JOURNAL OF VIROLOGY, Apr. 1997, p. 3312–3318 Vol. 71, No. 4
0022-538X/97/$04.00+0

Expression of a Murine Leukemia Virus Gag-*Escherichia coli* RNase HI Fusion Polyprotein Significantly Inhibits Virus Spread

MATTHEW VANBROCKLIN,[1] ANDREA L. FERRIS,[2] STEPHEN H. HUGHES,[2] AND MARK J. FEDERSPIEL[1]*

Molecular Medicine Program, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905,[1] and Advanced Bioscience Laboratories-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702[2]

Received 25 September 1996/Accepted 7 January 1997

The antiviral strategy of capsid-targeted viral inactivation (CTVI) was designed to disable newly produced virions by fusing a Gag or Gag-Pol polyprotein to a degradative enzyme (e.g., a nuclease or protease) that would cause the degradative enzyme to be inserted into virions during assembly. Several new experimental approaches have been developed that increase the antiviral effect of the CTVI strategy on retroviral replication in vitro. A Moloney murine leukemia virus (Mo-MLV) Gag-*Escherichia coli* RNase HI fusion has a strong antiviral effect when used prophylactically, inhibiting the spread of Mo-MLV and reducing virus titers 1,500- to 2,500-fold. A significant (~100-fold) overall improvement of the CTVI prophylactic antiviral effect was produced by a modification in the culture conditions which presumably increases the efficiency of delivery and expression of the Mo-MLV Gag fusion polyproteins. The therapeutic effect of Mo-MLV Gag–RNase HI polyproteins is to reduce the production of infectious Mo-MLV up to 18-fold. An Mo-MLV Gag–degradative enzyme fusion junction was designed that can be cleaved by the Mo-MLV protease to release the degradative enzyme.

The ability to efficiently introduce exogenous genes into cells has opened up new avenues that could be used to prevent or combat disease. Novel approaches for increasing resistance to viruses are needed as alternatives to the more traditional approaches of immunization and drug therapy. Alternative treatments would be especially useful against viruses that are not blocked by a preventative vaccine (e.g., human retroviruses), have a high rate of antigenic variation (e.g., influenza virus), or are refractive to antiviral drug treatments. We and others have demonstrated that foreign proteins fused to retroviral Gag or Gag-Pol polyproteins can be incorporated efficiently into virions during virus assembly (10, 13, 17, 18, 25, 29, 30). Foreign proteins fused to certain lentivirus accessory proteins known to coassemble with Gag polyproteins have also been used to insert foreign proteins into virions (32–34). The antiviral strategy of capsid-targeted viral inactivation (CTVI) was designed to disable newly produced virions by fusing a Gag or Gag-Pol polyprotein to a degradative enzyme that would cause the degradative enzyme to be inserted into virions during assembly. One advantage of incorporating a degradative enzyme into virions is that the delivery of the Gag-enzyme fusion can be relatively inefficient since, at least in theory, one active degradative enzyme could disable a virion. However, the degradative enzymes (e.g., proteases or nucleases) chosen for the CTVI antiviral strategy must be chosen to limit toxicity to the cell. The efficacy of the CTVI antiviral strategy has been demonstrated by using the retrotransposon Ty1 and the retrovirus Moloney murine leukemia virus (Mo-MLV), but the strategy may ultimately have broader applications to other virus classes.

We initially demonstrated the efficacy of the CTVI strategy for retroviruses with an Mo-MLV Gag-staphylococcal nuclease (SN) fusion polyprotein (18). The Mo-MLV *gag* gene encodes a 65-kDa polyprotein precursor, $Pr65^{gag}$, which is proteolytically cleaved into the four structural proteins by Mo-MLV protease MA (matrix)–p12–CA (capsid)–NC (nucleocapsid) (10). The Mo-MLV *gag* gene was fused to the SN gene at the carboxy terminus of the NC (MA-p12-CA-NC-nuclease). This orientation was chosen so that the nuclease would be inserted into the NC core of the particle, where it would have access to the viral RNA (3, 28). Construction of the expression system with the degradative enzyme as a domain of the Gag polyprotein results in approximately 20-fold higher protein levels than do constructions in which the degradative enzyme is part of a Gag-Pol fusion. The CTVI construct was expressed in chicken embryo fibroblasts (CEF) by an avian leukosis virus (ALV)-based retroviral vector system (5, 6, 8, 12, 21, 22). Genes encoding the Mo-MLV Gag and Mo-MLV Gag–SN fusion polyproteins were delivered to virtually all of the cells of a CEF culture by infection with the RCASBP expression vector. The retroviral vector gene delivery system efficiently generated populations of cells stably expressing Mo-MLV Gag and Gag-nuclease fusion polyproteins. In the prophylactic approach, the CTVI antiviral construct was delivered to cells before challenge with an amphotropic Mo-MLV to measure the ability of the construct to limit virus spread. Cells expressing the Mo-MLV Gag–SN fusion polyprotein inhibited virus spread and Mo-MLV titers were reduced ~30-fold (18). Recently, Schumann et al. (25) reported that delivery of the Mo-MLV Gag–SN fusion construct to cells chronically infected with Mo-MLV reduced the production of infectious virus 20- to 60-fold.

In this study, several new experimental approaches were tested in an effort to increase the antiviral effect of the CTVI strategy in vitro. First, we modified the cell culture conditions so that vigorous cell growth was maintained throughout the experiment. We have observed a slowing in cell growth, espe-

* Corresponding author. Mailing address: Molecular Medicine Program, Mayo Foundation, 200 First St., SW, Rochester, MN 55905. Phone: (507) 284-8895. Fax: (507) 266-4797. E-mail: federspiel.mark@Mayo.edu.

