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Spleen Necrosis Virus *gag* Polyprotein Is Necessary for Particle Assembly and Release but Not for Proteolytic Processing

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The nature of spleen necrosis virus *pol* gene expression and the role of *gag* and *gag-pol* polyproteins in virion assembly was investigated. The DNA sequence of the *gag-pol* junction revealed that the two genes occupy the same open reading frame but are separated by an in-frame amber stop codon. Biochemical analysis of *gag-pol* translational readthrough in vitro and in *Escherichia coli* suggests that, in a manner similar to that in other mammalian type C retroviruses, amber stop codon suppression is required for *pol* gene expression. Removal of the *gag* stop codon had little or no effect on synthesis or cleavage of the polyprotein but interrupted particle assembly. This block could be overcome by complementation with wild-type *gag* protein.

The general pattern of *gag* and *gag-pol* gene expression is highly conserved among retroviruses. Translation of genomic-length viral RNA results in two species of polyprotein precursors: a *gag* polyprotein, generated by translational termination at the end of the *gag* open reading frame (50), and a *gag-pol* polyprotein, formed by readthrough of the *gag* gene termination codon such that translation continues into the *pol* open reading frame (20, 37) (Fig. 1). Because the efficiency of translation of the *pol* gene ranges from only 4 to 10% that of the *gag* gene (20), intracellular levels of viral proteins are modulated such that there is a molar excess of *gag* proteins with respect to *pol* proteins. Similar strategies have been employed by other viral systems for reducing the expression of downstream genes present on polycistronic messages (8, 21, 31, 36, 46, 51, 52). These systems likely provide a way for viruses to produce an optimal balance of the different viral proteins during replication.

The molecular mechanism of translational readthrough has been studied extensively in several different retrovirus systems, including Rous sarcoma virus (19), murine leukemia virus (MLV), feline and bovine leukemia viruses (54–56), mouse mammary tumor virus (14, 30), and human immunodeficiency virus (HIV; 1). Readthrough occurs by ribosomal frameshifting for those viruses containing *gag* and *pol* genes in separate reading frames (14, 17–19, 30, 56). Jacks and co-workers have demonstrated that the frameshift event is initiated by ribosomal "slippage" at a consensus UUUA sequence located just upstream of a potentially stable stem-loop RNA secondary structure (17). The resulting frameshift bypasses the *gag* termination codon and thus aligns the ribosome in phase with the *pol* reading frame.

An alternative mechanism employed by those viruses with *gag* and *pol* genes in the same reading frame is amber stop codon suppression (37, 54, 55). Suppression was proposed for MLV following the observation that the level of *gag-pol* polyprotein synthesis generated in vitro could be specifically enhanced by the addition of yeast amber suppressor tRNA (37). Nucleotide sequence data (44) confirmed that the *gag* and *pol* genes of MLV shared the same reading frame but were separated by an in-frame amber stop codon. Direct evidence for suppression came from the amino acid sequence determined for the N terminus of purified MLV protease protein. When aligned with the known nucleotide sequence, the protein sequence revealed that the protease is encoded by both the *gag* and *pol* genes and that a glutamine residue is inserted at the site in the polypeptide corresponding to the amber stop codon (54). Further analysis in vivo and in vitro established that suppression is due to an intrinsic feature of a short *cis*-acting region of RNA flanking the *gag-pol* junction and that a normal cellular tRNA species can mediate suppression in the absence of *trans*-acting viral functions (10, 33).

While the phenomenon of suppression has been well established, little is known about its biological significance in viral replication. Following translation, the polyprotein precursors are proteolytically cleaved into mature *gag* and *pol* proteins by the virally encoded acid protease (45). The genetic arrangements of mature *gag* and *pol* proteins along a typical mammalian type C viral polyprotein are highlighted in Fig. 1 and from the N to the C terminus include the matrix (MA), phospho- (PP), capsid (CA), nucleocapsid (NC), protease (PR), reverse transcriptase (RT), and integration (IN) proteins (25). The protease cleaves itself from the *gag-pol* polyprotein following virion release through a poorly understood process believed to be coupled with core condensation or "maturation" (22, 57). Proteolytic processing of viral polyproteins is not essential for virion assembly or release since mutations that abrogate protease function retain the ability to release virion particles; however, these particles are uninfectious (4). Large deletion mutations in other *pol* domains are similarly unaffected in assembly (41, 43). In contrast, the majority of mutations in the *gag* gene, specifically in the MA and CA proteins, are blocked in virion assembly and release (15, 40, 42). Taken together, these results strongly implicate the unprocessed *gag* polyprotein as the nucleating unit in virion assembly, perhaps through oligomerization of the capsid domain (3). A recent report (9) described a mutation in MLV that specifically removed the *gag* termination codon. This mutant constitutively expressed the *gag-pol* polyprotein only and was blocked in virion assembly and proteolytic cleavage. The authors concluded that the molar ratio of *gag* to *gag-pol* polyproteins was critical for both processes and that steric hindrance, caused by the larger *gag-pol* polyprotein, may dominantly interfere with particle assembly.

In this report, we present the nucleotide sequence of the

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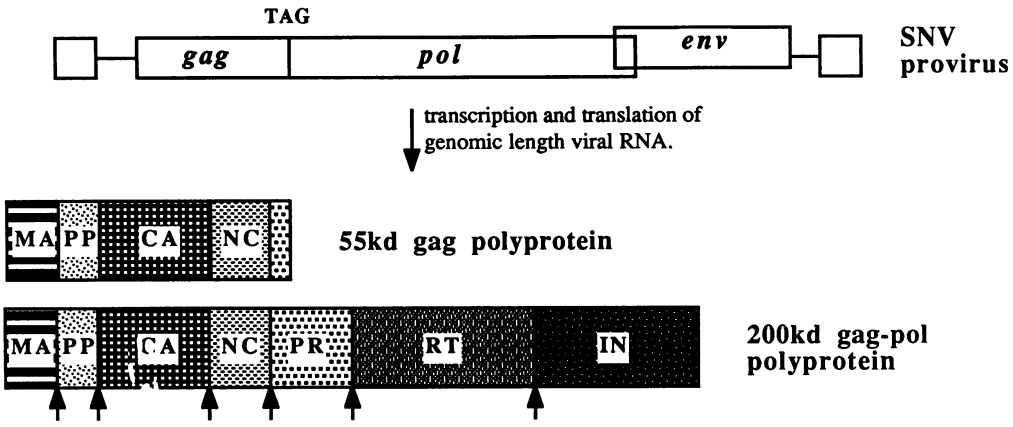

FIG. 1. Expression of SNV *gag* and *gag-pol* polyproteins. Translation of genomic length viral RNA yields a 55-kDa *gag* polyprotein at high efficiency (95%) and a 200-kDa *gag-pol* polyprotein at low efficiency (5%). Arrows delineate boundaries between the different *gag* and *pol* proteins, which from the N to the C terminus include MA (12 kDa), PP (18 kDa), CA (27 kDa), NC (10 kDa), PR (15 kDa), RT (84 kDa) and IN (44 kDa) (25). The NC-PR cleavage site is 5 residues upstream of the *gag* stop codon.

gag-pol junction of spleen necrosis virus (SNV), an avian type C retrovirus belonging to the reticuloendotheliosis virus group (39), and demonstrate that SNV *pol* gene expression results from amber stop codon suppression. In addition, we describe a mutation that specifically removes the *gag* stop codon such that only the *gag-pol* precursor is synthesized. Analysis of this mutant indicates that, in a manner similar to that of the MLV mutation, virion assembly and release are completely blocked. However, in contrast to the results with MLV, a high degree of intracellular proteolytic activity is observed. Therefore, polyprotein cleavage in SNV is not dependent on virion assembly and release. The readthrough mutation could be efficiently rescued by coexpression of a wild-type *gag* polyprotein. Thus, it appears that either the ratio of the *gag* to *gag-pol* proteins is crucial for replication or else there is a qualitative functional difference between the *gag* polyprotein that results from amber codon recognition and that which results from proteolytic scission.

