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Identification of Proteolytic Processing Sites within the Gag and Pol Polyproteins of Feline Immunodeficiency Virus

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N-terminal amino acid sequencing, ion spray mass spectrometry, and cleavage of synthetic peptide substrates were used to identify the N and C termini of the mature Gag and Pol proteins of feline immunodeficiency virus (FIV). The Gag polyprotein encodes matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. The Gag-Pol polyprotein encodes, in addition to the above proteins, protease (PR), reverse transcriptase (RT), dUTPase (DU), and integrase (IN). Secondary cleavage of RT at Trp-595–Tyr-596 of Pol yields a truncated form lacking the C-terminal RNase H domain. The observed and expected molecular masses of the viral proteins were in agreement, with three exceptions. (i) The molecular mass of MA was 14,735 Da, compared with a predicted mass of 14,649 Da, based on a single cleavage at Tyr-135–Pro-136 of Gag. The observed molecular mass is consistent with myristoylation of MA, which was confirmed by metabolic labeling of FIV MA with [^{3}H]myristic acid. (ii) The N terminus of the NC protein is generated via cleavage at Gln-366–Val-367 of Gag, which predicts a mass of 25,523 for CA and 9,101 for the major form of NC. The observed mass of CA was 24,569, consistent with loss of nine C-terminal amino acids by a second cleavage of CA at Leu-357–Leu-358. Synthetic FIV protease accurately cleaved synthetic peptide substrates containing this site. (iii) The actual mass of NC (7,120 Da) was approximately 2 kDa smaller than the mass predicted by synthesis to the stop codon at the end of Gag (9,101 Da). Experiments are in progress to characterize additional cleavage(s) in NC.

Feline immunodeficiency virus (FIV) is a lentivirus found in domestic cats with an AIDS-like disease (1, 4, 9, 14, 32, 43–45). As the smallest natural model for lentivirus infections, the feline-FIV system serves as a valuable model for development of both prophylactic and intervention therapies against lentivirus infections. Several molecular clones have now been produced and analyzed at the nucleotide sequence level (25, 29, 33, 41). The organization of the genome of FIV resembles that of other lentiviruses in that the *gag* gene encodes a polyprotein composed of matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. The *pol* gene is expressed as a Gag-Pol polyprotein via ribosomal frameshifting (26). The Pol portion of the polyprotein is the enzyme cassette of the virus and encodes protease (PR), reverse transcriptase (RT), dUTPase (DU) (10, 23), and integrase (IN) proteins. By analogy with other retroviruses, cleavage of both the Gag and Pol polyproteins of FIV into their constituent proteins is carried out by the viral PR during the budding process (7, 16, 20, 36). Unique properties and specificities of each of these proteins facilitate the various aspects of the virus life cycle. As such, they also offer potential targets for intervention therapies via rational drug design. Notably in human immunodeficiency virus (HIV), PR plays a highly specific and important role in virus maturation, and disruption of PR processing of the polyproteins results in noninfectious virions (3, 7, 20). It thus becomes important to define the boundaries of each respective constituent of Gag and Pol and therefore the cleavage junctions recognized by PR.

In the present study, we have carried out experiments to define the specific protease cleavage sites within the Gag and Pol polyproteins of FIV. These studies will thus facilitate the molecular cloning and characterization of individual proteins and will define the specificities of FIV PR. The latter information may then be used for synthesis of specific sequence-based PR inhibitors.

MATERIALS AND METHODS

Viruses and virus propagation. The 34TF10 molecular clone of the Petaluma isolate of FIV was used for these studies (41). Virus was propagated in Crandell feline kidney (CRFK) cells in Dulbecco's modified essential medium plus 10% fetal bovine serum. Supernatants from chronically infected cells were harvested at confluency and filtered through 0.45-μm-pore-size filters (Nalgene), and virions were precipitated for 30 min at 4°C with 7% polyethylene glycol 6000 in the presence of 400 mM NaCl. The supernatant was centrifuged at 8,000 × *g* for 30 min to pellet precipitated virions. The supernatant was neutralized with bleach and discarded, and the pellets were drained thoroughly and the tubes were wiped dry to minimize contamination by residual supernatant. Each pellet was then resuspended in 10 mM Tris-HCl (pH 7.5)–150 mM NaCl (TBS), loaded onto linear 0 to 60% Renografin gradients, and centrifuged at 100,000 × *g* for 16 h. Fractions were collected from the bottom of the tube, and the virus band was collected, diluted 1:2 with TBS, and pelleted at 100,000 × *g* for 1.5 h. The purity of the virus was assessed by sodium

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dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as detailed below.

SDS-PAGE analyses and N-terminal amino acid sequencing. Viral proteins were separated on 8 to 16% linear-gradient polyacrylamide gels (Novex) by the procedure of Laemmli (18). The proteins were transferred electrophoretically to ProBlot polyvinylidene difluoride membranes (Applied Biosystems) and stained with 0.1% Ponceau S–0.01% Coomassie blue in 50% methanol–0.5% aldehyde-free acetic acid to visualize the proteins. Bands corresponding to each of the viral proteins were excised and analyzed on a gas-phase amino acid sequencer (model 470A; Applied Biosystems). A minimum of three analyses were performed for each of the proteins identified. The N-terminal sequences thus obtained were then identified within the amino acid sequence predicted from the nucleotide sequence of the full-length infectious viral clone (41).

Ion spray mass spectrometry. Gradient-purified FIV was disrupted in 6 M guanidine-HCl and then applied to a Brownlee Aquapore OD-300 (pore size, 7 μm; 2.1 by 100 mm) reverse-phase high-pressure liquid chromatography (HPLC) column. Proteins were eluted over 45 min with a linear gradient from 0.04% trifluoroacetic acid in water to 60% CH_3CN containing 0.04% trifluoroacetic acid. The column effluent was injected directly into a Sciex API-III ion spray mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) by using a pneumatically assisted electrospray (ionspray) atmospheric-pressure ionization source. The interface sprayer was operated at a potential of +5,000 V. Positive ions generated by the ion evaporation process entered the analyzer of the mass spectrometer through the 100-μm sampling orifice. The instrument was operated at an orifice potential (OP) of +70 V. A total of 438 scans were acquired, with a single-scan duration of 7.00 s, from 700 to 2,400 *m/z* at a 0.5-amu mass interval with a 2.00-ms dwell time at each point. Raw data (intensity versus m/z) were used to calculate the molecular weight from the multiple charge states corresponding to each molecular species present. These measured molecular weights were compared with those from the predicted amino acid sequence, deduced from the nucleotide sequence of the 34TF10 clone of FIV (41), by using the program MacProMass (provided by Terry D. Lee and Sunil Vemuri, Beckman Research Institute, City of Hope, Duarte, Calif.).

