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Therapeutic Effect of Gag-Nuclease Fusion Protein on Retrovirus-Infected Cell Cultures

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Capsid-targeted viral inactivation is a novel protein-based strategy for the treatment of viral infections. Virus particles are inactivated by targeting toxic fusion proteins to virions, where they destroy viral components from within. We have fused *Staphylococcus* nuclease (SN) to the C-terminal end of Moloney murine leukemia virus Gag and demonstrated that expression of this fusion protein in chronically infected chicken embryo fibroblasts resulted in its incorporation into virions and subsequent inactivation of the virus particles by degradation of viral RNA. Release of particles incorporating Gag-SN fusion proteins into the extracellular milieu activates the nuclease and results in destruction of the virion from within. By comparing the effects of incorporated SN and SN*, an enzymatically inactive missense mutant form of SN, on the infectivity of virus particles, we have clearly demonstrated that nucleolytic activity is the antiviral mechanism. Expression of Gag-SN fusion proteins as a therapeutic agent causes a stable reduction of infectious titers by 20- to 60-fold. The antiviral effect of capsid-targeted viral inactivation in our model system, using both prophylactic and therapeutic approaches, suggests that a similar anti-human immunodeficiency virus strategy might be successful.

A general strategy for the treatment of infectious diseases termed intracellular immunization (3) is designed to render cells resistant to viral replication and to limit the spread of virus in a cell culture or an individual. In essence, intracellular immunization involves the efficient and stable transfer of genes that inhibit viral replication into cells that are potential targets for viral infections. The different inhibition strategies based on this principle can be subdivided into RNA-based and protein-based strategies (reviewed by Gilboa and Smith [14]).

We have developed a protein-based antiviral strategy called capsid-targeted viral inactivation (CTVI), in which virion structural protein-nuclease fusion proteins are targeted to virions, where they inactivate the virus by degrading its genomic RNA. The strategy takes advantage of the fact that Gag and Gag-Pol precursors are self-associating proteins capable of assembling into virions.

By using the yeast retrotransposon Ty1, an element whose transposition mechanistically resembles retroviral multiplication, this approach was tested for the first time as a novel means to interfere with viral replication (28). The strategy can inhibit transposition of yeast retrotransposon Ty1 by at least 98%.

The process of retroviral particle formation is still poorly understood. During assembly, Gag precursors appear to be incorporated into virions via Gag-Gag interactions, and the Gag portion of Gag-Pol is essential for targeting the polyprotein into the virion (21, 40, 41). The CA and NC domains of the precursors are involved in Gag-Gag interactions in human immunodeficiency virus type 1 (HIV-1) particles (9, 11, 25). Experiments have indicated that two regions, MA and CA, participate in murine leukemia virus (MuLV) assembly (16, 17, 20). Although Gag is the only viral protein required for assembly, it is likely that cellular proteins are also necessary in HIV-1 assembly (26).

Fusion proteins consisting of an N-terminal Gag moiety and a C-terminal foreign protein (e.g., cytochrome *c*, β-galactosidase, heterologous reverse transcriptases, and nucleases) coassemble with native Gag precursor proteins, forming chimeric retrovirus and retrotransposon particles (13, 17, 21, 28, 29, 38, 40).

We have fused *Staphylococcus* nuclease (SN) to the C-terminal end of MuLV Gag as part of a general strategy for destroying retroviruses from within. This nuclease degrades both RNA and DNA and, more importantly, depends on millimolar Ca^{2+} concentrations for activity (37). As cytoplasmic Ca^{2+} concentrations are typically in the nanomolar range and are always lower than 1 μM, SN is expected to be inactive intracellularly. Indeed, SN is nontoxic to *Saccharomyces cerevisiae* (28), chicken embryo fibroblasts (CEF) (29), and mammalian cells such as mouse fibroblasts (NIH 3T3 cells) and HeLa cells (42). Gag-SN fusion proteins incorporated into virions that are released into the extracellular milieu should become enzymatically active as a result of high Ca^{2+} concentrations and thus should degrade encapsidated viral RNA. The mechanism of calcium entry into the virion is unknown but is unlikely to be via a specific uptake system.

In a previous study, we developed a strategy for delivering and expressing murine Gag-SN fusion proteins by using the RCAS (replication-competent avian leukosis virus long terminal repeat splice acceptor) avian retrovirus gene expression system in avian cells (18, 32). Because the Gag moiety of the Gag-SN protein is derived from Moloney MuLV (M-MuLV), it neither coassembles with nor interferes with the avian retrovirus vector. Native avian retrovirus and MuLV-Gag proteins fail to coassemble, although certain chimeric versions of these proteins can interact (40a). These RCAS-infected CEF were then challenged with a murine retrovirus, Mo(4070A), that has a M-MuLV-derived *gag* gene and an *env* gene derived

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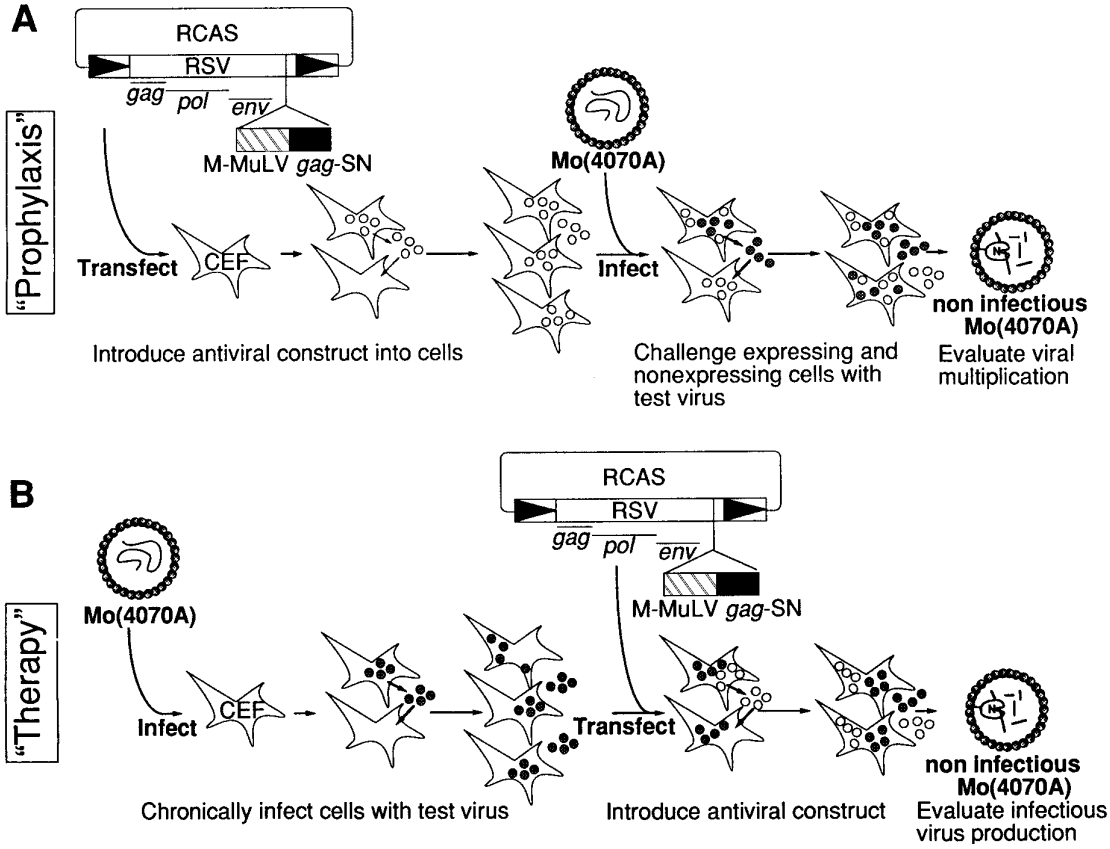