MATERIALS AND METHODS

Plasmid constructions. pPB101, a plasmid encoding an infectious proviral clone of SNV (1), was used as the source of SNV viral DNA in all constructs. In the generation of plasmid pTW44, a 3.8-kilobase *Xma*III-*Xho*I restriction fragment (encoding the entire SNV *gag* and most of the SNV *pol* gene) was blunt ended, modified with *Xho*I linkers (Pharmacia), and subcloned into the *Xho*I site in the polylinker of the bacterial expression vector pIC20R (26). pTW63 is a derivative of pTW44 in which sequences from an *Eco*RI site (located in the upstream polylinker sequence of pTW44) to a *Pst*I site (located downstream of the putative protease domain in *pol*) were subcloned into the equivalent restriction sites in the polylinker of the phagemid pTZ18R (Pharmacia). pTW76 contains a mutation which substitutes for Gln-stop Leu-Gln at the *gag* stop codon, whereas pTW133 contains a mutation which substitutes Arg for Asp at the highly conserved Asp residue 33 of the viral protease. Both were generated from pTW63 by site-directed mutagenesis. Proviral clones pTW84 and pTW148 were derived from pTW76 and pTW133, respectively, by subcloning an 850-base-pair *Eco*RV-*Apa*I restriction fragment into the equivalent sites of pPB101.

Induction of fusion proteins in *Escherichia coli*. Plasmids were transformed into *E. coli* JM83 and JM109 (26) and grown at 30°C in 50 ml of L broth until the cells reached an optical density at 560 nm of 0.7. Cells were induced for 6 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and then either total protein extracts or the insoluble inclusion body fraction of cell lysates (32) were isolated for further analysis.

Preparation of antisera raised against the SNV *gag* polyprotein. Rabbit polyclonal antisera were raised against a bacterially expressed SNV *gag* polyprotein. Constitutive expression of the plasmid pTW44 in JM83 cells resulted in high levels of a 55-kilodalton (kDa) *lacZ-gag* fusion polyprotein. This fusion polyprotein was gel purified from inclusion body preparations following preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 13). Polyclonal antisera directed against the fusion polyprotein were raised in New Zealand White female rabbits by standard methods (16).

In vitro mutagenesis. pTW63 was transformed into the *dut ung* mutant host RZ1032 (24). Bacteriophage replication was initiated by infection with the defective helper phage KO7 (Pharmacia), and the uracil-rich single-stranded phage DNA was used as the template for oligonucleotide-directed mutagenesis (24). Oligonucleotides were synthesized by the University of Wisconsin Biotechnology Center as follows: 5′-GA GGAATTACTGCAGGGCCGTCAG-3′ (pTW76) and 5′-GA TTTCTAGTACGTACGGGAGCG-3′ (pTW133). JM83 cells were transformed with the polymerization reaction, and plasmid DNAs from individual colonies were screened for the presence of the correct mutation by restriction enzyme polymorphism (*Pst*I for pTW76 and *Sna*BI for pTW133). The correct sequence was then confirmed by sequence analysis by using the sequenase enzyme (U.S. Biochemical Corp.).

Cells and viruses. The canine osteosarcoma cell line D17 (American Type Culture Collection, Rockville, Md.) and all derived lines were maintained in modified Eagle medium supplemented with 7% calf serum (Hyclone). Cells were cotransfected with 0.1 μg of pJD214hygro (6) and 5 μg of DNA bearing the mutation of interest by the polybrene-dimethylsulfoxide method, as previously described (5). Selection with 400 μg of hygromycin B (Boehringer Mannheim Biochemicals) per ml was initiated 24 h posttransfection. Viral infections were carried out on 1×10^6 cells in the

presence of 10 μg of polybrene per ml for 45 min (47). Chronically infected cells were established by cotransfecting D17 cells with pPB101 and pJD214hygro and pooling hygromycin-resistant cells. Reverse transcriptase assays were performed on purified virus preparations as previously described (34). Cell lines or bacteria were fixed with 3% glutaraldehyde and osmium tetroxide, cells were embedded in Epon 812, and uniform thin sections were prepared for transmission electron microscopy (53).

Metabolic labeling and immunoprecipitation of viral proteins. Cells (1×10^6) seeded on a 10-cm plate were incubated for 30 min in 3 ml of labeling medium (RPMI 1640 medium, supplemented with 2% dialyzed fetal calf serum and 5 mM Na_2HPO_4). This was followed by a 17-h pulse with [^{35}S] methionine-cysteine (Translabel; ICN Pharmaceuticals, Inc.) at a final concentration of 50 μCi/ml. Cells were lysed directly on the plate with ice-cold phospholysis buffer (0.5% deoxycholate, 1% Triton X-100, 10 mM NaH_2PO_4, 0.1% SDS, and 0.01% bovine serum albumin), and cell debris was removed by centrifugation. The supernatant was precleared with rabbit preimmune serum (1:200 dilution) and protein A-Sepharose (Sigma Chemical Co.; 50% in ddH_2O added at 5 times the volume of antiserum). *gag* antiserum was incubated (1:200 dilution) with the resulting supernatant for 1 h at 4°C, followed by pelleting with protein A-Sepharose. Samples were separated by SDS-PAGE, and following electrophoresis the separation gel was soaked for 20 min in 1 M salicyclic acid, dried under vacuum, and autoradiographed at −80°C.

