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SHORT COMMUNICATIONS

Post-translational Processing of the Feline Immunodeficiency Virus Envelope Precursor Protein

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The synthesis and processing of the envelope glycoprotein precursor of the feline immunodeficiency virus (FIV) isolate FIV-UT113 was investigated in a persistently infected Crandell feline kidney cell line (CRFK) and in an eukaryotic expression system. Pulse–chase studies showed two glycoproteins after a 5 min pulse-labeling: a gp150 and a gp130 species. During a 30-min chase the gp150 species disappeared almost completely while gp130 increased proportionally; it was subsequently processed into the surface glycoprotein (SU), gp100, and the transmembrane glycoprotein (TM), gp35. This final maturation step did also occur when the *env* gene was expressed independently, but at a much lower rate. The results indicate that FIV-UT113 envelope glycoprotein processing involves two successive proteolytic cleavages. The first cleavage removes a protein fragment of approximately 20 kDa and takes place post-translationally, at least in part. The deduced primary translation product of the *env* gene lacks an N-terminal signal sequence but instead contains two internal hydrophobic regions. Cleavage is predicted to occur behind the second region which would indeed release an N-terminal 20-kDa polypeptide. Thus, FIV glycoprotein processing resembles that found in the ungulate lentiviruses but differs from that in the primate lentiviruses, the envelope proteins of which possess a short N-terminal signal sequence.

Feline immunodeficiency virus (FIV) is a lentivirus first described in 1987 as the causative agent of an AIDS-like syndrome in domestic cats (*1*). Its genome has three open reading frames (ORFs) representing the *gag*, *pol*, and *env* genes and several ORFs capable of encoding regulatory genes (*2–6*). FIV resembles the human immunodeficiency virus (HIV-1) (*7*) in its molecular properties as well as in the immunological abnormalities it induces in cats (*8–11*). Because of these parallels the FIV infection in cats provides an attractive animal model for the study of AIDS.

The surface glycoprotein (SU) of HIV-1, gp120, is known to elicit neutralizing antibodies (*12, 13*) and to induce T-cell-mediated immunity in animals (*14*). The protein is therefore attributed a key role in viral pathogenesis. To establish such a role for the SU of FIV, this protein, designated gp120 (*15*) or gp130 (*16*), is the subject of ongoing investigation. The processing and glycosylation of the envelope precursor of the FIV-Petaluma isolate were described by Stephens *et al.* (*17*). They found that a glycoprotein with a M_r of 145 kDa (gp145) was gradually trimmed by glycosidases to a molecule of about 130 kDa (gp130) and subsequently cleaved into a SU of about 95 kDa (gp95) and a transmembrane glycoprotein (TM) with a M_r of 40 kDa (gp40).

Recently, we established a persistently infected Crandell feline kidney (CRFK; *18*) cell line using an FIV isolate from The Netherlands (FIV-UT113; *19*). In this study we report the synthesis and processing of its envelope protein and present evidence for a two-step cleavage process. Our results demonstrate that processing of the FIV glycoproteins is different from HIV-1 but resembles that described for the ungulate caprine arthritis–encephalitis lentivirus (CAEV; *20*).

CRFK cells persistently infected with FIV-UT113 were pulse-labeled with [^{35}S]methionine and FIV-specific proteins were immunoprecipitated (Fig. 1A). After a 5-min pulse (lane 2) four FIV-specific protein species were detectable with apparent MWs of 150, 130, 50, and 40 kDa, two of which (150 and 130 kDa) were glycosylated (Fig. 2), suggesting that they are *env* gene expression products. The 150-kDa glycoprotein (gp150) rapidly turned over during the chase and had disappeared almost completely after 30 min (lanes 2 to 4). Concomitantly, the amount of labeled 130-kDa glycoprotein (gp130) increased proportionally, suggest-

