Defective Endogenous Proviruses Are Expressed in Feline Lymphoid Cells: Evidence for a Role in Natural Resistance to Subgroup B Feline Leukemia Viruses

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Endogenous feline leukemia virus (FeLV)-related sequences (enFeLV) are a family of proviral elements found in domestic cats and their close relatives. These elements can recombine with exogenous, infectious FeLVs of subgroup A (FeLV-A), giving rise to host range variants of FeLV-B. We found that a subset of defective enFeLV proviruses is highly expressed in lymphoma cell lines and in a variety of primary tissues, including lymphoid tissues from healthy specific-pathogen-free cats. At least two RNA species were detected, a 4.5-kb RNA containing *gag, env*, and long terminal repeat sequences and a 2-kb RNA containing *env* and long terminal repeat sequences. Cloning of enFeLV cDNA from two FeLV-free lymphoma cell lines (3201 and MCC) revealed a long open reading frame (ORF) encoding a truncated *env* gene product corresponding to the N-terminal portion of $gp70^{env}$. Interestingly, all of three natural FeLV-B isolates include 3' *env* sequences which are missing from the highly transcribed subset and hence must be derived from other enFeLV elements. The enFeLV *env* ORF cDNA clones were closely similar to a previously characterized enFeLV provirus, CFE-16, but were polymorphic at a site corresponding to an exogenous FeLV neutralization epitope. Site-specific antiserum raised to a C-terminal 30-amino-acid peptide of the enFeLV *env* ORF detected an intracellular product of 35 kDa which was also shed from cells in stable form. Expression of the 35-kDa protein correlated with enFeLV RNA levels and was negatively correlated with susceptibility to infection with FeLV-B. Cell culture supernatant containing the 35-kDa protein specifically blocked infection of permissive fibroblast cells with FeLV-B isolates. We suggest that the truncated *env* protein mediates resistance by receptor blockade and that this form of enFeLV expression mediates the natural resistance of cats to infection with FeLV-B in the absence of FeLV-A.

Endogenous feline leukemia virus (FeLV)-related (enFeLV) sequences are found in the genomes of domestic cats and related small feline species (2, 3). enFeLV sequence family members are polymorphic, present at 8 to 12 copies per cell, and arranged as proviruses which generally retain both long terminal repeat (LTR) sequences (42, 43). Many of the enFeLV elements contain extensive deletions, while even the apparently full-length enFeLV proviruses appear to be defective; none have yielded infectious virus, and those which have been analyzed in detail have mutations in essential structural genes (27). However, enFeLV elements can participate in recombination with the common exogenous form of FeLV, subgroup A (FeLV-A), to generate envelope gene variants (FeLV-B) with an expanded host range (23, 27, 44). FeLV-B variants are more common in leukemic than in healthy cats (18, 19) and can, in some cases, act to accelerate disease induced by weakly oncogenic FeLV-A (46). The process of recombination is generally assumed to result from expression of endogenous viral sequences and copackaging of transcripts into exogenous virus particles (27, 46). However, the in vivo expression of enFeLV elements has not been fully described.

Earlier studies revealed low-level expression in placenta, fetal lymphoid tissues, and some FeLV-negative lymphomas (6, 24, 34). A limitation of these earlier studies is that they were performed with broadly reactive probes which could not be used to monitor expression of the endogenous elements in the presence of exogenous FeLV.

While feline embryonic fibroblasts are generally permissive for infection with FeLV-B (36, 37), cats are highly resistant to infection with FeLV-B in the absence of FeLV-A (19) and FeLV-B variants appear to depend on their associated FeLV-A forms for successful transmission (17, 18). Natural resistance to FeLV-B can be overcome by infection with phenotypic mixtures of FeLV-A and FeLV-B, but replication of FeLV-B is still restricted and delayed compared with the associated FeLV-A (16, 18, 19, 46).

These phenomena and the selective pressures favoring the retention of enFeLV sequences in the feline genome have previously been obscure. We now report that a subset of enFeLV sequences is expressed in healthy cat lymphoid tissues. Analysis of the sequence content of the expressed enFeLV rules these out as the immediate precursors of most natural FeLV-B recombinants. However, the expressed enFeLVs encode a truncated *env* gene product which appears to play a role in resistance to infection with exogenous FeLV-B. Our results therefore provide a rationale for the retention of actively transcribed enFeLV elements in the feline genome, since it appears that resistance to exogenous FeLV-B can be conferred by endogenous virus expression without necessarily increasing the risk of recombination to generate pathogenic virus variants.

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MATERIALS AND METHODS

Cell lines. AH927 (29) is a cell line derived from feline embryonic fibroblasts. 3201 (40) and MCC (7) are FeLV-negative lymphoid tumor cell lines derived, respectively, from a naturally occurring thymic lymphosarcoma and a large granular lymphoma. F422 (30) and FL74 (45) are FeLV-positive tumor cell lines established from naturally occurring thymic lymphosarcomas. Q201 is an interleukin 2-dependent, CD4$^+$ T-cell line derived from peripheral blood lymphocytes of a cat immunized with ovalbumin (47).

FeLV isolates and infectivity assays. Standard FeLV isolates were obtained from stably transfected BHK21 cells as previously described (32), including FeLV-A/Glasgow-1 from the pFGA-5 clone (44), FeLV-B/Gardner-Arnstein from pFGB (22), FeLV-B/Rickard from pFRB (22), and FeLV-C/Sarma from pFSC (31). Biologically cloned FeLV-B/Snyder-Theilen was propagated in mink lung (CCL64) cells (14), and uncloned FeLV-ABC/Kawakami-Theilen was obtained from the FL74 lymphoma cell line (45).

FeLV infectivity assays (see Tables 1 and 2) were carried out on clone 81 S$^+$ L$^-$ cells as previously described (11, 33).

Hybridization probes. The B/S *env* probe is a *Sau*3A fragment from the *env* gene of FeLV-B/Gardner-Arnstein which reacts with FeLV-B and enFeLV *env* sequences (see Fig. 3A). The 236-bp enU3 probe was generated by PCR from the LTR of an enFeLV proviral element, pLCM-1 (the kind gift of J. Mullins, Stanford University) by using primers 5'-GACC CCTGTCATAATATGC-3' (forward) and 5'-CCAGAAT GAGGGGAACAAAC-3' (reverse). The Δ*env* probe was also generated from pLCM-1, from primers flanking the 3' end of *env* (see Fig. 3A), i.e., 5'-AATGCCTCCATTGCCCCTCTA AGC-3' (forward) and 5'-ACTGCACCAACCGGTTAAGG ATGC-3' (reverse). The exU3 probe was a 0.9-kb *Eco*RI-*Hind*III fragment encompassing an exogenous FeLV-specific portion of the LTR U3 domain and host flanking sequences from the pFGB proviral clone of FeLV-B/Gardner-Arnstein (21). The *gag* probe was an *Sst*I-*Bgl*II fragment (634 to 1111) encompassing the *gag* leader sequence and most of the p15^{gag} coding sequence; the *pol* probe was a *Kpn*I-*Kpn*I fragment (3411 to 4995) covering most of the reverse transcriptase coding sequence and the 5' end of the integrase coding sequence. Both probes were derived from the pFGA-5 clone (44), and numbering is relative to FeLV-A/1161E (9). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 720-bp fragment of the rat GAPDH-encoding gene amplified by PCR of rat cDNA (gift of R. Hennigan, Beatson Institute for Cancer Research). [α-^{32}P]CTP-labelled probes with specific activities of 10^7 to 10^8 cpm/μg were produced by using nick translation kits (Amersham).

