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Immunological Characterization of the *gag* Gene Products of Bovine Immunodeficiency Virus

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The bovine immunodeficiency virus (BIV) *gag* gene encodes a 53-kDa precursor ($Pr53^{gag}$) that is involved in virus particle assembly and is further processed into the putative matrix (MA), capsid (CA), and nucleocapsid (NC) functional domains in the mature virus. Gag determinants are also found in the Gag-Pol polyprotein precursor. To immunologically identify the major precursors and processed products of the BIV *gag* gene, monospecific rabbit sera to recombinant BIV MA protein and $Pr53^{gag}$ and peptides predicted to correspond to the CA and NC proteins and the MA-CA cleavage site were developed and used in immunoprecipitations and immunoblots of BIV antigens. Monospecific antisera to native and recombinant human immunodeficiency virus type 1 proteins were also used to identify analogous BIV Gag proteins and to determine whether cross-reactive epitopes were present in the BIV Gag precursors or processed products. The BIV MA, CA, and NC Gag proteins were identified as p16, p26, and p13, respectively. In addition to BIV $Pr53^{gag}$, the major Gag precursor, two other Gag-related precursors of 170 and 49 kDa were identified that have been designated $pPr170^{gag\text{-}pol}$ and $Pr49^{gag}$, respectively; $pPr170^{gag\text{-}pol}$ is the Gag-Pol polyprotein precursor, and $Pr49^{gag}$ is the transframe Gag precursor present in $pPr170^{gag\text{-}pol}$. Several alternative Gag cleavage products were also observed, including p23, which contains CA and NC determinants, and p10, which contains a peptide sequence conserved in the CA proteins of most lentiviruses. The monospecific antisera to human immunodeficiency virus type 1 CA (p24) and NC (p7) proteins showed cross-reactivity to and aided in the identification of analogous BIV proteins. Based on the present data, a scheme for the processing of BIV Gag precursors is proposed.

Bovine immunodeficiency virus (BIV) is an infectious pathogenic lentivirus of cattle (12) that is purported to be associated with lymphadenopathy, lymphocytosis, central nervous system lesions, progressive weakness, and emaciation (4, 52). The animal lentiviruses are similar to their human counterparts in many important aspects of their biology (11, 13). Because of these biologic similarities and their genetic relationship to human immunodeficiency virus type 1 (HIV-1), the nonhuman lentiviruses have been recognized as potentially useful models for understanding the pathogenesis of HIV-1 and evaluating methods for effective treatment and control of viral infection (9, 14, 15).

As a first step towards understanding the molecular basis of BIV replication and the host response to BIV infection, two biologically active proviruses of BIV have been molecularly cloned (3) and sequenced (10). The BIV genome contains the *gag*, *pol*, and *env* structural genes and a complex central region that codes for the nonstructural/regulatory genes *vif*, *tat*, *rev*, *W*, and *Y* (10, 36). The peptide composition of the major precursor and processed products of BIV structural genes has been deduced from translations of their nucleotide sequences (10). The predicted BIV Gag and Env precursors, $Pr53^{gag}$ and gPr145, respectively, have been confirmed experimentally by selective expression of the BIV *gag* and *env* open reading frames (ORFs) in the baculovirus-insect cell system (40, 41). $Pr53^{gag}$ is believed to be processed into the following mature Gag proteins: matrix (MA), p16; capsid (CA), p26; and nucleocapsid (NC), p13 (10).

The identification of BIV proteins is an important step towards obtaining a better understanding of the immune response of virus-infected animals and determining the function of individual proteins. Only one of the putative mature BIV Gag proteins, the p26 CA protein, has been subjected to cross-serological analyses with antisera to HIV-1 and other lentiviruses (12). Using Western immunoblotting and immunofluorescence assays, Gonda et al. (12) demonstrated the existence of conserved epitopes between the CA proteins of BIV (p26) and HIV-1 (p24). Several additional studies have reported observing numerous low- and high-molecular-weight BIV-specific protein bands in immunoblots with sera from infected animals and animals hyperimmunized with whole virus or recombinant BIV $Pr53^{gag}$ (*rec* Pr53) (4, 27, 38, 40, 41, 56, 57). It is unclear from most of these studies whether many of the recognized bands were related to Gag or Env proteins. Further immunologic characterization requires the use of monospecific reagents made to the individual proteins, for example, through the development of monoclonal antibodies (27) and the use of antisera produced to individually expressed proteins and synthetic peptides.

In the present study, sera obtained from BIV-infected animals and antisera to purified virions, synthetic peptides, and recombinant proteins of BIV were used to identify the major BIV MA-, CA-, NC-related products expressed by BIV-infected bovine cells and virions. High-titered antisera to purified Gag proteins of HIV-1 were also used to determine whether cross-reactive epitopes were present and to assist in the identification of analogous proteins. These studies also identified the Gag-Pol polyprotein and the transframe Gag precursors. Processing of these precursors can be potentially quite complex and can yield a multitude of subunits, some with multiple Gag determinants. Using data derived from this study and previous observations (40), we

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TABLE 1. Amino acid compositions of BIV Gag synthetic peptides

Peptide	Amino acid positions[a] (nucleotide positions)	Amino acid sequence
MA-CA	123–145 (682–750)	KSIYPSLTQNTQNKKQTSNQTNT
CA	286–305 (1171–1230)	GPINIHQGPKEPYTDFINRL
NC*cys*	398–419 (1507–1572)	GPEDGRRCYGCGKTGHLKRNCK
NC*term*	442–461 (1639–1698)	KCSSAPYGQRSQPQNNFHQS

[a] Nucleotide and amino acid positions are those for BIV127 as reported by Garvey et al. (10). Amino acid positions are those for the *gag* ORF (Fig. 1).

have proposed a tentative mechanism for the cleavage of the BIV Gag-related precursors.

MATERIALS AND METHODS

Virus and cell culture. BIV127-infected bovine leukocyte adherent cells (BLAC-20) and uninfected BLAC-20 cultures were propagated in Dulbecco's modified Eagle's medium (GIBCO) as described previously (10, 38). BIV virions were purified and concentrated from the supernatants of BIV-infected cultures by previously published methods (2). Recombinant baculoviruses expressing BIV *rec* Pr53 (40) and recombinant HIV-1 Pr55^{gag} (*rec* Pr55) (51) were propagated in Sf-9 cells; pseudovirions containing the Gag precursors were purified and concentrated from infected insect cell culture supernatants, as described before (40).

Radiolabeling of native and recombinant BIV proteins. BIV127-infected BLAC-20 cultures were radiolabeled with 100 μCi each of [^{35}S]methionine and [^{35}S]cysteine (Amersham Corp.) per ml in methionine- and cysteine-deficient Dulbecco's modified Eagle's medium, chased in complete Dulbecco's modified Eagle's medium, and lysed in NET lysis buffer (400 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% Triton X-100, 0.01 IU of alpha-2-macroglobulin per ml, 10 μg of aprotinin per ml) as described before (40, 41). Radiolabeling times and chase periods are indicated in the figure legends. For radiolabeled virions and pseudovirions (virus-like particles containing *rec* Pr53), low-speed-clarified (250 × *g*) culture supernatants were sedimented (100,000 × *g*) through 20% (wt/vol) buffered sucrose cushions for 2 h at 4°C. The resultant pellets were resuspended in NET lysis buffer. Prior to radioimmunoprecipitation, virions and pseudovirions containing *rec* Pr53 were subjected to further denaturation by boiling in the presence of 1% sodium dodecyl sulfate (SDS) for 10 min, dilution (1:10) in NET lysis buffer, and clarification at 100,000 × *g* for 30 min at 4°C.

