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Design and construction of oncoretroviral vectors expressing a packageable ribonuclease for use in HIV gene therapy

Research Article

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Abbreviations: Acquired immunodeficiency syndrome, (AIDS); capsid, (CA); constitutive transport element, (CTE); cytomegalovirus, (CMV); *enhanced green fluorescence protein, (egfp);* enzyme linked immunosorbent assay, (ELISA); human immunodeficiency virus type-1, (HIV-1); internal ribosome entry site, (IRES); isopropylthio- -D-galactoside, (IPTG); long terminal repeat, (LTR); Luria-Bertani, (LB); mason-pfizer monkey virus, (MPMV); matrix, (MA); Moloney murine leukemia virus, (MoMuLV); murine stem cell virus, (MSCV); mutant, (mt); *neomycin phosphotransferase*, (*neo*); nucleocapsid, (NC); polyacrylamide gel electro-phoresis, (PAGE); regulator of expression of virion proteins, (Rev); Rev responsive element, (RRE); ribonucleases, (RNases); *RNase T1, (rt1);* sodium dodecyl sulphate, (SDS); staphylococcal nuclease, (SN); *thymidine kinase*, (*tk); trans*-activation response element, (TAR); Vesicular stomatitis virus-G, (VSV-G)

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Summary

A number of different strategies are being developed for inhibition of human immunodeficiency virus type-1 (HIV-1) replication via gene therapy. In this study, a packageable ribonuclease, Gag-RNase T1, was constructed. The Gag domain from HIV-1 should allow copackaging into HIV-1 virions and the RNase T1 domain from *Aspergillus oryzae* should allow cleavage of HIV-1 virion RNA. In order to have regulator of expression of virion proteins (Rev)-dependent and Rev-independent production of Gag-RNase T1, the HIV-1 Rev responsive element (RRE) and the mason-pfizer monkey virus (MPMV) constitutive transport element (CTE) were cloned downstream to the *gagt1* gene. Expression and enzymatic activity of the Gag-RNase T1 fusion protein was compared using the Moloney murine leukemia virus (MoMuLV)-based vector, MoTiN, and murine stem cell virus (MSCV)-based vector, MGIN. Very little amount of Gag-RNase T1 was present in the cell lysate and in the culture supernatant of cells co-transfected with the MoTiN-based vector. In contrast, the amount of Gag-RNase T1 present in the cell lysate and in the culture supernatant of cells co-transfected with the MGIN-based vector was ~20 fold better. HIV-based lentiviral vector particles produced from cells expressing Gag-RNase T1 or mutant Gag-RNase T1 were also analyzed. Gag-RNase T1 present in these samples was shown to be full-length (56 kDa) and was enzymatically active *in vitro*. However, the titer of these vector particles was not decreased. These results suggest that Gag-RNase T1 is only capable of homochimeric assembly and is excluded from vector particles containing the HIV-1 Gag and Gag-Pol proteins.

I. Introduction

Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus type-1 (HIV-1), which infects CD4+ T lymphoid and myeloid cells, and causes a slow and progressive destruction of the immune system. Despite advances in the understanding of

the biology of HIV-1, there is still no cure for this disease. Currently available antiretroviral drugs seem to slow down HIV-1 replication in infected persons, but are partially successful because of side effects associated with prolonged use and the development of viral resistance to these drugs (Max et al, 2000; Servais et al, 2001). Anti-HIV gene therapy offers an alternative treatment and is currently being developed. Many gene therapy strategies are being developed to inhibit HIV replication. These include the use of RNA decoys, antisense RNAs, ribozymes, *trans*-dominant negative mutants of viral proteins, and ribonucleases (RNases) (Lamothe and Joshi, 2000). RNases are proteins that cleave RNA molecules in a catalytic manner, resulting in a permanent loss of RNA function (Sorrentino and Libonati, 1997). Three types of anti-HIV RNases may be developed: targeted RNases (Melekhovets and Joshi, 1996; Singwi et al, 1999; Singwi and Joshi, 2000) to specifically recognize and cleave HIV-1 RNAs, co-localized RNases to be co-localized with and cleave HIV-1 RNAs, and cytotoxic RNases (Singwi and Joshi, 2000) to specifically kill HIV-infected cells.

Co-localized RNases may be designed to be packaged within the virions. It is expected that the virion genomic RNA and the co-packaged cellular $tRNA_3^{lys}$ that serves as a primer during reverse transcription will both be cleaved. This can be achieved by fusing a viral structural protein to a ribonucleolytic domain. Vpr and Vpx proteins have been used to package staphylococcal nuclease (SN) into HIV particles (Wu et al, 1995). Vpr-SN and Vpx-SN fusion proteins were shown to be incorporated into virus-like particles *via* association with HIV-1 and HIV-2 Gag, respectively, and to possess nuclease activity *in vitro* (Wu et al, 1995). However, in experiments where HIV protease was also present, the SN moiety was shown to be inactivated (Wu et al, 1995). The efficacy of a packageable nuclease based on Gag has been demonstrated using the Moloney murine leukemia virus (MoMuLV) Gag-SN and Gag-*E. coli* RNase H fusion proteins (Schumann et al, 1996; VanBrocklin et al, 1997; VanBrocklin and Federspiel, 2000). The Gag-SN and Gag-RNase H fusion proteins were shown to be incorporated into MoMuLV virions and to reduce their infectivity by degrading virion RNA.

In this paper, we have used HIV-1 Gag to develop a packageable RNase. We fused the HIV-1 *gag* gene to the *RNase T1 (rt1)* gene from *Aspergillus oryzae*. RNase T1 (104 amino acids) is an endoribonuclease that cleaves single stranded RNA 3' to the G residues (Takahashi, 1971). The HIV-1 Gag precursor polyprotein, $Pr55^{Gag}$, is cleaved by viral protease into MA (matrix), CA (capsid), p1, NC (nucleocapsid), and the C-terminal product p6 (Gottlinger et al, 1989). The NC protein is the major protein component of the virion nucleocapsid where it coats the RNA genome (Lapadt-Tapolski et al, 1995; Poon et al, 1998). It is required for viral genomic RNA dimerization, encapsidation, and initiation of reverse transcription (Lapadt-Tapolsky et al, 1993). The 51 amino acid-long p6 region, downstream of the NC domain in the wild type HIV-1 Gag precursor, was not included as this region is believed to bind to the envelope within the virion and is not necessary for packaging HIV-1 Gag into virus particles (Royer et al, 1991; Hockley et al, 1994). Also, no proteolytic cleavage site was inserted between the NC domain and the RNase T1 domain (**Figure 1a**).

