAtTTGGCTTAAAAACTTTCCCCaGGCATGGGCAGAAACAGGAGGTATGGAAT

A  501 GGCTCATTGcCAAGCCCCCaTcCTCATTCAACTTAAaGCTACTGCCACcCCAATCTCCATtCGgCAGTAcCCcATGCCCCATGAAGCtTACCAaGGAATT
B  498 GGCTCATTGtCAAGCCCCCgTtCTCATTCAACTTAAgGCTACTGCCACtCCAATCTCCATcCGaCAGTAtCCtATGCCCCATGAAGCcTACCAgGGAATT

A  601 AAACCcCATATAAGgAGAATGCTgGAcCAAGGCATCCTCAAGCCCTGCCgGTCCCCATGGAATACACCCcTATTACCTGTcAAaAAGCCAGGaACCggGG
B  598 AAACCtCATATAAGaAGAATGCTaGAtCAAGGCATCCTCAAGCCCTGCCaGTCCCCATGGAATACACCCtTATTACCTGTtAAgAAGCCAGGgACCaaGG

A  701 ATTACcGACCAGTGCAGGACTTAAGAGAAGTAAAtAAAAGgGTAGAgGACATCCATCCcACTGTGCCAAAcCCATAcAACCTCCTTAGCACCCTCCCaCC
B  698 ATTACaGACCAGTGCAGGACTTAAGAGAAGTAAAcAAAAGaGTAGAaGACATCCATCCtACTGTGCCAAAtCCATAtAACCTCCTTAGCACCCTCCCgCC

A  801 tTCTCACCCTTGGTACACcGTCCTAGATcTAAAAGAtGCTTTTTCTGCtTGCGACTACACcCTGAGAGcCAATTACTcTTTGCATTTGAATGGAaAGAT
B  798 gTCTCACCCTTGGTACACtGTCCTAGATtTAAAAGAcGCTTTTTCTGCcTGCGACTACACtCTGAGAGtCAATTACTtTTTGCATTTGAATGGAgAGAT

A  901 CCAGAgATAGGgCTGTCAGGGCAaCTgACCTGGACtCGCCTTCCaCAaGGGTTCAAGAACAGCCCCACCCTATTTGAcGAGGCcCTaCACTCAGACCTGG
B  898 CCAGAaATAGGaCTGTCAGGGCAgCTaACCTGGACaCGCCTTCCtCAgGGGTTCAAGAACAGCCCCACCCTATTTGAtGAGGCtCTgCACTCAGACCTGG

A 1001 CaGATTTCAGGGTgAGGTAtCCaGCTtTAGTCCTCCTACAATATGTAGATGACCTCTTGCTGGCTGCGGCAACCAaGACcGAAaGCCTaGAAGGGACTAA
B  998 CcGATTTCAGGGTaAGGTAcCCgGCTcTAGTCCTCCTACAATATGTAGATGACCTCTTGCTGGCTGCGGCAACCAgGACtGAAtGCCTgGAAGGGACTAA

A 1101 GGCACTCCTTGAGACTTTGGGCAATAAGGGtTACCGAGCCTCTGCAAAGAAGGCCCAgATTTGCCTGCAgaAAGTCACATACCTaGGGTACTCTTTAgAg
B 1098 GGCACTCCTTGAGACTTTGGGCAATAAGGGgTACCGAGCCTCTGCAAAGAAGGCCCAaATTTGCCTGCAagAAGTCACATACCTgGGGTACTCTTTAaAa

A 1201 GATGGtCAAAGaTGGCTTACCAAAGCTCGgAAAGAAGCCATtCTcTCCATCCCTGTGCCTAAAAACCCACGgCAAGTgAGAGAGTTCCTTGGAcCTGCAG
B 1198 GATGGcCAAAGgTGGCTTACCAAAGCTCGcAAAGAAGCCATcCTaTCCATCCCTGTGCCTAAAAACCCACGaCAAGTaAGAGAGTTCCTTGGAaCTGCAG

A 1301 GcTACTGCCGGCTGTGGATTCCCGGTTTTGCCGAGCTCGCtGCCCCGCTATAtCCTCTCACTCGACCAGGAACTCTGTTtCAGTGGGGAgCAGAaCAACA
B 1298 GtTACTGCCGGCTGTGGATTCCCGGTTTTGCCGAGCTCGCaGCCCCGCTATAcCCTCTCACTCGACCAGGAACTCTGTTcCAGTGGGGAaCAGAgCAACA

