

Expression of Feline Immunodeficiency Virus *gag* and *env* Precursor Proteins in *Spodoptera frugiperda* Cells and Their Use in Immunodiagnosis

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The *gag* and *env* genes of the feline immunodeficiency virus strain UT113 were cloned into a baculovirus transfer vector. The recombinant plasmids were used to create recombinant baculoviruses that expressed either the *gag* or the *env* precursor protein in insect cells (Sf9 cells). Leader sequence cleavage occurred in Sf9 cells expressing the envelope precursor, but further processing was not observed. Crude lysates of insect cells infected with the wild-type baculovirus or with the recombinant viruses were used to develop an enzyme-linked immunosorbent assay for the detection of feline immunodeficiency virus-specific antibodies in cat sera. The assay showed a higher sensitivity and specificity than immunofluorescence and Western blotting (immunoblotting).

Feline immunodeficiency virus (FIV) is a recently discovered lentivirus (13) that causes a syndrome in cats resembling AIDS in humans (3). The viral genome is typical for lentiviruses with three large open reading frames encoding the viral core (*gag*), polymerase (*pol*), and envelope proteins (*env*) as well as various smaller open reading frames encoding regulatory proteins (12). In cats experimentally infected with FIV, an antibody response against the structural proteins can be detected 2 to 4 weeks after infection (5). Antibodies directed against the envelope surface glycoprotein can be detected first, immediately followed by antibodies to the viral core proteins p15 and p24, respectively. Diagnosis of FIV infection is currently based on assays that primarily detect antibodies to the *gag* proteins (6, 11, 15), although some infected cats lack a significant antibody response to these proteins (5). In this article we describe the cloning of the *gag* and *env* genes of the virus isolate FIV-UT113 into the genome of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV). High levels of expression of both envelope and core precursor proteins were obtained in the baculovirus system. The *env* gene expression product was further characterized and used, in combination with the *gag* gene product, to develop an assay for the detection of anti-FIV antibodies in cat sera.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* Sf9 cells (ATCC CRL 1711) were grown in monolayer cultures at 27°C in TC100 medium (Gibco BRL) supplemented with 10% fetal calf serum, 50 µg of gentamicin per ml, and 2.5 µg of fungizone per ml. Cell culture procedures and virus manipulations were performed essentially as described by Summers and Smith (17).

Construction of baculovirus transfer vectors. All DNA techniques used were based on those of Ausubel (1). The *gag* and *env* genes of FIV-UT113 (EMBL data library accession numbers X68019 and X60725) were enzymatically amplified by using the polymerase chain reaction. Primers

corresponding to the 5' and the 3' ends of the *gag* and *env* genes of FIV-UT113 were used to amplify the coding sequences and added *Bgl*II or *Bam*HI restriction sites to the amplified fragments. The primers used to amplify the *gag* gene were as follows: 5' primer, GTGAGATCTATGGGG AACGGACAG; 3' primer, CGCAGATCTTACAAATCCA ATAGTTTCTCC. The primers used to amplify the *env* gene were as follows: 5' primer, CCCGGATCCATGGCAGAAG GATTTGC; 3' primer, CCCGGATCCTCTCCTCCTCT TTTTC. [*Bgl*II (AGATCT) and *Bam*HI (GGATCC) restriction sites are underlined.] The amplification products were digested with *Bgl*II or *Bam*HI, purified, and cloned into the *Bam*HI site of the baculovirus transfer vector pACYM1 (9). Resultant plasmids were used in cotransfection experiments to generate recombinant baculoviruses.

Generation of recombinant baculoviruses. Sf9 cells were cotransfected with a mixture of AcNPV DNA and transfer vector DNA (17). Recombinant baculoviruses were purified by several rounds of limiting dilution as described by Goswami and Glazer (7). High-titer stocks (>10⁷ PFU/ml) were prepared and used to infect Sf9 monolayers.

