JOURNAL OF VIROLOGY, Sept. 1997, p. 6727–6741 Vol. 71, No. 9
0022-538X/97/$04.00+0

Characterization of the Genome of Feline Foamy Virus and Its Proteins Shows Distinct Features Different from Those of Primate Spumaviruses

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Received 19 February 1997/Accepted 1 June 1997

The genome of the feline foamy virus (FeFV) isolate FUV was characterized by molecular cloning and nucleotide sequence analysis of subgenomic proviral DNA. The overall genetic organization of FeFV and protein sequence comparisons of different FeFV genes with their counterparts from other known foamy viruses confirm that FeFV is a complex foamy virus. However, significant differences exist when FeFV is compared with primate foamy viruses. The FeFV Gag protein is smaller than that of the primate spumaviruses, mainly due to additional MA/CA sequences characteristic of the primate viruses only. Gag protein sequence motifs of the NC domain of primate foamy viruses assumed to be involved in genome encapsidation are not conserved in FeFV. FeFV Gag and Pol proteins were detected with monospecific antisera directed against Gag and Pol domains of the human foamy virus and with antisera from naturally infected cats. Proteolytic processing of the FeFV Gag precursor was incomplete, whereas more efficient proteolytic cleavage of the pre125$^{Pro\text{-}Pol}$ protein was observed. The active center of the FeFV protease contains a Gln that replaces an invariant Gly residue at this position in other retroviral proteases. Functional studies on FeFV gene expression directed by the promoter of the long terminal repeat showed that FeFV gene expression was strongly activated by the Bel1/Tas transactivator protein. The FeFV Bel1/Tas transactivator is about one-third smaller than its counterpart of primate spumaviruses. This difference is also reflected by a limited sequence similarity and only a moderate conservation of structural motifs of the different foamy virus transactivators analyzed.

Increasing attention has been directed to the study of foamy virus (FV) replication and gene expression (for reviews, see references 10, 38, 47, 53, and 66). Features of FVs not shared by other retroviruses include the presence of an internal promoter for expression of the regulatory and accessory *bel* genes required for virus replication and the expression of the Pol proteins by a spliced mRNA and not as part of a Gag-Pol fusion protein (4, 14, 29, 39, 41, 53, 66). Furthermore, the absence of the Cys-His motif in the FV nucleocapsid (NC) protein sequences is noteworthy (45). Primate FV *gag* sequences instead encode Gly/Arg (GR)-rich repeats that were implicated in the nuclear targeting of Gag proteins and in genome binding (58, 67). The proteolytic processing of human FV (HFV) and other primate FV Gag protein is incomplete, resulting in two predominant high-molecular-weight precursor forms (2, 19, 23, 32).

While the in vitro study of FVs has led to the understanding of the above characteristic features, very little is known about the pathogenicity and biology of these viruses during naturally occurring infection. FVs are generally considered apathogenic, although this was not experimentally studied in detail (43). For instance, spumaviruses have been considered to be copathogens for cats infected with feline immunodeficiency virus (FIV), but conflicting data have been reported (63, 68). The role of HFV as a copathogen was supported by the finding that the Bel1 transactivator of HFV is capable of transactivating human immunodeficiency virus type 1 long terminal repeat (LTR)-directed gene expression in transfected cells (30, 36). Furthermore, transgenic mice carrying certain HFV proviral clones showed neurological symptoms with a well-defined pattern of gene expression and tissue damage predominantly in certain areas of the brain (1).

Feline FV (FeFV) was repeatedly isolated from naturally infected cats that were apparently healthy or suffered from diverse diseases (16, 22). Conflicting data were reported on whether the presence of FeFV is correlated with the infection with either FIV or feline leukemia virus or any other feline pathogen.

Current research on the molecular biology of FVs has been limited by the lack of a complete genomic sequence of a nonprimate FV, and for this reason, the phylogenetically distant FeFV was chosen for a detailed molecular biological characterization. As a first step to understanding the regulation and replication of FeFV in its natural host, the complete FeFV genome, its genomic organization, and the expression of FeFV proteins were investigated and compared with those of primate FVs (17, 26, 35, 45, 48, 51). A feline spumavirus has the added advantage of making possible analysis of FeFV replication in vivo.

MATERIALS AND METHODS

Cells and virus. The cultivation of Crandell feline kidney (CRFK) cells and the propagation of the FeFV isolate FUV were performed as described previously (16). Different FeFV field isolates were derived directly from domestic cats by cocultivating lymphocytes from FeFV-infected cats with permissive CRFK cells. Oropharyngeal swabs and blood samples were obtained from cats which had

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been put to death at the Animal Welfare Refuge, Adelaide, Australia. Cat blood from naturally FeFV-infected cats was also obtained from the small animal clinics of the Hannover Veterinary School, Hannover, Germany.

DNA and protein extraction and immunoblotting. Low-molecular-weight DNA was prepared by the method of Hirt (27). Total DNA from cultured cells was prepared with the QIAamp Tissue DNA extraction kit as specified by the manufacturer (Qiagen, Hilden, Germany). DNAs from FeFV-infected cats were harvested from peripheral blood lymphocytes with the QIAamp Blood DNA extraction kit (Qiagen). FeFV antigen from infected CRFK cells was harvested 4 to 6 days postinfection (p.i.). The preparation of cell-associated antigen, immunoblotting, and the sera used were described previously (31, 32, 39, 40, 42, 44, 49). In particular, the HFV CA/NC antiserum is directed against HFV Gag residues 388 to 636 and may therefore be specific for capsid (CA) and NC epitopes. The HFV RNase serum was raised against residues 676 to 765 of HFV Pro-Pol protein, including peptide sequences from the end of the reverse transcriptase (RT).

Construction of recombinant FeFV DNA clones. Molecular cloning was performed by standard techniques (57). DNA extracted from FeFV-infected cells by the method of Hirt (27) was digested with *Eco*RI and cloned into *Eco*RI-digested plasmid pBluescript KSII (Stratagene, Heidelberg, Germany) that had been dephosphorylated with shrimp alkaline phosphatase (Amersham-Buchler, Braunschweig, Germany). Recombinant bacteria derived upon transformation with FeFV Hirt DNA were transferred to nitrocellulose filters (BA85; Schleicher & Schüll, Dassel, Germany) and hybridized with an HFV provirus DNA probe. The probe corresponded to pHSRV13 nucleotides (nt) 3202 to 3870 that was obtained by *Pvu*II-digestion of pHSRV13 DNA (40). The isolated DNA fragment was randomly labelled with [α-^{32}P]dCTP (random-primed kit [Amersham-Buchler]). Hybridization was performed at 55°C overnight, nonspecifically bound radioactivity was removed by washing twice with low-stringency buffer (0.5% bovine serum albumin, 1 mM EDTA, 40 mM Na(Pi) [pH 7.2], 1% sodium dodecyl sulfate) at 55°C followed by washing twice in high-stringency buffer (0.5% bovine serum albumin, 1 mM EDTA, 40 mM Na(Pi), [pH 7.2], 5% sodium dodecyl sulfate) at 55°C. Positive colonies were identified by autoradiography. To obtain long FeFV-specific PCR amplification products, the PCR Expand kit (Boehringer, Mannheim, Germany) for long and high-fidelity PCR amplification was used as specified by the manufacturer (4). Total DNA from FeFV-infected CRFK cells was used as the template for PCR amplifications with sense primer Jps (5′-TTTGCTCAGTGGGCAAAGGAAAGGAATATACAATTGG-3′) (see Fig. 1A) and antisense primer Jla (5′-TTGACACTGATTTATATGGCA CAATAATTTCTCTC-3′) to amplify FeFV sequences from the end of the *pol* gene to the start of the 3′ LTR. For cloning the 3′ half of the FeFV genome, the PCR amplification product with primers Jps and Jla was digested with *Eco*RI. The generated fragments of about 3.1, 0.9, and 0.55 kbp were cloned into the *Eco*RI-digested pUC18 vector (New England Biolabs, Schwalbach, Germany) as described above. The sense primer F8636s (5′-GTGGAAATGGAACTGGTT CAGACTGCC-3′) and the antisense primer F11700a (5′-GCCATCGATGTCG GTGCCTATACCTGGGATAG-3′) were used to construct a FeFV DNA clone from FeFV nt 8636 to the end of the 3′ LTR. The Expand PCR system and total DNA from FeFV-infected cells were used. Terminal A residues were added to the ends of the blunt PCR fragment with *Taq* polymerase (Renner, Dannstadt, Germany). After the A addition, the ca. 3.0-kbp FeFV DNA was cloned into plasmid pCR2.1 (Invitrogen, Leek, The Netherlands).

PCR primers 2620s (5′-CTCTGATGTTCCCGAACAGAGAGA-3′) and 3039a (5′-GAAGCAAGTCCTTTGGAACACAGG-3′) span the FeFV *gag-pol* overlap region and were used with DNA samples from FeFV-infected cell cultures or feline lymphocytes. The reactions were performed with *Taq* polymerase for 35 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C, followed by a final step of 10 min at 72°C.

Analysis of FeFV proviral DNA clones and sequence comparisons. Both strands of the FeFV DNA inserts were automatically sequenced with a sequencing apparatus (LI-COR, Lincoln, Neb.). For determination of DNA and protein sequence homologies and for database searches, diverse programs of the Heidelberg Unix Sequence Analysis Resource (HUSAR) and the EMBLpredict secondary-structure determination program (56) were used. The accession numbers for the different FV genomes are as follows: HFV, U21247; SFV-1, EMBL X54482; SFV-3, GenBank M74895SFV; SFVcpz, GenBank U04327.