Western analysis of intracellular viral antigens. Cells (10^6) seeded on a 10-cm plate were rinsed three times in ice-cold phosphate-buffered saline (PBS) containing 0.1% D-glucose and then incubated for 10 min with 1 ml of ice-cold hypotonic buffer (10 mM Tris hydrochloride [pH 7.9], 10 mM NaCl, and 2 mM $MgCl_2$). The cells were collected and pelleted by centrifuging at 1,000 × *g* for 3 min. The pellet was then washed an additional two times before the cells were homogenized by 25 strokes in a Dounce homogenizer. The resulting homogenate was then separated by successive centrifugations into nuclear (3,000 × *g* for 10 min), microsomal (10,000 × *g* for 20 min), and smooth membrane (100,000 × *g* for 40 min) pellets. The final high-speed, smooth membrane pellet was solubilized in 50 μl of phospholysis buffer and heated at 100°C with an equal volume of 2× protein sample loading buffer. For Western blot (immunoblot) analysis, the sample was separated by SDS-PAGE and electrotransferred to nitrocellulose filter paper; prestained "rainbow" markers (Amersham Corp.) were used to monitor separation during electrophoresis and efficiency of transfer to nitrocellulose. Following transfer, the filters were incubated in blocking buffer (1% dry fat milk [Carnation] in 1× PBS), and primary antiserum (final dilution, 1:5,000) was added for 1 h at room temperature before the addition of ^{125}I-protein A (Dupont, NEN Research Products) (2×10^5 dpm/ml in blocking buffer). Filters were washed three times (0.3% Tween 20 in 1× PBS), air dried, and autoradiographed at room temperature.

In vitro transcription and translation of viral proteins. Uncut plasmid DNA (1 μg) was transcribed with T7 RNA polymerase under standard conditions (Promega Biotec). RNA was precipitated with 2.5 volumes of ice-cold ethanol in the presence of 2 M ammonium chloride and suspended in 10 mM Tris–1 mM EDTA (pH 7.9) to a final concentration of 1 μg/μl. Capped RNA (5 μg) was translated in a cell-free rabbit reticulocyte lysate (Promega) supplemented with 1 μg of calf liver tRNA (Boerhinger) per μl and 1 μCi of [^{35}S] methionine-cysteine (Translabel; ICN) per μl. The reaction was stopped by repeated freeze-thawing, and viral antigens were analyzed by immunoprecipitation by using *gag* antiserum.

Protein microsequencing. Accurate capsid proteolytic cleavage in bacteria was demonstrated by protein microsequencing of the 27-kDa *gag* protein found in JM109 cells expressing the *lacZ-gag* fusion protein encoded by plasmid pTW63. Total cellular lysates were separated by SDS-PAGE and transferred to Immobilon-P membrane filters (Millipore Corp.) by the methods of Matsudaira (27). The 27-kDa protein was identified by Western analysis, and the corresponding Coomassie blue-stained band in an adjacent lane was excised for automated protein microsequence analysis (performed by the University of Wisconsin Biotechnology Center). The 10 N-terminal amino acid sequence of this 27-kDa protein was identified as N-Pro-Leu-Arg-Glu-Thr-Gly-Glu-Arg-Asp-Met. This sequence corresponds to the N terminus previously determined for the REV-A CA protein (49).

RESULTS

A single in-frame amber codon separates the SNV *gag* and *pol* genes. In order to determine the nature of the junction between the *gag* and *pol* open reading frames for SNV, we molecularly cloned and sequenced a 1-kilobase *Bgl*II-*Sma*I restriction fragment known to encode the distal portion of the *gag* gene. Examination of the nucleotide sequence identified two long open reading frames, presumably *gag* and *pol*, that were separated by a single in-frame amber stop codon (Fig. 2). Analysis of the deduced amino acid sequence encoded in the 3′ portion of the *gag* gene revealed the presence of a conserved sequence found in all previously examined retrovirus NC proteins. One copy of the Cys-$(X)_2$-Cys-$(X)_4$-His-$(X)_4$-Cys motif, referred to as the Cys-His box by Meric and Goff (28), is present between residues 28 and 41 in the SNV NC protein (Fig. 2). This sequence might constitute a "zinc-finger" found in some nucleic acid-binding proteins (2) and has been implicated in NC protein function for MLV and Rous sarcoma virus. Mutations that disrupt this motif destroy NC protein binding to both the encapsidation region of viral RNA and to the primer tRNA. The former has been postulated to be required in RNA packaging (12, 28), whereas the latter may be required for dimerization with viral RNA and initiation of reverse transcription (38). The leucine residues at positions 1 and 52 define the N- and C-terminal ends of the SNV NC protein, as evidenced by the partial amino acid sequence of purified REV-A NC protein (49). The nucleotide sequence predicts the presence of a Glu residue at position 8 of the NC protein (Fig. 2); the corresponding residue in REV-A was identified as Gln (49). Otherwise, the predicted nucleocapsid sequence is the same for both viruses.

Examination of the *pol* open reading frame exposed several series of amino acids that are highly conserved among previously characterized retrovirus proteases and nonviral acid proteases (35). This includes the presence of the Asp-Thr(Ser)-Gly motif, containing the active site aspartic acid residue (Fig. 2, residue 32) and the X-X-Gly-X-Asp motif located in the C-terminal portion of the protein (29, 35) (Fig. 2, beginning at residue 88). We conclude from the location of these protease-specific sequences and by homology with MLV that the first five amino acids of the SNV protease are encoded by the last 15 base pairs of the *gag* open reading

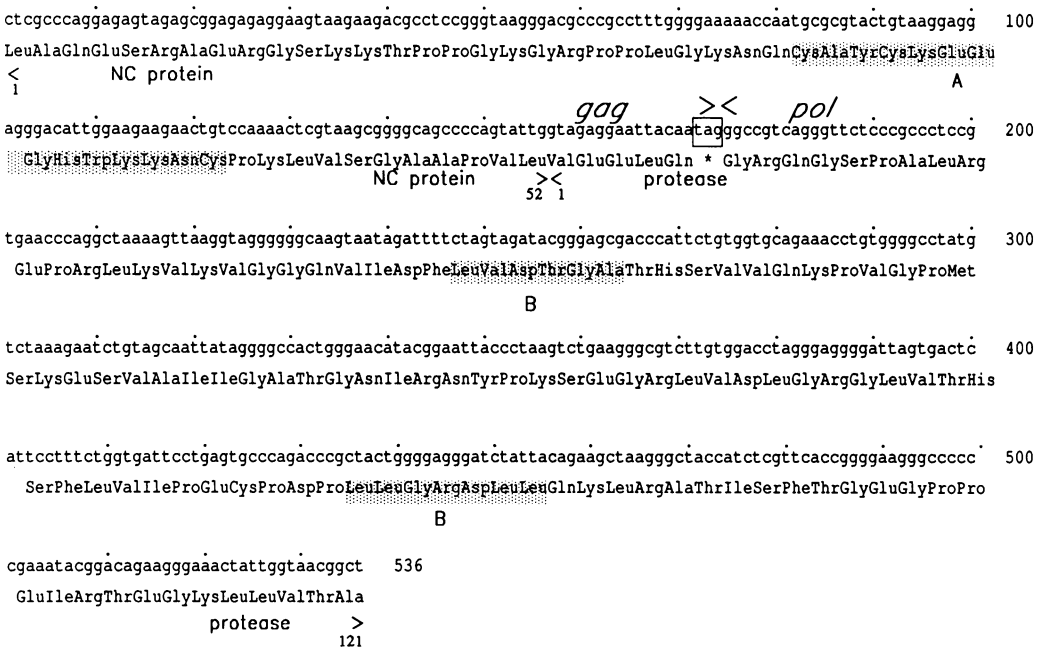

FIG. 2. Nucleotide sequence surrounding the SNV *gag-pol* junction. The DNA sequence is presented in the 5′-to-3′ direction in the same polarity as the genomic RNA and consists of one large open reading frame with the exception of an amber stop codon starting at nucleotide 172. The deduced amino acid sequence from the open reading frame is given below the nucleotide sequence, with key residues numbered according to their positions from the N terminus of the polypeptide. Large inverted arrows delineate the boundaries of the *gag* and *pol* genes, while small inverted arrows mark the termini of the NC and PR proteins as determined by amino acid sequence analysis of the NC protein (49). Shaded areas include a conserved amino acid sequence in the NC protein (Cis-His box) (A) and two conserved amino acid sequences in the protease (B).

frame, the sixth is derived by suppression of the amber stop codon, and the remaining amino acids are encoded by the *pol* open reading frame up to the Ala codon at position 121.

