3270–3278 *Nucleic Acids Research, 1998, Vol. 26, No. 13*

Co-packaging of sense and antisense RNAs: a novel strategy for blocking HIV-1 replication

Shi-Fa Ding, Jeffrey Noronha and Sadhna Joshi*

Department of Medical Genetics and Microbiology, Faculty of Medicine, University of Toronto, Toronto, Ontario M5S 3E2, Canada

Received February 13, 1998; Revised and Accepted May 6, 1998

ABSTRACT

Retroviral vectors were engineered to express either sense (MoTiN-TRΨ^{e+}) or sense and antisense (MoTN-TRΨ$^{e+/-}$) RNAs containing the human immunodeficiency virus type-1 (HIV-1) *trans*-activation response (TAR) element and the extended packaging (Ψ^{e}) signal. The Ψ^{e} signal includes the dimer linkage structure (DLS) and the Rev response element (RRE). Amphotropic vector particles were used to transduce a human CD4+ T-lymphoid (MT4) cell line. Stable transductants were then tested for sense and antisense RNA production and susceptibility to HIV-1 infection. HIV-1 production was significantly decreased in cells transduced with MoTiN-TRΨ^{e+} and MoTN-TRΨ$^{e+/-}$ vectors. Efficient packaging of sense and most remarkably of antisense RNA was observed within the virus progeny. Infectivity of this virus was significantly decreased in both cases, suggesting that the interfering RNAs were co-packaged with HIV-1 RNA. Vector transduction was not expected to occur and was not observed. Inhibition of HIV-1 replication was also demonstrated in human peripheral blood lymphocytes transduced with retroviral vectors expressing antisense RNA. These results suggest that (i) both sense and antisense RNAs were co-packaged with HIV-1 RNA, (ii) the co-packaged sense and antisense RNAs inhibited virus infectivity and (iii) the co-packaged sense and antisense RNAs were not transduced. Sense and antisense RNA-based strategies may also be used to co-package other interfering RNAs (e.g. ribozymes) to cleave HIV-1 virion RNA.

INTRODUCTION

Human immunodeficiency virus (HIV) gene therapy strategies have largely been based on sense RNAs, antisense RNAs, ribozymes and *trans*-dominant mutants of viral proteins (reviewed in 1–4). Many of these strategies are aimed at inhibiting HIV protein–RNA interactions between *trans*-activator of transcription (Tat)–*trans*-activation response (TAR) element, regulator of expression of virion proteins (Rev)–Rev-response element (RRE), and Pr55^{Gag}-packaging (Ψ) signal.

The Tat protein is produced early during the virus life cycle. It interacts with a 3 nt bulge in the HIV-1 TAR element. This 59 nt RNA element forms a stem–loop structure and is located within the 5′ and 3′ non-coding regions of all HIV-1 unspliced and spliced mRNAs (5,6). Tat–TAR interactions enhance the rate of transcription elongation from the HIV promoter (7,8). Inhibition of Tat–TAR interactions using Tat *trans*-dominant mutants (9,10), TAR RNA decoys (11–15) and antisense Tat/TAR RNAs (13,15–17) was shown to inhibit HIV-1 replication.

Rev is a phosphorylated nuclear protein that, like Tat, is also produced early during the virus life cycle (18,19). It binds to a 13 nt region within the HIV-1 RRE. The RRE is a 351 nt RNA element with a complex secondary structure that is located within the *env* coding region on HIV-1 singly spliced and unspliced mRNAs (20). Rev–RRE interactions facilitate nuclear export of these mRNAs (21,22). HIV gene therapy strategies targeted against Rev–RRE interactions include Rev *trans*-dominant mutants (23–26), RRE RNA decoys (27–31) and antisense Rev/RRE RNAs (15,17,28).

An essential step during retroviral replication is the encapsidation of two full-length viral genomic RNA copies amid spliced viral transcripts and cellular RNAs. Selective encapsidation depends upon the recognition of HIV-1 RNA by a zinc finger-like motif in the nucleocapsid domain of Pr55^{Gag} (32). The HIV-1 Ψ signal contains four stem–loops located near the 5′ major splice donor and the beginning of the *gag* open reading frame (33). This region is essential (34) and sufficient (35) for RNA packaging. However, the Ψ^{e} signal, as we define it, includes an additional 1.1 kb *cis*-acting packaging element located within the *env*-coding region containing the RRE. The *env* component appears to increase packaging efficiency (36). Interestingly, the dimer linkage structure (DLS) necessary for RNA dimerization (37) overlaps with the Ψ signal, implying that encapsidation and dimerization may be mechanistically linked. Interference at the level of the Ψ signal–Pr55^{Gag} interaction may be achieved by using sense and antisense RNAs targeted to the HIV-1 Ψ signal. RNA containing a 1.43 kb region antisense to the HIV-1 Ψ and *gag* coding sequence was previously shown by our laboratory to inhibit HIV-1 replication in stable MT4 transductants (28). However, RNA containing this region in the sense orientation conferred no protection (28). RNAs containing 1000 nt or more (in the antisense orientation) within this 1.43 kb region were shown to be sufficient to inhibit HIV replication (38). However, the best results were obtained with the 1.43 kb region in the antisense orientation. Furthermore, this antisense RNA strategy was also shown to inhibit HIV-1 replication better than the Rev M10 *trans*-dominant mutant (39). The mode of inhibition

*To whom correspondence should be addressed. Tel: +1 416 978 2499; Fax: +1 416 638 1459; Email: sadhna.joshi.sukhwal@utoronto.ca

underlying this antisense RNA mediated resistance was shown to occur pre-splicing (39,40).

In this study, we developed and tested Moloney murine leukemia virus (MoMuLV)-based retroviral vectors expressing interfering RNAs containing the HIV-1 TRΨ^{e} (TAR, RRE and Ψ^{e}) sequence in sense or in both sense and antisense orientations. Inhibition of HIV-1 replication was first assessed in stable transductants of a human CD4+ T-lymphoid cell line. Vectors allowing constitutive and Tat-inducible production of sense RNA or constitutive production of antisense RNA were both shown to confer resistance to HIV-1 replication, although better results were obtained using the antisense RNA approach. Vectors expressing antisense RNA were also shown to inhibit HIV replication in transduced human peripheral blood lymphocytes (PBLs). Sense and antisense RNAs were found to be packaged within the virus progeny. In both situations, loss of virus infectivity resulted, suggesting that these interfering RNAs must have been co-packaged with HIV-1 RNA. These results demonstrate that both sense and antisense RNA based strategies can be successfully used to inhibit HIV replication at the level of RNA packaging.

MATERIALS AND METHODS

Construction of retroviral vectors

For construction of the MoTiN-TRΨ^{e+} vector, a 1.8 kb *Sca*I–*Not*I fragment of the LRPL vector (36) containing the HIV-1 long terminal repeat (LTR) promoter, TAR, RRE and Ψ signal (including DLS) was ligated to an 8379 bp *Not*I–*Nru*I fragment (omitting the ribozyme) of MoTiN-Rz$_{Pro}$ vector (41). The correct clone, MoTiN-TRΨ^{e+}, was screened by restriction enzyme and polymerase chain reaction (PCR) analysis and confirmed by partial DNA sequencing. MoTN-TRΨ$^{e+/-}$ vector was constructed as follows: A pBluescript based vector (pBS-GE), which contains the HIV-1 5′-LTR-*gag*-intercistronic sequence-*env*-3′-LTR, was previously constructed in our laboratory. The intercistronic sequence composition is 5′-AGA TAG GGG GGC AAC TAA GAA TTC GGC GCC GCG GCC GCG TCG ACA GTG GCA-3′. The HIV-1 5′-LTR-*gag*-intercistronic region-*env* sequences were PCR amplified using SD-5′ (5′-GCC CGA GAG GCC AGC TGG GCC TTC GAA CGG CCG CTG ACA TCG AGC TTG C-3′) and SD-3′ (5′-ATA TAT ATG GCC ATA TGG GCC TGA TCA TGC TGG CTC AGC TCG TCT CTT TAT TTC C-3′) primers. The SD-3′ primer provided a transcription terminator. The SD-5′ and SD-3′ primers were both designed to contain an *Sfi*I site. These sites were designed to differ in their NNNNN sequence composition. The PCR product was digested with *Sfi*I and cloned in the antisense orientation in the MoTN vector, previously modified to contain two compatible *Sfi*I sites between *Xho*I and *Apa*I sites. The resultant vector, MoTN-TRΨ$^{e+/-}$ was selected and confirmed by PCR, restriction enzyme analysis and partial sequencing.