Southern blotting. Total DNA from CRFK cells was extracted 4 days after FeFV and mock infection, and 10 μg each was digested with *Bam*HI, *Hin*dIII, *Pst*I, and *Xba*I overnight under standard conditions. Digested and undigested DNA was separated on an 0.8% agarose gel, partially fragmented by acid hydrolysis in 0.25 M HCl for 30 min, and transferred onto Hybond-N+ membranes as recommended by the manufacturer (Amersham-Buchler). The immobilized DNA was probed with an FeFV-specific DNA probe prepared by PCR amplification of cloned FeFV DNA in the presence of [α-^{32}P]dCTP (Amersham-Buchler), using the sense primer Fgag-s (5′-TCATATGGCTCGAGAATTA AATCCTC-3′) and primer p-4 located in the 5′ end of FeFV *pol* (4). DNA hybridization and washing with high-stringency buffer were performed as recommended (Amersham-Buchler).

Construction of FeFV LTR reporter plasmids and FeFV Bel1 expression clones. FeFV LTR reporter plasmids directing the expression of the firefly luciferase (*luc*) gene were constructed by using the promoterless reporter construct pGL2-bas (Promega, Heidelberg, Germany). FeFV inserts were obtained by digesting plasmid 15 (containing FeFV nt 17 to 5811 [see Fig. 1A]) with either *Ecl*136II (cleavage at FeFV nt 1073 at the end of the U3 region), *Mun*I (FeFV nt 1208 at the end of the R region), and *Ehe*I (FeFV nt 1359 in the primer binding site [PBS] downstream of the 5′ LTR). The *Mun*I restriction site was blunt ended with Klenow enzyme. Then the different DNAs were digested with *Kpn*I (which cleaves in the vector backbone) and the isolated FeFV DNA fragments were inserted into the pGL2-bas vector, which was first digested with *Hin*dIII, then blunt-ended with the Klenow enzyme, and then digested with *Kpn*I. The resulting clones contained the FeFV LTR fragment in the sense orientation relative to the *luc* gene and were designated pFeFV-U3, pFeFV-U3R, and pFeFV-LTR according to the inserted FeFV DNA fragments. Plasmids pHFV-LTR and pHFV-IP containing the HFV LTR and internal promoter upstream of the *luc* gene have been described recently (64).

The FeFV *bel1* gene was amplified with *Pfu* polymerase (Stratagene), using primers Fbel1s (5′-GC**ACTAGT**ATGGCTTCAAAATACCCGGAAGAAGG-3′; the introduced *Spe*I restriction site is in boldface type) and Fbel1a (5′-GCCATCGATTTGTACCAGGCCTATTCCTGG-3′) as specified by the manufacturer. FeFV PCR clone 6 (FeFV nt 8636 to 11698) was used as the template. The PCR product of about 640 nt was purified, digested with *Spe*I, and inserted into the eukaryotic expression vector pBK-CMV (Stratagene) digested with *Spe*I and *Sma*I. The identity and integrity of the *bel1* gene were confirmed by restriction mapping and DNA sequencing. The corresponding DNA clone was designated pFeFV-Bel1.

DNA transfection and expression assays. Transfections by electroporation of 10 μg of plasmid DNA into CRFK or BHK-21 cells were performed at 975 μF and 160 V in 2-mm electroporation cuvettes as described previously (40, 41). Plasmid pCMVβgal directing the expression of β-galactosidase (β-gal) from the cytomegalovirus (CMV) immediate-early (IE) promoter (41) was used for normalization of transfection efficiency. *luc* reporter gene assays were performed and quantitated as described previously (64) with a Lumat LB 9501 luminometer (Berthold, Wildbad, Germany).

RESULTS

Molecular cloning and sequence determination of the FeFV provirus genome. To clone FeFV proviral DNA, subconfluent CRFK cells were infected with FeFV isolate FUV (16). Syncytium formation started 2 days p.i. and progressed upon further incubation. About 4 days p.i., low-molecular-weight DNA was prepared, digested with *Eco*RI, and ligated into the *Eco*RI-digested and dephosphorylated vector pBluescript KSII. FeFV-specific recombinant clones were identified with an HFV-specific DNA hybridization probe derived from the 5′ end of the HFV *pol* gene. DNA inserts from clones with positive hybridization signals were mapped by restriction digestion and partially sequenced with primers located on either side of the cloning site. Six independent recombinant clones contained FeFV-specific DNA inserts; clones 21 and 23 contained FeFV sequences from nt 17 to nt 3841; and clones 7, 8, 11, and 15 contained sequences from nt 17 to 5811 (Fig. 1B). The larger clones contained the intact *Eco*RI site at position 3841. Clones 7 and 15, which represent both orientations of the insert, were completely sequenced. Both clones contained most of the FeFV 5′ LTR, the complete *gag* gene, and part of *pol* (Fig. 1). Clone 7 and 15 were identical except that clone 15 contained an *Eco*RI-*Eco*RI insert of unknown origin exactly upstream of the FeFV DNA insert.

Based on these sequencing data, PCR amplification experiments were performed to obtain the remainder of the *pol*, *env*, *bel*, and 3′ LTR sequences. The sense primer Jps is located in the *pol* gene upstream of the terminal *Eco*RI site of plasmids 7 and 15, whereas the antisense primer Jla is located about 100 nt downstream of the *Eco*RI site in the U3 part of the LTR (Fig. 1A). The PCR Expand system for high-fidelity amplification of long DNA fragments was used. Reactions with total DNA from FeFV-infected cells resulted in a prominent band of about 4.7 kbp. Repeated attempts to clone this DNA into different vectors did not result in any stable recombinant clone. To clone fragments of the 4.7-kbp DNA individually, the PCR product was purified and digested with *Eco*RI (Fig. 1B). FeFV DNA bands of approximately 3.2, 0.9, and 0.55 kbp obtained upon digestion of the 4.7-kbp PCR product were cloned into

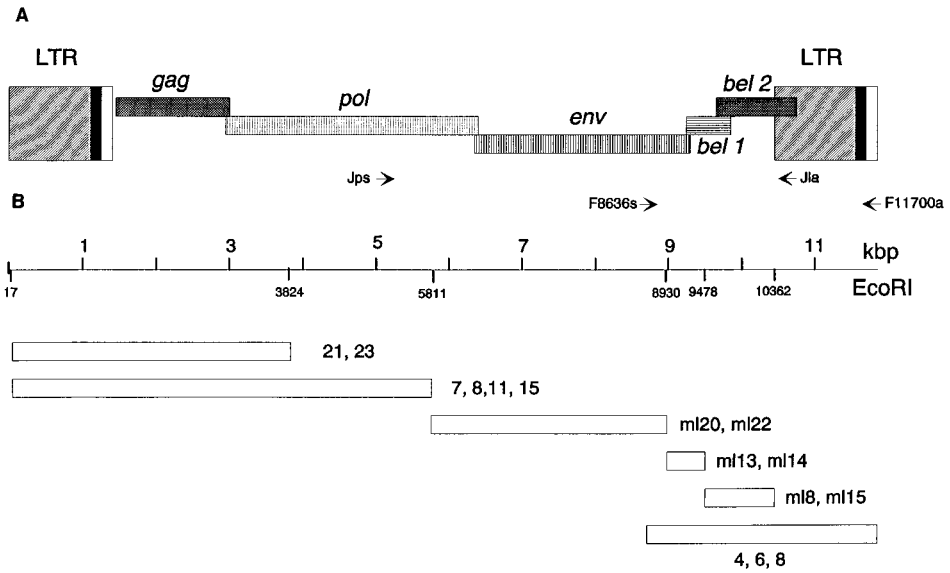

FIG. 1. Schematic presentation of the genomic organisation of the FeFV provirus (A) and cloning strategy (B). (A) To-scale presentation of the FeFV provirus with the LTRs divided into the U3, R, and U5 regions (hatched, solid, and open boxes, respectively); the structural *gag*, *pol*, and *env* genes; and the nonstructural *bel1* and *bel2* genes. The locations, orientations, and names of PCR primers used for cloning are shown below the map. The scale indicates the size in kilobase pairs (top) and the nucleotide positions (bottom) of the *Eco*RI restriction sites. (B) Approximate sizes and locations of FeFV DNA clones obtained either by cloning low-molecular-weight DNA from FeFV-infected cells (no. 7, 8, 11, 15, 21, and 23) or from PCR products with the PCR primers indicated. Clones ml8, ml13, ml14, ml15, ml20, and ml22 were constructed by cloning the *Eco*RI DNA fragments of the amplicon obtained with the primers Jps and Jla. Clones 4, 6, and 8 were obtained by direct cloning of PCR DNA with primers F8636s and F11700a.

the *Eco*RI site of plasmid pUC18. Recombinant clones were characterized by restriction enzyme digestion and DNA sequencing (Fig. 1B). The 3.1-kbp FeFV DNA fragment extended from the end of *pol* to the end of *env*. The 550-bp insert contained the remainder of *env* and extended into the *bel1* gene. The 880-bp FeFV DNA fragment contained the remainder of the *bel* genes with the polypurine tract (PPT) and the 5′-terminal 17 bp from the U3 region of the 3′ LTR.

To independently obtain the complete 3′ LTR sequence, PCRs with the PCR Expand system were performed with primers F8636s located in the carboxy-terminal region of *env* and F11700a complementary to the 3′ end of the U5 part of the 3′ LTR (Fig. 1A). A DNA product of 2.6 kbp was cloned into pCR2.1 after terminal addition of a single A residue by *Taq* DNA polymerase. The sequence of three independent clones confirmed the recently published nucleotide sequence of FeFV LTR (Fig. 1B). Furthermore, the sequence of the overlapping clones was verified (Fig. 1B). Based on the size of the fragments and the nucleotide sequence of these clones and by alignment with FV genomic sequences, we conclude that the clones described represent the complete FeFV DNA sequence.

The FeFV proviral DNA sequence from the PBS to the 3′ end of the 3′ LTR and the deduced amino acid sequences of the *gag*, *pol*, *env*, and *bel* genes are shown in Fig. 2. Landmarks of the FeFV genome are shown within the sequence. The nucleotide sequence of the 5′ LTR published previously is in complete agreement with the data (4).

