Bovine Leukemia Virus Protease: Purification, Chemical Analysis, and In Vitro Processing of *gag* Precursor Polyproteins

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Bovine leukemia virus protease was purified to homogeneity and assayed by using murine leukemia virus Pr65^{gag}, a polyprotein precursor of the viral core structural proteins, as the substrate. A chemical analysis of the protease, including an amino acid composition and NH₂- and COOH-terminal amino acid sequence analysis, revealed that it has an *M_r* of 14,000 and is encoded by a segment of the viral RNA located between the *gag* gene and the putative reverse transcriptase gene. As expected from the nucleotide sequence data (Rice et al., *Virology* 142:357-377, 1985), the reading frame for the protease is different from both the *gag* and reverse transcriptase reading frames. The 5' end of the protease open reading frame extends 38 codons upstream from the codon for the NH₂-terminal residue of the mature viral protease and overlaps the *gag* open reading frame by 7 codons. The 3' end of the protease open reading frame extends 26 codons beyond the codon for the COOH-terminal residue of the mature protease and overlaps 8 codons of the reverse transcriptase open reading frame. Several lines of evidence, such as protein mapping of the *gag* polyprotein precursor, the characteristic structure of the mRNA, and promotion of the synthesis of a *gag* polyprotein precursor by lysine tRNA in vitro, suggest that the protease could be translated by frameshift suppression of the *gag* termination codon. In vitro synthesized bovine leukemia virus *gag*-related polyproteins were cleaved by the protease into fragments which were the same size as the known components of bovine leukemia virus, suggesting that the specificity of cleavage catalyzed in vitro by the purified protease is the same as the specificity of cleavage found in the virus.

Bovine leukemia virus (BLV) is the etiologic agent of enzootic bovine leukemia (2). Structural analyses of the major viral core protein have allowed this virus, along with the recently discovered human T-cell leukemia virus (17, 26), to be classified as members of a new family of retroviruses (16). Chemical analyses of the viral structural proteins (4, 13, 14, 21) and nucleotide sequence analyses of the proviral DNAs (18-20) have shown that the gene order in the BLV genome is 5'-*gag-pol-env-x-3'*. The *gag* gene codes for core structural proteins p15, p24, and p12; the *pol* gene codes for reverse transcriptase; and the *env* gene codes for envelope proteins gp60 and p30. In addition to the structural proteins, several open reading frames have been observed, including two open reading frames in the *pol* gene and, as in human T-cell leukemia virus (23), two open reading frames downstream from the *env* gene (X region or long open reading frame region) (19, 20). Between the 3' end of the *gag* gene and the 5' end of the putative reverse transcriptase gene, the amino acid sequence deduced from the DNA sequence shows clear homology to the murine and avian viral proteases. Recently, we identified Moloney murine leukemia virus protease and reported that this protease is encoded by the *gag-pol* gene and is synthesized through suppression of the *gag* termination codon (UAG) (28). In this paper we describe the purification, chemical analysis, and biological specificity of the BLV protease. The possible mechanism of synthesis of the protease, which could be

translated by frameshift suppression of the *gag* termination codon, is discussed.

MATERIALS AND METHODS

Viruses. BLV was grown in fetal lamb kidney cells (24). Gazdar murine sarcoma virus (Gz-MSV) was grown in HTG-2 cells (6). The viruses were purified by sucrose density gradient centrifugation by the Biological Products Laboratory, Program Resources, Inc., National Cancer Institute Frederick Cancer Research Facility, Frederick, Md.

Assay of protease. Gz-MSV, which itself has no protease activity and contains uncleaved Pr65^{gag} (30), a homolog of the polyprotein precursor of the *gag* (group-specific antigen) gene products in other murine viruses, was used as a substrate in assays for the protease activity. Protease activity was assayed as previously described (29). In some cases, immune precipitates of BLV *gag* polyproteins synthesized in an in vitro translation system were incubated with the protease fractions under the same conditions.