Labeling of expression products. Sf9 cells were seeded in a culture dish and allowed to attach for 2 h. The cells were infected with recombinant baculovirus at a multiplicity of infection of 5 and incubated for 2 days at 27°C. The culture medium was removed and replaced by methionine-free medium. The cells were preincubated for 1 h, and the medium was replaced by 0.5 ml of methionine-free medium supplied with 50 µCi (for pulse-chase experiments: 100 µCi) of [³⁵S]methionine. The cells were incubated for 5 h at 27°C. When a chase was performed on the labeled proteins, the medium was removed after 1 h of incubation and the cells were washed with cold TC100 medium and further incubated in the same medium. After the labeling-chase period, the cells and medium were collected and centrifuged for 5 min at 2,000 × g. The cells were dissolved in 10 mM Tris-HCl (pH 7.4)–1 mM EDTA–150 mM NaCl–1% Triton X-100. The supernatant was transferred to a new tube and recentrifuged (5 min, 8,000 × g). An aliquot of 900 µl was transferred to a new tube, and 100 µl of 10% Triton X-100 was added. Labeled proteins were immunoprecipitated with 5 µl of

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immune serum as described previously (4). Immunoprecipitated proteins were either analyzed directly by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or treated with endoglycosidases prior to analysis.

Endoglycosidase treatment of immunoprecipitated glycoproteins. Treatment of immunoprecipitated glycoproteins with endoglycosidase H or endoglycosidase F/N-glycosidase F (Boehringer Mannheim) was performed as described previously (19).

Immunoblotting. Cells expressing the recombinant FIV proteins were disrupted in lysis buffer. Proteins were separated by SDS-10% PAGE and transferred to a nitrocellulose filter as described by Towbin et al. (18). Serum incubation was performed essentially as described by Egberink et al. (5).

Analysis of cat sera by using an ELISA based on recombinant proteins. To evaluate the levels of antibodies to FIV core and envelope proteins, we developed an enzyme-linked immunosorbent assay (ELISA) in microtiter plates coated with lysates of Sf9 cells expressing these products. Baculovirus-infected cells were harvested 72 h after infection by low-speed centrifugation. They were washed once with phosphate-buffered saline (PBS) and resuspended in PBS-0.25% SDS to a concentration of 2×10^7 cells per ml. The suspension was boiled for 5 min, followed by removal of cell debris (15 min, Eppendorf centrifuge, 15,000 rpm), and the supernatant was used for coating of the wells. Optimal antigen dilution was determined by titration; a 1:1,500 dilution of the antigen in PBS was routinely used. The format of this ELISA consisted of three wells coated with lysates of cells expressing the envelope glycoprotein precursor, expressing the *gag* precursor, or infected with the wild-type (WT) baculovirus, respectively. Each well was coated with 100 μ l of antigen dilution. The plates were incubated overnight at 4°C, washed three times with PBS-0.05% Tween 20 and blocked for 1 h with 10% skim milk powder-0.5% Tween 20-0.2 M NaCl in PBS (PTN) at 37°C. They were then washed three times with PBS-Tween, and 100 μ l of serum dilution (in PTN) was added to each well, followed by 1 h of incubation at 37°C. After three washes with PBS-Tween, a 1:7,500 dilution (in PTN) of peroxidase-conjugated goat anti-cat immunoglobulin G (Cappel Research Products) was added (100 μ l per well) and the plates were incubated 1 h at 37°C. Finally, they were washed five times with PBS and the substrate-chromogen solution (0.01% tetramethyl benzidine-0.006% H₂O₂-0.1 M Na acetate [pH 5.5]) was added to the wells (100 μ l per well). Color development was stopped with 50 μ l of 2 M H₂SO₄ per well, and the A₄₅₀ was measured with a Titertek Multiskan Plus spectrophotometer.

