

# A COMBINATION ANTI-HIV-1 GENE THERAPY APPROACH USING A SINGLE TRANSCRIPTION UNIT THAT EXPRESSES ANTISENSE, DECOY, AND SENSE RNAs, AND TRANS-DOMINANT NEGATIVE MUTANT GAG AND ENV PROTEINS

Shi-Fa Ding, Rocco Lombardi, Reza Nazari, and Sadhna Joshi

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

## TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
  - 3.1. Construction of plasmid vectors allowing Tat- and Rev-inducible expression of anti-HIV-1 genes
  - 3.2. Construction of bacterial expression vectors, pET-GE and pET-GmEm
  - 3.3. Bacterial expression of WT and TDM gag and env genes
  - 3.4. Construction of pTev-Puro expressing HIV-1 Tev
  - 3.5. Stable transfection of HeLa cell line with pTev-Puro
  - 3.6. Stable transfection of HeLa and HeLa-Tev cells
  - 3.7. RT-PCR analysis of RNA extracted from the transfected HeLa and HeLa-Tev cells
  - 3.8. Construction of retroviral vectors expressing anti-HIV-1 genes
  - 3.9. Production of amphotropic retroviral vector particles
  - 3.10. Production of stable MT4 transductants
  - 3.11. HIV-1 susceptibility of stable MT4 transductants
  - 3.12. RT-PCR analysis of RNA extracted from the uninfected and HIV-1 infected stable MT4 transductants, and from the progeny virus released from the HIV-1 infected stable MT4 transductants
  - 3.13. Transduction of human PBLs
  - 3.14. HIV-1 susceptibility of PBL transductants
4. Results
  - 4.1. Assessment of the full-length gag and env open reading frames
    - 4.1.1. Construction of pET-GE and pET-GmEm
    - 4.1.2. Testing for expression of WT and TDM proteins
  - 4.2. Assessment of Tat- and Rev-inducible gene expression
    - 4.2.1. Construction of pBS-Ti-GE-Ri-Ter and pBS-Ti-GmEm-Ri-Ter
    - 4.2.2. Testing for Tat-inducible expression of GE/GmEm mRNA and Tat- and Rev-inducible expression of WT/TDM Gag protein in HeLa and HeLa-Tev cells
  - 4.3. Assessment of anti-HIV-1 potential of oncoretroviral vectors expressing the anti-HIV-1 genes
    - 4.3.1. Construction of MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter
    - 4.3.2. Establishment of pools of stable MT4 transductants
    - 4.3.3. HIV-1 susceptibility of stable MT4 transductants
    - 4.3.4. HIV-1 susceptibility of human PBL transductants
5. Discussion
6. Acknowledgements
7. References

## 1. ABSTRACT

Oncoretroviral vectors were engineered to allow constitutive expression of an antisense RNA and the *trans*-activator of transcription (Tat)-inducible expression of a mRNA containing the *trans*-activation response (TAR) element, the Rev response element (RRE), and the efficient packaging signal ( $\Psi^e$ ) of human immunodeficiency virus-1 (HIV-1) RNA. Nuclear export of this mRNA by the regulator of expression of virion proteins (Rev) would allow its translation into wild type (WT) (MoTN-Ti-GE-Ri-Ter) or *trans*-dominant negative mutant (TDM) (MoTN-Ti-

GmEm-Ri-Ter) Gag and Env proteins. Thus, the antisense RNA produced in a constitutive manner would ensure that even if there is leaky expression, no WT/TDM Gag or Env protein would be produced in the uninfected cells. If cells become infected by HIV-1, the antisense RNA would inhibit HIV-1 replication. Failure on the part of antisense RNA to inhibit virus replication would allow GE/GmEm mRNA production. The GE/GmEm mRNA would cause partial inhibition of HIV-1 replication as it contains the TAR, RRE, and  $\Psi^e$  signal sequences. Translation of GmEm

## A combination approach to HIV-1 gene therapy

mRNA would give rise to TDM Gag and Env proteins, which would further decrease progeny virus infectivity. Tat- and Rev-inducibility was demonstrated in transfected HeLa and HeLa-Tev cells. Full-length WT/TDM Gag production was confirmed by Western blot analysis. Amphotropic vector particles were used to transduce a human CD4<sup>+</sup> T-lymphoid cell line, and the stable transductants were challenged with HIV-1. Virus replication was better inhibited by the MoTN-Ti-GE-Ri-Ter vector than by the MoTN-Ti-GmEm-Ri-Ter vector. Inhibition of HIV-1 replication was also demonstrated in transduced CD4<sup>+</sup> human peripheral blood T lymphocytes (PBLs). Moreover, our results suggest that cloning in the reverse transcriptional orientation must be avoided to prevent antisense RNA-mediated inhibition of transgene and endogenous gene expression.

## 2. INTRODUCTION

The development of retroviral vectors as a delivery vehicle for cloned genes into various target cells has contributed towards major advancements in human gene therapy. Since HIV-1 mainly infects T-lymphocytes and monocytes/macrophages, retroviral vectors carrying anti-HIV-1 genes may be used to infect patients' hematopoietic stem cells. Various anti-HIV-1 genes, which have thus far been proposed for use in retroviral vector-mediated gene transfer, give rise to an interfering RNA or protein molecule (1-4). Interfering RNAs include sense RNAs, antisense RNAs, and ribozymes (catalytic RNAs). Antisense RNAs act by inhibiting HIV-1 RNA function (upon hybridization), whereas ribozymes act by inactivating HIV-1 RNA (upon cleavage). Sense RNAs act either as decoys of HIV-1 regulatory protein binding sites or as competitors for HIV-1 RNA packaging within the progeny virus. Interfering proteins include TDMs of HIV-1 proteins, nucleases, and suicide proteins. TDMs of viral proteins act by inhibiting the function of WT HIV-1 proteins. HIV-1 RNA-specific (targeted) or virion-encapsidated (packageable) ribonucleases are designed to cleave HIV-1 RNA within the cell or viral progeny. Suicide proteins are designed to selectively destroy the HIV-infected cells. It is therefore evident, with the many options available that a number of stages within the HIV-1 life cycle may be disrupted by the use of retroviral vector-mediated transfer of anti-HIV genes. The aim of this study is to develop a single anti-HIV gene, which can inhibit HIV-1 replication using four different strategies. This gene is designed to express (i) an antisense RNA, (ii) TAR and RRE decoys, (iii) a sense RNA containing the  $\Psi^e$  signal, and (iv) TDMs of HIV-1 Gag and Env proteins.

A number of antisense RNAs were designed using various sites within the HIV-1 RNA (1). An antisense RNA spanning a 1430-nucleotide-long region within the HIV-1 packaging signal ( $\Psi$ ) and *gag* coding region inhibited HIV-1 replication (5). Antisense RNAs spanning 1000 nucleotides or more within this region conferred similar protection (6), although the best result was obtained when the 1430-nucleotide-long region was targeted. Several other antisense RNAs spanning a region of similar or greater length within the HIV-1 RNA were since developed (7, 8).

These antisense RNAs were shown to act at a pre-splicing step (7), and were also shown to be packaged, yielding non-infectious progeny virus (8).

The TAR element is a 59-nucleotide-long stem loop structure, located within the 5' and 3' non-coding regions of all HIV-1 mRNAs. A 3-nucleotide-long bulge within this element interacts with the viral Tat protein. Tat-TAR interaction enhances transcription from the HIV 5' long terminal repeat (LTR) promoter. Inhibition of Tat-TAR interaction using TDM Tat, TAR RNA decoys, and antisense Tat/TAR RNA was shown to inhibit HIV-1 replication (reviewed in 1).

Rev interacts with a 13-nucleotide-long region within the HIV-1 RRE. RRE is a 351-nucleotide-long RNA element with complex secondary structure. It is located within the *env* coding region of unspliced and singly spliced HIV-1 mRNAs. Rev-RRE interaction facilitates nuclear export, which is required for translation of these mRNAs. Gene therapy strategies targeted against Rev-RRE interaction include Rev TDMs, RRE RNA decoys, and antisense Rev/RRE RNA (reviewed in 1).

HIV-1 RNA encapsidation within the virions requires virion RNA recognition by the nucleocapsid domain of Pr55<sup>Gag</sup>. HIV-1 RNA contains a  $\Psi$  signal, which is made of four stem loops and is located near the 5' major splice donor and the beginning of the *gag* open reading frame. This region is essential and sufficient for HIV-1 RNA packaging. However, increased packaging efficiency was reported using the  $\Psi^e$  signal, which includes an additional 1.1-kb *cis*-acting element located within the *env*-coding region containing the RRE (9). The dimer linkage structure (DLS) necessary for RNA dimerization overlaps with the  $\Psi$  signal, suggesting that the RNA dimerization and encapsidation may be mechanistically linked. Interference at the level of HIV-1 RNA packaging was reported using sense RNAs containing the HIV-1  $\Psi^e$  signal and antisense RNAs targeted to the HIV-1  $\Psi$  and  $\Psi^e$  sequences (reviewed in 1).

A "dominant negative" or "trans-dominant" mutant is an altered form of a protein capable of inhibiting the WT protein in a cell, thus causing the cell to be deficient in the function of the WT protein in its presence. TDMs of HIV-1 Tat, Rev, Tat and Rev (10), Gag (11-13), and Env (14) were developed and tested for inhibition of HIV-1 replication.

Since the *gag* gene products exist in a highly multimerized state in the mature virion, TDM Gag represents a particularly suitable candidate for gene therapy. Gag TDM VI (a truncated Gag precursor, Pr49<sup>Gag</sup>) contains a deletion of the p17-p24 cleavage site (11). In the absence of WT HIV-1 Gag, this mutant did not produce any virus. However, when cells expressing this mutant were challenged with WT HIV-1, virus production was significantly reduced, suggesting that multimerization of Gag monomers occurs before processing, and that grossly modified precursors can still interact with the WT Gag. A retroviral vector expressing Gag TDM VI (with the p6

## A combination approach to HIV-1 gene therapy

region deleted) was also developed (13). In T-lymphocytes transduced with this vector, both HIV-1 and simian immunodeficiency virus (SIV) replication was significantly inhibited, suggesting that this mutant Gag protein may have anti-viral efficacy against a broad range of primate lentiviruses and HIV-1 isolates.

