ELSEVIER

Antiviral Research 32 (1996) 99–115

Antiviral Research

Transient protection of human T-cells from human immunodeficiency virus type 1 infection by transduction with adeno-associated viral vectors which express RNA decoys

Clay Smith[a,*], Seong-Wook Lee[b], Ellen Wong[c], Humilidad Gallardo[d], Kevin Page[e], Ofelia Gaspar[c], Jane Lebkowski[e], Eli Gilboa[c]

[a]*Division of Hematology/Oncology, MSRB 421, Box 2606, Duke University Medical Center, Durham, NC 27710, USA*
[b]*Program of Molecular Biology, Memorial Sloan Kettering Cancer Center, New York, NY, USA*
[c]*Division of Experimental Surgery, Duke University Medical Center, Durham, NC, USA*
[d]*Division of Hematology/Oncology, Memorial Sloan Kettering Cancer Center, New York, NY, USA*
[e]*Applied Immune Sciences, Santa Clara, CA, USA*

Received 18 June 1996; accepted 20 June 1996

Abstract

RNA decoys are oligonucleotides corresponding to the TAR and RRE sequences of HIV which inhibit the HIV-encoded regulatory proteins Tat and Rev, respectively. Adeno-associated viral vectors encoding RNA decoys stably transduced into the human T-cell line CEM-SS expressed transactivating region (TAR) and Rev-responsive element (RRE) RNA decoys from tRNA *pol*III promoters at high levels, without any apparent deleterious effects on cell growth or expression of CD4. DNA blot analysis indicated that RNA decoy-encoding vectors were not rearranged and were integrated into the genomic DNA of selected cell lines. Vector DNA with the appropriate TAR and RRE sequences was isolated from transduced cell lines after prolonged growth in culture, further confirming that the vector DNA was present in a stable form through multiple cell cycles. Cell lines expressing TAR and RRE decoys transiently inhibited HIV gene expression and replication by 70–99% as determined by measurement of intracellular and extracellular HIV p24 production. Adeno-associated vectors encoding RNA decoys may be useful for gene therapy of HIV infection.

Keywords: HIV; Gene therapy; Adeno-associated virus vectors; RNA decoys

* Corresponding author. Tel.: +919 6815255; fax: +919 6849424; e-mail: csmith@claydos.mc.duke.edu

PII S0166-3542(95)00987-4

1. Introduction

Treatment of human immunodeficiency virus (HIV) infection with anti-retroviral agents has not been curative, prompting the development of alternative treatment strategies, including gene therapy. One form of gene therapy for HIV infection, termed intracellular immunization, involves the introduction of genetic elements which inhibit HIV expression or replication into cells which are infectable by HIV (Baltimore, 1988). A number of alternative intracellular immunization strategies have been described including transdominant mutant forms of HIV proteins, antisense RNA, ribozymes, HIV-specific intracellular antibodies, and RNA decoys (Gilboa and Smith, 1994; Yu et al., 1994).

RNA decoys are short oligonucleotides which correspond to the transactivating region (TAR) or to the Rev-responsive element (RRE) of HIV RNA. TAR and RRE are involved in critical aspects of HIV expression and, consequently, viral replication (Cullen, 1991). Binding of the HIV-encoded regulatory protein Tat and one or more cellular factors to the TAR sequence enhances HIV transcription. Binding of the HIV-encoded protein Rev to RRE is critical for post-transcriptional HIV RNA processing and transport to the cytoplasm. HIV mutants defective in *tat* and *rev* are severely impaired in their ability to express viral gene products and replicate (Cullen, 1991). TAR and RRE decoys appear to inhibit HIV expression and replication by blocking the binding of Tat and Rev to the authentic TAR and RRE regions (Sullenger et al., 1990a,b; Smith et al., 1991; Lisziewicz et al., 1991; Lee et al., 1992, 1994, 1995; Bevec et al., 1994). In experiments using murine retroviral vectors to stably transduce cells to express RRE and TAR decoys, we have observed over 95% inhibition of HIV-encoded reverse transcriptase (RT) and extracellular p24 production in isolated transduced clones of the human T-cell line CEM-SS. (Sullenger et al., 1990b; Lee et al., 1992, 1994, 1995).