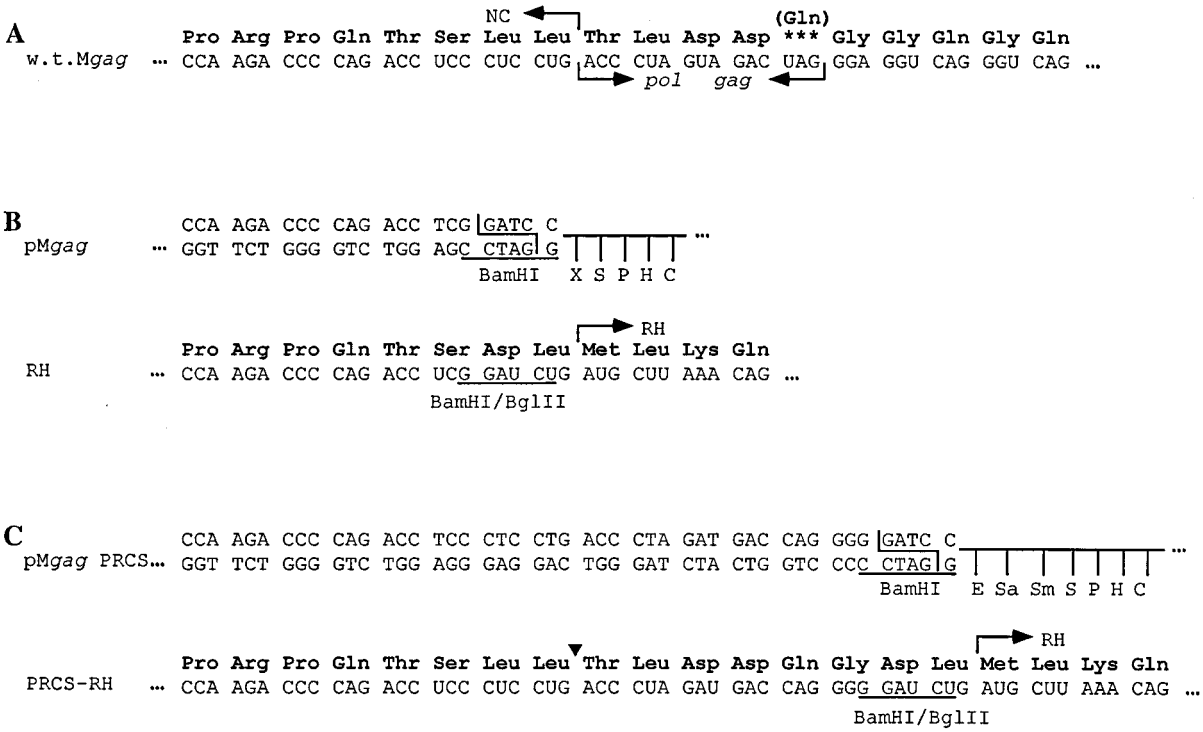

FIG. 1. Mo-MLV Gag–*E. coli* RNase HI fusion junctions. (A) The region encoding the C terminus of Gag from wild-type (w.t.) Mo-MLV is shown. The protein is released at the termination codon (UAG) 95% of the time this region is translated, yielding a Gag polyprotein. The Gag-Pol polyprotein is produced by nonsense suppression of this codon, which occurs 5% of the time. The Mo-MLV protease cleaves the Gag and Gag-Pol polyproteins four amino acids upstream of the *gag* termination codon. Cleavage produces the mature C terminus of the NC domain of the Gag polyprotein and the mature protease N terminus in the Gag-Pol polyprotein. (B) The cloning vector pM*gag* contains the Mo-MLV *gag* region in the pCla12NCO adaptor plasmid, followed by a *Cla*I site. A *Bam*HI site has been inserted two amino acids upstream of the NC PRCS, and four cloning sites *Xba*I (X), *Sal*I (S), *Pst*I (P), and *Hin*dIII (H), remain downstream for insertion of genes. The region encoding *E. coli* RNase HI (RH) was inserted on a *Bgl*II-*Sal*I fragment. The Mo-MLV Gag–RNase H fusion junction substitutes an Asp for the Leu two amino acids upstream of the NC C terminus, while the RNase HI coding region begins just downstream. (C) An Mo-MLV PRCS was inserted into the Gag-nuclease fusion by modifying the pM*gag* Mo-MLV *gag* region to include six codons of the Gag-Pol coding region (pM*gag*PRCS). A *Bam*HI cloning site was inserted, and six other cloning sites, *Eco*RI (E), *Sac*I (Sa), *Sma*I (Sm), *Sal*I (S), *Pst*I (P), and *Hin*dIII (H), remain downstream for insertion of genes, followed by a *Cla*I site. *E. coli* RNase H was inserted as described above. The predicted Mo-MLV PRCS is indicated (▼).

cially after MLV infection, in previous studies (25) (data not shown). We hypothesized that retroviral replication would only be optimal in rapidly growing cells, including the delivery and expression of the Mo-MLV Gag–nuclease fusion polyproteins. Second, we tested an Mo-MLV Gag–*Escherichia coli* RNase HI polyprotein for an antiviral effect in the CTVI strategy. RNase H is an endonuclease that specifically recognizes RNA-DNA hybrids, digesting only the RNA strand of the duplex. In the retrovirus life cycle, RNase H activity is required during reverse transcription to degrade the RNA strand. This degradation is necessary for transfer of the first viral DNA strand between templates and produces specific RNA primers that are used in the synthesis of the second DNA strand (2, 31). *E. coli* RNase HI has been well characterized both biochemically and by X-ray crystallography (4). The *E. coli* RNase HI enzyme has a specific activity several orders of magnitude greater than the RNase H domains of reverse transcriptases (RTs) (23) and has been shown to degrade the specific RNA primers generated by RNase H of RT in vitro (9, 24). Ma and Crouch (16) showed that purified *E. coli* RNase HI could inhibit the complete synthesis of a DNA copy of an RNA template in an in vitro assay using RT from AKR MuLV. They also showed that transposition of yeast retrotransposon Ty1 was inhibited >99% in cells expressing a Ty1 capsid-*E. coli* RNase HI fusion polyprotein in vivo. No cytotoxicity was observed in yeast expressing *E. coli* RNase HI. We hypothesized that uncontrolled RNase H activity in retroviral virions (i.e., RNase H activity independent of RT) could interfere with the generation of the double-stranded DNA copy of the viral genome and thereby disrupt the viral life cycle. Third, we designed an Mo-MLV Gag–degradative enzyme fusion junction that can be cleaved by the Mo-MLV protease, releasing the degradative enzyme. This technology may be useful in future developments of the CTVI strategy in which relatively toxic degradative enzymes are expressed in the form of zymogens.