FIG. 1. Schematic representation of the prophylactic and therapeutic approaches to demonstrate the antiviral effect of Gag-SN. (A) Prophylaxis. The antiviral construct was introduced into CEF by transfection. To examine the effects of the Gag-SN fusion protein on viral multiplication, CEF expressing this protein were infected with a murine retrovirus at low multiplicity of infection. (B) Therapy. CEF were first chronically infected with Mo(4070A) at high multiplicity of infection and passaged seven times to ensure spreading of the virus throughout the culture. Then the antiviral RCAS construct was introduced, and its effect on infectious-virus production was evaluated for 34 days. Hatched circles, Mo(4070A) virions; open circles, RCAS virions; heavy lines, viral nucleic acid; N, nuclease. (C) Multiplication of Mo(4070A) in CEF after infection at high multiplicity of infection. After infection, cells were passaged every 2 days and the supernatant of the Mo(4070A)-infected cells was harvested and replaced by fresh medium. Infectious titers were determined by the S^+L^- focus formation assay. The curve reaches a plateau 9 days after infection, indicating that the virus has spread throughout the culture. FIU, focus-inducing units.

from the amphotropic 4070A virus that replicates in CEF (31). These experiments demonstrated the feasibility of CTVI in such a "prophylactic" approach (29), in which expression of the *gag-SN* gene preceded the challenge with the target virus Mo(4070A) (Fig. 1A).

However, although Mo(4070A) infection was inhibited by preinfection with a vector expressing Gag-SN, these experiments did not explicitly demonstrate that SN enzymatic activity was responsible for the observed antiviral effect—some or all of the effect could in principle be due to steric interference of the fusion protein (29).

In this paper, we describe a comparison between missense mutant (Gag-SN*) and wild-type Gag-SN to more directly demonstrate that nuclease activity is responsible for the antiviral effect observed. SN* was generated by site-directed mutagenesis of SN and differs from wild-type SN by two amino acid substitutions. Changing Glu to Ser (position 43) and Arg to Gly (position 87) does not influence the folding of SN* but reduces its specific activity 10^6-fold relative to that of wild-type SN (39).

Furthermore, to investigate the therapeutic potential of this novel antiviral strategy, we designed an experiment to assess the effectiveness of Gag-SN in a tissue culture previously infected with Mo(4070A). This is referred to as the therapy approach (Fig. 1B). We chronically infected CEF with the target Mo(4070A) virus, introduced the antiviral RCAS construct, evaluated the effect on infectious virus production, and biochemically examined the virions produced. We showed that M-MuLV Gag-SN fusion proteins incorporated into virions reduce the infectious titer of chronically infected CEF cultures significantly and inactivate viral particles by degradation of its