The HIV-1 Env protein also exists in an oligomeric structure on the surface of the virion lipid membrane. Env glycoprotein (gp160) is synthesized as a 160-kD polypeptide precursor by membrane-bound ribosomes. During transport to the cell membrane via the secretory pathway, gp160 is cleaved by a cellular protease yielding the 120-kD surface glycoprotein (gp120) and the 41-kD transmembrane glycoprotein (gp41). A TDM Env (gp41.2) with a point mutation (from Val to Glu) at the second amino acid of gp41 was shown to interfere with the WT Env-induced formation of syncytia (14). Expression of WT and TDM Env glycoproteins had no detectable effect on Env processing. TDM Env did not block Env transport to the cell surface nor did it block gp160 or gp120 binding to CD4. It was postulated that the gp41.2 mutant incorporation within the Env oligomers dominantly interferes with the membrane fusion process.

We report here the development and testing of Moloney murine leukemia virus (MoMuLV)-based retroviral vectors allowing constitutive, Tat-inducible, or Tat- and Rev-inducible expression of anti-HIV-1 genes. The transcription units, allowing inducible expression, were cloned in a reverse orientation with respect to the retroviral vector. The vector RNA produced from the MoMuLV 5' LTR promoter would therefore contain the HIV-1 5' LTR, the 5' untranslated region, and the WT or TDM *gag* and *env* coding regions in the antisense orientation. Constitutive expression of this RNA, herein referred to as vector (antisense) RNA, would ensure that there is no leaky expression of WT/TDM *gag* and *env* genes in the absence of HIV-1 infection. However upon infection by HIV-1, Tat-induction would allow GE/GmEm mRNA production. These mRNAs were designed to inhibit HIV-1 replication through the HIV-1 TAR, RRE, and  $\Psi^e$  sequences. Upon Rev-mediated nuclear export, translation of these mRNAs would give rise to WT or TDM Gag and Env proteins of HIV-1. In order to allow translation of both *gag* and *env* open reading frames, the intercistronic region, which is the region between the stop codon of the *gag* open reading frame and the (AUG) start codon of the *env* open reading frame, was designed according to the bicistronic mRNA translation model described elsewhere (15). According to this model, the intercistronic region must contain between 15 and 78 nucleotides, and the only AUG codon present within this region must be the one where the translation is to be reinitiated.

Inhibition of HIV-1 replication was first assessed in stable transductants of a human CD4<sup>+</sup> T-lymphoid cell line. Both the MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors were shown to allow constitutive expression of vector (antisense) RNA. Both transcription units were shown to allow Tat-inducible expression of GE/GmEm mRNA in HeLa-Tev cells. The WT and TDM *gag* open

reading frames were also shown to be full-length. Both MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors inhibited HIV-1 replication in stable MT4 transductants, although the inhibition of HIV-1 replication was more prominent with the MoTN-Ti-GE-Ri-Ter vector than with the MoTN-Ti-GmEm-Ri-Ter vector. Both of these vectors also inhibited HIV-1 replication in transduced human PBLs.

## 3. MATERIALS AND METHODS

A pBlueScript-II-SK(+) (Stratagene, La Jolla, CA, USA) vector was first used to allow Tat- and Rev-inducible expression of anti-HIV-1 genes; these vectors were used to demonstrate the Tat- and Rev-inducibility upon transfection of a HeLa-Tev cell line. To assess the full-length open reading frame of the WT and TDM *gag* and *env* genes, these genes were cloned into a bacterial expression vector, pET-15b (Novagen, Madison, USA). Finally, in order to assess the anti-HIV-1 potential, the HIV-1 LTR-GE-Ri-Ter and LTR-GmEm-Ri-Ter cassettes were cloned, in the reverse transcriptional orientation, into the retroviral vector MoTN (16).

All PCRs were performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) for the purpose of cloning and *Taq* DNA polymerase (Life Technologies, Burlington, ON, Canada) for characterization of clones, as described previously (17, 18). PCR products used for cloning were precipitated with ethanol, digested with the restriction enzymes as indicated, and then gel-eluted using the Gene Clean Kit (BIO 101, Vista, CA, USA). Vector and insert fragments were then ligated using the T4 DNA ligase (Life Technologies, Burlington, ON, Canada), followed by transformation into *E. coli* DH5alpha (Life Technologies, Burlington, ON, Canada), as described elsewhere (19). The correct clones were identified by restriction enzyme digestions, as well as by PCR analysis and partial DNA sequencing.

### 3.1. Construction of plasmid vectors allowing Tat- and Rev-inducible expression of anti-HIV-1 genes

The HIV-1 3' LTR terminator was amplified from plasmid p*Env* using the Ter-F primer (5'-GGG-GGG-GGG-CCC-TGC-TGC-TTG-TGC-CTG-GC-3'; it contains an *Apa*I site) and the Ter-R primer (5'-GGG-GGG-GGT-ACC-TGC-TAG-AGA-TTT-TCC-AC-3'; it contains a *Kpn*I site). The 785-bp product was then digested with *Apa*I and *Kpn*I and inserted at the same sites within pBlueScript-II-SK(+). The correct clone was screened by digestion with *Kpn*I, *Apa*I and *Hind*III. This yielded pBS-Ter.

The next step involved the insertion of the HIV-1 5' LTR and WT *gag* gene upstream of the terminator in pBS-Ter. The LTR-5'*gag*-F primer (5'-TAT-AGG-ATC-CTG-GAA-GGG-CTA-ATT-CAC-T-3'; it contains a *Bam*HI site) and the 5'*gag*-R primer (5'-GCA-CTG-GAT-GCA-CTC-TAT-3') were used to amplify the HIV-1 5' LTR, the 5' untranslated region, and the 5' portion of the WT *gag* gene from the plasmid dE-neo (11). The 5'*gag*-R primer was designed against a sequence downstream of a

## A combination approach to HIV-1 gene therapy

unique *Pst*I site within the *gag* gene. The 1452-bp PCR product was digested with *Bam*HI and *Pst*I. The 3' *gag*-F primer (5'-ATG-AGG-AAG-CTG-CAG-AAT-3') and the 3' *gag*-I-R primer (5'-ATA-GAA-TTC-TTA-AGT-TGC-CCC-CCT-ATC-T-3'); it contains an *Eco*RI site and a portion of the intergenic region) were used to amplify the 3' portion of the *gag* gene from the plasmid dE-neo (11). The 3' *gag*-F primer was designed to complement a sequence upstream of the same unique *Pst*I site mentioned above. The resulting 913-bp PCR product was digested with *Pst*I and *Eco*RI. The 1419-bp *Bam*HI-*Pst*I and 896-bp *Pst*I-*Eco*RI PCR fragments were then inserted, in tandem, into the *Bam*HI and *Eco*RI sites of pBS-Ter. The correct clone was screened by *Hind*III and *Pvu*II digestions. This step created pBS-Ti-G-Ter.

The insertion of WT *env* gene downstream of the *gag* gene in pBS-Ti-G-Ter involved the ligation of a PCR-derived fragment and a restriction enzyme digested fragment. The I-5' *env*-F primer (5'-ATA-GAA-TTC-GGC-GCC-GCG-GCC-GCG-TCG-ACA-GTG-GCA-ATG-GGA-GT-3'); it contains *Eco*RI, *Nar*I, *Not*I, and *Sal*I sites and the rest of the intergenic region between *gag* and *env* coding regions) and the 5' *env*-R primer (5'-TCT-CCC-GCT-ACT-ACT-ATT-3') were used to PCR-amplify the 5' portion of the HIV-1 *env* gene from pHenv (20). The 5' *env*-R primer was designed against a sequence downstream of a unique *Apa*LI site within the *env* gene. The 474-bp PCR product was digested with *Eco*RI and *Apa*LI. The 3' portion of the *env* gene was isolated from pHenv by digestion with *Apa*LI and *Xho*I, producing a 2278-bp *Apa*LI-*Xho*I fragment. The 427-bp *Eco*RI-*Apa*LI PCR product and 2278-bp *Apa*LI-*Xho*I fragment were then inserted, in tandem, into the *Eco*RI and *Xho*I sites in pBS-Ti-G-Ter. The correct clone was screened by *Hind*III and *Pvu*II digestions. This step created pBS-Ti-GE-Ri-Ter. The intergenic sequence composition between *gag* and *env* coding regions was designed to be 5'-AGA-TAG-GGG-GGC-AAC-TTA-AGA-ATT-CGG-CGC-GGC-GGC-GTC-GAC-AGT-GGC-A-3'; this is in accordance with the intergenic sequence requirements for the bicistronic mRNA translation model (15).

Subsequent cloning steps were used to replace the WT *gag* and *env* genes with their respective TDM genes. The WT *env* gene was replaced first. pHenv41.2 (expressing HIV-1 TDM *env* gene) (14) was digested with *Nde*I and *Xho*I. The 2494-bp *Nde*I-*Xho*I fragment, containing the point mutation in the amino terminal coding region of gp41, was used to replace the corresponding *Nde*I-*Xho*I fragment of pBS-Ti-GE-Ri-Ter. The correct clone was screened by *Hind*III and *Pvu*II digestions. This step created pBS-Ti-GEm-Ri-Ter.