Synthetic peptides. Four oligopeptides were selected from the BIV *gag* gene translation (10) for commercial synthesis (Peninsula Laboratories, Inc., and Multiple Peptide Systems). The amino acid composition of the peptides is shown in Table 1, and the locations of these peptides within the *gag* ORF are shown in Fig. 1. The MA-CA peptide incorporates sequences predicted to overlap the MA-CA junction in the Gag precursor. The CA peptide was chosen because it contains the conserved CA epitope common to p26 of BIV and p24 of HIV-1 and the CA proteins of several other lentiviruses (10); this peptide is 45% identical and 90% similar (no gaps) to the corresponding peptide from HIV-1. Analysis was conducted with the GAP program in the University of Wisconsin Genetics Computer Group (UWGCG) Sequence Analysis Package (6). The NC*cys* peptide represents sequences in the first cysteine-rich region of the predicted BIV NC protein; these sequences are 45%

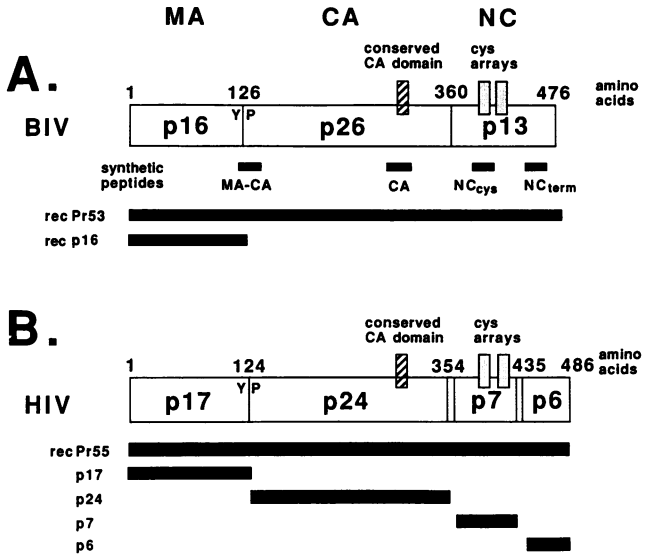

FIG. 1. Schematic representation of the BIV and HIV-1 *gag* ORFs, showing regions of Gag precursors and cleavage products that were used to produce monospecific reagents to immunologically characterize BIV Gag proteins. Standard nomenclature and acronyms for retrovirus Gag proteins are used (33). (A) The BIV *gag* ORF encodes Pr53, the Gag precursor (40), which is predicted to be processed into at least three mature Gag proteins: p16 (MA), p26 (CA), and p13 (NC) (10). (B) The HIV-1 *gag* ORF encodes Pr55, the Gag precursor, which is further processed into four mature Gag proteins: p17 (MA), p24 (CA), p7 (NC), and p6, another small protein at the carboxyl terminus of the NC protein (13). Dark bars below the Gag ORFs represent synthetic peptides and native and recombinant proteins used to make the monospecific antisera. For BIV (A), antisera were made to four Gag synthetic peptides (MA-CA, CA, NC*cys*, and NC*term*), recombinant Pr53^{gag} (*rec* Pr53), and recombinant p16 (*rec* p16). For HIV-1 (B), antisera to HPLC-purified native Gag subunits (p17, p24, p7, and p6) and to recombinant Pr55^{gag} (*rec* Pr55) were used. Boxes with hash marks represent the highly conserved CA domain in BIV (aa 293 to 302) and HIV-1 (aa 284 to 293) common to most lentiviruses (10). Shaded boxes represent the cysteine arrays or nucleic acid-binding domains found in the NC proteins of BIV (aa 405 to 418 and 423 to 436) and HIV-1 (aa 392 to 405 and 413 to 426), respectively (10). The Tyr-Pro (Y-P) cleavage sites are indicated.

identical and 82% similar (one gap; three residues) to those found in the p7 nucleocapsid NC protein of HIV-1 (10, 22). The NC*term* peptide contains sequences found in the carboxyl-terminal region of the BIV Gag precursor that were predicted not to be contained in the transframe Gag precursor; this peptide resides in the BIV Gag precursor region that is analogous to the carboxyl-terminal p6 protein of HIV-1. The translation of the BIV genome was scanned with each peptide sequence to ensure that the sequences chosen were unique to that region by using the BESTFIT program available in the UWGCG Sequence Analysis Package (6). Prior to making antibodies to the four synthetic Gag peptides, we determined whether they were immunologically reactive with antiserum made to BIV *rec* Pr53 in enzyme-linked immunosorbent assays (ELISAs); these initial data indicated that all four of the peptides were recognized by this Gag-specific antiserum, suggesting that they contained Gag epitopes.

Construction and expression of recombinant BIV MA protein. To derive an MA-specific antiserum, the predicted BIV

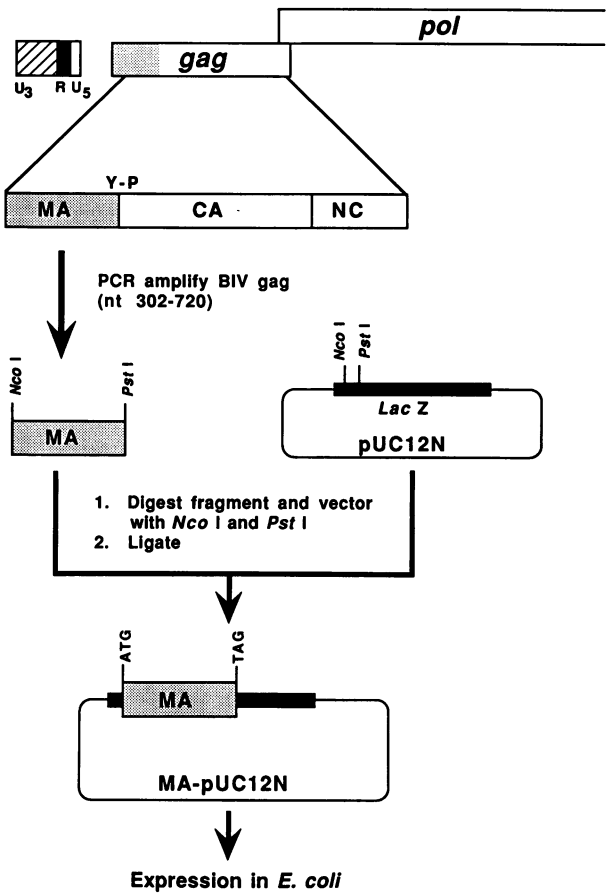

FIG. 2. Cloning strategy for construction of the plasmid for expression of recombinant BIV MA. The segment of the BIV genome encoding the 126 aa that comprise the MA coding region of the *gag* ORF was PCR amplified from a full-length functional BIV provirus. Oligonucleotide-directed mutagenesis was used to create two restriction sites (*Nco*I and *Pst*I) and a stop codon to facilitate the cloning and expression of the construct. The fragment was cloned into pUC12N and expressed in *E. coli* as a nonfusion protein.