A mutation destroying the *gag* stop codon results in efficient *gag-pol* readthrough in vitro and protease activity in *E. coli*. A main goal of this work was to characterize the role translational termination plays in viral replication. Therefore, site-directed mutagenesis was employed in order to change the SNV *gag* amber stop codon (TAG) into a glutamine codon (CAG). This particular mutation was chosen because of previous results indicating that a glutamine residue is inserted into the nascent polypeptide at the site corresponding to the *gag* stop codon during translation of MLV mRNA (54).

Site-directed mutagenesis (24) was used to create the desired readthrough mutation in the bacterial *gag-pol* expression vector pTW63 (Fig. 3A). This vector possesses an f1 origin of replication, allowing the generation of a single-stranded template and a phage T7 transcriptional promoter upstream of the viral insert, useful for synthesis of capped viral RNA in vitro. A correct mutant (pTW76) was initially detected by a *Pst*I restriction site polymorphism created at the mutated stop codon and then directly confirmed by DNA sequence analysis (data not shown). pTW76 should encode an 84-kDa *gag-pol* fusion protein resulting from efficient translation of the *pol* gene. This protein is expected to contain the glutamine residue (codon CAG) as well as a glutamine-to-leucine (codon CTG) substitution because of alteration of the codon immediately 5′ to the primary mutation (Fig. 3A).

We attempted to determine the level of *gag-pol* translational readthrough for the mutant pTW76 in vitro and in *E. coli* in order to ensure that efficient readthrough into the *pol* gene had been effected. Synthetic RNA was generated in vitro and used to program a rabbit reticulocyte lysate supplemented with calf liver tRNA and [^{35}S]methionine-cysteine. The presence viral proteins in each lysate was assayed by immunoprecipitation with antisera raised against the entire *gag* polyprotein (Materials and Methods). Translation of the control pTW63 RNA resulted in accumulation of both the 55-kDa *gag* polyprotein and to a lesser extent 84-kDa *gag-pol* readthrough polyprotein (Fig. 4, lane 2). In contrast, translation of the readthrough mutant pTW76 RNA resulted in synthesis of the 84-kDa *gag-pol* polyprotein (Fig. 4, lane 3). These data indicate that like MLV (10, 37), suppression of the SNV *gag* amber codon can be attained in vitro. Moreover, consistent with the mechanism of amber stop codon suppression, elimination of the *gag* amber stop codon in pTW76 caused complete readthrough into the *pol* gene.

For further characterization of the *gag* and *gag-pol* products of the primary pTW76 protein products, to determine whether readthrough of the natural amber stop codon can take place in *E. coli* and to assess whether the viral protease can function in a bacterial system, we examined expression of both pTW63 and pTW76 *E. coli*. Both pTW63 and pTW76 were transformed into *E. coli* JM109, and induced cell lysates were assayed for the presence of fusion proteins by Western analysis using *gag* antisera. To our surprise, a major *gag* protein found in the pTW76 as well as the pTW63 lysate (Fig. 5B, lane 3) was a 27- rather than the 84-kDa readthrough product observed in vitro. This 27-kDa protein comigrated with the mature viral CA protein found in chronically infected D17 dog osteosarcoma cells (Fig. 5B, lane 2). Moreover, direct N-terminal sequence analysis of the *E. coli*-produced 27-kDa protein revealed that the first 10 residues were identical to those previously determined for

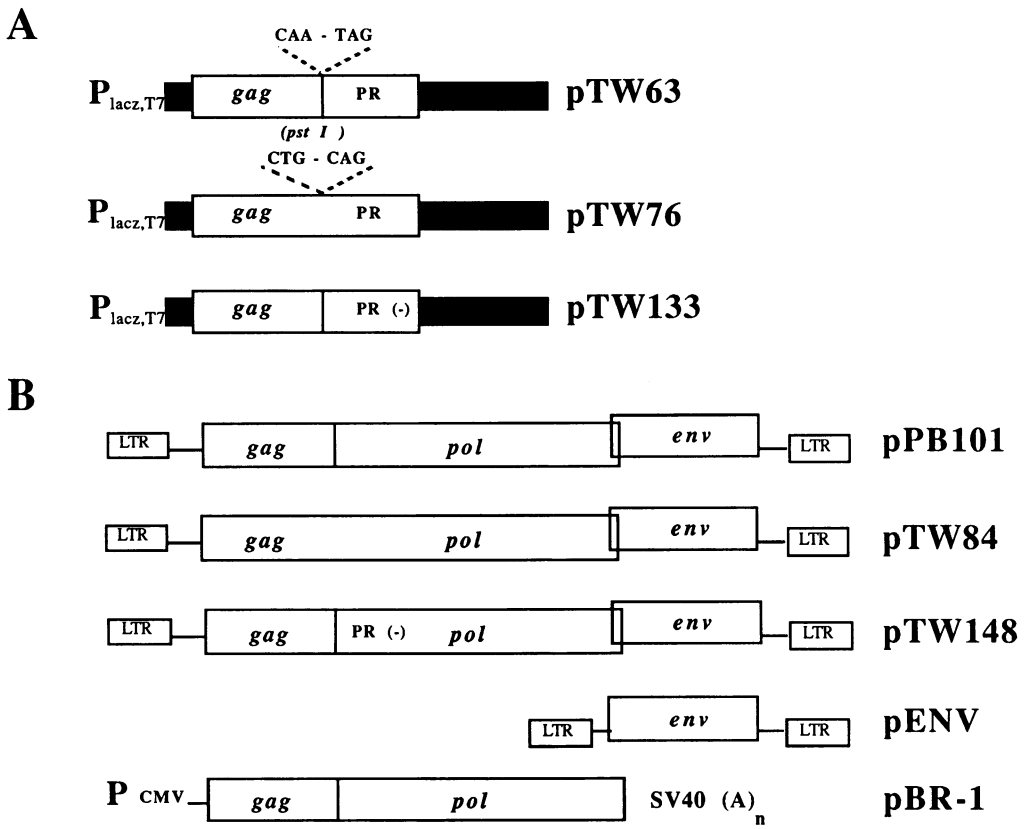

FIG. 3. (A) *E. coli* vectors used to express *gag* and the protease region of *pol* genes. A mutation in pTW76 creates a *Pst*I restriction site and a substitution of Leu-Gln for Gln-stop at the *gag* stop codon. pTW133 has a mutation introduced at the active site Asp residue at position 33 of the viral protease that destroys all cleavage activity (submitted). P, Bacterial and phage promoter. Bacterial coding sequences (■) were used for fusion with SNV viral sequences (□). (B) Eucaryotic expression vectors. pTW84 has the readthrough mutation derived from pTW76; pTW148 has the protease mutation derived from pTW133; and pPB101, pENV, and pBR1 have been previously described (1, 5, 7). Abbreviations: P, human cytomegalovirus promoter; SV40 $(A)_n$, polyadenylation signal from simian virus 40. SNV viral sequences are represented by open boxes.