The replacement of WT *gag* with TDM *gag* was carried out in the following manner. The pBS-Ti-GEm-Ri-Ter vector was digested with *Pst*I and *Xho*I, and the 3601-bp *Pst*I-*Xho*I fragment was isolated. This fragment contained the 3' portion of the *gag* gene and the TDM *env* gene. A second fragment was obtained by digesting pBS-Ti-GEm-Ri-Ter with *Xho*I and *Nar*I. The 4257-bp *Xho*I-*Nar*I fragment contained the HIV-1 3' LTR, ampicillin

resistance gene, and HIV-1 5'LTR. A third fragment was obtained by digesting plasmid VI-neo (containing the HIV-1 LTR driving the expression of TDM *gag* gene) (11) with *Nar*I and *Pst*I. The resulting 596-bp *Nar*I-*Pst*I fragment (containing the 5' untranslated region and a portion of the TDM *gag* gene) was then ligated in combination with the other two fragments. The correct clone was identified by *Hind*III and *Pvu*II digestions. This step created pBS-Ti-GmEm-Ri-Ter.

### 3.2. Construction of bacterial expression vectors, pET-GE and pET-GmEm

The 5'LTR-F primer (5'-ATA-TCC-ATG-GGT-GCG-AGA-GCG-TCA-GTA-3'); it contains a *Nco*I site) and the Ter-R primer were used to amplify the WT *gag* and *env* genes from pBS-Ti-GE-Ri-Ter and the TDM *gag* and *env* genes from pBS-Ti-GmEm-Ri-Ter. The 8953-bp WT and the 8772-bp TDM fragments were digested with *Xho*I and *Nco*I, creating 8114 and 7933-bp products, respectively. These were then inserted into the *Nco*I and *Xho*I sites of the bacterial expression vector, pET-15b. The correct clone was identified by digestion with *Eco*RI, *Bam*HI, and *Hind*III. The clone containing the WT *gag* and *env* genes was called pET-GE, and the clone containing the TDM *gag* and *env* genes was called pET-GmEm.

### 3.3. Bacterial expression of WT and TDM *gag* and *env* genes

The bacterial expression vectors, pET-GE and pET-GmEm, were used to transform competent *E. coli* BL21(DE3)LysS cells (Novagen, Madison, WI, USA). Following a 5 hr induction using 1 mM isopropylthio-beta-galactoside (IPTG), *E. coli* cell lysates from the uninduced and induced cultures were prepared as described previously (21). Uninduced and induced cell lysates were tested for the presence of WT or TDM HIV-1 Gag by enzyme-linked immunosorbent assay (ELISA) using a p24 antigen detection kit (Coulter, Burlington, ON, Canada).

An aliquot (0.015 ml) of the uninduced or induced *E. coli* lysates, as well as HIV-1 infected MT4 cell lysate (as a positive control), were analyzed on a 0.1% sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, as described elsewhere (22). Pre-stained 18 to 108-kD "wide range" molecular weight standards (Biorad, Mississauga, ON, Canada) were analyzed in parallel. Proteins were electroblotted onto nitrocellulose (BA-85, Schleicher and Schuell NC, Keene, NH, USA) using the Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad, Mississauga, ON, Canada), according to the instruction manual. The nitrocellulose filter was incubated overnight at 4°C in PBS-TM (phosphate buffered saline, 0.1% Tween-20, 12.5% powdered Carnation skim milk), with gentle agitation. The filter was incubated for 1 hr at 37°C in a 1:800 dilution of inactivated pooled anti-HIV-1 serum (from HIV+ individuals) in PBS-TM. The filter was washed three times and then incubated for 1 hr at 37°C in the presence of sheep anti-human IgG peroxidase-conjugated monoclonal antibody (1:1000 dilution; Boehringer Mannheim, Laval, PQ, Canada) in PBS-TM. The filter was again washed, followed by enhanced chemoluminescence reaction using a

## A combination approach to HIV-1 gene therapy

kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The blot was then exposed to a Kodak XAR-5 X-ray film (Kodak, Rochester, NY, USA) for approximately 15 sec.

### 3.4. Construction of pTev-Puro expressing HIV-1 Tev

The pTev-Puro vector was constructed by modifying the plasmid pNL4.1.7.D (containing a cDNA derived from the HIV-1 *tev* gene) (23) to contain a selectable marker. A 1055-bp fragment, containing the SV40 promoter and the puromycin acetyltransferase gene, was excised from pBabePuro (24) by digestion with *Bam*HI and *Cl*aI. The pNL4.1.4.6D.7 plasmid was linearized by digestion with *Xho*I. In order to create blunt ends, both the vector and the 1055-bp insert were filled-in using Klenow (Amersham Pharmacia Biotech, Buckinghamshire, England). The vector was then dephosphorylated using calf intestinal alkaline phosphatase (Boehringer Mannheim, Laval, PQ, Canada). The two fragments were ligated, then transformed into *E. coli* DH5alpha, and the correct clone was screened by *Hind*III and *Bam*HI digestions. The resulting vector was called pTev-Puro.

### 3.5. Stable transfection of HeLa cell line with pTev-Puro

HeLa cells ( $1 \times 10^6$ ) were transfected with 0.03 mg pTev-Puro using the calcium phosphate co-precipitation method as described previously (10). Stable HeLa-Tev transfectants were selected in media containing 500 ng/ml puromycin.

### 3.6. Stable transfection of HeLa and HeLa-Tev cells

HeLa and HeLa-Tev cells were co-transfected with 0.03 mg of pBS-Ti-GE-Ri-Ter or pBS-Ti-GmEm-Ri-Ter and 0.003 mg pSV2Neo (Clontech, Palo Alto, CA, USA) as described previously (10). The stable transfectants were selected in a medium containing 0.6 mg/ml G418 for HeLa cells and 0.6 mg/ml G418 and 500 ng/ml puromycin for HeLa-Tev cells. Transfected HeLa and HeLa-Tev cell lysates (0.3 ml each) were prepared as described earlier (18). These lysates were tested for the presence of WT and TDM HIV-1 Gag by ELISA using a p24 antigen detection kit (Coulter).

### 3.7. RT-PCR analysis of RNA extracted from the transfected HeLa and HeLa-Tev cells

Total cellular RNA was extracted from HeLa and HeLa-Tev cells following stable transfection with pBS-Ti-GE-Ri-Ter and pBS-Ti-GmEm-Ri-Ter, as described elsewhere (25). The RNA was incubated with 4 units of DNase RQ1 (Promega) in the presence of 20 mM  $MgCl_2$  and 10 mM DTT for 15 min at 37°C, followed by phenol/chloroform extraction and isopropanol precipitation. Lack of DNA contamination in the RNA samples was confirmed by direct PCR of RNA samples (without reverse transcription). RT-PCRs were performed, as described previously (26), using the reverse primers during reverse transcription and both forward and reverse primers during PCR. The Gap-F primer (5'-CCA-CCC-ATG-GCA-AAT-TCC-ATG-GCA-3') and the Gap-R primer (5'-TCT-AGA-CGG-CAG-GTC-AGG-TCC-ACC-3') were used to detect a 600-bp region within the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The Gag-F

(5'-ATA-GTA-TGG-GCA-AGC-AGG-G-3') and 5'*gag*-R primers, flanking the *gag* mutation site, were used to amplify a 555-bp region within the WT and a 374-bp region within the TDM *gag* coding region. The *env*-F primer (5'-ATC-ATA-TGT-AAT-ACG-ACT-CAC-TAT-AGG-GCG-AGA-AGG-AGA-AAT-ATC-AGC-3') and the 5'*env*-R primer were used to amplify a 463-bp region within the *env* coding region of GE and GmEm mRNAs. The RT-PCR products were analyzed by electrophoresis on a 1.5-2% agarose gel.

### 3.8. Construction of retroviral vectors expressing anti-HIV-1 genes

The retroviral vector MoTN (16) was first modified to generate MoCTN, as follows. A 1396-bp region, containing a promoter driving the chloramphenicol acetyl transferase (*cat*) gene expression, was PCR-amplified from pACYC184 (New England Biolabs, Mississauga, ON, Canada). The *Pcat*-F primer (5'-ATA-TAT-ATC-TCG-AGA-AGG-CCA-TAT-GGG-CCA-ACC-GC-T-AGC-GCT-GAT-GTC-3'; it contains *Xho*I and *Sfi*IIa sites) and the *cat*-Mo-R primer (5'-TGG-GTG-GTG-TAA-TCC-CAG-TCT-GGC-CAG-CTG-GGC-CGC-GCT-AAC-CGT-TTT-TAT-3'; it contains a *Sfi*IIb site and a region complementary to the Mo-F primer) were used for this purpose. The *Sfi*IIa and *Sfi*IIb sites were designed to differ in their NNNNN sequence composition to allow forced ligation and to avoid vector self-ligation. The Mo-F primer (5'-AGA-CTG-GGA-TTA-CAC-CAC-CCA-3') and the Mo-R primer (5'-GAT-TTT-GAT-CGA-TGA-GTG-GAT-CCG-GAC-CAA-3'; it contains *Cl*aI and *Bam*HI sites) were designed against MoTN vector DNA sequences upstream and downstream of the unique *Ap*aI and *Bam*HI sites. MoTN was used in a PCR using the Mo-F and Mo-R primers to amplify a 2126-bp product containing the MoTN vector sequences encompassing the *Ap*aI site, the herpes simplex virus (HSV) thymidine kinase (*tk*) promoter, the neomycin phosphotransferase (*neo*) gene, and the *Bam*HI site; the *cl*aI site present within the Mo-R vector would be located at the 3' end of this product. The two PCR products were then used in an overlapping PCR with the *Pcat*-F and Mo-R primers as described earlier (17, 18). The resulting 3501-bp PCR product was digested with *Xho*I and *Cl*aI and cloned at the same sites within the MoTN vector. This step created the MoCTN vector. This vector contained the *Xho*I and *Sfi*IIa sites, a promoter driving the *cat* gene expression, and the *Sfi*IIb and *Ap*aI sites, between the MoMuLV  $\Psi$  signal and the HSV *tk* promoter.