Purification of the protease. The method used to purify the protease was essentially the same method used previously for the mouse protease (28). To 60 mg of purified BLV suspended in 2 ml of 0.13 M NaCl-0.01 M Tris hydrochloride (pH 7.2)-0.001 M EDTA (STE buffer), 20 volumes of cold (-70°C) acetone was added. The precipitate was dried under reduced pressure. The resulting acetone powder was then extracted with 4 ml of 0.02 M Tris hydrochloride (pH 7.2)-5 mM dithiothreitol (Sigma Chemical Co., St. Louis, Mo.) (TD buffer) containing 1.0 M NaCl at 4°C for 30 min with constant stirring, and the preparation was centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant, which contained the protease activity, was then fractionated on a Sephacryl

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FIG. 1. Purification of protease by RP-HPLC. The protease-active fraction from Sephacryl S-200 chromatography (see text) was further fractionated by RP-HPLC on a μ Bondapak C_{18} column (0.39 by 30 cm; Waters Associates). The gradient conditions used were as follows: 0 to 35% acetonitrile over a period of 2 min; isocratic at 35% acetonitrile for 10 min; and 35 to 60% acetonitrile over a period of 50 min at a constant flow rate of 1.0 ml/min. One-tenth of each fraction was lyophilized and analyzed for protein composition. Staining was with Coomassie brilliant blue R-250. Lane V contained BLV (40 μ g).

S-200 chromatography column (2.5 by 90 cm). After the protease activity of each fraction (3 ml) was determined, fractions were pooled and subjected to reverse-phase high-pressure liquid chromatography (RP-HPLC) by using a μ Bondapak C_{18} column (Waters Associates, Inc., Milford, Mass.). Fractions (5 ml) were collected, and portions were removed for protein composition analysis, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and protease activity measurements.

SDS-PAGE. Various protein materials were analyzed by discontinuous SDS-PAGE (11). Proteins were visualized by staining with either Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, Calif.) or silver (25). Radiofluorography was done by using a sodium salicylate treatment (3).

Amino acid composition. Samples for amino acid analysis were hydrolyzed for 24 h in vacuo with 6 N HCl containing 0.1% liquid phenol and then dried by vacuum desiccation. Analyses were performed with a Durrum model D-500 amino acid analyzer by using ninhydrin to detect the eluted amino acids.

NH₂-terminal microsequence analysis. The purified protease was subjected to automated Edman degradation in a gas phase sequencer (9) by using the program supplied by the manufacturer (Applied Biosystems, Inc.). The amino acid anilinothiazolinones were converted to phenylthiohydantoin derivatives by using 25% trifluoroacetic acid in water. Phen-

ylthiohydantoin amino acids were identified and quantitated by RP-HPLC (8).

COOH-terminal sequence analysis. Protein samples were digested with carboxypeptidase Y for various times as previously described (14), and the amino acids released were quantitated by using the Durrum model D-500 analyzer.

In vitro translation of BLV RNA. A 20-mg portion of purified virus in STE buffer was disrupted with 1% SDS and extracted twice with water-saturated phenol. The RNA was precipitated by adding two volumes of cold (-20°C) ethanol and was collected by low-speed centrifugation. The RNA was then suspended in 0.5 ml of STE buffer containing 1% SDS and was fractionated by centrifugation on a 5 to 20% (wt/vol) sucrose density gradient (made up in STE buffer containing 0.1% SDS) at 30,000 rpm for 150 min in an SW40.1 rotor at 20°C . Fractions were collected, and the RNA was again precipitated by adding 2 volumes of cold (-20°C) ethanol. The fractions were kept overnight at -20°C , and precipitates were collected by centrifugation at 4°C . The precipitates were solubilized in 100 μ l of water and heated at 90°C for 2 min. A 5- μ l portion of each fraction was analyzed to determine the size of the RNA species on a 1% agarose gel (12) by using bovine liver RNA as a marker. The in vitro translation experiments were performed in 25- μ l incubation mixtures at 37°C for 60 min by using an in vitro translation kit (New England Nuclear Corp., Boston, Mass.)

FIG. 2. Cleavage of Gz-MSV Pr65^{gag} with purified BLV protease (fraction 11 in Fig. 1). Portions (2.5%) of fraction 11 from the experiment shown in Fig. 1 were lyophilized, solubilized in TD buffer (pH 7.0) containing 0.5% Nonidet P-40 (100 μ l), and then incubated in the presence or absence of Gz-MSV (1.5 μ g) as the substrate. After incubation at 20°C for 16 h, a 25- μ l portion of each sample was analyzed by SDS-PAGE. The gel was stained with silver nitrate. Lane 1, Moloney murine leukemia virus (7 μ g); lane 2, Gz-MSV (1.5 μ g) alone before incubation; lane 3, Gz-MSV (1.5 μ g) alone after incubation; lane 4, fraction 11 alone (0.3 μ g); lane 5, fraction 11 (0.3 μ g) plus Gz-MSV (1.5 μ g) after incubation; lane 6, BLV (7 μ g). The gel was stained with silver nitrate (25).

with 25 μ Ci of L-[35 S]methionine (New England Nuclear Corp.), a reduced concentration of potassium acetate (75 mM), 1.0 mM magnesium acetate, and RNA which was ice quenched following heating at 90°C for 2 min. Samples (1 μ l) were removed to determine the radioactivity incorporated into the proteins, and the samples were analyzed by SDS-PAGE.