Southern blot analysis of FeFV proviral DNA. Southern blotting to confirm that the cloned FeFV DNA was from an exogeneous virus and was not present in uninfected CRFK cells was performed. DNA from CRFK cells harvested 4 days after FeFV and mock infection was used. DNA from FeFV-infected cells was digested with *Bam*HI, *Hin*dIII, *Pst*I, and *Xba*I, which are predicted to cleave FeFV proviral DNA as shown in the upper part of Fig. 3. The DNAs were electrophoretically separated, transferred onto a nylon membrane, and hybridized with a DNA probe obtained by PCR amplification of cloned FeFV DNA in the presence of radioactively labeled dCTP. The probe spanned FeFV nt 1442 to 3107, as schematically shown in Fig. 3. Whereas no specific hybridization signal was obtained with DNA from mock-infected CRFK cells, the sizes of specifically detected restriction fragments from FeFV-infected cells corresponded fully to the predictions from the sequencing data. As expected, *Xba*I digestion resulted in a band of 2.4 kbp. The specifically detected FeFV restriction fragments for the other enzymes were as follows: *Bam*HI, a major band of about 6.7 kbp (a higher-molecular-weight band corresponds to partial digestion); *Hin*dIII, 9.7 and 0.67 kbp; *Pst*I, 6.8 kbp (faint) and 2.2 kbp. These results fully agree with the sequencing data and confirm the data that the infectivity of FeFV provirus DNA is, e.g., sensitive toward digestion with *Hin*dIII and *Xba*I (7).

Analysis of FeFV-specific proteins by immunoblotting. Immunoblotting was performed to identify and characterize FeFV proteins from infected CRFK with antisera directed against defined domains of HFV proteins and sera from FeFV-infected cats.

A clear reactivity of an antiserum directed against HFV Gag residues 388 to 636, designated HFV CA/NC, was obtained with protein samples from FeFV-infected cells (Fig. 4A). Two protein bands of 52 Da and 48 kDa were specifically detected in extracts from FeFV-infected cells (Fig. 4A, lanes 3 and 6). By analogy to HFV with predominant Gag-specific bands of 74 and 70 kDa (lanes 1 and 2), these proteins probably correspond to the unprocessed 52-kDa and the carboxy-terminally processed 48-kDa FeFV Gag proteins (32, 39). Additional processing forms of the FeFV Gag protein were not detectable under the conditions used; however, nonspecific reactivity with proteins from mock-infected CRFK cells (lane 7) or infected cells was obtained. To confirm that the HFV CA/NC antiserum detected FeFV Gag proteins, the experiment was repeated

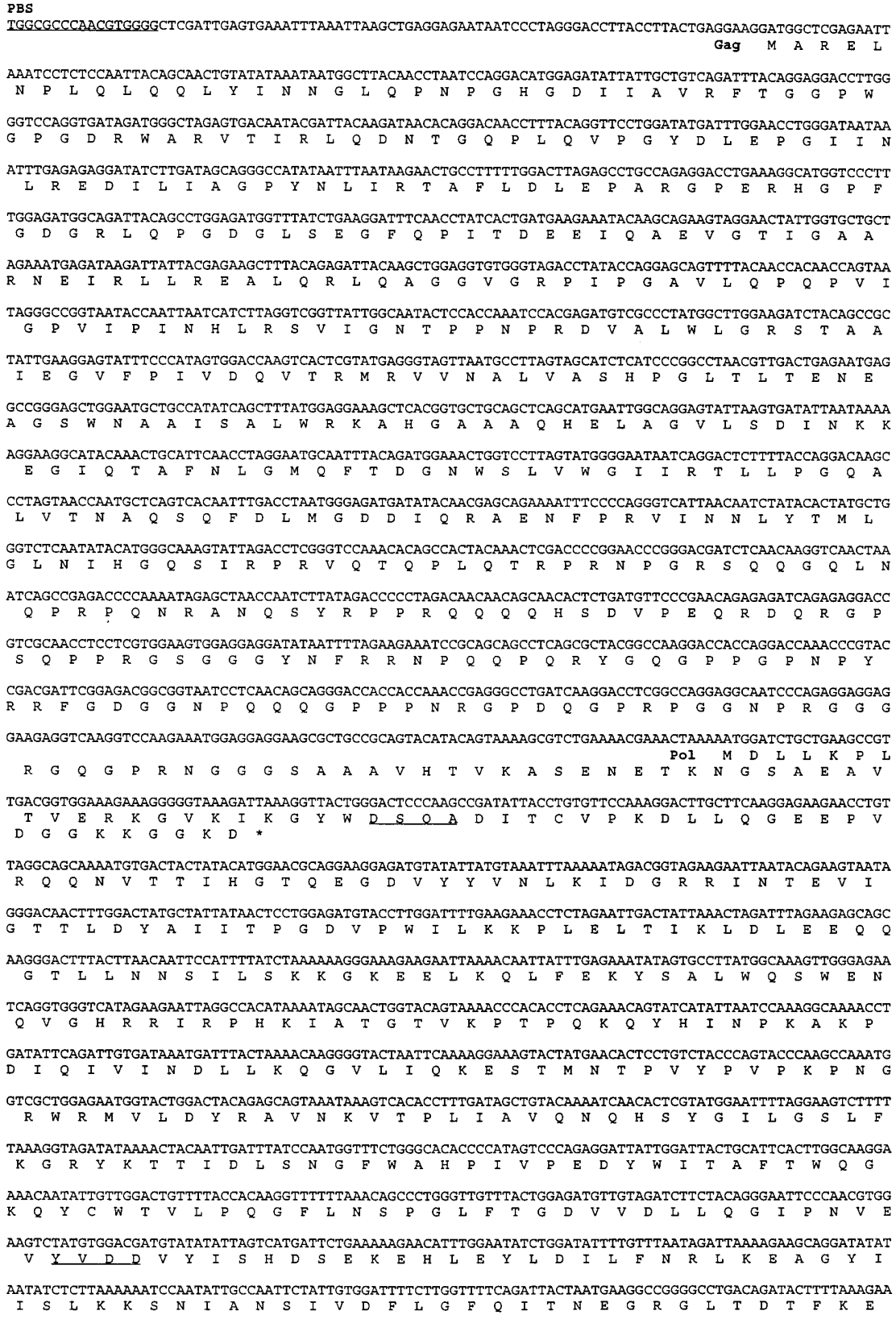

FIG. 2. FeFV provirus DNA sequence from the PBS directly downstream of the 5′ LTR to the end of the 3′ LTR and the deduced amino acid sequence in the single-letter code of the FeFV Gag, Pro-Pol, Env, Bel1, and Bel2 proteins. Hallmarks of the FeFV genome and proteins are marked (above the nucleotide sequence and below the deduced amino acid sequences, respectively). Residues in Pol conserved among FVs, corresponding to critical residues in Pro, RT, RNase H, and IN, are underlined. The conserved PPT at the end of *pol* and *env* are underlined. Arrowheads in Env mark postulated processing sites for the removal of the leader peptide, and the open arrow marks the cleavage site between the FeFV SU and TM domains. The membrane anchor of Env is underlined. The TATA box of the internal promoter is in boldface type, and the cap site is marked by a vertical arrow. The double Lys motif for type II membrane proteins proximal to the carboxy terminus of Env is in boldface type.

