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Co-packaging of non-vector RNAs generates replication-defective retroviral vector particles: a novel approach for blocking retrovirus replication

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ABSTRACT

A Moloney murine leukemia virus (MoMuLV)-derived packaging retroviral vector, pUCMoTN-PR3, was previously developed in which the packaging (ψ) signal was cloned within the 5′-long terminal repeat (LTR) U3-r and U5 sequences. The MoTN-PR3 vector particles released from a transfected packaging cell line contain RNAs with r-ψ-U5 sequences at the 5′-end and U3-r sequences at the 3′-end. Upon infection, these vector particles can efficiently transduce the neomycin phosphotransferase (*neo*) gene to the target cells. The structure of the proviral DNA synthesized in these cells was shown to contain modified 5′- and 3′-LTRs with U3-r-ψ-U5 sequences, indicating that this vector can undergo reverse transcription and integration. Analysis of ψ signal-containing RNAs revealed that in addition to vector RNA transcribed from the MoMuLV 5′-LTR promoter, readthrough *neo* RNA transcribed from the internal herpes simplex virus (HSV) thymidine kinase (*tk*) promoter and cellular RNAs transcribed from the MoMuLV 3′-LTR promoter are produced. Of these, the downstream cellular RNAs are also packaged within the vector particles. These vector particles containing the vector and non-vector RNAs carrying the MoMuLV ψ signal are non-infectious. It is proposed that intracellular expression of packageable non-viral RNAs may represent an effective strategy for inhibiting animal and plant virus replication.

INTRODUCTION

MoMuLV-based retroviral vector pUCMoTN (1) contains 5′- and 3′-long terminal repeats (LTRs) made up of U3-r-U5 sequences. Vector RNA is transcribed from the 5′-LTR promoter and contains r-U5 sequences at the 5′-end and U3-r sequences at the 3′-end. These sequences are required for reverse transcription. This vector also encodes for *neo* mRNA from the internal HSV *tk* promoter. In addition, downstream cellular RNAs may be transcribed from the 3′-LTR promoter (Fig. 1a). A ψ signal sequence is responsible for packaging RNA into the virions. A dimer linkage structure within the ψ signal mediates dimerization and co-packaging of two copies of full-length vector RNA molecules. Therefore, with the inclusion of the ψ signal, *neo* and cellular RNAs may also be packaged within the vector particles and impede reverse transcription of co-packaged vector RNA.

We have previously developed a packaging retroviral vector pUCMoTN-PR3 in which nt 211–1039 (forming the MoMuLV ψ signal) have been deleted from their original location and cloned between U3-r and U5 sequences within the 5′-LTR (2). In this study we investigate whether MoTN-PR3 vector particles can reverse transcribe and integrate to result in provirus DNA with the ψ signal duplicated in both the 5′- and 3′-LTRs and whether the HSV *tk* promoter and the proviral 3′-LTR promoter can give rise to transcripts that contain the ψ signal. We further investigate whether these non-vector RNAs can be packaged within the retroviral vector particles and, if so, whether these particles containing chimeric RNAs can efficiently replicate.

MATERIALS AND METHODS

Retroviral vectors

The packaging retroviral vector pUCMoTN-PR3 was constructed as described previously (3). The parent vector pUCMoTN (1) served as a control.

Cell lines

NIH 3T3, Psi-2 (4) and PA317 (5) cell lines were cultured in α-MEM medium supplemented with 2 mM L-glutamine, antibiotic–antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin; Gibco) and 10% fetal bovine serum (Hyclone) at 37°C in a humidified atmosphere with 5% CO_2. The CEM-10 cell line was cultured in a similar fashion, except that RPMI 1640 medium was used.

Transfection and infection of mammalian cell lines

Both transfection of the ecotropic Psi-2 packaging cell line using pUCMoTN and pUCMoTN-PR3 vector DNA and infection of the amphotropic PA317 packaging cell line using MoTN and MoTN-PR3 vector particles released from the stably transduced

