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Delayed Cytopathicity of a Feline Leukemia Virus Variant Is due to Four Mutations in the Transmembrane Protein Gene

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Two molecularly cloned, replication-defective variants of feline leukemia virus, called 61B and 61C, have both been shown to cause fatal immunodeficiency in cats when coinfected with a replication-competent, minimally pathogenic helper virus, but 61B exhibits a longer latency period between infection and disease (J. Overbaugh, E. A. Hoover, J. I. Mullins, D. P. W. Burns, L. Rudensey, S. L. Quackenbush, V. Stallard, and P. R. Donahue, Virology 188:558–569, 1992). Infection of the 3201 feline T-cell line with 61B plus helper virus also results in longer time from infection to cytopathic effect compared with 61C plus helper virus, providing an in vitro system with which to study the mechanism for this difference. We report that the primary determinant of cytopathicity of 61B maps to gp70, the extracellular envelope glycoprotein. The long latency of 61B, on the other hand, maps to the extracellular portion of the envelope transmembrane protein, in which there are only four predicted amino acid differences between 61B and 61C. These differences render 61B replication defective, and two of the predicted amino acid changes lie in a region that is highly conserved among many retroviruses. The eventual onset of 61B cytopathicity in cell culture was associated with the outgrowth of an apparent recombinant virus that encodes the pathogenic gp70 of 61B and replaces the transmembrane protein of 61B with that of the helper virus. Thus, during in vitro infection, a cytopathic virus evolved from a replication-defective virus and a nonpathogenic virus, suggesting that recombination between multiple variants in natural infection may influence progression of feline leukemia virus-associated immunodeficiency disease.

Retroviruses generate variant genomes at a high rate during replication, because of both error-prone transcription and frequent recombinations between RNA strands in the diploid virion (reviewed in reference 20). Genetic variation may result in different viral phenotypes, and in a natural infection, it is difficult to know how each variant contributes to disease progression. In particular, variation in the retroviral envelope gene (*env*), which governs viral interaction with the host cell surface, has been correlated with differences in pathogenicity (reviewed in references 19 and 41).

Feline leukemia virus (FeLV), a type C retrovirus, provides an opportunity to study, in vitro and in vivo, how defined retroviral variants act alone or in concert to cause clinical disease. FeLV infection in cats is associated with degenerative diseases such as anemia and T-cell depletion as well as proliferative diseases such as leukemia and lymphoma (15, 16, 18). Its most common disease manifestation is a fatal immunodeficiency syndrome similar to human immunodeficiency virus (HIV)-associated immunodeficiency in humans (16, 32). Previous studies have shown that the disease outcome depends in part on the viral molecular clone with which the cat is infected, suggesting that molecular genetic characteristics of the virus are determinants of pathogenesis (8, 27, 34).

FeLV variant genomes have previously been cloned directly from the tissue DNA of cats that developed fatal immunodeficiency after inoculation with a natural isolate of FeLV called FeLV-FAIDS (27). Variants analyzed to date show a high degree of nucleotide sequence homology but have distinct biological effects in vivo and in vitro (27, 28). Three clones isolated from a single cat with immunodeficiency will be discussed here. FeLV clone 61E was found to be replication competent and non-immunodeficiency inducing when inoculated into cats (27). Restriction fragment analysis suggested that 61E is representative of a common form that predominates in vivo throughout infection (17, 26, 27). Two other cloned variants, 61B and 61C, are both replication defective (27) and exhibit the restriction fragment pattern characteristic of variant viral DNA whose appearance in vivo was correlated with disease onset in FeLV-FAIDS-infected animals (17, 26, 27). These two variant genomes were characterized in vivo by coinfecting them with 61E as a helper virus; the viral mixtures are referred to as 61C(61E) and 61B(61E). Both virus mixtures caused immunodeficiency in cats, but with mean survival of 5 months for cats infected with 61C(61E) and 18 months for those infected with 61B(61E) (27, 28). That is, 61B(61E) had a longer clinical latency period between infection and disease onset than did 61C(61E), even though the 61B genome is detectable in bone marrow early after infection (28). The FeLV clones were also studied in vitro in a feline T-cell line called 3201. When this cell line is infected with 61E, the virus replicates but does not produce cytopathic effect (CPE) (9, 28). However, when 61B(61E) or 61C(61E) is used to infect the 3201 cell line, the cultures undergo CPE with death of many cells in the culture, after which the surviving cells resume growth at a rate close to that of uninfected cells. The latency time between infection and onset of CPE is approximately twice as long for 61B(61E) as for 61C(61E) (28). Thus, infection of the 3201 cell line accurately reflects the in vivo phenotype of the viruses in terms of both ability to cause cytopathicity and relative time from infection to CPE. Other experiments have also supported the ability of 3201 cell infection in vitro to reflect the replicative ability and pathogenic characteristics of FeLV molecular clones (9, 28).

Previous studies showed that a chimeric viral genome, EECC, containing the 5′ long terminal repeat (LTR) and the

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gag and *pol* genes of 61E combined with the *env* gene and 3′ LTR of 61C is both replication competent and highly cytopathic (9, 27). This finding indicates that the 5′ LTR/*gag*/*pol* portion of 61C confers a replication defect, while its *env*/3′ LTR portion is sufficient for pathogenicity. Further analysis of chimeras between 61E and 61C showed that a small region at the 3′ end of the portion of *env* that encodes the extracellular glycoprotein (called gp70 in FeLV) is the most critical determinant of cytopathicity in 3201 cells and of immunodeficiency disease in cats (9, 32). Within this segment, the major difference between the 61C and 61E gp70 sequences is a predicted six-amino-acid insertion in 61C. The gp70 of 61B contains a similar insertion relative to 61E (28). In fact, nucleotide sequence analysis of *env* and the 3′ LTR shows only a few differences between 61C and 61B, but the construct analogous to EECC, called EEBB, is replication defective, suggesting that the *env*/3′ LTR portion of 61B, unlike that of 61C, confers a replication defect (28). We undertook further studies to elucidate how 61B(61E) is eventually able to produce CPE and immunodeficiency disease. Our results show that a segment of the transmembrane portion of the 61B *env* gene (called p15E in FeLV), containing four predicted amino acid differences relative to 61C, is responsible for delayed cytopathicity as well as a replication defect. CPE in 3201 T cells is associated with outgrowth of an apparent recombinant virus that replaces the 61B p15E segment with that of the coinfecting helper virus, 61E.