Northern (RNA) blot analysis. Total cellular RNA was prepared by the RNazol method (8), resolved by electrophoresis in 1.2% agarose-formaldehyde gels, and transferred to nitrocellulose filters. After prehybridization at 42°C, the blots were hybridized to FeLV-specific probes or the GAPDH probe. For FeLV probes, blots were washed at high stringency (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.5% sodium dodecyl sulfate [SDS] at 65°C) or low stringency (42°C) for GAPDH, air dried, and autoradiographed.

DNA sequencing. The EnU3 probe was cloned into bacteriophage M13mp10, and both strands were sequenced by the dideoxy-chain termination method of Sanger et al. (35). The enFeLV *env* cDNA clones were sequenced as double-stranded plasmid DNA by using a combination of universal and custom-synthesized primers, also by the chain termination method.

cDNA synthesis. A 5-μg sample of poly(A)$^+$ RNA prepared from 3201 or MCC cells was used as the template for cDNA synthesis with a Pharmacia cDNA synthesis kit. The products of second-strand synthesis were purified on Sephacryl S-300 spun columns. The final DNA products were ethanol precipitated and resuspended in distilled water.

PCR amplification of enFeLV sequences. The Perkin Elmer-Cetus PCR kit was employed with recombinant *Taq* polymerase. The oligonucleotide sequences used as specific primers in the amplification of the enU3 probe were 5'-GACCCCCTGTCATAATATGC-3' and 5'-CCAGAATGAG GGGAACAAAC-3'. Reaction mixtures contained 1 ng of plasmid DNA, 1 μg of each primer (1 mg/ml), 10 μl of 10× reaction buffer, 500 mM deoxynucleotides, and water to a volume of 95 μl. After 10 min of incubation at 95°C, 0.5 U (5 μl) of *Taq* polymerase was added and amplification was achieved by 30 cycles of denaturation (92°C), annealing (50°C), and polymerization (72°C). For the amplification of *env* sequences from cDNA, the primers used were 5'-TCAGACA GACCCCAGCTCAGA-3' and 5'-CCAGAATGAGGGGAA CAAAC-3' and annealing was done at 60°C. The PCR products were resolved on a 6% nondenaturing acrylamide gel and subcloned into M13 or pIC20H for sequence analysis.

Expression cloning. A 278-bp *Hae*III fragment encompassing the 3' end of the enFeLV *env* open reading frame (ORF) and the adjacent LTR sequences was subcloned into pIC20H (*Eco*RV site) and then directionally into pGEX-2T (*Bam*HI-*Eco*RI). Clones were tested for production of glutathione *S*-transferase (GST) fusion proteins of the appropriate size and by Western blot (immunoblot) analysis with FeLV gp70-specific antiserum.

Production of GST fusion proteins. GST fusion proteins were prepared by established methods (39). Five to ten liters of exponentially growing bacteria containing the appropriate clone were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (23 μg/ml). The cells were lysed by sonication, and the insoluble cellular debris was removed by centrifugation at 27,200 × *g*. The GST protein was purified by binding to glutathione-bound agarose and elution with 10 mM glutathione after washing to remove unbound proteins.

Immunization of rabbits. Rabbits were immunized subcutaneously with 500 μg of GST fusion protein in Freund's complete adjuvant and then boosted 3 weeks later with the same dose in Freund's incomplete adjuvant.

Metabolic labelling and immunoprecipitation. Cells (10^6) were incubated in methionine-free medium for 30 min at 37°C, after which 100 μCi of [^{35}S]methionine-cysteine label (NEN) was added and cells were incubated for 30 min. The labelled cells were pelleted, rinsed in 1× Tris-buffered saline, and disrupted in lysis buffer (0.1 M Tris, 0.14 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1% aprotinin, pH 7.4). After removal of debris by centrifugation (10,000 × *g* for 5 min and then 50,000 × *g* for 30 min), lysates were incubated with sera for 60 min on ice. Immune complexes were captured on protein A-Sepharose beads. After successive washes with 0.5 M LiCl and lysis buffer (three times), the adsorbed proteins were released from the beads by incubation in protein gel sample buffer at 100°C for 3 min. Proteins were resolved by SDS–8 to 15% gradient polyacrylamide gel electrophoresis, and the gels were fixed in 5% methanol–7.5% acetic acid for 1 h before being washed in 5 volumes of autoradiographic enhancer solution (Enlightning; NEN) for 30 min prior to drying under a vacuum and autoradiography.

RESULTS

Generation of enFeLV-specific probes. A prerequisite for this study was the generation of probes which could detect the expression of enFeLV, even in the presence of closely related exogenous FeLV transcripts. As reported previously, a 296-bp *Sau*3A fragment derived from the *env* gene of FeLV-B/Gardner-Arnstein (designated B/S) detects enFeLV sequences but not FeLV-A under high-stringency Southern blot hybridization analysis (44). However, since the B/S probe also detects exogenous FeLV-B viruses, it could not be used to detect enFeLV expression where FeLV-B might be present. We generated an alternative probe for enFeLV from the U3 domain of enFeLV proviral clone pLCM-1 (enU3). By aligning the known enFeLV and exogenous FeLV LTR sequences, a segment of the U3 domain with a minimal sequence match was identified. PCR primers based on the CFE-6 LTR sequence (20) were used to amplify and clone the corresponding region of pLCM-1. The sequence of the 236-bp PCR-derived enU3 probe is closely similar to the three published enFeLV sequences and has little residual identity to exogenous FeLV (data not shown). The enU3 probe was found to be a highly sensitive probe for enFeLV and did not cross-react with exogenous FeLV sequences.

Truncated EnFeLV proviruses are highly expressed in feline lymphoma cell lines and in normal hemopoietic tissues. Endogenous FeLV expression was previously detected in placenta, fetal hemopoietic tissues, and some FeLV-negative lymphomas (6, 34), but the cDNA probes used in those early studies were derived from the entire FeLV genome and gave no detailed information on transcript structure. By using the enU3 and B/S *env* probes, we analyzed enFeLV expression in a range of feline cell lines and tissues. The two probes were equally effective in analysis of FeLV-free tissues, but the enU3 probe was obviously preferred where exogenous FeLV was present. As can be seen from the examples shown in Fig. 1, enFeLV expression was detected in a range of feline lymphoma cell lines, both FeLV infected (F422 and FL74) and FeLV free (3201 and MCC). However, no expression was detected in feline fibroblast cell lines AH927 and FEA.

Where enFeLV was found to be expressed, a 2-kb RNA was invariably present. Larger transcripts were more heterogeneous, and atypical RNA species were seen in the MCC and FL74 lymphoma cell lines. The most commonly observed pattern was that of the 3201 lymphoma cell line, with two major transcripts of 4.5 and 2 kb. This pattern of transcripts was also detected in a series of primary FeLV-induced lymphomas (data not shown). However, no expression of full-length enFeLV proviral elements was evident and the largest transcripts detected were 4 to 5 kb long.