A retroviral vector was then engineered to express various anti-HIV-1 genes. The HIV-1 5' LTR, the 5' untranslated region, the *gag* gene, and a portion of the intercistronic region were PCR-amplified from the pBS-Ti-GE-Ri-Ter vector using the LGI-F primer (5'-GCC-CGA-GAG-GCC-AGC-TGG-GCC-TTC-GAA-CGG-CCG-CTG-ACA-TCG-AGC-TTG-C-3'; it contains *Sfi*IIb site) and the LGI-R primer (5'-GCC-ACT-GTC-GTT-ACG-CGT-CCG-CGG-CGC-C-3'; it contains a *Mlu*I site). This yielded a 2046-bp product containing a *Sfi*IIb site at the 5' end and a *Mlu*I site at the 3' end. The remainder of the intercistronic region and the *env* gene was PCR-amplified from the pBS-Ti-GE-Ri-Ter vector using the IET-F primer (5'-GGC-GCC-GCG-GAC-GCG-TAA-CGA-CAG-TGG-C-3'; it

## A combination approach to HIV-1 gene therapy

contains a *MluI* site) and the IET-R primer (5'-ATA-TAT-ATG-GCC-ATA-TGG-GCC-TGA-TCA-TGC-TGG-CTC-AGC-TCG-TCT-CTT-TAT-TTC-C-3'); it contains a transcription terminator and the *SfiIa* site). The resulting 2700-bp product (containing the intercistronic region, the *env* gene, and a transcription terminator) was flanked with the *MluI* site at the 5' end and the *SfiIa* site at the 3' end. The two PCR products were digested with *SfiI* and *MluI*, and the *SfiIb-MluI* and *MluI-SfiIa* products were cloned (in the reverse orientation) at the compatible *SfiIa* and *SfiIb* sites in the MoCTN vector.  $\text{Cm}^S$  and  $\text{Km}^R$  colonies were selected, and the correct clone was identified by PCR, restriction enzyme analyses, and partial DNA sequencing. This vector was named MoTN-Ti-GE-Ri-Ter.

A similar strategy was used to generate the MoTN-Ti-GmEm-Ri-Ter vector. The HIV-1 5' LTR promoter, the 5' untranslated region, the TDM *gag* gene, and a portion of the intercistronic region were PCR-amplified from the pBS-Ti-GmEm-Ri-Ter vector using the LGI-F and LGI-R primers. The 1865-bp PCR product contained the *SfiIb* site at the 5' end and a *MluI* site at the 3' end. The remainder of the intercistronic region and the TDM *env* gene was PCR-amplified from the pBS-Ti-GmEm-Ri-Ter vector using the IET-F and IET-R primers. The resulting 2700-bp product contained the *MluI* site at the 5' end and a terminator and the *SfiIa* site at the 3' end. The two PCR products were digested with *SfiI* and *MluI*, and the *SfiIb-MluI* and *MluI-SfiIa* products were cloned (in the reverse orientation) at the compatible *SfiIa* and *SfiIb* sites in the MoCTN vector.  $\text{Cm}^S$  and  $\text{Km}^R$  colonies were selected, and the correct clone was identified by PCR, restriction enzyme analyses, and partial DNA sequencing. This vector was named MoTN-Ti-GmEm-Ri-Ter.

Note that the promoter and the *cat* gene were cloned between the *SfiI* sites in the MoCTN vector. However, they were deleted in the MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors. Consequently, the vector backbone in these vectors resembled the MoTN vector, which was used as a control.

### 3.9. Production of amphotropic retroviral vector particles

The ecotropic Psi-2 packaging cell line (27) was transfected with MoTN, MoTN-Ti-GE-Ri-Ter, and MoTN-Ti-GmEm-Ri-Ter vectors, as described previously (10). The ecotropic vector particles released from the pools of stable transductants were used to transduce the amphotropic PA317 packaging cell line (28). The amphotropic vector particles released from the pools of stable transductants were then collected. These particles were tested and confirmed to be free of helper virus contamination or recombination by RT-PCR using the RCR-F primer (5'-GAG-ATT-TGG-TTA-GAG-AGG-CA-3') and the RCR-R primer (5'-CTG-ACC-TCC-CTA-GTC-ATC-TA-3'), which were unique to the sequences present within the MoMuLV *pol* coding region.

### 3.10. Production of stable MT4 transductants

The amphotropic retroviral vector particles were used to transduce the human CD4+ T lymphoid (MT4) cell

line as described previously (10). The pools of stable G418<sup>R</sup> MT4 transductants were each selected and used without cloning.

### 3.11. HIV-1 susceptibility of stable MT4 transductants

Pools of actively dividing stable MT4 transductants ( $2 \times 10^6$  cells) were each infected with 30 ng (p24 equivalent) of the HIV-1 strain NL4-3 as described previously (26). Cells were routinely checked for syncytia formation. One third of the cell culture was collected every three days and replaced with fresh medium. The amount of HIV-1 p24 antigen produced in the cell culture supernatants was determined by ELISA using a p24 antigen detection kit (Abbott, Park, IL, USA). A standard curve (based on OD<sub>492</sub> as a function of p24 antigen concentration in pg/ml) was drawn using the p24 antigen provided by the supplier. The OD<sub>492</sub> values obtained from various samples (following an appropriate dilution) were converted to pg or ng of HIV-1 p24 antigen released per ml of cell culture.

### 3.12. RT-PCR analysis of RNA extracted from the uninfected and HIV-1 infected stable MT4 transductants, and from the progeny virus released from the HIV-1 infected stable MT4 transductants

Total cellular RNA was extracted from the uninfected and HIV-infected MT4 transductants and treated with DNase RQ1, as described above. The lack of DNA contamination was confirmed in these RNA samples by direct PCR analysis (without reverse transcription). RT-PCRs were then performed, as described previously (26), using the reverse primers during reverse transcription and both the forward and the reverse primers during PCR. As an internal control, the Gap-F and Gap-R primers were used to detect a 600-bp region within the GAPDH mRNA. The Vu-F primer (5'-GCC-AGC-AAC-TTA-TCT-GTG-3') and the Vu-R primer (5'-CGT-CTC-GCG-GCC-ATC-CG-3') were used to detect a 567-bp region (upstream of the *neo* coding region) within the vector RNA. The Neo-F primer (5'-CAA-GAC-CGA-CCT-GTC-CGG-3') and the Neo-R primer (5'-CTC-TTC-GTC-CAG-ATC-ATC-3') were used to detect a 337-bp region (of *neo* coding region) within the vector and *neo* RNAs. The 3'*gag*-F and LGI-R primers were used to detect a 941-bp region (downstream of the mutation site) within the *gag* coding region of GE/GmEm mRNA. The LGI-R primer binding site is absent in the HIV-1 RNA. Therefore, this primer pair will not detect the presence of HIV-1 RNA. The Vpr-F primer (5'-ATA-CTT-GGG-CAG-GAG-TGG-AAG-C-3') and Vpu-R primer (5'-CTA-TGG-ACC-ACA-CAA-CTA-TTG-C-3') were used to detect a 424-bp region unique to the HIV-1 RNA; these primers would not bind to the vector and GE/GmEm RNAs, and therefore would not detect their presence. RT-PCR products were analyzed by electrophoresis on a 1.5-2% agarose gel.

For RNA extraction from virus particles present in the cell culture supernatants, contaminating cells and cell debris were first removed by centrifugation. RNA was extracted by phenol-chloroform treatment, precipitated using ethanol, and resuspended in H<sub>2</sub>O. The Vpr-F and Vpu-R primers were used to detect a 424-bp region unique to the HIV-1 RNA, the Vu-F and Vu-R primers were used to detect a 567-bp region within the vector or vector (antisense)

## A combination approach to HIV-1 gene therapy

RNA, and the 3' *gag*-F and LGI-R primers were used to detect a 941-bp region within the GE/GmEm mRNA.

### 3.13. Transduction of human PBLs

Human peripheral blood mononuclear cells were isolated from fresh heparinized blood samples from healthy donors by Ficoll-Hypaque gradient centrifugation, as described previously (18). Over 85% of these cells represented T lymphocytes, as determined by flow cytometry analysis using an anti-CD4 monoclonal antibody (CD4RD1; Coulter, Burlington, ON, Canada). PBLs ( $1 \times 10^6$  cells) were then mixed with 1 ml of the amphotropic MoTN, MoTN-Ti-GE-Ri-Ter, and MoTN-Ti-GmEm-Ri-Ter vector particles in the presence of 0.016 mg/ml polybrene and 20 units/ml human recombinant interleukin (IL)-2 (Boehringer Mannheim, Laval, Quebec, Canada). The mixture was centrifuged at  $200 \times g$  for 1 hr at 32°C, and the cells were cultured for 16 hr at 32°C and for 6 hr at 37°C in IL-2 supplemented medium. The transduction procedure was carried out once daily for three consecutive days. Twenty-four hours after the third transduction, cells were cultured for eight days in a IL-2 supplemented medium containing G418 (0.5 mg/ml), and were periodically checked for growth and viability.

### 3.14. HIV-1 susceptibility of PBL transductants

Transduced PBLs ( $5 \times 10^6$  cells) were infected with 30 ng (p24 equivalent) of the HIV-1 strain NL4-3 for 16 hr at 37°C. Cells were then washed twice with RPMI 1640 medium, resuspended in 0.75 ml of the same medium containing 20 units/ml IL-2, and incubated at 37°C. The culture supernatants were collected every three days and replaced with fresh medium. The amount of HIV-1 p24 antigen released in the cell culture supernatant was measured by ELISA using a kit (Abbott).