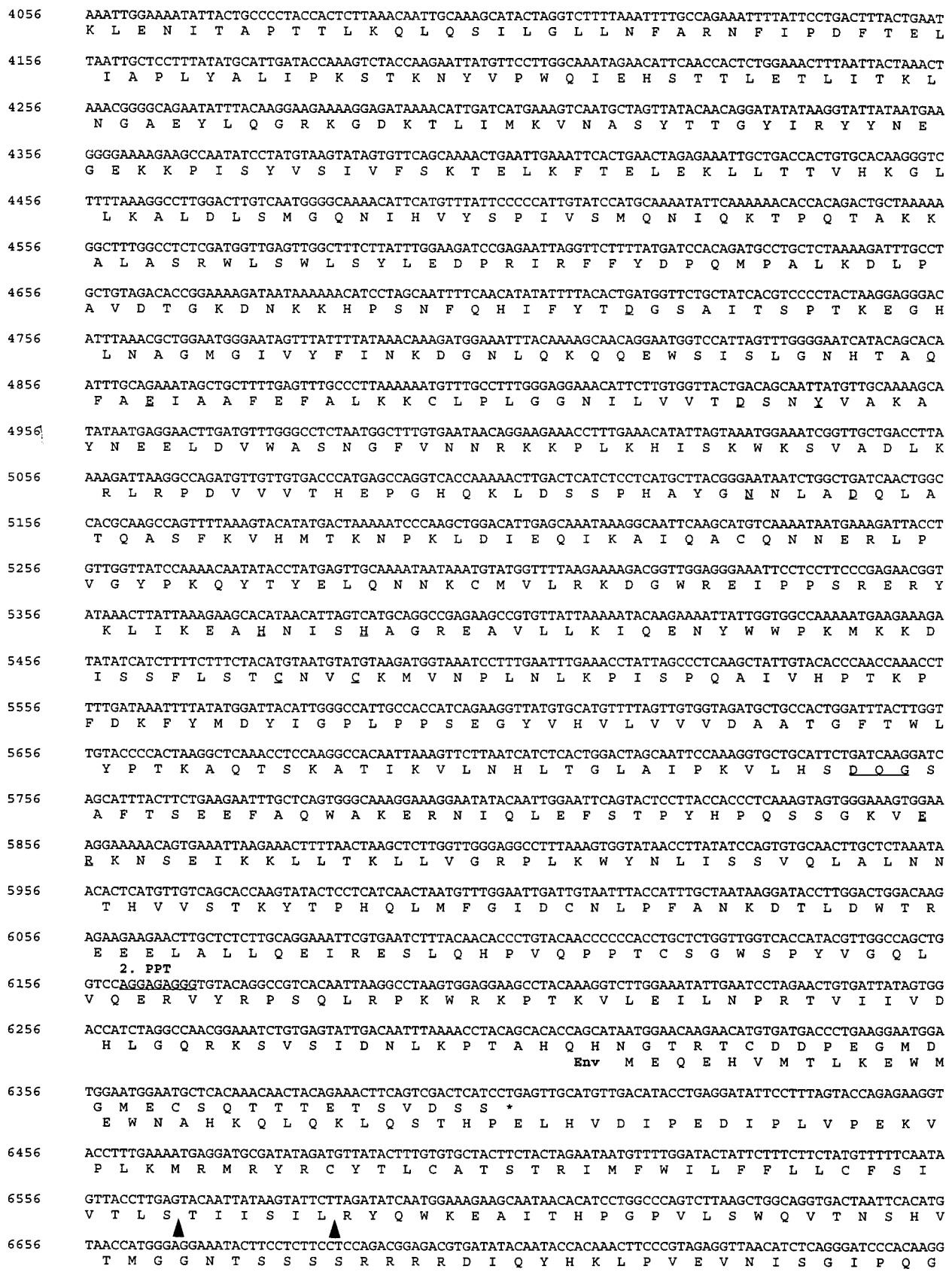

FIG. 2—*Continued.*

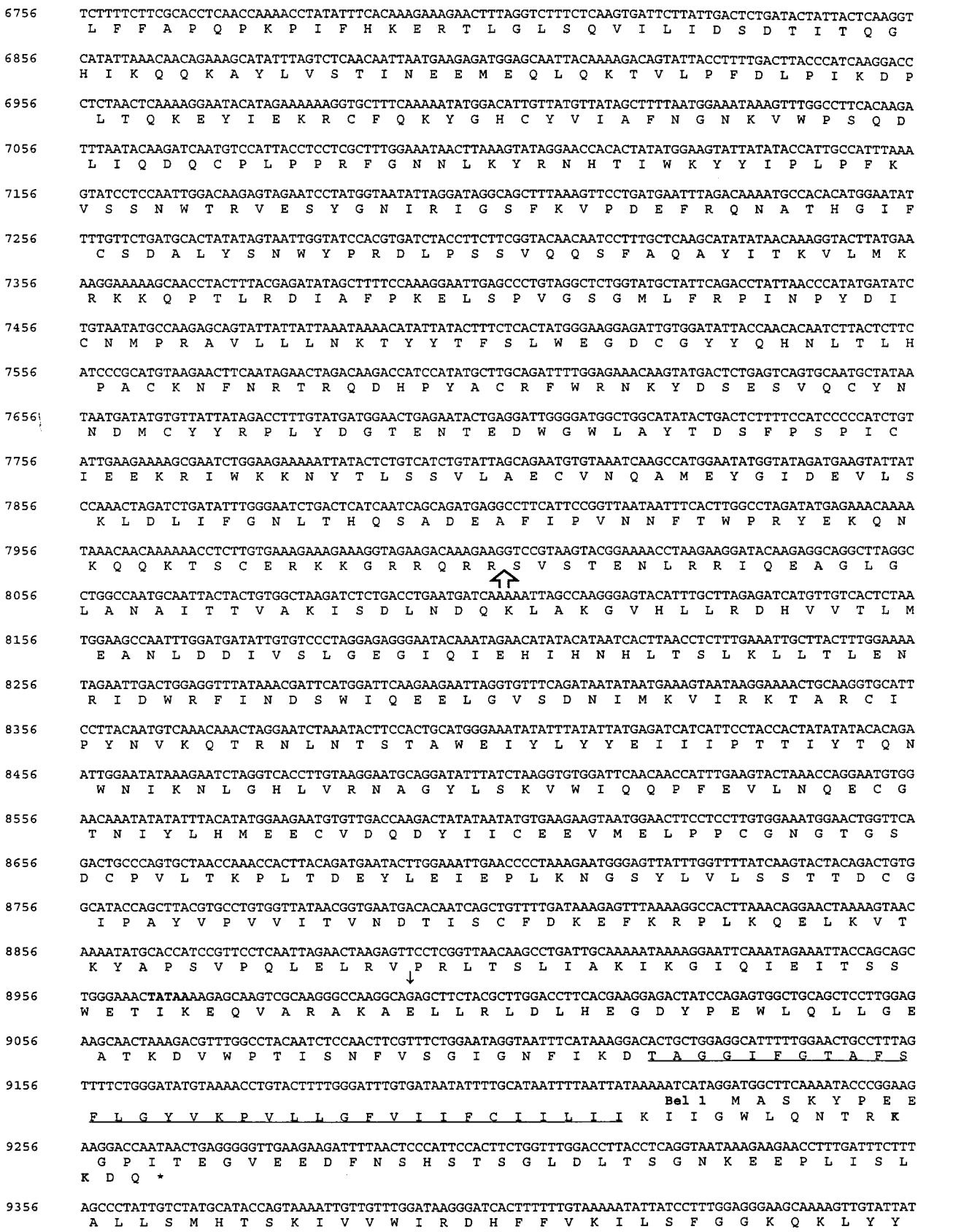

FIG. 2—*Continued.*

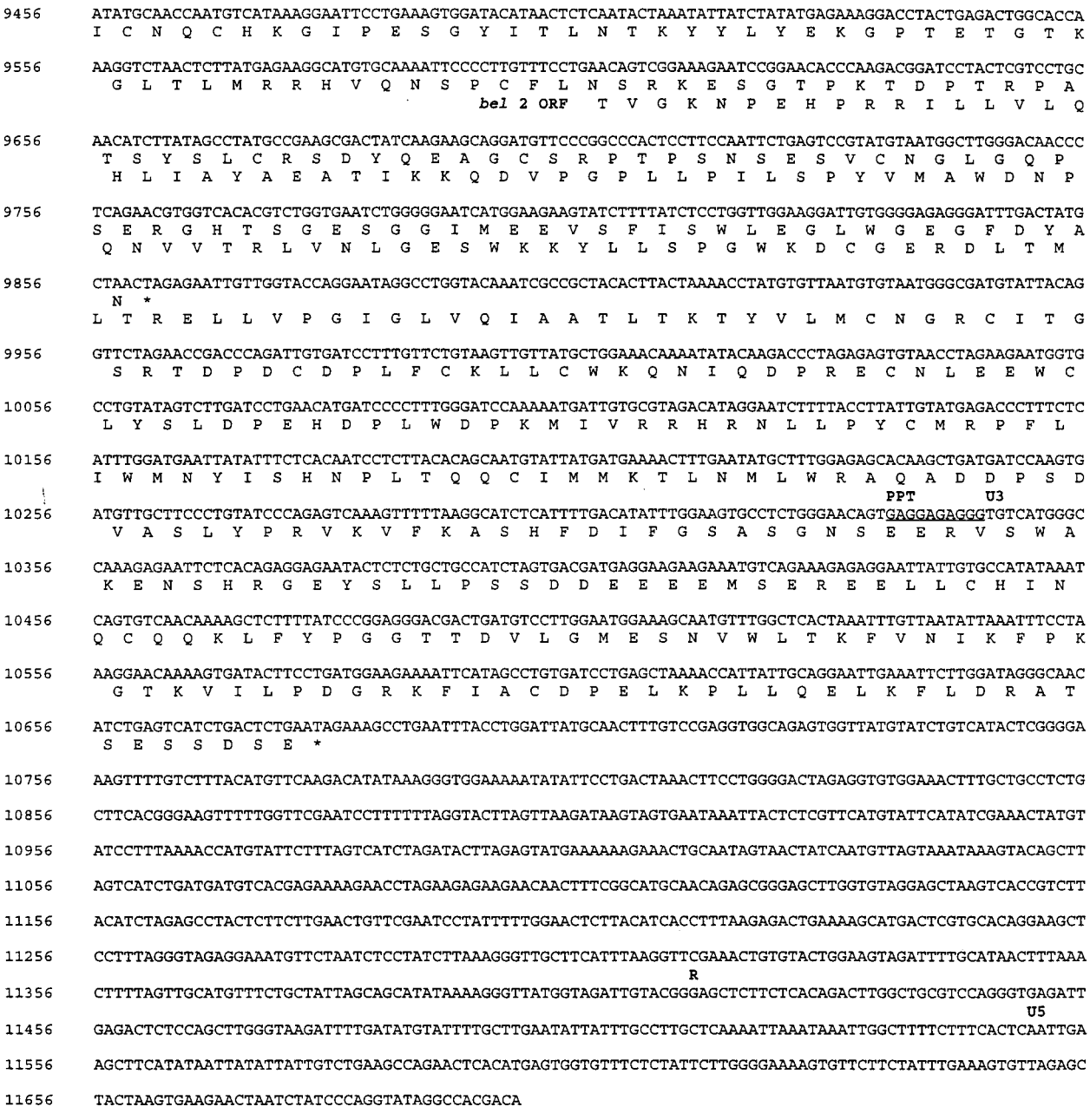

FIG. 2—*Continued.*

with an antiserum from a naturally FeFV-infected cat (Fig. 4B). This feline serum specifically reacted with the 52- and 48-kDa proteins also detected with the HFV CA/NC antiserum, confirming that they correspond to the FeFV Gag proteins. Furthermore, bands in the range of 43 kDa were specifically detected, possibly corresponding to FeFV Bet or further processing products of Gag or Env. The assumption that one of the 43-kDa proteins detected with the feline serum represents the FeFV Bet protein is consistent with our preliminary data on FeFV transcripts and the notion that Bel1/Bet-specific antibodies are reproducibly found in FV-infected primates (23). Obviously, *pol*-specific antigens were not detected by the feline antiserum used.

An HFV matrix (MA)-specific antiserum did not react with corresponding proteins in FeFV-infected cells. The HFV CA antiserum resulted in a very faint staining of proteins of about 52 to 48 kDa in extracts from FeFV-infected cells (data not shown).