MATERIALS AND METHODS

Cell culture. AH927 feline embryonic fibroblasts were maintained in minimum essential medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 2 mM L-glutamine. The feline T-cell line 3201 (a gift from W. D. Hardy, Jr. [37]) was maintained in 50% Leibovitz's L15–50% RPMI 1640 supplemented with 15% fetal calf serum, 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 2 mM L-glutamine.

Construction of chimeric viruses. The following restriction endonuclease sites were used in constructing chimeric viruses; base numbers (in parentheses) are based on the nucleotide sequence of the 61E genome (GenBank accession number M18247). *Bgl*II (1106) is in *gag*, 422 nucleotides downstream of the initiation codon. *Xho*I (5818) is in *pol*, 162 nucleotides upstream of the start of *env*. *Hga*I (7115) is in the gp70 gene. *Nco*I (7195) is in gp70, 121 nucleotides 5′ of the gp70-p15E cleavage site and immediately 3′ of the segment shown to be the minimal pathogenic determinant of 61C (9, 32). *Bal*I (7440) is in the gene encoding p15E. *Rsr*II (7898) is 9 nucleotides before the 3′ end of the p15E gene. *Pst*I (7997) is in the U3 portion of the LTR. *Sma*I (8330) is in U5, 110 nucleotides from the 3′ end of the proviral genome. Chimeric viruses are diagrammed in Fig. 2a and 3a.

Plasmids encoding the 61C, 61B, and 61E genomes have been described elsewhere (27); each genome was cloned into pUC18 at the *Eco*RI site. Several plasmids containing smaller portions of 61B or 61C in pUC were made in order to isolate desired viral genome fragments: 3′CC (27) and 3′BB were made by filling in the ends of the 3′ *Bam*HI-*Eco*RI fragment (base 5318 to the 3′ flanking DNA) of 61C and 61B, respectively, ligating it into pUC18 at the *Sma*I site, and selecting clones with the polylinker *Eco*RI site 5′ to the viral sequence. Subclones 3′C2.1K and 3′B2.1K contain a 2.1-kb *Kpn*I-*Kpn*I fragment (bases 6260 to 8336) of 61C and 61B, respectively, in pUC 18. Subclone 3′C0.9H/P contains a 882-bp *Hga*I-*Pst*I fragment of 61C in pUC18. For subsequent chimera construction, all fragments were isolated by electrophoresis through agarose. The convention for naming the chimeric viruses is as described in the legend to Fig. 2a and is similar to that used in previous studies (9, 28, 33).

The construction of EECC (27) and EEBB (28) has been described previously. CCCB, EEBC, and EECB were made by ligating three fragments: the 5′ *Eco*RI-*Xho*I fragment from 61E or 61C, the *Xho*I-*Nco*I fragment from 3′CC or 3′BB, and the *Nco*I-*Eco*RI pUC-containing segment from 3′CC or 3′BB, as appropriate. To make (EB)BBC, the *Bgl*II-*Xho*I fragment was exchanged from 61B into EEBC.

To make EEC(BC), the 703-bp *Nco*I-*Rsr*II fragment of 61B was isolated from 3′B2.1K and ligated into *Nco*I-*Rsr*II-digested 3′C2.1K. The resulting plasmid was digested with *Nco*I and *Sma*I, and the 1,135-bp fragment was ligated into *Nco*I-*Sma*I-digested 3′CC. This plasmid was then cut with *Eco*RI and *Xho*I, and the resulting 5.5-kb vector was ligated to a 6.5-kb *Eco*RI-*Xho*I 5′ fragment of 61E to give a complete viral genome. To make EEC(CB), the 8.7-kb *Eco*RI-*Rsr*II fragment was isolated from EECC and ligated to a 4.5-kb *Eco*RI-*Rsr*II vector from 3′BB.

To make EEC([CB]C), EEC([BC]C), and CCC([CB]C), 3′C0.9H/P was digested with *Bal*I and *Nco*I or *Bal*I and *Rsr*II to create two vectors into which *Nco*I-*Bal*I or *Bal*I-*Rsr*II fragments of 61B were ligated. The resulting two plasmids were then digested with *Rsr*II and *Sph*I; the latter cuts in the pUC polylinker 3′ to the viral sequence. A 0.7-kb *Rsr*II-*Sph*I fragment from 3′CC, encoding the 3′ end of 61C, was ligated into these two vectors. The resulting plasmids were digested with *Nco*I and *Eco*RI; the latter cuts in the pUC polylinker 5′ to the viral sequence. A 1.8-kb *Eco*RI-*Nco*I fragment was isolated from 3′CC and ligated into these vectors. The resulting plasmids contained the entire 3′ half of the viral genome with either the *Nco*I-*Bal*I or the *Bal*I-*Rsr*II segment donated by 61B and the remainder donated by 61C. The *Eco*RI-*Xho*I 5′ portion from 61E (6.5 kb) or 61C (6.3 kb) was ligated into this plasmid to yield full-length viral genomes.

The identity of the constructs was checked by restriction fragment analysis. Those constructs that were indistinguishable by this method were verified by nucleotide sequence analysis.

Virus origins, titration, transfection, and infection. FeLV molecular clones and chimeras, each in pUC vector, were transfected by electroporation into AH927 fibroblasts or 3201 T cells. Viruses were designated replication defective if transfection into 3201 or AH927 cells in duplicate failed to show viral replication, as measured by enzyme-linked immunosorbent assay (ELISA) detection of FeLV $p27^{gag}$ (Virachek/FeLV; Synbiotics, San Diego, Calif.) in the culture supernatant after 4 weeks of cultivation. Replication-defective viruses were cotransfected with 61E at a defective/61E DNA ratio of 5:1 to 10:1 (weight/weight). Cell-free viral supernatants were harvested, and their tissue culture infectious dose (TCID) was determined by endpoint dilution as follows. 3201 cell cultures were infected in quadruplicate with serial dilutions of supernatant, passaged for 4 weeks, and then tested for presence of virus by ELISA. Titered supernatants were then used to infect 3201 T cells at multiplicities of infection (MOIs) ranging from 0.0005 to 0.1. CPE was evaluated by counting viable cells in culture every 2 or 3 days and correcting for passage dilution to arrive at the total number of cells generated in the culture. Cellular DNA was extracted and purified from infected cells by phenol

extraction and alcohol precipitation, using standard methods.