## 4. RESULTS

The objective of this study was to develop and test the anti-HIV-1 potential of oncoretroviral vectors expressing various interfering RNAs and proteins from a single anti-HIV gene. This gene was designed to inhibit HIV-1 replication via four different strategies utilizing (i) antisense RNA, (ii) TAR and RRE decoys, (iii)  $\Psi^e$  RNA, and (iv) TDMs of HIV-1 Gag and Env proteins. The antisense RNA was produced in a constitutive manner, whereas the RNA containing the TAR and RRE decoys and the  $\Psi^e$  element was produced in a Tat-inducible manner. Upon Rev-induction, this RNA was designed to yield WT (as a control) or TDM Gag and Env proteins. The following aspects were studied: (i) the assessment of the full-length *gag* and *env* open reading frames, (ii) the assessment of Tat- and Rev-inducible gene expression, and (iii) the assessment of the anti-HIV-1 potential of oncoretroviral vectors expressing the anti-HIV-1 genes.

### 4.1. Assessment of the full-length *gag* and *env* open reading frames

#### 4.1.1. Construction of pET-GE and pET-GmEm

The pET-GE and pET-GmEm vectors were designed to allow the T7 promoter-driven expression of either WT or TDM *gag* and *env* genes (Figure 1a). These

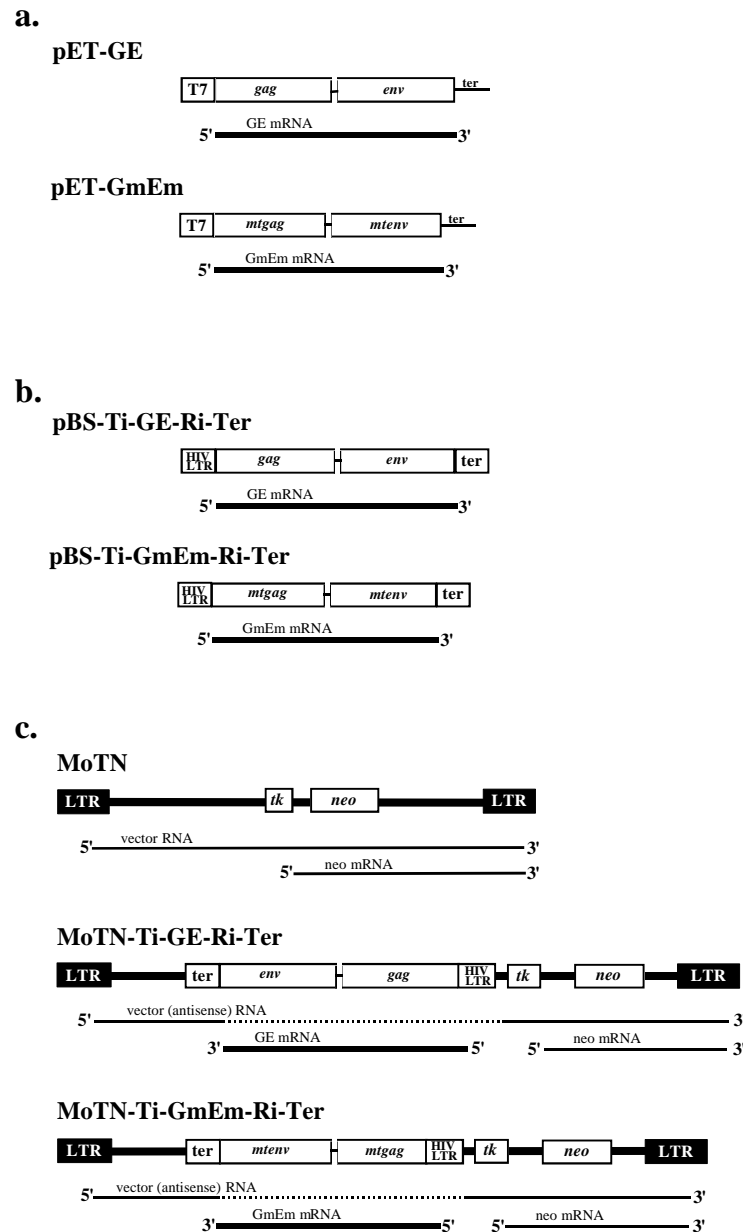
vectors were used to determine whether the open reading frames of the *gag* and *env* genes would be intact.

#### 4.1.2. Testing for expression of WT and TDM proteins

*E. coli* BL21(DE3)LysS cells were transformed with the pET-GE and pET-GmEm vectors. Following a 5 hr IPTG induction, uninduced and induced bacterial lysates were analyzed for p24 antigen production by ELISA. The uninduced and induced negative controls using the pET-15b vector yielded undetectable levels of p24 antigen. The lysate from the uninduced cells transformed with pET-GmEm contained a detectable amount of p24 (57 pg/ml), which was 4-fold less than that present in the induced cell lysate (205 pg/ml). On the other hand, the lysates from the pET-GE vector-transformed *E. coli* cells, whether induced or not, contained >250 pg/ml p24 antigen. It is possible that the TDM Gag is inefficiently recognized by specific antibodies, as compared to the WT Gag.

Bacterial protein lysates and a positive control lysate from HIV-1 infected MT4 cells were analyzed by Western blot analysis using anti-HIV serum and a peroxidase-conjugated secondary antibody. Bands corresponding to the Pr55<sup>Gag</sup> precursor and Gag p24 were clearly evident in the HIV-1 positive control (Figure 2, lane 1); these bands were absent in the uninduced and induced pET-15b negative controls (figure 2, lanes 2, 3) and in the uninduced samples from pET-GE and pET-GmEm (figure 2, lanes 4, 6). Several intense bands were present in the induced pET-GE sample (figure 2, lane 5); however, those of interest were the ones corresponding to the Pr55<sup>Gag</sup> precursor and possibly the Env protein gp41. No Gag protein cleavage products were produced because the HIV-1 protease was not present. The remaining bands may correspond to bacterial protease cleavage products of the Gag precursor. A band corresponding to the Pr49 TDM Gag precursor was detected in the induced pET-GmEm sample (figure 2, lane 7). However, the intensity of this band was noticeably weaker in comparison to the band corresponding to the WT Pr55<sup>Gag</sup> precursor. It is conceivable that the deletion of the region surrounding and including the HIV-1 protease cleavage site between p17 and p24 resulted in a conformational change in the TDM Gag, which poorly displayed the normal epitopes for antibody binding. As a result, the antibodies raised against WT Gag protein might not have recognized the TDM Gag protein effectively.

These results suggest that the WT and the TDM *gag* open reading frames were full-length. No conclusive indication could be obtained for whether the *env* open reading frame was functionally capable of expressing Env protein. The intercistronic region present between the *gag* and *env* open reading frames was designed as described previously (15). This sequence lacked the ribosome binding site for translation in *E. coli* (5'-AGGAGGU-3', four to ten nucleotides upstream of the start codon of the mRNA), and therefore might not have allowed internal initiation required for Env protein production in *E. coli*. Alternatively, the tRNA pool in the *E. coli* BL21(DE3)LysS cells may be sub-optimal for the codon usage in the HIV-1 *env* gene.



**Figure 1.** Schematic diagrams of (a) pET-GE and pET-GmEm, (b) pBS-Ti-GE-Ri-Ter and pBS-Ti-GmEm-Ri-Ter, and (c) MoTN, MoTN-Ti-GE-Ri-Ter, and MoTN-Ti-GmEm-Ri-Ter and transcripts produced. The MoMuLV 5' LTR and HSV *tk* promoter-driven transcripts are shown by a thin line. RNAs containing HIV-1 RNA sequences in sense or antisense orientation are shown by thick and dotted lines, respectively.

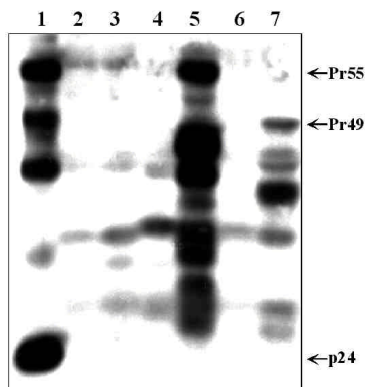
## 4.2. Assessment of Tat- and Rev-inducible gene expression

### 4.2.1. Construction of pBS-Ti-GE-Ri-Ter and pBS-Ti-GmEm-Ri-Ter

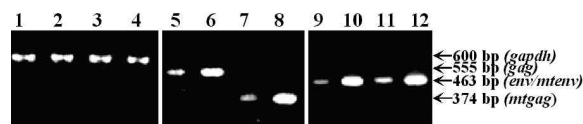
pBS-Ti-GE-Ri-Ter and pBS-Ti-GmEm-Ri-Ter were engineered to allow Tat-inducible expression of GE and GmEm mRNAs (Figure 1b). Rev-mediated nuclear export of these mRNAs should allow translation of these bicistronic mRNAs into WT/TDM Gag and Env proteins. Tat-inducibility was conferred by the HIV-1 5' LTR

(nucleotides -454 to +184, relative to the transcription start site). This segment includes the TATA box and the upstream Sp1 and NF- $\kappa$ B sites, which are required for transcription from the HIV-1 LTR promoter in the presence of Tat. Rev-inducibility was conferred by the *cis*-acting repressive sequences present within the HIV-1 *gag* and *env* coding regions and by the RRE present within the *env* coding region. A plasmid with a similar structure was also reported by others to allow for Tat- and Rev-inducible gene expression (29).





**Figure 2.** Western blot analysis of pET-15b, pET-GE, and pET-GmEm vector-transfected *E. coli* BL21(DE3)LysS cell lysates using anti-HIV serum. Lane 1, HIV-infected MT4 cell lysate (positive control); lane 2, uninduced pET-15b lysate (negative control); lane 3, induced pET-15b lysate (negative control); lane 4, uninduced pET-GE lysate; lane 5, induced pET-GE lysate; lane 6, uninduced pET-GmEm lysate; and lane 7, induced pET-GmEm lysate.