Production of amphotropic retroviral vector particles

The ecotropic Ψ-2 packaging cell line (42) was transfected with MoTN (43), MoTiN (44), MoTiN-TRΨ^{e+} and MoTN-TRΨ$^{e+/-}$ vector DNAs as described previously (45). The ecotropic vector particles released from the pools of transductants (containing at least 100 colonies) were then used to transduce the amphotropic PA317 packaging cell line (46). Amphotropic vector particles released from the pools of stable transductants (containing at least 100 colonies) were then collected. These particles were tested and confirmed to be free of helper virus contamination or recombination by reverse transcription (RT)-PCR using primers specific to the MoMuLV *pol* coding region (RCR-5′, 5′-GAG ATT TGG TTA GAG AGG CA-3′; and RCR-3′, 5′-CTG ACC TCC CTA GTC ATC TA-3′).

Transduction of stable MT4 transductants

Amphotropic retroviral vector particles were used to transduce the human CD4+ lymphocyte-derived MT4 suspension cell line (47,48) as described previously (25,28). The pools of G418-resistant stable MT4 transductants were each selected and used without cloning.

RT-PCR analysis of stable MT4 transductants

Total RNA, extracted (49) from stable MT4 transductants (5×10^6 cells), was incubated with 4 U DNase RQ1 (Promega) in the presence of 20 mM $MgCl_2$ and 10 mM DTT for 15 min at 37°C. The RNA was then phenol/chloroform extracted. RT-PCRs were performed as described previously (41) using various primer pairs shown in Table 1. RT-PCR using Gap-5′ and Gap-3′ primers was performed as an internal control to detect cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA. All RT-PCR products (10 µl) were analyzed by electrophoresis on a 1.5–2% agarose gel.

Flow cytometry analysis of transduced MT4 cells

Prior to HIV-1 infection, aliquots of transduced cells were analyzed for surface CD4 expression as described previously (50).

HIV-1 susceptibility of stable MT4 transductants

Pools of actively dividing stable MT4 transductants (2×10^6 cells) were each infected with 30 ng (p24 equivalent) of the HIV-1 strain, NL4-3 (51), as described previously (41). The amount of HIV-1 is expressed throughout in p24 equivalents. Cells were routinely checked for syncytium formation. One-third of the cultures were collected every 3 days and replaced with fresh medium. The amount of HIV-1 p24 antigen produced in the cell culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA; Abbott) as described (41). The experiment was repeated twice.

RT-PCR analysis of RNAs present within the cells and within the virus released from HIV-1 infected stable MT4 transductants

Total cellular RNA was extracted as described elsewhere (49). For RNA extraction from virus particles present in the cell culture supernatants, contaminating cells and cell debris were first removed by centrifugation at 200 *g*. RNA was extracted by phenol/chloroform treatment, ethanol precipitated and resuspended in H_2O. RT-PCRs were performed using various primer pairs (Table 1) as indicated. The Gap-5′/Gap-3′ primer pair was used as a control to detect cellular GAPDH RNA. Total cellular RNA from HIV-1 infected MoTN-TRΨ$^{e+/-}$ transduced MT4 cells was also analyzed by RT-PCR using the SD-3′ during reverse transcription and the TAR-5′ (5′-GGG TCT CTC TGG TTA GA-3′) and SD-3′ primers during PCR.

Table 1. Primer pair used during RT-PCR, RNAs detected, and the product size

Primer pair	RNAs detected	Product size
Gap-5' (5'-CCA-CCC-ATG-GCA-AAT-TCC-ATG-GCA-3') **Gap-3'** (5'-TCT-AGA-CGG-CAG-GTC-AGG-TCC-ACC-3')	GAPDH RNA	600 bp
Vu-5' (5'-GCC-AGC-AAC-TTA-TCT-GTG-3') **Vu-3'** (5'-CGT-CTC-GCG-GCC-ATC-CG-3')	v-RNA v-TRΨ^{e+} RNA v-TRΨ^{e-} RNA	567bp
Neo-5' (5'-CAA-GAC-CGA-CCT-GTC-CGG-3') **Neo-3'** (5'-CTC-TTC-GTC-CAG-ATC-ATC-3')	n-RNA n-TRΨ^{e+} RNA v-TRΨ^{e+} RNA v-TRΨ^{e-} RNA	337 bp
Vd-5' (5'-CCT-ATA-GAG-TAC-GAG-CC-3') **Vd-3'** (5'-AAC-AGA-AGC-GAG-AAG-CG-3')	v-RNA v-TRΨ^{e+} RNA v-TRΨ^{e-} RNA n-RNA n-TRΨ^{e+} RNA TRΨ^{e+} RNA[a]	457 bp
Gag-5' (5'-CGA-GAG-GAA-GCT-GCA-GAA-TGG-GA-3') **I-3'** (5'-GCC-ACT-GTC-GTT-ACG-CGT-CCG-CGG-CGC-C-3')	v-TRΨ^{e-} RNA[b] TRΨ^{e+} RNA[b,c]	935 bp
Psi-5' (5'-ATA-GTA-TGG-GCA-AGC-AGG-G-3') **Psi-3'** (5'-GAT-GTT-CTG-CAC-TAT-AGG-G-3')	v-ΨRNA[d] n-ΨRNA[d] HIV-1 RNA[e]	315 bp
Vpr-5' (5'-ATA-CTT-GGG-CAG-GAG-TGG-AAG-C-3') **Vpu-3'** (5'-CTA-TGG-ACC-ACA-CAA-CTA-TTG-C-3')	HIV-1 RNA	424 bp

[a]TRΨ^{e+} RNA produced from MoTiN-TRΨ^{e+} vector and not the one produced from MoTN-TRΨ$^{e+/-}$ vector.

[b]When Gag-5' primer is used during reverse transcription.

[c]TRΨ^{e+} RNA expressed from MoTN-TRΨ$^{e+/-}$ vector and not the one expressed from MoTiN-TRΨ^{e+} vector.

[d]When I-3' primer was used during reverse transcription.

[e]When Psi-5' primer was used during reverse transcription.

[f]When Psi-3' primer was used during reverse transcription.

Replication-competence of virus progeny released from the HIV-1 infected stable MT4 transductants

Virus progeny released from HIV-1 infected MoTiN and MoTiN-TRΨ^{e+} vector-transduced MT4 cells (containing 9.6 ng of p24) and from HIV-1 infected MoTN and MoTN-TRΨ$^{e+/-}$ vector-transduced MT4 cells (containing 1.2 ng of p24) was used to infect untransduced parent MT4 cells. The amount of HIV-1 p24 produced in the cell culture supernatants was determined by ELISA (Abbott). Infectivity was also checked at various time points by RT-PCR analysis of cellular RNA using the Vpu-5′/Vpr-3′ primer pair (Table 1). In addition, PCR with the Vd-5′/Vd-3′ primer pair (Table 1) was performed to check if virus progeny infection of MT4 cells resulted in vector transduction. Gap-5′ and Gap-3′ primers were used to detect GAPDH DNA and RNA as an internal control for all PCRs and RT-PCRs.