To translate the RNA decoy intracellular immunization strategy into an effective therapy for HIV-infected individuals, it will be necessary to transduce primary human hematopoietic stem cells at high efficiency and achieve durable high-level expression of the RNA decoys. In previous studies, attempts to effectively inhibit HIV in cultures of primary peripheral blood $CD4^+$ T-cells using murine retroviral vectors encoding TAR decoys were limited by relatively low gene transfer efficiencies (5–15%) and limited potency of the original TAR decoy (Sullenger et al., 1990a; Smith et al., 1991). Attempts to transfer genes into primate and human pluripotent hematopoietic stem cells capable of long term hematopoietic reconstitution following transplantation using retroviral vectors have also been limited by the low efficiency of gene transfer (van Beusechem et al., 1992). Retroviral vectors posses several additional inherent disadvantages which may limit their effectiveness in clinical applications to HIV infection. Retroviral vectors integrate randomly or semi-randomly leading to the possibility of insertional inactivation of tumor suppresser genes (Miller, 1992). The LTRs of retroviral vectors contain active transcription elements which may activate downstream genes, including oncogenes. Murine retroviral vector preparations may become contaminated with replication-competent murine retrovirus which may cause lymphoma (Miller, 1992). Retroviral vector-mediated gene transfer also requires cellular proliferation which may impair the biologic function of T-cells and hematopoietic stem cells following transplantation (Miller and Adam, 1990; Dumenil et al., 1989).

Recently, adeno-associated virus (AAV) vectors have been developed as an alternative vector system for achieving stable gene transfer in mammalian cells, including hematopoietic stem cells (Lebkowski et al., 1988; Chatterjee et al., 1992; Muzyczka, 1992; Zhou et al., 1994). AAV is a 4681-bp linear single-stranded DNA virus containing inverted terminal repeat sequences at each end of the genome. AAV vectors capable of stable transduction and integration have been constructed which contain the AAV ITRs and minimal sequences from the remainder of the AAV genome (Muzyczka, 1992). AAV vectors offer several potential advantages relative to retroviral vectors for stem-cell-based gene therapy of HIV infection. AAV vectors may contain few active viral transcriptional regulatory elements in the vector (Muzyczka, 1992). In addition, since wild-type AAV preferentially integrates into a defined

site on chromosome 19 (Kotin et al., 1992; Samulski et al., 1992), it may be possible to engineer recombinant AAV vectors so that they integrate specifically. AAV vector gene transfer may not require proliferation of the target cell so that stem cell function may be less compromised following gene transfer. Despite these potential advantages, currently there is conflicting data as to whether recombinant AAV vectors do undergo site-specific and stable integration into genomic DNA and whether nondividing primary hematopoietic cells can be transduced efficiently (Goodman et al., 1994).

In this study, we sought to determine whether AAV vectors should be considered for RNA decoy-based intracellular immunization approaches to treating HIV infection. AAV vectors were used to introduce RNA decoys into the human CD4$^+$ T-cell line CEM-SS. AAV vector-transduced CEM-SS cells stably expressed RRE and TAR decoys under the transcriptional control of a *pol*III tRNA$_i^{met}$ gene. The AAV vectors were integrated into genomic DNA and the RNA decoy-coding sequences were intact after prolonged passage of the transduced cells. Isolated CEM-SS cell lines expressing these RNA decoys significantly inhibited HIV expression and replication. These studies provide a foundation for examining the utility and safety of AAV vectors encoding RNA decoys in studies involving primary T-lymphocytes and hematopoietic stem cells.

2. Materials and methods

2.1. Construction of tRNA-RNA decoys and retroviral vectors

A 500-bp DNA fragment containing the Δ3-2 mutant tRNA$_i^{met}$ gene (Adeniyi-Jones et al., 1984) was excised from plasmid phH2D with the restriction enzymes *Stu*I and *Bam*HI and cloned into the *Sna*BI site in a 5′ *Apa*I-*Bg*III-*Sna*BI-*Sac*II-*Mlu*I-3′ polylinker in the 3′-LTR of the Moloney murine leukemia virus (MoMLV) N2A (Sullenger et al., 1990b). An oligonucleotide encoding a *Bam*HI site and a *pol*III transcription termination signal (Ter) was inserted downstream of the tRNA, between the *Sac*II and *Mlu*I sites of the polylinker. This vector was called DCT. An oligonucleotide encoding a *Sac*II site and a stem-loop structure was cloned between *Sac*II and *Bam*HI (hpI). A second oligonucleotide (hpII) harboring another stem-loop structure (hpII) was cloned into the *Bam*HI site, and a third oligonucleotide (L) encoding *Sac*II and *Bam*HI sites in the middle of the oligonucleotide was inserted between *Sac*II and *Bam*HI sites. An oligonucleotide containing the 13-nucleotide minimal Rev binding domain of RRE (Tiley et al., 1992), termed mRRE (Fig. 1(A)) was cloned between the *Sac*II and *Bam*HI sites of the modified DCT vector to generate DCT:RRE4 (Lee et al., 1994). An oligonucleotide containing the ARV-2 TAR sequence with hpI but lacking hpII (Fig. 1(A)) was cloned into DCT to generate DCT:TAR3 (Lee et al., 1995). The sequences of the oligonucleotides described above are as follows: Ter-5′-GGTGGATCCGGTTTTTTGCGCA-3′; hpI: 5′-TTAAGCTTGCACTAACCTGTGCAAGCTT-AAGCGGTACCGCGGAGG; hpII: 5′-GATC-CAAGCTTGTACTTGGTCAGGA; L: 5′-GAA-CTCGAGACCGCGGTACGTAGGATCCAG-GCCTAGAAGGCCT; mRRE: 5′-GTTCTGGG-CGCTTCGGCGGTACAGACCGCGGTACGTA G; TAR: 5′-GGGGTCTCTCTGGTTAGACCA-GATCTGAGCCTGGGAGCTCTCTGGCTAA-CTAGGGAACCCACG-3′. The correct sequence of the chimeric tRNA DNA templates was verified by sequencing. Vector DNA was converted to corresponding virus using established procedures (Sullenger et al., 1990a).