A 1401 ATTGGCCTTCGAaAACATTAaAAAaGCCCTCTTGAGcTCCCCTGCCCTGGGGTTGCCAGATATCACCAAGCCCTTTaAATTATTTATTGATGAGAgCTCA
B 1398 ATTGGCCTTCGAgAACATTAgAAAgGCCCTCTTGAGtTCCCCTGCCCTGGGGTTGCCAGATATCACCAAGCCCTTTgAATTATTTATTGATGAGAaCTCA

A 1501 GGgTTTGCGAAGGGGGTGTTgGTCCAAAAACTGGGACCCTGGAAAAGACCAGTTGCCTACCTATCAAAAAAaCTGGATACAGTGGCATCTGGATGGCCCC
B 1498 GGaTTTGCGAAGGGGGTGTTaGTCCAAAAACTGGGACCCTGGAAAAGACCAGTTGCCTACCTATCAAAAAAgCTGGATACAGTGGCATCTGGATGGCCCC

A 1601 CTTGTTTACGCATGGTTGCAGCCATCGCCATCCTAGTCAAGGATGCAGGGAAGCTAACtCTAGGACAGCCGCTAACTgTCCTGACCTCCCACCCAGTTGA
B 1598 CTTGTTTACGCATGGTTGCAGCCATCGCCATCCTAGTCAAGGATGCAGGGAAGCTAACcCTAGGACAGCCGCTAACTaTCCTGACCTCCCACCCAGTTGA

A 1801 ACgGTCTCCCTTAACCCTGCcACcTTGCTtCCCCTCCCCAacGaGaGAAgCCACCACGACTGcCTCCAGAtTTTAGCCGAGACCcATGGCACcagacCCG
B 1798 ACaGTCTCCCTTAACCCTGCtACtTTGCTcCCCCTCCCCA  GcGgGAAaCCACCACGACTGtCTCCAGA TTTAGCCGAGACC ATGGCAC  agaCCG
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FIG. 11. Nucleotide sequence of the CFE-6 *pol* gene (A) compared with the FeLV-A published sequence (B) (10). Arrows mark the beginning of the protease (PRO), RT, and INT genes. Uppercase letters indicate similarity, while lowercase letters represent divergence. Spaces indicate deletions or insertions.

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A 1901 ACTTAACTGACCAGCCGTTaCCGGATGCAGACCTGACCTGGTACACGGATGGTAGCAGCTTCATCCGTAACGGAGAGAGAAAAGCCGGAGCCGCgGTAAC
       ||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
B 1892 ACTTAACTGACCAGCCGTTgCCGGATGCAGACCTGACCTGGTACACGGATGGTAGCAGCTTCATCCGTAACGGAGAGAGAAAAGCCGGAGCCGCaGTAAC

A 2001 AACCGAATCTGAGGTAATCTGGGCTGCTTCCCTCCCACCCGGAACaTCAGCCCAGCGAGCCGAACTGATTGCCCTGACCCAGGCACTaAAGATGGCAAAA
       ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||| ||||||||||||
B 1992 AACCGAATCTGAGGTAATCTGGGCTGCTTCCCTCCCACCCGGAACgTCAGCCCAGCGAGCCGAACTGATTGCCCTGACCCAGGCACTgAAGATGGCAAAA

A 2101 GGTAAGAAGCTAACTGTCTATACGGACAGCCGATAcGCCTTTGCTACAGCTCATGTACAtGGGGAAATCTACAGGCGGCGGGGCCTGCTAACTTCAGAAG
       ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
B 2092 GGTAAGAAGCTAACTGTCTATACGGACAGCCGATAtGCCTTTGCTACAGCTCATGTACAcGGGGAAATCTACAGGCGGCGGGGCCTGCTAACTTCAGAAG