Polymerase chain reaction (PCR) amplification of viral sequences. Two primers were designed to amplify an 820-nucleotide segment extending from the 3′ end of gp70 to nearly the 3′ end of p15E. They do not recognize endogenous FeLV sequences that are present in all feline cells (1). FeLV-*env*5 (*GCGGGATCC*TGAAGTATCAGGGCAAGGAC) spans bases 7038 to 7058 in gp70 (numbered relative to 61E), with added bases (in italics) to create a 5′ *Bam*I restriction site. FeLV-*env*6 (*GCGGAATTC*AAATTAAGGCTTGTACCACA) spans bases 7840 to 7859 in p15E, with added bases (in italics) to create a 5′ *Eco*RI restriction site. Reaction mixtures contained 400 ng of cellular DNA and 1 mg of each of the oligonucleotide primers in a 100-ml volume. An initial 4-min 94°C denaturation was followed by 35 amplification cycles of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 2 min of extension at 72°C. Negative control reactions were done in parallel with no DNA and with DNA from uninfected feline cells. Reactions were assessed for successful amplification by ethidium bromide visualization of product electrophoresed on an agarose gel.

Cloning and nucleotide sequence analysis of PCR product. PCR product was pooled from four reactions of each genomic DNA sample. The 0.8-kb fragment was isolated by agarose electrophoresis and purified by phenol extraction and alcohol precipitation. The product was treated with Klenow enzyme to create blunt ends and ligated into *Hin*cII-digested M13 bacteriophage. Multiple clones were isolated for nucleotide sequence analysis of portions of the FeLV *env* fragment by the dideoxy method (34a), using the Sequenase enzyme and reagents (United States Biochemical).

RESULTS

Cytopathicity of 61C(61E) and 61B(61E) mixtures in 3201 T cells. When 61C(61E) and 61B(61E) viral supernatants of equal TCID, harvested from AH927 fibroblasts, were used to infect 3201 cells, both cultures exhibited CPE, as manifested by a decrease in viable cell number. However, cells infected with 61B(61E) showed a longer latency period before onset of cytopathicity than did those infected with 61C(61E) (Fig. 1). These findings are in agreement with previously published studies (28) that compared fibroblast-derived 61C(61E) and 61B(61E) viral supernatants with use of virus doses normalized for FeLV p27^{gag} antigen. This difference in latency between the two viral mixtures was observed at all MOIs tested; Fig. 1a to c show cultures infected at MOIs of 0.001, 0.025, and 0.1, respectively. The latency period for 61C(61E) was approximately the same at all MOIs, while that of 61B(61E) was somewhat shorter at high than at low MOIs but remained longer than that of 61C(61E) in all cases. In the experiments shown in Fig. 1a to c, we used virus from AH927 cells cotransfected with defective variant and 61E. Southern blot analysis of DNA from these fibroblasts showed the 61E provirus to be about 10-fold more prevalent than that of the variant (data not shown). Figure 1d shows results for an infection using 61C(61E) and 61B(61E) viral supernatants generated by infecting AH927 fibroblast cell clones bearing stably integrated 61B or 61C proviral genomes (27) with 61E to rescue the variant; the MOI in the infection shown was 0.001, the same as in Fig. 1a. Southern blot analysis of DNA from these fibroblasts showed a helper/variant provirus ratio of about 1:1 (data not shown). In this infection, the latency time of 61B(61E) is comparable to that seen in the infection shown in Fig. 1a and again is longer than that of 61C(61E). Thus, while the MOI of the infecting 61B(61E) mixture had a small effect on its latency period, the relative ratio of 61B to 61E provirus in the fibroblasts from which the viral mixture was harvested did not have a detectable effect on latency.

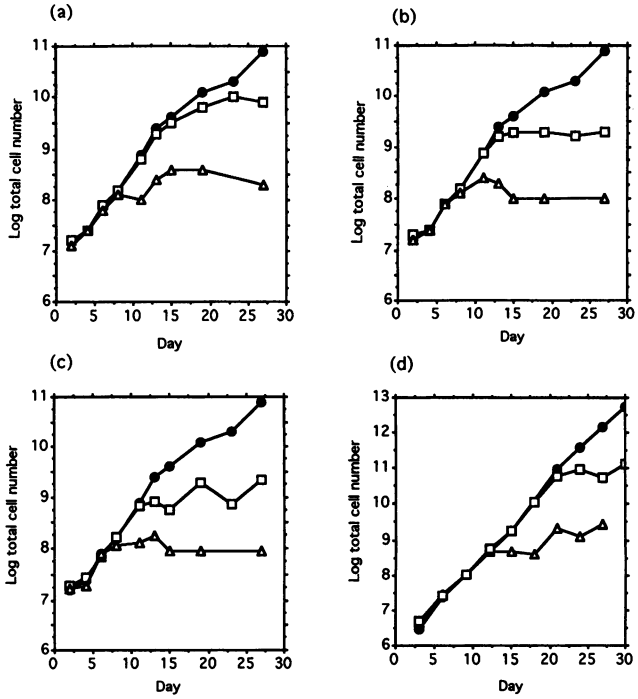

FIG. 1. Growth of 3201 feline T cells following infection with FeLV 61B(61E) and 61C(61E) viral supernatants. 3201 T cells were infected with cell-free viral supernatants; uninfected cells were cultured in parallel. Total number of cells (average of duplicate cultures) is plotted against days after infection. Cells were passaged as needed to maintain density below 10^7 cells per ml. Cells were infected with no virus (●), 61B(61E) (□), or 61C(61E) (△). In the infections depicted in panels a to c, the infecting 61B(61E) and 61C(61E) supernatants were derived from fibroblasts cotransfected with 61E plus variant at MOIs of 0.001 (a), 0.025 (b), and 0.1 (c). In the infection shown in panel d, 61B(61E) and 61C(61E) supernatants were generated by rescuing variant stably integrated into the cell genome with superinfecting 61E as described in the text. These supernatants were used to infect 3201 cells at an MOI of 0.001.