MA coding sequences were expressed as a nonfusion protein in bacteria. As shown in Fig. 2, the MA coding region was polymerase chain reaction (PCR)-amplified from a full-length functional BIV provirus; the map positions of the synthesized oligonucleotides were those of the nucleotide sequence of BIV127 (10). The MA-CA cleavage site was predicted to be the first tyrosine-proline (amino acids [aa] 125 and 126) residues in the amino-terminal end of the *gag* ORF translation; this is the consensus sequence cleavage site (aromatic aa-proline) predicted for the MA and CA Gag subunits found in retroviruses (19, 21, 23, 24). This new assignment changes our previous prediction for the MA-CA cleavage site (aa 133 and 134) that was based on alignments with HIV-1 (10). To subclone the predicted MA coding region (nucleotides [nt] 316 to 693), oligonucleotide-directed mutagenesis was used to create an *Nco*I site with the forward primer and a *Pst*I site and a stop codon (nt 694 to 696) with the reverse primer. The forward mutagenic oligonucleotide primer for PCR synthesis of the MA coding region was GTGTTTTCCCCGccATG gAGAG (5′→3′) and the reverse mutagenic oligonucleotide primer was ttCTGTGTcTgcaGTGTTAGGGActaGTAAAT ACTCTTAATTTCGCCTTCC (5′→3′); the lowercase letters identify nucleotide changes that were introduced during PCR amplification with these oligomers. The PCR-amplified MA product was cloned into the *Nco*I and *Pst*I sites of the expression vector pUC12N (25); this plasmid was designated MA-pUC12N. Prior to expression, the DNA sequences of the MA clones were confirmed by dideoxy sequencing (46); no additional mutations were introduced during the amplification and cloning.

To express the recombinant p16 (*rec* p16), bacterial cells (DH5α strain of *Escherichia coli*; Life Technologies, Inc.) were transformed with MA-pUC12N and grown in ampicillin-containing M9 medium for 16 h (37). Cells were pelleted, washed, and lysed as previously described (26). The expressed recombinant MA proteins from recombinant bacteria were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (32) on 16% polyacrylamide gels. The resolved proteins were visualized by silver staining (Polysciences, Inc.). For gel purification of the *rec* p16, unstained gel slices containing proteins of 14 to 20 kDa were excised from the gel, electroeluted, and concentrated with a Centricon-3 microconcentrator according to the manufacturer's instructions (Amicon Corp.).

Immunological reagents. Rabbit antiserum to BIV was obtained from an animal that had been experimentally infected with BIV127 (38). Bovine antiserum was obtained from an animal that had been naturally infected with BIV; this animal was free of bovine leukemia virus. Hyperimmune antiserum directed to BIV virions was generated by immunizing a rabbit with sucrose gradient-purified whole BIV (12). Antisera to baculovirus-expressed BIV *rec* Pr53 and HIV-1 *rec* Pr55 were produced in rabbits by previously described methods (40, 51). Each peptide used in the inoculations was conjugated separately to keyhole limpet hemocyanin with glutaraldehyde (1). Animals were first inoculated with 1 mg of conjugated peptide in complete Freund's adjuvant and then with 0.2 mg in incomplete Freund's adjuvant every 2 weeks thereafter for a total of 10 weeks. The BIV *rec* p16 was introduced into a rabbit similarly except that 0.1 mg of gel-purified protein was used for each inoculation. All animals were bled by protocols approved by the Institutional Animal Care and Use Committee, and the blood was processed for serum by standard protocols. Antisera directed to high-pressure liquid chromatography (HPLC)-purified p17 (MA), p24 (CA), p7 (NC), and p6 of HIV-1_{Mn} virions (20) were raised in rabbits. Secondary-antibody reagents were purchased from Organon Teknika Corp.-Cappel and Kirkegaard & Perry Laboratories, Inc.

Immunological assays. Radioimmunoprecipitation assays (RIPAs) of [^{35}S]methionine- and [^{35}S]cysteine-labeled antigens (cell lysates, virions, and *rec* Pr53) were performed with the various BIV and HIV-1 antisera as described before (40, 41). Briefly, immune complexes formed during antigen-antibody incubations were precipitated with protein A-Sepharose CL-4B beads (Sigma), washed extensively, resuspended in SDS-PAGE sample buffer (32), resolved by SDS-PAGE, and subjected to fluorography with Amplify (Amersham Corp.). Precast Tris-glycine-polyacrylamide gels (NOVEX) were used for Western blots. Proteins separated by SDS-PAGE were electrotransferred onto either Immobilon-P (Millipore Corp.) or nitrocellulose membranes, as described previously (40). The membranes were then incubated with various antisera, and bound antibody was detected by using horseradish peroxidase-conjugated anti-rabbit or anti-bovine immunoglobulin G, as appropriate, and the enhanced chemiluminescence system (Amersham Corp.). Preimmune serum from each immunized animal was

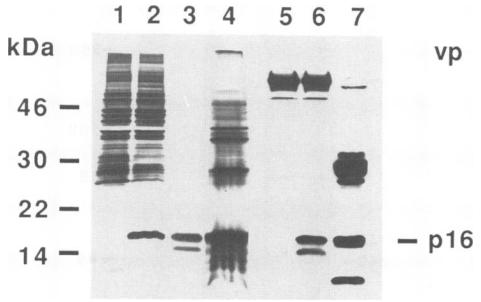

FIG. 3. SDS-PAGE and Western immunoblot analysis of recombinant BIV MA proteins expressed in *E. coli*. (Lanes 1 to 4) Silver-stained gel of *E. coli*, *E. coli*-produced recombinant BIV MA, and BIV virion proteins. (Lanes 5 to 7) Western immunoblotting of native and *E. coli*-produced proteins with a rabbit anti-*rec* Pr53 antibody. Lanes 1 and 5, pUC12N-transformed *E. coli*; lanes 2 and 6, MA-pUC12N-transformed *E. coli*; lane 3, gel-purified recombinant MA; lanes 4 and 7, BIV virions. Proteins were resolved on 16% polyacrylamide gels; the gel containing lanes 5 to 7 was transferred onto a nitrocellulose membrane prior to immunoblotting. Molecular mass markers are indicated on the left. The location of p16 (MA) from both recombinant (lanes 2, 3, and 6) and viral (lanes 4 and 7) antigens is indicated on the right side of the figure.

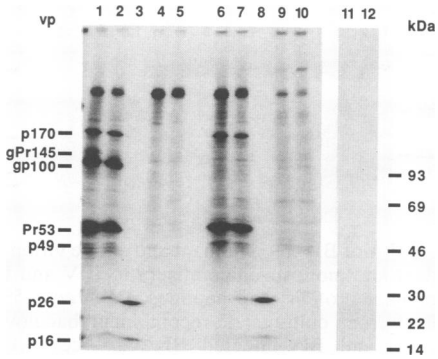

FIG. 4. RIPA of pulse-chase-labeled BIV-infected cell lysates and virions. BIV-infected BLAC-20 cells and virions were radiolabeled with ^{35}S-labeled methionine and cysteine, lysed, immunoprecipitated, and resolved by SDS-PAGE (5 to 20% polyacrylamide gradient gel), as described in Materials and Methods. Lanes 1, 4, 6, and 9, lysates from BIV-infected BLAC-20 cultures that were pulse-labeled for 20 min without chase; lanes 2, 5, 7, and 10, lysates from cultures that were pulse-labeled for 20 min followed by a 1-h chase; lanes 11 and 12, lysates from uninfected BLAC-20 cultures that were pulse-labeled for 20 min without chase; lanes 3 and 8, virions purified from cultures that were labeled for 1 h, followed by a 1 h chase. Antigens were immunoprecipitated with sera from (lanes 1 to 3 and 11) a bovine naturally infected with BIV, (lanes 4 and 5) a BIV-negative bovine, (lanes 6 to 8 and 12) a rabbit immunized with BIV *rec* Pr53, and (lanes 9 and 10) a negative-control rabbit. The BIV proteins (vp) p170 (Gag-Pol polyprotein), gPr145 (Env precursor), gp100 (surface Env protein), Pr53 (Gag precursor), p49 (Gag cleavage product from Gag-Pol precursor), p26 (CA), and p16 (MA) are designated on the left; molecular mass markers are indicated on the right side.