As anticipated from the initial detection of FeFV proviral DNA with a DNA probe from the HFV *pol* gene (see above), an antiserum directed against the RNase H domain of HFV reacted specifically with proteins of about 125 and 80 kDa present in FeFV-infected CRFK cells (Fig. 4C, lane 3). These FeFV *pol*-specific proteins comigrated with the pre125$^{Pro\text{-}Pol}$ and the 80 kDa RT/RNase H proteins from HFV-infected cells (lane 1), thus corresponding to the FeFV Pro-Pol precursor and the RT/RNase H. A *pol*-specific protein of about 190 kDa representing an FeFV Gag-Pol fusion protein was not detect-

able. A reaction with FeFV *pol*-specific proteins was also obtained with an HFV integrase (IN)-specific antiserum that reacted with proteins of about 125 and 42 kDa representing the FeFV Pro-Pol precursor and IN, respectively (data not shown). An HFV protease (Pro)-specific antiserum did not cross-react with proteins from FeFV-infected CRFK cells.

Sera directed against HFV Bel1-, Bel1/Bet-, and Bel 2/Bet-specific domains did not cross-react with FeFV proteins (data not shown). These data are consistent with a low sequence homology of the *bel* sequences of HFV and FeFV, as discussed below. An antiserum directed against the HFV SU and TM part of the Env protein did not show any reactivity with FeFV Env proteins.

FeFV Gag and 5′-untranslated region. By analogy to other known FVs, the PBS of FeFV (Fig. 2) is complementary to the 3′ end of the eukaryotic $tRNA^{Lys}_{1,2}$. The putative Met start codon of the FeFV *gag* gene is at nt 1442, which corresponds to nt 372 of the unspliced *gag*/genomic mRNA. This Met residue is the third codon of the *gag* reading frame and is in a consensus sequence context for efficient translational initiation (33). The presence of a long untranslated leader of *gag* mRNA is common to the other known FVs and may contain regulatory signals for Gag expression and genome encapsidation and dimerization.

The predicted FeFV Gag protein consists of 514 residues corresponding in size to the 52-kDa Gag protein detected in immunoblots. The FeFV Gag is about 130 residues smaller than that of the primate FVs but is similar to the bovine FV (BFV) Gag of 544. This is evident from the binary- and multiple-sequence comparison of the FeFV, BFV, HFV, and simian FV type 1 (SFV-1) Gag proteins (Fig. 5). HFV and SFV-1 Gag sequences used for the alignments are prototypic for other primate FVs, since they share extensive genetic conservation (38, 47, 48).

The amino-terminal MA domain of FV Gag proteins shows a cluster of conserved residues among the aligned sequences:

HFV:	**Gly**Trp**TrpGly**GlnIleGlu**Arg**PheGlnMetValArgLeuIle**LeuGlnAsp**
BFV:	**Gly**Pro**TrpGly**IleGlyAsp**Arg**TyrAsnLeuIleArgIleHis**LeuGlnAsp**
FeFV:	**Gly**Pro**TrpGly**ProGlyAsp**Arg**TrpAlaArgValThrIleArg**LeuGlnAsp**
Consensus:	**Gly** X **TrpGly** X X X **Arg** X X X X X X X **LeuGlnAsp**

This peptide motif, where X represents any residue, is reminiscent of the dominant morphogenetic signal of the type D retrovirus Mason-Pfizer monkey virus, which was shown to be responsible for the cytoplasmatic preassembly site of this type of retrovirus (54).

It is noteworthy that the primate FV Gag sequences between residues 130 and 300 do not show obvious sequence homology to either the FeFV or BFV Gag proteins. In addition, the domain of about 170 residues of primate FV Gag proteins is only about 40 residues in FeFV and 65 residues in BFV (Fig. 5).

Downstream of this primate FV-characteristic domain, distinct residues are conserved among FeFV, BFV, SFV-1, and HFV over a stretch of 170 amino acid residues. However, an amino acid sequence resembling the major homology region of other retroviruses is not present (10).

The remaining carboxy-terminal part of the FV Gag proteins, which corresponds to the NC domain of other retroviruses, is only marginally conserved among primate and nonprimate spumaviruses. This is reflected by several gaps that were introduced into the sequence alignments. It is remarkable that the alignments showed a highly conserved stretch of 6 residues in this region. This motif is part of the so-called GR box II (Fig. 5, HFV Gag residues 535 to 557), which was identified upon alignment of the primate FV Gag sequences and is responsible for nuclear localization (58). However, a new motif completely conserved in the known FV NCs is Gln-Pro-Gln-Arg-Tyr-Gly (Fig. 5). The primate FV GR boxes I and III (Fig. 5, HFV Gag residues 485 to 512 and 585 to 618 [58]) were not identified in homology searches performed with the primate and nonprimate sequences except for the sequence Gly-Arg-Gly, located in the center of GR box III (Fig. 5). Although the exact location of the Gly and Arg residues is not conserved among the known FVs, the NC domain of each known FV Gag contains not only many Arg and Gly residues but also more than the average number of Pro and Gln residues.

It is remarkable that the net number of positively charged amino acid residues is invariant between the NC domains for FeFV and BFV. This is reminiscent of the Semliki Forest virus NC protein, whose amino-terminal domain is responsible for interaction with viral RNA and virus assembly (18).

We postulate that the $pre52^{Gag}$ of FeFV is cleaved by the FeFV protease at a distinct site. The most likely cleavage site is marked by a vertical arrow in Fig. 5. Close examination of different alignments with FV Gag proteins shows that peptides of 26 to 31 residues would be released depending on the FV. The p4 peptides are of the correct size to explain the difference between the $pre74^{Gag}$ and the resulting $pre70^{Gag}$ (19, 32). Mass-spectrometric analyses of synthetic peptides flanking the presumptive Gag p74-p70 cleavage site after treatment with a recombinant HFV protease confirmed that proteolytic processing occurred close to the COOH terminus (50).

FeFV Pol. The FeFV *pol* gene is expressed from a spliced transcript as reported in a previous study (4). By analogy to HFV, the translational start site of Pol is the first Met codon of the Pol-specific FeFV mRNA. The location of this Met is conserved among the known FVs. FeFV *pol* contains the Pro, RT, RNase H, and IN domains.

The amino acid sequence of the active center of the Pro of FeFV isolate FUV was repeatedly sequenced from different DNA clones, and instead of Asp-Ser-Gly-Ala, the sequence was consistently found to be Asp-Ser-**Gln**-Ala. To date, the glutamine residue (shown in boldface type) has not been observed at this position in any wild-type retroviral Pro. Thus, in the FeFV catalytic center of the FeFV Pro, the apparently canonical Gly was replaced by a Gln residue.

Using the Bestfit sequence comparison program, the complete FeFV Pol sequence shows a degree of about 60% identical residues compared to primate FV and BFV Pol proteins. When the different domains of Pro-Pol are compared, the homology of the FeFV Pro domain to that of the primate FVs is 42 to 46% and the homology to BFV is 51%. In FeFV RT, 66 to 67% of the residues are identical compared to the other FVs whereas the RNase H domain has a homology of 51 to 54%. The homology of the FeFV IN to other FV IN proteins is 61 to 64%. In summary, the whole FeFV *pol* gene shows the protein motifs and features known and characterized in all retroviral *pol* gene products (Fig. 2), except for the unusual catalytic center of Pro.

FeFV Env. The FeFV Env protein is similar in size and domain structure to that of the other FVs. The FeFV surface (SU) protein has a degree of identity of 37 to 40% and the FeFV transmembrane (TM) protein is 49 to 53% identical to the corresponding domains of other known FVs. The high degree of homology of FV Env proteins even across host species and family borders contrasts remarkably with the situation in lentiviruses with a very high genetic diversity, especially of Env, but is also found in human T-cell leukemia viruses, Rous sarcoma virus, and murine leukemia viruses (10). It may reflect

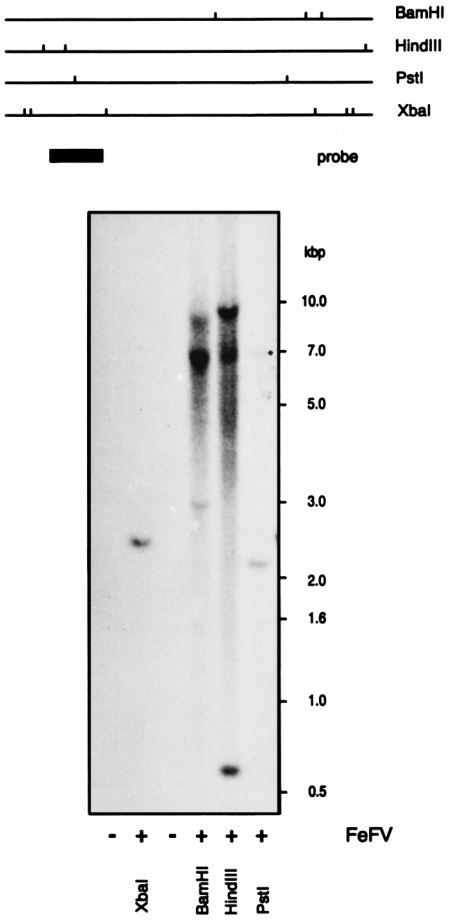

FIG. 3. Identification and characterization of FeFV provirus DNA by Southern blot hybridization. The schematic locations of different restriction enzyme recognition sites (short vertical lines) in the FeFV genome are shown above the autoradiogram. The DNA hybridization probe is indicated by the solid box. The autoradiogram of a Southern blot with DNA from mock-infected (minus signs) and FeFV-infected (plus signs) CRFK cells is shown. The positions and sizes of DNA markers are indicated to the right. The faint 6.8-kbp band resulting from *Pst*I digestion is indicated by a solid diamond.

a common feature of FV replication that sets them apart from other complex retroviruses.

The different sequence motifs described for FV Env proteins are shared by FeFV (38). By analogy to primate FVs, the cleavage site for proteolytic removal of the leader peptide can be located downstream of a large hydrophobic domain and upstream of a charged region (61a). Accordingly, the cleavage site is predicted to be located between residues 83 and 84 or residues 89 and 90 of FeFV Env (Fig. 2).