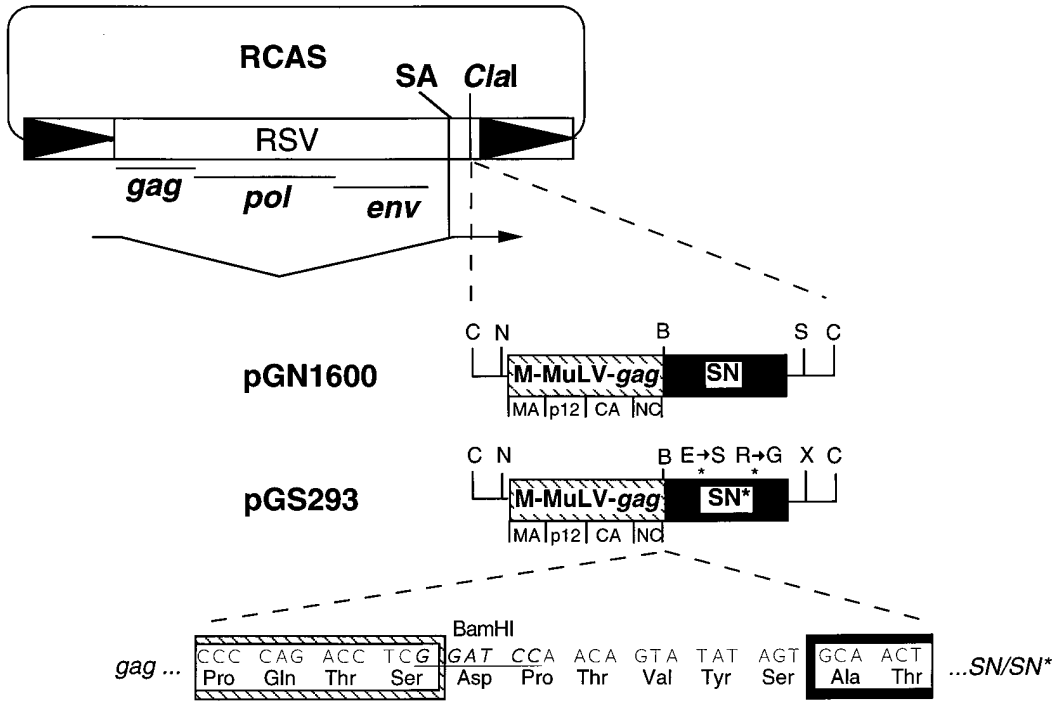

FIG. 2. Replication-competent retrovirus vector to introduce and express M-MuLV-*gag-SN* and M-MuLV-*gag-SN** fusions in CEF. The structure of the Rous sarcoma virus (RSV)-based RCAS vector has been described previously (32). Expression of M-MuLV-*gag-SN* (pGN1600) and M-MuLV-*gag-SN** (pGS293) is under control of the Rous sarcoma virus long terminal repeat. pGN1600 and pGS293 differ only in two missense mutations (∗) in the SN gene, which change Glu (E) to Ser (S) and Arg (R) to Gly (G) and result in inactive mutant enzyme (SN*). The four different domains of M-MuLV–Gag, matrix (MA), p12, capsid (CA), and nucleocapsid (NC) are indicated. Boxed triangles, long terminal repeats; open box, Rous sarcoma virus coding regions; hatched box, MuLV sequences; solid box, SN coding sequences; arrow with V, spliced mRNA produced in RCAS; SA, splice acceptor. Relevant restriction sites are indicated: *Bam*HI (B), *Cla*I (C), *Nco*I (N), *Sal*I (S), and *Xho*I (X).

encapsidated viral RNA. In addition, there appears to be a more modest "steric" antiviral effect afforded by both mutant and wild-type fusion proteins.

MATERIALS AND METHODS

Cell lines. CEF derived from 12-days-old chicken embryos of the EV-0 strain were grown at 39°C in 100-mm dishes containing M199 medium (Gibco) supplemented with 1% chicken serum, 1% fetal calf serum, 2% tryptose phosphate broth, penicillin, and streptomycin (Gibco no. 15140-015; 1:100 dilution). D56 cells used for the S^+L^- focus formation assay were maintained at 37°C in modified McCoy's 5A medium (Gibco no. 16600-025) supplemented with 10% fetal calf serum, 2 mM glutamine, and penicillin and streptomycin as above.

Construction of recombinant plasmids. The construction and detailed maps of the RCAS replication-competent avian retrovirus vector and its derivative pGN1600 have been described previously (19, 29). pGS293 was constructed by replacing the *Bam*HI-*Sal*I SN fragment in pGN1600 with the *Bam*HI-*Xho*I SN* fragment from pGN1709.3 (Fig. 2). The SN* gene was amplified by PCR from the double mutant E43S+R87G (39) and confirmed by DNA sequencing to contain no additional mutations.

S^+L^- focus formation assay. The assay we applied to determine infectious Mo(4070A) titers is based on the method described by Bassin et al. (4). D56 cells were plated at 9×10^4 cells per dish in 60-mm dishes containing 4 ml of medium. After 24 h of incubation at 37°C, the medium was aspirated and replaced by 2 ml of fresh medium containing 20 μg of DEAE-dextran (Pharmacia) per ml. The cells were incubated at 37°C for 20 min and rinsed with 2 ml of medium. Portions (500 μl) of six different dilutions (10^0 to 10^{-5}) of each viral supernatant were added to the D56 cells for infection. The cells were incubated again for at least 30 min. Then 6 ml of medium per dish was added, and the cells were grown for another 4 to 5 days. D56 cells were infected with supernatants from each CEF transfection series (including mock, *gag-SN*, and *gag-SN** transfection), and 4 to 5 days later, scoring of the resulting foci was performed for all samples on the same day. Scoring of the dishes was performed blind and subsequently decoded. To demonstrate the reliability and reproducibility of the three S^+L^- assay series, we used viral supernatant of known titer from Mo(4070A)-infected NIH 3T3 cells as a positive control.

Infections and transfections. Amphotropic Mo(4070A) produced from a proviral clone (30, 31) was used to infect CEF at a high multiplicity of infection as previously described (29). The titer of infectious particles was quantitated by the S^+L^- focus formation assay.

CEF cells were grown to a density of 7×10^6 to 1×10^7 cells per dish, passaged at a 1:3 dilution with trypsin, and transfected the next day with 10 μg of plasmid DNA by the calcium-phosphate precipitation method (24).

Zymograms. Viral particles were boiled for 2 min in Laemmli buffer and loaded on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel containing 10 μg of sheared herring sperm DNA per ml. Following electrophoresis at 100 V for 90 min in a Hoefer SE 260 slab gel electrophoresis unit, the gel was placed in water for 3 h and gently shaken in 40 mM Tris · HCl (pH 9.5)–0.1 mM $CaCl_2$–100 mM glycine–0.5 μg of ethidium bromide per ml at room temperature for 30 min to 2 days. The incubation allows protein refolding and enzymatic action, and the digested regions of the gel are detected under UV light as dark bands on a fluorescent background of ethidium bromide bound to the DNA. Gels were photographed with an Eagle Eye detection system (Stratagene) at 40 integrations. As little as 0.5 ng of purified SN was visible after 12 to 24 h of incubation.

Virus preparation and RNA isolation. Virions used for immunoblotting and zymograms were prepared from 30 ml of tissue culture supernatant as previously described (29). After removal of residual cells, virus particles were pelleted by centrifugation of the tissue culture supernatant for 30 min at 25,000 rpm and 4°C in a Beckman SW28 rotor, washed in 500 μl of buffer (50 mM Tris · HCl [pH 6.8], 100 mM NaCl), and resuspended in 30 μl of buffer. Virions used for RNA isolation were pelleted by centrifugation of 30 ml of sterile filtered tissue culture supernatant at 25,000 rpm for 30 min at 4°C in a Beckman SW28 rotor and resuspended in lysis buffer (50 mM Tris · HCl [pH 7.4], 10 mM EDTA, 1% SDS, 100 mM NaCl, 50 μg of yeast tRNA per ml, 100 μg of proteinase K per ml). Genomic RNA was extracted from the viral lysate with an equal volume of phenol followed by an equal volume of chloroform. After repetition of this extraction, the RNA was precipitated with 95% ethanol (12). Viral RNA isolated from 30 ml of tissue culture supernatant was dissolved in 30 μl of diethylpyrocarbonate-treated sterile water.