used as a negative control in RIPAs and immunoblots; only virus-specific proteins are identified in the reporting of the results. The percentages of acrylamide in the gels are indicated in the figure legends. ELISAs with BIV *rec* Pr53 and synthetic peptide antigens were performed as described by Rasmussen et al. (40).

RESULTS

Expression of recombinant BIV MA protein in *E. coli*. To facilitate the production of an MA-specific reagent, the segment of the BIV *gag* gene coding for the MA protein was synthesized by PCR and cloned into the bacterial expression vector pUC12N (Fig. 2). A BIV-specific 16-kDa nonfusion protein was expressed in bacteria by using the recombinant MA-pUC12N plasmid. The expressed recombinant protein appeared to migrate with the same mobility as the putative MA protein found in BIV virions; moreover, it was recognized by antiserum made to *rec* Pr53 (Fig. 3). The migration of the MA protein is consistent with its predicted molecular mass of 14.6 kDa (calculated by the PEPTIDESORT algorithm in the UWGCG Sequence Analysis Package [6]), isoelectric point of 8.34, and posttranslational modifications (e.g., fatty acid acylation). The presence of multiple BIV-specific MA bands (Fig. 3, lanes 3 and 6) may represent the initiation of translation from one or more of the six possible start codons located within the MA coding region or, alternatively, cleavage of the *rec* p16 by bacterial proteases. The *rec* p16 was gel-purified from bacterial lysates, electroeluted, and used to immunize a rabbit to produce an MA-specific antiserum for use in the studies described below.

Identification of putative BIV Gag-specific proteins. Antisera from a BIV-infected bovine (with reactivity to BIV Env and Gag determinants) and a rabbit immunized with BIV *rec* Pr53 were used to identify the native BIV Gag-related precursors and putative cleavage products in pulse and pulse-chase radiolabeling experiments (Fig. 4). In the pulse-labeling experiment (20 min), the serum from the BIV-infected bovine immunoprecipitated the putative Gag-Pol precursor (p170), Env glycoproteins (gPr145 and gp100), the major Gag precursor (Pr53^{gag}), and three bands in the 42- to 49-kDa range (Fig. 4, lane 1). In contrast, the rabbit anti-*rec* Pr53 serum did not recognize the Env glycoproteins but did immunoprecipitate the Gag-Pol precursor Pr53^{gag} and at least one of the bands (49 kDa) in the 42- to 49-kDa range (Fig. 4, lane 6). No virus-specific bands were detected when cell lysates of uninfected cultures were immunoprecipitated by antiserum from the BIV-infected bovine or the rabbit immunized with BIV *rec* Pr53 (Fig. 4, lanes 11 and 12, respectively).

In the pulse-chase labeling experiments with a 1-h chase, the serum from the BIV-infected bovine recognized the same proteins seen in the pulse-labeling experiment except for gPr145. In addition, two new proteins, p26 and p16, were observed in RIPAs with the BIV-infected bovine (Fig. 4, lane 2) and the anti-*rec* Pr53 (Fig. 4, lane 7) sera; the same two proteins were also recognized in virion antigen preparations (Fig. 4, lanes 3 and 8, respectively). Longer chase times reduced the intensity of all virus-specific bands in the lysates, indicating that the processed proteins were incorporated into virions and released from the cell. Therefore, a more accurate representation of the chase experiment is found in a comparison of lysates and virions. gPr145, gp100, p170, Pr53^{gag}, and p49 were notably absent in the virion preparation in RIPAs with either the infected bovine serum or the anti-*rec* Pr53 serum (Fig. 4, lanes 3 and 8, respectively). BIV Env glycoproteins are rarely immunoprecipitated from virions, suggesting that they are either readily shed or present in low abundance. Thus, the Gag-specific serum allowed us to discriminate between proteins containing Gag or Env epitopes in virions and cells. Furthermore, a comparison of the RIPA data with virions and pulse- and pulse-chase-labeled cell antigens suggests that the three

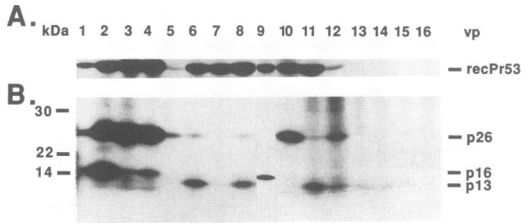

FIG. 5. RIPA of BIV recombinant and native virion Gag proteins by polyvalent and monospecific antisera to BIV and HIV-1 native and recombinant proteins and peptides. BIV *rec* Pr53 and virions were purified from cultures of recombinant baculovirus-infected Sf-9 cells (A) and BIV-infected BLAC-20 cells (B) that were radiolabeled with [^{35}S]methionine and [^{35}S]cysteine for 18 h (40). These antigens were immunoprecipitated with sera from a BIV-infected bovine (lane 1), BIV-infected rabbit (lane 2), and rabbits immunized with purified BIV virions (lane 3), BIV *rec* Pr53 (lane 4), BIV CA peptide (lane 5), BIV NC*cys* peptide (lane 6), BIV MA-CA peptide (lane 7), BIV NC*term* peptide (lane 8), BIV *rec* p16 (lane 9), HIV-1 *rec* Pr55 (lane 10), HIV-1 p7 (lane 11), HIV-1 p24 (lane 12), HIV-1 p6 (lane 13), and HIV-1 p17 (lane 14) and a negative-control rabbit (lane 15) and a BIV-negative bovine (lane 16). The precipitated proteins were resolved on 5 to 20% polyacrylamide gradient gels. Molecular mass markers are indicated on the left; BIV proteins (vp) *rec* Pr53, p26, p16, and p13 are designated on the right side.

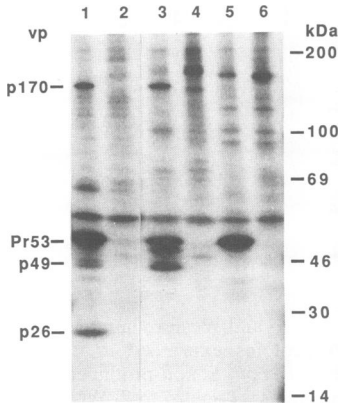

FIG. 6. RIPA of BIV-infected cell lysates with polyvalent and monospecific antisera to BIV Gag proteins. Uninfected (lanes 2, 4, and 6) and BIV-infected (lanes 1, 3, and 5) BLAC-20 cells were radiolabeled for 1 h with ^{35}S-labeled methionine and cysteine, lysed, immunoprecipitated, and resolved by SDS-PAGE (5 to 20% polyacrylamide gradient gel) as described in Materials and Methods. Antigens were immunoprecipitated with antisera from rabbits immunized with BIV *rec* Pr53 (lanes 1 and 2), BIV MA-CA peptide (lanes 3 and 4), and BIV NC*term* peptide (lanes 5 and 6). Molecular mass markers are indicated on the right; BIV proteins (vp) p170, Pr53, p49, and p26 are designated on the left side.

largest proteins (p170, Pr53^{gag}, and p49) immunoprecipitated by the Gag-specific serum are precursors that are further processed into the mature proteins found in virions as in other lentiviruses. p26 and p16 will be shown to be the CA and MA proteins, respectively, with the use of monospecific antisera in the experiments described below.