A replication defect mapped to a portion of the 61B p15E gene. To determine the molecular basis for the difference in latency between 61B and 61C, we constructed chimeric viral genomes containing regions where the two variants are known to differ in sequence and determined the replicative and cytopathic characteristics of the chimeras in the 3201 cell line. In an effort to make replication-competent chimeras analogous to EECC, constructs were made with the 5′ LTR, *gag*, and *pol* contributed by 61E (Fig. 2a), since these genes encode functional viral proteins and are not determinants of immunodeficiency (9, 27, 32). Chimeras were transfected into 3201 cells, and virus production was monitored by ELISA detection of p27^{gag} protein in the supernatant. Previously described chimeras EECC and EEBB are shown for comparison. Chimera EEBC, which bears a portion of 61B encompassing most of gp70, was replication competent, suggesting that the gp70 of 61B is not responsible for that variant's replication defect. The reciprocal construct, EECB, containing the segment of 61B that encodes the 39

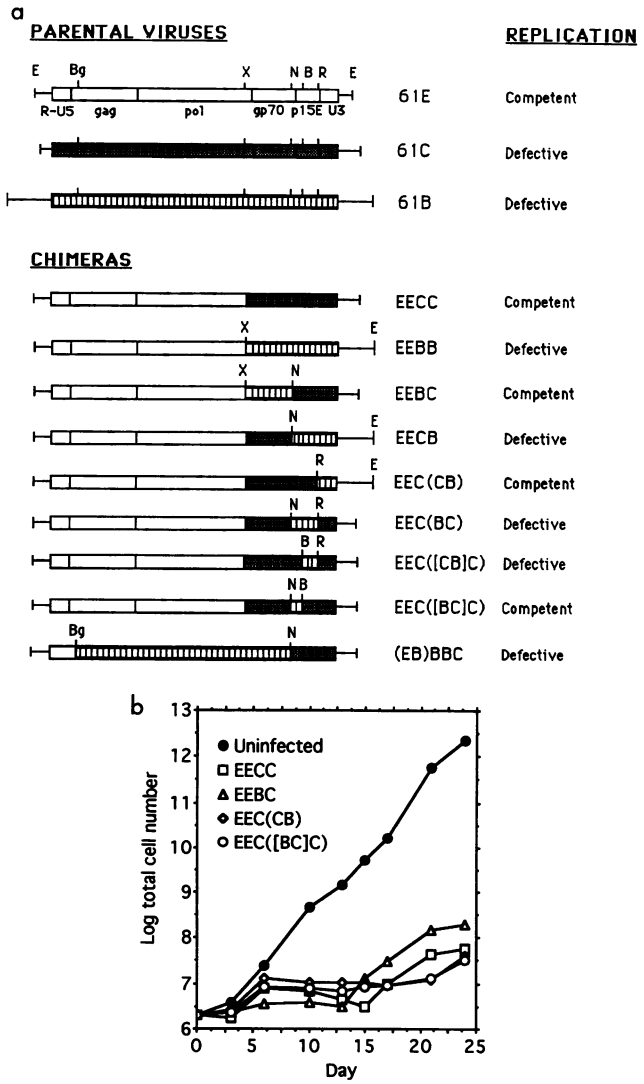

FIG. 2. (a) Schematic representation of chimeras constructed from portions of 61E, 61C, and 61B viral genomes. Parental viruses are shown for comparison. Exchanges were made at the restriction endonuclease sites indicated, abbreviated E (*Eco*RI), Bg (*Bgl*II), X (*Xho*I), N (*Nco*I), B, (*Bal*I), and R (*Rsr*II). The system for naming chimeras is similar to that used previously (9, 28, 33): for the chimeras with four-letter names, the letters designate, respectively, the 5′ LTR/*gag* segment; the *pol* segment; the *Xho*I-*Nco*I fragment encoding a small segment of 3′ *pol* and most of gp70; and the *Nco*I-*Eco*RI fragment encoding the C-terminal end of gp70, p15E, and 3′ LTR. Parentheses in the names of subsequent chimeras indicate a subdivision of the fourth segment, and brackets denote a further subdivision of part of that segment. For each chimera, the restriction sites bordering the portion donated by 61B are indicated. Chimeras were designated replication competent if $p27^{gag}$ protein was detectable in the culture supernatant by ELISA 4 weeks after transfection or infection and were designated replication defective if no $p27^{gag}$ was detected a 4 weeks. (b) Growth of 3201 feline T cells after infection with replication-competent chimeras. Viral supernatants, harvested from 3201 cells transfected with chimeric genomes, were used to infect naive 3201 cells at an MOI of 0.001. Cells were cultured in duplicate as described for Fig. 1.

C-terminal amino acids of gp70 as well as p15E and the 3′ LTR, was, like EEBB, replication defective, indicating that 61B contains a replication defect in that segment. This portion of 61B was further subdivided by using the *Rsr*II site near the 3′ end of the p15E gene. EEC(CB), which is like EECC except in carrying the 3′ LTR of 61B, was replication competent, while EEC(BC), bearing a small portion of 3′ gp70 and the p15E of 61B, was replication defective, showing that the defect lay in the 3′ gp70/p15E envelope fragment of 61B, not in the 3′ LTR. Chimeras EEC([CB]C) and EEC([BC]C) were then constructed by using the *Bal*I site in the 5′ portion of p15E and found to be replication defective and replication competent, respectively, further narrowing down the location of 61B's replication defect to a fragment encoding a 154-amino-acid portion of the p15E gene. Within this fragment, there are four predicted amino acid differences between 61B and 61C (28) (see Fig. 6).

Cytopathicity of replication-competent chimeras. To assess the cytopathic properties of those chimeras that were replication competent, an equal TCID of each virus, generated in 3201 cells, was used to infect naive 3201 cells (Fig. 2b). Construct EEBC was cytopathic, showing that the 61B gp70 gene can substitute for that of 61C in causing cytopathicity. In EEBC, the 34-amino-acid segment known to be the major determinant of pathogenicity for 61C (9, 32) was donated by 61B. EEC(CB) and EEC([BC]C) were also cytopathic, and all three constructs caused CPE with a latency period similar to that of EECC (Fig. 2b), suggesting that the 3′ LTR and the 3′ gp70-5′p15E segments of 61B do not encode determinants responsible for its delayed CPE.