Analysis of tissues from healthy cats revealed similar transcripts in primary lymphoid organs (thymus, spleen, and lymph nodes) and tissues with extensive lymphoid compartments (e.g., bone marrow and intestines) but not in other tissues, such as muscle, brain, and kidney tissues. Figure 1 shows representative examples in which total cellular RNA prepared from kidney, lymph node, and thymus tissues of two healthy FeLV-infected cats (P111 and P112) and an uninfected specific-pathogen-free cat were analyzed for enFeLV expression by using the enU3 probe. Individual animals in the outbred cat population display minor differences in the pattern of enFeLV expression, as shown by a variant 3-kb transcript in the FL74 lymphoma line and in P112 thymus tissue.

Genetic content of enFeLV transcripts. To characterize further the genetic content of enFeLV RNA transcripts, Northern blot strips of total RNA from 3201 cells were
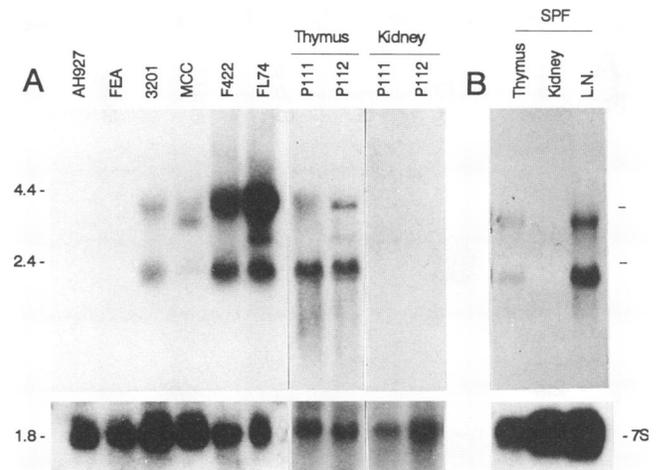
FIG. 1. Expression of enFeLV sequences in lymphoma cell lines and normal feline lymphoid tissues. Total RNA samples (20 μg) were separated by electrophoresis on a 0.8% agarose gel in the presence of 2.2 M formaldehyde and blotted onto a GeneScreen membrane. The upper blots were hybridized with the enU3 (A) or B/S (B) probe and washed under high-stringency conditions (three washes with 0.1× SSC, 65°C). The lower part of each panel shows parallel blots hybridized with GAPDH (A) and murine 7S RNA-specific (1) (B) probes to serve as RNA loading controls. The relative positions of RNA ladder markers are also indicated, and sizes are given in kilobases. The RNA samples were derived from feline fibroblasts (AH927 and FEA), FeLV-negative T-lymphoma cell lines (3201 and MCC), FeLV-positive T-lymphoma cell lines (T3 and FL74), and tissues from healthy FeLV-infected cats (P111 to P114) and a specific-pathogen-free (SPF) domestic cat. L.N., lymph node.

hybridized with FeLV-derived subgenomic probes. As shown in Fig. 2A, the larger transcript hybridized with enU3, *gag*, and *env* sequences while the smaller contained *env* and enU3 sequences only. It is possible that this pattern arises from the expression of one or more enFeLV loci substantially deleted in *pol* and removal by splicing of the packaging signal and truncated *gag-pol* domain to yield a 2-kb *env* mRNA-like form. Support for this interpretation is provided by the fact that only the larger transcript was packaged into FeLV-A virions harvested from 3201 cells, albeit at low levels (data not shown).

enFeLV cDNAs contain ORFs for closely related *env* gene products but lack 3' *env* sequences present in three natural FeLV-B isolates. The enFeLV transcripts were not expected to encode *gag-pol* products, since the *gag* genes of the enFeLV family which have been restriction mapped or sequenced contain large deletions or premature stop codons (20), MCC and 3201 cells do not express FeLV *gag*-related antigens (7, 41), and the enFeLV transcripts have a large deletion that affects *pol* (Fig. 2A). In contrast, enFeLV proviruses have ORFs in *env* (20) and there has been a previous report that FeLV *env* determinants were expressed on the surface of 3201 cells (41). For these reasons, we decided to examine the *env* coding potential of the enFeLV transcripts.

cDNA was prepared from poly(A)+ RNAs isolated from 3201 and MCC cells. Primers were designed to amplify the putative enFeLV *env* genes by using the published sequence of a cloned enFeLV element, CFE-6 (20). The 5' amplimer (A) corresponded to a region of CFE-6 *env* immediately upstream of the *env* initiation codon, while 3' amplimers were derived from the LTR (B and C).

As shown in Fig. 2B, these primers directed the amplification of 1.3-kb (A + B) or 1.2-kb (A + C) *env* fragments from

A

Probe: gag pol B/S env enU3 exU3

4.4 -

2.4 -

B

M pLCM-1 3201 MCC pLCM-1 MCC 3201

4.4 -

2.25 -
1.96 -

1.36 -

1.08 -

Primers: A+B   A+C

[LTR] CFE-6 (3')
env

A —————————— B 2.3 kb
A —————————— C 2.2 kb
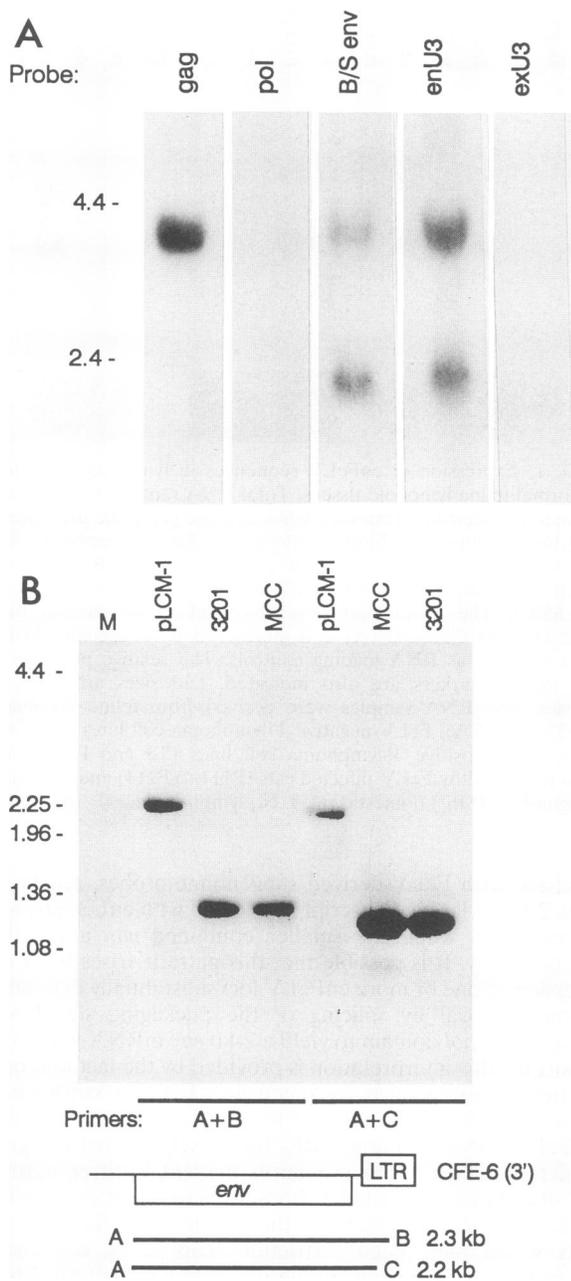
FIG. 2. (A) Composition of enFeLV transcripts in 3201 FeLV-negative lymphoma cells. Northern blot strips were prepared from 20-μg aliquots of 3201 cell total RNA and hybridized with subgenomic FeLV probes as explained in Materials and Methods. (B) PCR amplification of enFeLV *env* sequences. Five milligrams of poly-(A)$^+$ RNA from two FeLV-negative lymphoma cell lines (3201 and MCC) was used to prepare cDNA. PCR amplification was carried out with primers spanning the *env* gene and LTR of enFeLV CFE-6 as shown at the bottom of panel B. The positive control for amplification of the full-length enFeLV gene was proviral clone pLCM-1. Products were separated by polyacrylamide gel electrophoresis and detected by hybridization analysis with the B/S probe. As can be seen, the two cDNA preparations yielded a homogeneous 1.2-kb product but no full-length enFeLV *env* products were detected, by either ethidium bromide staining (data not shown) or hybridization. The numbers to the left are molecular sizes in kilobases. Lane M contained molecular size standards.