Thus, upon maturation in virus particles, the Gag-RNase T1 fusion protein should give rise to MA, CA, and NC-RNase T1. NC-RNase T1 is expected to degrade HIV RNA as well as the cellular $tRNA_3^{lys}$ that is packaged within the virions. Since the *gag* coding region contains several *cis*-acting repressive sequences (Fukumori et al, 1999; Brighty and Rosenberg, 1994), the HIV-1 regulator of expression of virion proteins (Rev) response element (RRE) was included downstream of the *gagt1* gene to allow Rev-dependent production of Gag-RNase T1. To

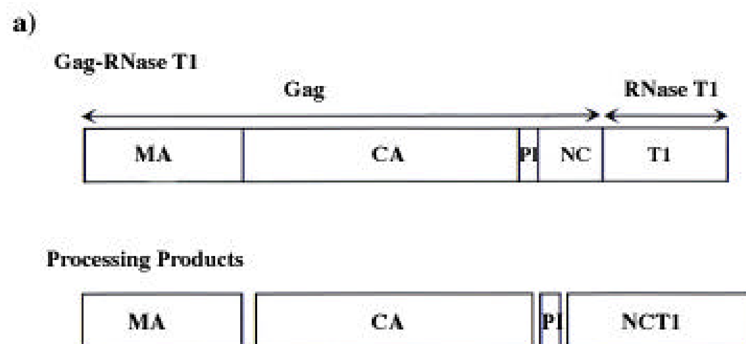

Figure 1a. Schematic diagram of the Gag-RNase T1 fusion protein and of its processing products. The Gag-RNase T1 fusion protein contains two domains Gag and RNase T1. The Gag domain contains MA, CA, p1, and NC regions of HIV-1 Gag protein; p2 and p6 domains of HIV-1 Gag were not included as they are not required for virion assembly. The RNase T1 domain consists of the entire 104 amino acids of RNase T1 from *A. oryzae*. The HIV-1 protease cleavage site at the NC-RNase T1 junction was deleted.

b)

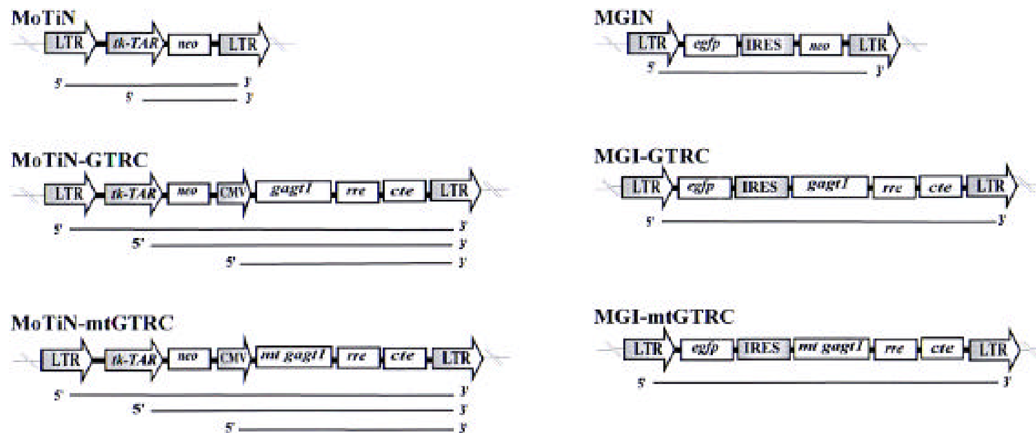

Figure 1b. Schematic diagram of MoTiN- and MGIN-derived oncoretroviral vectors. The MoMuLV-based MoTiN vector expresses the *neo* gene under control of the HSV *tk*-HIV-1 *TAR* fusion promoter. MoTiN-GTRC and MoTiN-mtGTRC vectors allow *gagt1* and *mt gagt1* gene expression under the control of the CMV promoter in an HIV-1 Rev-dependent and Rev-independent fashion. MSCV-based MGIN vector contains the *egfp* gene, an IRES, and the *neo* gene. MGI-GTRC and MGI-mtGTRC vectors allow *gagt1* and *mt gagt1* gene expression under the control of 5' LTR promoter in an HIV-1 Rev-dependent and Rev-independent manner. Transcripts produced from the 5' LTR and internal promoters are also shown.