the REV-A CA protein (49). Therefore, in cells expressing pTW76, efficient suppression of the *gag* gene stop codon caused by the readthrough mutation apparently resulted in activation of the viral protease in *E. coli* and release of the viral CA protein from the *gag* precursor polyprotein. Furthermore, the protease can efficiently process the 84-kDa *gag-pol* polyprotein in the absence of the wild-type 55-kDa *gag* polyprotein substrate.

We next used the protease activity in *E. coli* as a functional assay for *pol* gene expression to investigate the mechanism of translational readthrough employed by SNV. When the fusion protein encoded by plasmid pTW63, containing the wild-type *gag* termination codon, was expressed in amber suppressor-negative *E. coli* JM83, no protease activity was detected by Western blot analysis (Fig. 5A, lane 3). In contrast, protease activity was observed when the same fusion protein was induced in amber suppressor-positive *E. coli* JM109 (Fig. 5A, lane 2). Cleavage of the *gag* polyprotein in the amber suppressor-positive *E. coli* strain was shown to be dependent on the viral protease by demonstrating that a protease-negative derivative of pTW63 (pTW133) did not exhibit cleavage activity when induced in JM109 cells (Fig. 5B, lane 5). These results are consistent with the mechanism of amber stop codon suppression for translational readthrough into the *pol* gene.

Removal of the *gag* amber stop codon abrogates viral

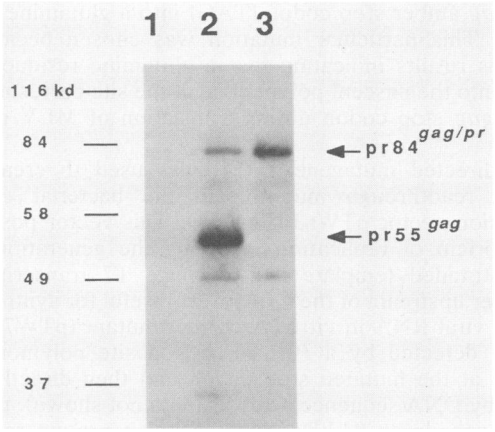

FIG. 4. Immunoprecipitation of *gag-pol* fusion proteins translated in vitro. RNA was translated in a cell-free rabbit reticulocyte lysate for 1 h in the presence of [^{35}S]methionine-cysteine. Lysates were precleared with preimmune serum and then immunoprecipitated with *gag* antisera. The proteins were analyzed by SDS-PAGE followed by 17-h autoradiography. Lane 1, No RNA; lane 2, RNA synthesized from pTW63 (wild type); lane 3, RNA synthesized from pTW76 (readthrough mutant). $pr84^{gag/pr}$, Polyprotein encoded by pTW76; $pr55^{gag}$, polyprotein encoded by pTW63.

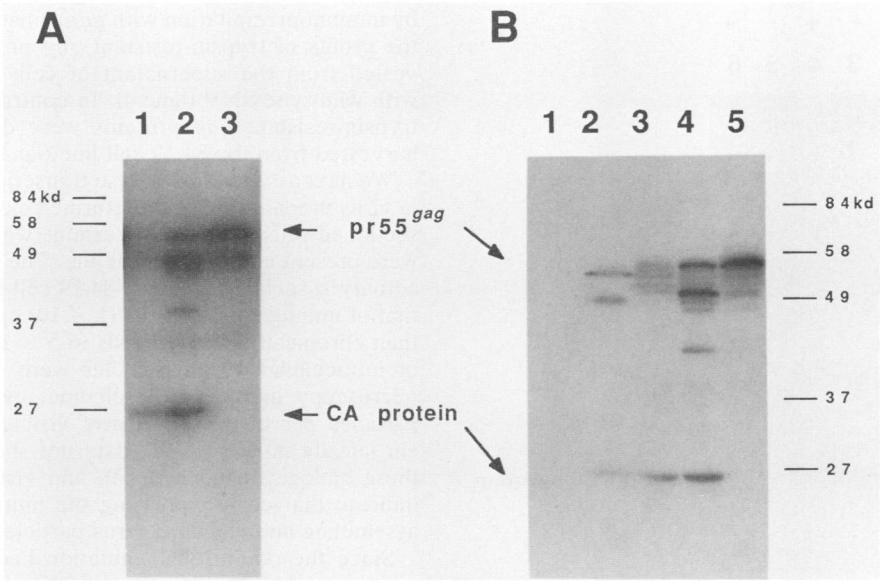

FIG. 5. Western blot analysis of *gag* and *gag-pol* fusion proteins synthesized in *E. coli* cell lysates. Vectors pTW63, pTW76, and pTW133 were transformed into *E. coli* JM83 and JM109 and induced for 6 h with 1 mM IPTG. Total cellular lysates were separated by SDS-PAGE, and viral antigens were visualized by ^{125}I-protein-A Western blotting by using *gag* antisera (see Materials and Methods). (A) Lane 1, D17 cells chronically infected with SNV; lane 2, pTW63 in *E. coli* JM109 (amber suppressor positive); lane 3, pTW63 in *E. coli* JM83 (amber suppressor negative). (B) Lane 1, Uninfected D17 cells; lane 2, D17 cells chronically infected with SNV; lane 3, pTW76 in JM83; lane 4, pTW63 in JM109; lane 5, pTW133 in JM109. pr55^{gag}, viral *gag* polyprotein.

replication. The readthrough mutation from pTW76 was subcloned into a full-length viral DNA (designated pTW84, Fig. 3B) and tested for infectivity in D17 cells. pTW84 and pJD214hygro (a retrovirus vector expressing the hygromycin resistance [Hyg^r] gene) (6) were cotransfected into D17 cells, and the medium harvested 7 days posttransfection was used as a potential source of virus to infect fresh D17 cells. If the readthrough mutant is replication competent, it should allow its own propagation and also serve as a helper virus for the mobilization of the Hyg^r vector. Possible transmission of the readthrough mutant was assayed by immunofluorescence with *gag*-specific antisera, whereas mobilization of the retroviral vector was assessed by scoring Hyg^r colonies following 2 weeks of selection with hygromycin B. As positive and negative controls for this replication assay, equivalent concentrations of either the wild-type SNV proviral DNA (pPB101) (1) or no DNA were used in parallel cotransfections. The results from this experiment showed that the titer of the Hyg^r virus was reduced by at least 5 orders of magnitude when the readthrough mutant was used as helper virus (Table 1). In addition, less than 0.1% of the cells cotransfected with the readthrough mutant DNA expressed viral antigens. Therefore, the readthrough mutation completely interrupts virus replication.