Western blot analyses. Viral proteins were identified by using antisera directed to synthetic peptides corresponding to amino acid sequences of each respective protein, as predicted from the nucleotide sequence of the 34TF10 clone (41). Peptide selection was biased toward regions predicted to contain reverse turns (5), and antisera to peptides conjugated to keyhole limpet hemocyanin were prepared in rabbits, as previously described (19). Western immunoblots were performed on proteins transferred to either Immobilon or nitrocellulose membranes after SDS-PAGE, essentially as described previously (15). Blots were developed either by reaction of peroxidase-conjugated secondary antibodies with 4-chloro-1-naphthol dye (8) or by enhanced chemiluminescence procedures (ECL; Amersham).

Metabolic labeling of virus-infected cells with [^{3}H]myristic acid. CRFK cells, chronically infected with the 34TF10 molecular clone of FIV, were grown to near confluency in a T175 tissue culture flask containing Dulbecco's modified Eagle medium plus 10% fetal bovine serum. They were then switched to Dulbecco's modified Eagle medium plus 1% fetal bovine serum and 200 μCi of [^{3}H]myristic acid (31 Ci/mmol; New England Nuclear) and incubated for 16 h. The supernatant from this culture was then combined with the supernatants from five identical unlabeled flasks, and virus was prepared as detailed above. The virus was disrupted in SDS-PAGE sample buffer and electrophoresed as described above. The gel was permeated with En^{3}Hance (New England Nuclear), dried, and subjected to autoradiography at −70°C to detect labeled protein species.

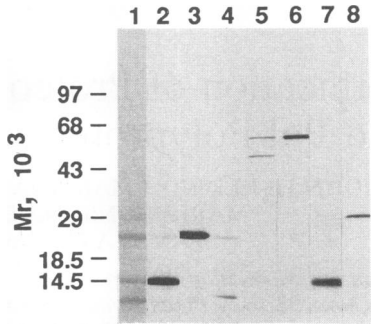

FIG. 1. Western blot analysis of Gag and Pol proteins of FIV. Gradient-purified FIV was disrupted and subjected to SDS-PAGE as detailed in Materials and Methods. The viral proteins were transferred to nitrocellulose membranes, reacted with site-directed antisera to specific viral proteins by Western blot, developed by chemiluminescence, and subjected to autoradiography. Lanes: 1, amido black staining of FIV proteins; 2, proteins reacted with rabbit anti-MA antipeptide antiserum; 3, proteins reacted with rabbit anti-CA antipeptide antiserum; 4, proteins reacted with rabbit anti-synthetic PR antiserum; 5, proteins reacted with a rabbit antipeptide antiserum to the N-terminal region of RT (10); 6, proteins reacted with an antipeptide antiserum to the RNase H domain of RT (10); 7, proteins reacted with a rabbit antipeptide antiserum to DU (10); 8, proteins reacted with a rabbit anti-peptide antiserum to IN (10). No antibodies were available for reaction specifically with NC. However, the N-terminal sequence of NC was the predominant signal present in the major protein species immediately below the 14.5-kDa marker (lane 1).

RESULTS

N-terminal amino acid sequences of Gag and Pol proteins. In Fig. 1, the total protein pattern of gradient-purified FIV is shown, along with Western blots in which site-directed antibodies were used to identify individual protein species (with the exception of NC [not shown]). As is typical of retroviruses, the Gag proteins of FIV are approximately 20-fold more abundant than the Pol proteins and thus dominate the staining pattern (Fig. 1, lane 1). However, with the exception of DU and MA, each protein was distinctly separated by SDS-PAGE and was thus sufficiently pure for interpretable N-terminal sequence analyses. The MA protein proved to be blocked and was therefore unavailable for Edman degradation (see below), which facilitated obtaining the N-terminal sequence of the comigrating DU protein.

Table 1 lists the N-terminal sequences of each protein species examined; these sequences were obtained by gas-phase N-terminal amino acid sequence analyses of proteins transferred to polyvinylidene difluoride membranes, as described in Materials and Methods. The prediction made earlier for the N terminus of CA (41), based on alignment with HIV CA, proved to be correct and verified results reported by others (40). However, the junctions between CA and NC of Gag, as well as between the Pol proteins, differed from that predicted by alignments with HIV sequences. The

TABLE 1. N-terminal sequences of Mature Gag and Pol proteins of FIV, as derived from Edman degradation

Protein	Amino acid residue[a] at position:				
	1	2	3	4	5
MA	–[b]	–	–	–	–
CA	Pro (136G)[c]	Ile	Gln	Thr	Val
NC	Val (367G)	Val	Gln	Ser	Lys
PR	Tyr (35P)	Asn	Lys	Val	Gly
RT	Ala (151P)	Gln	Ile	Ser	Asp
DU	Met (707P)	Ile	Ile	Glu	Gly
IN	Ser (840P)	Ser	Trp	Val	Asp

[a] Sequences shown were determined from at least three different viral protein preparations by Edman degradation as detailed in Materials and Methods.

[b] –, blocked.

[c] The numbering system corresponds to that used in Fig. 4; G, Gag polyprotein sequence numbering; P, Pol polyprotein sequence numbering.

NC N terminus found here also varied by one residue from that previously reported by others (40).

Ion spray mass spectrometry analyses of viral proteins. (i) Gag proteins. In most cases, the apparent molecular size of each viral protein, as judged by SDS-PAGE, agreed with sizes predicted from the N termini as described above. Detailed analyses by ion spray mass spectrometry, however, revealed discrepancies (Fig. 2 and 3; Table 2). The predicted mass of MA, based on cleavage to the right of Tyr-135 of Gag (see Fig. 4), agreed with the ion spray mass spectrometry value precisely, once modified by the removal of the N-terminal Met and addition of the mass corresponding to myristic acid. The N terminus of MA was also unavailable for Edman degradation, consistent with N-terminal myristoylation characteristic of many other lentiviruses (6, 11, 12; see below).

The molecular mass of CA (24,568 Da [Fig. 3]) was smaller by 954 Da than predicted if the C terminus of the protein was generated by the cleavage that yielded the N terminus of NC (see Fig. 4). The findings are thus consistent with a double cleavage between CA and NC, similar to that observed in HIV (11, 24, 31) and murine leukemia virus (13, 30).