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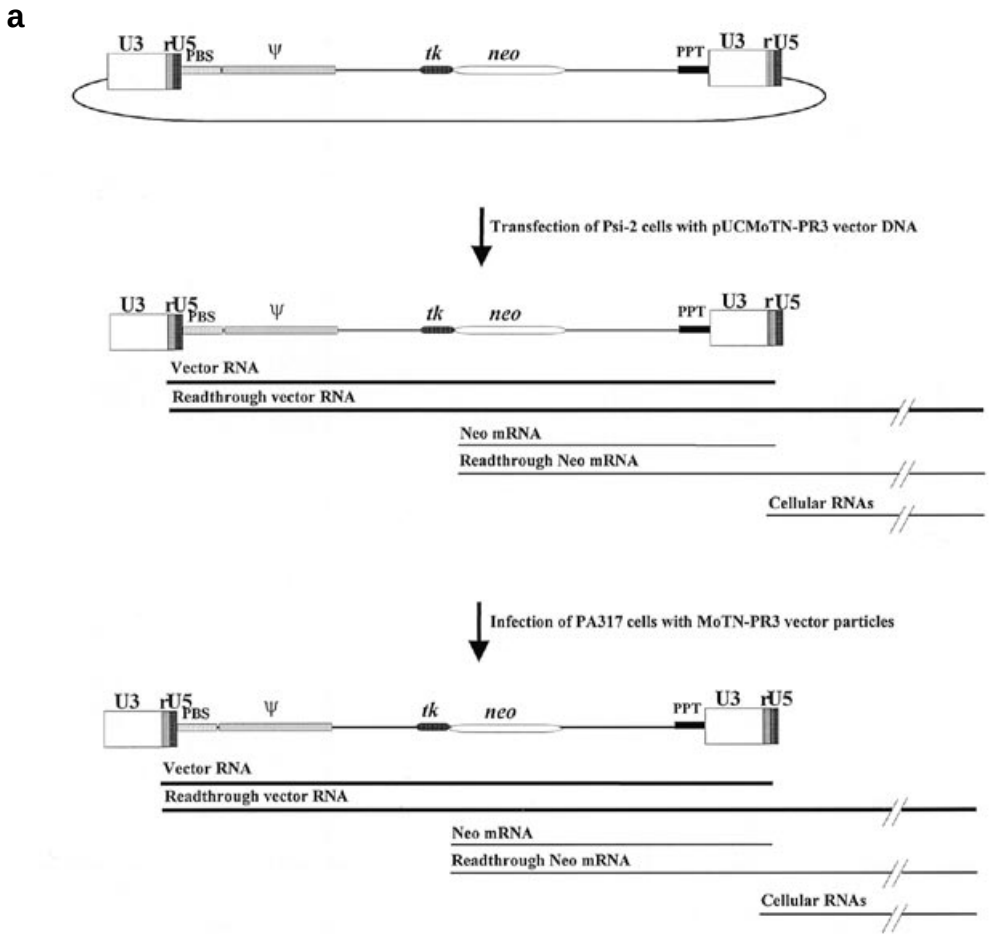

Psi-2 cells were carried out as described previously (3). Vector particles released from both transfected Psi-2 and infected PA317 cells were then analysed as described below.

Vector particle titre determination

The titre of vector particles released from Psi-2 and PA317 transductants (50–100% confluent) was determined (3) by counting the number of G418^R colonies obtained 2–3 weeks post-infection of NIH 3T3 cells. The titre of vector particles released from PA317 cells was also determined using CEM-10 cells.

Genomic DNA cloning and Southern blot analysis

Genomic DNA isolated from PA317 cells infected with MOTN–PR3 vector particles was digested with *Xho*I and cloned into the pUC18 vector at the compatible *Sal*I site. A kanamycin-resistant (Km^R) clone was selected. Cloned DNA was digested with *Sty*I and analysed by Southern blot analysis (6). The blots were probed with a ^{32}P-labelled 210 bp *Sty*I fragment of pUCMoTN containing the r-U5 sequence.

Polymerase chain reaction (PCR) analysis

Genomic DNA was isolated (6) from PA317 cells infected with MoTN and MoTN-PR3 vector particles. PCR was performed using the PPT-5′ (5′-CCT ATA GAG TAC GAG CC) and Psi-3′ (5′-ACA GAT AAG TTG CTG GC) primer pair as described previously (7).

Reverse transcription (RT)-PCR analysis

Total RNA isolation from PA317 cells infected with MoTN and MoTN-PR3 vector particles and from the Psi-2 cells transfected with pUCMoTN-PR3 vector DNA was carried out as described (8). Ten millilitre culture supernatants from 50–100% confluent cells were centrifuged in a Ti 70.1 rotor at 60 000 r.p.m. for 4 h to pellet MoTN and MoTN-PR3 vector particles, which were then used to extract RNA (8). RNA extracted from cells and vector particles was treated with DNase and used in RT-PCR as described previously (7) using Psi-3′ and Neo-3′ (5′-TCT TTC ATC CAG ATC ATC-3′) primers for reverse transcription and the PPT-5′/Psi-3′ and Neo-5′ (5′-CAA GAC CGA CCT GTC CGG 3′)/Neo-3′ primer pairs for PCR.