Transduction of human PBLs

Human peripheral blood mononuclear cells were isolated from fresh heparinized blood samples from healthy donors by Ficoll-Hypaque gradient centrifugation. Cells were washed once with phosphate buffered saline (PBS) and cultured at a density of 1×10^6 cells/ml in RPMI 1640 containing 10% fetal bovine serum, 20 U/ml human recombinant interleukin (IL)-2 (Boehringer Mannheim), and 5 µg/ml of phytohemagglutinin (Sigma) for 1 day at 37°C. The adherent layer was removed and the suspension cells were cultured for a further 2 days. Over 85% of these cells represented T lymphocytes as determined by flow cytometry analysis using specific monoclonal antibodies. PBLs (1×10^6 cells) were then mixed with 1 ml of amphotropic MoTN, MoTN-TRΨ$^{e+/-}$ and MoTN-Ψ$^-$ vector particles (with 16 µg/ml polybrene and 20 U/ml IL-2) and centrifuged at 200 *g* for 1 h at 32°C and then cultured for 16 h at 32°C. Cells were then incubated for 6 h at 37°C in the presence of IL-2 supplemented medium. The transduction procedure was carried out a total of three times. Twenty-four hours after the third transduction, cells were cultured for 8 days in IL-2 supplemented medium containing G418 (500 µg/ml). Cells were checked periodically for growth and viability.

HIV-1 susceptibility of PBL transductants

Transduced PBLs (5×10^6 cells) were infected with 30 ng (p24 equivalent) of the HIV-1 strain, NL4-3, for 16 h at 37°C. Cells were then washed twice with RPMI 1640 medium, resuspended in 750 µl of the same medium containing 20 U/ml IL-2 and incubated at 37°C. The culture supernatants were collected every 3 days and replaced with fresh medium. The amount of HIV-1 p24 antigen released in the cell culture supernatant was measured by ELISA (Abbott).

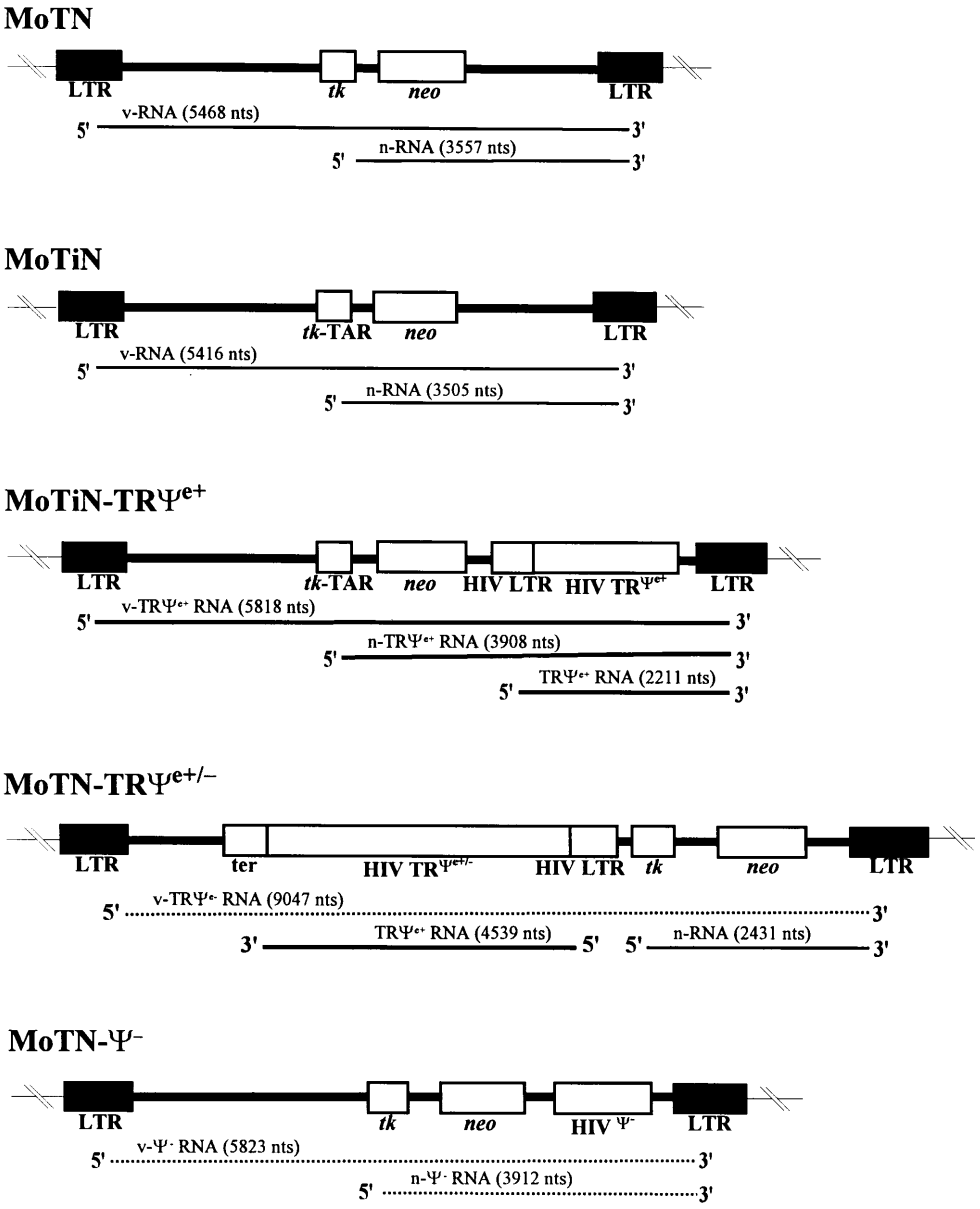

Figure 1. Schematic diagrams of MoTN, MoTiN, MoTiN-TRΨ^{e+}, MoTN-TRΨ$^{e+/-}$ and MoTN-Ψ^{-} vectors and transcripts produced. MoMuLV 5′-LTR, HIV-1 LTR, HSV *tk* and *tk*–TAR fusion promoter-driven transcripts are shown by the thin line. RNAs containing HIV-1 RNA sequences in sense or antisense orientation are shown by thick and dotted lines, respectively.

RESULTS

Retroviral vectors expressing the HIV-1 TAR-RRE-Ψ^{e} (TRΨ^{e}) sequence in sense or in sense and antisense orientations

All retroviral vector constructs examined here are based on the MoMuLV-derived MoTN or MoTiN vectors. The MoTN vector (43) contains the MoMuLV 5′-LTR and herpes simplex virus thymidine kinase (*tk*) promoters that allow constitutive transcription of vector (v-) and neomycin phosphotransferase (*neo*, n-) RNAs, respectively (Fig. 1). The MoTiN vector was previously constructed (44) by modifying the MoTN vector to contain a *tk*–TAR fusion promoter to allow low level constitutive gene expression with upregulation upon Tat-induction.

A cassette containing the HIV-1 LTR promoter driving expression of TRΨ^{e} (consisting of the HIV-1 5′ leader sequence, part of *gag* coding region and a 1.1 kb fragment of *env*-coding region which includes the RRE) was cloned into the MoTiN vector downstream of the *neo* gene in the sense orientation. In the resulting MoTiN-TR-Ψ^{e+} vector, v-TRΨ^{e+} RNA is produced constitutively from the MoMuLV 5′-LTR promoter, n-TRΨ^{e+} RNA is produced in a constitutive low level but Tat-inducible manner from the *tk*–TAR fusion promoter, and TRΨ^{e+} RNA is produced in a Tat-inducible manner from HIV-1 LTR promoter (Fig. 1).