**Figure 3.** RT-PCR analysis of RNAs produced in HeLa and HeLa-Tev cells transfected with the pBS-Ti-GE-Ri-Ter and pBS-Ti-GmEm-Ri-Ter vectors. Detection of GAPDH RNA sequence (lanes 1-4) as an internal control (using the Gap-F/Gap-R primer pair), of WT (lanes 5 and 6) or TDM (lanes 7 and 8) *gag* sequences within the GE/GmEm mRNA (using the Gag-F/5'*gag*-R primer pair), and of WT (lanes 9 and 10) or TDM (lanes 11 and 12) *env* sequences within the GE/GmEm mRNA (using the *env*-F/5'*env*-R primer pair). RT-PCR products were analyzed from HeLa (lanes 1, 3, 5, 7, 9, and 11) or HeLa-Tev (lanes 2, 4, 6, 8, 10, and 12) cells following stable transfection with pBS-Ti-GE-Ri-Ter (lanes 1, 2, 5, 6, 9, and 10) or pBS-Ti-GmEm-Ri-Ter (lanes 3, 4, 7, 8, 11, and 12).

#### 4.2.2. Testing for Tat-inducible expression of GE/GmEm mRNA and Tat- and Rev-inducible expression of WT/TDM Gag protein in HeLa and HeLa-Tev cells

The pTev-Puro vector was constructed to demonstrate that the pBS-Ti-GE-Ri-Ter and pBS-Ti-GmEm-Ri-Ter vectors allow Tat- and Rev-inducible gene expression in mammalian cells. This vector expresses the *tev* cDNA containing the HIV-1 Tat coding exon 1 and the Rev coding exon 2. The Tev protein can therefore substitute for Tat function, and also partly for the Rev function (23, 30). The pTev-Puro vector was transfected into the HeLa cells. Stable transfectants were selected and were called HeLa-Tev.

HeLa and HeLa-Tev cells were used to test the Tat- and Rev-inducible expression from the pBS-Ti-GE-Ri-Ter and pBS-Ti-GmEm-Ri-Ter vectors. These vectors were

transfected into HeLa and HeLa-Tev cells with pSV2Neo. The *neo* gene expression in pSV2Neo, under the control of a weak SV40 promoter, allowed for the selection of stable transformants.

The presence of the *gag* and *env* genes was confirmed by PCR using specific primer pairs (results not shown). Total cellular RNA was then analyzed by RT-PCR to detect 555- or 374-nucleotide-long regions within the WT or TDM *gag* coding regions, respectively. The difference in size between the amplified WT and TDM *gag* regions is due to the 181-bp deletion in the TDM *gag* gene. RT-PCRs were also performed to detect a 463-nucleotide-long region within the *env* coding region. The *env* mutation, being a single point mutation, did not produce a PCR product of a different size. Expected products were obtained for the WT (a 555-bp product; Figure 3, lanes 5, 6) and TDM (a 374-bp product; figure 3, lanes 7, 8) *gag* coding regions and for the *env* coding region (a 463-bp product; figure 3, lanes 9-12) of GE/GmEm mRNAs. Band intensities of samples obtained from the transfected HeLa-Tev cells were much stronger than those obtained from the HeLa cells, suggesting that the GE and GmEm mRNAs were transcribed in a Tat-inducible manner. Note that Rev is only required for nuclear export and not for RNA synthesis. The faint bands detected in the transfected HeLa cells may correspond to leaky transcription from the HIV-1 5' LTR promoter. As a control, RT-PCRs were also performed to detect a 600-bp region within the GAPDH mRNA; the expected products with similar intensities were obtained from all transfected HeLa and HeLa-Tev samples (figure 3, lanes 1-4).

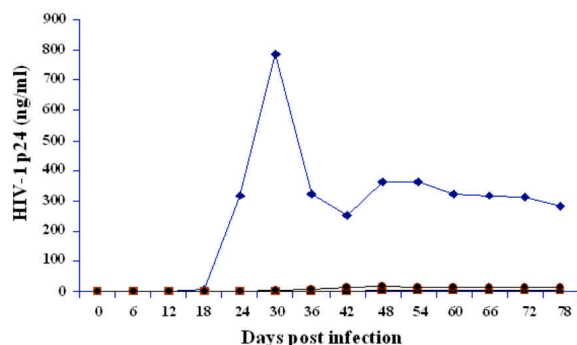
Cell lysates were also analyzed using p24 antigen ELISA. The results indicated that the WT Gag protein was expressed by the pBS-Ti-GE-Ri-Ter vector. Evidence for the Tev-inducible expression of the Gag protein was derived from the fact that 90.17 pg/ml p24 was detected when HeLa-Tev cells were transfected with pBS-Ti-GE-Ri-Ter, compared to 21.85 pg/ml p24 in transfected HeLa cells. The apparent elevated p24 levels in HeLa cells could be due to leaky expression, even though both Tat and Rev (or Tev) proteins were required for Gag expression. HeLa-Tev cells transfected with pBS-Ti-GmEm-Ri-Ter were negative for p24 antigen. As mentioned earlier, the 181-bp deletion of the region surrounding the p17 and p24 cleavage site in the TDM Gag protein, could have resulted in a conformational change in the protein which may not be efficiently recognized by the monoclonal antibody used in the Coulter p24 ELISA.

#### 4.3. Assessment of anti-HIV-1 potential of oncoretroviral vectors expressing the anti-HIV-1 genes

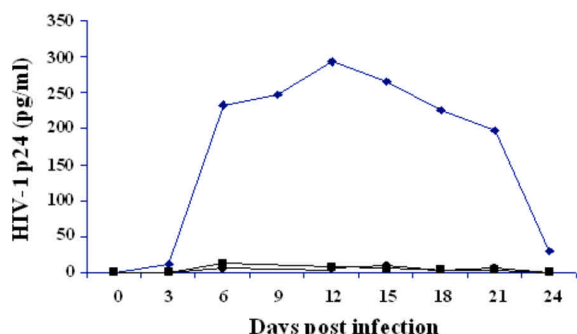
##### 4.3.1. Construction of MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter

MoMuLV-derived MoTN vector was used in this study. This vector contains the MoMuLV 5' LTR and HSV *tk* promoters that allow constitutive transcription of vector and *neo* RNAs, respectively (figure 1c).

The MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors were engineered to allow Tat-inducible expression of GE/GmEm mRNAs and Tat- and Rev- inducible



**Figure 4.** 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 MoTN (—◆—), MoTN-Ti-GE-Ri-Ter (—■—), and MoTN-Ti-GmEm-Ri-Ter (—●—) vector-transduced MT4 cells.



**Figure 5.** HIV-1 susceptibility of transduced human PBLs. HIV-1 p24 antigen production was measured at various time intervals following HIV-1 challenge of MoTN (—◆—), MoTN-Ti-GE-Ri-Ter (—■—), and MoTN-Ti-GmEm-Ri-Ter (—●—) vector-transduced human PBLs.

expression of WT/TDM Gag and Env proteins. For construction of this vector, a cassette containing the HIV-1 5' LTR promoter expressing WT or TDM *gag* and *env* genes, and a terminator was cloned downstream of the MoMuLV 5' LTR promoter (figure 1c). This cassette was cloned in a reverse transcriptional orientation. As a result, the vector RNA transcribed from the MoMuLV 5' LTR promoter would contain the HIV-1 sequences in an antisense orientation. This vector (antisense) RNA will serve two purposes: (i) it will prevent leaky expression of WT/TDM Gag and Env proteins in the uninfected cells, and (ii) it will act as an antisense RNA and prevent virus replication in the HIV-infected cells. HIV-1 Tat protein would allow the production of GE and GmEm mRNAs containing the TAR, RRE, and  $\Psi^e$  sequences. Rev-mediated nuclear export would then allow translation of these mRNAs into the WT/TDM Gag and Env proteins.

#### 4.3.2. Establishment of pools of stable MT4 transductants

The amphotropic MoTN, MoTN-Ti-GE-Ri-Ter, and MoTN-Ti-GmEm-Ri-Ter vector particles were used to

transduce MT4 cells. Stable MT4 transductants were selected and tested without cloning. Total cellular RNA was analyzed by RT-PCR. Expected products were detected in each case, confirming vector RNA production from the MoTN, MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors (results not shown). Production of neo mRNA was indirectly demonstrated as cells were resistant to G418.

#### 4.3.3. HIV-1 susceptibility of stable MT4 transductants

Pools of stable MT4 transductants were challenged with the HIV-1 strain NL4-3 (Figure 4). The MoTN vector-transduced cells allowed 783 ng/ml HIV-1 p24 production by day 30; virus production then decreased due to cell death. Cells transduced with the MoTN-Ti-GE-Ri-Ter or MoTN-Ti-GmEm-Ri-Ter vectors demonstrated a very strong inhibition with no detectable HIV-1 p24 until day 21 (MoTN-Ti-GE-Ri-Ter) or day 18 (MoTN-Ti-GmEm-Ri-Ter). HIV-1 p24 levels then gradually increased until day 48 to 2.52 ng/ml in MoTN-Ti-GE-Ri-Ter vector-transduced cells and to 6.8 ng/ml in MoTN-Ti-GmEm-Ri-Ter vector-transduced cells. Thereafter, virus replication remained below this value until day 78, when the experiment was terminated (figure 4).

Upon the HIV-1 challenge of stable MT4 transductants, vector and GE/GmEm RNA production was assessed by RT-PCR analysis of RNA extracted from the infected cells. All HIV-1 infected MT4 transductants produced HIV-1 RNA, the respective retroviral vector or vector (antisense) RNA, and the GE/GmEm mRNA (results not shown).

RT-PCR analysis of RNA extracted from the progeny virus produced from all MT4 transductants revealed that HIV-1 RNA was packaged. While the vector (antisense) RNA produced from MoTN-Ti-GE-Ri-Ter vector-transduced cells was also shown to be packaged, the GE mRNA was not (results not shown). As expected, vector RNA produced from the HIV-infected MoTN (control) vector-transduced cells was not packaged. The cellular GAPDH mRNA was also not found to be packaged, confirming that the virion RNA was not contaminated with cellular RNA (results not shown).