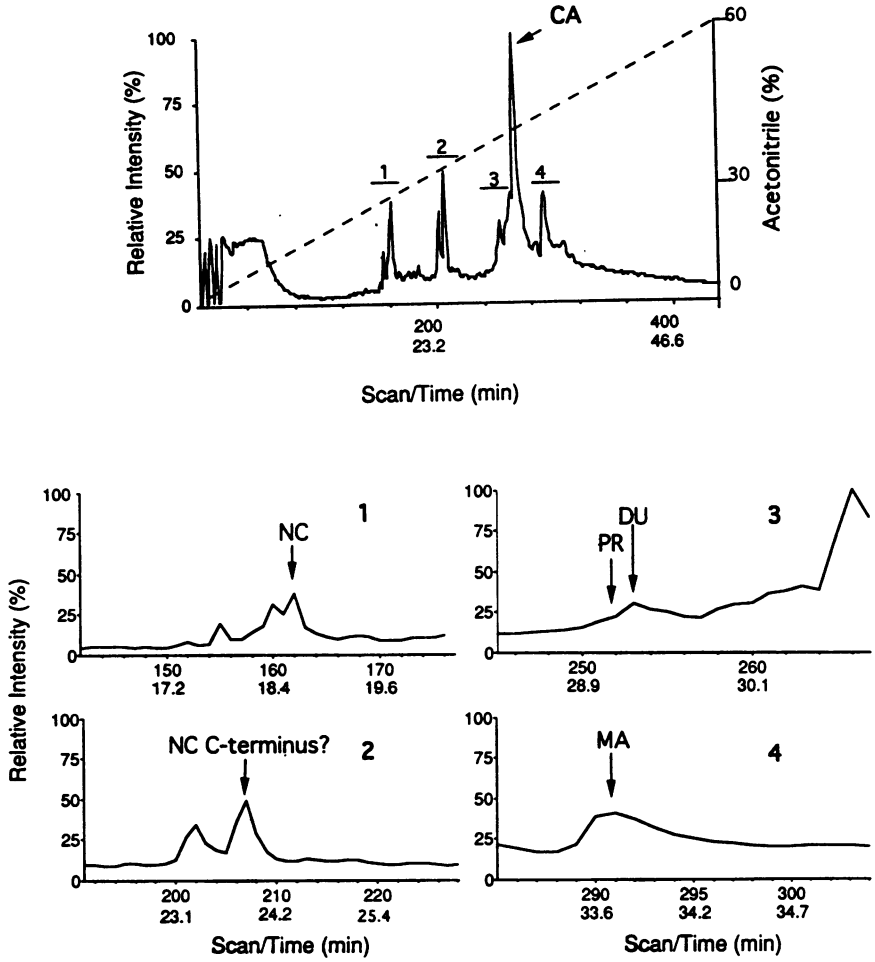

FIG. 2. HPLC separation of proteins from gradient-purified FIV. Virus was purified, disrupted in 6 M GnHCl, and applied to a reverse-phase column as detailed in Materials and Methods. The proteins were then eluted on a linear 0 to 60% CH_3CN–0.04% trifluoroacetic acid gradient and subjected to ion spray mass spectrometry. The upper panel represents the total profile of proteins eluted from the column; graphs 1 to 4 represent enhancements of regions 1 to 4, respectively, shown in the upper panel. Arrows denote points in the gradient where ion spray mass spectrometry data corresponding to each of the viral proteins were analyzed (detailed in Fig. 3).