The denatured RNA was subjected to electrophoresis in a horizontal 1% agarose gel containing morpholinepropanesulfonic acid (MOPS) buffer and 6% formaldehyde (34). GeneScreen Plus membranes (Du Pont) were stripped by being boiled for 30 min in a solution of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–1 mM SDS. Northern (RNA) blot analysis under nondenaturing conditions was performed as previously described (22). The probes were a 1,150-bp *Sac*I-*Sal*I fragment from M-MuLV proviral DNA obtained from plasmid pNCA

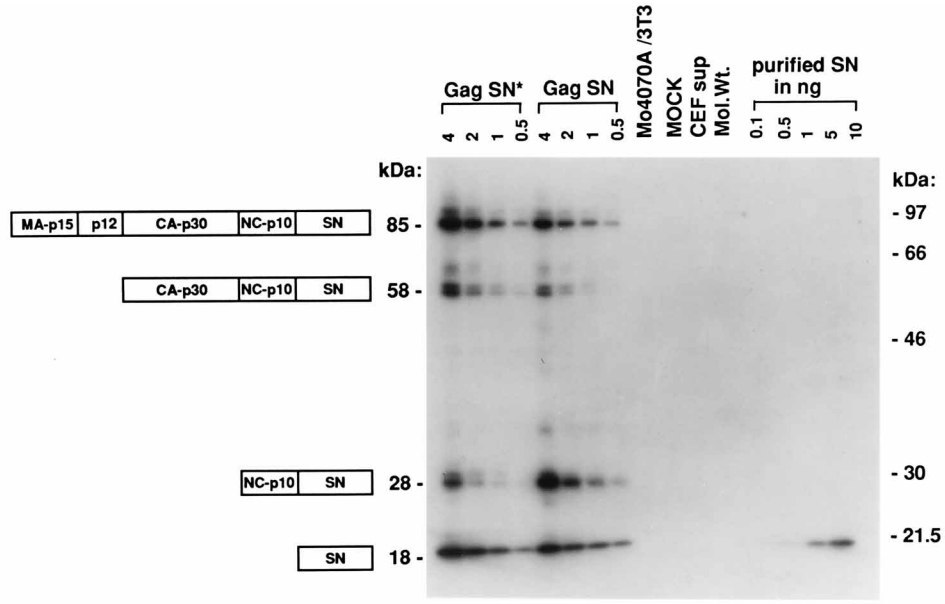

FIG. 3. Incorporation of Gag-SN and Gag-SN* fusion proteins into Mo(4070A) virions. Mo(4070A)-infected CEF were transfected with pGN1600 and pGS293. After transfection, 30-ml culture supernatants were collected every 18 to 24 h and virions were prepared and resuspended in identical volumes of buffer. Various volumes (4, 2, 1, and 0.5 μl) of viral pellet fractions from *gag-SN*- and *gag-SN**-transfected CEF cells (Gag-SN, Gag-SN*) harvested 9 days after transfection were subjected to SDS-polyacrylamide gel electrophoresis and examined by immunoblot analysis with anti-SN antibody as the probe. Control lanes were loaded with identical volumes (4 μl) of resuspended viral pellets from Mo(4070A)-infected NIH 3T3 cells (Mo4070A/3T3); mock-transfected, Mo(4070A)-infected CEF cells (MOCK); and supernatant from uninfected CEF cultures (CEF sup). Products of proteolytic processing of Gag-SN or Gag-SN* precursors are depicted as boxes. Purified SN (0.1 to 10 ng) was loaded to prepare a standard curve to quantitate SN expression. Molecular mass markers and estimated masses of the detectable processing products of Gag-SN and Gag-SN* fusion proteins are shown.

and a 2,940-bp *Sst*I-*Sna*BI fragment from RCAS derivative pGN1600. The DNA probes were labeled for hybridization with [α-^{32}P]dCTP by random priming (10).

Immunoblot analysis. Resuspended viral pellets were mixed with 2× Laemmli buffer, boiled, and separated on 10% polyacrylamide gels containing SDS. Following electrophoresis, the proteins were transferred by electroblotting, by standard methods (36), to an Immobilon-P membrane (supplied by Millipore) prewetted with 100% methanol and preincubated in transfer buffer for 10 min. Protein transfer was performed in a Genie apparatus (Idea Scientific) at 24 V for 60 min. After the transfer, blocking buffer (4% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]) was preabsorbed for 1 h at room temperature onto the filters, which were then incubated with the appropriate polyclonal antibody diluted in 2% BSA–PBS, washed with PBS, incubated with ^{125}I-protein A in 2% BSA–PBS, washed with PBS, and prepared for autoradiography. When goat anti-MuLV p30^{CA} was used as the primary antibody, the filter was incubated with ^{125}I-protein G instead of ^{125}I-protein A (ICN). Crude rabbit anti-SN antiserum, detecting SN and SN*, was used at a 1:2,000 dilution. Goat anti-p30^{CA} antibody, detecting Mo(4070A) capsid proteins, was used at a 1:1,000 dilution. The phosphorimager and the program ImageQuant used for phosphorimager analysis were supplied by Molecular Dynamics.

RESULTS

Delivery and expression of murine Gag-SN and Gag-SN* fusion genes in Mo(4070A)-infected cells. A CEF culture was infected with Mo(4070A) and passaged every other day for 14 days to ensure that the target retrovirus would spread throughout the culture; accumulation of infectious virus in the supernatant was monitored by the S^+L^- focus formation assay (4) (Fig. 1C). In three transfection series, each started from a single dish of infected confluent CEF culture, cells were transfected with pGN1600 or pGS293 or mock-transfected (Fig. 2). These constructs carry two fusion genes in which the C terminus of M-MuLV *gag* is joined in frame to *SN* (pGN1600) or the mutant *SN** (pGS293).

Transfected CEF cells were passaged every 3 to 6 days over a 34-day period, and viral supernatants were harvested every 18 to 24 h and replaced with fresh medium. The cultures were assayed for production of fusion protein and its incorporation into virions by immunoblotting with anti-SN antibodies (Fig. 3). Transfection of pGN1600 and pGS293 resulted in the expression of 85-kDa Gag-SN and Gag-SN* fusion proteins. Inefficient cleavage at the proteolytic cleavage sites in the Gag-SN/SN* precursors by the viral protease generated 58- and 28-kDa products; these presumably represent CA-NC-SN/SN* and NC-SN/SN*, respectively, since they are detectable with anti-SN antibody. Quantitation of the immunoblot signals by phosphorimager analysis showed that only 24 and 27% of the virion-associated Gag-SN and SN* fusion protein, respectively, was processed to an 18-kDa SN or SN* protein. Processing of the Gag-SN precursor was somewhat more efficient than for Gag-SN* and caused a 2.7-fold difference in the amount of the 28-kDa NC-SN intermediate. Because this difference in processing was reproducible, it must result from the amino acid substitutions at positions 43 and 87, where the SN* mutant differs from wild-type SN.