A 2201 GAAAAGAAATTAAAAATAAAAATGAAATCCTCGCCCTATTAGAGGCGTTATTCTTACCCAAAAGACTGAGTATCATCCATTGCCCGGGACACCAAAAAGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B 2192 GAAAAGAAATTAAAAATAAAAATGAAATCCTCGCCCTATTAGAGGCGTTATTCTTACCCAAAAGACTGAGTATCATCCATTGCCCGGGACACCAAAAAGG
                                                                                                  INT
A 2301 tGATAGTCCCCAGGCGAAAGGAAACAGATTAGCCGATGATACAGCAAAGAAAGCCGCCACAGAGACTCAATCATCACTAACCATCTTACCCACaGAACTT
        |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
B 2292 cGATAGTCCCCAGGCGAAAGGAAACAGATTAGCCGATGATACAGCAAAGAAAGCCGCCACAGAGACTCAATCATCACTAACCATCTTACCCACtGAACTT

A 2401 ATAGAGGGTCCCAAAAGGCCTCCATGGGAATATGATGACAGTGATTTAGACCTTGTGCAGAAACTCGAAGCTCATTATGAGCCgAAAAGAGGTACCTGGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
B 2392 ATAGAGGGTCCCAAAAGGCCTCCATGGGAATATGATGACAGTGATTTAGACCTTGTGCAGAAACTCGAAGCTCATTATGAGCCaAAAAGAGGTACCTGGG

A 2501 AGTACCaAGGaAAAACTATAATGCCTGAAAAATACGCAAAaGAGTTGATTAGCCATCTGCATAAGTTAACACACCTCAGTGCTAGAAAAATGAAAACTTT
       |||||| ||| ||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B 2492 AGTACCgAGGgAAAACTATAATGCCTGAAAAATACGCAAAgGAGTTGATTAGCCATCTGCATAAGTTAACACACCTCAGTGCTAGAAAAATGAAAACTTT

A 2601 ACTAGAAAGgGAAGAAACTGGGTTTTACCTCCCTAACAGAGACTTACACCTCCGGCAAGTAACAGAGAGCTGCCGGGCATGTGCTCAAATaAACGCAGGg
       ||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
B 2592 ACTAGAAAGaGAAGAAACTGGGTTTTACCTCCCTAACAGAGACTTACACCTCCGGCAAGTAACAGAGAGCTGCCGGGCATGTGCTCAAATcAACGCAGGa

A 2701 AAaATAAAGTTTGGACCTGATGTgAGaGCCCGAGGCCaCCGGCCCGGAAtACATTGGGAAGTAGACTTCACTGAAATCAAGCCAGGAATGTATGGATATA
       || |||||||||||||||||||| || |||||||||| ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
B 2692 AAgATAAAGTTTGGACCTGATGTaAGgGCCCGAGGCCgCCGGCCCGGAAcACATTGGGAAGTAGACTTCACTGAAATCAAGCCAGGAATGTATGGATATA

A 2801 AATACCTCTTGGTGTTCATAGACACCTTCTCTGGCTGGGCCGAaGCTTACCCCGCCAAACATGAAACAGCAAAAGTTGTTGCCAAGAAACTCTTAGAAGA
       ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B 2792 AATACCTCTTGGTGTTCATAGACACCTTCTCTGGCTGGGCCGAgGCTTACCCCGCCAAACATGAAACAGCAAAAGTTGTTGCCAAGAAACTCTTAGAAGA

A 2901 gATTTTTCCtCGtTACGGGATCCCTCAGGTATTGGGTTCAGATAATGGACCCGCCTTcATCTCCCAGGTAAGTCAGTCTGTGGCCACCCTACTGGGGATT
        |||||||| || |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
B 2892 aATTTTTCCcCGcTACGGGATCCCTCAGGTATTGGGTTCAGATAATGGACCCGCCTTtATCTCCCAGGTAAGTCAGTCTGTGGCCACCCTACTGGGGATT

A 3001 AATTGGAAaTTACATTGTGCATAcCGACCCCAAAGTTCAGGTCAGGTAGAAAGAATGAATAGATCAATTAAGGAGACTTTAACTAAATTAACGCTAGAAA
       |||||||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B 2992 AATTGGAAgTTACATTGTGCATAtCGACCCCAAAGTTCAGGTCAGGTAGAAAGAATGAATAGATCAATTAAGGAGACTTTAACTAAATTAACGCTAGAAA