Expression of an Mo-MLV Gag–*E. coli* RNase H fusion polyprotein in CEF. A gene encoding an Mo-MLV Gag–*E. coli* RNase HI fusion was constructed by linking the RNase HI coding region to the Mo-MLV Gag polyprotein six codons upstream of the Gag termination codon (Fig. 1A and B). The RNase HI coding sequence was isolated from *E. coli* DNA by PCR amplification with a 5′ primer containing a *Bgl*II site (5′-GCG CAT GCA GAT CTG ATG CTT AAA CAG GTA GAA ATT TTC ACC GAT GG-3′) and a 3′ primer containing a *Sal*I site (5′-GCT GCT GCG TCG ACT TAA ACT TCA ACT TGG TAG CCT GTA TCT TCC-3′). The reagents and conditions used for the PCR were described previously (7). The *E. coli* RNase HI fragment was sequenced and contained one silent nucleotide difference from the sequence published by Kanaya and Crouch (14), at nucleotide 470 (A→G). The Mo-MLV Gag–RNase H fusion construct was based on the design of the Mo-MLV Gag–SN fusion which has been used to introduce SN into virions (18). The genes encoding the CTVI constructs were delivered into CEF by the RCASBP(A) ret-

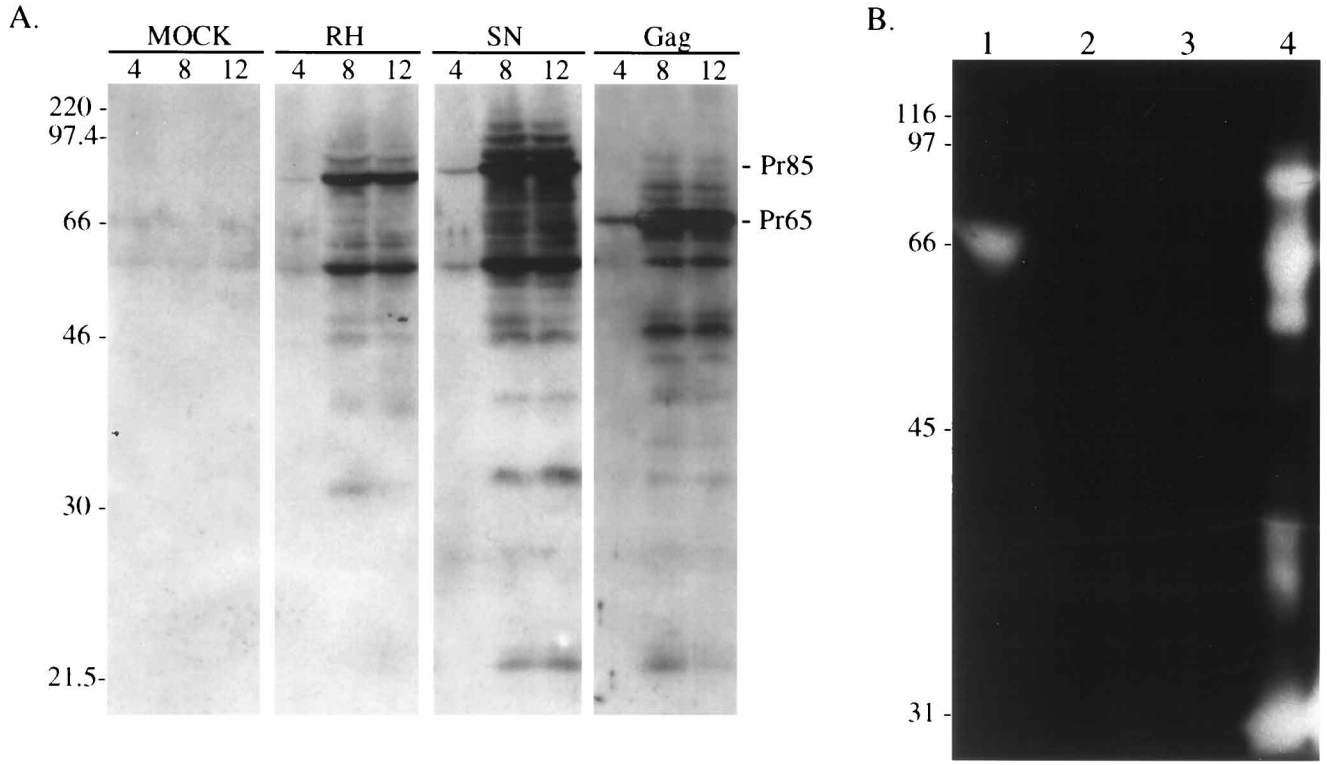

FIG. 2. Expression of Mo-MLV Gag fusion polyproteins in CEF. (A) Mo-MLV Gag fusion genes were delivered and expressed by the replication-competent retroviral vector RCASBP(A) in CEF. RCASBP(A) infections were initiated by transfection of plasmids containing the vector in proviral form. Virions were pelleted from 5 ml of infected cell supernatants at 4, 8, and 12 days posttransfection. The proteins were denatured, separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis, and analyzed by Western transfer (6). The filter was probed with anti-MuLV CA serum (1:5,000 delution), and the bound proteins were visualized by chemiluminescence on Kodak X-Omat film. MOCK, uninfected CEF; RH, Mo-MLV Gag–*E. coli* RNase HI; SN, Mo-MLV Gag–SN; Gag, Mo-MLV Gag. The locations of the ~85-kDa Mo-MLV Gag–SN and Mo-MLV Gag–RNase H polyproteins (Pr85) and the ~65-kDa Mo-MLV Gag polyprotein (Pr65) are indicated. (B) *E. coli* RNase HI activity was detected by an in situ polyacrylamide gel assay described previously (11, 26). Viral proteins prepared from day 14 infected-cell supernatants were separated on a sodium dodecyl sulfate–9.5% polyacrylamide gel containing a ^{32}P-labeled RNA-DNA hybrid substrate. Viral protein from 2 ml of supernatant was loaded per lane. After electrophoresis, the gel was put through a series of washes to allow the proteins to renature and RNase H to hydrolyze the ^{32}P-labeled RNA. The gel was dried and exposed to Kodak X-Omat film. RNase H activity appears as clear bands on a black background. Lanes: 1, purified human immunodeficiency virus type 1 RT (10 μg) used as an RNase H activity positive control; 2, Mo(4070A)-infected CEF; 3, Mo-MLV Gag-expressing CEF; 4, Mo-MLV Gag–RNase H-expressing CEF. The values to the left are molecular sizes in kilodaltons.