Most importantly, phosphorimager analysis demonstrated that nearly identical amounts of SN and SN* fusion proteins had been incorporated into virions (49 and 52 ng of SN and SN* equivalents per ml, respectively).

Identification of enzymatically active processing products of the Gag-SN fusion protein. Previous experiments demonstrated that virions containing coassembled Gag-SN fusion proteins have Ca^{2+}-dependent nuclease activity (29). To determine which processing product(s) in the virions had nucleolytic activity, we analyzed activity gels (zymograms). The principle of this method for detecting enzymatically active SN and its fusion proteins as bands on the gel is based on the fact that ethidium bromide fluoresces only when bound to DNA molecules (5, 23, 33, 43). DNase activity can be detected as dark bands by incorporating DNA into the gel prior to electrophoresis and incubating it in ethidium bromide containing buffer after electrophoresis.

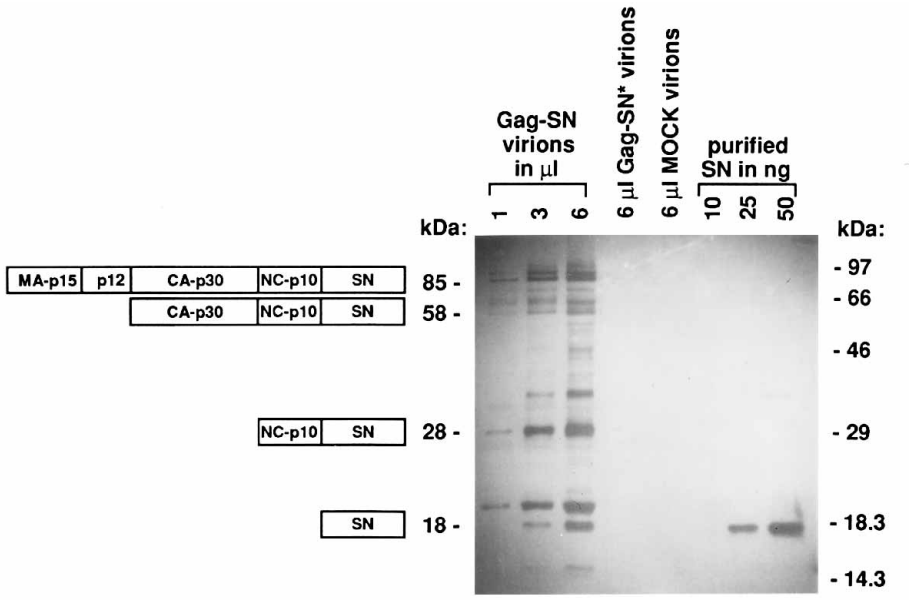

FIG. 4. Zymogram analysis of Mo(4070A) virions containing Gag-SN and Gag-SN* fusion proteins. An SDS–15% polyacrylamide gel containing herring sperm DNA was loaded with the indicated volumes of resuspended viral particles containing Gag-SN fusion proteins or purified SN. A 6-µl sample of the viral pellet fraction from mock-transfected cells served as the negative control; 10, 25, and 50 ng of purified SN served as the positive control. Methods for incubation, staining, and photography of the gel are described in Materials and Methods.

An advantage of the zymogram method is that it can simultaneously detect different processing products of the Gag-SN fusion proteins which have retained nucleolytic activity. The zymogram in Fig. 4 shows that in contrast to Gag-SN* particles, virions that incorporated Gag-SN fusion proteins exhibited abundant nuclease activity. All processing products of the Gag-SN fusion protein detected in the anti-SN immunoblot were enzymatically active. Different signal intensities indicated that the 18-kDa SN protein, the 28-kDa NC-SN fusion protein, and the 85-kDa Gag-SN precursor protein had the largest amount of enzymatic activity. A 20-kDa protein which demonstrates very high enzymatic activity suggests the existence of a further protease cleavage site at or near the NC/SN junction. An additional band of approximately 33 kDa is visible in the purified SN sample (Fig. 4, right-hand lane). This has been previously described as the dimeric form of SN (33); the SN dimer band is also present in these virion preparations.

Incorporation of Gag-SN fusion proteins into Mo(4070A) virions reduces their infectivity. To test the ability of the coassembled Gag-SN fusion proteins to inactivate Mo(4070A) particles released from infected CEF, we assayed the titers of infectious particles directly and quantitatively by the S^+L^- focus formation assay (Fig. 5). The titers of infectious virus in the supernatants were determined blind and subsequently decoded. The supernatants of triplicate cultures of *gag-SN*-, *gag-SN**-, and mock-transfected cells were harvested daily, and the cultures were passaged every 3 to 6 days for 34 days after transfection.

The length of this type of experiment is limited by the fact that CEF cells are primary cells, which can be passaged a limited number of times. Moreover, their generation time and virus production efficiency change as the cells are passaged. After four passages, all cultures were split every 4 days and then, as the cell generation time increased further, every 6 days (Fig. 5). Limited viability in combination with the fact that the supernatant was harvested daily and replaced with fresh medium explains the general decrease of all infectious titers beginning approximately 12 days after transfection.

Uniformly, at every time point tested after day 6 posttransfection, infectious virus titers of Gag-SN-expressing cells were reduced 10- to 21-fold relative to Gag-SN*-expressing cells and 10- to 33-fold relative to mock-transfected cells. This dramatic reduction in infectious-virus output was readily apparent even though these cell lines had been chronically infected with Mo(4070A) virus (Fig. 1C). This antiviral effect was stable during the entire lifetime of the cultures, demonstrating that there was no selection against Gag-SN-expressing CEF cells.