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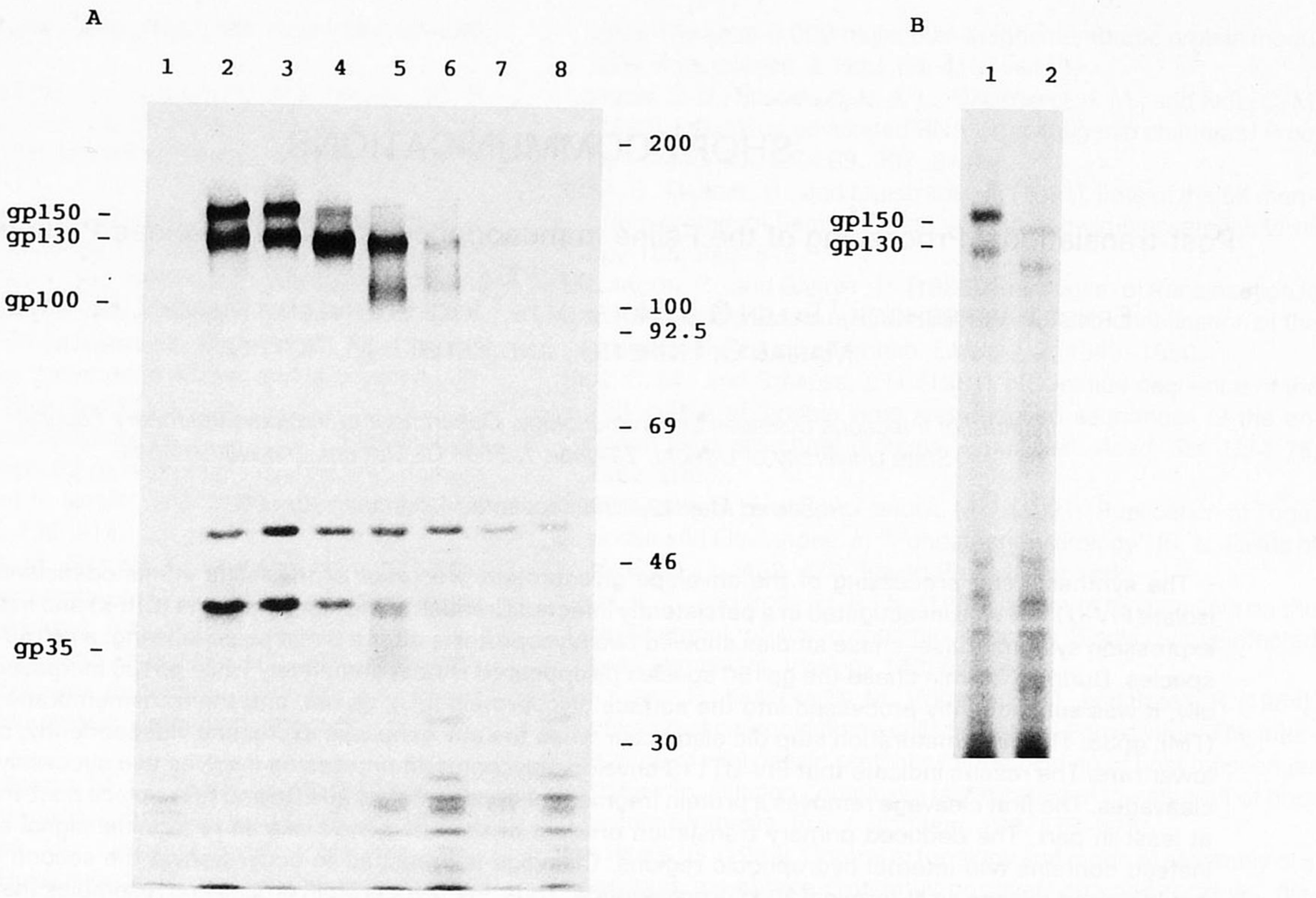