roviral vector and expressed under the control of the viral promoter-enhancer in the long terminal repeat. CEF were cultured and passaged every 2 days when they reached confluence, as described previously (6). ALV retroviral vector propagation was initiated by transfection of plasmid DNA that contained the retroviral vector in a proviral form by the calcium phosphate precipitation method (15). The course of the retroviral infection was monitored by assaying culture supernatants from confluent cells for the viral Gag protein. Three plasmids that had been previously described were used in this study for comparison: Mo-MLV Gag–SN plasmid pGN1600 (18); Mo-MLV Gag plasmid pGN1601 (18), which produces only the Mo-MLV Gag polyprotein; and Mo-MLV Gag-SN* plasmid pGS293 (25), which differs from Mo-MLV Gag–SN in two missense mutations in the SN gene that result in an inactive SN enzyme. Maximum levels of Mo-MLV Gag, Mo-MLV Gag–RNase H, Mo-MLV Gag–SN, and Mo-MLV Gag–SN* were seen 10 to 14 days posttransfection (Fig. 2A), which coincided with maximum RCASBP(A) production (data not shown). Immunoblot analysis indicates that the Mo-MLV Gag–RNase H polyprotein was expressed at a lower level than Mo-MLV Gag or Mo-MLV Gag–SN (Fig. 2A). Several viral proteins that were smaller than the expected full-length polyproteins were also observed. We believe that these proteins are the result of partial degradation of the Pr85 or Pr65 polyprotein associated with the virion isolation procedure, since smaller Gag-associated proteins were also seen in the Mo-MLV Gag control. Southern blot analysis of genomic DNA isolated from day 24 cultures detected only intact proviruses (data not shown). An in situ RNase H assay showed that virus particles isolated from CEF expressing the Mo-MLV Gag–RNase H polyprotein contained high levels of RNase H activity (Fig. 2B). Little or no RNase H activity was detected in any other culture supernatants (Fig. 2B).

CEF expressing the Mo-MLV Gag–RNase H polyprotein significantly inhibit Mo-MLV spread. The antiviral effect of the CTVI fusions expressed by the CEF cultures was measured first in a prophylaxis assay. The cultures were challenged with a low dose (multiplicity of infection [MOI] of 0.05 to 0.1 focus-forming units [FFU] per cell) of amphotropic Mo-MLV strain Mo(4070A) (19, 20). Mo(4070A) is an engineered hybrid Mo-MLV that contains the *env* gene and a portion of the *pol* gene of the amphotropic 4070A virus. The Mo(4070A) virus stock was produced on NIH 3T3 cells (1×10^6 to 2×10^6 FFU/ml). Infectious virus was quantitated by the S+L− focus assay on D56 cells (1). The CEF cultures, which were dividing rapidly, were passaged for 14 days after Mo(4070A) infection. Infectious Mo(4070A) was quantitated at day 14 by the S+L− focus assay (Table 1). CEF expressing the Mo-MLV Gag–RNase H polyprotein reduced the level of infectious Mo(4070A) pro-

TABLE 1. Inhibition of Mo-MLV spread by CTVI[a]

Construct used for treatment	Titer (FFU/ml)[b] (fold inhibition)[c]	
	Expt 1	Expt 2
None (mock treatment)	1.6×10^5	2.1×10^5
Vector alone	2.4×10^4 (6.6)	ND[d]
Mo-MLV Gag	2.5×10^4 (6.3)	4×10^4 (5)
Mo-MLV Gag–RNase H	1×10^2 (1,550)	9×10^1 (2,330)
Mo-MLV Gag–SN	5×10^1 (3,100)	1.6×10^1 (13,125)
Mo-MLV Gag–SN*	1.9×10^4 (8.4)	ND

[a] In a prophylaxis assay, CEF cultures seven passages after transfection with plasmids containing the RCASBP(A) retroviral vector-CTVI constructs were challenged with Mo(4070A) (multiplicity of infection, 0.05 to 0.1 FFU per cell). Infectious Mo(4070A) levels were quantitated from supernatants 14 days postinfection by S+L− focus assays. This experiment was performed twice.

[b] Titers are averages of duplicate assays.

[c] Fold inhibition was determined by comparing the Mo(4070A) titers of the experimental constructs to the Mo(4070A) titer on mock-treated CEF.

[d] ND, not done.

duced by more than 1,500-fold compared to the Mo(4070A)-infected CEF control.