The MoTN-TRΨ$^{e+/-}$ vector was constructed to allow constitutive expression of antisense RNA and Tat-inducible expression of sense RNA. For construction of this vector, a cassette containing the HIV-1 LTR promoter expressing the HIV-1 5′ leader sequence, the *gag* and *env* coding regions, and a terminator was cloned

downstream of the MoMuLV 5′-LTR (Fig. 1). This cassette was cloned in the antisense orientation such that the MoMuLV 5′-LTR promoter will transcribe constitutively v-TRΨ^{e-} RNA containing TRΨ^{e} sequences in the antisense orientation. Sense TRΨ^{e+} RNA will be produced in a Tat-inducible manner from the HIV-1 LTR promoter. Since the HIV-1 LTR-TRΨ^{e} sequences were not cloned downstream of the *neo* gene, the MoTiN vector containing the *tk*-TAR fusion promoter was not needed.

The MoTN-Ψ^{-} vector was previously constructed (28) by cloning HIV-1 sequences (that include the Ψ signal near the 5′ splice donor until the beginning of *pol* overlap in the *gag* coding region) in the MoTN vector downstream of the *neo* gene in the antisense orientation. This vector should allow constitutive production of v-Ψ^{-} RNA from the MoMuLV 5′-LTR promoter and n-Ψ^{-} RNA from the HSV *tk* promoter.

Establishment of pools of stable transductants of human CD4+ T-lymphoid (MT4) cells

Amphotropic MoTN, MoTiN, MoTiN-TRΨ^{e+} and MoTN-TRΨ$^{e+/-}$ vector particles were generated and were used to transduce a human CD4+ T-lymphoid (MT4) cell line. Stable MT4 transductants were selected and tested without cloning. RT-PCR analysis of total cellular RNA using various primer pairs (Table 1) was performed to detect vector RNA sequences upstream and downstream of the *neo* coding region, *neo* coding sequence and the HIV-1 Ψ signal sequence. Products of the expected sizes were detected in each case, confirming v-TRΨ^{e+} RNA production from the MoTiN-TRΨ^{e+} vector, and v-TRΨ^{e-} RNA production from the MoTN-TRΨ$^{e+/-}$ vector (results not shown; see later RT-PCR results from HIV-1 infected cells). Production of n-TRΨ^{e+} RNA from MoTiN-TRΨ^{e+} vector and of n-RNA from MoTN-TRΨ$^{e+/-}$ vector was indirectly demonstrated, as cells were resistant to G418. Production of TRΨ^{e+} RNAs from both MoTiN-TRΨ^{e+} and MoTN-TRΨ$^{e+/-}$ vectors was not expected to take place in the absence of HIV-1 Tat protein and was not observed in MoTN-TRΨ$^{e+/-}$ vector-transduced cells (results not shown). Transduced MT4 cells were analyzed by flow cytometry using anti-CD4 monoclonal antibodies and, as expected, over 90% of the cells were CD4 positive.

HIV-1 susceptibility of stable MT4 transductants

Each pool of stable MT4 transductants was challenged with the HIV-1 strain, NL4-3 (Fig. 2). The MoTiN control vector-transduced cells produced 1009.8 ng/ml of HIV-1 p24 by day 24, after which virus production decreased due to cell death. The MoTiN-TRΨ^{e+} vector-transduced MT4 cells displayed no noticeable HIV-1 p24 production until day 21 post-infection. From then onwards, p24 levels increased gradually to 53.7 ng/ml by day 54 and then remained below this value until day 78 (Fig. 2a).

The MoTN vector-transduced cells allowed 855.5 ng/ml p24 production by day 30. Virus production then decreased due to cell death. Cells transduced with the MoTN-TRΨ$^{e+/-}$ vector demonstrated very strong inhibition, with no detectable p24 until day 21 post-infection. HIV-1 p24 levels then gradually increased to 2.4 ng/ml until day 48 and then remained below this value until day 78 (Fig. 2b).

Packaging of sense and antisense RNAs within virus progeny

Upon HIV-1 challenge of stable MT4 transductants, sense and antisense RNA production and packaging was assessed by

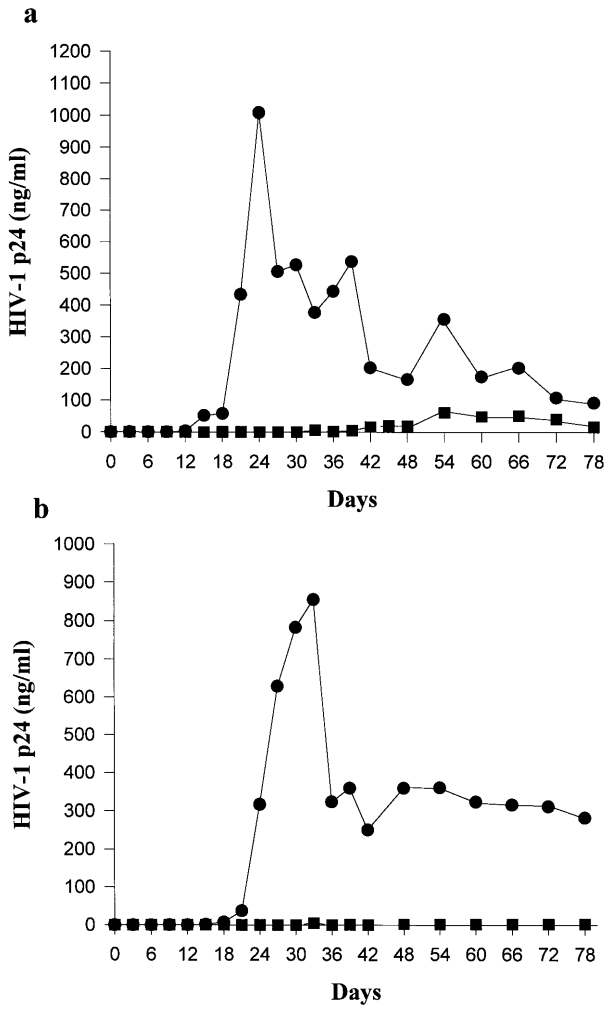

Figure 2. HIV-1 susceptibility of stable MT4 transductants. The level of HIV-1 p24 produced in the culture supernatants was determined at various time intervals following HIV-1 challenge of (**a**) MoTiN (●) and MoTiN-TRΨ^{e+} (■) or (**b**) MoTN (●) and MoTN-TRΨ$^{e+/-}$ (■) vector-transduced MT4 cells.

RT-PCR analysis of RNA extracted from infected cells and the virus progeny (Tables 1 and 2). All HIV-1 infected MT4 transductants were shown (Figs 3a and 4a) to produce HIV-1 RNA (Vpu-5′/Vpr-3′ primer pair) and the respective retroviral vector RNAs (Vu-5′/Vu-3′ primer pair for the vector sequences upstream of the *neo* coding region, Neo-5′/Neo-3′ primer pair for *neo* coding region and Vd-5′/Vd-3′ primer pair for vector sequences downstream of *neo* coding region). The TRΨ$^{e+/-}$ RNA produced from the MoTN-TRΨ$^{e+/-}$ vector was shown not to be spliced as no product corresponding to spliced RNA amplification was detected using the TAR-5′/SD-3′ primer pair (results not shown). RT-PCR analysis of virion RNAs revealed that v-TRΨ^{e+} RNA produced from the MoTiN-TRΨ^{e+} vector (Fig. 3b) and of v-TRΨ^{e-} RNA produced from MoTN-TRΨ$^{e+/-}$ vector were packaged (Fig. 4b). Packaging of n-TRΨ^{e+} and TRΨ^{e+} RNAs produced from the MoTiN-TRΨ^{e+} vector could not be demonstrated by RT-PCR. The TRΨ^{e+} RNA produced from the MoTN-TRΨ$^{e+/-}$ vector was shown not to be packaged (Fig. 4b). As expected, no v- or n-RNA was packaged from HIV-1 infected cells transduced with MoTN or MoTiN control vectors (Figs 3b and 4b). RT-PCR analysis of virion RNAs did not detect GAPDH RNA, confirming