One hypothesis which could account for the replication-defective phenotype of the readthrough mutant is that the increased level of *pol* gene expression is lethal to the host cell. If so, the transfected cells might die before virus budding. We tested this hypothesis by determining whether the transfection efficiency of pJD214hygro could be substantially lowered when excess readthrough mutant viral DNA is present. D17 cells were either transfected with pJD214hygro alone or cotransfected with pJD214hygro and a 50-fold excess of pTW84 or pPBR322. The results of this experiment indicate that pTW84 did not significantly reduce the number of stable Hyg^r transfectants (Table 1). The defect must therefore be intrinsic to viral replication and not due to toxicity of one of the *pol* gene products to the host cell.

The readthrough mutant retains the ability to undergo intracellular proteolytic cleavage but is unable to produce virion particles. A stable cell line harboring pTW84 and pJD214-hygro DNA and expressing viral proteins was isolated in order to investigate the step(s) of viral replication affected by the readthrough mutation. This line (84.19) was tested for the ability to release trypsin-resistant virion par-

TABLE 1. Effects of readthrough mutation on viral replication and host cell viability

DNA	Hyg^r colonies/μg of pJD214hygro	Virus titer
Effect of readthrough mutation on virus titer[a]		
None	ND[b]	0
pPB101	ND	10^5
pTW84	ND	0
Effect of increased *pol* gene expression on cell viability[c]		
None	170	ND
pBR322	30	ND
pTW84	100	ND

[a] Seven days posttransfection, the titer of the Hyg^r vector (pJD214hygro) present in the culture supernatant was determined by serial dilution and infection of parallel cultures of 10^6 D17 cells. The number of Hyg^r colonies resulting from successful infection was counted after 3 weeks of selection with hygromycin B.

[b] ND, Not determined.

[c] To test the possibility that efficient expression of the *pol* gene was lethal to the host cell and thus indirectly affected virus infectivity, we cotransfected the Hyg^r vector (pJD214hygro) along with a molar excess of either nonspecific pPBR322 or mutant viral DNA into D17 cells. The average number of Hyg^r colonies from five independent transfections observed after 3 weeks of selection with hygromycin B was used as a measure of host cell viability.

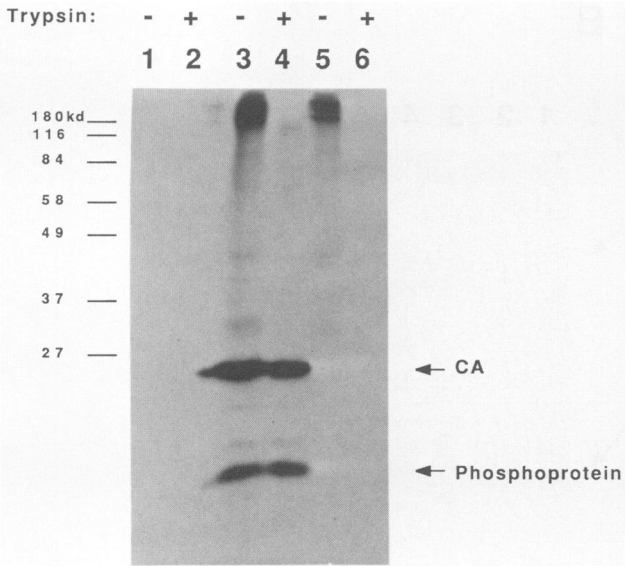

FIG. 6. Immunoprecipitation of trypsin-resistant virion particles. Virons were purified from tissue culture supernatant after 17-h metabolic labeling with [^{35}S]methionine-cysteine. Each preparation was split in two and treated (+) or not treated (−) with trypsin (1 μg/ml) for 30 min at 37°C. Proteins were immunoprecipitated with *gag* antisera, separated by SDS-PAGE, and autoradiographed for 17 h. Lanes 1 and 2, Uninfected D17 cells; lanes 3 and 4, chronically infected D17 cells; lanes 5 and 6, 84.19 cells. The identity of the *gag* PP (phosphoprotein) was determined by labeling cells with [^{32}P]phosphate and immunoprecipitation (data not shown).

ticles into the culture supernatant by first growing cells in the presence of [^{35}S]methionine-cysteine for 17 h and then treating harvested virus with 1 μg of trypsin per ml for 30 min. The resulting trypsin-resistant particles were analyzed by immunoprecipitation with *gag* antiserum. Figure 6 shows the profile of trypsin-resistant *gag* proteins in virions harvested from the supernatant of cells chronically infected with wild-type SNV (lane 4). In contrast to this pattern, no trypsin-resistant *gag* proteins were detected in the virus harvested from the 84.19 cell line (lane 6).

We next assayed for reverse transcriptase activity present in virus preparations and performed electron microscopy on sectioned cells to further determine whether virion particles were present in the 84.19 cell line. The reverse transcriptase activity associated with the 84.19 cell line was the same as that of uninfected D17 cells (1×10^4 cpm) and 650-fold less than chronically infected cells (6.5×10^6 cpm). No budded or intracellular virion particles were detected by electron microscopy in the 84.19 cell line, even though up to 50 particles per cell were clearly visible in thin sections of chronically infected cells (data not shown). The results of these biological, biochemical, and visual assays uniformly indicate that cells expressing the mutant provirus are not assembling and releasing virus particles.

Since the readthrough mutation had a drastic effect on virus assembly, we attempted to determine whether proteolytic processing of the *gag-pol* polyprotein was taking place within the cell. Western blot analysis using *gag* antisera was performed on intracellular smooth membrane preparations obtained from 84.19 and chronically infected cell homogenates following high-speed centrifugation (Materials and Methods); we found that this preparation greatly enriches the recovery of viral antigens and increases the sensitivity of the Western assay. Figure 7A illustrates the intracellular cleavage pattern of wild-type and readthrough mutant *gag* polyproteins. The 55-kDa *gag* polyprotein within chronically infected cells accumulated to roughly 20 times the level of the 200-kDa *gag-pol* precursor (Fig. 7A, lane 2). This 55-kDa protein actually represents two species of *gag* precursors, an abundant species that is formed by translational termination