Immunoprecipitation. Immunoprecipitation was done as follows. Protein A-Sepharose (15 mg; Pharmacia Fine Chemicals, Piscataway, N.J.) in 0.5 ml of Dulbecco phosphate-buffered saline and 10 μ l of serum were incubated for 1 h at 4°C. After washing with phosphate-buffered saline twice, the beads were incubated with a translation mixture, which was diluted 10-fold with immunoprecipitation buffer (0.02 M Tris hydrochloride pH 7.5, 0.1 M NaCl, 1% Nonidet P-40), for 16 h at 4°C. Then the beads were washed three times with immunoprecipitation buffer and once with water and analyzed by SDS-PAGE.

RESULTS

Detection of protease activity in BLV. To detect protease activity, the virus was incubated under the conditions used previously for the mouse protease assay (28). The optimum pH for protease activity was determined by incubating 30- μ g portions of purified virus at various pHs in TD buffer containing 0.5% Nonidet P-40 for 16 h at 22°C in the presence or absence of the exogenous substrate Gz-MSV Pr65^{gag}. It appeared reasonable to use Gz-MSV Pr65^{gag} as the BLV protease substrate because the COOH-terminal amino acid sequence of the BLV *gag* proteins (-Pro-Ala-Ile-Leu-COOH) has been shown to be similar to the sequences of the mouse *gag* proteins (13, 15). When purified BLV alone was incubated under these conditions, no precursor-product relationship like that seen in the mouse system was observed (29, 30). However, when Gz-MSV Pr65^{gag} was added to BLV preparations in the assay described above, the Pr65^{gag} content decreased and there was a concomitant increase in

TABLE 2. N-terminal amino acid analysis of BLV protease

Cycle	Amino acid	Amt recovered (pmol) ^a
1	Leu	210
2	Ser	26
3	Ile	203
4	Pro	155
5	Leu	167
6	Ala	146
7	Arg	65
8	X	
9	Arg	93
10	Pro	100
11	Ser	9
12	Val ^b	
13	Ala	90
14	Val	
15	Tyr	79
16	Leu	94
17	Ser	5
18	Gly	37
19	Pro	56
20	Trp	11
21	Leu	83
22	Gln	26
23	Pro	55
24	(Ser)	2
25	Gln	55
26 ^c		
27	Gln	43
28	Ala	21
29	Leu	37
30	Met	12
31	Leu	46

^a Based on the phenylthiodihydantoin amino acid derivatives.

^b Valine assignments were qualitative only due to the presence of an artifactual peak in the chromatographic analysis of the phenylthiodihydantoin amino acids.

^c Sample was lost during workup.

TABLE 1. Amino acid composition of BLV protease^a

Amino acid	No. of residues per protein determined	Predicted no. of residues ^b
Asp	10.32 (10)	10
Thr	4.86 (5)	5
Ser	7.75 (8)	9
Glu	13.29 (13)	11
Pro	12.07 (12)	14
Gly	10.03 (10)	9
Ala	9.56 (10)	9
Val	10.02 (10)	11
Met	0.66 (1)	2
Ile	6.78 (7)	8
Leu	15.83 (16)	17
Tyr	2.79 (3)	3
Phe	2.08 (2)	2
His	0.95 (1)	0
Lys	4.21 (4)	3
Arg	8.00 (8)	9
Cys	ND ^c	0
Trp	ND	4

^a The determined molecular weight was 13,763, and the predicted molecular weight was 14,035.

^b Amino acid composition predicted from the DNA sequence (18). Based on the determined NH₂ and COOH termini, the sequence from nucleotide 1677 to nucleotide 2054 was used for this comparison.

^c ND, Not determined.

the smaller proteins (data not shown). The activity was highest around pH 7.0 to 7.2. Thus, we decided to use Gz-MSV Pr65^{gag} as a substrate in BLV protease assays to monitor activity in the procedures developed for the purification of the enzyme.