A 3101 CTGGCTCTAAGGATTGGGTGCTCCTCCTGCCCCTGGTTTTATACCGGGTACGTAACACGCCAGGtCCCCACGGGTTAACTCCTTTTGAAATtCTGTACGG
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||| ||||||||
B 3092 CTGGCTCTAAGGATTGGGTGCTCCTCCTGCCCCTGGTTTTATACCGGGTACGTAACACGCCAGGcCCCCACGGGTTAACTCCTTTTGAAATcCTGTACGG

A 3201 GGCACCtCCACCTATGGCTCACTTCTTTGATtCTGAcATCTCTggtTTCGCTACaTCCCCCACcATGCAGGCACATTTACGCGCCCTGCAGCTGGTCCAA
       |||||| |||||||||||||||||||||||| |||| ||||||   |||||||| |||||||| ||||||||||||||||||||||||||||||||||||
B 3192 GGCACCcCCACCTATGGCTCACTTCTTTGATaCTGAtATCTCTacgTTCGCTACcTCCCCCACtATGCAGGCACATTTACGCGCCCTGCAGCTGGTCCAA

A 3301 GAAGAaATCCAGAGACCTCTAGCGGCaGCCTACCGAGAAAAGCTCGAAACCCCGGTTGTGCCTCACCCCTTCAAACCAGGAGACTCCGTCTGGGTTCGGA
       ||||| |||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B 3292 GAAGAgATCCAGAGACCTCTAGCGGCgGCCTACCGAGAAAAGCTCGAAACCCCGGTTGTGCCTCACCCCTTCAAACCAGGAGACTCCGTCTGGGTTCGGA

A 3401 GACATCAAACCAAGAACCTCGAGCCACGGTGGAAgGGACCACATATCGTCCTCCTGACCACCCCCACgGCCTTAAAaGTAGACGGAGTTGCTGCtTGGAT
       |||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||| |||||||| ||||||||||||||||| |||||
B 3392 GACATCAAACCAAGAACCTCGAGCCACGGTGGAAaGGACCACATATCGTCCTCCTGACCACCCCCACaGCCTTAAAgGTAGACGGAGTTGCTGCcTGGAT

A 3501 cCACGCCTCtCATGTaAAgGCTGCAGGgCCAACCACCgATCAgGACCcCTCaGACgaCCCCAGCTCAGACGATCtATCaAGATGGAAgGTCCAACGCACC
        |||||||| ||||| || |||||||| ||||||||| |||| |||| ||| |||  ||||||||||||||||| ||| |||||||| ||||||||||||
B 3492 tCACGCCTCcCATGTgAAaGCTGCAGGaCCAACCACCaATCAaGACCtCTCgGACagCCCCAGCTCAGACGATCcATCgAGATGGAAaGTCCAACGCACC

A 3601 CAAAACCCTCTAAAGATAAGACTtTCTCGTGGgACcTAa
       ||||||||||||||||||||||| |||||||| || ||
B 3592 CAAAACCCTCTAAAGATAAGACTcTCTCGTGGaACtTAg
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FIG. 11—*Continued.*

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PRO →
A    1 LNLED*EgQGQDPPPpsPg*p*KwGGnq*TFLVDTGAQHSVLTRPDGPLSDRsALVQGATGSKNYRWTTDRRVQLATrKVTHSFLYVPECPYPLLGRDLL
B    1 LNLED*EsQGQDPPP ePritlKiGGqpvTFLVDTGAQHSVLTRPDGPLSDRtALVQGATGSKNYRWTTDRRVQLATgKVTHSFLYVPECPYPLLGRDLL
                                RT →
A  101 TKLKAQIHFTGEGANVVGPmGsPLQVLTLQLEEEYRLFEPkSelKQgMDsWLKNFPrAWAETGGMGMAHCQAPiLIQLKATATPISIRQYPMPHEAYQGI
B  100 TKLKAQIHFTGEGANVVGPrGlPLQVLTLQLEEEYRLFEPeStqKQeMDiWLKNFPqAWAETGGMGMAHCQAPvLIQLKATATPISIRQYPMPHEAYQGI