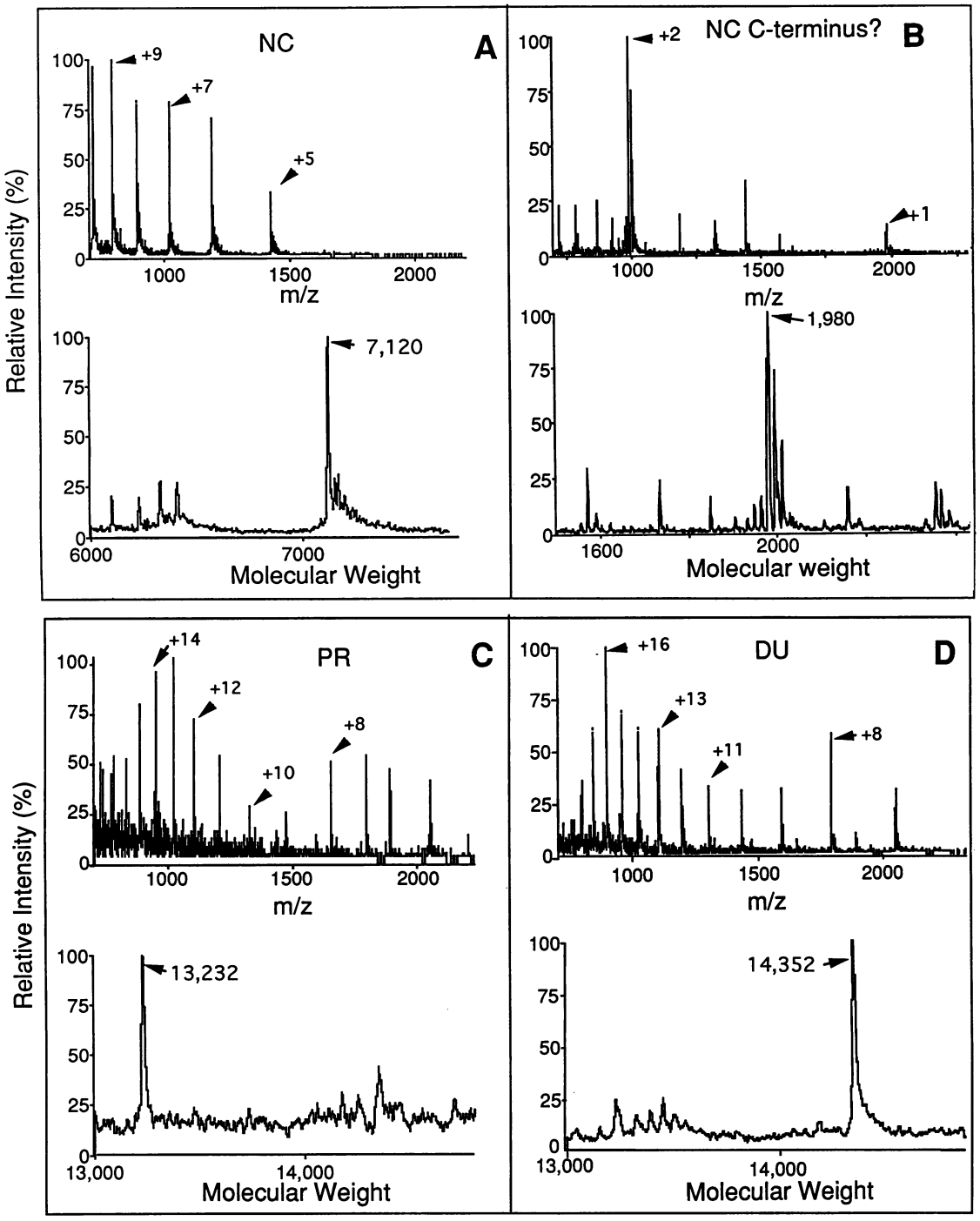

FIG. 3. Ion spray mass spectrometry of FIV proteins separated and resolved by HPLC. FIV proteins were separated as detailed in the legend to Fig. 3 and then subjected directly to ion spray mass spectrometry. The analyses shown were derived from data collected at 18.7 min for NC (A), 23.8 min for the potential NC N terminus (B), 29.1 min for PR (C), 29.2 min for DU (D), 30.4 min for CA (E), and 34.7 min for MA (F), as illustrated in Fig. 3. In each panel, the upper graph represents the data as multiple charge states of the individual protein species and the lower graph represents the reconstructed mass spectrum of each protein species after an algorithm (21, 34) was applied to convert the family of ion peaks into a single peak corresponding to the neutral molecule.

Similarly, the observed mass of NC was smaller than would be predicted if the C terminus of the protein coincided with the end of the Gag polyprotein (Table 2). The results of ion spray mass spectrometry (Fig. 3A and B) predict a cleavage at Met-432–Gln-433, on the basis of an observed NC protein of 7,120 Da and a C-terminal fragment of 1,980 Da. A peptide which may coincide with the C-terminal fragment of NC was identified in FIV virions (Fig. 3B, Table 2). Experiments are under way to further characterize this product.

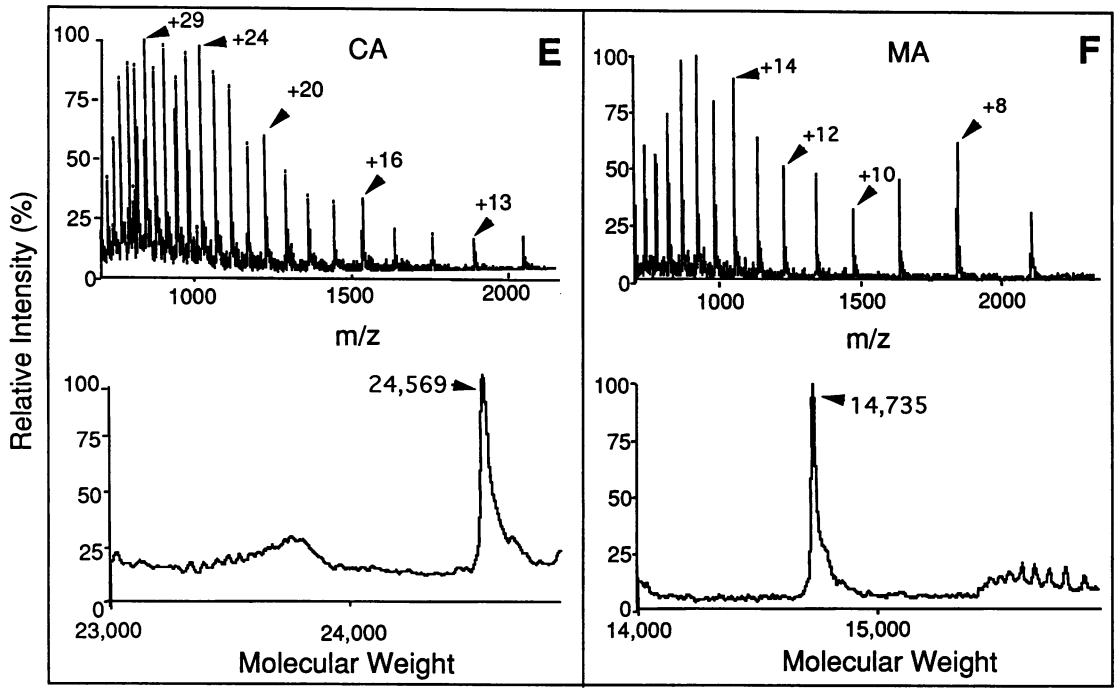

FIG. 3—*Continued.*

(ii) Pol proteins. The sizes of the Pol proteins PR, RT, DU, and IN are consistent with single cleavage events at the sites defined by the N termini of each species (Tables 1 and 2). Ion spray mass spectrometry of PR (Fig. 3) revealed a molecular mass of 13,320 Da, which is identical to the molecular mass predicted by a C-terminal cleavage after Met-150 of Pol (see Fig. 4). Likewise, the mass of DU obtained from ion spray mass spectrometry, 14,352 Da, agrees with the predicted molecular mass based on cleavages after Met-706 and Phe-839 of Pol. Although not analyzed by mass spectrometry, the molecular size of IN as judged by SDS-PAGE (Fig. 1) is consistent with a cleavage event after Phe-839 and synthesis to the end of the *pol* open reading frame.

Approximately half the RT present in virions was further processed to a truncated form lacking the C-terminal RNase H domain (Fig. 1, lane 5) (10), as with HIV RT. A summary of all the defined cleavage sites within the context of the Gag-Pol amino acid sequence is presented in Fig. 4.

Metabolic labeling of FIV with [^{3}H]myristic acid. To verify the results described above for the apparent acylation of MA, we metabolically labeled FIV-infected cells with [^{3}H]myristic acid and purified the virus by density gradient centrifugation in the presence of unlabeled virus from parallel cultures. SDS-PAGE followed by autoradiography revealed that a single radiolabeled species corresponding in molecular weight to MA was present in intact virions. Specific immune precipitation of MA by feline sera from FIV-positive cats but not by control feline sera from healthy cats verified the viral specificity of the myristoylated protein (Fig. 5).