Northern blot analysis

Total cellular and vector particle RNAs (5 µg) were subjected to Northern blot analysis using a ^{32}P-labelled ψ signal-specific probe (5′-TAA ATC AGA CAT AGA CA) as described (6).

RESULTS AND DISCUSSION

The packaging retroviral vector pUCMoTN-PR3 (2) contains the MoMuLV ψ signal (nt 211–1039) inserted between U3-r and U5 sequences within the 5′-LTR. The modified 5′-LTR contains

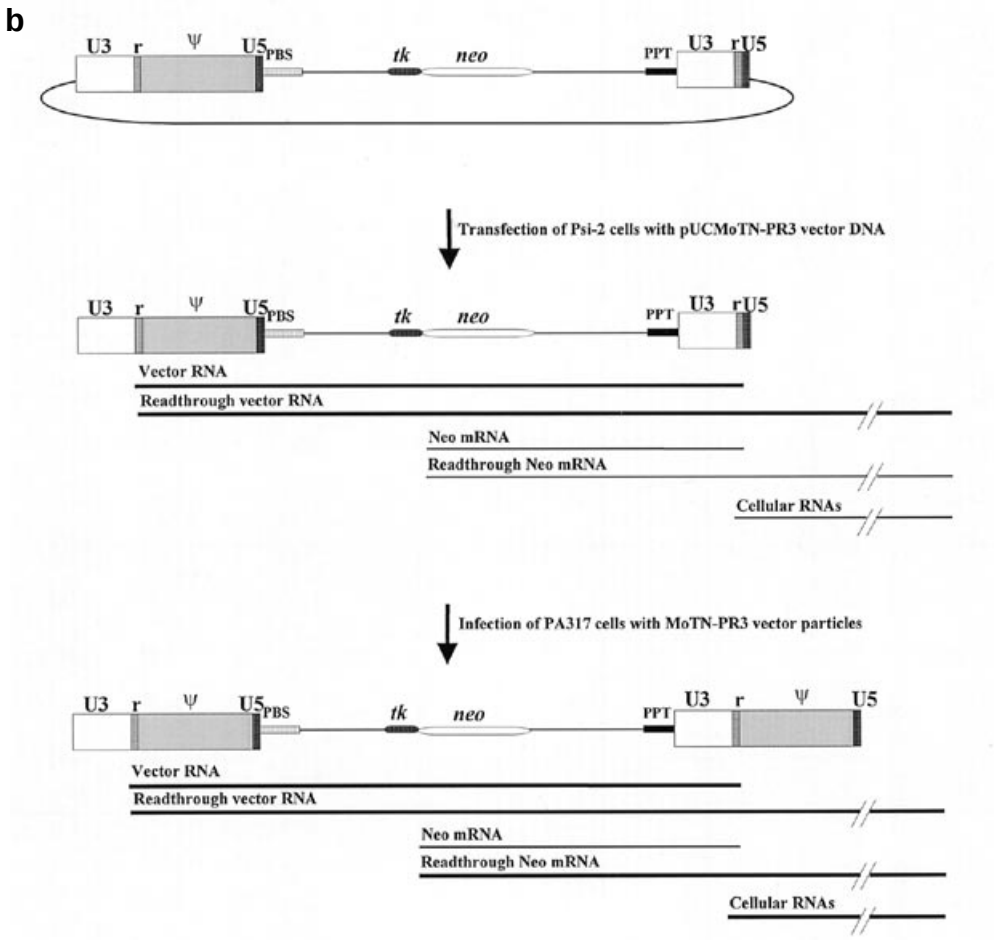

Figure 1. Structure of transcripts produced in packaging cell lines transfected/infected with MoTN (**a**) and MoTN-PR3 (**b**) vector particles. Transcripts containing the ψ signal are shown with a thick line.