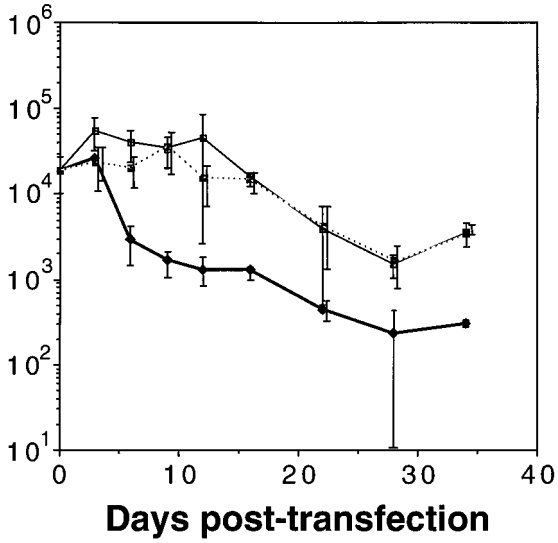

FIG. 5. Therapeutic effect of the Gag-SN fusion protein. Infectious Mo (4070A) titers of the supernatants of *gag-SN*-transfected (pGN1600) (◆), *gag-SN**-transfected (pGS293) (· · ■ · ·), and mock-transfected (—■—) cells were assayed by S^+L^- focus formation. The mean titers were determined from three independent transfection series and monitored every 3 to 6 days for 34 days after transfection. Error bars indicate standard deviations. The vertical axis shows focus-inducing units per ml.

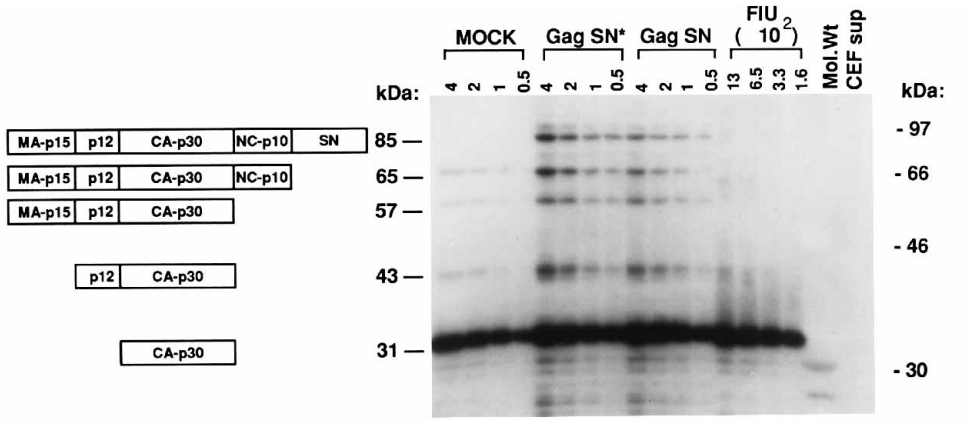

FIG. 6. Quantitation of bulk Mo(4070A) virions. Various volumes of the same viral pellet fractions from *gag-SN*-, *gag-SN**-, and mock-transfected cells (Gag-SN, Gag-SN*, and MOCK), used for the immunoblot analysis in Fig. 3, were examined by immunoblot analysis with anti-$p30^{CA}$ antibody as the probe. Dilutions of a Mo(4070A) stock of known titer were loaded as positive controls. The quantity of infectious particles that corresponds to the amount of loaded bulk particles is indicated by FIU (10^2). Supernatant from an uninfected and untransfected CEF culture served as the negative control (CEF sup). Processing intermediates and final products resulting from processing of the wild-type Gag precursor and the Gag-SN and Gag-SN* fusion proteins are indicated.

To clearly demonstrate that the reduced infectious-virus titer in the supernatant of Gag-SN-expressing cells resulted from inactivated particles and not from a decrease of bulk particle production in these cells, the total quantities of virions in the supernatants of the three differently transfected cell lines were compared. The quantity of $p30^{CA}$ in these particles was used as a standard for the amount of bulk Mo(4070A) particles. Immunoblotting of viral pellets, purified from equal volumes of culture medium, with anti-CA antibody identified various protein species attributed to incomplete processing of the Gag-SN fusion protein or, for particles from mock-transfected cells, to incomplete processing of $Pr65^{Gag}$ (Fig. 6). The same viral pellets analyzed in this experiment had previously been characterised by the immunoblot analysis shown in Fig. 3. Quantitation by phosphorimager analysis demonstrated that the sum of CA protein and its incompletely processed fusion proteins is identical in the supernatants from Gag-SN- and Gag-SN*-expressing cells. Since the amount of CA is proportional to the number of bulk particles in the supernatants, the quantities of bulk particles released by the two differently transfected cell lines were nearly identical. The supernatant of Gag-SN- and Gag-SN*-expressing cells contained 1.7-fold more bulk particles than did the supernatant of mock-transfected CEF cells. Additional CA proteins (proteolytically) derived from Gag-SN* or Gag-SN fusion proteins are presumably responsible for the higher yield of particles in the supernatants of the transfected cell lines. Considering that identical quantities of Gag-SN and Gag-SN* fusion proteins are incorporated into similar numbers of bulk particles released by the two differently transfected CEF cultures, the 10- to 21-fold antiviral effect of Gag-SN fusion proteins relative to Gag-SN* must be caused by the enzymatic activity of SN.

A minor steric effect of the fusion proteins. Interestingly, the 1.7-fold increase in the number of bulk particles for Gag-SN*-expressing cell lines did not increase the quantity of infectious particles. Rather, it had the opposite effect and reduced the absolute titers 3, 6, and 12 days after transfection by an average of two- to threefold. Normalizing this to the number of bulk particles therefore suggests that this presumed steric effect of the fusion proteins decreases infectivity by three- to fivefold.

Active SN degrades virion RNA. The zymogram described above clearly shows that SN and the different processing intermediates that contain the SN moiety and are assembled into virions retain nucleolytic activity. If the enzyme is active inside the virion core and the antiviral effect is based on degradation of viral genomic RNA, this can be confirmed by RNA blot analysis. RNA was extracted from virions isolated from the same supernatant as the virions used for immunoblot and zymogram analysis. After separation of virion RNAs by gel electrophoresis under denaturing conditions and blotting with an M-MuLV probe (Fig. 7A), the filter was stripped and rehybridized with an RCAS-specific probe. This control experiment was performed to examine the status of the RCAS-RNA, which is expected to be intact in both Gag-SN and Gag-SN* supernatants because there should be no M-MuLV Gag-SN protein incorporated into the RCAS virions (Fig. 7B).