Delayed latency also mapped to the p15E gene segment of 61B. The chimeras described above revealed the location of a replication defect in 61B and indicated that differences in the 3′ LTR and gp70 of 61B were not associated with its increase in latency relative to 61C. To positively show that the p15E segment of 61B was the determinant of long latency, we constructed a set of chimeras that contained segments of 61B *env* in a virus otherwise isogenic with 61C (Fig. 3a). Chimeras CCCB and CCC([CB]C) were cotransfected with 61E into AH927 cells to generate viral supernatants, whose TCID was determined. These stocks, along with similarly produced and titered 61B(61E) and 61C(61E) stocks for comparison, were used to infect 3201 T cells in duplicate at the same MOI (Fig. 3b). Both chimera-helper mixtures produced CPE after a latency period comparable to that of 61B(61E) and longer than that of 61C(61E), mapping the location of the determinant for long latency to the same p15E-encoding segment shown to confer the replication defect in the 3′ half of 61B.

The *gag/pol* region of 61B confers a separate replication defect. Previous analysis of chimeras between 61C and 61E had shown that the 5′ half of 61C encodes a defective *gag* and/or *pol* gene (27). To determine whether 61B had a similar defect in this 5′ region, we prepared a construct that contained the 5′ LTR of 61E, *gag*, *pol*, and gp70 of 61B, and the p15E and 5′ LTR of 61C. This chimera, (EB)BBC, was replication defective, showing that 61B bears a defect in *gag* and/or *pol* as well as in p15E.

In summary, analysis of chimeric virus phenotypes in 3201 cells demonstrated that (i) the 5′LTR/*gag/pol* region of 61B, like that of 61C, confers a replication defect; (ii) the gp70 of 61B can substitute for that of 61C in conferring a cytopathic phenotype; (iii) the difference between 61B and 61C in latency in vitro is determined by a 154-amino-acid portion of the transmembrane protein gene, p15E; and (iv) that segment of the 61B transmembrane protein gene also confers a replication defect on genomes that contain it.

Difference in latency between virus stocks from different cell lines. Our initial experiments to characterize the in vitro phenotypes of 61B(61E) and 61C(61E) were done by infect-

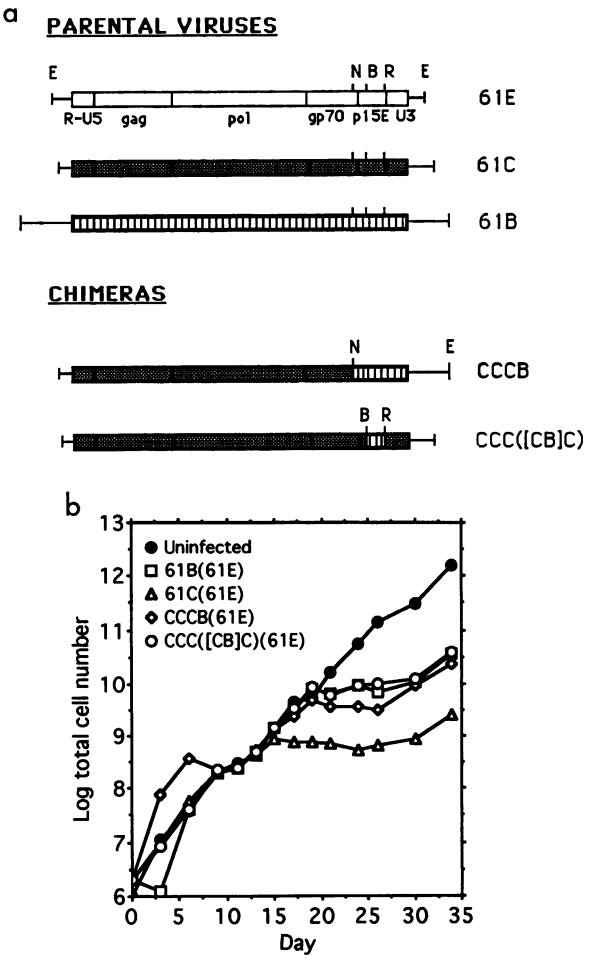

FIG. 3. (a) Schematic representation of replication-defective chimeras made with portions of 61C and 61B. Chimeras were named by using the convention described in the legend to Fig. 2a. (b) Growth of 3201 T cells infected with 61C/61B chimeras. 3201 T cells were infected with viral supernatants generated by cotransfecting 61B, 61C, CCCB, or CCC([CB]C) DNA (50 μg) with 61E DNA (5 μg) into AH927 fibroblasts as described in Materials and Methods. Cultures were infected in duplicate at an MOI of 0.0005 and cultured as described for Fig. 1.

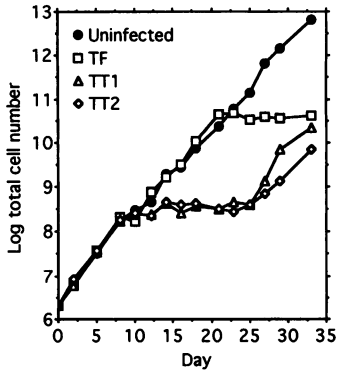

FIG. 4. Growth of 3201 T cells infected with 61B(61E) viral supernatant generated in different cell lines. 3201 T cells were infected in duplicate, each at an MOI of 0.0005, with 61B(61E) virus from TF [fibroblast-derived 61B(61E) supernatant] and from TT1 and TT2 [two independently generated 61B(61E) supernatants from 3201 T cells]. Cells were cultured as described for Fig. 1.

ing 3201 T cells with virus stocks generated in AH927 fibroblasts. However, when we used supernatants from chronically infected 3201 cells to infect naive 3201 cells, we found that the phenotype of the 61B(61E) supernatants had changed: this virus mixture now exhibited a short latency like that of 61C(61E). Infection of 3201 cells with fibroblast-derived 61B(61E), 3201 T-cell-derived 61B(61E), and fibroblast-derived 61C(61E) confirmed that the 61B(61E) virus mixture that had been passaged in 3201 cells acquired 61C(61E)-like short latency. One such experiment is shown in Fig. 4, in which two different 61B(61E) supernatants taken from 3201 cells after CPE had occurred caused CPE in naive 3201 cells after a short latency rather than the long latency produced by fibroblast-derived 61B(61E) supernatant. 3201 cells were also infected with 61B(61E) supernatant taken from 3201 cells 10 days after infection, before the onset of CPE. This supernatant caused CPE after a latency period of about 16 days (data not shown), intermediate between that of fibroblast-derived 61B(61E) (22 days) and that of 61B(61E) from 3201 cells after CPE (12 days). This intermediate result suggested that the change that occurs when 61B(61E) is passaged in 3201 cells develops over time in those cells, rather than being a biochemical difference between virus expressed in T cells and that expressed in fibroblasts. In other experiments (not shown), we found that when the 61C(61E) virus mixture is passaged in 3201 cells, the resulting supernatant causes CPE in naive 3201 cells slightly faster than does an equal TCID of fibroblast-derived 61C(61E), but the magnitude of this difference was modest compared with that of 61B(61E). At a given MOI, the times to CPE produced by T-cell-derived 61B (61E) and T-cell-derived 61C(61E) supernatants are equivalent and are as short as that of supernatant from 3201 cells infected with replication-competent chimeras such as EECC.