U3-r-ψ-U5 sequences. Upon transfection of the Psi-2 (4) packaging cell line, expressing the MoMuLV *gag*, *pol* and *env* genes, the pUCMoTN-PR3 vector DNA should be transcribed to produce vector RNA from the 5′-LTR promoter and *neo* mRNA from the HSV *tk* promoter. These RNAs should terminate within the 3′-LTR at the end of the U3-r sequence. In addition, readthrough vector, readthrough *neo* and 3′-LTR promoter-driven cellular RNAs ending at the next available transcription termination site may also be generated (Fig. 1b). Of these, only the vector and the readthrough vector RNAs will contain the ψ signal and therefore be packaged into vector particles. Subsequent infection of another packaging cell line, PA317 (5), with these vector particles should result in reverse transcription (9), generating provirus DNA with the ψ signal duplicated within both the 5′- and 3′-LTRs. Transcripts produced from this proviral DNA that have one or duplicate copies of the ψ signal include vector RNA, readthrough vector RNA, readthrough *neo* RNA and 3′-LTR-driven downstream cellular RNAs. These vector and non-vector RNAs should be encapsidated into vector particles. Hence, it will be interesting to examine if the presence of chimeric non-vector RNAs interferes with packaging and/or reverse transcription.

Accuracy of reverse transcription and integration of packaging retroviral vectors

To this end, the vector particles released from transfected Psi-2 cells were used to infect PA317 cells. The structure of the 3′-LTR present within the proviral DNA produced as a result of reverse transcription and integration in these cells was then analysed as described above by Southern blot analysis of cloned 3′-LTR DNA sequences and by RT-PCR. Specific probe/primers were used to distinguish between 3′-LTRs containing U3-r-U5 versus U3-r-ψ-U5 sequences.

X*ho*I-digested genomic DNA from PA317 cells infected with MoTN-PR3 vector particles was cloned into the pUC18 vector at the compatible *Sal*I site. A Km^R clone, containing the *neo* gene and the 3′-LTR sequences of the proviral DNA and the downstream cellular DNA sequence up to the next available *Xho*I site, was selected. The structure of the 3′-LTR present in this clone was confirmed to be U3-r-ψ-U5 by Southern blot analysis of *Sty*I-digested DNA. *Sty*I cleaves within the r and the U5 sequences, but not within the ψ sequence (results not shown).

Genomic DNA from PA317 cells infected with MoTN and MoTN-PR3 vector particles was also analysed by PCR (Fig. 2a).

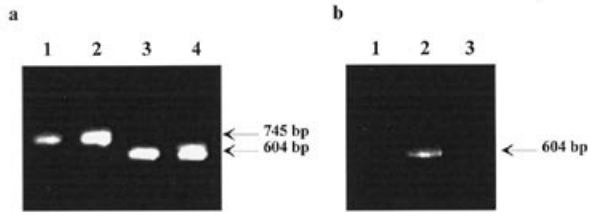

Figure 2. PCR and RT-PCR analysis to detect the presence of modified 3′-LTR sequences within the genome of PA317 cells infected with MoTN-PR3 vector particles. (**a**) PCR analysis of genomic DNA from PA317 cells infected with MoTN (lane 1) and MoTN-PR3 (lane 3) vector particles using the PPT-5′/Psi-3′ primer pair. As a control, PCR was also performed using pUCMoTN (lane 2) and pUCMoTN-PR3 (lane 4) vector DNA. (**b**) RT-PCR analysis of total cellular RNA from PA317 cells infected with MoTN (lane 1) and MoTN-PR3 (lane 2) vector particles and from Psi-2 cells transfected with pUCMoTN-PR3 vector DNA (lane 3). Reverse transcription was performed using the Psi-3′ primer followed by PCR using the PPT-5′/Psi-3′ primer pair.

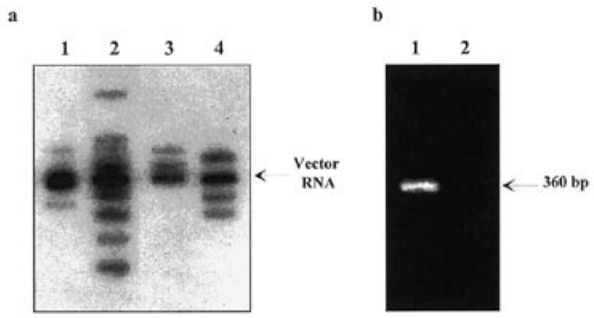

Figure 3. Northern blot and RT-PCR analysis of ψ signal-containing RNAs. (**a**) Northern blot analysis of RNA isolated from MoTN- (lanes 1 and 3) and MoTN-PR3-infected (lanes 2 and 4) PA317 cells (lanes 1 and 2) and released vector particles (lanes 3 and 4). A ^{32}P-labelled Psi probe was used to detect the presence ψ signal-containing RNAs. (**b**) RT-PCR analysis to characterize RNAs packaged in the vector particles released from MoTN-PR3-infected PA317 cells. Two sets of primers were used to detect RNAs containing the *neo* (lane 1) and the PPT-U3-r-ψ (lane 2) sequences.