FIG. 1. (A) Synthesis and processing of the FIV-UT113 envelope glycoproteins. CRFK cells persistently infected with FIV-UT113 were pulse-labeled with 200 μCi/ml [^{35}S]methionine for 5 min and chased for various time periods up to 24 hr. FIV-specific proteins were immunoprecipitated and analyzed on a 10% SDS–PAGE gel as previously described (*15*). Lane 1, sample of pulse-labeled uninfected CRFK cells. Lanes 2 to 8, sample of FIV-UT113-infected CRFK cells chased for 0, 5, 30 min and 3, 6, 12, and 24 hr, respectively. The positions of marker proteins (in kDa) are given at the right. (B) Comparative analysis of envelope precursor synthesis of the FIV isolates UT113 (lane 1) and Petaluma (lane 2). CRFK cells persistently infected with either isolate were pulse-labeled in parallel for 5 min with 200 μCi/ml [^{35}S]methionine. Envelope glycoproteins were immunoprecipitated and analyzed in a 10% SDS–PAGE gel.

ing that this protein is a processing product of gp150. Subsequently, the intensity of the 130-kDa species started to decline while a new glycoprotein of about 100 kDa (gp100) became evident after a 3-hr chase (lane 5). Longer chases resulted in the disappearance of both the 130-kDa and the 100-kDa protein from the cell lysate. In some experiments the appearance of gp100 coincided with the detection of a glycoprotein of about 35 kDa (gp35; lane 5). The latter did not show up clearly, probably as a result of heterogenous glycosylation, since a distinct 25-kDa protein became detectable after deglycosylation (Fig. 2, lane 6). The 50-kDa nonglycosylated protein represents the gag precursor (*15*). The virus specificity of the 40-kDa protein has yet to be confirmed as it was not consistently precipitated.

These observations suggest two successive steps in the processing of the *env* gene translation product. First the 150-kDa glycoprotein appears to be processed into a 130-kDa species, which in turn is cleaved into a 100-kDa and a 35-kDa protein. The first processing step was not observed by Stephens *et al.* (*17*) using the FIV-Petaluma isolate. To compare both isolates under similar conditions we performed a 5-min pulse-labeling in parallel on both isolates grown in CRFK cells. Apart from slight differences in apparent molecular weight, explained by the absence of two potential N-glycosylation sites in the Petaluma envelope protein (*4*), a similar glycoprotein pattern was found (Fig. 1B). Thus, the synthesis of a full-size primary translation product seems to be a general feature of FIV.

To further characterize the FIV-specific proteins they were treated with endoglycosidases (Fig. 2). Endo H treatment of the pulse-labeled sample (lane 1) converted gp150 and gp130 completely into proteins of about 100 and 75 kDa (lane 2). Endo F/glycopeptidase F treatment had the same effect, although, for unknown reasons, both had a slightly lower electrophoretic mobility than their endo H treated counterparts (lane 4). Endo F/glycopeptidase F treatment of the 3-hr chase sample showed the conversion of the gp130 and gp100 (lane 5) into 80- and 55-kDa species, respectively (lane 6). The protein band of about 25 kDa visible after glycosidase treatment probably originates from the diffuse 35-kDa glycoprotein band mentioned above.

Since the processing pattern of the envelope precursor of our FIV isolate was different from that of most other lentiviruses published so far, we determined the sequence of the FIV-UT113 *env* gene. Genomic DNA