both lymphoma cell lines. A single major band was resolved in each case, and there was no detectable amplification of DNA fragments corresponding to full-length enFeLV *env* genes. The full-length genes from enFeLV proviral clones (e.g., pLCM-1) were readily amplified by using the same primers and conditions.

The amplified fragments from both cell lines were cloned into M13 vectors and sequenced on both strands. In both cases, we found an ORF with the potential to code for a 273-amino-acid polypeptide including the full *env* signal peptide and the N-terminal half of gp70^{env}. The DNA and predicted protein sequences of the enFeLV cDNA clones were very similar to those of a previously characterized enFeLV proviral element, CFE-16 (20). A line-bar diagram (Fig. 3A) shows DNA sequence differences from CFE-16, with coding sequence changes further marked with circles. The enFeLV clones and CFE-16 were much more similar to each other than to another enFeLV proviral clone, CFE-6, which contains a full-length *env* gene (20). Moreover, the cDNAs contained a 457-bp deletion relative to CFE-6 encompassing the 3' half of the gp70 coding sequence and leaving only the terminal 15 bp of the p15E coding sequence. This deletion in the cDNA PCR clones corresponded precisely to that found in enFeLV provirus CFE-16 (20). The MCC and 3201 clones differed from each other and from CFE-16 by a few scattered point mutations. Although *Taq* polymerase errors or cloning artifacts could not be ruled out for individual point mutations, the reading frames were all intact and the presence of some mutations in more than one clone suggested that these are genuine sequence polymorphisms.

Northern blot analyses (Fig. 1) revealed no detectable full-length enFeLV transcripts, and PCR analysis (Fig. 2B) revealed no evidence of full-length *env* genes in the abundant transcripts. However, since PCR amplification may bias towards detection of the shorter defective *env* species, we could not completely exclude the possibility that a full-length *env* gene was present among the truncated proviral transcripts. We therefore decided to look for evidence of full-length enFeLV *env* expression by a different approach. PCR primers were designed from within the domain of CFE-6 *env* sequences which are missing from the cDNA clones, and a probe (delta *env*) was generated by amplification of the pLCM-1 enFeLV clone. This probe should hybridize selectively with full-length *env* (diagram in Fig. 3A). The probe was used in Northern blot hybridization analysis (Fig. 3B) and found to give a strong signal when exogenous FeLV species were present. The typical pattern of full-length and subgenomic FeLV RNA species was detected in 3201 lymphoma cells infected with FeLV. Similar transcripts were seen in FL74 lymphoma cells, in which defective exogenous FeLV predominates, but the 4 to 4.5-kb and 2-kb RNA species detected with the B/S probe (Fig. 1) were not seen and no transcripts were detected in uninfected 3201 cells. We conclude that the abundant enFeLV transcripts in 3201 cells do not contain a full-length *env* gene.

In addition to their similar overall structure, the coding potentials of the MCC and 3201 enFeLV *env* ORFs were also closely similar to that of CFE-16 (Fig. 4). The 3201 *env* ORF had a coding potential identical to that of the CFE-16 ORF, while the MCC cell clone had a single asparagine-to-aspartic acid substitution at position 240. Again, this difference was clearly not a PCR artifact since it was present in two independent clones and corresponds to a known polymorphism among enFeLV proviral elements. Intriguingly, this difference occurs within the core of a previously mapped neutralizing epitope (MGPNL) common to exogenous FeLV-A, FeLV-B, and FeLV-C (10, 25).
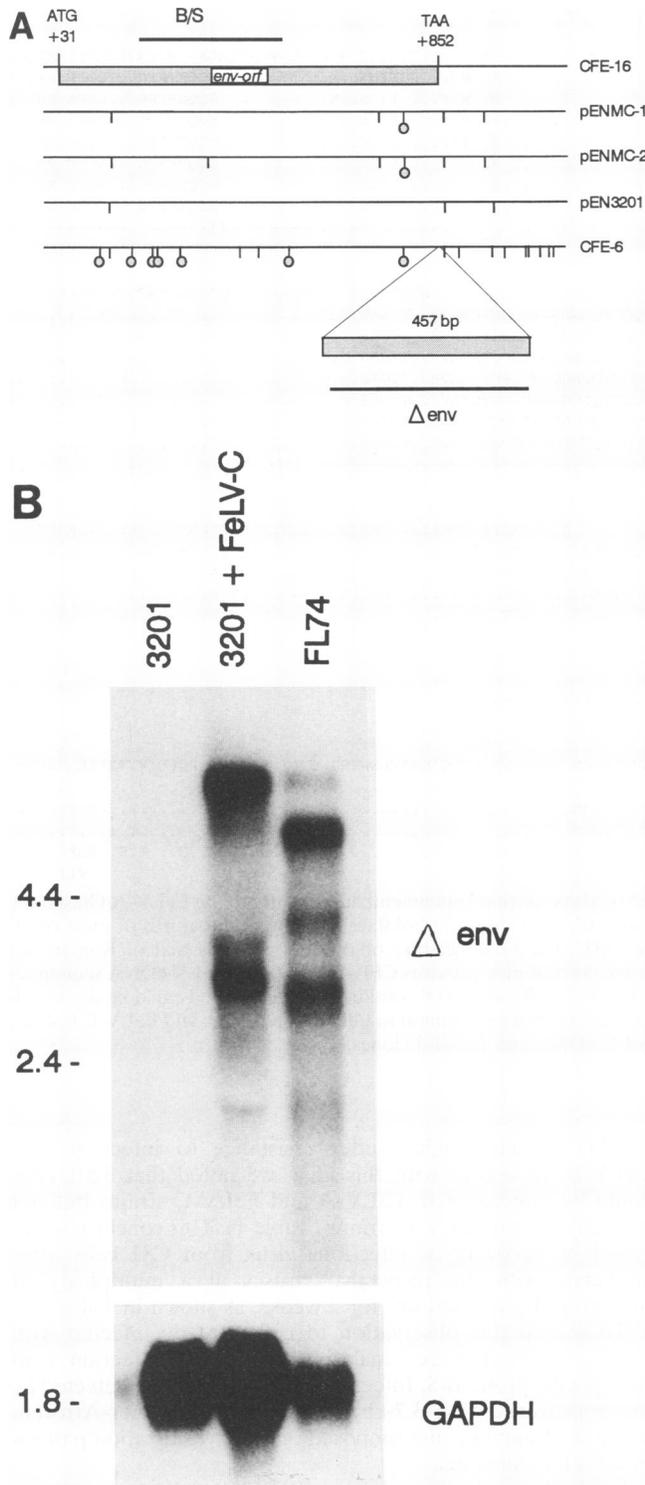
FIG. 3. (A) Alignment diagram showing the relationship of en-FeLV cDNA sequences with enFeLV proviral elements CFE-16 and CFE-6. The PCR products shown in Fig. 2 were cloned and completely sequenced on both strands. The full 1,234-bp sequences of two MCC cDNA clones and one 3201 cDNA clone are represented and aligned with the published sequences of enFeLV elements CFE-6 and CFE-16 (20). Point mutational differences are represented by vertical bars under the line, and shaded circles denote coding sequence changes. The CFE-16 and enFeLV cDNA sequences have an identical 457-bp deletion relative to the full-length CFE-6 clone and terminate out of