Characterization of BIV virion proteins with monospecific Gag antisera. To examine the various Gag proteins in more detail, radiolabeled *rec* Pr53 antigens and BIV virions were immunoprecipitated with a panel of virus-specific antisera (Fig. 5). This panel consisted of antisera from BIV-infected animals (bovine and rabbit) and rabbits that were hyperimmunized with BIV virions, individual proteins (including *rec* Pr53 and MA), and conjugated synthetic peptides representing domains in the CA and NC and the predicted MA-CA cleavage site. In addition, rabbit antisera made to *rec* Pr55 and HPLC-purified MA, CA, NC, and p6 HIV-1$_{Mn}$ proteins were used to detect cross-reactive epitopes between BIV and HIV-1 that would be useful in determining the identity of BIV Gag proteins. The locations of each of these Gag proteins and peptides in relation to the BIV and HIV-1 *gag* ORFs are shown in Fig. 1.

These antisera were first used to immunoprecipitate BIV *rec* Pr53 to determine whether they could recognize epitopes in the BIV Gag precursor. All of the antisera immunoprecipitated BIV *rec* Pr53 except the anti-HIV-1 p17 and p6 sera; these two anti-HIV-1 sera also did not recognize BIV virion proteins (Fig. 5). When BIV virions were used as antigens and immunoprecipitated proteins were resolved on 5 to 20% acrylamide gradient gels, a band migrating at a rate consistent with a 26-kDa protein was recognized by sera from BIV-infected animals and from rabbits immunized with BIV virions, *rec* Pr53, CA, NC*cys*, and NC*term* peptides and HIV-1 *rec* Pr55, NC, and CA proteins (Fig. 5B, lanes 1 to 6, 8, and 10 to 12, respectively). A 16-kDa protein was recognized by five antisera from the panel; these include the sera from BIV-infected animals and sera from rabbits immunized with virions and the *rec* Pr53 and *rec* MA proteins (Fig. 5B, lanes 1 to 4 and 9, respectively). A 13-kDa protein was strongly recognized by sera from rabbits immunized with the BIV NC*cys* and NC*term* peptides and HIV-1 p7 (Fig. 5B, lanes 6, 8, and 11, respectively) and weakly recognized by the BIV-infected rabbit serum and rabbit antisera to BIV virions, *rec* Pr53, and HIV-1 p24 (Fig. 5B, lanes 2 to 4 and 12, respectively). The unexpected precipitation of a 26-kDa BIV protein by NC-specific antiserum and a 13-kDa protein by HIV-1 CA antiserum could be due to a complexing of the NC and CA proteins during immunoprecipitation; however, the results of immunoblots of virion proteins maintained under more stringent denaturing conditions and run in SDS-PAGE, shown below, suggest that this is probably not the case. Negative-control rabbit and bovine sera did not immunoprecipitate BIV *rec* Pr53 or any BIV virion proteins (Fig. 5A and B, lanes 15 and 16, respectively).

Radiolabeled BIV-infected cell lysates were also immunoprecipitated with all antisera in the panel to determine whether epitopes recognized in the BIV Pr53^{gag} would also be recognized in the putative Gag-Pol precursor (p170). All antisera to BIV recognized the Gag-Pol precursor and p49 except the antiserum to the BIV NC*term* peptide. In Fig. 6, the nonrecognition of p170 and p49 by the BIV NC*term* peptide serum (lane 5) and, for comparison, the positive reactivity of anti-BIV *rec* Pr53 (lane 1) and anti-BIV MA-CA peptide (lane 3) sera are shown. The HIV-1 MA and p6 sera also did not recognize these two proteins (data not shown).

Western blot analysis of BIV Gag proteins contained in virions. The reactivity of BIV-infected animal sera to whole virus was determined by Western blots (Fig. 7). To resolve the entire antigenic spectrum contained in BIV virions, 8 to 16% polyacrylamide gradient gels are used routinely in diagnostic immunoblots with BIV reactive sera. In our experience with a multitude of BIV-infected bovine and rabbit sera, we typically find BIV protein bands in Western blots representing the Env precursor, surface, and transmembrane proteins (gPr145, gp100, and gp45, respectively); these viral glycoproteins have been previously identified and characterized by using Env-specific sera (41). In addition, protein bands related to Pr53^{gag}, p49, p26, p16, p13, and two

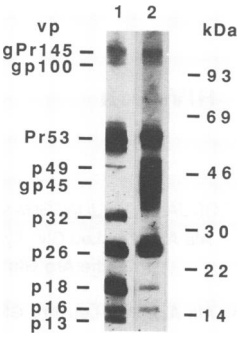

FIG. 7. Diagnostic Western immunoblot of BIV proteins. Purified BIV virions were resolved on an 8 to 16% polyacrylamide gradient gel and transferred onto an Immobilon-P membrane. Antisera used were from a naturally infected bovine (lane 1) and a rabbit experimentally infected with BIV (lane 2). BIV proteins (vp) gPr145, gp100, Pr53, p49, gp45, p32, p26, p18, p16, and p13 are designated on the left; molecular mass markers are indicated on the right side.

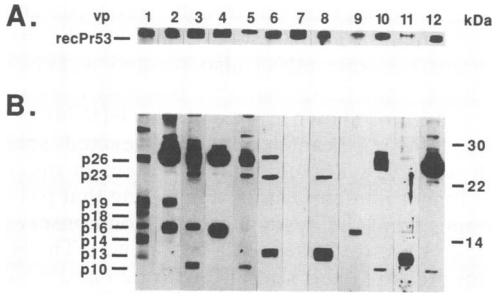

FIG. 8. Western immunoblot analysis of recombinant and native BIV Gag proteins. The *rec* Pr53 protein (A) and purified BIV virions (B) were resolved on 12 and 16% polyacrylamide gels, respectively, and transferred onto Immobilon-P membranes. These membranes were reacted with antisera from a BIV-infected bovine (lane 1), a BIV-infected rabbit (lane 2), and rabbits immunized with purified BIV virions (lane 3), BIV *rec* Pr53 (lane 4), BIV CA peptide (lane 5), BIV NC*cys* peptide (lane 6), BIV MA-CA peptide (lane 7), BIV NC*term* peptide (lane 8), BIV *rec* p16 (lane 9), HIV-1 *rec* Pr55 (lane 10), HIV-1 p7 (lane 11), and HIV-1 p24 (lane 12). Molecular mass markers are indicated on the right; BIV proteins (vp) *rec* Pr53, p26, p23, p19, p18, p16, p14, p13, and p10 are designated on the left side.

unidentified viral proteins (p32 and p18) are present. The serum from the BIV-infected bovine used in this study recognized gPr145, gp100, Pr53^{gag}, p49, p32, p26, p18, p16, and p13 (Fig. 7, lane 1). The serum from the BIV-infected rabbit recognized a similar set of proteins except that the p32 and p13 bands were not present, and gp45 was observed (Fig. 7, lane 2). Although the above-mentioned virus-specific protein bands are typically recognized, there is considerable diversity among antisera obtained from BIV-infected animals in terms of relative reactivity for individual proteins. This diversity can even be seen in sequential serum samples from individual animals (data not shown).