Full-length genomic RNA from MuLV and RCAS particles was quantitated by phosphorimager analysis. Hybridization with an M-MuLV probe demonstrates that the number of RNA molecules extracted from MuLV particles released from mock-transfected cells made up only 60% of the genomic RNA molecules from particles released from Gag-SN*-expressing cells, consistent with the difference observed in bulk CA protein. The difference in the amount of full-length genomic RNA isolated from particles carrying Gag-SN and Gag-SN* fusion proteins is 55-fold, although titers of bulk MuLV particles were nearly identical. Rehybridization of the same filter with the RCAS-specific probe showed only a 1.7-fold difference in full-length RCAS RNA levels (Fig. 7B), clearly demonstrating that the observed difference in Fig. 7A is limited to the RNA extracted from MuLV particles. This strongly suggests that the major reason for the loss of infectivity of the Mo(4070A) virions released by pGN1600-transfected cells is the degradation of encapsidated Mo(4070A) genomic RNA. Residual SN activity during RNA extraction from viral pellet suspensions might be responsible for the 1.7-fold difference seen for the RCAS genomic RNA. Aliquots of the same RNA preparations used in the experiment in Fig. 7A were also loaded on a nondenaturing gel and blotted with the same M-MuLV probe (Fig. 7C).

The RNA blot experiments demonstrate that the very small quantity of residual RNA from Mo(4070A) particles released from *gag-SN*-transfected cells exists in the dimer form, as is the case in particles from mock- and Gag-SN*-transfected cells. No degradation products are visible, implying that coassembly of Gag-SN fusions leads to complete degradation of genomic

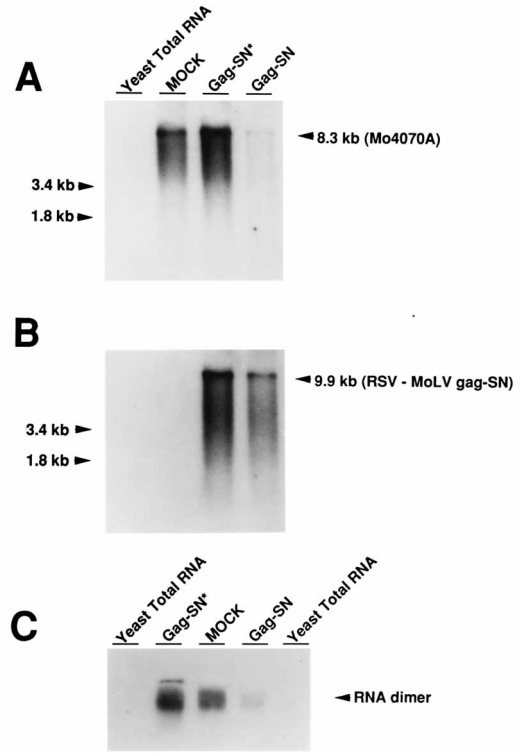

FIG. 7. Virion RNA analysis. Northern blot analyses of genomic RNA from viral pellet fractions from mock-, *gag-SN**-, and *gag-SN*-transfected CEF under denaturing (A and B) and nondenaturing conditions (C) are shown. RNA was extracted from virions isolated from the same supernatants as the virions used in the immunoblot analyses. Electrophoresis and transfer of the viral RNA were performed as described in Materials and Methods. (A) The denatured genomic RNA was first hybridized to the M-MuLV *pol* probe. The arrowhead indicates the position of 8.3-kb Mo(4070A) full-length genomic RNA. (B) Subsequently, the membrane was stripped and hybridized to a 2.9-kb RCAS *gag-pol* probe. The 9.9-kb full-length RCAS-*gag*-SN RNA is indicated (RSV, Rous sarcoma virus). Total RNA from *Saccharomyces cerevisiae* cells (4 μg) served as negative control. As the virions were harvested at intervals of 18 to 24 h, the viral RNA extracted from M-MuLV and RCAS particles contains some spontaneous breaks that are hidden under native conditions by the secondary structure of the dimeric RNA genome (6). Broken RNAs cause a smear below the full-length RNA band in the denaturing gel used in the RNA blot analysis. (C) Samples of the three different viral pellet fractions were subjected to electrophoresis in a nondenaturing 1% agarose gel. After transfer of the RNA, the membrane was hybridized with the same M-MuLV *pol* probe described above. Residual RNA from particles released from Gag-SN-expressing cells exists primarily as dimeric genomic RNA.

RNA down to small RNA fragments and single ribonucleotides. We interpret these results to mean that the residual full-length RNA observed in the RNA blots of Gag-SN particle RNA derives primarily from a small population of infectious particles, which have failed to incorporate active Gag-SN.

DISCUSSION

In this report, we describe the therapeutic application of capsid-targeted viral inactivation. We introduced an antiviral *gag-SN* fusion gene into cell cultures chronically infected with Mo(4070A) retrovirus and demonstrated the mechanisms by which Gag-SN fusion proteins incorporated into virions cause a 20- to 60-fold inactivation of the virus particles. That SN-mediated RNA degradation is the major virus-inactivating mechanism is indicated by two observations: (i) incorporated, enzymatically inactive Gag-SN* fusion proteins have only a slight antiviral effect; and (ii) greatly reduced amounts of genomic RNA are observed in virions containing Gag-SN fusion proteins. The possibility of inefficient packaging of viral RNA as the reason for the reduced RNA level observed in Gag-SN virions is excluded because the enzymatically inactive Gag-SN* fusion has no effect on the amount of encapsidated genomic RNA. As the same quantities of Gag-SN and enzymatically inactive Gag-SN* fusion proteins are incorporated into virions, the Gag-SN-dependent reduction of infectious-virus titers almost certainly results from the enzymatic action of the nuclease. Interestingly, all virion-incorporated processing intermediates that include an SN moiety retain nuclease activity (Fig. 4). Therefore, we regard the very slight difference in processing of Gag-SN and Gag-SN* (Fig. 3) as insignificant.

The antiviral effect of Gag-SN fusion proteins actually has two components, nuclease-mediated RNA degradation and a steric component. The geometric mean of the difference in infectivity between Gag-SN- and Gag-SN*-expressing cultures between day 6 and day 34 after transfection is 11. This represents the nuclease activity component of the antiviral effect. In addition, there is a three- to fivefold reduction in specific infectivity (focus-inducing units per milliliter of bulk particles) between Gag-SN* and mock-transfected cells between days 6 and 12. We attribute this latter effect to steric problems caused by incorporation of large amounts of Gag fusion protein.