The amino acid sequence alignment in Fig. 4 also reveals that three FeLV-B viruses (Gardner-Arnstein, Rickard, and Snyder-Theilen) have more extensive enFeLV-derived sequences than are present in the enFeLV cDNA clones. All three FeLV-B isolates have all of variable region V, and two have region VI derived from enFeLV.

A truncated *env* gene product is detected in cells expressing enFeLV transcripts and is shed into culture supernatants. Metabolic labelling and immunoprecipitation analysis of 3201 and MCC cells with a polyclonal goat antiserum raised to partially purified FeLV gp70^{env} (derived from the F422 lymphoma cell line [30]) is shown in Fig. 5. This antiserum was highly reactive with FeLV gp70^{env} but also detected a protein of 35 kDa in cell lines expressing enFeLV RNA. This size is compatible with the coding potential of the enFeLV cDNAs. For additional evidence of the identity of the 35-kDa protein, it was decided to raise new antisera specific for the putative *env* ORF product.

A 278-bp *Hae*III fragment from the 3' end of the MCC *env* ORF, encompassing the terminal 90 bp of *env* and noncoding LTR sequences, was cloned into bacterial expression vector pGEX-2T. This vector allows the expression in *Escherichia coli* of foreign peptides as C-terminal fusions with GST under the control of an IPTG-inducible promoter (39). The GST-*env* fusion protein containing the terminal 30 amino acids of the enFeLV truncated *env* gene was found to be stably expressed. A 5-mg sample of the fusion protein was purified by affinity chromatography on glutathione-agarose beads and used to immunize rabbits.

Immune precipitation analysis with the new antiserum confirmed the 35-kDa protein as the candidate *env* ORF product (Fig. 5). As expected, the product was not detected in the AH927 fibroblast cells, which did not express significant levels of enFeLV RNA. Expression was high in 3201 cells but rather lower in MCC cells (data not shown), in accord with the lower levels of the 2-kb presumptive *env* mRNA in MCC cells (Fig. 1).

The new antiserum also precipitated FeLV-B gp70, although much more weakly than did goat anti-gp70 serum (Fig. 5A). This might be explained by the fact that the relevant peptide is at the C terminus in both the env ORF product and the GST fusion protein but internal to FeLV-B gp70. Alternatively, amino acid substitutions within this region which distinguish FeLV-B from enFeLV sequences (Fig. 4) might account for the weaker reactivity against FeLV-B gp70. Anti-*env* ORF serum precipitated the 35-kDa protein as efficiently as did goat anti-gp70 serum. The identity of a 75-kDa 3201 cell protein precipitated by goat anti-gp70 serum was unclear, since it was not detected with anti-env ORF serum nor did it react with a panel of monoclonal antibodies to FeLV gp70 (data not shown). We consider it most likely that the goat immunogen

frame within the last codons of *env*. (B) Further analysis of transcripts in 3201 FeLV-negative lymphoma cells. The Northern blot was probed initially with the PCR-generated Δ*env* probe as described in Materials and Methods and as depicted in panel A and then stripped and reprobed with a GAPDH probe as for Fig. 1. The lanes contained 10-μg aliquots of RNA from 3201 cells, either uninfected or after infection with FeLV-C/Sarma, or 2 μg of RNA from the FL74 lymphoma cell line, which contains multiple exogenous FeLV transcripts and releases viruses of subgroups A, B, and C. The sample in the middle lane was prepared from a 20-μg aliquot of 3201 cell total RNA and hybridized with a subgenomic FeLV probe as explained in Materials and Methods. The numbers to the left are molecular sizes in kilobases.

```
         <Leader                        <Start
                          .         .              .          .        50      .        *       .          .         100      .
A-Glasgow MESPTHPKPKPSKDKTLSWNLAFLVGI    LFTIDIGMANPSPHQIYNVTWVITNVQTNTQANATSMLGTLTDAYPTLHVDLCDLVGDTWEPIVLNPTNVKHGARYSSSKYGCKTTDRKKQQQ
B-GA      ..................V.....       ...............V.....T...LV.G.K.............F..MYF....II.N..N.       SDQEPFPG...DQPM.RW..R
B-ST      ..G..........F..D.MI...V       .LRL.V...............T...LV.G.K.............F..MYF....II.N..N.       SDQEPFPG...DHPM.RW..R
B-Rickard ..........................     ................T...LV.G.K.............F..MYF....II.N..N.            SDQEPFPG...DQPM.RW..R
en-CFE-6  ..G..........F..D.II...V       .LRL.A.........V..I..T...LV.GIK............TF..IYF....II.N..N.       SDQEPFPG...DQPM.RW..R
en-CFE-16 ..G..........F..D.II...V       .LRL.V.........V..I..T...LV.G.K.............F...YF....II.N..N.       SGQEPFPG...DQPM.RW..R
pEN-MC-1  ..G..........F..D.II...V       .LRL.V.........V..I..T...LV.G.K.............F...YF....II.N..N.       SGQEPFPG...DQPM.RW..R
pEN-32-1  ..G..........F..D.II...V       .LRL.V.........V..I..T...LV.G.K.............F...YF....II.N..N.       SGQEPFPG...DQPM.RW..R
          ----------I-----------------   -----------IA--                        ----------------------II-----------------


              .*         .        150        .*       .                                  .         .            200       .
A-Glasgow TYPFYVCPGHAPSLGPKGTHCGGAQDGFCAAWGCETTGETWWKPTSSWDYITVKRG                        SSQDNSCE GKCNPLVLQFTQKGRQASWDGPKMWGLRLYRTG
B-GA      NT.........NRKQ    ...P......V.........Y.R............K.VTQGIYQCSGGGWCGPCYDKAVH..TTGAS.G.R....I.........T......S.......S.
B-ST      NT.........NRKQ    ...P......V.........Y.R............K.VTQGIYQCSGGGWCGPCYDKAVH..ITGAS.G.R....I.........T......S.......S.
B-Rickard NT.........NRKQ    ...P......V.........Y.R............K.VTQGIYQCSGGGWCGPCYDKAVH..TTGAG.G.R....I.........T......S.......S.
en-CFE-6  NTA........NRKQ    ...P......V.........Y.............K.VTQGIYQCNGGGWCGPCYDKAVH..TTGAS.G.R...I.........T......S.......S.
en-CFE-16 NTA........NRKQ    ...P......V.........Y.............K.VTQGIYQCSGGGWCGPCYDKAVH..TTGAS.G.R....I.........T......S.......S.
pEN-MC-1  NTA........NRKQ    ...P......V.........Y.............K.VTQGIYQCSGGGWCGPCYDKAVH..TTGAS.G.R....I.........T......S.......S.
pEN-32-1  NTA........NRKQ    ...P......V.........Y.............K.VTQGIYQCSGGGWCGPCYDKAVH..TTGAS.G.R....I.........T......S.......S.
          ---      ---III---                              ---------------IV--------------------


              .         .          .*    250        .                .          .          .         300        .          .
A-Glasgow YDPIALFTVSRQVSTITPPQAMGPNLVLPDQKPPSRQSQTGSKVATQRPQTNES          APRSVAPTTMGPKRIGTGDRLINLVQGTYLALNATDPNKTKDCWLCLVSRPPYYEG
B-GA      .......S.....M........................IE.R.TPHHS.G.GGTPGITLVNASI..L.TPV.PAS............................R.................
B-ST      .......S.....M........................IE.R.TPHHS.G.GGTPGITLVNASI..L.TPV.PAS.......N..............V.N.................
B-Rickard .......S.....M........................IE.R.TPHHS.G.GGTPGITLVNASI..L.TPV.PAS.......N..............V.N.................
en-CFE-6  .......S.....M...........P............IE.R.IPHHP.G.GGTPGITLVNASI..L.TPV.PAS.......N...........T..V.N.................
en-CFE-16 .......S.....M...........DP...........IE.R.IPHPS#(term.)
pEN-MC-1  .......S.....M...........P............IE.R.IPHPS#
pEN-32-1  .......S.....M...........DP...........IE.R.IPHPS#
                                                   -----------------------V----------


              .         .          350        .         .          .          400        .          .          .         .
A-Glasgow IAILGNYSNQTNPPPSCLSIPQHKLTISEVSGQGLCIGTVPKTHQALCNKTQQGHTGAHYLAAPNGTYWACNTGLTPCISMAVLNWTSDFCVLIELWPRVTYHQPEYVYTHFAKAVRFRR
B-GA      ...............................................E...........................................................A....
B-ST      ..V.............D...............................K...K..K.T......S.........I...................................D.TV.L..
B-Rickard ..V.............D...............................K...K..K.T....V.S...................................................
en-CFE-6  ..V.............V...............A...............K..R.T...V.....................................T.....I...E...I.S..ENKP..K.
                                                       --VI--                                                              -VII
```