A potential cleavage site for proteolytic processing of the FeFV Env precursor into the SU and TM domains is well conserved among FVs. This postulated cleavage site (Fig. 2) will result in a mature SU protein of about 475 residues and a TM protein of 418 residues. Since many of the Cys residues in the SU protein are conserved, a similar overall structure of the different FV Env proteins seems likely. Remarkably, the long and hydrophobic membrane-spanning sequence and the short and hydrophilic cytoplasmic domain of 14 residues are shown in Fig. 2 and meet all criteria found for FV Env proteins (38). The highly charged carboxy-terminal sequence, Arg-Lys-Lys-Asp-Gln-Stop, resembles the retrieval signal for retrograde transport into the endoplasmatic reticulum (ER) that is characterized by two Lys residues 3 and 4 or 3 and 5 residues directly upstream of the carboxy terminus of the protein (28, 59). The ER retrieval signal has been detected in the cytoplasmic domain of FV Env proteins and recently characterized in HFV Env (20, 21). Since this signal is well conserved, it may play a role in retaining Env inside the cell to prevent syncytium formation and/or to allow evasion of immune surveillance. The ER retrieval signal may also have functions in FV particle assembly, which takes place inside the cell, where preformed particle budd into intracellular membrane compartments (20, 21, 40). In addition, the conserved FV Trp residue that corresponds to a Phe in cellular proteins is followed by either Leu or Ile located between the transmembrane anchor and the ER retrieval signal of FVs. This motif may be an anterograde transport signal for plasma membrane expression of Env, as shown for eukaryotic p24 cargo receptors (15). Thus, localization of Env may be governed by two apparently opposite signals controlling the level of Env proteins present on the cell surface and inside the cell.

In the 3′ end of *env* about 240 bp upstream of *bel1*, a typical TATA box motif is present and is shown in Fig. 2 in boldface type in the *env* sequence. About 30 bp downstream, the nucleotide sequence motif 5′-GAGCTTC-3′, resembling the FV transcriptional start site consensus sequence 5′-GAGCTC-3′, probably represents the transcriptional start site of an internal promoter that has been detected in different primate FVs (Fig. 2) (5, 41, 46).

FeFV *bel* genes. In addition to *gag*, *pol*, and *env*, two *bel* genes are located between *env* and the 3′ LTR of the FeFV provirus (Fig. 1A). A third reading frame corresponding to the HFV *bel3* gene is not present in FeFV by analogy to SFV-1, SFV-3, and BFV.

The *bel2* gene of FeFV corresponds to an open reading frame of 357 amino acid residues that has a remarkably low degree of identity of 18% (HFV) to 23% (SFVs) to the corresponding *bel2* genes of the other FVs.

FeFV Bel1 is the viral transactivator for LTR-directed gene expression. To determine whether FeFV codes for a functional transactivator of LTR-directed gene expression, three different LTR promoter constructs that direct *luc* reporter gene expression were constructed. Plasmid pFeFV-U3 contains the complete U3 region and lacks the 5′-terminal 17 bp, pFeFVU3R contains most of the FeFV U3R region, and pFeFV-LTR contains the LTR plus some base pairs from the PBS upstream of the *luc* gene. Initially, CRFK cells were transfected with the different FeFV LTR reporter plasmids, the vector backbone pGL2-bas, and a CMV–β-gal expression plasmid and were seeded in duplicate, and 16 h after transfection, one plate was inoculated with FeFV. After 2 days of incubation, the cells were harvested to determine β-gal and luciferase activities. The normalized values of luciferase activity expressed showed a strong *luc* expression in samples from FeFV-infected compared to uninfected cultures when the different FeFV LTR promoter constructs had been transfected (Table 1). Without FeFV infection, *luc* expression by the FeFV U3 and U3RU5 constructs was in the range of that by the promoterless vector backbone pGL2-bas either with or without FeFV infection. Plasmid pFeFV-U3R showed an increase of three to fourfold in the basal activity. The 150- to 380-fold transactivation of the LTR-based reporter plasmids as a result of FeFV superinfection indicates that FeFV contains a transactivator for LTR-directed gene expression. The target sites for transactivation are in the U3 region, as in other FVs.

To determine whether the FeFV *bel1* gene with the coding capacity for a 209-residue protein with limited sequence homology to the HFV and BFV Bel1 transactivator is capable of

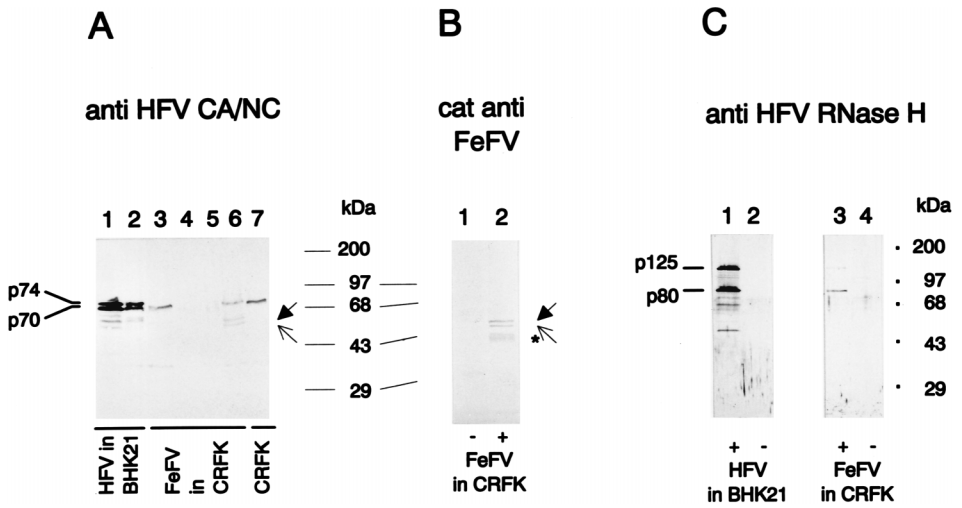

FIG. 4. Detection of FeFV-specific proteins by polyclonal monospecific antisera directed against the HFV CA/NC (A) and the RNase H domains (C) and a serum sample from an FeFV-infected cat (B). (A) Extracts from BHK-21 cells 4 days after HFV infection (lanes 1 and 2) or CRFK cells harvested 2, 3, 5, and 6 days after FeFV infection (lanes 3, 4, 5, and 6, respectively) or uninfected CRFK cells (lane 7) were immunoblotted and reacted with the 1:100-diluted HFV CA/NC-specific antiserum (directed against HFV Gag residues 388 to 636). The positions of the HFV p74 and p70 Gag proteins and the FeFV Gag proteins of 52 kDa (solid arrow) and 48 kDa (open arrow) are shown. The positions and sizes of marker proteins are shown to the right. (B) Extracts from CRFK cells harvested 6 days after FeFV infection (lane 2) or uninfected CRFK cells (lane 1) were immunoblotted and reacted with a 1:50-diluted serum from a naturally FeFV-infected cat. The positions of the FeFV Gag proteins of 52 kDa (solid arrow) and 48 kDa (open arrow) are shown; the asterisk marks viral proteins of about 43 kDa. The positions and sizes of marker proteins are shown on the left. (C) Extracts from BHK-21 cells 4 days after HFV infection (lane 1) or mock-infected BHK-21 cells (lane 2) and CRFK cells harvested 6 days after FeFV infection (lane 3) or uninfected CRFK cells (lane 4) were immunoblotted and reacted with the 1:100-diluted HFV RNase H-specific antiserum. The positions of the HFV pre125$^{Pro\text{-}Pol}$ and p80$^{RT\text{-}RNase\ H}$ and the comigrating proteins from FeFV-infected cells are shown. Positions and sizes of marker proteins are indicated to the right.

transactivating the FeFV LTR promoter constructs, the CMV-IE promoter-based FeFV Bel1 expression plasmid pFeFV-Bel1 was constructed by PCR cloning. The FeFV Bel1 expression clone pFeFV-Bel1 was used for cotransfection experiments in CRFK cells with the reporter plasmids described above. As expected, in the presence of the FeFV Bel1 expression clone, U3-, U3R-, and FeFV LTR-directed *luc* expression was increased 900- to 8,300-fold (Table 1). Again, plasmid pFeFV-U3R showed an elevated basal activity in the absence of Bel1. The *luc* vector backbone did not respond to FeFV Bel1 coexpression.

To extend the studies on FeFV gene expression, cross-transactivation experiments were performed with BHK-21 cells that are fully permissive for HFV replication to analyze whether FeFV Bel1 can transactivate the HFV LTR promoter in plasmid pHFV-LTR or the IP in plasmid pHFV-IP (64). In addition, feline CRFK cells were used to study whether the FeFV LTR-directed gene expression is transactivated by HFV Bel1 (Table 2). In BHK-21 and CRFK cells, the FeFV Bel1 expression plasmid induced a strong increase of *luc* expression from the FeFV LTR *luc* construct whereas significant transactivation was not observed with either the HFV expression vectors or the empty vector control pGL2-bas. In both cell types, the HFV Bel1 induced a strong increase in *luc* expression directed by the HFV LTR and the HFV IP. The promoter transactivator systems showed a clear cell specificity: the feline LTR/Bel1 system showed the highest activity in CRKF cells, whereas both HFV promoters were maximally transactivated in BHK-21 cells.