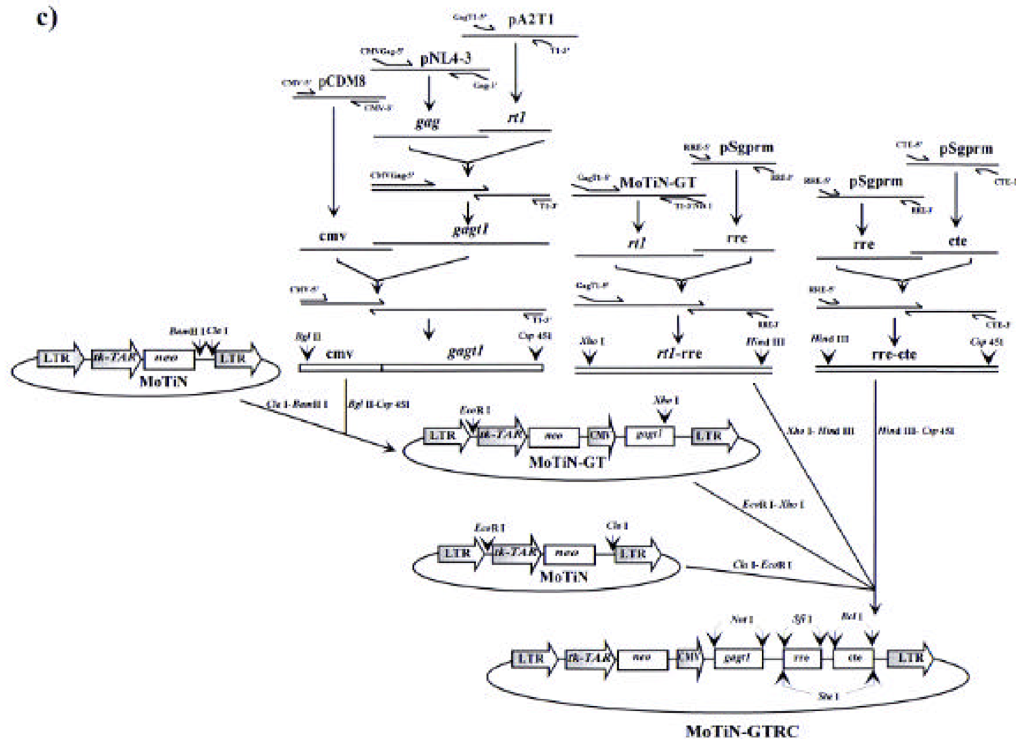

Figure 1c. Schematic diagram showing various steps involved in the construction of the MoTiN-GTRC vector. Firstly, the cmv-*gagt1* insert was constructed using a three-step overlap PCR strategy. The cmv-*gagt1* insert was digested with *Bgl* II and *Csp* 451 and ligated with the *Cla* I to *Bam*H I fragment of MoTiN to generate the MoTiN-GT vector. A tetrapartite ligation strategy was used to construct the MoTiN-GTRC retroviral vector. The *rt1*-rre and rre-cte fragments were derived by overlap PCRs and double digested with *Xho* I/*Hin*d III and *Hin*d III/*Csp*451, respectively. These fragments were ligated to the *Eco*R I to *Xho* I fragment of the MoTiN-GT vector and the *Cla* I to *Eco*R I fragment of the MoTiN vector. The resulting vector, MoTiN-GTRC, contained the *gagt1* gene flanked by *Not* I sites, the rre element flanked by *Sfi* I sites, the cte element flanked by *Bcl* I sites, and both the rre and the cte elements flanked by *Stu* I sites.

allow Rev-independent production of Gag-RNase T1, the constitutive transport element (CTE) from the mason-pfizer monkey virus (MPMV) was inserted 3' to the RRE. CTE has been shown to promote nuclear export of incompletely spliced HIV RNAs in a Rev-independent manner (Bray et al, 1994); RRE and CTE together were shown to allow even higher levels of expression. A mutant (mt) Gag-RNase T1 fusion protein with an inactive RNase T1 domain served as a control.

We demonstrate here that Gag-RNase T1 expression is better from the murine stem cell virus (MSCV)-based MGIN vector than from the MoMuLV-based MoTiN vector. The fusion protein formed vector particles. HIV-based vector particles produced from cells expressing Gag-RNase T1 were also analyzed. Gag-RNase T1 present in these samples was full-length and displayed RNase activity *in vitro*. However, the titer of the HIV-based vector particles produced from cells expressing Gag-RNase T1 was not decreased compared to the controls.

II. Results

A. Design and construction of vectors expressing Gag-RNase T1 and mt Gag-RNase T1

Oncoretroviral vectors MoTiN (Joshi et al, 1993) and MGIN (Cheng et al, 1997) were used in this study. MoTiN expresses the *neomycin phosphotransferase* (*neo*) gene under control of the herpes simplex virus *thymidine kinase* (*tk)*-HIV-1 *trans*-activation response element (TAR) fusion promoter (**Figure 1b**). MGIN contains the *enhanced green fluorescence protein (egfp)* gene, an internal ribosome entry site (IRES), and the *neo* gene (**Figure 1b**). The inclusion of IRES allows translation of the two proteins from the 5' long terminal repeat (LTR) directed RNA.

To construct the *gagt1* gene, the *rt1* gene encoding RNase T1 was cloned in frame immediately downstream of sequences coding for the NC domain within the HIV-1 *gag* gene (**Figure 1a**). The p6 domain located downstream of the NC domain was not included in Gag-RNase T1 fusion protein. The *gagt1* gene was also designed to exclude the HIV-1 protease cleavage site between the NC and the RNase T1 domains. A *mt gagt1* gene (as a control) was similarly designed to produce Gag-RNase T1 with an inactive RNase T1 domain. Glu_{58} and His_{40} are essential for RNase T1 activity (Heinemann and Saenger, 1982; Steyaert et al, 1990). Therefore, these amino acids were substituted by Ala in the *mt gagt1* gene. The HIV-1 *gag-coding* region within the *gagt1* and *mt gagt1* genes contains *cis*-acting repressive sequences. Therefore, expression of these genes in mammalian cells is Rev-dependent and requires inclusion of HIV-1 RRE. Alternatively, MPMV CTE (Bray et al, 1994) can be used to allow Rev-independent gene expression. Therefore, Rev-dependent production was accomplished by including the HIV-1 RRE (Brighty and Rosenberg, 1994) and Rev-independent production was achieved by including the MPMV CTE (Bray et al, 1994) within the mRNA encoding Gag-RNase T1 or mt Gag-RNase T1. Inclusion of both RRE and CTE within the 3' untranslated region has been shown to produce high levels of HIV-1 Gag (Bray et al, 1994). The CTE element was cloned near the polyadenylation signal, as it functions in a position-dependent manner (Rizvi et al, 1997).

In order to demonstrate that the *gagt1* open reading frame is intact and that the fusion protein is functionally active, the pET-GTRC vector was constructed to express *gagt1* gene under the control of T7 lac promoter in the bacterial system.

The MoTiN-GTRC vector was constructed to express the *gagt1* gene in mammalian cells (**Figure 1c**). This vector was designed to allow *gagt1* gene expression under control of the cytomegalovirus (CMV) promoter in an HIV-1 Rev-dependent and Rev-independent manner (**Figure 1c**). The MoTiN-mtGTRC vector was constructed to allow expression of the *mt gagt1* gene encoding a mtGag-RNase T1 fusion protein with an inactive RNase T1 domain (**Figure 1b**). MGI-GTRC and MGI-mtGTRC vectors were constructed to express *gagt1* and *mt gagt1* genes in mammalian cells. Both of these vectors were designed to allow *gagt1* and *mt gagt1* gene expression under the control of LTR promoter in an HIV-1 Rev-dependent and -independent manner (**Figure 1b**).