To serologically identify the individual viral proteins further, we used a panel of polyvalent and monospecific antisera in Western blots of BIV *rec* Pr53 and disrupted BIV virions, which were resolved on 12% and 8 to 16% polyacrylamide gels, respectively. As observed in RIPAs, all of the antisera reacted with *rec* Pr53 in Western blots (Fig. 8A) except the anti-HIV-1 p17 and p6 sera (data not shown). The results obtained with virions resolved on gradient gels in Western blots were also similar to the previous RIPA results (Fig. 5), including the recognition of a 26-kDa and a 13-kDa protein by the NC- and CA-specific antisera, respectively (data not shown). These data suggested to us that several of the individual lower-molecular-weight viral protein bands resolved on gradient gels contained a mixture of viral Gag proteins related to the BIV CA or NC protein, obscuring the identification of these proteins by monospecific sera in these assays.

To test this hypothesis and to better resolve the lower-molecular-weight proteins in the 13- to 26-kDa range observed in the gradient gels, we repeated immunoblots with 16% polyacrylamide gels (Fig. 8B). Data from this experiment confirmed the complexity of the BIV-specific bands. Serum from the BIV-infected bovine reacted with p26, p19, p18, p16, p14, and p13 (Fig. 8B, lane 1). The p19 and p18 viral proteins (Fig. 8B, lane 1) appear to have resolved from the single p18 band identified in the previous immunoblots with the BIV-infected bovine serum (Fig. 7, lane 1); they were not immunoprecipitated from virions with any of the sera in the panel (Fig. 5B). Similarly, the p14 and p13 bands (Fig. 8B, lane 1) were resolved from the single p13 band (Fig. 7, lane 1). Serum from the BIV-infected rabbit was strongly reactive with p26, p19, and p16 (Fig. 8B, lane 2). Antisera to purified BIV virions and *rec* Pr53 also recognized p26 and p16 (Fig. 8B, lanes 3 and 4, respectively). A viral protein of 10 kDa (p10) was also identified by the antiserum to BIV virions (Fig. 8B, lane 3) and antisera from many other BIV-infected animals (data not shown). The 26-kDa band resolved by the BIV-infected rabbit serum and antisera to BIV virions and *rec* Pr53 appeared to be a complex of viral proteins (Fig. 8B, lanes 2, 3, and 4). One protein with an apparent molecular mass of 23 kDa (p23) was strongly recognized by the antiserum to BIV virions (Fig. 8B, lane 3) but only weakly recognized by the BIV-infected rabbit and anti-BIV *rec* Pr53 sera (Fig. 8B, lanes 2 and 4, respectively). A 24-kDa BIV protein was observed previously in immunoblots with antisera from BIV-infected animals, but its composition had not been elucidated (56, 57). Data obtained with the monospecific sera in Western blots (shown below) will demonstrate that both p23 and p10 contain BIV CA epitopes and that p23 contains both CA and NC determinants.

To further characterize each of the mature BIV Gag proteins and to confirm the cross-reactivity between BIV and HIV-1 observed in RIPAs, we reacted monospecific rabbit antisera made to BIV Gag peptides and proteins and HPLC-purified HIV-1 Gag proteins with BIV virions in immunoblots. As expected, the serum made to the BIV CA peptide reacted with p26, the CA protein; in addition, it also reacted with p23 and p10 (Fig. 8B, lane 5). The BIV NC*cys* peptide antiserum reacted with a 26-kDa protein, p23, and p13 (Fig. 8B, lane 6). This antiserum also demonstrated reactivity with an 11-kDa protein in longer exposures of this blot (data not shown); the significance of p11 will be discussed below. The BIV NC*term* peptide antiserum reacted strongly with p23 and p13 (Fig. 8B, lane 8). The reactivity of the NC*cys*, NC*term*, and CA antisera to p23 strengthens the notion that p23 contains determinants from both the conserved CA domain and the amino and carboxyl termini of the BIV NC protein. Again, as in the RIPA, the MA-CA peptide antiserum did not react with any BIV virion proteins in the 16- to 26-kDa region (Fig. 8B, lane 7). In contrast, the BIV *rec* p16 antiserum was reactive with p16, the putative MA protein (Fig. 8B, lane 9).

The HIV-1 *rec* Pr55 antiserum reacted strongly with BIV p26 and p10 (Fig. 8B, lane 10). The rabbit anti-HIV-1 p7

antiserum, however, reacted strongly to BIV p13 and weakly to bands at 26 and 16 kDa (Fig. 8B, lane 11); the preimmune serum from the same rabbit also reacted weakly with the 16-kDa band, indicating that this was not a virus-specific reaction (data not shown). As with the BIV NC*cys* antiserum, the HIV-1 p7 antiserum also reacted specifically, although weakly, with the 11-kDa protein in other experiments at longer film exposures, suggesting that p11 contains NC epitopes from the cysteine-rich region conserved in the NC proteins of retroviruses (data not shown). The HIV-1 CA antiserum cross-reacted strongly with p26 and weakly with p23 and p10 (Fig. 8B, lane 12).

Analysis of the putative BIV Gag-Pol transframe coding region. The *pol* gene product precursor of all retroviruses analyzed to date has been shown to be expressed as a Gag-Pol polyprotein (29, 53, 55). In some retroviruses, like Moloney murine leukemia virus, the *gag* and *pol* genes are separated by a UAG codon. In 5 to 10% of the translations, the termination is read through, with the insertion of a glutamine residue (31, 58). In other retroviruses, such as Rous sarcoma virus, the *gag* and *pol* genes overlap and are in different reading frames, with *pol* being in a −1 phase with respect to *gag* (30, 48). The *gag* and *pol* gene topography of lentiviruses is similar to that of Rous sarcoma virus. For example, in HIV-1, the *gag* and *pol* gene ORFs overlap by 241 nt (29, 42, 54). HIV-1 Pol is expressed as a Gag-Pol polyprotein of about 160 kDa by a −1 ribosomal frameshift (29) occurring at the UUA codon at nt 2094 (22); the transframe peptide of HIV-1_{Mn} has been isolated and sequenced (22). Jacks et al. (29) proposed that a stem-loop structure located downstream of the frameshift site may be important in facilitating frameshifting in HIV-1. In addition, a pseudoknot structure can potentially form between nucleotides in the loop and a short sequence located further downstream of the frameshift (5). Both the stem-and-loop and pseudoknot structures have been implicated in promoting frameshifting in retroviruses, since secondary structures in the RNA may form translational barriers that lead to low-frequency frameshifting.