RESULTS

Construction of recombinant baculoviruses vBG12 and vBE23. Genomic DNA isolated from feline thymocytes infected with the Dutch isolate FIV-UT113 was digested with *Nhe*I, and the DNA fragments were cloned into the *Xba*I site of the vector λ ZAPII (Stratagene). Two clones were isolated, one clone containing a 3-kb DNA fragment from the 5' end and another, 6-kb clone spanning the 3' end of the FIV genome. Both clones were sequenced, and DNA isolated from the λ ZAPII clones was used as a template for the amplification of the *gag* and *env* genes of FIV-UT113. Amplification products were digested with either *Bgl*II or *Bam*HI and cloned into the baculovirus transfer vector pACYM1. Two plasmid clones, pBG12 and pBE23, harboring the *gag* and *env* genes, respectively, were isolated. DNA

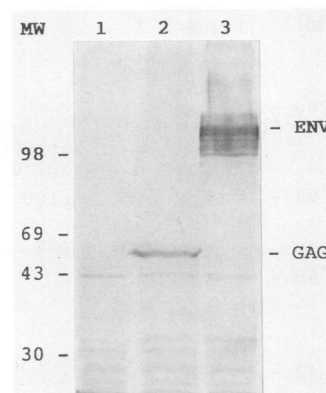


FIG. 1. Expression of the FIV-UT113 *gag* and *env* genes in Sf9 cells. Sf9 cells were infected with WT or recombinant AcNPV as described in Materials and Methods. Proteins were separated on a SDS-10% polyacrylamide gel and analyzed by immunoblotting by using an anti-FIV serum. Lane 1, Sf9 cells infected with WT AcNPV. Lanes 2 and 3, Sf9 cells infected with recombinant baculoviruses vBG21 and vBE23, respectively. MW, molecular weight markers (10^3).

of these plasmids was cotransfected into Sf9 cells with AcNPV DNA, and recombinant viruses were isolated by several rounds of limiting dilution. Finally, two recombinants (vBG12 and vBE23) were plaque purified once, and large recombinant virus stocks were prepared.

Expression of recombinant *gag* and *env* proteins in Sf9 cells. Expression of the *gag* and *env* gene products in insect cells is shown in Fig. 1. The *gag* precursor protein migrated as a band with an estimated size of 50 kDa (p50) which corresponds to the native molecule in FIV-infected cells (4, 16) and to data from Morikawa et al. (10). The *env* gene expression product migrated as a broad band and had an estimated size of 130 kDa. No recombinant envelope glycoproteins could be detected in the culture medium; however, a small amount of p50 was found (not shown) probably representing virus-like particles consisting of unprocessed p50 (10).

The envelope precursor of FIV is N-glycosylated and partially processed in insect cells. The *env* gene expression product was characterized by endoglycosidase digestion (Fig. 2). Expression products were labeled and incubated with endoglycosidase H (lane 2) or endoglycosidase F/N-glycosidase F (lane 3). Labeling of cells infected with vBE23 resulted in the synthesis of two glycoprotein species with estimated molecular sizes of 150 and 130 kDa, respectively. Two other protein species of 65 and 35 kDa are detectable, the latter slightly glycosylated, but these cannot be identified as FIV related. Glycosidase treatment of the gp150 and gp130 glycoproteins resulted in a shift in molecular sizes to about 100 and 80 kDa, respectively. Slight molecular size differences observed between lanes 2 and 3 are due to different enzymatic activities of the glycosidases used.

To investigate whether gp150 and gp130 are related, we performed pulse-chase labeling on vBE23 infected cells. The results are shown in Fig. 3. Longer chase periods (up to 24 h) resulted in the gradual disappearance of gp150 while the amount of gp130 appeared to increase in time, suggesting that gp150 is a precursor molecule of gp130.

Immune responses to FIV in experimentally infected cats. Sera from four experimentally infected cats obtained at