B. Inducible expression and analysis of Gag-RNase T1 produced in BL21 (DE3)pLysS and BL21 RIL Codon Plus strains of *E. coli*

The pET-GTRC vector was transformed into the BL21 (DE3)pLysS strain for isopropylthio- -D-galactoside (IPTG)-inducible expression of Gag-RNase T1. The pET15b vector was used as a control. However, upon IPTG-induction, no Gag-RNase T1 could be detected by sodium dodecyl sulphate (SDS)-polyacrylamide gel electro-phoresis (PAGE) (results not shown). Expression of -galactosidase, which served as an induction control, could be detected in the induced samples, indicating that the induction conditions were appropriate. Poor translation of Gag-RNase T1 in *E. coli* could be due to the fact that codon usage and the respective tRNA pools are different in bacterial and mammalian cells. Analysis of the *gagt1* open reading frame revealed a high occurrence of certain codons, which are rarely found in highly expressed bacterial genes. High level expression (upon IPTG-induction) of Gag-RNase T1 containing these rare codons could have resulted in the depletion of the corresponding tRNA pools, which in turn, could have slowed down or aborted translation (Kane, 1995; Kleiber-Janke and Becker, 2000).

BL21-Codon Plus-RIL *E.coli* cells contain a ColE1-compatible plasmid with extra copies of the rare *argU*,

ileY, and *leuW* tRNA genes. These tRNAs recognize Arg (AGA/AGG), Ile (AUA), and Leu (CUA) codons. Therefore, pET15b and pET-GTRC vectors were transformed into BL21 RIL Codon Plus *E. coli* strain. Bacterial cell lysates were analyzed 2, 4, 6, and 19 hours post-IPTG induction by SDS-PAGE, followed by Coomassie blue staining. The induced sample obtained from BL21 RIL Codon Plus *E. coli* transformed with pET-GTRC revealed an intense dark band corresponding to Gag-RNase T1. Expression was maximal at 19 hours post-induction. This band was absent in the samples obtained from uninduced *E. coli* transformed with pET-GTRC and in the uninduced and induced *E. coli* transformed with pET15b (results not shown). As expected, the -galactosidase was detectable in the induced induction control sample but not in the corresponding uninduced sample. These results were further supported by Western blot analysis of the induced pET15b and pET-GTRC samples. Gag-RNase T1 could be detected after immunostaining with HIV-1 positive human polyclonal serum in the induced pET-GTRC sample; no such reactivity was observed with the induced pET15b sample (results not shown).

Immunostaining with anti-RNase T1 antibodies demonstrated a 56-kDa band corresponding to Gag-RNase T1 (**Figure 2**). This band was only detected in the induced pET-GTRC sample. The induced pET15b sample did not show any reactivity with anti-RNase T1 antibodies.

To determine if the Gag-RNase T1 produced in *E.coli* was enzymatically active, induced pET15b and pET-GTRC samples from transformed BL21 RIL Codon Plus *E. coli* were analyzed on a Zymogram. Following electrophoresis and protein renaturation, the RNase T1 activity was detected by incubating the gel in the presence of ethidium bromide. A zone of clearance, indicating localized enzymatic digestion of the RNA, became visible in the lane containing the induced pET-GTRC sample; no such activity was observed in induced pET15b sample (result not shown).

These results demonstrate that the *gagt1* open reading frame is intact, and that the Gag-RNase T1 produced in BL21 RIL codon plus strain can cleave RNA *in vitro*.

C. Characterization of Gag-RNase T1 and mt Gag-RNase T1 produced in mammalian cells

The retroviral vectors MoTiN-GTRC, MoTiN-mtGTRC, MGI-GTRC, and MGI-mtGTRC were transiently transfected into 293T cells to determine Gag-RNase T1 and mt Gag-RNase T1 production level, assembly and release into vector particles. The parent retroviral vectors, MoTiN and MGIN, were used as negative controls. Cells were co-transfected with pMD.G and pHRG, and with pCMV 8.2 where indicated. pMD.G (Ory et al, 1996) was used to express the Vesicular stomatitis virus-G (VSV-G) envelope protein. pHRG (modified by replacing the *lacZ* gene with the *egfp* gene in pHR'CMVlacZ) was used to express HIV-based vector RNA. And, pCMV 8.2 (Naldini et al, 1996a) was used to express HIV-1 Gag, Gag-Pol, Tat, and Rev proteins. Transfections were performed both in the presence and absence of pCMV 8.2. In the absence of pCMV 8.2, VSV-G envelope-pseudotyped vector particles should be produced that contain Gag-RNase T1 (or mt Gag-RNase T1) and the HRG vector RNA. In the presence of pCMV 8.2, VSV-G envelope pseudotyped HIV-based vector particles should be produced that contain either Gag-RNase T1 (or mt Gag-RNase T1) and HIV-1 Gag/Gag-Pol proteins and the HRG vector RNA. Heterochimeric assembly should result in maturation of both HIV-1 Gag/Gag-Pol and Gag-RNase T1 proteins. Processing of Gag-RNase T1 by HIV-1 protease is expected to give rise to MA, CA, and NC-RNase T1 products. Gag-RNase T1 and NC-RNase T1 are both expected to contain the RNase T1 activity.

To determine whether *gagt1* and *mt gagt1* are expressed, cell lysates and cell culture supernatants from co-transfection experiments performed in the absence of pCMV 8.2 were analyzed by enzyme linked immunosorbent assay (ELISA) using p24 antibodies (**Table 1**). The p24 ELISA results from cell lysates and culture supernatants indicate that Gag-RNase T1 and mt Gag-RNase T1 are produced from both MoTiN- and MGIN-based vectors. However, Gag-RNase T1/mt Gag-RNase T1 expression is ~20 fold better from the MGIN-based vectors than from the MoTiN-based vectors. The p24 antigen detection from the cells transduced with the parental retroviral vectors MoTiN and MGIN is negligible,

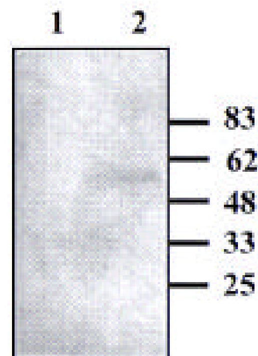

Figure 2. Western blot analysis of Gag-RNase T1 produced in *E. coli* BL21 RIL codon plus strain. Western Blot analysis was performed using anti-RNase T1 antibodies. Cell lysate from *E.coli* transformed with pET15b (lane 1) or pET-GTRC (lane 2) was analyzed 19 hours post-induction with 1 mM IPTG. Molecular weight markers are in kDa.

as expected. These results show that the *gagt1* and *mt gagt1* genes are better expressed from the MGIN-based vectors than from the MoTiN-based vectors, and Gag-RNase T1 and mt Gag-RNase T1 are also capable of homochimeric assembly resulting in the production of vector particles. Co-transfection with the pCMV 8.2 plasmid resulted in high levels of Gag and Gag-Pol production (result not shown).