Cleavage of synthetic peptide substrates. Definition of the limits of FIV PR allowed chemical synthesis of the entire protein and renaturation to an active form, similar to previous studies with HIV PR (37, 42). A complete description of the synthesis and cleavage specificity of synthetic FIV PR is the subject of a separate report (38a). For the purposes of the present study, the synthetic enzyme was used to verify the secondary C-terminal cleavages of CA and NC, predicted by ion spray mass spectrometry. Synthetic peptides that bridged the predicted cleavage junctions were prepared. In addition, a peptide predicted to contain the site of cleavage between the polymerase and RNase H domains of RT was synthesized, along with several of the sites defined by N-terminal sequence analyses. These peptides were then

TABLE 2. Ion spray mass-spectrometric analysis of FIV Gag-Pol proteins

Protein	Predicted mass (Da)	Observed mass (Da)	Comments
MA	14,649	14,735 ± 2	+ myrystic acid − N-terminal Met = 14,738 Da
CA	25,523	24,569 ± 3	−9 C-terminal aa,[a] = 24,569 Da
NC	9,101	7,120 ± 1	Truncated by 1,981 Da
NC C terminus (?)	1,980	1,980 ± 1	
PR	13,234	13,232 ± 2	
DU	14,352	14,352 ± 2	

[a] aa, amino acids.

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MA►
*
MGNGQGRDWKMAIKRCSNVAVGVGGKSKKFGEGNFRWAIRMANVSTGREPGDIPETLDQL   60
RLVICDLQERREKFGSSKEIDMAIVTLKVFAVAGLLNMTVSTAAAAENMYSQMGLDTRPS  120
             CA►
MKEAGGKEEGPPQAYPIQTVNGVPQYVALDPKMVSIFMEKAREGLGGEEVQLWFTAFSAN  180
               ▲
LTPTDMATLIMAAPGCAADKEILDESLKQLTAEYDRTHPPDAPRPLPYFTAAEIMGIGLT  240
QEQQAEARFAPARMQCRAWYLEALGKLAAIKAKSPRAVQLRQGAKEDYSSFIDRLFAQID  300
QEQNTAEVKLYLKQSLSIANANADCKKAMSHLKPESTLEEKLRACQEIGSPGYKMQLLAE  360
       NC►                                                ▲ KL    2
ALTKVQVVQSKGSGPVCFNCKKPGHLARQCREVKKCNKCGKPGHLAAKCWQGNRKNSGNW  420
      ▲                              PR►
EGGASCSPSESNAASSNAICTSNGGETIGFVNYNKVGTTTTLEKRPEILIFVNGYPIKFL   62
KAGRAAAPVNQMQQAVMPSAPPMEEKLLDLstop                           450
                               ▲
LDTGADITILNRRDFQVKNSIENGRQNMIGVGGGKRGTNYINVHLEIRDENYKTQCIFGN  122
                            RT►
VCVLEDNSLIQPLLGRDNMIKFNIRLVMAQISDKIPVVKVKMKDPNKGPQIKQWPLTNEK  182
                            ▲
IEALTEIVERLEKEGKVKRADSNNPWNTPVFAIKKKSGKWRMLIDFRELNKLTEKGAEVQ  242
LGLPHPAGLQIKKQVTVLDIGDAYFTIPLDPDYAPYTAFTLPRKNNAGPGRRFVWCSLPQ  302
GWILSPLIYQSTLDNIIQPFIRQNPQLDIYQYMDDIYIGSNLSKKEHKEKVEELRKLLLW  362
WGFETPEDKLQEEPPYTWMGYELHPLTWTIQQKQLDIPEQPTLNELQKLAGKINWASQAI  422
PDLSIKALTNMMRGNQNLNSTRQWTKEARLEVQKAKKAIEEQVQLGYYDPSKELYAKLSL  482
VGPHQISYQVYQKDPEKILWYGKMSRQKKKAENTCDIALRACYKIREESIIRIGKEPRYE  542
                                                   RNase H►
IPTSREAWESNLINSPYLKAPPPEVEYIHAALNIKRALSMIKDAPIPGAETWYIDGGRKL  602
                                                    ▲
GKAAKAAYWTDTGKWRVMDLEGSNQKAEIQALLLALKAGSEEMNIITDSQYVINIILQQP  662
                                            DU►
DMMEGIWQEVLEELEKKTAIFIDWVPGHKGIPGNEEVDKLCQTMMIIEGDGILDKRSEDA  722
                                            ▲
GYDLLAAKEIHLLPGEVKVIPTGVKLMLPKGYWGLIIGKSSIGSKGLDVLGGVIDEGYRG  782
                                                          IN►
EIGVIMINVSRKSITLMERQKIAQLIILPCKHEVLEQGKVVMDSERGDNGYGSTGVFSSW  842
                                                         ▲
VDRIEEAEINHEKFHSDPQYLRTEFNLPKMVAEEIRRKCPVCRIIGEQVGGQLKIGPGIW  902
QMDCTHFDGKIILVGIHVESGYIWAQIISQETADCTVKAVLQLLSAHNVTELQTDNGPNF  962
KNQKMEGVLNYMGVKHKFGIPGNPQSQALVENVNHTLKVWIQKFLPETTSLDNALSLAVH 1022
SLNFKRRGRIGGMAPYELLAQQESLRIQDYFSAIPQKLQAQWIYYKDQKDKKWKGPMRVE 1028
YWGQGSVLLKDEEKGYFLIPRRHIRRVPEPCALPEGDEstop                   1066
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FIG. 4. Amino acid sequence of the Gag and Pol polyproteins of FIV. The amino acid sequence was inferred from the nucleotide sequence as previously reported (41). Amino acid no. 1 of the Gag polyprotein corresponds to a methionine residue lost commensurate with myristoylation of Gly-2 (asterisk). Numbering for the Pol region commences with an Asn residue thought to be the first residue after the Gag-Pol frameshift (26). Arrows denote cleavage sites of FIV PR, defined in the present study by direct N-terminal sequence analyses and/or specific cleavage of synthetic peptides. The underlined Asp (Pol residue 64) corresponds to the Asp in the active site of FIV PR.

cleaved with synthetic FIV PR, and the products were analyzed by HPLC followed by ion spray mass spectrometry. In Fig. 6, the results of cleavage of the secondary site at the C terminus of CA are shown. Size measurements by ion spray mass spectrometry (Fig. 3E) indicated a molecular mass of 24,568 Da for the major form of CA in virions. This would coincide with cleavage between Leu-357 and Leu-358 of Gag (Fig. 4). The data in Fig. 6 show that FIV PR cleaves specifically and efficiently at this junction.