Comparison of the FeFV Bel1 to the transactivators of the other spumaviruses. Remarkably, the FeFV Bel1 transactivator for LTR-directed gene expression consists of only 209 amino acid residues compared to the ca. 300-residue Bel1 of HFV or the Tas (transactivator of spumavirus) proteins of SFVs (69). The FeFV Bel1 is about 100 residues smaller than the primate FV transactivator and is still 40 residues smaller than its BFV counterpart. As determined by the Bestfit alignment program, the degree of identity of the FeFV Bel1 to the FV Bel1/Tas proteins is only 17 to 23% and is limited to residues that are also conserved between BFV and the primate FVs. Since the degree of protein sequence homology between the known FV transactivators is low (53), computer-based analyses of the secondary structure of Bel1/Tas proteins were performed with a neural network program for secondary-structure analysis (EMBLpredict [56]). HFV, SFV-1, and SFV-3 transactivators showed a similar overall pattern of β-sheets, helical structures, and unstructured secondary structures. The major difference between SFV and HFV transactivators is the presence of either only one (HFV), two (SFV-3), or three (SFV-1) α-helical regions in the amino-terminal domain of about 90 residues (data not shown). When the predicted secondary structures of the FeFV and BFV Bel1 proteins were aligned to that of the prototypic HFV Bel1 (Fig. 6), significant differences became apparent. Seemingly, both the BFV and FeFV Bel1 proteins lack about 60 residues that comprise the amino-terminal domain present in the primate viruses. This domain of HFV Bel1 was reported to be virtually dispensable for transactivation (24, 37). In addition, the FeFV Bel1 lacks a β-sheet in the amino-terminal domain. A long helical region in the HFV Bel1 from residues 85 to 112 is interrupted in BFV and almost absent in FeFV Bel1 sequences. The amino acid motif Tyr-X-Cys-X-X-Cys, of unknown biological function, is present in all FV transactivators analyzed so far (53). This motif is located in either the center or the carboxy terminus of a β-sheet. The spacing of the Tyr-X-Cys-X-X-Cys motif to a dibasic Arg/Lys-His motif is constant in the different transactivators. In addition, this motif is always located in a comparably short α-helical region predicted to be exposed on the surface of the protein. The region between residues 200 and 225 of HFV Bel1 contains a bipartite nuclear localization sig-

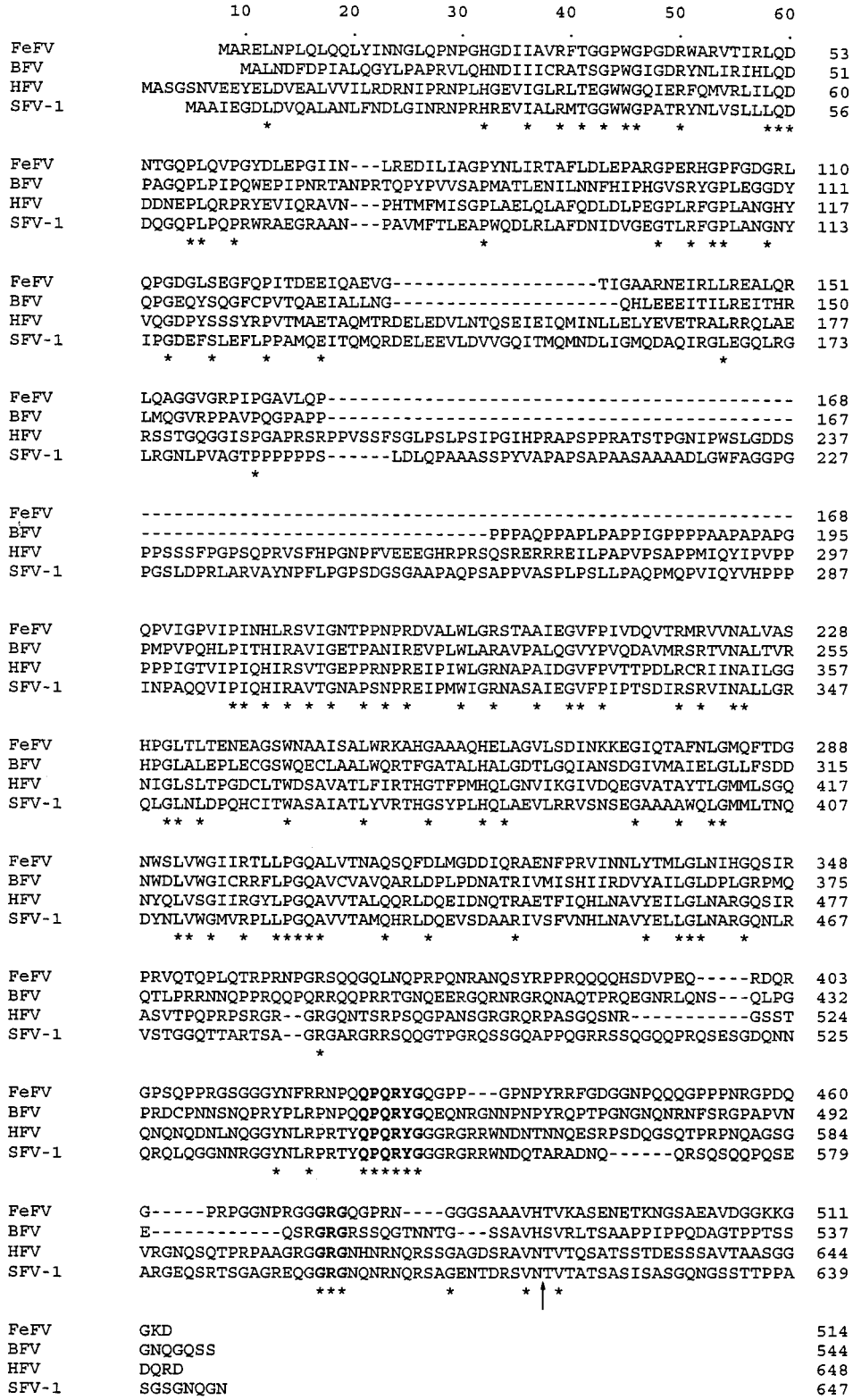

FIG. 5. Alignment of the amino acid sequences (in single-letter code) of the FeFV, BFV, HFV, and SFV-1 (from top) Gag proteins by using the Malign program with a modified alignment parameter to reduce the size and number of gaps introduced. The two conserved motifs in the putative NC domains of the Gag proteins are in boldface type. Asterisks mark the position of conserved residues. The postulated carboxy-terminal cleavage site of FV Gag proteins is indicated by a vertical arrow.

TABLE 1. Transactivation of FeFV LTR promoter-based luciferase reporter plasmids by FeFV infection and cotransfection of FeFV Bel1 expression plasmids in CRFK cells

Plasmid	Luciferase activity[a] after:					
	FeFV infection			FeFV Bel1 cotransfection		
	Mock infected	FeFV infected	Fold increase	pBKCMV	pFeFV-Bel1	Fold increase
pGL2-bas	26.5	37.1	1.4	8.7	25.1	2.9
pFeFV-U3	26.8	4,111.0	153.0	50.3	44,448.0	884.0
pFeFV-U3R	102.2	16,638.0	163.0	184.0	269,839.0	1,467.0
pFeFV-LTR	34.0	12,830.0	377.0	25.1	209,148.0	8,333.0

[a] Data are expressed as relative luciferase units normalized to cotransfected β-gal activity.

nal (NLS) that is highly enriched in basic amino acid residues (6, 24). This basic NLS region of HFV seems to be completely absent in BFV and FeFV. The remaining one-third of the different FV transactivators do not show strong conservation of secondary structure and are in general characterized by flexible loops or β-turn regions. A β-sheet and an α-helix in the carboxy-terminal activation domain of HFV Bel1 is also present in FeFV Bel1, but the α-helix is not present in BFV Bel1.

DISCUSSION

The molecular cloning and characterization of the FeFV genome confirm that FeFV is a member of the subfamily of *Spumavirinae* within the *Retroviridae* (7–9, 22, 55). Our data show that the FeFV genome encodes *bel* genes and the classical retroviral *gag*, *pol*, and *env* genes similar to other known FVs and that FeFV fulfills the criteria for a complex retrovirus (12). The FeFV genes and predicted gene products show unambiguous homologies to the corresponding genes of prototypic HFV. Importantly, the Bel1 protein of FeFV is the transactivator for FeFV LTR-directed gene expression.

The availability of sequence information and experimental data not only from the closely related primate FVs but also from the nonprimate FeFV and BFV allows us to distinguish between conserved and nonconserved features of spumaviruses. These comparative studies identify a conserved sequence in the amino terminus of FV Gag proteins that is reminiscent of the morphogenic signal of type D retroviruses dominantly directing the capsid assembly to the cytoplasm (54). Since FVs also preassemble their capsids in the cytoplasm of infected cells, this protein motif may serve a similar function in FVs (40). Whereas the amino-terminal and central parts of the MA domain of known FVs show sequence and secondary-structure conservation (data not shown), a primate FV-specific domain extends into the central CA domain that is not present in FeFV and BFV. The function of this highly unordered Pro-rich sequence of primate FVs is not known. It is possible that these sequences are differentially processed in the different FVs.

The three GR boxes that are present in the primate FV Gag proteins at the carboxy terminus corresponding to the NC domain of other retroviruses are not conserved when aligned with BFV and FeFV Gag sequences (58, 67). In contrast, the motif Gln-Pro-Gln-Arg-Tyr-Gly is present in the known FVs. The related motif Asn-Leu-Gln-Arg-Gln-Gly was recently detected at a corresponding position in the Gag sequence of an endogenous FV-related retrovirus (11). Despite this sequence conservation, the deletion of the Gln-Pro-Gln-Arg-Tyr-Gly motif in HFV reduced viral infectivity only 10-fold (67).

The FV NC domains are characterized by different arrangements of positively charged regions, notably of distinctly spaced Arg residues. The FV-specific arrangements are reminiscent of cellular Y-box proteins that bind RNAs of defined lengths and sequences (for a review, see reference 60). It is noteworthy that most of the Arg residues conserved between FeFV and HFV NC domains are equidistant (data not shown). Overall, the FV NC domains are histone-like but distinct, and because of their charge distribution, they are predicted to bind single-stranded nucleic acids.