Purification of the protease. The protease (molecular weight, 14,000 [14K]) eluted from the Sephacryl-200 column (see Materials and Methods) was further purified by RP-HPLC, using a shallow acetonitrile gradient on a μ Bondapak C₁₈ column (Fig. 1). Fractions (5 ml) were collected and lyophilized for determinations of purity and activity. The peak activity was eluted in fraction 11 at an acetonitrile concentration of about 52 to 54%. This activity was clearly separated from proteins p12, p10, and p15. The purified protein produced a single band in SDS-PAGE gels (Fig. 1, fraction 11). The amount of total protein recovered in RP-HPLC fraction 11 was 35 μ g. The total recovery of enzyme activity was 40%, as determined from a 50% reduction in the Pr65^{gag} band density after 16 h of incubation (28). Relatively large amounts of activity were lost during extraction from the acetone powder of the virus. This was probably caused by the hydrophobic nature of the protein, as indicated by the position of its elution in the acetonitrile gradient. However, this step was essential to remove the bulk of hydrophobic and low-molecular weight contaminants. To identify the protein responsible for the protease activity, the purified protease fraction from RP-HPLC was incubated with Gz-MSV Pr65^{gag}. As shown in Fig. 2, the activity was found in the 14K protein (fraction 11). In this fraction Pr65^{gag}

FIG. 3. Alignment of the determined NH₂- and COOH-terminal amino acid sequences (underlined) of the protease with the amino acid sequence deduced from the DNA sequence (18). RT, Reverse transcriptase; fr1, frame 1.

was cleaved into 44K, 40K, and 20K major bands (all doublets), including minor proteins after incubation (Fig. 2, lane 5). The BLV protease purified in this way apparently did not process Gz-MSV Pr65^{gag} to the usual final products (p15, p30, p12, and p10). It remains to be determined which sites in Gz-MSV Pr65^{gag} are cleaved by the BLV protease. This could be accomplished by an NH₂- and COOH-terminal analysis of each product.

Chemical analysis of the protease. The amino acid composition data for the protease are shown in Table 1. The total number of amino acids, not including cysteine and tryptophane, was 126, and the molecular weight calculated from these data was 13,763. The composition which we determined is in good agreement with the composition predicted from the nucleotide sequence (Table 1). To determine the NH₂-terminal amino acid sequence of the purified protease recovered from fraction 11, 0.25 nmol of protein was subjected to analysis on a gas phase sequenator. The amino acids identified at each cycle (of the first 31 cycles) are shown in Table 2, together with the quantitative yields. To determine whether the protease is virus encoded, we aligned the NH₂-terminal amino acid sequence with the amino acid sequence deduced from the nucleotide sequence of the DNA. As shown in Fig. 3, the protease amino acid sequence starts with a leucine residue encoded by the triplet at nucleotides 1677 to 1679 in the *pol* gene (18), 70 nucleotides downstream from the end of *gag* gene, and located, as expected, in a reading frame different from that of *gag* (18). The following two lines of evidence suggested that the protease COOH terminus is at nucleotides 2052 to 2054 and not at nucleotides 2130 to 2132 of this open reading frame adjacent to the termination codon (UAG): (i) the molecular weight estimated from the SDS-PAGE profile and the amino acid composition data (Fig. 1 and Table 1) was about 14,000; and (ii) the data from a preliminary analysis showed that

the COOH-terminal sequence of this protein was -Val-Gly-COOH. The protein and nucleotide sequence data together clearly showed that the protease-encoding region located in the genome from nucleotide 1677 to nucleotide 2056 is in a different frame than *gag* and the putative reverse transcriptase gene.

In vitro processing of BLV *gag* precursors by the protease. To determine whether the BLV protease cleaves the BLV polyprotein precursor accurately, we used the polyprotein substrates synthesized in vitro. BLV RNA-directed cell-free protein synthesis yielded three major proteins, designated the 66K, 44K, and 14K proteins. The 66K and 44K proteins were related to the *gag* gene, as demonstrated by immunoprecipitation with anti-p24 antiserum (Fig. 4B, lane 5, and Fig. 4C, lane 7). The 14K protein is not considered here; it is described elsewhere (31). After the immune complexes (antigen-antibody-protein A-Sepharose complexes) of the 66K and 44K proteins were incubated with the partially purified protease under the assay conditions described above, the cleavage products were analyzed by SDS-PAGE. As shown in Fig. 4, the same sizes of BLV *gag* proteins (p24, p15, p12, and p14 [protease]) were generated by the protease treatment (see below) (Fig. 5). Additional bands, labeled bands m and n, which were also present in the purified virus preparation, were detected as cleavage products (Fig. 4C, lane 9). We identified band m as p10, an NH₂-terminal polypeptide of *gag* polyproteins. From these results, we concluded that the purified protease processes the BLV *gag* precursors in a specific manner.