2.2. Construction of AAV vectors and preparation of recombinant AAV stocks

AAV vectors were constructed by isolating a 1.7-kb *Eco*RI fragment containing the RRE4 and the TAR3 sequences from the 3′ LTR region of the corresponding N2A-based vector (Lee et al., 1994, 1995). The *Eco*RI fragment was converted to a blunt-ended sequence by treatment with the Klenow fragment of DNA polymerase I and cloned into the *Xho*I site of the AAV plasmid pA1Tneo in both forward and reverse orientations relative to the AAV backbone (Fig. 1(B)).

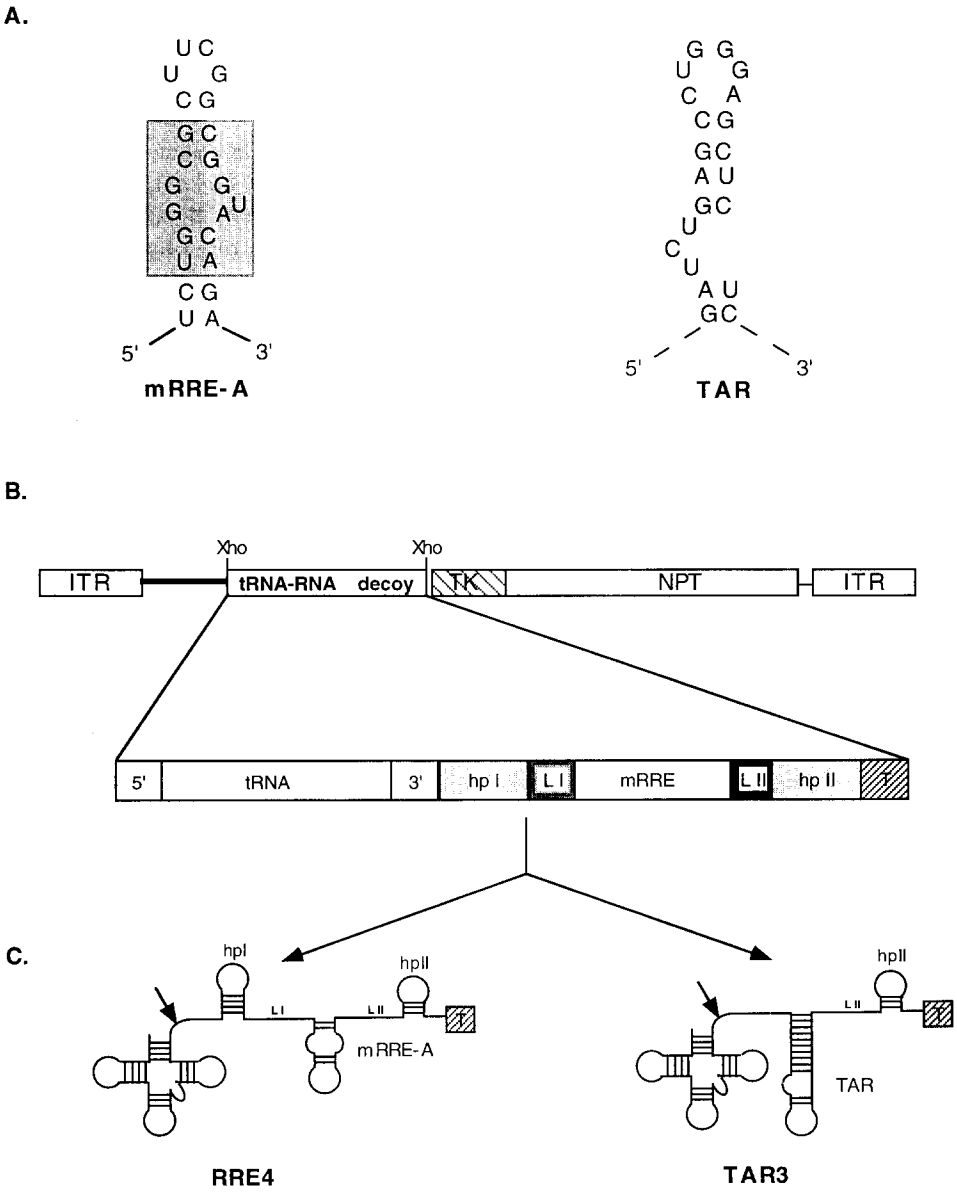

Fig. 1. RNA decoy-encoding AAV vectors. (A) Sequence and predicted secondary structure of the RNA decoy oligonucleotides (the grey box in mRRE-A contains the minimal rev binding domain); (B) structure of the AAV vectors derived from pA1Tneo. The ITRs and the 5′ sequences delineated by the bold line are derived from AAV-2; (C) sequences and schematic secondary structures of the chimeric tRNA–RNA decoys. The arrow indicates the tRNA processing site.