Because we found that the putative Gag-Pol precursor protein (p170) was recognized by the BIV NC*cys* peptide antiserum but not by the BIV NC*term* peptide antiserum, we predicted that the BIV Gag-Pol frameshift was located in the nucleotide sequences between those coding for the two peptides. Therefore, we analyzed this 164-nt overlap in the BIV *gag* and *pol* coding sequences for the presence of RNA elements that have been implicated in the translational frameshifting of other lentiviruses and compared it with that of HIV-1_{Mn} (22, 50). The HIV-1_{Mn} frameshifting signal is the UUUUUUA heptanucleotide, which is followed by a predicted stem-loop structure (Fig. 9A). At BIV nt 1629, we found a putative heptanucleotide frameshift signal (AAAA AAC) that was immediately followed by a potential stem-loop structure (Fig. 9B). The BIV stem-loop structure, which contains 12 bp in the stem and 5 nt in the loop, is predicted to form at 8 nt downstream from the heptamer. A potential pseudoknot structure may also form between four nucleotides in the loop (TTAT) and the four nucleotides starting at nt 1761 (ATAA). The BIV heptanucleotide, AAA AAAC, is identical to the equine infectious anemia virus (EIAV) heptanucleotide (50). In HIV-1, the p6 protein sequences are omitted from the Gag-Pol polyprotein as a result of the frameshift (13, 22). In BIV, the predicted frameshift would delete approximately 4 kDa from the carboxyl terminus of $Pr53^{gag}$, yielding a protein of 49 kDa; a protein of that size has been detected in immunoblots (Fig. 7)

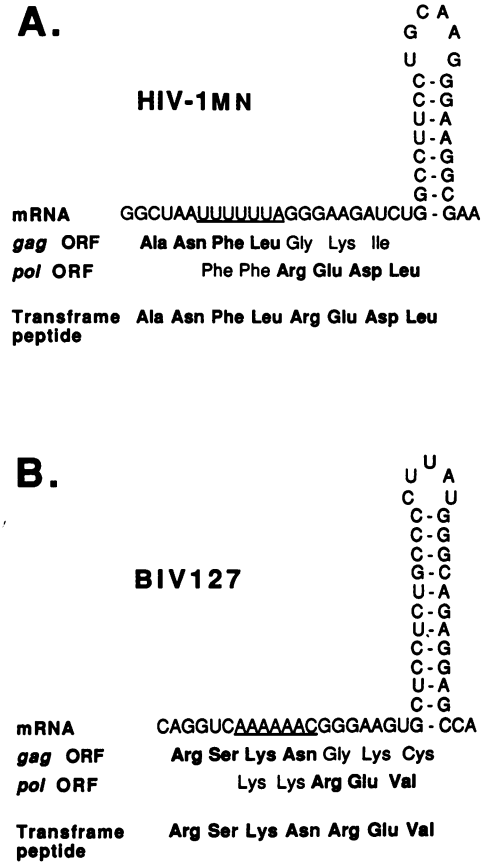

FIG. 9. Predicted HIV-1_{Mn} and BIV127 Gag-Pol transframe peptides and RNA secondary structures at the Gag-Pol frameshift. (A) HIV-1_{Mn} Gag-Pol transframe peptide deduced from translation of viral RNA, based on a −1 frameshift from the *gag* into the overlapping *pol* ORF at the underlined heptanucleotide frameshift sequence. (B) BIV127 Gag-Pol transframe peptide predicted by comparison to HIV-1_{Mn} and other predicted lentivirus frameshift regions. Amino acid residues deduced from translation of the *gag* and *pol* ORFs that contribute to the Gag-Pol polyprotein are shown in boldface.

and RIPAs (Fig. 4) in the present report and in previous studies (40). Interestingly, the first two amino acids in the amino termini of both the HIV-1_{Mn} and BIV Pol sequences in their respective Gag-Pol polyproteins would be Arg-Glu before the sequences diverge (Fig. 9).

DISCUSSION

Lentivirus *gag* and *pol* gene products are translated from the full-length viral RNA and are believed to be synthesized as a Gag precursor and a larger Gag-Pol polyprotein precursor. These precursors are cleaved by the aspartic acid-type viral protease into various Gag and Pol products (49). The Gag precursor is processed into at least three major proteins, representing the MA, CA, and NC functional domains. In the case of HIV-1 and EIAV, the Gag precursor is a protein of 55 kDa (24, 28, 34, 45); four major products of these Gag precursors have been identified. Three of these proteins represent the functional MA, CA, and NC proteins; the fourth protein, p6 and p9 in HIV-1 and EIAV, respectively, is derived from amino acids at the carboxyl end of the NC protein (8, 21, 24). Sequences coding for these proteins are located in the *gag* ORF in the region overlapping the *pol*

ORF. The Gag-Pol polyprotein precursors of HIV-1 and EIAV have been identified (29, 44). Fewer Gag proteins are derived from cleavage of the Gag-Pol polyprotein precursor than from the Gag precursor in HIV-1 or EIAV because the Gag-Pol polyprotein precursor does not contain a p6 (as in HIV-1) or a p9 (as in EIAV). The p6 and p9 polypeptides are missing because the site for the Gag-Pol frameshift in HIV-1 and EIAV, respectively, precedes the sequences encoding them. Although the functions of the HIV-1 p6 and EIAV p9 domains have not been confirmed, one study has suggested that HIV-1 p6 is involved in virus particle formation (16).

To immunologically identify and gain insight into the processing of Gag-containing precursors and products, we used a panel of antisera from BIV-infected animals and animals immunized with BIV virions, *rec* Pr53, *rec* p16, synthetic peptides, and HIV-1 *rec* Pr55, p17, p24, p7, and p6 in RIPAs and Western blots. Our present data demonstrate that processing of the BIV $Pr53^{gag}$ is complex. The three major cleavage products, p16, p26, and p13, of the BIV Gag precursor, which correspond to the MA, CA, and NC proteins, respectively, previously predicted from the nucleotide sequence (10), were identified. In addition, there are two protein bands (p26 and p23) that appear to contain multiple Gag determinants and a protein (p10) that contains CA determinants. The 23-kDa band appears to be a single protein which contains CA and NC determinants, while the 26-kDa band appears to be two distinct proteins, one with CA determinants and the other with NC determinants. We believe that the 26-kDa protein with NC determinants is a dimer of the BIV p13 NC protein; dimers of the HIV-1 NC protein in immunoblots of HIV-1 virions have been observed with the HIV-1 p7 antiserum, and HPLC-purified p7 has been observed to form dimers in vitro (18).

The MA proteins of retroviruses are known to be modified by fatty acids, such as myristic acid, which are believed to be required for transport of the Gag precursor to the plasma membrane (47). Myristic acid is specifically incorporated at the amino-terminal end of the Gag precursor of HIV-1 and several other retroviruses (7, 17, 35, 43). Such a modification would therefore appear in the Gag precursor and cleavage products containing the MA protein. We used this technique to localize the Gag precursors and MA cleavage product; myristic acid was not incorporated into any BIV proteins (data not shown). These findings are consistent with the substitution of a Lys residue for the obligate Gly as the second amino acid in the Gag precursor, which appears to be necessary for myristylation. EIAV and visna virus Gag precursors are also not myristylated (47). The exact fatty acid modification of the BIV MA protein remains to be determined. The antisera to BIV *rec* p16 and the CA peptide specifically recognized the MA and CA proteins of BIV; however, the antiserum made to the MA-CA peptide was nonreactive to either the MA or CA protein. The MA-CA antiserum did recognize Gag precursors, which indicates that this peptide spans the MA-CA cleavage site. This conclusion is further supported by the identification of the aromatic (Tyr)-Pro sequence as the MA-CA cleavage site in all lentivirus Gag proteins characterized to date (19, 21).