A  201 KPHIRRMLDQGILKPCrSPWNTPLLPVKKPGTgDYRPVQDLREVNKRVEDIHPTVPNPYNLLSTLPPSHPWYTVLDLKDAFFCLRLHpESQLLFAFEWkD
B  200 KPHIRRMLDQGILKPCqSPWNTPLLPVKKPGTkDYRPVQDLREVNKRVEDIHPTVPNPYNLLSTLPPSHPWYTVLDLKDAFFCLRLHsESQLLFAFEWrD

A  301 PEIGLSGQLTWTRLPQGFKNSPTLFDEALHSDLADFRVRYPALVLLQYVDDLLLAAATkTEsLEGTKALLETLGNKGYRASAKKAQICLQkVTYLGYSLe
B  300 PEIGLSGQLTWTRLPQGFKNSPTLFDEALHSDLADFRVRYPALVLLQYVDDLLLAAATrTEcLEGTKALLETLGNKGYRASAKKAQICLQeVTYLGYSLk

A  401 DGQRWLTKARKEAILSIPVPKNPRQVREFLGpAGYCRLWIPGFAELAAPLYPLTRPGTLFQWGaEQQLAFENIkKALLSSPALGLPDITKPFkLFIDEsS
B  400 DGQRWLTKARKEAILSIPVPKNPRQVREFLGtAGYCRLWIPGFAELAAPLYPLTRPGTLFQWGtEQQLAFENIrKALLSSPALGLPDITKPFeLFIDEnS

A  501 GFAKGVLVQKLGPWKRPVAYLSKKLDTVASGWPPCLRMVAAIAILVKDAGKLTLGQPLTvLTSHPVEALVRQPPNKWLSNARMTHYQAMLLDAERVHFGP
B  500 GFAKGVLVQKLGPWKRPVAYLSKKLDTVASGWPPCLRMVAAIAILVKDAGKLTLGQPLTiLTSHPVEALVRQPPNKWLSNARMTHYQAMLLDAERVHFGP

A  601 TVSLNPATLLPLPnershhdclqiLAEThgtrpDLTDQPLPDADLTWYTDGSSFIRNGERKAGAAVTTESEVIWAASLPPGTSAQRAELIALTQALKMAK
B  600 TVSLNPATLLPLP sgkpprlspdLAET maqtDLTDQPLPDADLTWYTDGSSFIRNGERKAGAAVTTESEVIWAASLPPGTSAQRAELIALTQALKMAK
                                                                                                  INT →
A  701 GKKLTVYTDSRYAFATAHVHGEIYRRRGLLTSEGKEIKNKNEILALLEALFLPKRLSIIHCPGHQKGDSPQAKGNRLADDTAKKAATETQSSLTILPTEL
B  698 GKKLTVYTDSRYAFATAHVHGEIYRRRGLLTSEGKEIKNKNEILALLEALFLPKRLSIIHCPGHQKGDSPQAKGNRLADDTAKKAATETQSSLTILPTEL

A  801 IEGPKRPPWEYDDSDLDLVQKLEAHYEPKRGTWEYqGKTIMPEKYAKELISHLHKLTHLSARKMKTLLEREETGFYLPNRDLHLRQVTESCRACAQINAG
B  798 IEGPKRPPWEYDDSDLDLVQKLEAHYEPKRGTWEYrGKTIMPEKYAKELISHLHKLTHLSARKMKTLLEREETGFYLPNRDLHLRQVTESCRACAQINAG

A  901 KIKFGPDVRARGhRPGiHWEVDFTEIKPGMYGYKYLLVFIDTFSGWAEAYPAKHETAKVVAKKLLEEIFPRYGIPQVLGSDNGPAFISQVSQSVATLLGI
B  898 KIKFGPDVRARGrRPGtHWEVDFTEIKPGMYGYKYLLVFIDTFSGWAEAYPAKHETAKVVAKKLLEEIFPRYGIPQVLGSDNGPAFISQVSQSVATLLGI

A 1001 NWKLHCAYRPQSSGQVERMNRSIKETLTKLTLETGSKDWVLLLPLVLYRVRNTPGPHGLTPFEILYGAPPPMAHFFDsDISgFATSPTMQAHLRALQLVQ
B  998 NWKLHCAYRPQSSGQVERMNRSIKETLTKLTLETGSKDWVLLLPLVLYRVRNTPGPHGLTPFEILYGAPPPMAHFFDtDIStFATSPTMQAHLRALQLVQ