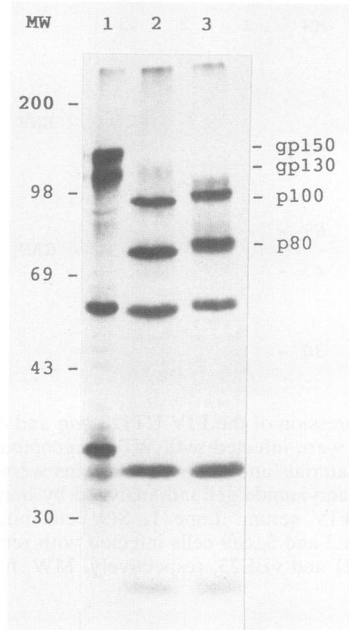


FIG. 2. Endoglycosidase treatment of FIV-UT113 envelope glycoproteins expressed in insect cells. Cells were infected with vBE23 at a multiplicity of infection of 5 and labeled with 50 μ Ci of [35 S]methionine for 5 h. FIV-specific proteins were immunoprecipitated, treated with endoglycosidases, and analyzed by SDS-10% PAGE and fluorography. Lane 1, untreated proteins. Lanes 2 and 3, FIV-specific proteins treated with endoglycosidase H and endoglycosidase F/N-glycosidase F, respectively. MW, molecular weight markers (10^3).

different time points were examined by ELISA for antibodies directed against the envelope and core proteins of FIV. Cats 320 and 340 were infected with a Dutch isolate (FIV-UT48), while cats 199 and 201 were infected with FIV-Petaluma, the prototype strain isolated in the United States (13). As can be seen in Fig. 4, all cats seroconverted within 3 to 4 weeks, with anti-envelope antibodies detectable first. Three out of four cats showed comparable immune responses during the course of infection. In these cats the anti-envelope antibody titers remained higher than those against the core. Cat 320 had a different antibody response, with the anti-core antibody titer showing a very strong increase until 10 weeks postinfection, even exceeding the anti-envelope titers. The core-antibody titers dropped after 4 months, while anti-envelope titers continued to increase.

Use of the ELISA for screening of sera from naturally infected cats. To evaluate the ELISA for its use in routine antibody screening, we tested 206 serum samples from naturally infected cats. All sera had been prescreened in our laboratory by an immunofluorescence assay (IFA) on FIV-UT113-infected Crandell feline kidney (CRFK) cells (2). Positive sera and sera that had given ambiguous results in the fluorescence assay were also screened by Western blot (immunoblot) (WB). Sucrose gradient-purified FIV that had lost most of its envelope surface glycoprotein was used to prepare the nitrocellulose strips. Thus, WB analysis was primarily based on the detection of antibodies directed against the other structural proteins, primarily core proteins. To reduce the effects of aspecific binding of feline antibodies to the microtiter plate, we used a high serum dilution (1:500) in combination with a blocking solution containing 10% skim milk powder, 0.5% Tween 20, and 0.35 M NaCl.

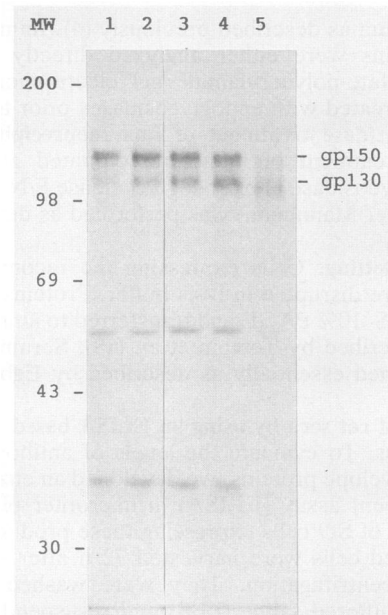


FIG. 3. Synthesis and processing of the FIV-UT113 envelope precursor protein in insect cells. Sf9 cells infected with vBE23 were labeled with 100 μ Ci of [35 S]methionine for 1 h and chased for various time periods up to 24 h. FIV-specific expression products were immunoprecipitated and analyzed by SDS-10% PAGE. Lanes 1 to 5, samples of vBE23-infected Sf9 cells chased for 0, 1, 3, 6, and 24 h, respectively. MW, molecular weight markers (10^3).