We identified BIV $Pr53^{gag}$ and observed a larger protein, with an apparent molecular mass of 170 kDa, that demonstrated reactivity to both BIV-infected animal sera and antiserum to BIV *rec* Pr53; this protein was previously identified as p174 and was suggested to be the Gag-Pol polyprotein by Rasmussen et al. (40, 41). The apparent 170-kDa molecular mass of the Gag-Pol polyprotein corresponds well to the 168-kDa molecular mass calculated by algorithms of the PEPTIDESORT program in the UWGCG Sequence Analysis Package (6). Although our present study has not definitely shown that p170 contains Pol products, the incorporation of BIV *gag-pol* sequences into a recombinant baculovirus resulted in the synthesis of p170 in infected insect cells in addition to $Pr53^{gag}$, p49, and processed proteins; moreover, BIV-specific reverse transcriptase activity has been demonstrated in these cultures (39). The molecular mass of the Gag contribution to the BIV Gag-Pol polyprotein was similarly calculated and found to be 49 kDa. In the present study, BIV p170 and p49 were recognized by all the sera in the panel except the BIV NC*term* and HIV-1 p17 and p6 antisera in RIPAs with cell lysates. In Western blots of virions reacted with BIV-infected animal sera, the p49 band was also recognized but not as strongly as $Pr53^{gag}$ (Fig. 7). These data suggest that p170 is the Gag-Pol polyprotein precursor ($pPr170^{gag\text{-}pol}$) and p49 is the Gag precursor ($Pr49^{gag}$) derived from the Gag-Pol polyprotein.

We also detected an 11-kDa protein in Western blots with the BIV NC*cys* antiserum but not the NC*term* antiserum, suggesting that this is the truncated form of the BIV NC protein predicted to be found in $Pr49^{gag}$. Cleavage of 4 kDa from the 13-kDa NC protein would yield a protein of 9 kDa with an isoelectric point of 10.3, which would migrate relatively slowly in SDS-PAGE. The p11 would be bounded by the CA on its amino terminus and the viral proteinase on its carboxyl terminus. In the present report, proteins of 18 and 19 kDa were also observed in immunoblots with sera from infected animals; an 18-kDa band has also been observed by others, but its identity was not ascertained (4, 56, 57). Neither of these proteins are recognized by the monospecific Gag antisera, and thus they appear to be non-Gag. Their identification is in progress.

As shown in Fig. 10, we propose a proteolytic-processing scheme for Gag proteins from both the BIV Gag precursor and the Gag-Pol polyprotein precursor. This scheme is based on our current results with monospecific antisera and previously published data (27, 40, 41) and the premise that cleavage products migrate faster than their precursors in SDS-PAGE. While we have immunologically identified the BIV $pPr170^{gag\text{-}pol}$, $Pr53^{gag}$, $Pr49^{gag}$, p26, p23, p16, p13, p11, and p10 shown in Fig. 10, the remaining putative Gag cleavage products, p42, p39, p37, p32, and p21, as well as the p16 related to the BIV CA, have not been characterized in this study or, in some cases, observed experimentally. This could be due to (i) the relative abundance of these products in virions and cell lysates; (ii) the kinetics of proteolytic processing; (iii) the affinity of the antibody; (iv) the lack of appropriate monospecific reagents; or (v) the relative immunogenicity of the protein. Moreover, it should also be noted that small peptides in the 0.5- to 2-kDa range are known to be cleaved from the HIV-1, EIAV, and simian immunodeficiency virus Gag precursors during their processing into mature proteins (19, 21, 24). Because these small peptides are not readily recognized immunologically, such potential cleavage products in the BIV Gag precursor would not have been observed in the present study.

Alignments of the predicted amino acids of the CA proteins of BIV, simian immunodeficiency virus, EIAV, and HIV-1 revealed a highly conserved domain spanning 10 aa (Fig. 1) (10). In the present report, we prepared an antiserum to a 20-aa stretch in the BIV CA protein that encompasses this conserved domain. This antiserum and an antiserum to HIV-1 p24 were both shown to immunoprecipitate BIV p26 and detect BIV p26, p23, and p10 in immunoblots. These results suggest that these three proteins contain the con-

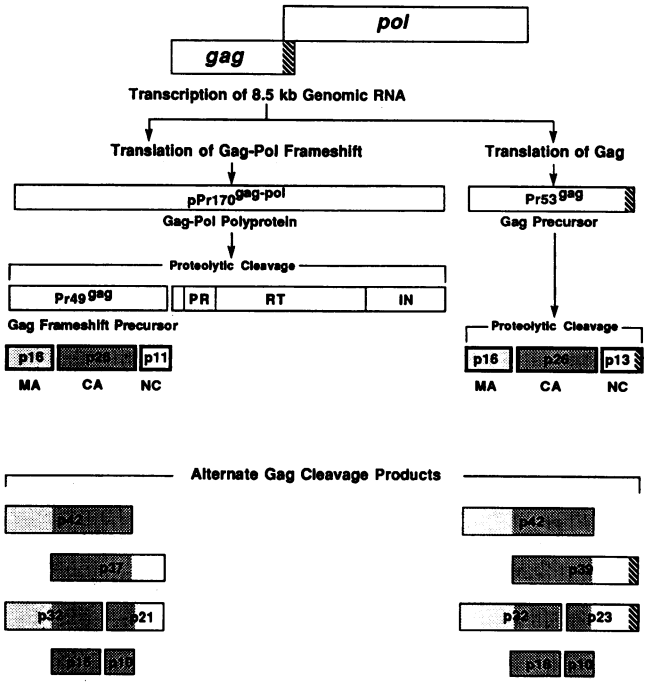

FIG. 10. Proposed scheme for the processing of BIV Gag and Gag-Pol polyprotein precursors translated from genome-length RNA. Light-shaded, dark-shaded, and open boxes in the processed products of the Gag precursors represent the MA, CA, and NC domains, respectively. Diagonal hash marks in the region of overlap between the *gag* and *pol* ORFs denote sequences which are translated in the Gag precursor and products but not the Gag-Pol polyprotein precursor and products. The p23 and p10 BIV proteins indicated in the alternative Gag cleavage products have been immunologically identified in the present study. The viral protease (PR), reverse transcriptase (RT), and integrase (IN) domains of *pol* are shown but have not been identified in this study.

served CA epitope. Since the p10 protein is a component of both p26 and p23 (Fig. 10), our data further suggest that the highly conserved CA domain is within the p10 protein. Previous studies have shown immunological cross-reactivity between the MA proteins of HIV-1 and EIAV (24). In the present study, the antiserum to the HIV-1 NC protein bound to BIV p13. These results extended the cross-reactivity between HIV-1 and BIV to the NC domains and suggest that other determinants may be conserved.

The development of additional monospecific reagents and the isolation, purification, and amino acid sequence analysis of the individual processed Gag proteins will be useful in confirming our assignments and in more precisely identifying the exact cleavage sites recognized by the viral protease. Preliminary data obtained from N-terminal sequence analyses of BIV Gag proteins are in agreement with our present observation that only three large Gag products result from proteolytic processing of the Gag precursor (18). A better understanding of the structural and nonstructural/regulatory proteins of BIV will enable a more thorough analysis of the viral proteins involved in the host immune response to and pathogenesis of BIV, which may lead to effective methods for developing protective host immunity to BIV infections.

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