DISCUSSION

BLV protease was purified, and its NH₂- and COOH-terminal amino acid sequences were determined. Our results show that the gene for the BLV protease (*M_r*, 14,000) is located between the *gag* gene and the putative reverse

FIG. 4. In vitro cleavage of the BLV *gag* precursor proteins with the BLV protease. Whole incubation mixtures (100 μ l; labeled with L-[35 S]methionine or L-[35 S]cysteine) of the BLV RNA-directed in vitro translation system were immunoprecipitated with anti-p24 rabbit antiserum (see text). The immune complex was then incubated with the BLV protease fraction under the assay conditions described in the text. After incubation, proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue R-250, and then autoradiography was performed by using sodium salicylate. (A) Stained gel of the L-[35 S]methionine labeling experiment. Lane 1, BLV whole virus (40 μ g); lane 2, partially purified protease (the RP-HPLC fraction used in this experiment contained some p24 in addition to protease p14); lane 3, Gz-MSV (7.5 μ g); lane 4, Gz-MSV plus partially purified protease; lane 5, the immune complex alone; lane 6, the immune complex plus partially purified protease. (B) Autoradiogram of the L-[35 S]methionine labeling experiment. The stained gel (panel A) was exposed to X-ray film. Lanes 1' to 4', Tracings of lanes 1 to 4 in the stained gel (panel A); lane 5', the immune complex alone; lane 6', the immune complex plus partially purified protease. (C) Autoradiogram of the L-[35 S]cysteine labeling experiment. Lane 7, The immune complex alone (no incubation); lane 8, the immune complex alone (incubated); lane 9, the immune complex plus partially purified protease; lane 10, partially purified protease; lane 11, BLV whole virus (40 μ g) (tracings of the stained gel).

transcriptase gene and is coded in a reading frame that is different from the reading frames of the *gag* and reverse transcriptase genes (18). In both Moloney murine leukemia virus and feline leukemia virus (FeLV) the protease gene is located in the *pol* gene in the same reading frame as the *gag* gene (27, 28); and in the avian retroviral system, the protease gene is located in the 3' region of the *gag* gene (22). Thus, in BLV the organization of the protease gene in the viral genome is different from the organization in both murine leukemia virus and FeLV systems and avian systems. However, within the protease-encoding region the translated BLV sequence shows homology with the avian and mammalian type C virus sequences over the entire length of the region. The BLV protease is most closely related to mouse protease (18).

Based on an NH₂- and COOH-terminal analysis of the *gag* proteins, we suggested previously (13, 27) that the cleavage sites in human T-cell leukemia virus and FeLV *gag* precursors are very similar to the cleavage sites in the mouse *gag* polyprotein. The cleavage sites in BLV are also similar in the COOH-terminal amino acid sequences of each *gag* protein to the cleavage sites in the above-mentioned retroviruses (4, 13, 14, 27). Based on this similarity, we used Gz-MSV Pr65^{gag} as the substrate for the BLV protease during purification steps. Purified BLV protease was capable of cleaving

the mouse *gag* precursor but apparently did not readily produce the correct final products (Fig. 2 and 4). However, we found that purified or partially purified BLV protease cleaved in vitro synthesized BLV polyproteins into the expected viral *gag* components without yielding other major products. Furthermore, when we used other proteins, such as bovine serum albumin or the mouse immunoglobulin heavy chain, as the protease substrate, no cleavage was observed. The exact specificity of the in vitro protease cleavage remains to be determined.