IFA and WB analysis of the 206 serum samples resulted in 108 negative reactions, 84 positive reactions, and 14 ambiguous reactions. The last group were from sera that had given weak reactions with p10 or p24 in WB. The ELISA results are summarized in Table 1. Most FIV-positive sera (98%) had an optical density at 450 nm in the *env* or *gag* ELISA which was 2 to 10 times the background value measured in the wells coated with lysate from WT AcNPV-infected cells. However, 2% of the sera gave high background values (optical density at 450 nm > 0.5). This prevented the use of more commonly accepted criteria such as a cutoff limit of twice the background value, since this would falsely assign FIV-positive, high-background sera as FIV negative. To circumvent this problem, we subtracted the WT optical density readings from the *env* and *gag* ELISA values and used the resultant Δ ENV or Δ GAG figures to define the cutoff limit. The limit was empirically set at 0.04. When this low cutoff value was used, the ELISA results closely correlated with the IFA-WB results. Ninety-three serum samples were scored ELISA positive: 45 serum samples by both Δ ENV and Δ GAG ELISA, 46 serum samples by Δ ENV ELISA, and 2 serum samples by the Δ GAG ELISA only. However, four serum samples scored positive by ELISA and negative in the IFA-WB tests and two serum samples tested negative by ELISA and positive in the IFA-WB tests. Of the 14 serum samples reacting ambiguously by IFA-WB, 7 tested positive and 7 tested negative by ELISA.

All 20 serum samples (14 ambiguously reacting in IFA-WB, 4 ELISA positive and IFA negative, and 2 ELISA negative and IFA and WB positive) were further examined by using a radioimmunoprecipitation assay (RIPA) with labeled FIV-UT113 virus. The 14 serum samples with ambiguous reactions by IFA-WB gave RIPA results that were in agreement with the ELISA scores; also, the seropositivity