Expression of fusion protein by MGI-GTRC and MGI-mtGTRC vectors was further confirmed by Western blot analysis using anti-RNase T1 antibodies. Concentrated vector particles from cells co-transfected with pMD.G, pHRG, pCMV 8.2, and either MGIN, MGI-GTRC or MGI-mtGTRC were analyzed for this purpose. The 56 kDa full-length Gag-RNase T1 and mt Gag-RNase T1 fusion proteins were detected in the concentrated samples from cells co-transfected with MGI-GTRC or MGI-mtGTRC vectors (**Figure 3**). No such protein was detected in samples analyzed from cells co-transfected with pMD.G, pCMV 8.2, and MGIN. This result indicates that Gag-RNase T1 and mt Gag-RNase T1 are not processed by the viral protease, suggesting homochimeric assembly.

Table 1. Amount of Gag-RNase T1/mt Gag-RNase T1 present within the cell lysate and cell culture supernatant of 293-T cells cotransfected with pMD.G, pHRG, and MoTiN- or MGIN-based vectors.

Gag RNase T1/ mt Gag RNase T1 (pmoles)*	MoTiN	MoTiN-GTRC	MoTiN-mt GTRC	MGIN	MGI-GTRC	MGI-mtGTRC
Cell lysate	6.2x10^{-4} pmoles	16.6x10^{-4} pmoles	14.5x10^{-4} pmoles	6.2x10^{-4} pmoles	312x10^{-4} pmoles	126x10^{-4} pmoles
Cell culture supernatant	5.8x10^{-4} pmoles	5.8x10^{-4} pmoles	46.6x10^{-4} pmoles	5.8x10^{-4} pmoles	163x10^{-4} pmoles	122x10^{-4} pmoles

*The amount of p24 antigen (in pg/ml) as determined by ELISA was converted to pmoles/ml. Each pmole of p24 antigen corresponds to one pmole of Gag-RNase T1/mt Gag-RNase T1. Total amount of Gag-RNase T1/mt Gag-RNase T1 produced from MoTiN- or MGIN-based vectors in cell lysates or cell culture supernatants was calculated by multiplying the pmoles/ml values with the total volume of cell lysates and cell culture supernatants.

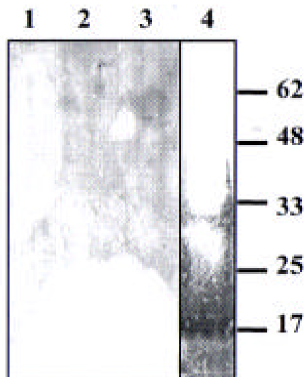

Figure 3. Western blot analysis of Gag-RNase T1 present within the vector particles produced from mammalian cells. Concentrated vector particles were analyzed from 293-T cells co-transfected with MGIN (lane 1), MGI-mtGTRC (lane 2), or MGI-GTRC (lane 3). These cells were also co-transfected with pMD.G, pHRG, and pCMV 8.2. Purified RNase T1 was analyzed as a positive control (lane 4). Analysis was performed using RNase T1 antibodies. Molecular weight markers are in kDa.

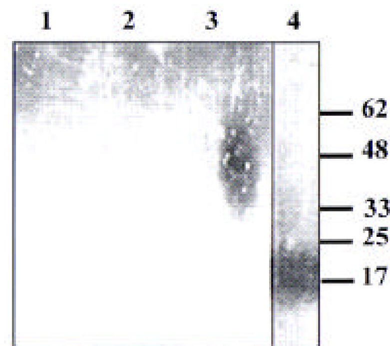

Figure 4. Zymogram displaying RNase activity of Gag-RNase T1 or mt Gag-RNase T1 present within the vector particles produced from mammalian cells. Concentrated vector particles released from 293-Tcells co-transfected with MGIN (lane1), MGI-mtGTRC (lane2), or MGI-GTRC (lane 3), along with pMD.G, pHRG, and pCMV 8.2, were analyzed on a 15% SDS-PAGE containing RNA. Purified RNase T1 served as a positive control (lane 4).

Concentrated vector particles from cells co-transfected in a serum-free medium with pMD.G, pHRG and pCMV 8.2 along with MGIN, MGI-GTRC or MGI-mtGTRC, were analyzed by a Zymogram. To preserve enzymatic activity, loading samples were prepared in the absence of -mercaptoethanol and were not boiled although this resulted in smearing. A diffused zone of clearance, indicating localized enzymatic digestion of the RNA, became visible in the lane containing the concentrated vector particles from MGI-GTRC co-transfected cells (**Figure 4**). No RNase activity was observed in lanes containing concentrated vector particles from cells co-transfected with MGIN or MGI-mtGTRC. Pure RNase T1 showed specific zone of clearance, as expected. This result indicates that the RNase T1 domain of the Gag-RNase T1 fusion protein is enzymatically active. However, since heterochimeric assembly could not be demonstrated, we cannot conclude that the RNase T1 domain of Gag-RNase T1 is not inactivated by the HIV-1 protease.

Vector particles from cells co-transfected with pMD.G, pHRG, pCMV 8.2 and either MGIN, MGI-GTRC or MGI-mtGTRC were also analyzed for their titer. Heterochimeric assembly of Gag-RNase T1 within the lentiviral vector particles followed by HRG vector RNA cleavage should decrease the vector titer. However, vector titer was not decreased (results not shown). This result is consistent with a homochimeric assembly model for Gag-RNase T1.

III. Discussion

In this study, we investigated the use of HIV-1 Gag to incorporate an RNase into HIV-1 virions. We designed the fusion protein Gag-RNase T1 which contains the HIV-1 Gag domain and the *A. oryzae* RNase T1 domain.