Additional cleavages were also defined and verified by using synthetic substrates. Results obtained by using peptides corresponding to the MA-CA, NC-PR, PR-RT, and DU-IN junctions verified predictions from N-terminal sequencing coupled with ion spray mass spectrometry (data not shown). In addition, a synthetic peptide that was predicted to contain the cleavage site (shown in boldface type) between the polymerase and RNase H domains of RT (GAE**TWY**IDKR) was prepared. The peptide was cleaved by FIV PR, coinciding to cleavage at Trp-594–Tyr-595 of Pol (Fig. 4).

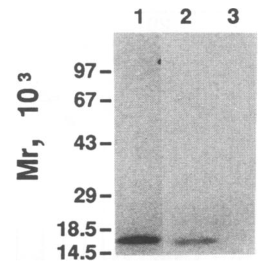

FIG. 5. FIV-infected cells were labeled with [^{3}H]myristic acid, and virus was gradient purified as detailed in Materials and Methods. Extracts were also prepared from the metabolically labeled cells, and immune precipitations were performed with sera from control and FIV-infected cats. SDS-PAGE analyses of gradient-purified FIV revealed a single radiolabeled species coinciding in mass with MA (lane 1). FIV-infected cat serum specifically precipitated the 14.5-kDa MA protein (lane 2). Immune precipitation from labeled extracts with control cat serum failed to precipitate any labeled species (lane 3). These results directly confirm analyses by ion spray mass spectrometry of MA in virions (Fig. 3).

DISCUSSION

In the present study, we have carried out analyses to define the cleavage junctions within the Gag and Pol polyproteins of FIV. These analyses revealed the specific boundaries of each *gag*- and *pol*-encoded protein species and thus detailed the specific cleavage sites used by FIV PR. The study is a necessary prelude to more detailed attempts to develop specific intervention therapies directed at the en-

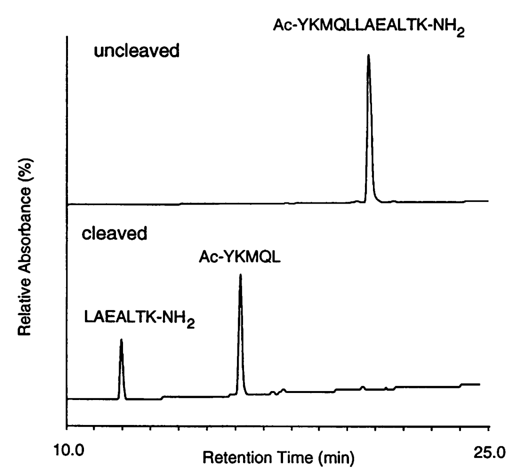

FIG. 6. Specific cleavage of a synthetic peptide corresponding to the putative C terminus of FIV CA by using synthetic FIV PR. Both the substrate peptide and the synthetic FIV PR were prepared by highly optimized stepwise solid-phase peptide synthesis (38). Ion spray mass spectrometry of the virion-borne CA had indicated that an additional cleavage occurred between Leu-357 and Leu-358 of Gag (Fig. 3; Table 2). Synthetic peptide was treated with synthetic FIV PR (pH 5.25) (0.05 M citrate, 0.1 M P_i)–1 M NaCl–0.1 mM EDTA–1 mM dithiothreitol overnight at RT. The upper panel shows reverse-phase HPLC of the substrate peptide, whose sequence is acetyl-Tyr-Lys-Met-Gln-Leu-Leu-Ala-Glu-Ala-Leu-Thr-Lys-amide. The lower panel shows products after overnight digestion with FIV PR. The two fragments resulting from cleavage by PR were characterized by mass spectrometry. The synthetic peptide was cleaved specifically at the Leu-Leu peptide bond, corresponding to the cleavage observed by mass spectrometry to have occurred in the virion.

zymes encoded by FIV, via both development of PR inhibitors and subgenomic expression of viral Gag and Pol protein constituents.

In addition to defining specific cleavage sites for PR, the above studies have defined secondary cleavages and posttranslational modifications characteristic of FIV Gag and Pol. In particular, results of ion spray mass spectrometry coupled with direct labeling with [^{3}H]myristic acid confirmed that FIV MA is myristoylated, similar to most members of the family *Retroviridae*, including HIV and simian immunodeficiency virus (6, 11, 12, 39). However, it is interesting that FIV appears to be an exception among nonprimate lentiviruses in that viruses such as equine infectious anemia virus and visna virus are not myristoylated (39). The relevance of this distinction awaits a better understanding of the role of myristoylation in the virus life cycle.

The present study revealed that the major capsid protein, CA, is processed from the polyprotein in a manner very similar to that of HIV. The N-terminal cleavage junction, from P3 to P3′, is identical to that of HIV in four of the six positions. Such a degree of homology was not observed at other cleavage junctions, suggesting that this region may afford the most appropriate template for developing peptide mimetics that may serve as general inhibitors for HIV and FIV PRs. The results further indicate a double cleavage at the C terminus of CA, similar to that observed with HIV CA (11, 24, 31).

The results with NC were particularly intriguing. Cleavage at Gln-366–Val-367 should have yielded a predominant species of 9,101 Da, terminating at the stop codon for Gag. However, the results of ion spray mass spectrometry (Fig. 3; Table 2) and matrix-assisted laser desorption (not shown) indicated that the major form of NC in virions has a molecular mass of 7,120 Da. Thus, a second cleavage event at Met-432–Gln-433 was predicted. A small peptide of appropriate molecular mass for the N terminus of NC was identified in virions (Fig. 3). Further experimentation is in progress to characterize this secondary NC cleavage by using FIV PR and synthetic peptide substrates. It will also be of interest to determine whether other secondary cleavage events occur in NC after virus infection of the target cell, similar to reports with EIAV NC (36) and possibly HIV-1 NC (3).

Defining the N and C termini of the Pol-encoded proteins has already proved useful in the synthesis and/or expression cloning of the individual protein species. These proteins can now be investigated as potential targets for virus intervention therapies. As outlined above, we have successfully synthesized FIV PR and reconstituted the enzyme in active form (38a). The enzyme proved particularly useful in the present study to verify and define certain cleavage junctions within Gag and Pol. We have also cloned and expressed active forms of FIV RT, DU, and IN in *Escherichia coli*. DU is of particular interest, since it has only recently been identified as a constituent of certain members of the family *Retroviridae* (10, 23). This genomic segment had previously been referred to as a "protease-like" region on the basis of distant homologies with aspartic proteinases (22). Why this enzyme is maintained in certain retroviruses and not in others is a point of interest and continued investigation. The results of the present study have allowed us to perform expression cloning of DU in the absence of other viral proteins (41a). The overexpressed protein can now be used in detailed kinetic and inhibitor studies as well in defining, via mutagenesis, residues critical to enzyme function.