Another interesting question is the mechanism of the synthesis of BLV protease. In murine leukemia virus and FeLV, the protease is synthesized by readthrough of an amber termination codon at the end of the *gag* gene to produce a *gag-pol* fusion polyprotein. After being synthesized under translational control in a quantity that is small relative to the amount of *gag* precursor (Pr65^{gag}), Pr180^{gag-pol} is processed by proteolytic cleavage, and the protease is generated as Thr-Leu-Asp-Asp (from the *gag* gene)-Gln-(*pol* gene product). An intriguing hypothesis has been proposed for BLV protease synthesis (18); according to this hypothesis a -1 frameshift might occur near the end of the *gag* gene. In that region, a guanine-cytosine-rich hairpin structure and six consecutive adenine residues are found; both characteristic sequences have been thought to be

FIG. 5. Model for the synthesis of *gag* and protease proteins. Based on the DNA sequence data and the results obtained from the cleavage patterns in the processing of the *gag*-related 66K and 44K polypeptide precursor by the protease, a model for the origin of the BLV protease was made. (A) Coding frame of the *gag* and protease in the BLV genome. Symbols: ☆, termination codon; ●, methionine residue found in the DNA sequence; ▲, cysteine residue found in the DNA sequence. fr., Frame. (B) Translated product from coding frames 1 and 3. The partitions determine the NH₂ and COOH termini (cleavage sites) of each protein. Symbols: ★, point where a frameshift (from frame 1 to frame 3) could occur; ▼, another possible cleavage by BLV protease. (C) Final cleavage products, detected by labeling with either L-[³⁵S]methionine or L-[³⁵S]cysteine. All of these products were apparently identical to the products found in the purified virus. Both p12 (six cysteines) and band "n" (p12 + x; seven cysteines) are seen in Fig. 4. Only "n" is indicated above.

important for translational control of mRNA in procaryotes as well as eucaryotes (1, 5, 10). Not only the precise localization and reading frame assignment as described in this paper, but also the results of our biochemical studies with in vitro synthesized proteins are also compatible with this hypothesis. As reported previously by Ghysdale et al. (7) and as shown in Fig. 4, the 66K and 44K proteins were *gag*-related precursors. If frameshift suppression of the *gag* termination codon is the mechanism involved in the synthesis of the 66K polypeptide, we can expect the final cleavage products which we show in Fig. 5C. These products were obtained after incubation of the in vitro synthesized 66K and 44K polypeptides labeled with either [³⁵S]methionine or [³⁵S]cysteine. As shown in Fig. 4, we detected p14 protease, p24, and p12 labeled with [³⁵S]methionine (Fig. 4B, lane 6') and p24, p15, band n, p12, and band m labeled with [³⁵S]cysteine. p24, p15 and p12 are known structural proteins of BLV. Band n, which migrated slower than p12 (Fig. 4), is what would be expected to be a product of [³⁵S]cysteine-labeled 66K protein after incubation with protease consisting of a major portion of p12 plus downstream sequences in the protease reading frame. As for the fastest-migrating band (band m) detected in the [³⁵S]cysteine labeling experiment, we determined that this protein was p10, an NH₂-terminal piece of p15. This protein was also found to be myristylated in the virus (unpublished data). Therefore, all of these products, including the band n and m proteins, which were obtained after cleavage of the 66K and 44K precursors with the protease (Fig. 4), match our model (Fig. 5) for the synthesis and cleavage of *gag* and the protease. From these results it seems likely that the protease is synthesized by frameshift suppression of the *gag* termination codons.

The preliminary results of our in vitro translation experiments with lysine-specific tRNA (tRNA^{Lys}; a gift from B. J. Ortwerth, University of Missouri, Columbia) may support this conclusion. In these experiments, the synthesis of the

gag polypeptide precursor 66K was promoted by the addition of lysine-specific tRNA to the BLV genomic RNA-directed cell-free translation system. The degree of promotion was proportional to the amount of the tRNA added, and about two- to threefold stimulation was obtained with 0.02 U of optical density at 260 nm. Synthesis of the 44K and 14K proteins was not promoted at this concentration of tRNA.

A direct amino acid sequence analysis of *gag*-protease fusion protein could elucidate further details of the translational control and processing of BLV proteins. The other possibility, that a spliced mRNA is required for the synthesis of the *gag*-protease polypeptide, cannot be ruled out. In any case, the diversity of translational control mechanisms at the *gag-pol* junction of retroviruses, such as avian virus, murine leukemia virus, FeLV, BLV, and human T-cell leukemia virus, provides useful models for examining features of gene expression in eucaryotic cells. It also remains to be determined whether the protease itself cleaves all of the sites in *gag*-protease (66K) polypeptide or whether a cellular protease triggers the initial cleavage event. As shown in Fig. 4, we incubated the 66K and 44K polypeptides after immunoprecipitation with antibody without added protease and did not observe lower-molecular-weight products. Despite our inability to demonstrate self-cleavage in vitro of the 66K protein which contains the protease sequence, the possibility of autocatalytic cleavage under natural conditions cannot be ruled out.

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