The proteolytic processing of HFV and FeFV Gag and Pol proteins is incomplete, resulting in high concentrations of unprocessed precursor proteins or partially processed intermediates, as shown for different primate FVs (2, 19, 23). For HFV and most probably also for FeFV, the predominant Gag-specific proteins correspond to an unprocessed and a carboxy-terminally processed Gag protein (19, 32, 39). Further processing products are present in very small amounts. The incomplete processing of FeFV Gag was confirmed by Western blotting with an antiserum, derived from an FeFV-infected cat, that detected the 52- and 48-kDa Gag bands and additional proteins in the range of 43 kDa. The detection of smaller proteins corresponding to fully processed Gag proteins was not reproducible. The absence or low abundance of mature Gag proteins in infected cells does not allow us to define the domain structure of FV Gag proteins. FV-specific proteolysis of the pre125$^{Pro\text{-}Pol}$ protein seems to be less incomplete than that of Gag, since partial processing resulted in the 40-kDa IN, 80-kDa RT/RNase H, and p62 proteins that are detectable by Western blotting or in situ enzymatic assays (31, 49).

The question is whether the low degree of processing in cell cultures reflects the fact that HFV and FeFV are grown on cells not fully permissive for all steps of replication. This is, however, in contrast to the relatively high titer obtained in cell culture. Alternatively, the low processing of FV Gag and Pol proteins may be the consequence of the unique mechanism of FV Pro-Pol expression by a spliced transcript and not as part of a Gag-Pol fusion protein that complicates targeting of Pro-Pol into virions (4, 14, 29, 39, 66). It may be that the levels of Pro-Pol expressed by a spliced transcript are too low for complete proteolytic processing and for integration as well. Alternatively, the extra targeting steps required for FV Pro-Pol packaging may limit the amounts of protease incorporated into FV particles. The limited proteolytic processing of FV Gag proteins may indicate an inefficient targeting of protease to Gag. The incorporation of the complete set of Pro-Pol proteins is, furthermore, complicated in that protease may be enzymatically active when expressed as Pro-Pol proteins even in the absence of Gag proteins (39). To avoid premature Pol and Gag cleavages, thereby incorporating truncated Gag and Pro-Pol proteins that would lead to defective particles, it seems likely

TABLE 2. Transactivation of FeFV and HFV promoter-based luciferase reporter plasmids by FeFV Bel1 and HFV Bel1 in CRFK and BHK-21 cells

Plasmid	Bel1-mediated increase in *luc* expression[a] in:			
	CRFK cells		BHK-21 cells	
	FeFV Bel1	HFV Bel1	FeFV Bel1	HFV Bel1
pGL2-bas	0.7	0.2	0.3	0.6
pFeFV-LTR	4,243.1	1.0	322.5	3.3
pHFV-LTR	1.3	46.5	1.0	637.0
pHFV-IP	1.8	37.5	0.4	37.8

[a] Data are expressed as increase relative to cotransfection with the empty vector control pBKCMV normalized to cotransfected β-gal activity.

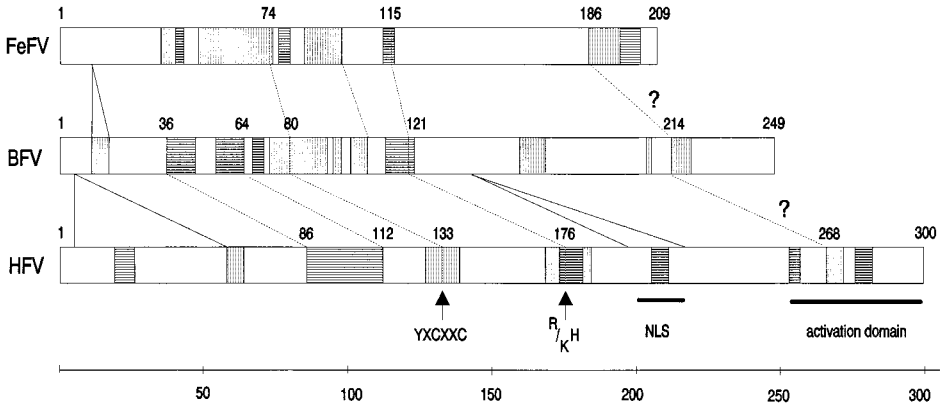

FIG. 6. Alignment of the predicted secondary structures of the Bel1 transactivators of FeFV (top), BFV (middle), and HFV (bottom). Extended secondary structures (β-sheet) are marked by stippled boxes, and regions predicted to be helical are marked by horizontally hatched boxes. The location of the Tyr-X-Cys-X-X-Cys motif is indicated. The NLS of HFV and the carboxy-terminal transcriptional activation domain of HFV are underlined (6, 24, 37). The Arg/Lys-His motifs in a helical region preserved in all FV transactivator proteins are shown. Dashed lines mark similar motifs in the proteins, and solid lines denote the borders of insertions or deletions. The position of conserved amino acid residues and a scale are shown at the bottom.

that proteolytic processing is downregulated in FVs. The limited processing of Gag ensures that complete or only carboxy-terminally trimmed Gag proteins are incorporated into virions.

Since sequencing revealed that the active center of FeFV Pro consists of the sequence Asp-Ser-**Gln**-Ala instead of Asp-Ser-Gly-Ala found in the known FV Pro sequences, we examined whether the substitution of the Gly by a Gln residue represents a special feature of the FeFV isolate FUV, e.g., an adaptation of FeFV-FUV to cell culture conditions. Therefore, DNA was isolated from different cell culture-adapted FeFV isolates and, in addition, directly from peripheral blood lymphocytes from FeFV-positive cats. Sequencing of cell culture-adapted and cat-derived FeFV DNAs showed that the sequences obtained of FeFV Pro, and especially that of the active-center motif, were identical in all DNAs analyzed and that Gln replaced the Gly residue in the DNA products. It is noteworthy that the incomplete processing of FeFV Gag and Pol proteins strongly resembles that of HFV that contains the canonical Gly residue in the catalytic center of Pro.

A Pol-specific protein of about 190 kDa representing an FeFV Gag-Pol fusion protein was not detected. This is in agreement with our previous data and shows that FeFV utilizes a spliced transcript for Pro-Pol protein expression and not a Gag-Pol fusion protein as all other retroviruses do except for the members of the *Spumavirinae* (4, 14, 29, 39, 66). Furthermore, the Met residue in the beginning of Pol, assumed to be encoded by the start codon for Pro-Pol protein translation, is also present and conserved in FeFV.

The strong transactivation of the FeFV LTR reporter constructs by FeFV Bel1 implies that the FeFV Bel1 transactivator is absolutely required for efficient LTR-directed transcription in the context of the complete provirus. Consistent with the idea that Bel1 is absolutely required for FeFV gene expression, the basal, Bel1-independent activity of the FeFV LTR was found to be low or even absent. This also holds for HFV, where Bel1 is absolutely required for replication (3, 40, 65). This is in contrast to the situation with FIV, where the ORF2 protein induces only a minimal increase in FIV LTR-directed transcription whereas the LTR has a high basal activity in the absence of the transactivator (61, 62). The FIV ORF2 protein is not required for viral infectivity in certain cells but is necessary in others, thus modulating cell tropism (62).

The target site for Bel1 to transactivate FeFV LTR-directed gene expression resides in the U3 part of the LTR as in the other known spumaviruses, indicating that a similar mechanism for targeting Bel1 is used in FVs (25, 53, 69). Therefore, the FeFV Bel1 protein may also interact with its DNA site(s) directly, as recently described for HFV and SFV-1 (25, 69). Within the lentiviruses, different mechanisms for targeting the transactivator are used: the primate lentiviruses use an RNA structure encoded in R, whereas the target for the viral transactivator of visna virus is located in the U3 region of the LTR (10, 13). Since the different LTR reporter plasmids showed a comparable gene expression, major *cis*-acting regulatory sequences are not present in the R and U5 regions of the FeFV LTR.

The FeFV LTR and the Bel1 protein functioned well in cells of different origins, consistent with the observation that the range of cells permissive for FeFV replication in vivo and in vitro is broad (22). However, the listed similarities of the biological activities of Bel1 proteins of different FVs are not reflected in a strong conservation of the size or primary amino acid sequence. This is also shown by the lack of cross-reactivity of two different HFV Bel1-specific sera with FeFV Bel1 and a lack of cross-transactivation of the HFV and FeFV promoters by the heterologous Bel1 proteins.

Computer-based predictions for the secondary structure of the Bel1/Tas proteins indicated that the overall structure shows some segments of homologous secondary structures in the center of the protein. In this region, a conserved short helical region was identified that contains the amino acid motif Arg/Lys-His present in all known FVs. The Lys-His dipeptide is essential for HFV Bel1 function but not for nuclear localization (37). Whereas this protein motif is not involved in nuclear targeting of the primate FV Bel1/Tas, it may be part of the FeFV and BFV NLS, since it resides in both transactivators in a domain rich in basic residues; however, a typical NLS is not present in either FeFV or BFV Bel1 (52). A comparable 30-residue sequence of Rous sarcoma virus IN that also does not encode a classical NLS has recently been shown to act as an NLS (34).

It is noteworthy that independently obtained DNA sequences from FeFV-FUV-infected CRFK cells were close to identical to each other and that the divergences found are in the range of the error rate of the PCR enzyme. This finding reflects a very low genetic diversity of the FUV isolate under cell culture conditions. This is in line with observations made during the study of other FVs. Having established the genomic

structure of a nonprimate FV, it will be interesting to analyze different and common strategies of FV molecular biology and to examine FeFV as a retroviral vector for targeted gene delivery in its indigenous host. Studies to determine the infectivity of the FeFV clones are under way.

ACKNOWLEDGMENTS

I.W. and J.B. contributed equally to the manuscript.

I.W. was supported by the Deutsche Akademische Austauschdienst, and J.B. was supported by the Wilhelm-Sander-Stiftung.

Different FeFV field isolates derived directly from domestic cats were kindly provided by Tim Harder, Hannover Veterinary School, Hannover. We thank James Casey and Don Holzschu for communicating the BFV sequence prior to publication and Harald zur Hausen for support.

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