Several cultures were further characterized over the course of the 14-day Mo(4070A) infection by quantitating the levels of infectious Mo(4070A) by S+L− focus assay (Fig. 3A) and analyzing the expression levels of the Mo-MLV Gag and RCASBP(A) Gag polyproteins by Western immunoblotting (Fig. 3B and C). The amount of infectious Mo(4070A) produced peaked 7 to 8 days after infection in the control cultures (CEF alone and CEF expressing Mo-MLV Gag), and the titer remained relatively constant over the next 6 days (Fig. 3A). In contrast, the amount of infectious Mo(4070A) produced by CEF expressing either the Mo-MLV Gag–RNase H or Mo-MLV Gag–SN polyprotein was significantly inhibited, although the levels of infectious virus slowly increased over the course of the experiment. Mo(4070A) virus production was also monitored by the appearance of the Mo-MLV CA protein (~30 kDa) on immunoblots (Fig. 3B). The levels of expression of the

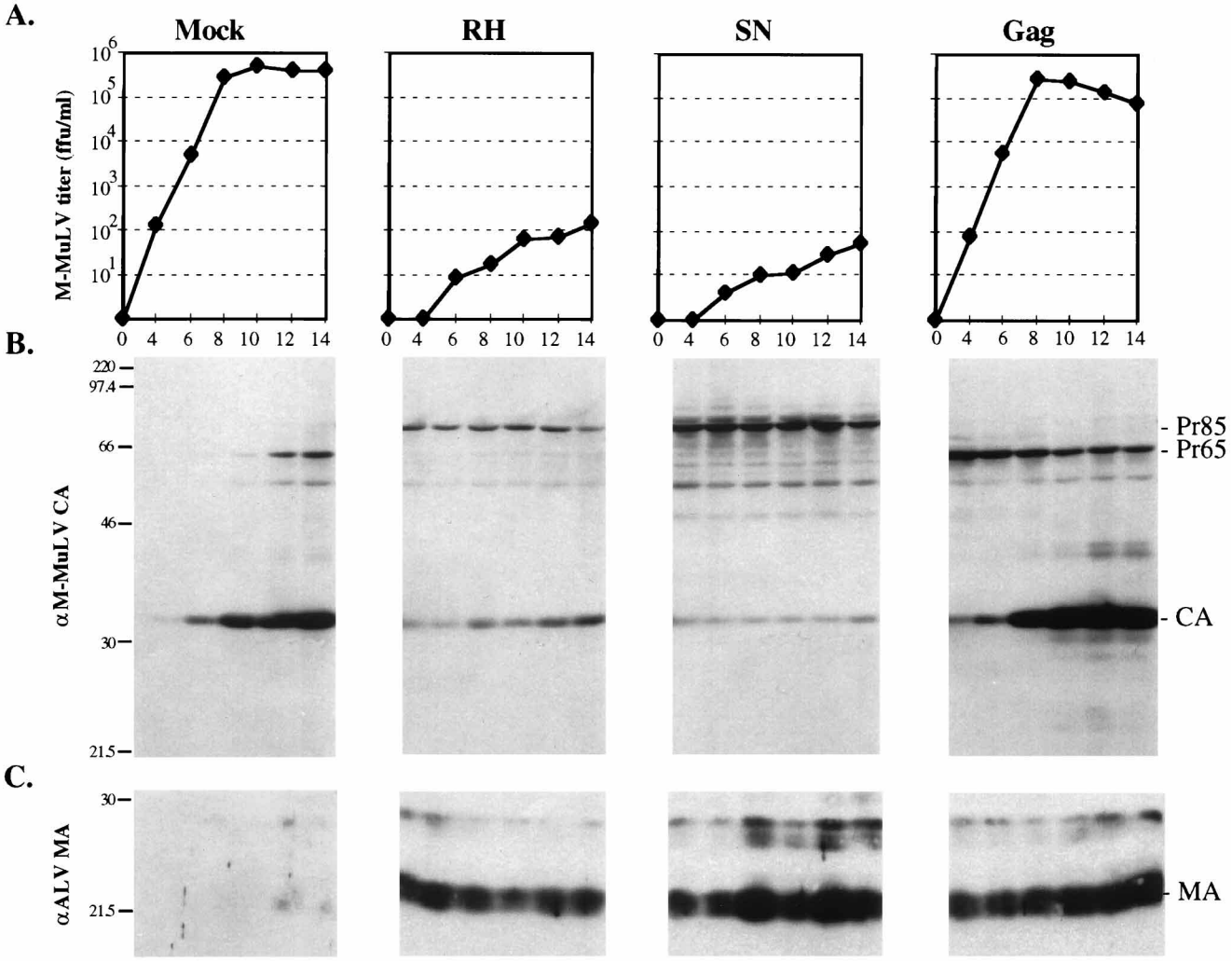

FIG. 3. Prophylactic antiviral effect of Mo-MLV Gag fusion polyproteins. CEF cultures expressing no Mo-MLV Gag (Mock), Mo-MLV Gag–RNase H (RH), Mo-MLV Gag–SN (SN), and Mo-MLV Gag (Gag) were challenged with Mo(4070A) (0.05 to 0.1 FFU/cell) at day 0. (A) Infected-cell supernatants from days 0, 4, 6, 8, 10, 12, and 14 postinfection were analyzed for infectious Mo(4070A) by S+L− focus assay. (B and C) Virions were pelleted from equal volumes of infected-cell supernatants from days 4, 6, 8, 10, 12, and 14 post-Mo(4070A) infection, and the Mo-MLV and RCASBP(A) Gag protein levels were analyzed. (B) Autoradiograms of Western immunoblots probed with the anti-MuLV CA serum, which detects the ~30-kDa MuLV CA protein (CA), the 65-kDa Gag polyprotein precursor of Mo-MLV (Pr65), and the ~85-kDa Gag polyprotein precursors of Mo-MLV Gag–RNase H and Mo-MLV Gag–SN (Pr85). Viral proteins obtained from 5 ml of supernatant were loaded on each lane. (C) Autoradiograms of Western blots probed with anti-avian myeloblastosis virus MA serum (1:5,000 dilution) (27), which binds the ~19-kDa ALV MA protein (MA). Viral proteins obtained from 0.5 ml of supernatant were loaded on each lane.

Mo-MLV Gag, Mo-MLV Gag–RNase H, and Mo-MLV Gag–SN polyproteins remained relatively constant throughout the experiment (Fig. 3B). At late time points post-Mo(4070A) infection (days 12 to 14), the Mo-MLV Gag polyprotein levels may decrease slightly due to proteolytic processing by protease from the infectious Mo(4070A). The levels of the MA protein (~19 kDa) from the RCASBP(A) retroviral vector remained relatively constant throughout the experiment (Fig. 3C).