To produce recombinant AAV stocks, 293 cells (American Tissue Culture Collection, CRL1573) were split and grown to an approximate confluency of 60%. The cells were infected with adenovirus type 5 at a multiplicity of infection (MOI) of 5 and incubated for 2 h at 37°C. Each infected cell dish (diameter 10 cm) was then cotransfected with 25 μg of the appropriate pA1Tneo/RNA

decoy recombinant plasmid and 10 μg of pABal, a plasmid encoding the AAV2 Rep and Cap proteins. Transfection was performed using calcium phosphate coprecipitation (Wigler et al., 1979). The transfected cells were incubated at 37°C for a further 16 h; then the medium was removed and replaced with 5 ml of Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). The cells were collected in medium after 60 h of incubation and freeze–thawed three times to lyse the cells. Cellular debris was removed by centrifugation and the clarified supernatant was heated at 56°C for 1 h to inactivate adenovirus. The supernatant was recentrifuged to remove any precipitate and the viral stocks aliquoted and stored at −20°C until needed.

The AAV vector titer was determined by adding serial dilutions of 200 μl of AAV-containing supernatants to 10-cm plates containing 60–70% confluent 293 cells in 10 ml DMEM/10% FCS. After 3 days, the media were replaced with DMEM/10% FCS containing 0.5 mg/ml G418 (Gibco). After an additional 7 days, the media were replaced with DMEM/10% FCS containing 1 mg/ml G418. Subsequently, selection media were changed every 7 days until discrete colonies appeared.

2.3. Transduction of CEM-SS cells

CEM-SS cells were grown in RPMI-1640 medium (Gibco) supplemented with 10% FCS (Hyclone). CEM-SS cells were transduced with retroviral vectors by a 2-h exposure to viral supernatants in the presence of 8 μg/ml polybrene. CEM-SS cells were transduced with AAV vectors by suspending 10^4 cells in 1 μl AAV supernatant (MOI of 5:1) at 37°C for 1 h. The supernatant was removed by centrifugation and the cells were resuspended in RPMI-1640/10% FCS and incubated for an additional 72 h prior to selection in G418. Clonal cell lines were isolated following G418 selection by plating surviving cells at 0.2 cells/well in a 96-well plate.

Viable cell number was determined by Trypan blue exclusion staining and microscopic examination. Measurement of CD4 expression was performed by staining 1×10^6 cells with 20 μl of PE-conjugated OKT4 (Ortho Diagnostics) in phosphate buffered saline (PBS)/4% FCS at 0°C for 30 min, then analyzing washed cells with a FACScan apparatus (Becton Dickenson). All cell samples were over 99% CD4 positive. The mean fluorescence intensity was determined by collecting 10 000 events.

2.4. DNA blot analysis

DNA was isolated from 10^7 cells using the IsoQuick kit (Microprobe) following kit instructions. 10 μg of DNA was digested with restriction endonucleases, fractionated on a 1% agarose gel, transferred to a Biotrans nylon membrane (ICN) with an electroblotter (BioRad), and hybridized with a *neo* gene-specific probe. This probe is a 969-bp DNA fragment excised with the restriction enzymes *Eco*RI and *Sal*I from pA1Tneo and ^{32}P-labeled with an oligolabeling kit (Pharmacia) following kit instructions.

2.5. PCR analysis of tRNA–RNA decoy DNA and RNA from transduced CEM-SS cells

DNA and total nucleic acid were extracted using the IsoQuick kit (Microprobe) following kit instructions. For RNA polymerase chain reaction (PCR), approximately 1 μg of total nucleic acid was treated with 1 unit DNAseI (Promega RQ1 Dnase) to eliminate contaminating DNA. cDNA was prepared using random hexamers, and reverse transcriptase using the Perkin-Elmer Gene Amp kit and following kit instructions. The PCR reaction contained 200 ng of DNA, 0.25 mM dNTPs, 0.75 μM primers, and 0.5 U Taq Polymerase (Perkin-Elmer) in a final volume of 100 μl. For studies involving autoradiography, 5 μCi of ^{32}P-dCTP (Amersham) was added.

The primers for PCR amplification of the tRNA–RNA decoy oligonucleotides were: upstream primer: 5′-AACCTGTGGTAGCCAAAGAAGTAGG-3′; downstream primer: 5′-CGGATCTCCTGACCAAGTACAAGC-3′. PCR was performed using the Ampliwax (Perkin-Elmer) hot start technique in a Perkin-Elmer 4800 Thermal Cycler. Cycle parameters were as fol-

lows: cycle # 1: 5 min at 95°C (denaturation), 1 min at 64°C (annealing) and 1 min at 72°C (extension); Cycle # 2–30: 1 min at 95°C (denaturation), 1 min at 64°C (annealing) and 1 min at 72°C.