Analysis of viral sequences from cell cultures. We hypothesized that the 61B(61E) virus mixture was undergoing some genetic or biochemical change during the course of 3201 cell infection; for example, a mutation may repair the defect conferred by the 61B transmembrane protein, allowing CPE to occur. To determine whether there were viral genetic changes in *env* during 61B(61E) infection of 3201 cells, we analyzed proviral sequences early and late after infection. Cellular DNA was extracted from the 3201 T-cell cultures shown in Fig. 4 as follows. Cellular DNA was taken 5 days after 3201 cells were infected with fibroblast-derived 61B(61E) mixture, before CPE had begun; this sample is called TFE (for T cells infected with fibroblast-derived supernatant, early time point). The same culture was sampled again at day 33, after CPE had occurred; this DNA is labeled TFL (for late time point). Cellular DNA was extracted from the two 3201 cell cultures infected with independently derived T-cell-passaged 61B(61E) supernatants at day 5, before the onset of CPE; these samples are called TT1E and TT2E. In addition, DNA was extracted from AH927 fibroblasts that had been cultured for 6 months following infection with fibrolast-derived 61B(61E); this sample is designated FFL. PCR was used to amplify a portion of the proviral *env* region from these DNA samples, and the product was cloned into M13 for nucleotide sequence analysis of multiple clones from each DNA sample.

The diagram at the top of Fig. 5 shows schematically the locations of the differences between the sequences of 61B and 61E in the amplified segment, which was designed to

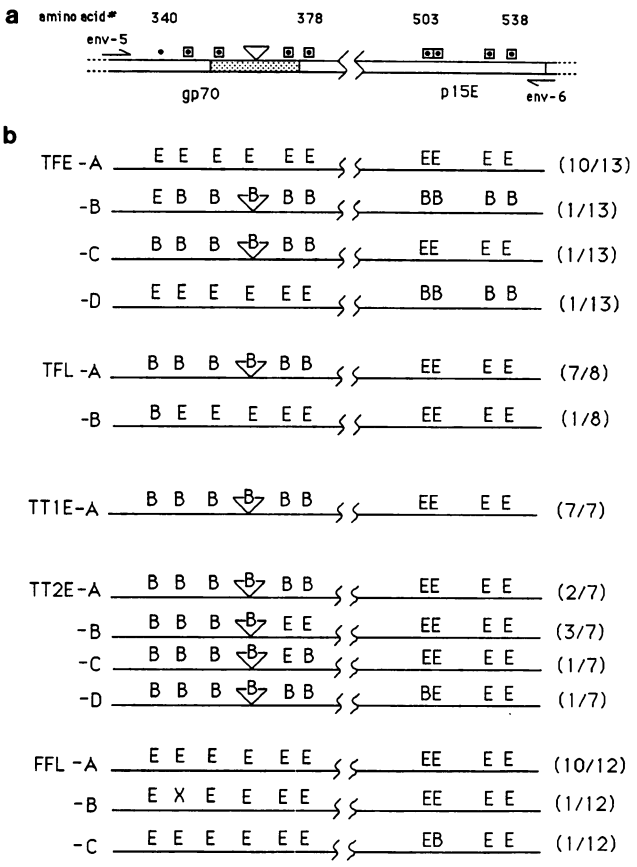

FIG. 5. Schematic representation of viral sequences amplified and cloned from cellular DNA after infection with 61B(61E) viruses generated in different cell lines. (a) Locations of differences between 61B and 61E sequences in the amplified region. A dot indicates a nucleotide change not resulting in a predicted amino acid change; a box dot indicates a nucleotide change resulting in a predicted amino acid change. The 18-nucleotide insertion in 61B and 61C relative to 61E is denoted by a triangle. The shaded area corresponds to the region of 61C shown to be the minimal pathogenic determinant for CPE in 3201 cells and immunodeficiency disease in vivo (9, 32). Amino acid numbers (numbered relative to 61E, counting from the start of gp70) of some of the differences are shown above the diagram. For reference, the putative gp70/p15E cleavage site is at amino acid 414 (25, 31). ≀≀ denotes a region of sequence that is identical for 61B and 61E and for which sequence was not determined. (b) 61B- or 61E-like nature of the sites in individual clones isolated from the genomic DNA of infected 3201 cells whose growth is shown in Fig. 4 and from chronically infected fibroblasts. Sequences designated TFE were amplified from genomic DNA of cell culture TF, 5 days after infection with fibroblast-derived 61B(61E). Sequences TFL are from DNA taken at day 33 from the TF cell culture. Sequences TT1E are derived from the DNA of culture TT1, 5 days after infection with 3201-derived 61B(61E). Sequences TT2E are derived from culture TT2, 5 days after infection with an independently generated 3201-derived 61B(61E) supernatant. Sequences FFL were isolated from the DNA of AH927 fibroblasts chronically infected with fibroblast-derived 61B(61E) supernatant. B indicates that a 61B-like nucleotide was found in the clone at the site of a difference between the sequences of 61B and 61E; E denotes a 61E-like nucleotide; X denotes a nucleotide different from that of 61B or 61E; ▽ denotes the 18-nucleotide insertion of 61B. Numbers at the end of each line represent the number of clones with the indicated sequence out of the total number of clones from each DNA sample.

include the 34-amino-acid region in gp70 that is the primary determinant of pathogenicity for 61C (9, 32); because 61B has a similar sequence in this region, we postulated that this segment might be an important determinant of 61B's T-cell tropism and/or pathogenicity. The amplified segment extends through the p15E region containing the four differences (Fig. 6) that we have shown to determine the latency difference between 61B and 61C. Below this diagram are shown schematic representations of the individual proviral sequences that were cloned from amplified cellular DNA, in which the 61E-like or 61B-like identify of the base at each site of a sequence difference is shown by E or B, respectively, as described in the figure legend. Several of the PCR-derived clones had occasional point mutations at one or more other sites within this segment, but only the 61B-like or 61E-like nature of the relevant sites is shown, because we wanted to focus on sequences identified as important for pathogenicity and latency of 61B and 61C.