The previously characterized Mo-MLV Gag–SN fusion polyprotein was tested in parallel with the Mo-MLV Gag–RNase H fusion to provide a direct comparison to a characterized CTVI construct known to have antiviral activity. Unexpectedly, the antiviral effect of Mo-MLV Gag–SN was 100-fold greater in the experiments reported here (>3,000-fold [Table 1]) than that previously observed (~30-fold [18]). We hypothesize that the increase in the antiviral effect is due to an increase in the efficiency of producing CEF cultures that express higher levels of the fusion polyprotein. The CEF themselves grew at a faster rate, presumably due to changes in the growth media including different serum lots. This difference in growth rate was especially apparent in CEF infected with Mo(4070A). In previous experiments, Mo(4070A) infection dramatically slowed the growth of CEF and caused some cytotoxicity. In the experiments reported here, all CEF cultures grew at the same rate until natural senescence. We also saw a consistent six- to eightfold inhibition of Mo(4070A) titer in cells expressing the control constructs, including cultures that expressed the RCASBP(A) vector (Table 1), that was not detected previously (18, 25). Although this may be related to the "steric effect" (three- to fivefold) of the Mo-MLV Gag–SN* polyprotein on Mo(4070A) virus production observed by Schumann et al. (25), the decrease in the Mo(4070A) titers produced in cultures that expressed RCASBP(A) suggests that the RCASBP vector may alter cells in ways that interfere with Mo-MLV virus production, leading to a slight overestimate of the CTVI effect. While several CTVI constructs have significant antiviral effects when used prophylactically, the levels of infectious virus do increase during the experiment. The production of a small number of viruses that do not contain CTVI-derived polyproteins most likely accounts for the small number of infectious viruses needed to spread the infection. We believe that mutations in the *gag* gene capable of circumventing this antiviral strategy would be exceedingly rare since they would require the selection of Gag mutants that would still assemble normally but be able to exclude Gag-degradative enzyme polyproteins from virions.

Delivery of the Mo-MLV Gag–RNase HI fusion polyprotein to Mo-MLV-infected CEF has a therapeutic effect. To assess the therapeutic potential of the CTVI strategy, CEF chronically infected with Mo(4070A) were transfected with the RCASBP(A) retroviral vector plasmids containing the Mo-MLV Gag fusions and passaged for 16 days. For the therapy assay, CEF chronically infected with Mo-MLV were generated by infecting 4×10^6 cells with 2×10^6 FFU of Mo(4070A) (1 ml of virus stock) and passaging the culture four to six times to allow maximum spread of the virus. The Mo(4070A)-infected CEF cultures produced a maximum titer of 3×10^5 to 4×10^5 FFU/ml as quantitated by the S+L− focus assay. The RCASBP virus spread through Mo(4070A)-infected CEFs with kinetics similar to the spread on CEF that had not been infected with Mo(4070A), reaching maximum levels after ~8 days as determined by Western analysis of ALV MA levels (data not shown). The levels of infectious Mo(4070A) were quantitated throughout the experiment. The Mo(4070A) titers at 14 days posttransfection in two experiments are shown in Table 2. The level of infectious Mo(4070A) was reduced 7.5- to 18-fold in CEF expressing the Mo-MLV Gag–RNase H polyprotein and 15- to 38-fold in CEF expressing the Mo-MLV Gag–SN polyprotein. Infection with the RCASBP(A) vector alone or RCASBP(A) expressing the Mo-MLV Gag polyprotein lowered the titer of Mo(4070A) produced by a chronically infected culture two- to threefold compared to Mo-MLV-infected CEF. This suggests that only a slight therapeutic effect is directly attributable to the CTVI constructs once the infection is established.

TABLE 2. Inhibition of infectious Mo-MLV produced after CTVI treatment[a]

Construct used for treatment	Titer (FFU/ml)[b] (fold inhibition)[c]	
	Expt 1	Expt 2
None (mock treatment)	3×10^5	3.8×10^5
Vector alone	1.5×10^5 (2)	ND[d]
Mo-MLV Gag	1×10^5 (3)	1.6×10^5 (2.4)
Mo-MLV Gag–RNase H	4×10^4 (7.5)	2.1×10^4 (18)
Mo-MLV Gag–SN	2×10^4 (15)	1.3×10^4 (29)

[a] In a therapy assay, CEF chronically infected with Mo(4070A) were transfected with plasmids containing the RCASBP(A) retroviral vector-CTVI constructs. Levels of infectious Mo-MLV in supernatants were quantitated 14 days posttransfection by S+L− focus assays. The experiment was performed twice.
[b] Titers are averages of duplicate assays.
[c] Fold inhibition was determined by comparing the Mo-MLV titers of the experimental constructs to the Mo-MLV titer on mock-treated CEF.
[d] ND, not done.

Cleavage of the Mo-MLV Gag–nuclease fusion junction by the incoming Mo-MLV protease. We designed an Mo-MLV Gag polyprotein fusion junction that could be cleaved by the protease of an incoming infectious Mo-MLV by including the region six amino acids on either side of the normal Mo-MLV protease cleavage site (PRCS) (Fig. 1C). The new Gag fusion plasmid moves the *Bam*HI site to a position just downstream of the Gag termination codon (pMGagPRCS). The termination codon was changed to a codon for glutamine, the same amino acid that Mo-MLV inserts during suppression of the stop codon when the Gag-Pol polyprotein is produced. We constructed genes encoding the Mo-MLV Gag PRCS–RNase H and Mo-MLV Gag PRCS–SN polyproteins to determine if cleavage of the RNase HI or SN domain from the polyprotein would increase the antiviral effect. A prophylaxis assay was used to measure Mo(4070A) production in cells expressing the Mo-MLV Gag PRCS fusions and the original Mo-MLV Gag fusions. Addition of the PRCS did not alter the level of inhibition by the RNase HI or SN polyprotein (data not shown), nor did inclusion of the PRCS alter the therapeutic antiviral effect of either the Mo-MLV Gag–RNase H or the SN polyprotein (data not shown). Virion proteins isolated from day 16 of the therapy assay of Mo-MLV-infected CEF and CEF expressing Mo-MLV Gag, Mo-MLV Gag–RNase H, and Mo-MLV Gag PRCS–RNase H were assayed for RNase H activity by in situ RNase H assay. Only virions from Mo-MLV Gag–RNase H and Mo-MLV Gag PRCS–RNase H cultures contained measurable RNase H activity (Fig. 4). A new protein with RNase H activity at ~22 kDa was observed in virions obtained from cultures expressing the Mo-MLV Gag PRCS–RNase H polyprotein. Presumably, this protein, which corresponds in size to *E. coli* RNase HI, is the RNase HI domain cleaved from the Mo-MLV Gag PRCS–RNase H polyprotein by the Mo-MLV protease. While the Mo-MLV Gag PRCS cleavage site construction did not increase the antiviral effect of RNase HI or SN, we have proven the feasibility of the concept. The ability to release a degradative enzyme from the