For RNA PCR, control reactions lacking reverse transcriptase were performed to confirm that contaminating DNA was eliminated by the DNAse treatment. The PCR products were analyzed by electrophoresis on either 1.2% NuSieve agarose gel (FMC) or 8% polyacrylamide (for ^{32}P-dCTP labeled samples). Agarose gels were visualized with ethidium bromide staining and acrylamide gels were dried and visualized by autoradiography.

For sequencing of PCR products, approximately 10–40 fmol of the PCR DNA product was sequenced using the fmol™ DNA deoxy/dideoxy sequencing kit according to kit instructions (Promega). Sequencing products were electrophoresed on a 6% polyacrylamide–urea denaturing gel and dried gels were visualized with autoradiography.

2.6. RNA blot analysis

Total RNA was isolated by the acid guanidine thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987) and equivalent amounts from each sample were fractionated on an acrylamide–urea gel. RNA was then transferred to a Biotrans nylon membrane (ICN) and prehybridized in 5 × SSC/10 × Denhardt's solution/20 mM Na_2PO_4 (pH 7.2)/7% SDS/100 μg/ml sonicated salmon sperm DNA for 4 h at 65°C. ^{32}P-end-labeled specific oligonucleotide probes and 10% dextran sulfate were added directly to the prehybridization solution, and hybridization was carried out for 16 h at 65°C. Hybridized membranes were washed once in 3 × SSC/5% SDS/10% Denhardt's solution/20 mM Na_2PO_4 (pH 7.2) at 65°C and once in 1 × SSC/1% SDS at 65°C. Total tRNA was detected by hybridizing stripped blots with a human $tRNA_i^{met}$ DNA probe as previously described (Lee et al., 1994).

The sequences of the RNA probes were: RRE4 probe: 5′-GATCCTACGTACCGCGGTCTG-TACCGCCGAAGCGCCCAGAACGC-3′; TAR-3 probe: 5′-GATCCGTGGGTTCCCTAGTT-AGCCAGAGAGCTCCCAGGCTCAGATCTG-GTCTAACCAGAGAGACCCCGC-3′.

2.7. Infection of CEM-SS cells with ARV-2

The ARV-2 isolate of HIV-1 (provided by Dr Cheng and Dr Levy) was propagated in HUT 78 cells and harvested as previously described (Sullenger et al., 1990b). 2×10^5 CEM-SS cells were infected with 200 $TCID_{50}$ units of ARV-2 virus for 2 h at 37°C in the presence of 5 μg/ml of polybrene. Cells were washed once and resuspended in the original volume of RPMI-1640/10% FCS. Cells were passaged at a 1:4 dilution in fresh media every 3–4 days.

2.8. FACS analysis of HIV-infected CEM-SS cells for intracellular p24

10^6 cells were washed twice in PBS/2% FCS, resuspended in 50 μl of PBS/2% FCS and 20 μl of PE-conjugated OKT4 (Ortho Diagnostics) was added. Cells were then washed twice with PBS, then resuspended in 195 μl of 0.5 mg/ml digitonin (Sigma) and 5 μl of a p24-specific FITC-conjugated antibody (KC-57, Coulter). Cells were held on ice for thirty min, then washed twice with a 10-fold volume of 5% Tween-20 (Sigma). Stained cells were resuspended in 0.05% paraformaldehyde and 10 000 events were analyzed on a FACScan apparatus. Irrelevant FITC-conjugated isotypic mAbs were used as controls for each experiment. Cursors were set to define p24 positive cells so that less than 1% of the isotypic control stained cells were detected as positive.

2.9. Extracellular p24 measurements

The concentration of HIV-1 p24 antigen was determined in 100 μl of cell-free supernatants using a p24-specific enzyme-linked immunosorbent assay (ELISA) (Dupont NEK-060) following kit instructions. Supernatants obtained at progres-

sive time points were serially diluted and the p24 values were determined from the dilution yielding measurements on the linear part of a standard p24 curve.

2.10. In situ immunofluorescence staining for HIV

10^5 cells were washed once with PBS, applied to microscope slides, and fixed with cold methanol acetone (2:1). Cells were incubated with human anti-HIV serum diluted 1:80 for 1 h at 37°C, washed with PBS, and incubated with goat anti-human antibody (Cappel) diluted 1:50. Cells were washed with PBS, counterstained with 0.03% Evan's blue, and visualized with a confocal microscope (Biorad). Pseudocolor was applied to the digitized images using the Aldus Photoshop program (Aldus). All manipulations to digitized images were applied equally to control and experimental samples.