Of the 13 *env* segments analyzed from DNA sample TFE, taken from 3201 cells at day 5 after infection with fibroblast-derived 61B(61E) supernatant, 10 were 61E-like at all sites where there are sequence differences between 61E and 61B (sequence TFE-A in Fig. 5). Another clone (TFE-B) was 61B-like except for one 61E-like base in gp70. One clone (TFE-C) appeared to be a recombinant with a 61B-like gp70 and a 61E-like p15E, while another (TFE-D) had the opposite pattern, namely, 61E-like sequence in 3′ gp70 and 61B-like sequence in p15E. DNA taken from the same culture on day 33, after CPE had occurred (TFL), was found to contain predominantly *env* segments that were 61B-like in the 3′ end of gp70 and 61E-like in p15E (TFL-A; seven of eight sequenced). The other clone (TFL-B) was 61E-like except for a single 61B-like nucleotide in gp70. In two infections of 3201 cells with T-cell-passaged 61B(61E) viral supernatant, all 14 clones (TT1E and TT2E) isolated at day 5 were 61B-like in most or all of 3′ gp70 and 61E-like in p15E; all had the 18-nucleotide insertion characteristic of 61B gp70. One clone (TT2-D) had a 61B-like nucleotide at one of the sites in p15E, but the other p15E sites were 61E-like. These apparent recombinant genomes were present at day 5, before the onset of CPE in these two cultures that showed rapid CPE compared with cells infected with fibroblast-derived 61B(61E) (Fig. 4). In the 12 sequences isolated from chronically infected AH927 fibroblasts (FFL), minor variations occurred but all sequences were predominantly 61E-like. We did not observe any wild-type 61B sequences in the FFL clones; this is in accordance with Southern blot analysis of the cellular DNA sample from which the clones were derived, which showed a 61E/61B ratio of approximately 10:1 to 20:1 (data not shown).