Table 2. RT-PCR results from cellular and virion RNAs from HIV-1 infected stable MT4 transductants

Primer pairs	Cellular RNA				Virion RNA			
	MoTiN	MoTiN-TRΨ^{e+}	MoTN	MoTN-TRΨ$^{e+/-}$	MoTiN	MoTiN-TRΨ^{e+}	MoTN	MoTN-TRΨ$^{e+/-}$
Gap-5' Gap-3'	+	+	+	+	-	-	-	-
Vu-5' Vu-3'	+	+	+	+	-	+	-	+
Neo-5' Neo-3'	+	+	+	+	-	+	-	+
Vd-5' Vd-3'	+	+	+	+	-	+	-	+
Gag-5' I-3'	NA	NA	-/-[a]	+/+[a]	NA	NA	-/-[a]	+/-[a]
Vpr-5' Vpu-3'	+	+	+	+	+	+	+	+

[a]Results obtained using either Gag-5' or I-3' primer during reverse transcription.

[b]+, When Gag-5' was used during reverse transcription; -, when I-3' was used during reverse transcription.

NA, Not applicable.

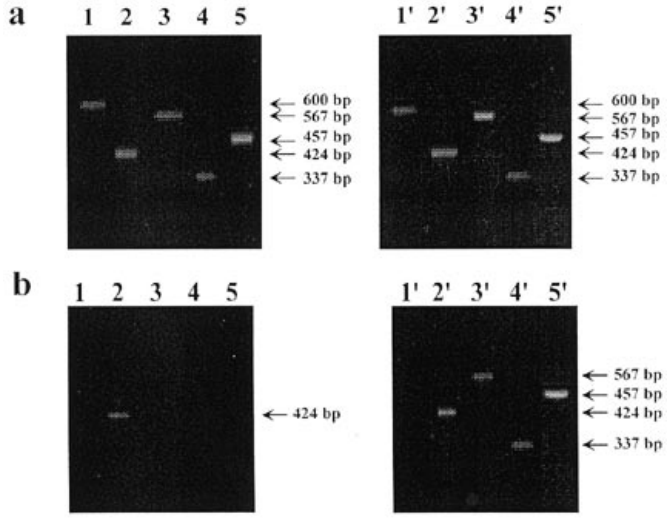

Figure 3. RT-PCR analysis of cellular (**a**) and virion (**b**) RNAs from HIV-1 infected MT4 cells transduced with MoTiN (lanes 1–5) and MoTiN-TRΨ^{e+} (lanes 1′–5′) vectors. Primer pairs used: lanes 1 and 1′, Gap-5′/Gap-3′; lanes 2 and 2′, HIV-1 Vpu-5′/Vpr-3′; lanes 3 and 3′, Vu-5′/Vu-3′; lanes 4 and 4′, Neo-5′/Neo-3′; lanes 5 and 5′, Vd-5′/Vd-3′.

the lack of cellular RNA contamination (Figs 3b and 4b). Antisense RNA packaging was also confirmed by RT-PCR analysis of RNA from virus progeny of HIV-1 infected stable MT4 transductants with the previously constructed vector, MoTN-Ψ^{-} (Fig. 5).

Replication-competence of virus progeny

Equal amounts of virus progeny from HIV-1 infected pools of MT4 cells transduced with MoTiN and MoTiN-TRΨ^{e+} vectors (9.6 ng of p24) or MoTN and MoTN-TRΨ$^{e+/-}$ vectors (1.2 ng of p24) were used to infect untransduced MT4 cells. Using the HIV-1 Vpu-5′/Vpr-3′ primer pair, RT-PCRs on cellular RNA extracted from the MT4 cells infected with virus progeny from either MoTN or MoTiN vector-transduced MT4 cells, gave positive results by day 21. RT-PCR analysis of cellular RNA from MT4 cells infected with virus progeny from HIV-1 infected MoTiN-TRΨ^{e+} and MoTN-TRΨ$^{e+/-}$ vector-transduced MT4 cells gave negative results at all time points tested until the end of the experiment (day 48; Fig. 6). RT-PCRs with Gap-5′/Gap-3′ primers were positive for all samples. HIV-1 p24 antigen detection by ELISA on days 36 and 48 post-infection also gave negative results for samples obtained from MT4 cells infected with virus progeny from HIV-1 infected MoTiN-TRΨ^{e+} and MoTN-TRΨ$^{e+/-}$ vectors, although results from virus progeny obtained from the MoTiN and MoTN vector-transduced cells were positive. PCR analysis of genomic DNA on day 36 with the Vd-5′/Vd-3′ primer pair gave negative results for all samples (results not shown), suggesting lack of vector transduction by the virus progeny.

HIV-1 susceptibility of human PBL transductants

Human PBLs were transduced with amphotropic MoTN, MoTN-TRΨ$^{e+/-}$ and MoTN-Ψ^{-} vector particles. The pools of PBL transductants were then challenged with 30 ng (p24 equivalent) of HIV-1 strain NL4-3. The amount of HIV-1 p24 antigen released in the cell culture supernatants was measured over time. Virus production was inhibited in both MoTN-TRΨ$^{e+/-}$ and MoTN-Ψ^{-} vector transduced PBLs, compared with PBLs transduced with the MoTN vector (Fig. 7). These results further demonstrate the efficacy of antisense RNA-mediated inhibition of virus replication in human PBLs.

DISCUSSION

HIV-1 TAR and RRE decoy RNAs inhibit HIV-1 replication by providing competitive protein binding sites and/or by sequestering key cellular factors (reviewed in 2–4). In addition, RNAs

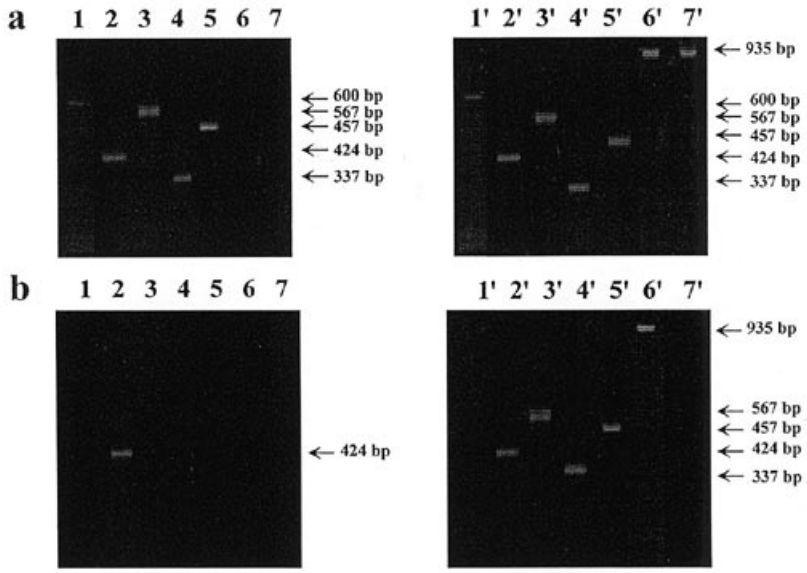

Figure 4. RT-PCR analysis of cellular (**a**) and virion (**b**) RNAs from HIV-1 infected MT4 cells transduced with MoTN (lanes 1–7) and MoTN-TR$\Psi^{e+/-}$ (lanes 1′–7′) vectors. Primer pairs used: lanes 1 and 1′, Gap-5′/Gap-3′; lanes 2 and 2′, HIV-1 Vpu-5′/Vpr-3′; lanes 3 and 3′, Vu-5′/Vu-3′; lanes 4 and 4′, Neo-5′/Neo-3′; lanes 5 and 5′, Vd-5′/Vd-3′; lanes 6 and 6′, Gag-5′/I-3′, when Gag-5′ was used during reverse transcription; lanes 7 and 7′, Gag-5′/I-3′, when I-3′ was used during reverse transcription.