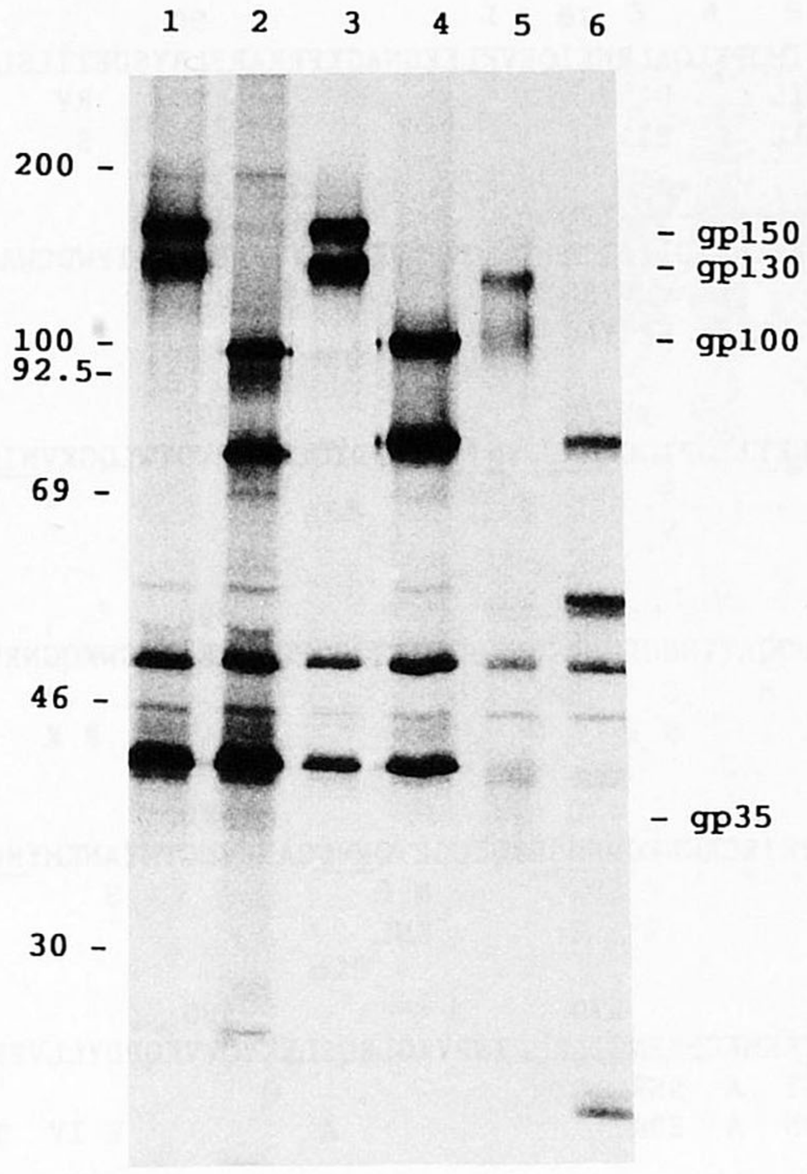

FIG. 2. Glycosylation of FIV-UT113 envelope proteins. Lanes 1 to 6, endoglycosidase treatments of samples obtained from the experiment of Fig. 1A. For endo H digestions immunoprecipitated material was resuspended in 20 μl 50 m*M* Tris–Cl (pH 6.8)/1% SDS and boiled for 5 min. Samples were split into two portions and to each 15 μl of 150 m*M* sodium citrate (pH 5.5) with or without 1 mU endo H was added. The mixtures were then incubated for 18 hr at 37°. For endo F/glycopeptidase F digestions immunoprecipitated material was resuspended in 32 μl endo F buffer (0.05 *M* KP_i, pH 6.8, 20 m*M* EDTA, 1% Triton X-100, 0.2% SDS, 1% 2-β-mercaptoethanol) and boiled for 5 min. The boiled samples were divided into two portions and incubated for 18 hr at 30° in the absence or presence of 125 mU of endo F/glycopeptidase F. Samples were analyzed on a 10% SDS–PAGE gel. Lanes 1 and 2, 0-min chase sample incubated in the absence (lane 1) or presence (lane 2) of endo H. Lanes 3 and 4, 5-min chase sample incubated in the absence (lane 3) or presence (lane 4) of endo F/glycopeptidase F. Lanes 5 and 6, 3-hr chase sample incubated in the absence (lane 5) or presence (lane 6) of endo F/glycopeptidase F.