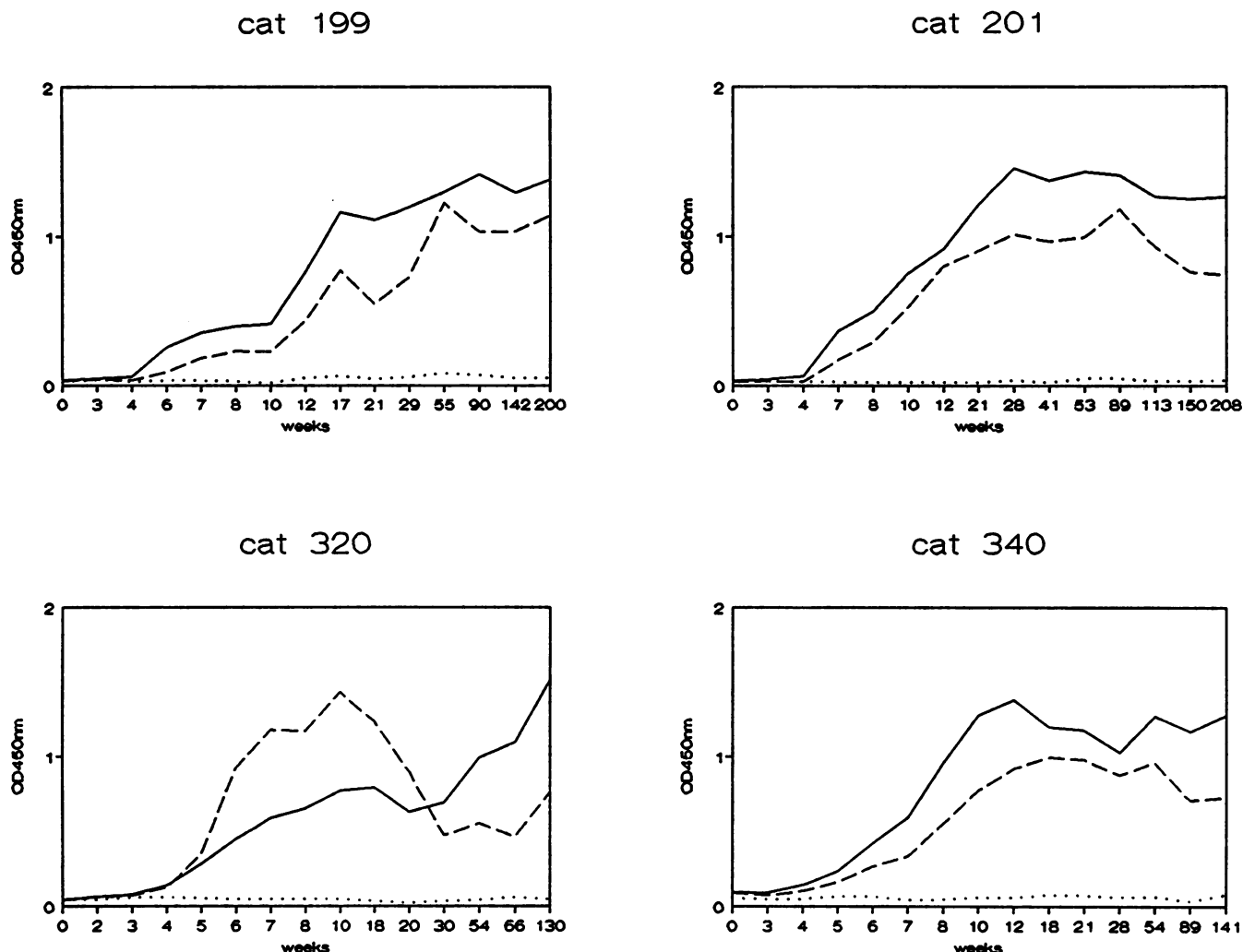


FIG. 4. Time course of the immune responses to FIV proteins in experimentally infected cats. Serum samples taken at various time points after experimental infection with FIV-UT48 (cat 320 and 340) or FIV-Petaluma (cats 199 and 201) were assayed at a 1:200 dilution by ELISA. —, anti-*env* protein antibody response; ---, anti-*gag* protein antibody response; ···, binding to wells coated with lysates from WT AcNPV-infected Sf9 cells. OD450nm, optical density at 450 nm.

of two of the four ELISA-positive and IFA-WB-negative sera was confirmed. Both serum samples that had tested negative by ELISA but positive by IFA-WB were confirmed as negative in the RIPA. In conclusion, only 2 out of 206 samples (0.97%) were falsely FIV seropositive, and no serum from FIV-infected cats escaped detection.

Interestingly, RIPA analysis of these 20 serum samples

resulted in the characterization of several serum samples that had no or only low levels of antibodies directed against the p17 and p24 core proteins (Fig. 5, lanes 4 to 10). In the ELISA, 2 of the 206 sera were ΔENV negative but ΔGAG positive, thereby suggesting a lack of anti-envelope antibodies. However, in RIPA they precipitated the p17/p24 *gag* proteins and also reacted with the envelope glycoprotein (Fig. 5, lane 3).

TABLE 1. Comparison of IFA-WB and ELISA results from 206 serum samples from naturally infected cats

ELISA result ^a	No. of samples in group ^b		
	I	II	III
Negative	104	2	7
Positive	4	82	7

^a ELISA values were scored positive when the ΔENV and/or ΔGAG values were ≥0.04.

^b Group I scored FIV negative by IFA and/or WB; group II scored FIV positive in IFA and/or WB; group III had ambiguous results (weak reaction on WB with p10 or p24).

DISCUSSION

The construction of a FIV-UT113 *gag* gene containing recombinant baculovirus resulted in a high-level expression of the *gag* precursor p50 in insect cells. Our results are in agreement with the findings of Morikawa et al. (10) who also showed the absence of processing of the *gag* precursor to the core proteins p10, p17, and p24. The *env* gene translation product was also expressed at a high level in our insect cell system. The envelope precursor appeared a glycoprotein of 150 kDa that was slowly processed to a glycoprotein of 130 kDa. Removal of a polypeptide of 20 kDa is identical to the