The 5′ primer (PPT-5′) used in this PCR was designed against a sequence close to the polypurine tract (PPT) located upstream of the 3′-LTR. The 3′ primer (Psi-3′) was designed against a sequence present within the ψ signal. Thus, if the 3′-LTR was modified in MoTN-PR3-infected PA317 cells, a 604 bp product containing PPT-U3-r-ψ sequences would be amplified. A 604 bp PCR product was detected in samples analysed from PA317 cells infected with MoTN-PR3 vector particles (Fig. 2a, lane 3). However, a 604 bp product was also detected when pUCMoTN-PR3 vector DNA was PCR amplified (Fig. 2a, lane 4). Similarly, a 745 bp PCR product was detected in samples analysed from PA317 cells infected with MoTN vector particles (Fig. 2a, lane 1) as well as in samples analysed from pUCMoTN vector DNA (Fig. 2a, lane 2). No PCR product was expected in the last three samples. Careful analysis revealed that these products must have resulted from hybridization due to extensive sequence complementarity between the 5′ and the 3′ primer extention products followed by PCR amplification. Therefore, from this experiment alone it could not be concluded whether the 604 bp product detected in samples from MoTN-PR3-infected PA317 cells is due to PCR amplification of a modified 3′-LTR.

As readthrough RNAs containing PPT-U3-r-U5 sequences are likely to be synthesized in MoTN-PR3-infected PA317 cells but not in MoTN-infected PA317 cells or pUCMoTN-PR3 vector DNA-transfected Psi-2 cells, RNA produced in these cells was reverse transcribed using the Psi-3′ primer and PCR amplified using the PPT-5′/Psi-3′ primer pair (Fig. 2b). Since only the 3′ primer is used during cDNA synthesis, only sequences present on the cDNA can be amplified during PCR. A 604 bp product was detected only in samples analysed from PA317 cells infected with MoTN-PR3 vector particles (Fig. 2b, lane 2). No PCR product was detected in samples analysed from PA317 cells infected with MoTN vector particles (Fig. 2b, lane 1) or from Psi-2 cells transfected with pUCMoTN-PR3 vector DNA (Fig. 2b, lane 3).

These results demonstrate that packaging retroviral vector particles containing RNAs with a r-ψ-U5 sequence at the 5′-end and a U3-r sequence at the 3′-end can, upon reverse transcription in the infected target cells, give rise to modified 5′- and 3′-LTRs containing U3-r-ψ-U5 sequences.

Expression and co-packaging of ψ signal-containing non-vector RNAs

The composition of ψ signal-containing RNAs present in PA317 cells infected with MoTN and MoTN-PR3 vector particles and in vector particles released from these cells was determined by Northern blot analysis using a ^{32}P-labelled probe specific for the ψ signal (Fig. 3a). This probe should hybridize to the vector and readthrough vector RNAs produced in MoTN-infected PA317 cells (Fig. 1a) and to the vector, readthrough vector, readthrough *neo* and 3′-LTR-driven cellular RNAs produced in MoTN-PR3-infected PA317 cells (Fig. 1b). Our results indicate that the vector and readthrough vector RNAs transcribed from the 5′-LTR promoter are produced in MoTN-infected PA317 cells (Fig. 3a, lane 1) and are also packaged within vector particles released from these cells (Fig. 3a, lane 3). Several additional bands corresponding to readthrough *neo* RNA transcribed from the HSV *tk* promoter and/or cellular RNAs transcribed from the modified 3′-LTR promoter can be detected in RNA analysed from MoTN-PR3-infected PA317 cells (Fig. 3a, lane 2). Since a MoMuLV ψ signal-specific probe was used in this Northern blot analysis, all of these RNAs must contain the ψ signal. The vector RNA, readthrough vector RNA and some *neo* readthrough and/or downstream cellular RNAs are also packaged within the vector particles released from MoTN-PR3-infected cells (Fig. 3a, lane 4).