isolated from FIV-UT113-infected feline thymocytes was digested with *Nhe*I. Fragments of 3 and 6 kb, hybridizing with a 1-kb *pol* gene-specific DNA probe of FIV-Petaluma (kindly provided by Dr. J. Elder), were isolated and cloned into the *Xba*I site of the λZAPII vector (Stratagene). A 6-kb clone, p6.10, hybridizing both with the *pol* probe and with a cDNA probe specific for the U3-R region of the FIV-LTR, was subcloned and sequenced. The deduced amino acid sequence of the *env* gene is shown in Fig. 3. The gene encodes a polypeptide of 856 amino acid residues with a calculated molecular weight of 98.471. Twenty-three potential N-glycosylation sites (N-X-S/T) are present, resulting in a theoretical molecular weight of the glycoprotein of 146.8 kDa, taking 2.1 kDa per site (*21*). These figures are in good agreement with our interpretation that the 150-kDa glycoprotein (Fig. 1A) is indeed the primary translation product of the *env* gene. Comparison of the FIV-UT113 envelope protein sequence with those of other FIV isolates reveals a high level of homology (Fig. 3) with percentages similarity of 91.9 and 92.2 to the Petaluma and PPR proteins, respectively. Clearly, the envelope glycoproteins of FIV are more similar to one another than has been found for HIV isolates (*22*). Hydrophobicity analysis (*23*) revealed that the protein lacks an N-terminal signal sequence. Two internal hydrophobic regions located around residues 100 and 160 each might serve this function. Another hydrophobic region (residues 616–640) occurs immediately C-terminal to the putative processing site between the SU and TM moiety (RRKR, residues 608–611), providing the TM with a characteristic hydrophobic N-terminal fusion peptide also described for other lentiviruses (*24, 25*). Finally, a hydrophobic domain is found close to the C-terminus (residues 786–812) which presumably represents the membrane anchor of the envelope protein. Prediction of signal sequence cleavage sites (*26*) invariably locates a processing site in the C-terminal region of the second hydrophobic domain of either isolate (Fig. 3). Cleavage of the precursor protein at that site would remove a leader peptide of about 20 kDa, leaving an envelope precursor of 79.7 kDa, i.e., 125–130 kDa in its glycosylated form.

Figure 4 shows the processing of the envelope glycoprotein expressed in CRFK cells using a T7 RNA polymerase-driven transient expression system (*27*). Four FIV-specific proteins with M_r of 150, 130, 98, and 92 kDa, respectively, could be detected after a pulse-labeling of 5 min (Fig. 4, lane 2). The 150- and 130-kDa species were N-glycosylated as they were susceptible to endo F treatment, whereas the 98- and 92-kDa protein species were not affected (lane 9). A chase was performed on the 5-min pulse sample (Fig. 4, lanes 2 to 7) and endo F/glycopeptidase F treatment was again used to identify the glycoproteins (lanes 9 to 14). The results were essentially identical to those shown in Fig. 1A, confirming the precursor–product relationships proposed for the envelope proteins in FIV-infected cells. In contrast to the latter situation cleavage of gp130 was not readily detectable, but the glycosidase treatments of the samples chased for 3 hr or longer revealed the 55- and 25-kDa cleavage products (lanes 12 to 14). Apparently, processing of the expressed precursor protein does occur but at a much lower rate, giving rise to envelope products which are heterogeneously glycosylated and which are therefore not detectable upon direct analysis.

Collectively, our data show that the envelope protein of FIV is synthesized as a full-length uncleaved precursor which is post-translationally processed by proteolytic cleavage in two successive steps to yield the viral