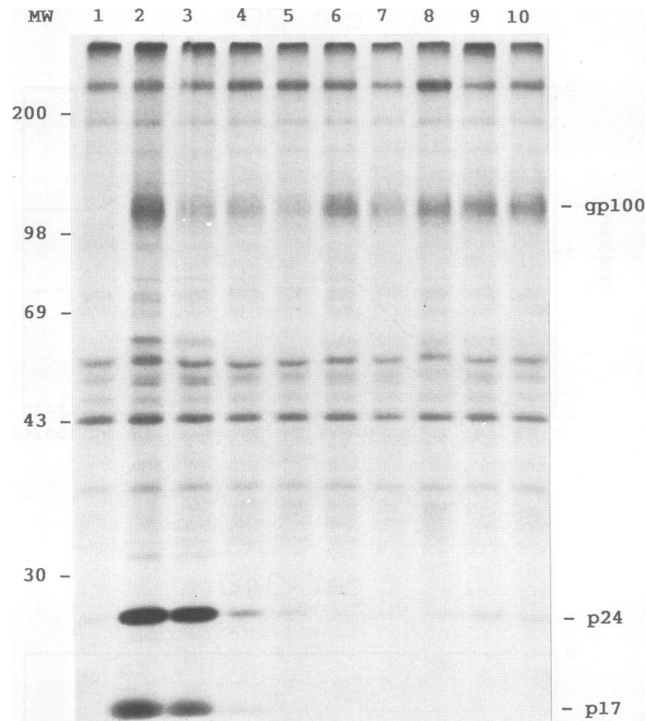


FIG. 5. RIPA analysis of cat sera using cell lysates of [³⁵S]methionine-labeled FIV-infected CRFK cells. Lane 1, negative control serum. Lane 2, positive control serum. Lanes 3 to 10, sera from naturally infected cats. Lane 3, *env*-ELISA-negative and *gag*-ELISA-positive serum. Lanes 4, 5, 8, 9, and 10, IFA-WB-indeterminate, ELISA-positive sera. Lanes 6 and 7, IFA-negative, ELISA-positive sera. MW, molecular weight markers (10³).

processing of the primary *env* gene translation product to the envelope precursor, gp130, in FIV-infected CRFK cells (19) but occurs at a much slower rate in insect cells. No evidence of further cleavage of the envelope precursor into the surface envelope (gp100) and transmembrane glycoproteins (gp35) was found. The latter cleavage was also lacking in the HIV-1 envelope precursor expressed in insect cells (8) but was apparent in the bovine immunodeficiency virus envelope precursor (14).

The recombinant core and envelope proteins were used to develop an ELISA for antibody detection in FIV-infected cats. A low cutoff value was defined for the detection of FIV-positive sera. Although this may result in erroneously identifying cats as FIV positive, this was not the case in our assay. The test had a greater fidelity than the IFA combined with the confirmatory WB assay. Only 2 out of 206 serum samples (0.97%) tested falsely positive, whereas all FIV seropositive cats, as examined by IFA, WB, and/or RIPA, were detected. This resulted in a very high sensitivity (percentage of positive sera that tested positive) and specificity (percentage of negative sera that also scored negative in the ELISA) for this test: 100% and 99%, respectively. It is very important to avoid false-negative results. Seronegative animals are not routinely retested in a confirmatory assay, and FIV-infected cats that escape detection at the first testing can continue to be a source of FIV infection in the cat population. In contrast, IFA-WB screening of the sera resulted in 4 false-positive or false-negative serum samples (1.9%) and 14 serum samples that could not be classified positive or negative (6.8%).

The results obtained with our ELISA and in confirmatory RIPA emphasize the necessity to include the screening for anti-envelope and anti-core protein antibodies in one diagnostic assay. Enlarging upon previously reported data (5), we have clear evidence for FIV-infected cats that possess very low amounts of antibodies directed against the core proteins p17 and p24 or even completely lack anti-core antibodies. Seven out of 91 FIV-positive serum samples (7.7%) lacked anti-core antibodies (or had low titers) and were thus prone to be overlooked in assays that are based on the detection of these antibodies (6, 11, 15). The presence of sera that did not react with the envelope expression product in the ELISA but had a strong anti-core response (2.2%) underscores the necessity of screening for antibodies to both viral proteins.

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