We expressed the *gagt1* gene from a bacterial expression vector pET15b to confirm that the fusion protein can be expressed and is enzymatically active. Initial transformation of BL-21 (DE3) pLysS with pET-GTRC did not show Gag-RNase T1 production upon IPTG induction. This low/undetectable level of the fusion protein could have been due to rare codon usage. A subset of codons, mainly Arg codons AGA and AGG are the least used codons in *E. coli*, and Ile AUA, Leu CUA, and Pro CCC codons are also known to affect the amount and quality of heterologous proteins produced in *E. coli*. These codons are decoded by rare tRNAs. An excess usage of any of these codons in a gene expressed in a bacterial system is known to result in very little/absence of full-length protein (Kane, 1995; Klaber-Janke and Beckor, 2000). Therefore we used BL21 RIL Codon Plus cells to allow Gag-RNase T1 expression from the pET-GTRC vector. The highest amount of recombinant protein was produced at 19 hours post-IPTG-induction. Western blot analysis resulted in specific reactivity of the induced pET-GTRC sample with HIV-1 positive human polyclonal serum (results not shown) and with anti-RNase T1 antibodies (**Figure 2**). Gag-RNase T1 produced in *E. coli* was also shown to be active *in vitro* (results not shown).

For expression in mammalian cells and for testing the homo-/heterochimeric assembly and RNase activity of the fusion protein, a MoMuLV-based MoTiN vector (Joshi et al, 1993) was designed to express the *gtrc* cassette containing the *gagt1* gene, rre and cte elements under control of the CMV promoter. Very little Gag-RNase T1 was detected in cell lysates and in the cell culture supernatants from the 293T cells co-transfected with the MoTiN-GTRC or MoTiN-mtGTRC vectors (**Table 1**). Poor expression could be due to promoter interference as the 5' LTR promoter has been shown to exert a negative effect on other promoters directly downstream of it (Emerman and Temin, 1984).

Next, the *gtrc* and *mt gtrc* expression cassettes were cloned in the MSCV-based MGIN vector (Cheng et al, 1997), which lacks an internal promoter. The second open reading frame in this vector is translated by internal initiation at the IRES element. Gag-RNase T1 and mt Gag-RNase T1 was detected in both cell lysates and culture supernatants (by ELISA using p24 antibodies; (**Table 1**). HIV-based vector particles from cells co-transfected with MGIN, MGI-GTRC or MGIN-mtGTRC vectors were also analyzed. Western blot analysis using RNase T1 antibodies (**Figure 3**) revealed that Gag-RNase T1 present in these samples is 56 kDa in size. Zymogram for the RNase activity revealed that Gag-RNase T1 present in this sample is active (**Figure 4**). No such activity was observed in the samples obtained from the MGI-mtGTRC or MGIN vector co-transfected cells, suggesting that the cleavage activity in the samples analyzed from the MGI-GTRC co-transfected cells is due to Gag-RNase T1. The titer of HIV-based vector particles produced from cells co-transfected with pMD.G, pHRG and pCMV 8.2, along with MGIN, MGI-GTRC or MGI-mtGTRC, was also determined. Similar titers were obtained from all three samples. Thus, HIV-1 Gag-RNase T1 fusion protein can be expressed in mammalian cells, can form vector particles, is not processed/inactivated by HIV-1 protease, and is enzymatically active *in vitro*. Also the titer of HIV-based vector particles produced from cells co-transfected with MGI-GTRC is not decreased. Taken together, these results suggest that Gag-RNase T1 is capable of homochimeric, but not heterochimeric, assembly. This study also demonstrated that MGIN vector with IRES elements allows higher level of expression of Gag-RNase T1 fusion protein than the MoTiN vector which contains an internal promoter.

IV. Materials and Methods

A. Vectors and oligonucleotides

The nucleotide sequence of various oligonucleotides used in this study was as follows. **CMV-5':** 5'-GGGCGCGG*AGATCT*-CGGGCCAGATATACGCGTTGAC-3'; **CMV-3':** 5'-TCTCTCTCCT*GCGGCCGC*GG-GTCTCCCTATAGTGAGTCGTAT-3'; **CMVGag-5':** 5'-TCACTATAGGGAGACCC-*GCGGCCGC*AGGAGAGAG<u>AT-</u>

GGGTGC-3'; **Gag-3':** 5'-AGCCTGTCTCTCAGTA-CAA-3'; **GagT1-5':** 5'-GATTGTACTGAGAGACAGGCTGCTTGCGACTACACTT-GC-3'; **T1-3':** 5'-ATATATAT*TTCGAA*TCGATTACTATGTACATTCAACGAAGT-3'; **T1-3'*Not*I:** 5'-GA*GGCCATTTTGGCCAGGCCTGCGGCCGC*AATTACTATGTACA-TTCAACGA-3'; **CTE-5':** 5'-A*GGCCAAAATGGCCTGATCA*CCCTCCCCTGTGAGC-TAGACT-3'; **CTE-3':** 5'-ATATATAT*TTCGAAAGGCCTTGATCA*CGACATCATCC-3'; **RRE-5':** 5'-TT*GCGGCCGGCCTGGCCAAAATGGCC*TCGGAGTAGCACCCAC-CAGG-3'; **RRE-3':** 5'-G*TGATCAGGCCATTTTGGCC*TAAGGAGTGTATTAAGCTT-GT-3'; **mtT1-5':** 5'-A-AACTGTT*GGATCC*AATTCTTACC-CAGCCAAATACAACA-ACTACGAAGGTTTTGATTTCTG-3'; **mtT1-3':** 5'-CATCACCGC*TCGAG*AGGATA-GGCC*ACGCGT*AGTAGGGAGAGCTCACAGAGAAATCAAAACCTT-3'; **GTRC-5':** 5'-ATATAT*CCATGG*CTG-CGAGAGCGTCAGTATTAA-3'; and **GTRC-3':** 5'-GCG-CGC*AGATCTGAATTC*AGGCCTTGATCACCA-3'. The restriction sites are shown in Italics. The beginning or end of an open reading frame is underlined. And, mutations resulting in an amino acid change are double underlined.