3. Results

3.1. Construction of AAV vectors encoding RNA decoys and transduction of CEM-SS cells

The recombinant AAV vectors constructed for these studies are described in Fig. 1. These vectors were derived from pA1Tneo which contains 625 nucleotides of the upstream region of AAV-2 including the left-hand ITR, the neomycin resistance gene fused to the herpes simplex virus thymidine kinase promoter, and the right hand ITR of AAV-2. The RNA decoy coding sequences were fused to a tRNA sequence to drive RNA decoy expression, since previous studies indicated that tRNA polymerase III promoters generate high levels of RNA oligonucleotides (Sullenger et al., 1990a; Lee et al., 1992, 1994). An RRE decoy containing the minimal 13-nucleotide primary Rev binding domain of RRE (mRRE) was used rather than the full-length RRE sequence, since the short RRE-derived decoy may not bind to cellular factors like the full-length RRE and therefore may be safer (Lee et al., 1994). The TAR decoy contains the entire TAR sequence since prior experiments indicated this was required for inhibition of Tat (Sullenger et al., 1991). The tRNA–RNA decoy sequences contain a wild-type tRNA processing site to facilitate cleavage of the chimeric molecule to yield a liberated RNA decoy sequence. The RNA decoy sequences were constructed to contain additional hairpin sequences in order to increase the stability of the liberated RNA decoy following processing from the chimeric tRNA–RNA decoy transcript. Prior studies indicate that this strategy may improve the potency of the RNA decoys (Lee et al., 1994, 1995).

The tRNA–RNA decoy sequences were cloned into the pA1Tneo vector in both forward and reverse orientations relative to the vector. Unless otherwise indicated, all experiments described below were performed with vectors containing the tRNA–RNA decoy sequence in the forward orientation. The AAV vector derived from the pA1Tneo backbone was termed A1TN. The AAV vector expressing the minimal RRE sequence in the forward orientation was termed A1TN/RRE4 and the vector expressing the 63-nucleotide sequence containing TAR in the forward orientation was termed A1TN/TAR3. The total length of A1TN/RRE4 and A1TN/TAR3 is about 3.7 kb, 80% of the length of the wild-type genome.

Recombinant AAV vectors expressing RRE4 and TAR3 were produced following transient transfection of 293 cells. AAV vector titers were determined to be 10^4–10^5 transducing infectious virions per ml as measured by resistance to G418. These titers are consistent with titers of other pA1Tneo-derived AAV vectors (data not shown). Since pA1Tneo contains a portion of the AAV coding sequence for the *rep* proteins Rep68 and Rep78 and these proteins have been associated with cellular toxicity (Muzyczka, 1992), transduced cells were analyzed for abnormalities in growth or expression of CD4. Three clonal lines for each vector were derived by single-cell cloning following transduction with A1TN, A1TN/TAR3 and A1TN/RRE4 and G418 selection. No significant differences in the mean growth rates and mean CD4 expression were noted between the transduced cell lines and nontransduced CEM-SS cells for the duration of the 30-day cultures (Fig. 2(A) and (B)). These characterized lines were used