The composition of RNAs packaged in the vector particles released from MoTN-PR3-infected PA317 cells was further characterized by RT-PCR analysis using *neo* and PPT-U3-r-ψ region-specific primers (Fig. 3b). In addition to vector RNA, either readthrough vector and readthrough *neo* RNAs or downstream cellular RNAs contained the ψ signal. RT-PCR analysis using the PPT-5′/Psi-3′ primer pair revealed that although present in the cells (Fig. 2b, lane 2), readthrough RNAs are not packaged in the vector particles released from MoTN-PR3-infected PA317 cells (Fig. 3b, lane 2). Control RT-PCR using *neo* region-specific primers confirmed the presence of vector RNA in these particles (Fig. 3b, lane 1). Since

no readthrough RNA can be detected by RT-PCR, the ψ signal-containing RNAs (other than the vector RNA) detected in the Northern blot experiment (Fig. 3a) must correspond to downstream cellular RNAs.

Packaging retroviral vectors containing cellular RNAs fail to transduce the *neo* gene during subsequent rounds of infection

The titre of MoTN-PR3 vector particles was compared with that of MoTN vector particles released from transfected Psi-2 and infected PA317 cells (Table 1). The titres of MoTN and MoTN-PR3 vector particles released from the transfected Psi-2 cells (from which no *neo* or cellular RNA should be packaged) were 1.6×10^6 and 2.8×10^5 colony forming units (c.f.u.)/ml respectively (2). In contrast, the titre of MoTN-PR3 vector particles released from infected PA317 cells was 0.0 c.f.u./ml; the titre of MoTN vector particles was 3.4×10^6 c.f.u./ml (Table 1).

These results indicate that the vector particles containing chimeric RNAs released from infected PA317 cells are non-infectious. Co-packaging of cellular RNAs in MoTN-PR3 vector particles released from infected PA317 cells is likely to interfere with reverse transcription, resulting in a 0.0 c.f.u./ml titre.

Table 1. Titre (c.f.u./ml) of MoTN and MoTN-PR3 vector particles released from Psi-2 cells transfected with vector DNAs and PA317 cells infected with retroviral vector particles

Vector particles	Released from transfected Psi-2 cells	Released from infected PA317 cells
MoTN	1.6×10^6	3.4×10^6
MoTN-PR3	2.8×10^5	0

Packaging retroviral vector particles released from infected PA317 cells do not interfere with the titre of infectious vector particles

The human CD_4^+ lymphocyte-derived adherent CEM-10 cell line was infected with MoTN vector particles mixed with increasing concentrations of MoTN-PR3 vector particles. Both types of particles were obtained from PA317 cells infected with virus derived from transfected Psi-2 cells. As shown in Table 2, the infectivity of MoTN vector particles is not affected by the presence of non-infectious MoTN-PR3 vector particles produced from PA317 cells. Up to a 5-fold excess of MoTN-PR3 vector particles failed to alter the titre of infectious MoTN vector particles. These results are not surprising, assuming that one infectious vector particle may be sufficient to infect a cell carrying perhaps 10 000 receptor molecules on its surface.

Table 2. Titre (c.f.u./ml) of MoTN vector particles in the presence or absence of non-infectious MoTN-PR3 vector particles

Vector particles	Titre
MoTN	3.4×10^6
MoTN + MoTN-PR3 (1:2)	4.4×10^6
MoTN + MoTN-PR3 (1:5)	4.4×10^6
MoTN-PR3	0

In summary, proviral 3′-LTR-driven cellular RNAs are transcribed in MoTN-PR3 packaging retroviral vector-infected cells. These RNAs contain the ψ signal and are therefore packaged into vector particles. Furthermore, the released vector particles are non-infectious. It is hypothesized that cells expressing non-retroviral RNAs containing a ψ signal could, upon infection by a retrovirus capable of recognizing this signal, release virus particles that fail to replicate in subsequent rounds of infection. We are now testing this hypothesis in an attempt to inhibit replication of human immunodeficiency virus type-1, which is responsible for acquired immune deficiency syndrome. This strategy may also be useful in inhibiting replication of other animal and plant RNA viruses.

In addition, the packaging retroviral vector pUCMoTN-PR3, allowing transcription and packaging of downstream cellular DNA sequences, would be of interest for identifying proviral DNA integration sites. Cell lines allowing expression of an insertionally activated gene(s) would also be useful for characterizing the structure/function of that gene (3).

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