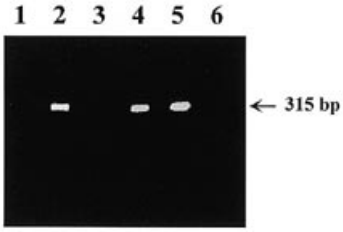

Figure 5. RT-PCR analysis of virion RNA from HIV-1 infected MT4 cells transduced with MoTN (lanes 1–3) or MoTN-Ψ^{-} (lanes 4–6) vectors using the Psi-5′/Psi-3′ primer pair. Lanes 1 and 4, PCR following cDNA synthesis with Psi-5′ primer; lanes 2 and 5, PCR following cDNA synthesis with Psi-3′ primer; lanes 3 and 6, PCR following cDNA synthesis without any primer.

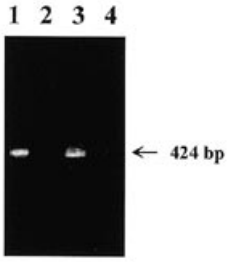

Figure 6. RT-PCR on day 48 post-infection of MT4 cells to detect the replication-competence of the virus progeny from HIV-1 infected stable MT4 transductants using primer pair HIV Vpu-5′/Vpr-3′. Lane 1, MT4 cells infected with virus progeny from MoTiN transductants; lane 2, from MoTiN-TRΨ^{e+} transductants; lane 3, from MoTN transductants; lane 4, from MoTN-TR$\Psi^{e+/-}$ transductants.

containing the HIV-1 Ψ^{e} signal sequence in the sense orientation may be used to inhibit HIV replication at the level of RNA packaging. Co-packaging with MoMuLV vector RNA of MoMuLV Ψ signal-containing RNAs was recently shown to result in the production of replication-defective vector particles (52).

We have previously demonstrated (28) significant inhibition of HIV-1 replication with the MoTN-Ψ^{-} vector that constitutively produced RNAs containing a 1.43 kb region antisense to the HIV-1 Ψ signal and *gag* coding sequences. Production of RNA containing this region in the sense orientation by MoTN-Ψ^{+} vector did not confer protection (28). This may have resulted from the lack of interference at the level of HIV-1 RNA packaging. In the present study, we improved the MoTN-Ψ^{+} vector with a 5′ extension of the HIV-1 Ψ signal until the +1 nucleotide of HIV RNA to include the TAR element, along with the Ψ signal and the DLS. We further added downstream a 1.1 kb fragment of the *env*-coding region that includes the RRE. Both Ψ and *env* sequences are important for efficient HIV-1 RNA packaging (33,36). Since lack of inhibition by MoTN-Ψ^{+} vector may also result from insufficient transcription, interfering RNA production was improved by adding an HIV-1 LTR and an internal *tk*–TAR fusion promoter (44). The new vector, MoTiN-TRΨ^{e+}, was thus designed to produce the HIV-1 TRΨ^{e+} sequence in abundance with three distinct RNA species transcribed from the MoMuLV 5′-LTR, *tk*–TAR and HIV-1 LTR promoters (Fig. 1). HIV-1 challenge of MoTiN-TRΨ^{e+} vector-transduced MT4 cells produced significantly less p24 relative to controls (Fig. 2a). Analysis of the released virus progeny by RT-PCR using Vu-5′/Vu-3′, Neo-5′/Neo-3′, and Vd-5′/Vd-3′ primer pairs showed that v-TRΨ^{e+} RNA is packaged (Fig. 3b). Whether n-TRΨ^{e+} and TRΨ^{e+} RNAs are also packaged could not be determined from these results. Hence the HIV-1 Ψ^{e+} sequence, irrespective of its location within the RNAs, allows efficient packaging of heterologous RNAs even in the presence of HIV-1 RNA. As expected, MoTiN vector-derived RNAs were not packaged. The lack of GAPDH RNA in virion RNA preparations confirmed the absence of any contamination with cellular RNAs.

Inhibition of HIV-1 replication by the interfering RNAs produced from the MoTiN-TRΨ^{e+} vector is likely to result from the Ψ^{e} signal, which acts by competing with HIV-1 RNA for binding to the $Pr55^{Gag}$ and the TAR and RRE which act by competing with HIV-1 RNA for binding to HIV-1 Tat and Rev proteins. Thus, inhibition at the level of encapsidation, *trans*-activation and HIV RNA export will take place. Previous studies have shown that RNAs containing

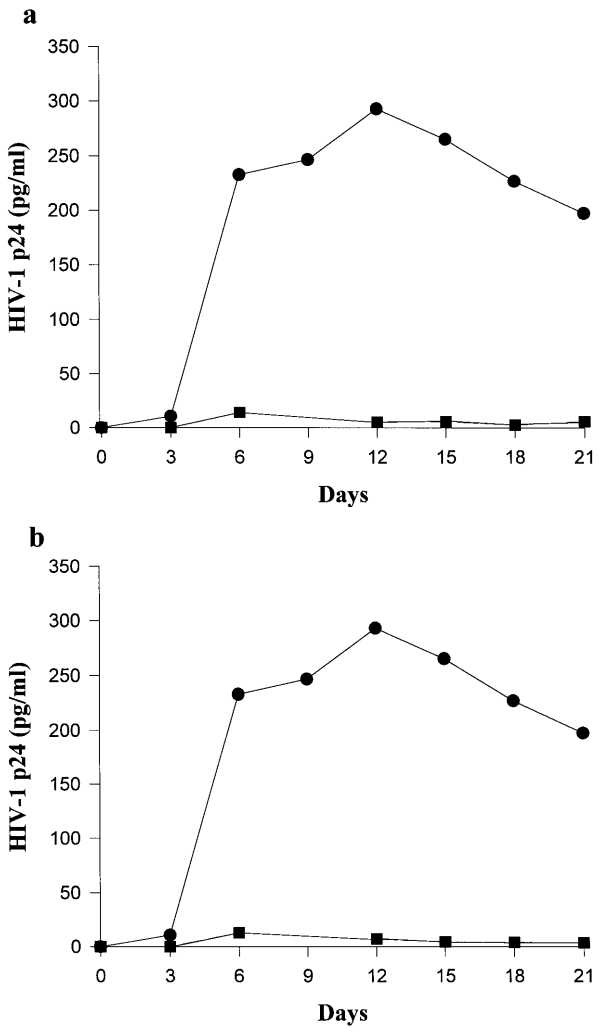

Figure 7. HIV-1 susceptibility of human PBL transductants. HIV-1 p24 antigen production was measured at various time intervals following HIV-1 challenge of (**a**) MoTN (●) and MoTN-TRΨ$^{e+/-}$ (■) or (**b**) MoTN (●) and MoTN-Ψ^{-} (■) vector-transduced human PBLs.

a single TAR and a single RRE inhibited HIV-1 replication by only a few days (28). Thus, the observed inhibition is expected to result mainly from interference at the level of viral RNA packaging. The RRE may also improve cellular trafficking of interfering RNAs and thus enhance co-packaging of the interfering RNA. The three types of RNAs produced from the MoTiN-TRΨ^{e+} vector contain HIV-1 R-U5 and PBS sequences either at an internal location or at the 5′ end. However, none of these RNAs contains the HIV-1 polypurine tract (PPT) and U3-R sequences that are required for provirus DNA synthesis. Co-packaging within HIV-1 particles of non-HIV RNAs lacking these sequences may result in abortive reverse transcription and prevent subsequent infection. Thus, if co-packaging of interfering RNAs was to render HIV non-infectious, subsequent rounds of infection should have been inhibited. This may well be the case, as virus production which is likely to result from multiple rounds of infection in MoTiN-TRΨ^{e+} vector-transduced cells was significantly reduced and delayed (Fig. 2a).