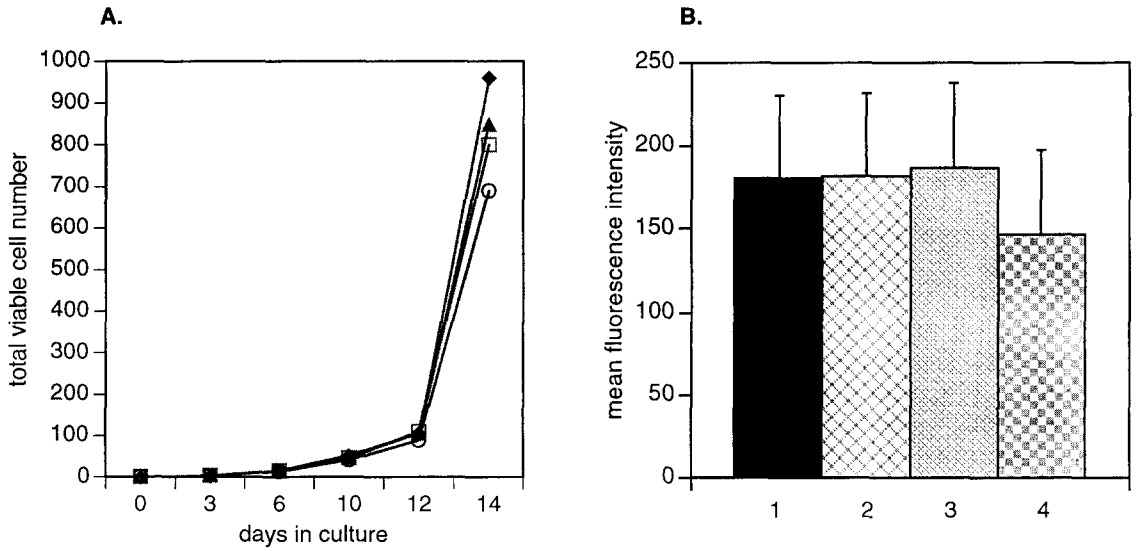

Fig. 2. Cell growth and CD4 expression following transduction with RNA decoy-encoding AAV vectors. (A) The total viable cell number of clonal lines derived from untransduced cells (○) and bulk selected cells transduced with A1TN (□), A1TN/RRE4 (▲), and A1TN/TAR3 (◆) was measured at progressive time points. (B) Expression of CD4 was determined by measuring the mean fluorescence intensity of OKT4 stained untransduced cells (1) and clonal lines transduced with A1TN (2), A1TN/RRE4 (3), and A1TN/TAR3 (4). In both figures, the data represent the mean and standard deviation of measurements performed on three independant cell lines derived from each bulk population.

for all other studies described below which utilized selected clonal cell lines.

3.2. Characterization of RNA decoy-encoding AAV vectors in CEM-SS cells

Previous studies have found that recombinant AAV vectors may not stably integrate into genomic DNA or may yield rearranged proviral genomes following transduction and cell proliferation (Goodman et al., 1994; Winocour et al., 1992). To evaluate these concerns, we examined the proviral structure and RNA decoy coding sequence in representative clonal cell lines after prolonged passage in culture. DNA blotting performed on DNA digested with the restriction enzymes *Eco*RI and *Sal*I yielded the expected 969-bp fragment from all three vectors and no other bands, indicating that large-scale rearrangements of the vector had not occurred (Fig. 3(B)). Digestion of DNA obtained from the A1TN/RRE4 and A1TN/TAR3 transduced lines with *Eco*RI alone yielded bands which were larger than any fragments which would be predicted from episomal genomes including concatemerized episomes (Fig. 3(A) and (C)). This finding indicates that the RNA decoy AAV vectors had integrated into chromosomal DNA. The clonal line transduced with A1TN/TAR3 yielded only one band at over 23 kb, indicating that only one A1TN/TAR3 vector had integrated into the host chromosomal DNA. The clonal line transduced with A1TN/RRE4 yielded several bands indicating that more than one vector may have integrated. Interestingly, the clonal line transduced with the control vector A1TN yielded an intense band which corresponds in size to the intact vector in addition to a larger band (Lane 2, Fig. 3(C)). This indicates that the vector may have persisted in this line both as an integrated provirus and either a circular monomeric episome or as an integrated head-to-tail concatemer. The intensity of the band indicates that in either form, multimerization has occurred.

To further confirm that the integrated proviral sequence was intact and stable, the tRNA–RNA