Likewise, expression cloning of an active FIV RT has now been completed by using the data generated in this study (13a). The feline enzyme can now be modeled on the detailed structures recently established for HIV RT (2, 17) as a prelude to development of generalized inhibitors of RT and possibly of RNase H. In previous studies, the feline system has been used to analyze RT inhibitors and drug-resistant mutants (27, 28, 35). The molecular cloning of FIV RT will facilitate defined inhibitor and mutagenesis studies.

In summary, the present study provides a detailed analysis of the processing sites within the Gag and Gag-Pol polyproteins of FIV. These results can now be applied to more direct studies of the development of efficacious intervention therapies against retrovirus infections.

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REFERENCES

1. **Ackley, C. D., J. K. Yamamoto, N. Levy, N. C. Pedersen, and M. D. Cooper.** 1990. Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. J. Virol. **64**:5652–5655.
2. **Arnold, E., A. Jacobo-Molina, R. G. Nanni, R. L. Williams, X. Lu, J. Ding, A. D. Clark, Jr., A. Zhang, A. L. Ferris, P. Clark, A. Hizi, and S. H. Hughes.** 1992. Structure of HIV-1 reverse transcriptase/DNA complex at 7 Å resolution showing active site locations. Nature (London) **357**:85–89.
3. **Baboonian, C., A. Dalgleish, L. Bountiff, J. Gross, S. Oroszlan, G. Rickett, C. Smith-Burchnell, P. Troke, and J. Merson.** 1991. HIV-1 proteinase is required for synthesis of pro-viral DNA. Biochem. Biophys. Res. Commun. **179**:17–24.
4. **Barlough, J. E., C. D. Ackley, J. W. George, N. Levy, R. Acevedo, P. F. Moore, B. A. Rideout, M. D. Cooper, and N. C. Pedersen.** 1991. Acquired immune dysfunction in cats with experimentally induced feline immunodeficiency virus infection: comparison of short-term and long-term infections. J. Acquired Immune Defic. Syndr. **4**:219–227.
5. **Barlow, D. J., M. S. Edwards, and J. M. Thornton.** 1986. Continuous and discontinuous protein antigenic determinants. Nature (London) **322**:747–750.
6. **Bryant, M. L., L. Ratner, R. J. Duronio, N. S. Kishore, B. Davadas, S. P. Adams, and J. I. Gordon.** 1991. Incorporation of 12-methoxydodecanoate into the human immunodeficiency virus 1 gag polyprotein precursor inhibits its proteolytic processing and virus production in a chronically infected human lymphoid cell line. Proc. Natl. Acad. Sci. USA **88**:2055–2059.
7. **Crawford, S., and S. P. Goff.** 1985. A deletion mutation of the 5′ part of the *pol* gene of Moloney murine leukemia virus blocks proteolytic processing of the *gag* and *pol* polyproteins. J. Virol. **53**:899–907.
8. **Douillard, J. Y., and T. Hoffman.** 1983. Enzyme-linked immunosorbent assay for screening monoclonal antibody production using enzyme-labeled second antibody. Methods Enzymol. **92**: 168–174.
9. **Dow, S. W., M. L. Poss, and E. A. Hoover.** 1990. Feline immunodeficiency virus: a neurotropic lentivirus. J. Acquired Immune Defic. Syndr. **3**:658–668.
10. **Elder, J. H., D. L. Lerner, C. S. Hasselkus-Light, D. J. Fontenot, E. Hunter, P. A. Luciw, R. C. Montelaro, and J. R. Phillips.** 1991. Distinct subsets of retroviruses encode dUTPase. J. Virol. **66**:1791–1794.
11. **Henderson, L. E., M. A. Bowers, R. C. Sowder II, T. D. Copeland, G. Smythers, and S. Oroszlan.** 1992. Gag proteins of the highly replicative MN strain of human immunodeficiency virus type 1: posttranslational modifications, proteolytic pro-