In summary, proviral DNA from chronically 61B(61E)-infected AH927 fibroblasts, as well as from 3201 T cells early after infection with fibroblast-derived 61B(61E), contained a predominance of *env* genes representing the infecting viruses, mostly the 61E helper virus. In contrast, 3201 cells that had undergone CPE after infection with fibroblast-derived 61B(61E) contained a predominance of proviral genomes with 61B-like sequence in the 3′ region of gp70 and 61E-like sequence in p15E. This apparent recombinant genome was also the predominant provirus detected in 3201 cells infected with the highly cytopathic 61B(61E) mixture derived from 3201 cells. Thus, the predominance of a provirus with a 61B-like gp70 and a 61E-like p15E gene correlated with the development of CPE in 3201 T cells.

```
FeLV-61C  LQNRRGLDILFLQEGGLCAALKEECFYADHTGLVRDNMAKLRERLKQRQQLFDSQQGWFEGWF
FeLV-61B  ......................KK.............I.................R......
FeLV-61E  ..........................................................
FeLV-GA   ..........................................................
enFeLV    ................T.....................................DGL....

Mo,MCF    ........L...K.......................S........N...K..E.T......L.
MuLV-AKV  ........L...K.......................S........S...K..E.Q......L.
Friend    ........L...K.......................S........T...K..E.S......L.

MPMV      ........L.TAEQ..I.L..Q.K.....NKS.I...KIKN.QDD.ER.RRQLIDNPF.TSFHG

HTLV-I    A.......L..WEQ....K..Q.Q.R.PNITNSH.PILQERPPLENRVLTGWGLNWDLGLSQ.A

HTLV-II   A.......L..WEQ....K.IQ.Q...LNISNTH.SVLQERPPLEKRVITGWGLNWDLGLSQ.A

HIV-1     .AVE.Y.KDQQ.LGIWGCSGKLICTTAVPWNASWSNKSLEQIWNNMTWMEWDREINNYTSLIHS
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FIG. 6. Comparison of predicted amino acid sequences in a conserved region of retroviral transmembrane proteins. Dots denote amino acids identical to those in the reference 61C sequence (27), which begins at amino acid 481, counting from the N terminus of gp70. The four amino acids that differ between 61C and 61B are underlined. Predicted amino acid sequences of other retroviral transmembrane proteins are shown, aligned to the conserved cysteines, which are boxed (13). In the case of HIV, which is quite divergent from FeLV in this region, alignment was based solely on the cysteines. Abbreviations and references for sequences: FeLV-61E, -61C, and -61B (27, 28); enFeLV (endogenous feline leukemia virus) (22); GA (FeLV group B, Gardner-Arnstein isolate) (11); Mo (Moloney murine leukemia virus) (36); MCF (Moloney mink cell focus-forming virus) (3); MuLV-AKV (AKV murine leukemia virus) (23); Friend (Friend murine leukemia virus) (21); MPMV (Mason-Pfizer monkey virus) (39); HTLV-I (human T-lymphotropic virus type I) (35); HTLV-II (human T-lymphotropic virus type II) (38); HIV-1, (HIV type 1, Bru isolate) (42).

DISCUSSION

A molecular clone of FeLV, 61B, has an increased latency period relative to another clone, 61C, in terms of both time to disease in cats and time to CPE in 3201 cell culture (28). These two replication-defective variants are otherwise similar: they were cloned from the same immunodeficient cat, have a high degree of sequence homology, and are both able to cause fatal immunodeficiency disease in vivo and T-lymphocyte death in vitro (27, 28). By analysis of viral chimeras, we have now shown that the gp70 of 61B confers cytopathicity in vitro. A segment of gp70, encoding 34 amino acids, was previously found to be the minimal determinant of 61C's pathogenicity (9, 32). The sequences of 61B and 61C in this region both have a six-amino-acid insertion (differing from each other by one amino acid) relative to a noncytopathic FeLV clone, 61E, but they are all quite similar otherwise. Thus, this insertion is likely to be the major determinant of cytopathicity for 61B as well as 61C.

While the cytopathicity of 61B for 3201 T cells mapped to the gp70-encoding region of *env*, the difference in latency between 61B and 61C mapped to a portion of *env* encoding the transmembrane protein, p15E. The four differences in this segment of 61B relative to 61C and 61E are all G-to-A substitutions clustered within 110 nucleotides in the predicted extracellular domain of p15E (28). Two of the differences yield a predicted change in charge from negative (glutamic acid) in the consensus residues to positive (lysine) in 61B (Fig. 6). These two nonconservative 61B amino acid differences lie between two cysteine residues, adjacent to an immunosuppressive peptide sequence (5), in a region of the transmembrane protein that is conserved among many retroviruses (6, 30, 39). The location of the cysteines is conserved among both oncoviruses and lentiviruses (7, 13), although the five or six amino acids between the cysteines differ between the two classes, as exemplified by FeLV and HIV, respectively (Fig. 6). Mutation of these cysteines in HIV impairs processing of the gp160 precursor to mature gp41 and gp120 (7, 40). In Mason-Pfizer monkey virus, which is somewhat more homologous to FeLV in this region, a 35-amino-acid deletion spanning the cysteines has also been shown to abolish cleavage of the *env* precursor, while a smaller deletion that did not include the cysteines did not affect cleavage (4). Preliminary evidence (31a) suggests that the 61B gp85^{env} precursor is blocked in its processing to p15E and gp70. The inability of 61B gp85 to the cleaved might explain the replication defect conferred by that virus's p15E-encoding region, because mutations that prevent *env* cleavage have been shown to abolish or greatly reduce infectivity in several retroviruses (2, 4, 7, 10, 12, 14, 24, 25, 40). These findings suggest that one or both of the changes within this highly conserved region may prove to be the most important in determining the long latency of 61B.

We found that during infection of 3201 T cells with 61B(61E) viral supernatant, a new proviral *env* sequence that contained 3′ gp70 sequence characteristics of 61B and a transmembrane protein-encoding sequence like that of the replication-competent helper, 61E, became predominant. Because of the overall similarity between 61E and 61B sequences in this region, we cannot say whether this virus arose from recombination or multiple mutation, but recombination appears likely because most of the hybrid sequences contained all 61B-like nucleotides in the 5′ region of the segment sequenced and all 61E-like nucleotides at the 3′ end. In fibroblasts infected with 61B(61E), only 61E-like proviral *env* genes were detected in 12 clones sequenced. Southern blot analysis of the fibroblast DNA from which these sequenced fragments were derived shows that the 61E provirus was 10- to 20-fold more prevalent than that of 61B. However, the relatively low level of the 61B genome is not sufficient to explain the delayed cytopathicity of fibroblast-derived virus, because 61B(61E) viral supernatant harvested from a fibroblast clone bearing roughly equal amounts of 61B and 61E provirus also showed delayed latency when used to infect 3201 cells. Conversely, 61C(61E) supernatant derived from fibroblasts with an approximately 10:1 ratio of 61E to 61C proviral DNA caused CPE with a short latency.

We believe that the emergence of the recombinant in 3201 T cells but not AH927 fibroblasts represents a differential selective advantage for the virus in T cells, not simply a lack of generation of recombinants in the fibroblast line. Previous studies (29) have shown that recombinants between 61E and endogenous FeLV are generated in AH927 fibroblasts; re-

combination of 61E with 61B may be even more likely since the exogenous viruses are expressed and thus copackaged at higher levels than is endogenous FeLV. Virions bearing 61C or 61B gp70 (i.e., EECC and EEBC chimeras) replicate to high levels in 3201 cells, in part because of their failure to establish superinfection interference (9). In contrast, these viruses do not replicate well in fibroblasts. Thus, once a virus bearing the gp70 of either 61B or 61C arises, it would be predicted to have a growth advantage in 3201 cells but not in AH927 fibroblasts. A high level of variant DNA in tissues of FeLV-FAIDS-infected cats is associated with the onset of immunodeficiency disease in vivo (17, 26) and with CPE in vitro (9). Thus, a variant that can replicate to high copy number may also be more cytopathic. The association of CPE with increased amounts of viral DNA in cells has been seen in other retroviral infections (reviewed in reference 41).

While it is possible that differences in infectivity and cytopathicity of 61B(61E) expressed in T cells compared with fibroblasts is the result of biochemical differences between viruses expressed in different cells, our data support the conclusion that selection of virus with the recombinant envelope gene accounts for the shortened latency of T-cell-derived 61B(61E). First, the recombinant was highly prevalent in cells producing short-latency supernatant. Second, 3201 cells early after infection with fibroblast-derived 61B(61E) produced virus with an intermediate latency, and such cells contained the recombinant as a minority of the proviral population.

We propose the following mechanism for the latency difference between 61B(61E) and 61C(61E) infection in 3201 T cells. As long as the 61B genome cannot produce a functional variant envelope glycoprotein, CPE does not occur. During replication in culture, a recombination event occurs that creates an *env* with features of 61B's gp70 and a p15E like that of 61E, allowing expression of mature variant glycoprotein. Once this recombinant arises, it is positively selected because it replicates to high levels in 3201 cells; this high level of replication is associated with CPE. Virus from such a culture, when used to infect 3201 cells, is cytopathic after a short latency period. To use terminology analogous to that used to label our chimeric constructs, the most successful recombinant would be of the form EEBE. A recombinant BBBE, although still requiring 61E *gag* and *pol* in *trans*, should still have a replication advantage over any recombinant bearing the p15E of 61B, because the latter is apparently unable to produce a functional glycoprotein.

Previous studies of 61B(61E) versus 61C(61E) infection of cats demonstrated that the genotype of the infecting FeLV variants is a determinant of disease latency (28). Our findings and those of previous studies have shown that the cytopathic properties of FeLV variants of 3201 cell culture are correlated with their ability to induce immunodeficiency disease in vivo (9, 29). Therefore, we have exploited the 3201 cell assay to define the viral molecular basis of differences in latency between 61B and 61C. Cloned retroviral genomes are usually studied in isolation, but we have characterized a system in which a minimally pathogenic virus and a replication-defective virus recombine to form a cytopathic virus which is positively selected for replication in T lymphocytes. Such mixed infections are probably a more accurate representation of in vivo infection, as exemplified by the cloning of the 61B, 61C, and 61E genomes and other genomes varying in pathogenicity from a single cat inoculated with the FeLV-FAIDS isolate (27, 28). Our data support the idea that retroviral pathogenesis may be influenced by the interaction of multiple viral genetic variants over time within the infected individual and that the emergence of certain variants may be significant in disease progression.

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