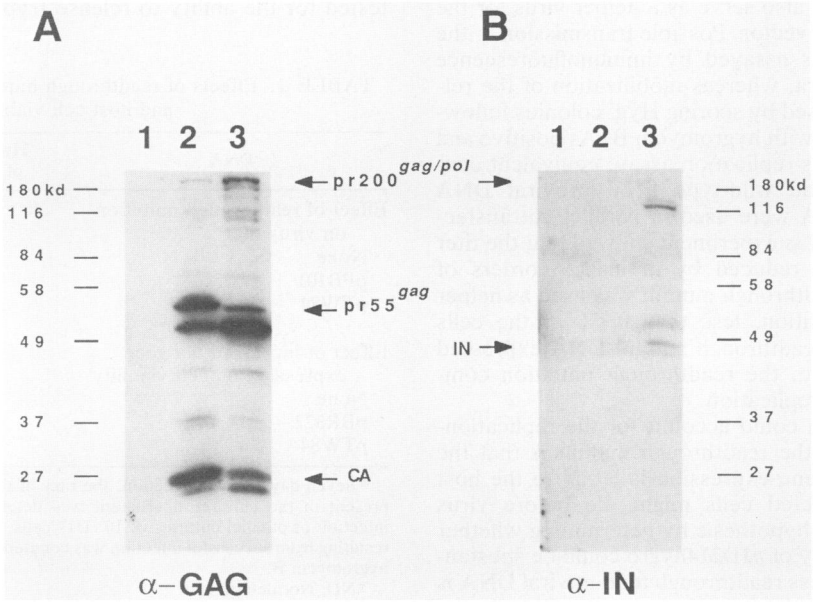

FIG. 7. Pattern of intracellular *gag-pol* polyprotein cleavage in cells expressing wild-type and readthrough mutant viral polyproteins. (A) *gag* antiserum; (B) IN protein antiserum. The smooth membrane fractions prepared from cell homogenates following high-speed centrifugation were analyzed by Western analysis and 17-h (A) or 3-day (B) autoradiography. Samples were prepared from uninfected D17 cells (lane 1), chronically infected D17 cells (lane 2), and 84.19 cells (lane 3). pr55^{55}, Viral *gag* polyprotein; pr200$^{gag\text{-}pol}$, viral *gag-pol* polyprotein.

at the end of the *gag* gene and a less abundant species generated by posttranslational proteolytic cleavage at the N terminus of the protease domain of the polyprotein. These two species differ only at their C terminus, where the proteolytically cleaved species lacks five residues that are present just upstream of the termination codon (Fig. 2). In 84.19 cells, a 55-kDa *gag* precursor protein was apparent (Fig. 7, lane 3) and most likely represents the proteolytically cleaved species of *gag* precursor. The increased level of *pol* expression in the readthrough mutant could also be seen when *pol*-specific IN protein antiserum was used to probe an identical Western blot (Fig. 7B, lanes 2 and 3). The large precursor identified by the IN protein antiserum had an approximate molecular mass of 120 kDa and may represent an unprocessed RT (80 kDa)-IN (44 kDa) polyprotein cleavage intermediate. Both the wild-type and readthrough mutant polyproteins undergo proteolytic cleavage as evidenced by the accumulation of smaller *gag* and *pol* specific proteins that comigrate with mature virion proteins (Fig. 7, lanes 2 and 3).

The readthrough mutation can be rescued in *trans* by coexpression of the *gag* polyprotein. Attempts at rescuing the readthrough mutant by genetic complementation were undertaken by introducing into the 84.19 cell line a retroviral vector that expresses a wild-type SNV *gag* polyprotein. The vector TW148 (Fig. 3B) was derived from pTW133 and constitutes a full-length SNV virus containing a mutant protease that causes a total block in viral replication (submitted for publication). We determined whether the wild-type *gag* polyprotein encoded by pTW148 could complement in *trans* the *gag-pol* polyprotein present in 84.19 cells by assaying for mobilization of the endogenous JD214hygro vector following transient transfection with pTW148. Supernatant harvested 7, 14, and 21 days posttransfection was used to infect fresh D17 cells. Any Hyg^r colonies obtained following this infection were pooled and expanded. The supernatant from these pooled colonies was then used for a second infection of fresh D17 cells. The growth of Hyg^r colonies after this second round of infection would indicate that the virus transferring the Hyg^r phenotype was the result of recombination or reversion rather than complementation in *trans*. As a control for the efficiency of rescue that might be obtained by complementation, we transfected an envelope glycoprotein expression vector (pEnv; Fig. 3B) (5) into a cell line expressing *gag* and *pol* genes (made with pBR1, Fig. 3B) (7). Because this cell line also contains an endogenous JD214hygro vector, it was possible to measure the level of complementation by wild-type genes in the same manner as that for complementation of 84.19 cells by TW148.

The results from this analysis are summarized in Table 2. Infectious virus was detected at 7-, 14-, and 21-day harvests. The virus titers at 7 and 14 days posttransfection were equivalent to that obtained when the envelope glycoprotein was introduced into the *gag-pol* cell line (Table 2). At these early times of virus harvest, no recombination between viral sequences or reversion took place, as evidenced by the lack of replication-competent virus and viral antigens (detected by immunofluorescence). The titer of Hyg^r virus obtained from the pTW148-84.19 transfection increased drastically between 14 and 21 days posttransfection. One hundred percent of the Hyg^r colonies derived from the day 21 infection expressed viral antigens and there was passage of the JD214hygro vector through a second round of replication. Therefore, we believe that a replication-competent virus originated in the pTW148-84.19 transfected cells sometime between day 14 and 21 posttransfection. The helper virus functions seen before that time were the result of complementation between the readthrough mutant *gag-pol* polyprotein and the *gag* polyprotein encoded by TW148.

TABLE 2. Rescue of readthrough mutant phenotype by genetic complementation[a]

DNA	Cell line	Viral harvest at: Day 7 Titer	Day 7 Recombination	Day 14 Titer	Day 14 Recombination	Day 21 Titer	Day 21 Recombination
None	D17	0	ND	0	ND	0	ND
	84.19	0	ND	0	ND	0	ND
pTW148	D17	0	ND	0	ND	0	ND
	84.19	10^2	−	10^3	−	$>10^6$	+
pEnv	D17	0	ND	0	ND	0	ND
	BR-2	10^2	−	10^2	−	10^3	−

[a] Transfections were performed with 5 μg of DNA (see Materials and Methods), and titers of the virus were determined at 7, 14, and 21 days posttransfection in the same manner as described in Table 1. Recombination was measured in two ways, either by detection of viral antigens in the Hyg^r cells or by passage of JD214hygro virus through a second round of replication.

DISCUSSION

Our DNA sequence analysis indicates that the *gag* and *pol* genes of SNV are in the same open reading frame but are separated by a single amber stop codon. Moreover, regions of amino acid homology adjacent to the *gag-pol* junction indicate that like other mammalian type C retroviruses, the NC protein maps to the C-terminal portion of the *gag* gene, whereas the PR protein is derived predominantly from the amino terminal portion of the *pol* gene. Although the precedent set by MLV leads to the a priori assumption that *pol* gene expression is attained through amber codon suppression, RNA spicing or multiple ribosomal frameshifting are alternative mechanisms and cannot be ruled out solely on the basis of sequence analysis. We have shown that *gag-pol* translational readthrough is achieved when synthetic viral RNA is translated in an in vitro rabbit reticulocyte system (Fig. 3). In addition, efficient *gag-pol* readthrough in *E. coli*, as measured by the activity of the downstream protease, only occurs in amber suppressor-positive hosts (Fig. 5A). These data indicate that translational suppression of the amber codon is responsible for *pol* gene expression. We believe that suppression in infected cells is mediated by a naturally occurring tRNA species, since a limited *cis*-acting component of the AK virus *gag* amber codon is sufficient to allow suppression (33) and since the array of $tRNA^{Gln}$ species is the same in both uninfected and infected cells (10).