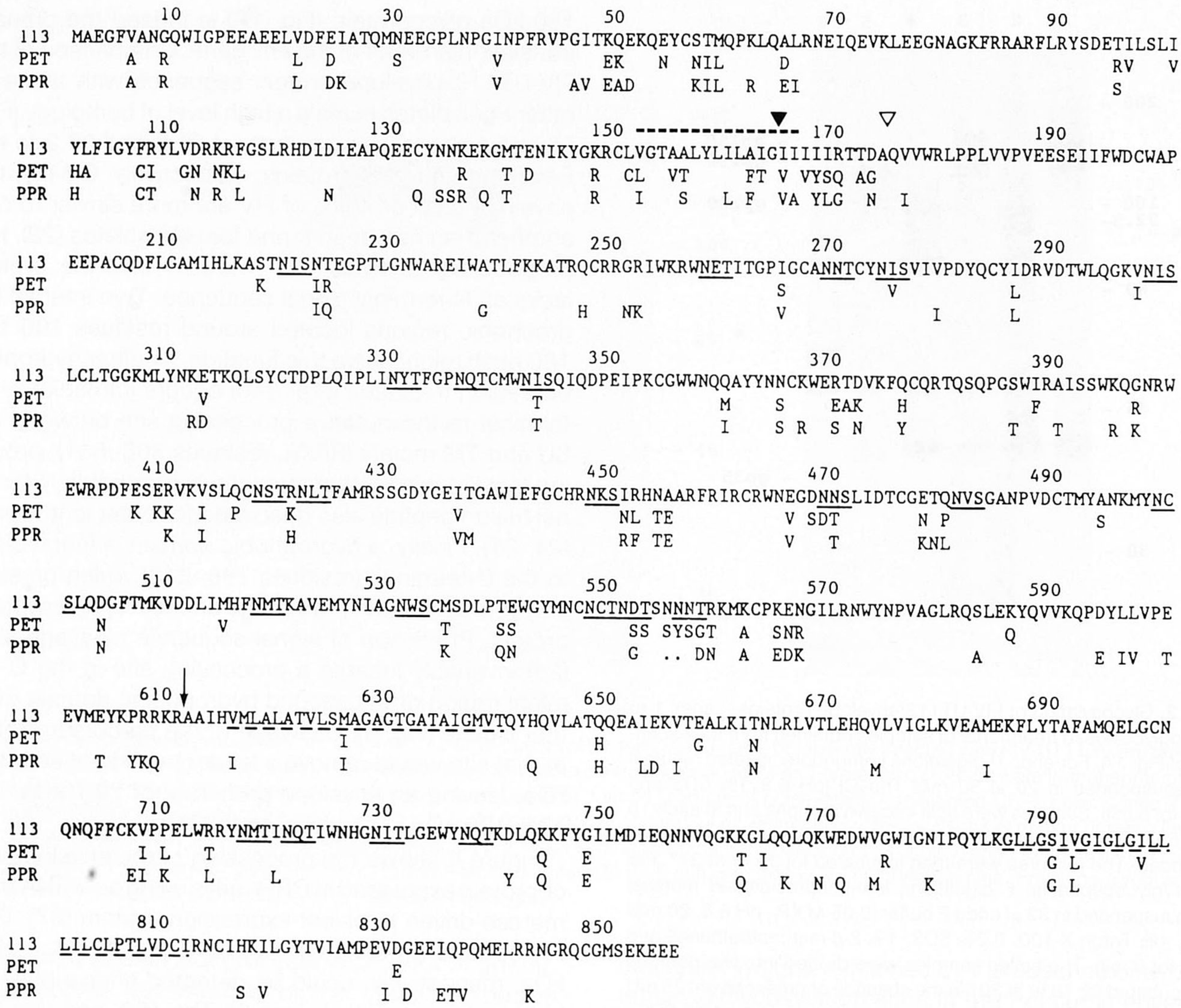

FIG. 3. Comparison of the amino acid sequences of envelope glycoprotein precursors of different FIV isolates. Sequence differences of the isolates FIV-Petaluma (PET; *4*) and FIV-PPR (PPR; *5*) with the FIV-UT113 sequence are indicated. Potential N-glycosylation sites in the FIV-UT113 sequence are underlined. The predicted signal sequence is indicated by ----. The putative signal sequence cleavage site is indicated by a closed (FIV-UT113) or open triangle (FIV-Petaluma and PPR). The putative processing site between SU and TM moiety is marked by an arrow. Hydrophobic domains representing the putative fusion peptide (residues 616–640) and membrane anchor (residues 786–812) are indicated by dashed underlines. The EMBL Data Library accession number of the FIV-UT113 envelope gene sequence is X60725.

envelope glycoproteins. The full-size *env* gene precursor has not been described earlier. The largest species observed by Stephens *et al.* (*17*) after pulse-labeling of FIV-Petaluma-infected cells was a 145-kDa glycoprotein that was gradually converted into the 130-kDa immediate envelope precursor by the trimming of its oligosaccharide side chains. We also identified this precursor (though of slightly different size) including its trimming (data not shown) but in addition detected the analogue of the FIV-UT113 gp150 polyprotein (Fig. 1B). Thus, a two-step post-translational processing pattern of the *env* gene product seems to be a general feature of FIV.