A 1101 EEIQRPLAAAYREKLETPVVPHPFKPGDSVWVRRHQTKNLEPRWKGPHIVLLTTPTALKVDGVAAWIHASHVKAAGPTTdQDpSDdPSSDDlSRWKVQRT
B 1098 EEIQRPLAAAYREKLETPVVPHPFKPGDSVWVRRHQTKNLEPRWKGPHIVLLTTPTALKVDGVAAWIHASHVKAAGPTTnQDlSDsPSSDDpSRWKVQRT

A 1201 QNPLKIRLSRGT*
B 1198 QNPLKIRLSRGT*
```

FIG. 12. Deduced amino acid sequence comparison of the *pol* gene of CFE-6 (A) with that of FeLV-A F6A (B) (10). The positions of the protease (PRO), RT, and INT genes are indicated as in Fig. 11. All nonsense codons are indicated with asterisks, of which the one at position 6 represents the shared amber codon.

ciencies of both recombinants are comparable in feline and human fibroblastic cells. This type of variability in replication ability may be related to the extent of cell receptor recognition or cell tropism and consequently presents a significant biological issue. In the region exchanged, there are six amino acid changes between CFE-16 and CFE-6 proviral loci. Five of these changes are internal to gp70, and one is in the signal peptide region (19). Although one of the internal changes is quite conservative (Leu→Ile), others (Thr→Ile, Ala→Thr, Gly→Asp, and Ser→Asn) are less so. The variation at isolated amino acid positions in the *env* gene of the endogenous provirus loci may become considerably more meaningful when viewed in the context of potential differential transcriptional activity of these loci. There is evidence from studies of endogenous FeLV LTR activity in vitro that the control of expression may be largely *cis* acting, mediated by cellular DNA sequences upstream of the LTR (3). In this regard, regulation of expression of some of these loci may be analogous to regulation of certain tissue-specific genes which contain 5′ negative regulatory elements. Therefore, it is not unreasonable to think that infectivity and target cell tropism of chimeric FeLVs resulting from interactions between infectious and endogenous viral sequences may be influenced by the specific endogenous provirus loci expressed in the exogenous-virus-infected cells. The actual number of contributing loci may, however, be limited to some but not all of the approximately 15 endogenous loci conserved in the cat genome (1, 2, 27). This is because several of these loci are known to be severely truncated (35, 36) and because we show here that coexpression of infec-

tious FeLV with the transcriptionally active proviral clone CFE-16, which is partially deleted in the *env* gene, fails to yield viable recombinants.

Another point to consider is that FeLV-B may recombine with divergent endogenous loci and continue to change in a dynamic fashion. This is particularly noteworthy in relation to the origin of FeLV-B. Data primarily based on restriction mapping and host range properties have previously implied that a FeLV-A virus could give rise to a FeLV-B-like virus by propagating the subgroup A agent in feline cells in culture (25). This important observation, combined with the inference that FeLV-B viruses are more highly leukemogenic than subgroup A (17), points to the evolution of pathogenic agents by recombination with endogenous FeLV elements. Our results of endogenous-provirus locus-specific effects extend this concept further in that FeLV-B may be continuously evolving by new recombinations with endogenous partners.

Because of the high level of nucleotide sequence similarities at the C-terminal half of gp70, it has been postulated that FeLV-C-Sarma may also have acquired endogenous FeLV sequences (22). Here we show that this FeLV-C can variably recombine with endogenous FeLV sequences when allowed to coexpress in transfected cells. In this respect, all FeLV subgroups may retain the capacity to interact with the homologous endogenous sequences. To monitor the events of recombination and analyze the precise locations of the crossover points, we have applied a PCR technique that addresses the size variations between the endogenous and FeLV-C-related sequence domains in the gp70 gene. In the past, hybridization to stretches of nucleotides in the U3 region of the LTR that are nonhomologous between exogenous and endogenous FeLVs has been primarily used to distinguish one class from the other (6). The PCR products of the present study, however, offer a highly sensitive approach by which not only can infectious and endogenous proviruses be distinguished but also the generation of recombinants can be ascertained.