The readthrough mutation created at the *gag* stop and adjacent Leu codon resulted in efficient translation of the *pol* gene and subsequent proteolytic cleavage when assayed in *E. coli* and in tissue culture. Therefore, the Gln-to-Leu substitution adjacent to to stop codon mutation does not affect protease function, even though the mutation is in the protease coding sequence. Repeated attempts to detect proteolytically mature *gag* proteins in the in vitro translation lysate were unsuccessful (data not shown). The in vitro lysate was incubated under the same conditions that were optimized for an in vitro retrovirus protease assay (23), but it still failed to produce mature *gag* proteins. The addition of mature viral protease in *trans* to the lysate (in the form of NP40-disrupted virions) also did not induce cleavage (data

not shown). Although it is possible that the conformation of the in vitro-synthesized *gag-pol* polypeptide is incorrect, we believe that the block in proteolytic activity in vitro is unlikely to be due to an intrinsic failure of the polyprotein substrate since the same protein is capable of proteolytic cleavage when expressed in *E. coli*. Instead, we favor the hypothesis that this result was due to a relatively low concentration of polyprotein substrate (and also viral protease) when expressed in vitro. High-level expression of the fusion polyprotein in *E. coli* resulted in the accumulation of insoluble particles known as inclusion bodies (32). Inclusion bodies were visible in the electron microscope as densely staining, roughly spherical particles of a relatively constant size (40 nm) (data not shown). It is possible that the formation of these particles allowed the concentration of polyprotein substrates to the level needed for dimerization and activation of the viral protease.

Analysis of the 84.19 cell line demonstrated that expression of the SNV *gag-pol* polyprotein in the absence of any *gag* polyprotein resulted in loss of particle assembly and virion release. This block in viral assembly was overcome by introducing a wild-type *gag* polyprotein in *trans*. Therefore, the unprocessed *gag* polyprotein is an essential organizing unit in retrovirus particle assembly, a result that is consistent with previous observations made with retrovirus mutants that express only *gag* (11, 41, 43) or *gag-pol* polyproteins (9).

Interestingly, Western blot analysis of the 84.19 cell line revealed that the *gag-pol* polyprotein undergoes wild-type intracellular proteolytic cleavage in the absence of intact *gag* polyprotein or particle release. A similar mutation created in MLV that changed the *gag* amber stop codon into a glutamine codon was also shown to abolish viral assembly and release; however, no intracellular proteolytic activity was observed in a cell line expressing mutant MLV DNA (9). These apparently conflicting results most probably reflect a genuine difference between the two virus species. In particular, the requirement for polyprotein cleavage in SNV may differ from MLV to the extent that only the SNV protease is activated under the conditions described in these experiments. Consistent with this idea is the observation that, unlike SNV, the MLV protease does not readily function when overexpressed as part of a *pol* polyprotein in *E. coli* (S. Goff, personal communication).

The processing pattern of *gag-pol* polyproteins in 84.19 cells revealed that a 55-kDa *gag* polyprotein was generated (Fig. 7A, lane 3). It is possible that this protein species resulted from translational termination near the end of the *gag* gene following ribosomal pausing induced by some intrinsic structural feature of the RNA transcript near the mutated *gag* termination signal. However, the same 55-kDa *gag* precursor was not observed when mutant viral RNA was translated in vitro (Fig. 4). Instead, we believe that this species of *gag* polyprotein was released from the *gag-pol* polyprotein following cleavage between the nucleocapsid and protease domains. It is surprising that this *gag* precursor did not participate in virion assembly. However, since five residues are removed from the C terminus of this species of *gag* polyprotein during proteolysis (Fig. 1 and 2), a change in the overall polyprotein conformation may interfere with its ability to participate in multimeric interaction during particle assembly. In support of this hypothesis is the recent report of C-terminal deletion of the HIV *gag* polyprotein found to be incapable of appropriate particle assembly (11). The mutation which substitutes Leu for Gln adjacent to the stop codon represents a further alteration that could disrupt normal assembly; however, a site-specific mutation that changes the amber codon into a Gln codon without altering the C-terminal Gln has been made and also found to be blocked in replication and virion release (data not shown).

An alternative hypothesis that may explain the inability of the proteolytically cleaved 55-kDa *gag* precursor to participate in multimeric assembly is that a specific stoichiometry between the 55-kDa *gag* protein and the 200-kDa *gag-pol* polyprotein is absolutely required. The ratio of 55-kDa *gag* polyprotein to 200-kDa *gag-pol* polyprotein in 84.19 cells (Fig. 7A, lane 3) was significantly less than that observed in chronically infected cells (Fig. 7A, lane 2). It is possible that at this reduced level, the 55-kDa *gag* polyprotein cannot participate in particle assembly. It has also been suggested that the *gag-pol* polyprotein may interfere with virion assembly through steric hindrance of the necessary protein-protein contacts (9). A recent study investigating the nature of HIV particle assembly in an insect cell line overexpressing the *gag* polyprotein also described a drastic decrease in particle release when the wild-type *gag-pol* polyprotein was present (11). This interesting result could be explained in two ways. Perhaps the protease encoded by the *gag-pol* polyprotein was activated to the extent that it cleaved the *gag* precursors before they were able to participate in assembly. Alternatively, the large *gag-pol* polyprotein may dominantly interfere with unprocessed *gag* polyprotein during multimer formation. Dominant interference affecting particle assembly has since been demonstrated by using mutations in the HIV CA protein (48). It should be noted however that in the experiments presented here, the virus titer obtained by coexpression of wild-type polyproteins and envelope glycoprotein was equivalent to the virus titer obtained during rescue of the readthrough mutant *gag-pol* polyprotein (Table 2). Therefore, if any dominant interference was induced by an excess of *gag-pol* polyprotein, it was not drastic enough to decrease the levels of viral replication appreciably.

In addition to detailing the mechanism of SNV *gag-pol* readthrough, we have shown an absolute requirement for intact *gag* polyprotein during particle assembly. The presence of proteolytically cleaved *gag* precursor is not enough to make up for the lack of the full-length *gag* polyprotein, possibly because of the lack of the C-terminal portion of the polyprotein or an altered *gag* to *gag-pol* polyprotein stoichiometry. One experiment that could distinguish between these two possibilities would be to artificially introduce a stop codon in place of the nucleocapsid-protease cleavage site in pTW84. Efficient termination at this site would mimic the conditions present in the 84.19 cell line without overabundant expression of the *gag-pol* polyprotein.

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