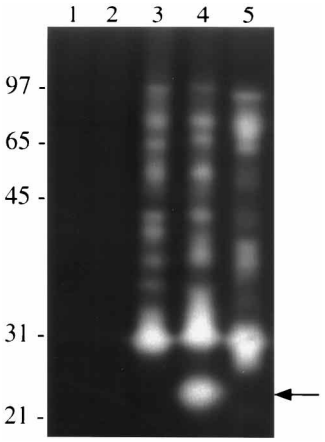

FIG. 4. *E. coli* RNase HI activity in virions from a therapeutic assay. An in situ RNase H assay was performed on denatured viral proteins from day 16 supernatants of a therapy assay separated on a sodium dodecyl sulfate–12% polyacrylamide gel containing a ^{32}P-labeled RNA-DNA substrate (see the legend to Fig. 2). Viral proteins from 0.5 ml of supernatant were analyzed per lane. CEF chronically infected with Mo(4070A) (lane 1) and expressing Mo-MLV Gag (lane 2), Mo-MLV Gag–RNase H (lane 3), or Mo-MLV Gag PRCS–RNase H (lane 4) and CEF expressing the Mo-MLV Gag–RNase H polyprotein (lane 5) but not Mo(4070A) infected were analyzed. The cleaved *E. coli* RNase H protein is indicated by the arrow. The numbers on the left are molecular sizes in kilodaltons.

polyprotein should make it possible to design future CTVI fusions in which activation of the degradative enzyme is controlled by protease cleavage (e.g., a zymogen). This should allow the use of degradative enzymes whose expression would otherwise be too toxic for the cell.

Conclusions. If we are to seriously consider using CTVI to treat viral diseases in humans, two significant problems must be solved. (i) If the host is immunocompetent, then the host's immune system is likely to eliminate cells that express the CTVI construct. This problem is exacerbated in diseases, like AIDS, in which the immune system of the host reacts strongly with the viral pathogen (human immunodeficiency virus type 1). (ii) There is no simple method that can be used to deliver genes into an adult animal (or human) stably and efficiently. Both of these limitations can be overcome in animal model systems, in which it is possible to deliver genes, by using either DNA microinjection or retroviral infection, into an embryo. This eliminates the host's immune response, and such methods can be used to deliver genes efficiently. The mouse experimental system can be used to test the CTVI strategy in vivo. If such experiments are successful, and a mouse expressing an appropriate Mo-MLV Gag–RNase H or Mo-MLV Gag–SN fusion is protected from Mo-MLV infection, then two types of additional projects can be contemplated. One is to adapt CTVI to other animal systems in which viral infections have important economic consequences (e.g., agriculture). This should be relatively straightforward. The more complex project would be to develop CTVI protocols that can be adapted to human patients. To be successful, such protocols would need to solve not only the problem of efficient delivery but also the problems posed by the presence of the patient's immune system.

We gratefully thank Jane Mirro and Alan Rein for technical advice, the D56 cells for the S+L− focus assay, and the rabbit anti-MuLV CA (p30) serum; Lance Stewart and Volker Vogt for the rabbit anti-avian myeloblastosis virus MA (p19) serum; and Maureen Craft for expert preparation of the manuscript.

This work was supported by the National Cancer Institute, under a contract with Advanced Bioscience Laboratories (S.H.H.); the Siebens Foundation, under the Harold W. Siebens Research Scholar Program (M.J.F.); and the Mayo Foundation (M.J.F.).