FIG. 4. Alignment of gp70^env amino acid sequences. Gaps were introduced to allow maximal alignment, and identity to the FeLV-A/Glasgow-1 sequence is indicated by dots. As discussed in the text, residues marked by asterisks and shown in boldface are common features of most or all natural FeLV-B isolates but divergent from FeLV-A and the enFeLV env ORF. The nomenclature of these domains is that of Kumar and coworkers (20), except that an additional subdomain (Ia) is delineated. The truncated enFeLV provirus CFE-16 and the enFeLV cDNA sequences from MCC and 3201 cells have an extensive deletion from domains V to X. The FeLV-B isolates all contain enFeLV-derived env domains which are deleted in CFE-16 and the enFeLV cDNA sequences. The core of a neutralization epitope common to FeLV-A, FeLV-B, and FeLV-C isolates (MGPNL) (10, 25) is underlined. This is a polymorphic site within the enFeLV cDNA and proviral clones.

contained contaminating feline cellular antigens or other viral components (e.g., RD-114) with sizes and properties similar to those of FeLV gp70. The presence of an FeLV env-related protein of this size seems unlikely from the RNA expression data (Fig. 1 to 3), but the possibility cannot be excluded.

From the enFeLV cDNA sequences it is predicted that the truncated env ORF protein will retain the N-terminal signal sequence directing membrane insertion but lack the trans-membrane anchor domain of p15E, suggesting that the protein is likely to be shed from cells. To examine this possibility, we pulse-chase labelled 3201 cells and carried out immunoprecipitation analysis on intracellular and extracellular fractions. As can be seen from Fig. 5B, the 35-kDa protein was detected in culture supernatants after a 4-h chase period. Electrophoretic mobility was unaltered, and we saw no evidence of further processing or degradation of the extracellular protein.

Resistance to infection with FeLV-B in vitro is associated with expression of the 35-kDa protein. Retroviral env gene products expressed in uninfected cells can bind to host cell receptors, thereby conferring resistance to exogenous virus infection. Since enFeLV env sequences are closely matched to FeLV-B (20, 44) and presumably carry the determinants of FeLV-B-specific receptor binding, it was considered that the

35-kDa protein might confer resistance to infection with FeLV-B. In accord with this idea, we noted that 3201 cells could be infected with FeLV-A and FeLV-C strains but not with any of four FeLV-B strains (Table 1). This conclusion was based on recovery of infectious virus from C81 cells after challenge with the respective viruses at a multiplicity of infection of 1 and culture for 2 weeks, as shown in Table 1.

To extend this observation to cells already infected with FeLV, we used direct analysis for FeLV-B infection with env-specific probe B/S. Infection with FeLV-B was detected by the appearance of a 3.7-kb KpnI FeLV-B/Gardner-Arnstein signature fragment superimposed on the hybridization pattern of enFeLV elements.