Infectivity of virus progeny (at fixed p24 concentrations) from HIV-1 infected MoTiN and MoTiN-TRΨ^{e+} vector-transduced MT4 cells was tested by infecting untransduced MT4 cells. HIV RNA could be detected in MT4 cells infected with virus progeny from MoTiN control vector-transduced cells. However, no viral RNA (Fig. 6) or p24 could be detected in MT4 cells infected within virus progeny from MoTiN-TRΨ^{e+} vector-transduced cells. Samples tested negative even after 48 days post-infection. A decrease in viral infectivity suggests that co-packaging of the sense RNA with HIV RNA took place. It is reasonable to suggest that RNA heterodimers consisting of an interfering RNA molecule and an HIV RNA molecule were packaged since empty particles or packaging of homodimers would not be expected to interfere with HIV-1 infectivity.

The properties of both MoTN-Ψ^{-} (28) and MoTiN-TRΨ^{e+} vectors were combined into the MoTN-TRΨ$^{e+/-}$ vector. The MoTN-TRΨ$^{e+/-}$ vector contained the HIV-1 5′ leader and the entire *gag* and *env* coding sequences, which include TAR, RRE and Ψ^{e+} sequences. This vector was designed to allow constitutive production of v-TRΨ^{e-} antisense RNA, and Tat-inducible production of TRΨ^{e+} sense RNA. Therefore, the antisense RNA should be present prior to HIV challenge and produced throughout HIV replication. Incomplete inhibition of HIV-1 Tat mRNA production by the complementary sequences present within the 5′ leader and *env* component of the antisense RNA may result in some Tat production which in turn would induce HIV-1 LTR driven expression of both sense and HIV-1 genomic RNAs. Sense RNA production from this vector would therefore depend on the level of antisense RNA-mediated inhibition of Tat protein production. HIV-1 challenge of MT4 cells transduced with MoTN and MoTN-TRΨ$^{e+/-}$ vectors demonstrated that virus production from MoTN-TRΨ$^{e+/-}$ vector-transduced cells was significantly inhibited (Fig. 2b). RT-PCR analysis revealed that only antisense v-TRΨ^{e-} RNA was packaged within the virus progeny (Fig. 4b). TRΨ^{e+} RNA produced from the MoTN-TRΨ$^{e+/-}$ vector could easily be detected amongst cellular RNAs (Fig. 4a) and was not found to be spliced (results not shown), but was not packaged (Fig. 4b). As expected, v- and n-RNAs produced from the MoTN vector were not packaged. Also the GAPDH RNA was not detected in the virus progeny, confirming the lack of contamination by the cellular RNA.

Compared with the infectivity of virus progeny from HIV-1 infected MoTN vector-transduced cells, the infectivity of virus progeny from HIV-1 infected MoTN-TRΨ$^{e+/-}$ vector-transduced cells was greatly reduced. This was demonstrated by the lack of HIV-1 RNA (Fig. 6) and virus p24 production until day 48 post-infection of MT4 cells lacking any interfering RNA. This decrease in virus progeny infectivity suggests antisense RNA co-packaging with HIV-1 RNA.

The TRΨ^{e+} RNA produced from MoTN-TRΨ$^{e+/-}$ vector contained the HIV-1 Ψ^{e} signal and therefore should have been packaged either by itself as a homodimer, or in association with HIV-1 RNA. Lack of 4.54 kb TRΨ^{e+} RNA packaging as homodimers cannot be explained because of the size restriction for the minimum length of the RNAs that may be packaged by HIV-1 Pr55^{Gag}, as HIV-1 based 4.3 kb vector RNAs produced from pHR′-CMV-Luc (53) can be packaged. The lack of packaging of TRΨ^{e+} RNA may also be due to its hybridization with excess v-TRΨ^{e-} RNA. Of the two TRΨ^{e+}/v-TRΨ^{e-} and HIV-1/v-TRΨ^{e-} sense/antisense RNA hybrids, only the latter was packaged into virus particles. Antisense v-TRΨ^{e-} RNA interaction is a perfect match over the entire length of TRΨ^{e+} RNA, which might prevent Ψ^{e} signal recognition by Pr55^{Gag}. On the other hand, the interaction between v-TRΨ^{e-} and HIV-1 RNA is

non-contiguous and therefore may form the required secondary structure needed for packaging.

Evaluation of the RNA composition of virus progeny from the HIV-1 infected MoTN-Ψ^- vector-transduced MT4 cells revealed that antisense RNAs containing HIV-1 Ψ and *gag* sequences were also packaged (Fig. 5). This result confirms that the antisense RNAs can indeed be packaged by HIV-1.

Both antisense RNA expression vectors, MoTN-TR$\Psi^{e+/-}$ and MoTN-Ψ^-, were also tested and shown to inhibit HIV-1 replication in human PBLs (Fig. 7).

This is the first time that packaging of antisense RNA has been shown to occur. This raises many interesting questions such as whether antisense RNA would be packaged as a heterodimer with HIV-1 RNA, in association with an HIV-1 RNA dimer (as a trimer), or as an antisense RNA dimer. The mechanism of antisense RNA packaging is yet to be determined. Antisense RNA used in this study may inhibit HIV-1 replication at the level of HIV-1 RNA export, translation and packaging. In addition, the co-packaged antisense RNA is likely to limit viral spread by inhibiting subsequent rounds of infection. Inhibition may also occur at the level of reverse transcription during subsequent rounds of replication or as a result of recombination leading to virus inactivation.

In summary, we demonstrate here that interfering sense and antisense RNAs can be co-packaged with HIV-1 RNA. Co-packaging of these RNAs was shown to inhibit HIV-1 replication through the production of defective/non-infectious virus progeny. Both sense and antisense RNA based strategies may therefore be used to co-package other types of anti-HIV-1 RNAs (e.g. ribozymes; 54).

ACKNOWLEDGEMENTS

This work is supported by grants from the Medical Research Council of Canada and National Health and Research Development Program of Canada. We thank R.A.Lombardi for pBS-GE vector construction and A.Jain for helpful assistance during preparation of this manuscript. LRPL vector was obtained from Dr A.M.L.Lever. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 (NL4-3 strain) produced from A3.01 cells from Dr R.C.Gallo and CD4+ lymphocyte-derived MT4 cell line from Dr D.Richman. Flow cytometry was performed at the Hospital for Sick Children's flow cytometry facility.