There are several possible hypotheses for the observed steric effect. One hypothesis is that Gag-SN and Gag-SN* fusion proteins might compete with native Gag-Pol polyproteins during assembly and reduce the infectivity of the particles by reducing the Gag-Pol/Gag ratio. Figure 6 demonstrates that proteolytic processing in particles containing Gag-SN or Gag-SN* fusion proteins is less efficient than in native Mo(4070A) particles released from CEF. It is not clear whether the excess of the MA-p12-CA-NC, MA-p12-CA, and p12-CA intermediates detectable in Gag-SN- and Gag-SN*-containing virions comes exclusively from incompletely processed Gag-SN and Gag-SN* precursor proteins or also from wild-type Gag and Gag-Pol proteins. This is consistent with the possibility that there is less protease in Gag-SN and Gag-SN* particles. It is also possible that dimerization of the Gag-Pol precursor, which is required for protease activation (2, 7, 16), is less efficient as a result of incorporated Gag-SN and Gag-SN* fusion proteins.

For Gag-nuclease fusion proteins to be useful in real antiviral therapy in vivo, the antiviral effect must be (i) sufficiently robust to reduce viral titers significantly, (ii) maintained over a long period, and (iii) nontoxic to the host organism. We observed a net reduction in specific infectivity of about 20- to 60-fold in the Gag-SN-transfected relative to the mock-transfected culture. We emphasize that this represents an effect on a fully infected producer cell line. In a low-multiplicity infection, an effect of this magnitude would be exerted in every growth cycle of the virus, potentially giving rise to a much larger net effect. As the stability of the antiviral effect throughout this period (Fig. 5) demonstrates, there was no selection against Gag-SN-expressing cells, and, furthermore, the cell cultures transfected with *gag-SN* and *gag-SN** did not differ in their generation time from mock-transfected CEF cells (data not shown). Importantly, a significant antiviral effect was maintained throughout this period. Gag-SN expression caused no discernible toxic effect in CEF during the 34 days after transfection.

By using both the therapeutic approach demonstrated here and the previous prophylactic study (29), a subset of Mo(4070A) virus particles somehow avoided inactivation. The persistence of small amounts of full-length viral RNA suggests that there is a small subpopulation of nuclease-free virions. Several possible mechanisms for this can be envisioned and will require further experimentation to unravel. (i) The residual infectious

Mo(4070A) particles might be shed from CEF which were rendered refractory to infection by the RCAS retroviral vector for some reason. (ii) Insufficient incorporation of RNA-degrading Gag-SN fusion proteins into some virus particles, e.g., as a result of inadequate expression of the antiviral protein, could also result in residual infectious particles. Inadequate expression of the antiviral protein could be caused by rearrangement of RCAS sequences in a subset of infected cells, leading to the loss of intact *gag-SN* fusion genes and a population of cells that do not express intact Gag-SN. Another possibility is that the intracellular translation of Gag-SN is not uniform or not congruent with the expression patterns of the Mo(4070A) proteins. These possibilities will be addressed by immunofluorescence analysis of infected cells by using anti-RCAS and anti-SN antibodies.

The proportion of Gag-SN and Gag-SN* fusion proteins coassembled with native Gag monomers can be calculated by combined analysis of the phosphorimager data resulting from the anti-SN- and anti-MuLV $p30^{CA}$ immunoblots of the two different viral pellet fractions. The fraction of unprocessed Gag-SN and Gag-SN* fusion proteins relative to the total amount of SN and SN* equivalents incorporated into the particles was ascertained by evaluating the anti-SN immunoblot analysis. Of all incorporated SN or SN* equivalents, 20 and 39%, respectively, are represented by full-length Gag-SN and Gag-SN* fusion proteins. Therefore, the $p30^{CA}$ phosphorimager signal resulting from full-length Gag-SN and Gag-SN* fusion proteins also represents 20 and 39%, respectively, of the CA derived from Gag-SN and Gag-SN* fusion proteins. By calculating the signal volume contributed by CA proteins derived from Gag-SN and Gag-SN* fusions and dividing it by the total CA protein signal measured in each viral pellet, the ratio of Gag-SN and Gag-SN* monomers to native Gag- and Gag-Pol monomers, respectively, was determined. The proportion of Gag-SN and Gag-SN* monomers incorporated into the virions during particle formation corresponds to 24 and 25.7%, respectively, of the total Gag protein in each virus preparation. This represents a relatively high absolute level of expression of Gag-SN and Gag-SN* fusion proteins from the RCAS constructs (corresponding to ~50 ng of incorporated SN and SN* per ml of viral supernatant).

The application of this antiviral strategy to HIV-infected cells is an important final goal of this work. However, HIV Gag fusion proteins are difficult to express because of negative regulatory sequences in the RNA that severely restrict their expression (35). The small virion proteins Vpr and Vpx represent an alternative route into HIV virions. A recent study demonstrated that HIV-1 Vpr and HIV-2 Vpx can direct the packaging of functionally active SN into virions when expressed as heterologous fusion molecules (42). The successful packaging of Vpr1-SN and Vpx2-SN fusion proteins into virions indicates their potential use for accessory protein-targeted viral inactivation.

The protein-based CTVI strategy we used has several advantages over other antiviral strategies involving RNA-based inhibitors, like antisense RNA or ribozymes. Viral escape mutants, i.e., mutants which are capable of performing their normal functions nearly as well as the wild type but are resistant to a given antiviral strategy, are a major difficulty in antiretroviral therapy. However, the Gag polyprotein plays a wide variety of roles in virus assembly and infection. This fact raises the possibility that mutants with alterations in *gag* will be profoundly impaired with respect to one or more of these functions. Thus, fully functional escape mutants may not arise in the case of CTVI. In particular, the region between residues 240 and 430 of HIV-1 $Pr55^{gag}$, comprising the C-terminal half of CA plus the NC domain, includes the major homology region (27). This region is known to be required for efficient assembly and is clearly involved in Gag-Gag interaction (9, 11, 25). However, this region also participates in cyclophilin binding (26) and in packaging of genomic RNA (1, 8, 15). Since the Gag-Gag interaction domain overlaps with several regions with different essential functions, there may be very little latitude for escape mutations. The feasibility of capsid-targeted viral inactivation of MuLV demonstrated in both the prophylactic and therapeutic approaches highlights the potential of this novel antiviral strategy.

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