In HIV and SIV the envelope precursor protein possesses an N-terminal signal sequence which is cotranslationally cleaved off to yield the mature envelope precursor. Data on the biogenesis of envelope glycoproteins of other lentiviruses are scarce, but recently the nucleotide sequences of the ungulate lentiviruses visna virus, caprine arthritis-encephalitis virus (CAEV) and South African ovine maedi visna virus (SA-OMVV) have been published (*28–30*). Their deduced *env* gene products resemble the FIV homologue in that they also have a predicted internal signal sequence. Recently it was shown that the CAEV envelope precursor is cleaved at a site located 60 to 80 amino acid residues from the N-terminus of the *env* ORF (*20*); an older report on visna virus (*31*) is also in line with this picture. The parallel between FIV and the ungulate viruses is not surprising. Phylogenetic analyses indicated that FIV is more closely related to nonprimate lentiviruses than to HIV and SIV (*3*, *4*).

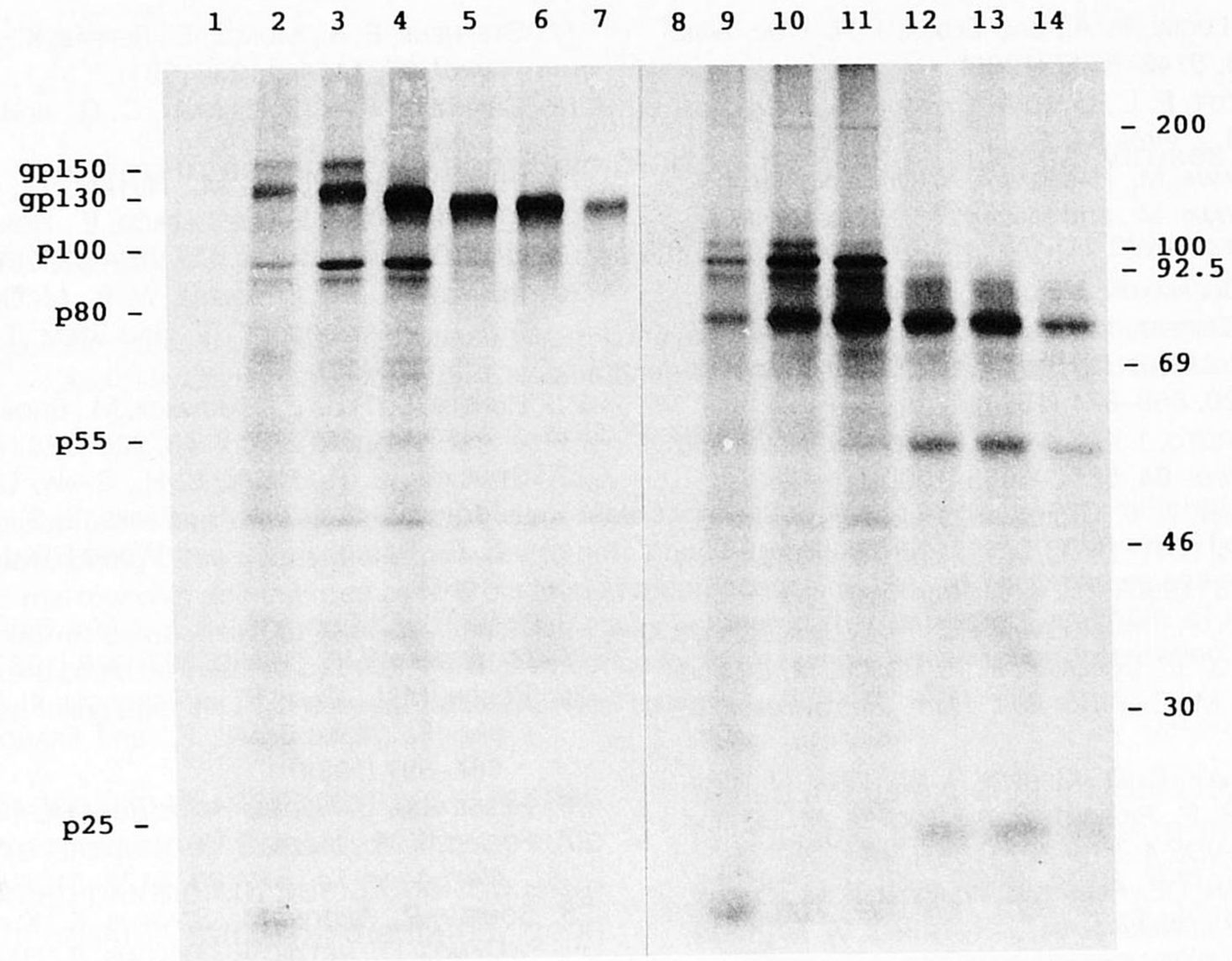

FIG. 4. Synthesis and processing of the expressed FIV-UT113 envelope protein. Clone p6.10 was used as a template for the enzymatic amplification of the putative *env* gene using the PCR primers 5′-CTCCCATGGCAGAAGGATTTGCAGCC-3′, located at the 5′ end of the gene with the putative ATG initiation codon contained within a *Nco*I recognition sequence, and 5′-CTCTCTAGACCTCTTTTTCAGACATGCCACATTGCC-3′, which corresponds to the 3′ end of the gene. The latter primer added a *Xba*I site to facilitate cloning and its use resulted in the loss of the two C-terminal glutamine residues of the protein in exchange for a valine residue. The PCR fragments were digested with *Nco*I and *Xba*I and subsequently cloned into pTM1 (*34*), which had been linearized with *Nco*I and *Spe*I. The resultant recombinant plasmid, pENV5, was used in the expression studies. Subconfluent monolayers of CRFK cells were infected with the vaccinia virus recombinant vTF7-3 (*27*) at a multiplicity of infection of 10 for 45 min at 20°. After a further incubation of 45 min at 37° the cells were