REFERENCES

1 Bridges,S.H. and Sarver,N. (1995) *The Lancet*, **345**, 427–432.
2 Medina,M.F., Ramezani,A. and Joshi,S. (1996) *Transfusion Science*, **17**, 109–120.
3 Poeschla,E.M. and Wong-Staal,F. (1995) *AIDS Clin. Rev.*, 1–45.
4 Smith,C. and Sullenger,B.A. (1995) In Dickson,G. (ed.), *Molecular and Cell Biology of Human Gene Therapeutics*. Chapman and Hall, Vol. 5, pp. 195–236.
5 Zacharias,M. and Hagerman,P.J. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 6052–6056.
6 Verhoef,K., Tijms,M. and Berkhout,B. (1997) *Nucleic Acids Res.*, **25**, 496–502.
7 Parada,C.A. and Roeder,R.J. (1996) *Nature*, **384**, 375–378.
8 Okamoto,H., Sheline,C.T., Corden,J.L., Jones,K.A. and Peterlin,B.M. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 11575–11579.
9 Green,M., Ishimo,M. and Loewenstein,P.M. (1997) *Cell*, **58**, 215–223.
10 Caputo,A., Grossi,M.P., Bozzini,R., Rossi,C., Betti,M., Marconi,P.C., Barbanti-Brodano,G. and Balboni,P.G. (1996) *Gene Ther.*, **3**, 235–245.
11 Sullenger,B.A., Gallardo,H.F., Ungers,G.E. and Gilboa,E. (1990) *Cell*, **63**, 601–608.
12 Lisziewicz,J., Rappaport,J. and Dhar,R. (1991) *The New Biol.*, **3**, 82–89.
13 Lisziewicz,J., Sun,D., Trapnell,B., Thomson,M., Chang,H.K., Ensoli,B. and Peng,B. (1995) *J. Virol.*, **69**, 206–212.
14 Lee,S.W., Gallardo,H.F., Gaspar,O., Smith,C. and Gilboa,E. (1995) *Gene Ther.*, **2**, 377–384.
15 Sczakiel,G., Oppenlander,M., Rittner,K. and Pawlita,M. (1992) *J. Virol.*, **66**, 5576–5581.
16 Biasolo,M.A., Radaelli,A., Del Pup,L., Franchin,E., De Giuli-Morghen,C. and Palu,G. (1995) *J. Virol.*, **70**, 2154–2161.
17 Peng,H., Callison,D., Li,P. and Burrell,C. (1996) *Virology*, **220**, 377–389.
18 Sakai,H., Furuta,R.A., Tokunaga,K., Kawamura,M., Hatanaka,M. and Adachi,A. (1995) *FEBS Lett.*, **365**, 141–145.
19 Dundr,M., Leno,G.H., Hammarskjold,M.L., Rekosh,D., Helga-Maria,C. and Olson,M.O. (1995) *J. Cell. Sci.*, **108**, 2811–2823.
20 Mann,D.A., Mikaelian,I., Zemmel,R.W., Green,S.M., Lowe,A.D., Kimura,T., Singh,M., Butler,P.J., Gait,M.J. and Karn,J. (1994) *J. Mol. Biol.*, **241**, 193–207.
21 Brighty,D.W. and Rosenberg,M. (1994) *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 8314–8318.
22 Nasioulas,G., Zolotukhin,A.S., Tabernero,C., Solomin,L., Cunningham,C.P., Pavlakis,G.N. and Felber,B.K. (1994) *J. Virol.*, **68**, 2986–2993.
23 Malim,M.H., Bohnlein,S., Hauber,J. and Cullen,B.R. (1989) *Cell*, **58**, 205–214.
24 Malim,M.H., Freimuth,W.W., Liu,J., Boyle,T.J., Lyerly,H.K., Cullen,B.R. and Nabel,G.J. (1992) *J. Exp. Med.*, **176**, 1197–1201.
25 Liem,S.E., Ramezani,A., Li,X. and Joshi,S. (1993) *Hum. Gene Ther.*, **4**, 625–634.
26 Bahner,I., Zhou,C., Yu,X.J., Guatelli,J.C. and Kohn,D.B. (1993) *J. Virol.*, **67**, 3199–3207.
27 Lee,T.C., Sullenger,B.A., Gallardo,H.F., Ungers,G.E. and Gilboa,E. (1992) *The New Biol.*, **4**, 66–74.
28 Cohli,H., Fan,B., Joshi,R.L., Ramezani,A., Li,X. and Joshi,S. (1994) *Antisense Res. Dev.*, **4**, 19–29.
29 Bevec,D., Volc-Platzer,B., Zimmermann,K., Dobrovnik,M., Hauber,J., Veres,G. and Böhnlein,E. (1994) *Hum. Gene Ther.*, **5**, 193–201.
30 Lee,T., Gallardo,H.F., Gilboa,E. and Smith,C. (1994) *J. Virol.*, **68**, 8254–8264.
31 Bahner,I., Kearns,K., Hao,Q.L., Smogorzewska,E.M. and Kohn,D.B. (1996) *J. Virol.*, **70**, 4352–4360.
32 Aldovini,A. and Young,R.A. (1990) *J. Virol.*, **64**, 1920–1926.
33 Clever,J., Sasetti,C. and Parslow,T.G. (1995) *J. Virol.*, **69**, 2101–2109.
34 Lever,A., Gottlinger,H, Haseltine,W. and Sodrowski,J. (1989) *J. Virol.*, **63**, 4085–4087.
35 Hayashi,T., Shioda,T., Iwakura,Y. and Shibuta,H. (1992) *Virology*, **188**, 590–599.
36 Richardson,J.H., Child,L.A. and Lever.A.M. (1993) *J. Virol.*, **67**, 3997–4005.
37 Awang,G. and Sen,D. (1993) *Biochemistry*, **32**, 11453–11457.
38 Veres,G., Escaich,S., Baker,J., Barske,C., Kalfoglou,C., Ilves,H., Kaneshima,H. and Böhnlein,E. (1996) *J. Virol.*, **70**, 8792–8800.
39 Veres,G., Kalfoglou,C., Beck,M., Chang,P., Ilves,H. and Böhnlein,E. (1998) In Karlsson,S. and Humphries,K. (organizers) *Keystone Symposium on Molecular and Cellular Biology: Gene Therapy Strategies for Hematopoietic Cells*, p. 63.
40 Veres,G., Junker,U., Baker,J., Barske,C., Kalfoglou,C., Ilves,H., Escaich,S., Kaneshima,H. and Bohnlein,E. (1998) *J Virol.*, **72**,1894–1901.
41 Ramezani,A. and Joshi,S. (1996) *Antisense and Nucleic Acid Drug Dev.*, **6**, 229–235.
42 Mann,R., Mulligan,R.C. and Baltimore,D. (1983) *Cell*, **33**, 153–159.
43 Magli,M.C., Dick,J.E., Huszar,D., Berstein,A. and Phillips,R.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 789–793.
44 Joshi,S., Davison,L., Van Brunschot,A. and Van der Elst,I. (1993) *Mol. Biol. Life Sci. Adv.*, **12**, 135–189.
45 Joshi,S., Van Brunschot,A., Robson,I. and Bernstein,A. (1990) *Nucleic Acids Res.*, **18**, 4223–4226.
46 Miller,A.D. and Buttimore,C. (1986) *Mol. Cell. Biol.*, **6**, 2895–2902.
47 Pauwels,R., De Clercq,E., Desmyter,J., Balzarini,J., Goudau,P., Herdewijn,P., Van der Haeghe,H. and Van de Putte,M. (1987) *J. Virol. Meth.*, **16**, 171–185.
48 Larder,B.A., Darby,G. and Richman,D.D. (1989) *Science*, **243**, 1731–1734.
49 Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
50 Ramezani,A., Ding,S.F. and Joshi,S. (1997) *Gene Ther.*, **4**, 861–867.
51 Adachi,A., Gendelman,H.E., Koenig,S., Solks,T., Willey,R., Rabson,A. and Martin,M.A. (1986) *J. Virol.*, **59**, 284–291.
52 Joshi,S., Ding,S.F. and Liem,S.E. (1997) *Nucleic Acids Res.*, **25**, 3199–3203.
53 Naldini,L., Blomer,U., Gallay,P., Ory,D., Mulligan,R., Gage,F.H., Verma,I.M. and Trono,D. (1996) *Science*, **272**, 263–267.
54 Sullenger,B.A. and Cech,T.R. (1993) *Science*, **262**, 1566–1569