B. Vector constructions

The MoMuLV-based MoTiN vector (Joshi et al, 1993), MSCV-based MGIN vector (Cheng et al, 1997), and a bacterial expression vector pET15b (Novagen, Madison, WI, USA) were used in this study. First, an expression cassette containing the CMV immediate early promoter and the *gagt1* gene was constructed and cloned into the MoTiN vector, downstream of the *neo* gene (**Figure 1c**). All polymerase chain reactions (PCRs) were performed as described earlier (Medina and Joshi, 1999), except that Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) was used for all PCRs and overlap PCRs performed for cloning. These products were gel purified using the Gene Clean kit before restriction enzyme digestion and subsequent use in cloning. Taq DNA polymerase was used for PCRs performed for characterization of clones.

The cmv-*gagt1* expression cassette was constructed using an overlap PCR strategy where the 365 bp *rt1* gene was amplified from the pA2T1 (Quaas et al, 1988) plasmid using the GagT1-5'/T1-3' primer pair. The 1341 bp *gag* gene was amplified from pNL4-3 (Adachi et al, 1986) using the CMVGag-5'/Gag-3' primer pair. The CMV-5'/CMV-3' primer pair was used for the amplification of a 703 bp region containing the CMV immediate early promoter and enhancer elements using the pCDM8 plasmid (Invitrogen, Faraday Ave, CA, USA). The *Gag* and *rt1* PCR products were combined in an overlap PCR using the CMVGag5'/T1-3' primer pair to construct the *gagt1* gene. The PCR amplified cmv promoter and *gagt1* gene were combined in an overlap PCR using the CMV-5'/T1-3' primer pair. The PCR amplified cmv-*gagt1* gene was digested with *Bgl* II and *Csp* 451 and was ligated with the *Cla* I to *Bam*H I fragment of MoTiN to generate the MoTiN-GT vector (**Figure 1c**).

Next, the MoTiN-GTRC vector was constructed (**Figure 1c**). In order to produce the *rt1*-rre PCR product, the 365 bp *rt1* gene was amplified from the MoTiN-GT plasmid using the GagT1-5'/T1-3'*Not*I primer pair which also created a *Not* I restriction site 3' to the *rt1* gene. The HIV-1 rre sequence (504 bp) was amplified using the pSgprm (pSV-*gag-pol*-rre-mpmv) vector (Bray et al, 1994) and the RRE-5'/RRE-3' primer pair. The PCR amplified *rt1* gene and rre sequence were combined to produce the 869 bp *rt1*-rre insert *via* an overlap PCR using the GagT1-5'/RRE-3' primer pair. To produce the rre-cte insert, the MPMV cte element (283 bp) was PCR amplified using the pSgprm vector and the CTE-5'/CTE-3' primer pair. PCR amplified rre and cte were combined in an overlap PCR to generate a 787 bp rre-cte insert using the RRE-5'/CTE-3' primer pair. The *Xho* I and *Hin*d III digested *rt1*-rre product and the *Hin*d III and *Csp* 451 digested rre-cte product were then ligated with the *EcoR* I to *Xho* I fragment of MoTiN-GT and the *Cla* I to *Eco* RI fragment of MoTiN (**Figure 1c**). MoTiN-GTRC clones were identified by extensive restriction enzyme and PCR analyses.

In order to produce the MoTiN-mtGTRC vector, His_{40} and Glu_{58} within the *rt1-coding* region were mutated to Ala_{40} and Ala_{58} via PCR followed by cloning. Nucleotide changes leading to amino acid substitutions were introduced within the synthetic primers. The mtT1-5' primer contained point mutations within the His_{40} codon, and the mtT1-3' primer contained point mutations within the Glu_{58} codon. The *Bam*H I and *Xho* I site were inserted in mtT1-5' and mtT1-3' primers, respectively. These primers were used to amplify the *mt t1* gene using MoTiN-GTRC. The resulting PCR product was digested with *Bam*H I and *Xho* I and was cloned at the same sites within MoTiN-GTRC to construct MoTiN-mtGTRC. A *Mlu* I restriction site was introduced into the mtT13' primer while maintaining the amino acid composition. *Mlu* I digestion was therefore performed for screening the MoTiN-mtGTRC clone.

In order to construct the pET-GTRC, the *gtrc* cassette was amplified from the MoTiN-GTRC vector (using GTRC-5'/GTRC-3' primer pair). The amplified product was digested with *Nco* I and *Bgl* II and cloned at the *Nco* I and *Bam*H I sites within the *E. coli* expression vector, pET15b.

A similar strategy was used to generate MGI-GTRC and MGI-mtGTRC vectors. Essentially, the *gtrc* and *mt gtrc* cassettes were PCR amplified from MoTiN-GTRC and MoTiN-mtGTRC vectors using the GTRC-5'/GTRC-3' primer pair. The PCR products containing *gtrc* and *mt gtrc* sequences were digested with *Nco* I and *Bgl* II and cloned at the *Nco* I and *Bam*H I sites within the MGIN vector (Cheng et al, 1997). As a result, the *neo* gene within the MGIN vector was deleted. Ampicillin resistant, kanamycin sensitive colonies containing the MGI-GTRC and MGI-mtGTRC clones were selected. Correct clones were further characterized by extensive restriction enzyme and PCR analyses.

C. Inducible expression and analysis of Gag-RNase T1 produced in *E. coli* BL21 (DE3)pLysS and BL21 RIL Codon Plus strains

The bacterial expression vectors, pET15b and pET-GTRC, were used to transform competent *E. coli* strains BL21 (DE3)pLysS (Novagen) and BL21 RIL Codon Plus (Stratagene, La Jolla, CA, USA) Transformed *E. coli* cells were grown in 10 ml of Luria-Bertani (LB) broth containing 50 µg/ml of ampicillin, 20 µg/ml chloramphenicol, and 1% glucose at 37°C overnight. 500 µl of the overnight culture was used to inoculate two separate flasks (for each plasmid) containing 100 ml of the same media (one of the two flasks was used as an uninduced control), and incubation continued at 37°C. To test the induction conditions for fusion protein expression, the diluted overnight cultures were grown for 3-4 hours at 37°C until mid-log phase (OD_{600} = 0.6). IPTG was then added at a final concentration of 1

mM, and incubation was continued at 37°C for 2, 4, 6, and 19 hours. No IPTG was added to the uninduced cells, which were cultured in a similar manner. Following IPTG induction, the cells were pelleted by centrifugation.