FeLV-B was harvested from BHK21S cells stably transfected with molecularly cloned FeLV-B/Gardner-Arnstein-(pFGB) (22). A range of cell lines was exposed (multiplicity of infection, 1), and the cells were cultured for 2 weeks. DNA was then extracted, and the presence of exogenous FeLV-B sequences was assessed by Southern blot hybridization with the B/S probe. After 2 weeks of propagation, only two of the cell lines showed the 3.7-kb signature fragment, denoting infection with FeLV-B/GA, i.e., AH927 fibroblasts, which are highly permissive for FeLV-B, and the Q201 helper T-cell line. The
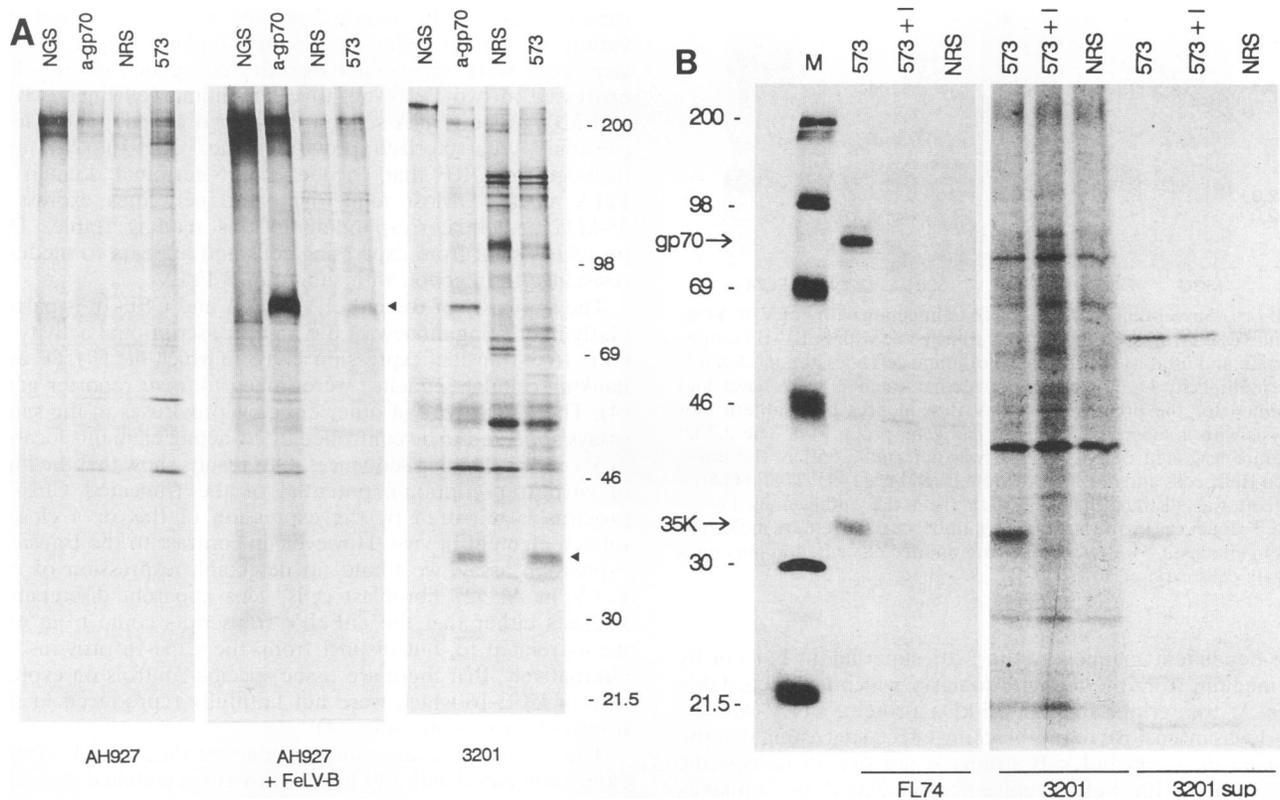
FIG. 5. (A) Expression of a candidate enFeLV *env* ORF product in feline lymphoma cell line 3201. Cells were metabolically labelled with [^{35}S]methionine and immunoprecipitated with the sera indicated above the lanes. The cells analyzed were feline fibroblast cells (AH927), either uninfected or infected with FeLV-B/Sarma, and FeLV-negative lymphoma cell line 3201. The sera used were a goat antiserum to FeLV gp70^{env} purified from the F422 cell line (kindly supplied by V. Moennig) and a rabbit antiserum (573) raised to a GST fusion protein carrying the C-terminal 30 amino acids of the MCC *env* ORF (this study). The controls used were normal goat serum (NGS) and preimmune serum from rabbit 573 (NRS). The 35-kDa candidate *env* ORF gene product is indicated by an arrowhead, as is the FeLV gp70^{env} protein detected in infected AH927 cells. Immune complexes were collected on protein A-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. (B) Specificity of 573 serum reactivity and evidence of shedding of the 35-kDa protein (35K) into the culture supernatant. Cells were metabolically labelled as for panel A and immunoprecipitated with 573 antiserum or preimmune serum (NRS). Further evidence of the antigenic specificity of 573 serum immunoprecipitation was provided by inhibition of the reaction by preincubating the serum aliquot (5 μl) with 50 μg of a GST-30-mer fusion protein (573 + I). In addition to the cell extracts from FeLV-positive lymphoma cells (FL74) and FeLV-negative 3201 cells (3201), culture supernatant was harvested from labelled 3201 cells after a 4-h chase with cold medium and analyzed in the same way (3201 sup). The numbers between the panels are molecular sizes in kilodaltons.

lack of superinfection of FL74 cells was not unexpected, since this line contains FeLV of all three subgroups (37). The exogenous FeLV-B component is revealed by a novel 5-kb signature fragment in the FL74 cell line (Fig. 6). However,

TABLE 1. Replication of FeLV isolates in 3201 lymphoma cells[a]

FeLV	FFU[b]/10⁶ cells at postinfection day:		
	0	7	15
None	<5	<5	<5
A/Glasgow-1(pFGA-5)	<5	86	1.2×10^3
B/Sarma	<5	<5	<5
B/Gardner-Arnstein(pFGB)	<5	<5	<5
B/Rickard (pFRB)	<5	<5	<5
B/Snyder-Theilen	<5	<5	<5
C/Sarma(pFSC)	<5	6.4×10^3	5.1×10^4
ABC/Kawakami-Theilen	<5	4.7×10^2	2.5×10^4

[a] 3201 cells were exposed to a series of FeLV isolates at a multiplicity of infection of 1 and cultured for 15 days. Aliquots of medium were titrated for FeLV infectivity on C81 S⁺ L⁻ cells at days 0, 7, and 15.
[b] FFU, focus-forming units.

F422 cells release only FeLV-A (26) and display a pattern of enFeLV sequences similar to that of the FeLV-free MCC and 3201 cell lines. The resistance of these three cell lines to FeLV-B is associated with expression of the 35-kDa protein.

In contrast to the resistant lines, we found no detectable expression of the 35-kDa enFeLV product in the Q201 cell line (data not shown). The susceptibility of Q201 cells to FeLV-B demonstrates that feline lymphoid cells may express receptors for FeLV-B and suggests further that resistance mediated by the 35-kDa protein is by blockade or down-regulation of these receptors.

Evidence that the 35-kDa protein is a soluble mediator of resistance to subgroup B FeLV. Since the 35-kDa protein is shed into culture supernatants from 3201 cells, we were interested in the possibility that the protein acts as a soluble mediator of interference to FeLV-B. To test this hypothesis, fibroblast cells (C81) permissive for all FeLV subgroups were infected with standard FeLV strains in the presence of culture supernatant from 3201 lymphoma cells or FEA fibroblasts at a range of dilutions (1/2 to 1/16). As can be seen from Table 2, infection with the two subgroup B isolates was inhibited in a
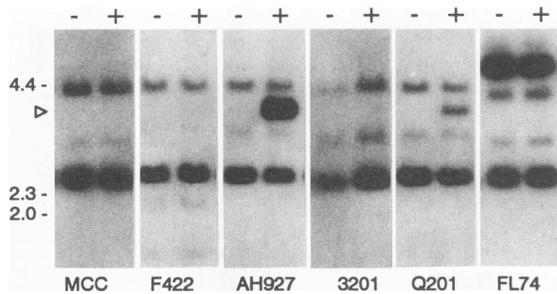
FIG. 6. Susceptibility of feline cells to infection with FeLV-B. Virus was harvested from BHK cells stably transfected with FeLV-B/Garner-Arnstein and used to infect a range of feline cell lines at a moderately high multiplicity (>1). Cell DNA was harvested 2 weeks later and examined for the presence of FeLV-B sequences by Southern blot analysis with a subgroup B-specific env gene probe (44). The 3.7-kb signature fragment of FeLV-B/GA was detectable only in the transfected BHK cells and in two of the feline cell lines (AH927 and Q201), where it was distinguishable by size from the endogenous FeLV-related sequences (arrowhead). The numbers on the left are molecular sizes in kilobases. Minus signs indicate uninfected cells, and plus signs indicate cells exposed to FeLV-B.

dose-dependent manner by the 3201 supernatant but not by the medium from the feline fibroblasts, which lack detectable enFeLV transcripts and the 35-kDa protein. FeLV-A/Glasgow-1 was insensitive to this blocking effect, suggesting that the blocking effect on FeLV-B strains is not due to nonspecific interference with FeLV replication mediated by cytokines released by the 3201 cells. These results suggest that the soluble env ORF product shed from 3201 cells is in an active conformation and is capable of binding receptors on nonexpressing cells.