#### 4.3.4. HIV-1 susceptibility of human PBL transductants

Human PBLs were transduced with the amphotropic MoTN, MoTN-Ti-GE-Ri-Ter, and MoTN-Ti-GmEm-Ri-Ter vector particles. The pools of transduced PBLs were then challenged with the 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 (below 12.7 pg/ml p24) in both MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vector-transduced PBLs, whereas 293.2 pg/ml p24 production was detected in the MoTN vector-transduced PBLs (Figure 5). These results further demonstrate that both MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter can efficiently inhibit HIV-1 replication.

## 5. DISCUSSION

As a safety precaution, the expression of anti-HIV genes encoding RNAs or proteins with cytotoxic or

## A combination approach to HIV-1 gene therapy

cytotoxic effect should be limited to HIV-1 infected cells. TAR and RRE decoys are known to sequester cellular factors required for host transcription (31, 32). Also, the HIV-1 Env protein has been found to be cytotoxic (14). Therefore, MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors were designed to allow constitutive expression of vector RNA (containing antisense sequences) and Tat-inducible expression of GE/GmEm mRNA (containing the TAR and RRE decoys and the  $\Psi^e$  signal). Upon Rev-mediated export, the GE/GmEm mRNA would give rise to the WT/TDM Gag and Env proteins. Thus, the vector (antisense) RNA would be present prior to HIV-1 infection and would be produced throughout virus replication. This RNA would inhibit HIV-1 replication at the level of viral RNA splicing, export, translation, and packaging. The co-packaged antisense RNA could also limit viral spread by inhibiting reverse transcription during subsequent rounds of replication or as a result of recombination leading to virus inactivation.

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. This in turn would induce HIV-1 5' LTR promoter-driven expression of both the GE/GmEm mRNA and the HIV-1 genomic RNA. Similarly, incomplete inhibition of Rev mRNA production would allow nuclear export of GE/GmEm mRNA and of singly spliced or unspliced HIV-1 RNA. Nuclear export and translation of GE/GmEm mRNA would therefore depend on the lack of inhibition of Tat and Rev protein production by the antisense RNA. The vector (antisense) RNAs produced from both MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors are expected to inhibit HIV-1 replication. However, as the vector (antisense) RNA expressed from the MoTN-Ti-GmEm-Ri-Ter vector contains a 181-bp deletion, it may not inhibit HIV-1 replication as well as the corresponding RNA produced from the MoTN-Ti-GE-Ri-Ter vector.

The GE and GmEm mRNAs produced from the MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors contain nucleotides ranging from +1 to the end of the WT/TDM *gag* coding region, which includes the TAR element along with the  $\Psi$  signal and the DLS. The WT/TDM *env* coding region includes the RRE and the sequences required for efficient HIV-1 RNA packaging. HIV-1 TAR and RRE, which are present on both GE and GmEm mRNAs, should confer partial (but similar) inhibition of HIV-1 replication by providing competitive protein binding sites and/or by sequestering key cellular factors (1). The HIV-1  $\Psi^e$  signal present in GE mRNA should inhibit HIV-1 replication by competing with HIV-1 RNA for packaging within the progeny virus (8, 33). However, the HIV-1 packaging signal sequences present within the GmEm mRNA (due to a 181-bp deletion within the TDM *gag* coding region) may not allow this RNA to efficiently compete with HIV-1 RNA for packaging within the virions. Thus, for inhibiting HIV-1 replication at the level of RNA packaging, the MoTN-Ti-GE-Ri-Ter vector may prove to be more effective than the MoTN-Ti-GmEm-Ri-Ter vector.

Translation of GE/GmEm mRNA would give rise to WT or TDM Gag and Env proteins. Of these, the WT Gag and Env proteins are not likely to inhibit HIV-1 replication. However, the TDM Gag and Env proteins, through incorporation within the viral progeny, are expected to render it non-infectious.

The pBS-Ti-GE-Ri-Ter and pBS-Ti-GmEm-Ri-Ter vectors were designed to allow Tat-inducible expression of GE/GmEm mRNA, and Tat- and Rev-inducible expression of WT or TDM Gag and Env proteins in the absence of antisense RNA production (figure 1b). Tat- and Rev-inducibility was demonstrated by RT-PCR analysis (figure 3) and p24 ELISA following stable transfection of HeLa and HeLa-Tev cells with these vectors. The assessment of the full-length open reading frames of the WT and TDM *gag* genes was done by Western blot analysis (figure 2) of the samples obtained from the *E. coli* DH5alpha cells, which were transformed with the inducible bacterial expression vectors, pET-GE and pET-GmEm (figure 1a).

In order to assess the anti-HIV-1 potential, the Ti-GE-Ri-Ter and Ti-GmEm-Ri-Ter cassettes were cloned in a reverse transcriptional orientation in the retroviral vector, MoTN. Amphotropic vector particles were used to allow stable transduction of a CD4+ T lymphoid (MT4) cell line. Constitutive expression of vector (control) or vector (antisense) RNA was confirmed by RT-PCR (results not shown).

HIV-1 challenge of transduced MT4 cells revealed that virus production from MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vector-transduced cells was significantly delayed and inhibited (figure 4). RT-PCR analysis showed that vector (antisense), GE/GmEm, and HIV-1 RNAs were produced in these HIV-1 infected cells (results not shown). The vector (antisense) RNA was also found to be packaged within the virus progeny, whereas the GE mRNA was not. The lack of GE mRNA packaging may be due to its hybridization with excess vector (antisense) RNA. The GAPDH mRNA was not detected in the progeny virus, confirming that the virion RNA was not contaminated with the cellular RNA. As expected, the vector RNA produced from the MoTN vector was not packaged, whereas the HIV-1 RNA was packaged within the progeny virus. Note that the ELISA used to detect progeny virus production would also detect the WT/TDM Gag produced by the MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors. However, the presence of HIV-1 RNA and vector (antisense) RNA within the progeny virus indicates that the progeny virus was produced in the transduced cells.

The TDM Gag and Env incorporate and interfere with the oligomeric protein structures of new viral particles and do not necessarily inhibit progeny virus production. Similarly,  $\Psi^e$  and vector (antisense) RNA-mediated interference at the level of HIV-1 RNA packaging is not likely to inhibit progeny virus production. However, the progeny virus produced was expected to be non-infectious in both cases.

## A combination approach to HIV-1 gene therapy

Both MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors were also tested and shown to inhibit HIV-1 replication in human PBLs (figure 5).

Taken together, we have demonstrated that a combination gene therapy approach based on antisense RNA, both decoy and sense RNAs, as well as TDM Gag and Env proteins can confer excellent inhibition of HIV-1 replication. Better inhibition of HIV replication was observed in MT4 cells transduced with the WT Gag and Env-expression vector (MoTN-Ti-GE-Ri-Ter) than in cells transduced with the TDM Gag and Env-expression vector (MoTN-Ti-GmEm-Ri-Ter). The TAR and RRE decoys could not account for this difference as they were expressed by both MoTN-Ti-GE-Ri-Ter and MoTN-Ti-GmEm-Ri-Ter vectors, and also because we have previously demonstrated that a single TAR or RRE decoy can only confer a partial delay in virus replication (5, 34). As GE mRNA containing the HIV-1  $\Psi^e$  signal was not packaged,  $\Psi^e$  RNA-mediated inhibition of HIV-1 RNA packaging was also not the likely cause of the observed inhibition of HIV-1 replication. As better inhibition of HIV-1 replication was observed in the MT4 cells transduced with the WT Gag and Env-expression vector, inhibition through TDM Gag and Env was ruled out. Therefore, of all the interfering strategies tested in this study, we believe that the use of antisense RNA expressed in a constitutive manner conferred the best inhibition.

In fact, better inhibition of HIV-1 replication was expected from the MoTN-Ti-GmEm-Ri-Ter vector than from the MoTN-Ti-GE-Ri-Ter vector. The observed results do not necessarily indicate that the level of inhibition of HIV-1 replication through TDM Gag and Env was insignificant. Instead, it is possible that TDM Gag and Env protein production was prevented by the vector (antisense) RNA. Note that in the absence of an antibody specific to the TDM Gag or Env proteins, it was not possible to demonstrate whether these proteins were produced in the HIV-1 infected cells. Nonetheless, our results caution against cloning genes in a reverse transcriptional orientation.

A number of retroviral vectors have been described in the literature, in which the genes of interest are cloned in a reverse transcriptional orientation. Reverse transcriptional orientation is chosen mainly for two reasons: to avoid leaky expression of genes that may be cytotoxic or cytotoxic (i.e. Diphtheria toxin) (29, 35), and to avoid the loss of introns from genes (i.e. *beta-globin* gene) (36), which require their presence for efficient expression. Therefore, they cannot be cloned as a cDNA in the forward orientation. Unless self-inactivating vectors were used, the vector RNA produced from the retroviral 5' LTR promoter would contain antisense sequences that would hybridize to the RNA produced from the transcriptional unit cloned in the reverse orientation. This vector (antisense) RNA could inhibit not only the transgene expression, but also the expression of the endogenous gene present within the target cells. Therefore, it is recommended that the vector (antisense) RNA production be avoided for efficient transgene and endogenous gene expression.

## 6. ACKNOWLEDGEMENTS

This work is supported by grants from the Canadian Institutes of Health Research. We thank Mr. P. Rajagopalan for critical proofreading of this article. Flow cytometry was performed by Mr. S. Asad in the laboratory of Dr. S.E. Read at the Hospital for Sick Children. We thank Mr. Azim Ladha for pTev-Puro construction, Dr. D. Trono for dE-neo and VI-neo vectors, Dr. B. Felber for pNL4.1.7.D, and Dr. H. Land for pBabePuro. Pooled anti-HIV serum was obtained from the Ontario Ministry of Health. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 NL4-3 strain from Dr. R.C. Gallo; CD4+ lymphocyte-derived MT4 cell line from Dr. D. Richman; pHenv and pHenv41.2 from Dr. E.O. Freed and Dr. R. Risser; and pHIV-LacZ from Dr. J. Maio.