By using the methods of induced coexpression and PCR, we show that several classes of recombinants are generated in the selection process utilized. At least four distinct classes of recombinants can be readily recognized. They vary in the crossover sites between FeLV-C and endogenous FeLV sequences. The results also show that, while up to 75% of the 5′ gp70 gene could be replaced by endogenous FeLV sequences to produce biologically active recombinant viruses, in no case could the complete gp70 of endogenous origin be detected. This observation, combined with the replication inability of the r6gp chimeric construct, suggests that the amino acid divergences at the C-terminal part of the surface glycoprotein (19) may not be compatible with the production of a viable virus with a full complement of endogenous gp70. Viruses containing different crossover sites behave differently in host cell tropism. This is illustrated by the inability of the FeLV-C recombinants containing E bands in the mid-gp70 region to replicate in human T cells (CEM) although these cells are permissive for the growth of variants containing E^- bands. In contrast, all recombinants appear to replicate well in human HT1080 fibrosarcoma cells. Thus, besides the replication restriction imposed by the locus of the endogenous provirus, a second restriction is apparent in the extent of the endogenous gp70 sequence captured during recombination.

As an explanation for the transduction of endogenous gp70 sequences by the infectious-FeLV partner either from the previously reported feline fibroblastic cells (25) or from the T-lymphoma cells described here, we present the nucleotide sequence of the *pol* gene of the endogenous CFE-6 provirus locus. Data revealing a high degree of nucleotide conservation and retention of an open reading frame in almost the entire *pol* region upstream of the *env* gene are of twofold interest. First, while the *gag* of the endogenous FeLV provirus is severely mutated, this finding of *pol* conservation suggests that homologous recombination may potentially begin at a point in that large area to include the *env* gene from its 5′ coding sequences. However, the validity of this issue would depend on the demonstration of retention of functional activity of the proteins encoded by the endogenous *pol* gene, since the possibility exists that even small numbers of amino acids changes could lead to functional inactivation. Second, as far as we know, the results show for the first time that the RT gene sequence of an endogenously inherited provirus locus has an open reading frame. The defect for RT translation seems to be at the level of *gag-pol* polyprotein synthesis of the endogenous provirus examined. While the presence of additional nonsense codons near the N-terminal protease function appears to severely limit the chances of RT protein expression from this locus in uninfected cells, it remains to be seen if events of alternative splicing or utilization of cryptic translation initiation signals may lead to synthesis of the endogenous RT protein in certain cell types. Further, such clustering of nonsense mutations may not be present in the *pol* gene of all endogenous proviral loci.

Finally, it is noteworthy that, besides alterations in in vitro cell tropism, recombinant FeLVs produced in this study may also manifest other interesting biological properties. For example, FeLV-C-Sarma and the derived recombinant viruses can induce a characteristic aggregation of the feline 3201B T-lymphoma cells. Since the recombinants do contain a major part of the gp70 from the endogenous *env* gene, it may be argued that a cell aggregation-inducing moiety may not be confined to the FeLV-C surface glycoprotein. Alternatively, the FeLV-C-induced cell clumping phenomenon may presumably be a result of in situ production of recombinant viruses. Indeed, recombinants are seen to appear spontaneously soon after infection of the 3201B cells by the FeLV-C virus. If this is the case, specificity in the induction of aggregation may reside in the chimeric protein produced by incorporating parts from both FeLV-C and the endogenous *env*. This is a relevant consideration, since recombinant viruses with FeLV-A and endogenous components fail to induce such characteristic clustering of the cells. In an independent investigation, Rojko et al. (personal communication) have observed similar clustering and inhibition of proliferation, although considerably higher than what we found, of these T-lymphoma cells by FeLV-C infection. Additionally, those authors found extensive lymphocytotoxicity that was not apparent in our experiments. The difference in observations in this matter may lie in the amount of serum used in the culture medium. Our study was done with 20% serum, while the Rojko group used only 5% serum. In fact, those investigators report partial protection against cytopathicity by increasing serum concentrations. In general, however, the observations on cell aggregation and cell death may be relevant to pathogenicity in vivo. Appropriately, it is worth mentioning that our preliminary investigations in collaboration with L. Mathes of Ohio State University indicate that newborn cats inoculated with certain mixtures of recombinant FeLV-C viruses rapidly develop severe anemia. It will be important now to establish and analyze the components involved in both cell killing and erythroid hypoplasia by using molecularly cloned individual recombinant FeLVs.

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