DISCUSSION

In this study, specific probes were used to analyze the distribution and structure of enFeLV transcripts in feline tissues and cell lines. Truncated enFeLV proviral transcripts

TABLE 2. Inhibition of FeLV-B focus formation by spent medium from 3201 lymphoma cells[a]

Origin of medium and dilution	Mean no. of foci ± SE		
	FeLV-A (pFGA)	FeLV-B/GA (pFGB)	FeLV-B/ST
FEA			
1:2	60 ± 0[b]	15 ± 2	45 ± 3
1:4	49 ± 1	33 ± 9	51 ± 5
1:8	60 ± 0[b]	37 ± 6	60 ± 0[b]
1:16	47 ± 3	ND[c]	60 ± 0[b]
3201			
1:2	60 ± 0[b]	0 ± 1[d]	4 ± 1[d]
1:4	60 ± 0[b]	3 ± 2[d]	15 ± 8[d]
1:8	40 ± 4	29 ± 5	21 ± 0[d]
1:16	44 ± 10	33 ± 4	35 ± 4[d]

[a] FeLV at approximately 50 focus-forming units was suspended in spent medium diluted (1/2 to 1/16) in C81 cell growth medium and then applied to adherent C81 S⁺ L⁻ cells. The cell growth medium was changed after 1.5 h and foci were counted 10 days later. The results shown are based on at least three replicate assays.
[b] The maximal number of countable foci in this assay is 60.
[c] ND, not done.
[d] Different from value for FEA cell supernatant at $P < 0.05$ (t test).

were found to be abundantly expressed in lymphomas and in a variety of normal feline tissues of lymphoid origin. cDNA sequences were determined for the env genes of enFeLV expressed in two FeLV-negative lymphoma cell lines (MCC and 3201). The cDNA sequences showed a close match to a previously characterized proviral clone, CFE-16 (20), and included an ORF that encodes the N-terminal domain of FeLV gp70^{env}. Most feline lymphoid cell lines express a 35-kDa protein corresponding to this reading frame. The protein is shed from expressing cells and appears to mediate resistance to infection with subgroup B FeLV.

The similarity of the enFeLV cDNA and CFE-16 is particularly interesting in view of the high transcriptional activity of CFE-16 in transient expression assays in which the 5′ LTR and flanking cellular sequence were linked to a cat reporter gene (4). The low activity of other enFeLV proviruses in the same assays appeared to be controlled by cis-acting elements located in 5′ cellular flanking sequences. Our results show that the high in vitro transcriptional potential of the truncated CFE-16 provirus is mirrored by the expression of this or a closely related element in vivo. However, in contrast to the transient expression assay, we found no detectable expression of enFeLV in AH927 fibroblast cells. This apparent discrepancy suggests either that the enFeLV transcripts come from elements related to, but distinct from, the CFE-16 provirus or, alternatively, that there are tissue-specific controls on expression of CFE-16 which were not faithfully reproduced in the transient expression assays (4).

The 457-bp deletion in the env genes of the enFeLV cDNA clones compared with full-length proviruses matched precisely with the CFE-16 proviral clone, providing strong evidence that this deletion is a feature of the transcriptionally active proviruses rather than a result of posttranscription processing. It follows that the enFeLV proviruses normally expressed in feline lymphoid cells contain an insufficient coding sequence to be the endogenous viral parents of the three natural FeLV-B isolates sequenced to date. Moreover, there are several polymorphic amino acid positions within the enFeLV-derived domains of FeLV-B which are shared by the exogenous viruses but differ from the known enFeLV sequences. These characteristic differences may have arisen by convergent evolution of the nascent FeLV-B viruses; more likely, they are the conserved hallmarks of a common but unidentified enFeLV progenitor.

The enFeLV transcripts carrying domains I to IV are copackaged with FeLV-A, albeit inefficiently, providing ample opportunity for recombination. Why, then, are the highly transcribed enFeLVs not the major source of FeLV-B recombinants? The possible explanations include a requirement for enFeLV sequences distal to region IV or other constraints on the structure of exogenous FeLV which render simple FeLV-A × env ORF recombinants defective. In this respect, it is interesting that cotransfection of the CFE-6 proviral clone with replication-competent FeLV-C/Sarma(pFSC) gave rise to an infectious recombinant virus capable of growing on human HT1080 cells (a hallmark of FeLV-B), while CFE-16 did not (28). While the possibility that the highly transcribed enFeLV proviruses act as intermediates in a multistep recombinational process cannot be ruled out, it is clear that other enFeLV elements are involved in the genesis of FeLV-B recombinants. Since we have not detected expression of the major class enFeLV proviral elements which retain full-length env genes, the mechanism by which FeLV-B recombinants are generated in vivo remains obscure.

It is interesting that the single-amino-acid polymorphism that distinguishes the enFeLV cDNAs of MCC and 3201 cells

is within the region corresponding to the core of the neutral-ization epitope (MGPNL) common to all exogenous FeLV strains. If this site was also a target for neutralizing antibody in the ancient progenitor of enFeLV, the polymorphism may be the remnant of an immune selection process which was operative at the time of insertion of enFeLV into the feline germ line. However, this sequence occurs within the proline-rich "hinge" region of gp70 and there may be structural, as well as immunological, selection on this site, as shown by the emergence of novel mutations within the epitope in recombinant viruses derived in vitro after cotransfection with FeLV-A and CFE-6 proviral clones (38).

By using an antiserum raised to a 30-amino-acid C-terminal fragment of the MCC *env* ORF, we detected a 35-kDa protein corresponding to the presumptive *env* ORF product. All of the cells that expressed this protein were found to be resistant to infection with FeLV-B, while those that did not express the protein were permissive. These observations strongly suggest that the 35-kDa protein mediates resistance to infection, most likely by a receptor blockade mechanism. Support for the notion that a truncated *env* protein could bind to its receptor is provided by the observation that a deliberately truncated form of Friend MuLV gp70^{env} of very similar length to the 35-kDa *env* ORF (245 versus 235 amino acids for enFeLV) was expressed and interfered efficiently with exogenous virus infection, even when the protein was retained in the endoplasmic reticulum by linkage to a KDEL sequence (13).

Cats are highly resistant to infection with FeLV-B in the absence of FeLV-A as a helper virus (18, 19). It seems likely that enFeLV expression plays a role in this natural resistance phenomenon in a manner similar to murine retroviral resistance genes *Fv-4* (15) and *Rmcf* (12), although the enFeLV phenomenon differs in that resistance to the murine viruses involves endogenous expression of complete rather than truncated gp70 molecules (5, 15).

The susceptibility of the Q201 helper T-cell line to FeLV-B shows that enFeLV-mediated resistance of feline lymphoid cells is not ubiquitous. It is possible that the Q201 cell line, which does not express the 35-kDa protein, was derived from one of a subset of cats naturally susceptible to FeLV-B (19). Alternatively, the protein may be expressed differentially within the lymphoid compartment and absent from mature helper T cells. However, since the 35-kDa enFeLV *env* protein is shed from cells and appears to be capable of acting as a soluble mediator of resistance to subgroup B FeLV, it is conceivable that it confers a measure of resistance on nonexpressing cells in vivo. This novel possibility merits further investigation, as does the effect of constitutive expression of a related endogenous antigen on the feline immune response to exogenous FeLV.

ACKNOWLEDGMENTS

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