## 7. REFERENCES

1. Lamothe B. & S. Joshi: Current developments and future prospects for HIV gene therapy using interfering RNA-based strategies. *Front Biosci* 1, D527-D555 (2000)
2. Singwi S. & S. Joshi: Potential nuclease-based strategies for HIV gene therapy. *Front Biosci* 5, 556-579 (2000)
3. Buchschacher J.R. & F. Wong-Staal: Approaches to gene therapy for HIV infection. *Hum Gene Ther* 12, 1013-1020 (2001)
4. Kohn D.B.: Gene therapy for genetic haematological disorders and immunodeficiencies. *J Internal Med* 249, 379-390 (2001)
5. Cohli H, B. Fan, R. L. Joshi, A. Ramezani, X. Li, & S. Joshi: Inhibition of HIV-1 multiplication in a human CD4+ lymphocytic cell line expressing antisense and sense RNA molecules containing HIV-1  $\Psi$  signal and RRE(s). *Antisense Re. Dev* 4, 19-29 (1994)
6. Veres G, S. Escaich, J. Baker, C. Barske, C. Kalfoglou, H. Ilves, H. Kaneshima & E. Böhnlein: Intracellular expression of RNA transcripts complementary to the HIV-1 *gag* gene inhibits viral replication in human CD4+ lymphocytes. *J Virol* 70, 8792-8800 (1996)
7. Veres G, U. Junker, J. Baker, C. Barske, C. Kalfoglou, H. Ilves, S. Escaich, H. Kaneshima & E. Böhnlein: Comparative analyses of intracellularly expressed antisense RNAs as inhibitors of HIV-1 replication. *J Virol* 72, 1894-1901 (1998)
8. Ding S.F, J. Noronha & S. Joshi: Co-packaging of sense and antisense RNAs: a novel strategy for blocking HIV-1 replication. *Nucleic Acids Res* 26, 3270-3278 (1998)
9. Richardson J.H, L.A. Child & A.M. Lever: Packaging of HIV-1 RNA requires *cis*-acting sequences outside the 5' leader region. *J Virol* 67, 3997-4005 (1993)

## A combination approach to HIV-1 gene therapy

10. Liem, S.E, A. Ramezani, X. Li & S. Joshi: The development and testing of retroviral vectors expressing TDMs of HIV-1 proteins to confer anti-HIV-1 resistance. *Hum Gene Ther* 4, 625-634 (1993)
11. Trono D, M.B. Feinberg & D. Baltimore: HIV-1 Gag mutants can dominantly interfere with the replication of the WT virus. *Cell* 59, 113-120 (1989)
12. Mammano F, A. Ohagen, S. Hoglund & .H. G. Gottlinger: Role of the major homology region of HIV-1 in virion morphogenesis. *J Virol* 68, 4927-4936 (1994)
13. Smythe J. A, D. Sun, M. Thomson, P. D. Markham, M. S. Jr. Reitz, R. C. Gallo & J. Lisiewicz: A Rev-inducible mutant gag gene stably transferred into T lymphocytes: an approach to gene therapy against HIV-1 infection. *Proc Natl Acad Sci USA* 91, 3657-3661 (1994)
14. Freed E.O, E. L. Delwart, G. L. Jr. Buchsacher & A. T. Panganiban: A mutation in the HIV-1 transmembrane glycoprotein gp41 dominantly interferes with fusion and infectivity. *Proc Natl Acad Sci USA* 89, 70-74 (1992)
15. Levine F, J. K. Yee & T. Friedmann: Efficient gene expression in mammalian cells from a dicistronic transcriptional unit in an improved retroviral vector. *Gene* 108, 167-174 (1991)
16. Magli M. C, J. E. Dick, D. Huszar, A. Berstein & R. A. Phillips: Modulation of gene expression in multiple hematopoietic cell lineages following retroviral vector gene transfer. *Proc Natl Acad Sci USA* 84, 789-793 (1987)
17. Medina M.F. & S. Joshi: Design, characterization and testing of tRNA<sub>3</sub><sup>Lys</sup>-based hammerhead ribozymes. *Nucl Acids Res* 27, 1698-1708 (1999)
18. Singwi S, A. Ramezani, S. F. Ding & S. Joshi: Targeted RNases: a feasibility study for use in HIV gene therapy. *Gene Ther* 6, 913-921 (1999)
19. Sambrook J, E F Fritsch & T Maniatis: Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, New York (1989).
20. Freed E.O, D. J. Myers & R. Risser: Characterization of the fusion domain of the HIV-1 envelope glycoprotein gp41. *Proc Natl Acad Sci USA* 87, 4650-4654 (1990)
21. Melekhovets Y.F. & S. Joshi: Fusion with an RNA binding domain to confer target RNA specificity to an RNase: design and engineering of Tat-RNase H that specifically recognizes and cleaves HIV-1 RNA *in vitro*. *Nucl Acids Res* 24, 1908-1912 (1996)
22. Laemmli U.K: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685 (1970)
23. Benko D.M, S. Schwartz, G. N. Pavlakis & B. K. Felber: A novel HIV-1 protein, Rev, shares sequences with Tat, and Rev proteins. *J Virol* 64, 2505-2518 (1990)
24. Morgenstern J.P. & H. Land: Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucl Acids Res* 18, 3587-3596 (1990)
25. Chomczynski P. & N. Sacchi: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162, 156-159 (1987)
26. Ramezani A. & S. Joshi: Comparative analysis of five highly conserved target sites within the HIV-1 RNA for their susceptibility to hammerhead ribozyme-mediated cleavage *in vitro* and *in vivo*. *Antisense and Nucl Acid Drug Dev* 6, 229-235 (1996)
27. Mann R, R. C. Mulligan D. Baltimore: Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 33, 153-159 (1983)
28. Miller A.D. & C. Buttimore: Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol Cell Biol* 6, 2895-2902 (1986)
29. Harrison G.S, C. J. Long, F. Maxwell, L. M. Glode & I. H. Maxwell: Inhibition of HIV production in cells containing an integrated, HIV-regulated diphtheria toxin A chain gene. *AIDS Res Hum Retroviruses* 8, 39-45 (1992)
30. Salfeld J, H. G. Gottlinger, R. A. Sia, R. E. Park, J. G. Sodroski & W. A. Haseltine: A tripartite HIV-1 Tat-Env-Rev fusion protein. *EMBO J* 9, 965-970 (1990)
31. Sullenger B.A, H. F. Gallardo, G. E. Ungers & E. Gilboa: Analysis of TAR decoy RNA-mediated inhibition of HIV-1 *trans*-activation. *J Virol* 65, 6811-6816 (1991)
32. Lee, T.C, B. A. Sullenger, H. F. Gallardo, G. E. Ungers & E. Gilboa: Over-expression of RRE-derived sequences inhibits HIV-1 replication in CEM cells. *New Biol* 4, 66-74 (1992)
33. Joshi S, S. F. Ding & S. E. Liem: Co-packaging of non-vector RNAs generates replication-defective retroviral vector particles: A novel approach for blocking retrovirus replication. *Nucl Acids Res* 25, 3199-3203 (1997)
34. Joshi S, A. Van Brunschot, S. Asad, I. Van der Elst, S. E. Read A. Bernstein: Inhibition of HIV-1 multiplication by antisense and sense RNA expression. *J Virol* 65, 5524-5530 (1991)
35. Ragheb J.A, L. Couture, C. Mullen, A. Ridgway & R. A. Morgan: Inhibition of HIV-1 by Tat/Rev-regulated expression of cytosine deaminase, interferon alpha2, or diphtheria toxin compared with inhibition by *trans*-dominant Rev. *Hum Gene Ther* 10, 103-112 (1999)

## A combination approach to HIV-1 gene therapy

36. Sabatino, D.E, N. E. Seidel, G. J. Aviles-Mendoza, A. P. Cline, S. M. Anderson, P. G. Gallagher & D. M. Bodine: Long-term expression of beta-globin mRNA in mouse erythrocytes from retrovirus vectors containing the human *beta-globin* gene fused to the ankyrin-1 promoter. *Proc Natl Acad Sci USA* 97, 13294-13299 (2000)

**Abbreviations:**  $\Psi$ : packaging signal,  $\Psi^e$ : efficient packaging signal, *cat*: chloramphenicol acetyl transferase, DLS: dimer linkage structure, ELISA: enzyme-linked immunosorbent assay, Gag TDM VI: a truncated Gag precursor Pr49<sup>Gag</sup>, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, gp120: 120-kD surface glycoprotein, gp160: 160-kD Env glycoprotein, gp41: 41-kD transmembrane glycoprotein, gp41.2: a TDM Env with a point mutation (from Val to Glu) at the second amino acid of gp41, HIV-1: human immunodeficiency virus-1, HSV: herpes simplex virus, IL-2: recombinant interleukin, IPTG: isopropylthio-beta-galactoside, LTR: long terminal repeat, MoMuLV: Moloney murine leukemia virus, *neo*: neomycin phosphotransferase, PBLs: peripheral blood T lymphocytes, PBS: phosphate buffered saline, Rev: regulator of expression of virion proteins, RRE: Rev response element, SIV: simian immunodeficiency virus, TAR: *trans*-activation response, Tat: *trans*-activator of transcription, TDM: *trans*-dominant negative mutant, *tk*: thymidine kinase, WT: wild type

**Key words:** Antisense RNA, Decoy RNA, Gene therapy, HIV-1, Retroviral vector, sense RNA, *Trans*-Dominant Negative Mutants

**Send correspondence to:** Sadhna Joshi, Department of Medical Genetics and Microbiology, 150 College Street, #212, Faculty of Medicine, University of Toronto, Toronto, Ontario M5S 3E2, Canada. Tel: 416-978-2499, Fax: 416-638-1459, E-mail: sadhnajoshi@yahoo.com