cessings, and complete amino acid sequences. J. Virol. **66**:1856–1865.
12. **Henderson, L. E., H. C. Krutzsch, and S. Oroszlan.** 1983. Myristyl amino-terminal acylation of murine retrovirus proteins: an unusual posttranslational protein modification. Proc. Natl. Acad. Sci. USA **80**:339–343.
13. **Henderson, L. E., R. Sowder, T. D. Copeland, G. Smythers, and S. Oroszlan.** 1984. Quantitative separation of murine leukemia virus proteins by reversed-phase high-pressure liquid chromatography reveals newly described *gag* and *env* cleavage products. J. Virol. **52**:492–500.
13a. **Hughes, S., A. Farris, and J. Elder.** Unpublished data.
14. **Ishida, T., T. Washizu, K. Toriyabe, S. Motoyoshi, and N. C. Pedersen.** 1989. Feline immunodeficiency virus infection in cats of Japan. J. Am. Vet. Med. Assoc. **194**:221–225.
15. **Johnson, D. A., J. W. Gautsch, R. Sportsman, and J. H. Elder.** 1983. Improved technique utilizing non-fat dry milk for analysis of protein and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. **1**:3–7.
16. **Katoh, I., Y. Yoshinaka, A. Rein, M. Shibuya, T. Odaka, and S. Oroszlan.** 1985. Murine leukemia virus maturation: protease region required for conversion from "Immature" to "Mature" core form and for virus infectivity. Virology **145**:280–292.
17. **Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz.** 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256**:1783–1790.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Liu, F. T., M. Zinnecker, T. Hamaoka, and D. H. Katz.** 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry **18**:690–693.
20. **Loeb, D. D., C. A. Hutchison III, M. H. Edgell, W. G. Farmerie, and R. Swanstrom.** 1989. Mutational analysis of human immunodeficiency virus type 1 protease suggests functional homology with aspartate proteinases. J. Virol. **63**:111–121.
21. **Mann, M., C. K. Meng, and J. B. Fenn.** 1989. Interpreting mass spectra of multiply charged ions. Anal. Chem. **61**:1702–1708.
22. **McClure, M. A., M. S. Johnson, and R. F. Doolittle.** 1987. Relocation of a protease-like gene segment between two retroviruses. Proc. Natl. Acad. Sci. USA **84**:2693–2697.
23. **McGeoch, D. J.** 1990. Protein sequence comparisons show that the 'pseudoproteases' encoded by poxviruses and certain retroviruses belong to the deoxyuridine triphosphatase family. Nucleic Acids Res. **18**:4105–4110.
24. **Mervis, R. J., N. Ahmad, E. P. Lillehoj, M. G. Raum, F. H. R. Salazar, H. W. Chan, and S. Venkatesan.** 1988. The *gag* gene products of human immunodeficiency virus type 1: alignment within the *gag* open reading frame, identification of posttranslational modifications, and evidence for alternative *gag* precursors. J. Virol. **62**:3993–4002.
25. **Miyazawa, T., M. Fukasawa, A. Hasegawa, N. Maki, K. Ikuta, E. Takahashi, M. Hayami, and T. Mikami.** 1991. Molecular cloning of a novel isolate of feline immunodeficiency virus biologically and genetically different from the original U.S. isolate. J. Virol. **65**:1572–1577.
26. **Morikawa, S., and D. H. L. Bishop.** 1992. Identification and analysis of the *gag-pol* ribosomal frameshift site of feline immunodeficiency virus. Virology **186**:389–397.
27. **North, T. W., R. C. Cronn, K. M. Remington, R. T. Tandberg, and R. C. Judd.** 1990. Direct comparisons of inhibitor sensitivities of reverse transcriptases from feline and human immunodeficiency viruses. Antimicrob. Agents Chemother. **34**:1505–1507.
28. **North, T. W., G. L. North, and N. C. Pedersen.** 1989. Feline immunodeficiency virus, a model for reverse transcriptase-targeted chemotherapy for acquired immune deficiency syndrome. Antimicrob. Agents Chemother. **33**:915–919.
29. **Olmsted, R. A., V. M. Hirsch, R. H. Purcell, and P. R. Johnson.** 1989. Nucleotide sequence analysis of feline immunodeficiency virus: genome organization and relationship to other lentiviruses. Proc. Natl. Acad. Sci. USA **86**:8088–8092.
30. **Oroszlan, S., L. E. Henderson, J. R. Stephenson, T. D. Copeland, C. W. Long, J. N. Ihle, and R. V. Gilden.** 1978. Amino- and carboxyl-terminal amino acid sequences of proteins coded by *gag* gene of murine leukemia virus. Proc. Natl. Acad. Sci. USA **75**:1404–1408.
31. **Oroszlan, S., and R. B. Luftig.** 1990. Retroviral proteinases. Curr. Top. Microbiol. Immunol. **157**:153–185.
32. **Pedersen, N. C., E. W. Ho, M. L. Brown, and J. K. Yamamoto.** 1987. Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. Science **235**:790–793.
33. **Phillips, T. R., R. L. Talbott, C. Lamont, S. Muir, K. Lovelace, and J. H. Elder.** 1990. Comparison of two host cell range variants of feline immunodeficiency virus. J. Virol. **64**:4605–4613.
34. **Reinhold, B. B., and V. N. Reinhold.** 1992. Electrospray ionization mass spectrometry: deconvolution by an entropy-based algorithm. J. Am. Soc. Mass Spectrom. **3**:207–215.
35. **Remington, K. M., B. Chesebro, K. Wehrly, N. C. Pedersen, and T. W. North.** 1991. Mutants of feline immunodeficiency virus resistant to 3′-azido-3′-deoxythymidine. J. Virol. **65**:308–312.
36. **Roberts, M. M., T. D. Copeland, and S. Oroszlan.** 1991. *In situ* processing of a retroviral nucleocapsid protein by the viral proteinase. Protein Eng. **4**:695–700.
37. **Schneider, J., and S. B. H. Kent.** 1988. Enzymatic activity of a synthetic 99 residue protein corresponding to the putative HIV-1 protease. Cell **54**:363–368.
38. **Schnolzer, M., P. Alewood, D. Alewood, and S. B. H. Kent.** *In situ* neutralization in boc-chemistry solid phase peptide synthesis. Int. J. Peptide Protein Res., in press.
38a. **Schnölzer, M., J. H. Elder, and S. B. H. Kent.** Unpublished data.
39. **Schultz, A. M., L. E. Henderson, and S. Oroszlan.** 1988. Fatty acylation of proteins. Annu. Rev. Cell Biol. **4**:611–647.
40. **Steinman, R., J. Dombrowski, T. O'Connor, R. C. Montelaro, Q. Tonelli, K. Lawrence, C. Seymour, J. Goodness, N. C. Pedersen, and P. R. Andersen.** 1990. Biochemical and immunological characterization of the major structural proteins of feline immunodeficiency virus. J. Gen. Virol. **71**:701–706.
41. **Talbott, R. L., E. E. Sparger, K. M. Lovelace, W. M. Fitch, N. C. Pedersen, P. A. Luciw, and J. H. Elder.** 1989. Nucleotide sequence and genomic organization of feline immunodeficiency virus. Proc. Natl. Acad. Sci. USA **86**:5743–5747.
41a. **Wagaman, P. C., and J. H. Elder.** Unpublished data.
42. **Wlodawer, A., M. Miller, M. Jaskolski, B. K. Sathyanarayana, E. Baldwin, I. T. Weber, L. M. Selk, L. Clawson, J. Schneider, and S. B. H. Kent.** 1989. Crystal structure of synthetic HIV-1 protease: conserved fold in retroviral proteases. Science **245**:616–621.
43. **Yamamoto, J. K., H. Hansen, E. W. Ho, T. Y. Morishita, T. Okuda, T. R. Sawa, R. M. Nakamura, W. P. Kau, and N. C. Pedersen.** 1989. Epidermiologic and clinical aspects of feline immunodeficiency virus infection in cats from the continental United States and Canada and possible mode of transmission. J. Am. Vet. Med. Assoc. **194**:213–220.
44. **Yamamoto, J. K., T. Okuda, C. D. Ackley, H. Louie, E. Pembroke, H. Zochlinski, R. J. Munn, and M. B. Gardner.** 1991. Experimental vaccine protection against feline immunodeficiency virus. AIDS Res. Hum. Retroviruses **7**:911–922.
45. **Yamamoto, J. K., E. Sparger, E. W. Ho, P. R. Andersen, P. O'Connor, C. P. Mandell, L. Lowenstine, R. Munn, and N. C. Pedersen.** 1988. The pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. Am. J. Vet. Res. **49**:1246–1258.