transfected with 6 μg of plasmid DNA and incubation was continued for 18 hr at 37°. Mock-transfected and pENV5-transfected CRFK cells were pulse-labeled and envelope expression products were immunoprecipitated, incubated in the absence (lanes 1 to 7) or presence (lanes 8 to 14) of endo F/glycopeptidase F, and analyzed by SDS–PAGE as described in Fig. 1. Lanes 1 and 8, samples from mock-transfected cells chased for 0 min. Other lanes, samples from pENV5-transfected cells chased for 0 min (lanes 2 and 9), 5 min (lanes 3 and 10), 30 min (lanes 4 and 11), 3 hr (lanes 5 and 12), 6 hr (lanes 6 and 13), and 12 hr (lanes 7 and 14). Marker proteins (in kDa) are given at the right. Envelope glycoproteins and deglycosylated products are indicated at the left.

An intriguing question is whether the 20-kDa presequence which is removed from the FIV envelope precursor during the first processing step plays a role in the viral life cycle. FIV might generate a functional protein through the use of an internally located signal sequence. In addition, other proteins might be specified using the FIV envelope presequence, analogous, e.g., to the rev-like protein of visna virus (*32*). The visna orf-L gene encoding this 167-amino-acid protein starts at the AUG initiation codon of the envelope precursor and shifts reading frame by a splice in the mRNA after the first 48-amino-acid residues (*33*).

The final processing step, cleavage of gp130 into its SU and TM moieties is not different from that described for other lentiviruses. The putative cleavage site (RRKR, residues 608–611) is similar to the consensus sequence RXKR used by cellular proteases in most retroviral envelope proteins. Processing of the envelope precursor to gp100 and gp35 also occurs when the protein is expressed independently, though at a much lower rate. The difference probably relates to a slow transport of the expressed precursor to the site of cleavage, as immunofluorescence experiments suggested that the protein accumulates in the ER region of the cell (data not shown).

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