REFERENCES

1. **Bassin, R. H., N. Tuttle, and J. R. Fischinger.** 1971. Rapid cell culture assay technique for murine leukaemia viruses. Nature (London) **229:**564–566.
2. **Champoux, J. J.** 1993. Roles of ribonuclease H in reverse transcription, p. 103–117. *In* A. M. Skalka and S. P. Goff (ed.), Reverse transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
3. **Coffin, J. M.** 1990. Retroviridae and their replication, p. 1437–1500. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York, N.Y.
4. **Crouch, R. J.** 1990. Ribonuclease H: from discovery to 3D structure. New Biol. **2:**771–777.
5. **Federspiel, M. J., P. Bates, J. A. T. Young, H. E. Varmus, and S. H. Hughes.** 1994. A system for tissue-specific gene targeting: transgenic mice susceptible to subgroup A avian leukosis virus-based retroviral vectors. Proc. Natl. Acad. Sci. USA **91:**11241–11245.
6. **Federspiel, M. J., and S. H. Hughes.** 1994. Effects of the *gag* region on genome stability: avian retroviral vectors that contain sequences from the Bryan strain of Rous sarcoma virus. Virology **203:**211–220.
7. **Federspiel, M. J., D. A. Swing, B. Eagleson, S. W. Reid, and S. H. Hughes.** 1996. Expression of transduced genes in mice generated by infecting blastocysts with avian leukosis virus-based retroviral vectors. Proc. Natl. Acad. Sci. USA **93:**4931–4936.
8. **Greenhouse, J. J., C. J. Petropoulos, L. B. Crittenden, and S. H. Hughes.** 1988. Helper-independent retrovirus vectors with Rous-associated virus type O long terminal repeats. J. Virol. **62:**4809–4812.
9. **Guo, J., W. Wu, Z. Y. Yuan, K. Post, R. J. Crouch, and J. G. Levin.** 1995. Defects in primer-template binding, processive DNA synthesis, and RNase H activity associated with chimeric reverse transcriptase having the murine leukemia virus polymerase domain joined to Escherichia coli RNase H. Biochemistry **34:**5018–5029.
10. **Hansen, M., L. Jelinek, S. Whiting, and E. Barklis.** 1990. Transport and assembly of Gag proteins into Moloney murine leukemia virus. J. Virol. **64:**5306–5316.
11. **Hizi, A., S. H. Hughes, and M. Shaharabany.** 1990. Mutational analysis of the ribonuclease H activity of human immunodeficiency virus 1 reverse transcriptase. Virology **175:**575–580.
12. **Hughes, S. H., J. J. Greenhouse, C. J. Petropoulos, and P. Sutrave.** 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. **61:**3004–3012.
13. **Jones, T. A., G. Blaug, M. Hansen, and E. Barklis.** 1990. Assembly of *gag*-β-galactosidase proteins into retrovirus particles. J. Virol. **64:**2265–2279.
14. **Kanaya, S., and R. J. Crouch.** 1983. Low levels of RNase H activity in *Escherichia coli* FB2 *rnh* result from a single-base change in the structural gene of RNase H. J. Bacteriol. **154:**1021–1026.
15. **Kingston, R. E., C. A. Chen, and H. Okayama.** 1989. Introduction of DNA into eukaryotic cells, p. 911–919. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 1. Wiley, New York, N.Y.
16. **Ma, W.-P., and R. J. Crouch.** 1996. Escherichia coli RNase HI inhibits murine leukemia virus reverse transcription *in vitro* and yeast retrotransposon Ty1 transcription *in vivo*. Genes Cells **1:**581–593.
17. **Natsoulis, G., and J. D. Boeke.** 1991. New antiviral strategy using capsid-nuclease fusion proteins. Nature (London) **352:**632–635.
18. **Natsoulis, G., P. Seshaiah, M. J. Federspiel, A. Rein, S. H. Hughes, and J. D. Boeke.** 1995. Targeting a nuclease to murine leukemia virus capsids inhibits viral multiplication. Proc. Natl. Acad. Sci. USA **92:**364–368.
19. **Ott, D., R. Fredrich, and A. Rein.** 1990. Sequence analysis of amphotropic and 10A1 murine leukemia viruses: close relationship to mink cell focus-inducing viruses. J. Virol. **64:**757–766.
20. **Ott, D. E., J. Keller, K. Sill, and A. Rein.** 1992. Phenotypes of murine leukemia virus-induced tumors: influence of 3′ viral coding sequences. J. Virol. **66:**6107–6116.
21. **Petropoulos, C. J., and S. H. Hughes.** 1991. Replication-competent retrovirus vectors for the transfer and expression of gene cassettes in avian cells. J. Virol. **65:**3728–3737.
22. **Petropoulos, C. J., W. Payne, D. W. Salter, and S. H. Hughes.** 1992. Appropriate in vivo expression of a muscle-specific promoter by using avian retroviral vectors for gene transfer. J. Virol. **66:**3391–3397.
23. **Post, K., J. Guo, E. Kalman, T. Uchida, R. J. Crouch, and J. G. Levin.** 1993. A large deletion in the connection subdomain of murine leukemia virus reverse transcriptase or replacement of the RNase H domain with Escherichia coli RNase H results in altered polymerase and RNase H activities. Biochemistry **32:**5508–5517.
24. **Randolph, C. A., and J. J. Champoux.** 1994. The use of DNA and RNA oligonucleotides in hybrid structures with longer polynucleotide chains to probe the structural requirements for Moloney murine leukemia virus plus strand priming. J. Biol. Chem. **269:**19207–19215.
25. **Schumann, G., L. Qin, A. Rein, G. Natsoulis, and J. D. Boeke.** 1996. Ther-

apeutic effect of Gag-nuclease fusion protein on retrovirus-infected cell cultures. J. Virol. **70:**4329–4337.
26. **Spanos, A., S. G. Sedgwick, G. T. Yarranton, U. Hubscher, and G. R. Banks.** 1981. Detection of the catalytic activities of DNA polymerases and their associated exonuclease following SDS-polyacrylamide gel electrophoresis. Nucleic Acids Res. **9:**1825–1839.
27. **Stewart, L., G. Schatz, and V. M. Vogt.** 1990. Properties of avian retrovirus particles defective in viral protease. J. Virol. **64:**5076–5092.
28. **Varmus, H., and P. Brown.** 1989. Retroviruses, p. 53–108. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
29. **Wang, C.-T., J. Stegeman-Olsen, Y. Zhang, and E. Barklis.** 1994. Assembly of HIV Gag-β-galactosidase fusion proteins into virus particles. Virology **200:**524–534.
30. **Weldon, R. A., C. R. Erdie, M. G. Oliver, and J. W. Wills.** 1990. Incorporation of chimeric Gag protein into retroviral particles. J. Virol. **64:**4169–4179.
31. **Whitcomb, J. M., and S. H. Hughes.** 1992. Retroviral reverse transcription and integration: progress and problems. Annu. Rev. Cell Biol. **8:**275–306.
32. **Wu, X., H. Liu, H. Xiao, J. A. Conway, and J. C. Kappes.** 1996. Inhibition of human and simian immunodeficiency virus protease function by targeting Vpx-protease-mutant fusion protein into viral particles. J. Virol. **70:**3378–3384.
33. **Wu, X., H. Liu, H. Xiao, J. Kim, P. Seshaiah, G. Natsoulis, J. D. Boeke, B. H. Hahn, and J. C. Kappes.** 1995. Targeting foreign proteins to human immunodeficiency virus particles via fusion with Vpr and Vpx. J. Virol. **69:**3389–3398.
34. **Wu, X., H. Lui, H. Xiao, and J. C. Kappes.** 1996. Proteolytic activity of human immunodeficiency virus vpr- and vpx-protease fusion proteins. Virology **219:**307–313.