A positive induction control strain provided by Novagen matching in promoter, selectable marker, and other vector elements was also included in the induction experiments. This strain contains a pET15b plasmid with an insert encoding 116-kDa -galactosidase.

SDS-PAGE and Western blot analysis: Cell pellets obtained from 100 µl of the pET15b and pET-GTRC transformed cell cultures (19 hours post-induction) were resuspended in SDS-PAGE gel loading buffer (Sambrook et al, 1989) containing -mercaptoethanol. Samples were boiled for 3 minutes and loaded on a denatured SDS-15% polyacrylamide gel. For immuno-blotting, proteins were transferred following electrophoresis, from polyacrylamide gels to Biotrans Nylon membrane (ICN, Irvine, CA) and prewetted in Tris/glycine electroblotting buffer (20% methanol) for 15 min. Trans-Blot semi dry electrophoretic transfer cell (Bio-Rad Laboratories, Mississauga, ON, CA) was used for 25 min at 22 volts to allow complete protein transfer. Anti-RNase T1 antibodies raised in rabbits were used (1:200 dilution) as the source of primary antibodies. The blot was then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2500 dilution; Sigma Chemical Co., St Louis, MO, USA). Finally, the blot was placed with the substrate BCIP (5-bromo-4-chloro-3-indolyl phosphate), which generates an alcohol insoluble dark blue/purple stain, and NBT (nitro blue tetrazolium) which enhances the product colour. Purified RNase T1 (Sigma) was used as a positive control.

Zymogram: The Zymogram was performed as described earlier (Singwi et al, 1999). Briefly, 100 µg/ml *E. coli* RNA was included in the 15% resolving gel. The protein samples to be loaded on this gel were prepared in the same manner as for SDS-PAGE, except that no -mercaptoethanol was added in the loading buffer and the samples were not boiled. Following electrophoresis, the gel was washed 3 times with distilled water while allowing gentle shaking and was then immersed in Zymogram buffer (Tris-Cl, pH 7.4 50 mM; EDTA, 2 mM; ethidium bromide, 2.5 µg/ml) and incubated overnight at 37ºC. The gel was then placed under ultraviolet light to detect the zone of clearance due to RNase activity.

D. Transient expression and analysis of Gag-RNase T1 and mt Gag-RNase T1 produced in 293T cells

VSV-G pseudotyped vector particles were generated by co-transfection of plasmid DNAs into 293 T cells as described previously (Joshi et al, 1990). Briefly, transfections were done in 10 cm cell culture dishes using 30 µg of each retroviral vector construct, 5 µg of the *env* expression vector pMD.G (Ory et al, 1996), 10 µg of the *gag/gag-pol*, *tat*, and *rev* expressing vector pCMV 8.2 (Naldini et al, 1996a), and 15 µg of the transfer vector pHRG. pHRG was constructed by replacing the *lacZ* gene with *egfp* gene in the pHR'CMVlacZ vector (Naldini et al, 1996b). 293 T cells ($4{\times}10^6$) were plated for 8 hours at 37°C. Plasmid DNAs were mixed together in a 450 µl volume. 50 µl of 2.5 M $CaCl_2$ was added to the DNA mix. 500 µl of hepes buffered saline solution (pH 7.05) was then added drop-wise, while bubbling with a plastic pipette. The tube was left at room temperature for 20 min and DNA was added gently over the cells while shaking the medium. Cells were incubated at 37°C overnight. The next day, medium was replaced with 7 ml of fresh medium. Transfections were also performed using a serum-free medium (Gibco BRL, Burlington, ON, CA).

1. ELISA using p24 antibodies

The presence of Gag-RNase T1 or mt Gag-RNase T1 in cell lysates and in the vector particles released in the cell culture supernatants was determined by ELISA using HIV-1 p24 antibodies (Abbott, Chicago, IL, USA). Cell lysates were prepared by lysing cells on day 3 post-transfection. Cell pellets were resuspended in 250 µl lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.02% NaN3, and 100 µg/ml phenylmethylsulfonyl fluoride, 1% NP-40), kept at 4°C for 20 min, and then centrifuged at 10,000 x g for 2 min to remove cell debris. The supernatant was diluted 10 fold in the culture media and used for p24 antigen determination using instructions provided by the supplier. p24 antigen levels were also determined in the cell culture supernatants.

2. Western blot and Zymogram analyses

Vector particles present in the supernatant of the co-transfected cells were first concentrated as follows. Samples were centrifuged at 1500 rpm for 5 minutes to remove the cell debris and then ultracentrifuged through a 20 % sucrose (prepared in phosphate buffer saline) cushion for 2 hours at 35,000 rpm, 4ºC (Schumann et al, 1997). The pellet was resuspended in a buffer containing 50 mM Tris-Cl, pH 6.8, 100 mM NaCl.

Concentrated vector particles from cells co-transfected with MGIN-based vectors, pMD.G, pCMV 8.2, and pHRG were analyzed by Western blot analysis using anti-RNase T1 antibodies. Assuming that the amount of Gag-RNase T1 or mt Gag-RNase T1 present in these vector particles is the same as when the particles are produced in the absence of pCMV 8.2, concentrated vector particles containing ~50-100 pg equivalent of Gag-RNase T1 (or mutant Gag-RNase T1) were loaded in each well. Purified RNase T1 (0.4 µg) was used as positive control.

In order to determine RNase activity of the fusion protein, vector particles were obtained as described above from cells co-transfected with MGIN-based vectors, pMD.G, pCMV 8.2, and pHRG and cultured in the serum-free medium. Concentrated vector particles containing ~50-100 pg equivalent of Gag-RNase T1 (or mt Gag-RNase T1) were analyzed by the Zymogram assay. Purified RNase T1 (0.4 µg) was analyzed in parallel to serve as a positive control

3. HIV-based vector titer

Vector particles released from the 293-T cells co-transfected with MGIN-based vectors, pMD.G, pHRG, and pCMV 8.2 were analyzed for their titer. Equal amount of vector particles was used to transduce 293-T cells as described previously (Joshi et al, 1990). The number of $EGFP^+$ and $EGFP^-$ cells was then determined on day 5 post-transduction and used to calculate vector titer.

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