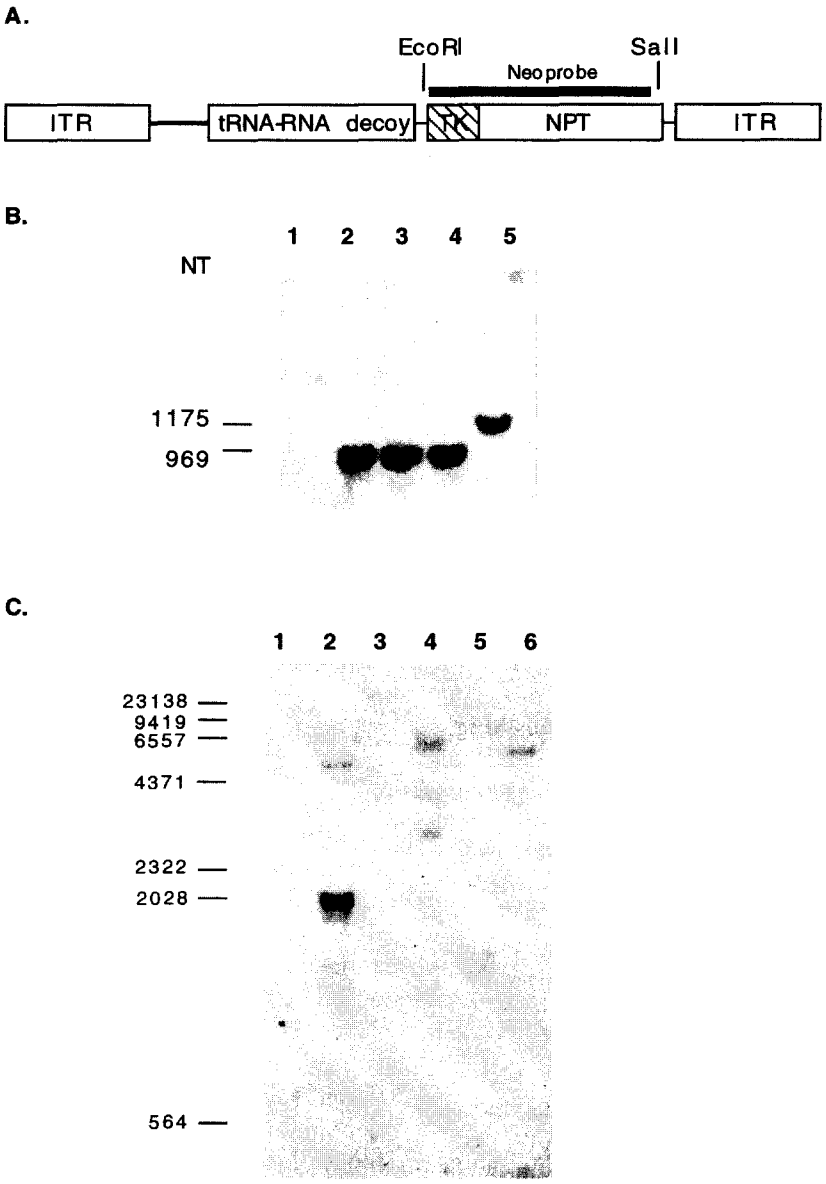

Fig. 3. AAV vector stability in transduced CEM cells. (A) Expected structure of the integrated AAV RNA decoy provirus. *Eco*RI and *Sal*I both cut at single sites within the vector; (B) DNA blot analysis of *Eco*RI and *Sal*I digested DNA extracted from untransduced CEM-SS cells (lane 1), A1TN clone #2 (lane 2), A1TN/TAR3 clone #1 (lane 3), A1TN/RRE4 clone #2 (lane 4) and CEM-SS transduced with the retroviral vector DCT/RRE5 (lane 5); (C) DNA blot analysis of DNA digested with *Eco*RI alone extracted from untransduced CEM-SS cells (lane 1), A1TN clone #2 (lane 2), A1TN/TAR3 clone #1 (lane 3—a weak band at >23 kb), A1TN/RRE4 clone #2 (lane 4), and CEM-SS transduced with the retroviral vector DCT (expected size 1.55 kb) (lane 5) and the plasmid pA1Tneo (expected size 5.2 kb) (lane 6).

decoy coding sequence from the three characterized A1TN/TAR3 and A1TN/RRE4 transduced clonal cell lines was amplified by DNA PCR following passage of the cell lines for over 60 days

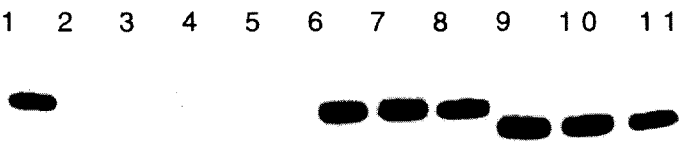

Fig. 4. RNA decoy DNA stability in AAV-transduced CEM cells. DNA PCR analysis of CEM-SS transduced with the retroviral vector DCT:RRE4 (lane 1), a negative control containing only reagents (lane 2), and A1TN clones # 1–3 (lanes 3–5), A1TN/RRE4 clones # 1–3 (lanes 6–8) and A1TN/TAR3 # 1–3 (lanes 9–11). In lanes 1 and 6–9, the bands migrate at the expected location of 354 bp and in lanes 9–11, the bands migrate at the expected location of 325 bp.

(over 30 population doublings). Amplified sequences were the expected size from all cell lines (Fig. 4) and sequencing of these fragments confirmed that the RNA decoy coding sequences were intact (data not shown).

3.3. Expression and processing of tRNA–RNA decoys in AAV vector transduced cells

RNA decoy expression from AAV vectors was evaluated initially by RNA PCR analysis of G418 selected bulk populations of CEM-SS cells (Fig. 5(A)). RNA of the appropriate size was detected in bulk cells transduced with A1TN/RRE4 and A1TN/TAR3, indicating that no significant deletions had occurred in the RNA decoy RNA sequence. In order to assess RNA expression more quantitatively, RNA blot analysis of G418 selected bulk CEM-SS cells transduced with A1TN/RRE4 and A1TN/TAR3 was performed and compared to CEM-SS cells transduced with the retroviral vectors DCT:RRE4 and DCT:TAR3. Expression of TAR3 decoy RNA was equivalent between bulk AAV vector transduced cells and a cell line transduced with a murine retroviral vector previously characterized as expressing high levels of TAR3 (Fig. 5(B)). Expression of mRRE was also similar in CEM-SS cells transduced with A1TN/RRE4 and in a cell line transduced with the murine retroviral vector DCT:RRE4 (data not shown).

In AAV vector transduced cells, processing of the chimeric tRNA-TAR decoy molecule occurred identically to processing in retrovirally transduced cells (Fig. 5(B)). Two bands were visualized with RNA blot analysis, the 209-nucleotide unprocessed tRNA-TAR decoy oligonucleotide and a second 137-nucleotide band representing the processed TAR decoy sequence. Processing of the chimeric tRNA-mRRE molecule also occurred appropriately (data not shown).

3.4. Inhibition of HIV in AAV vector transduced cells

In order to quantitate HIV infection and late gene expression within individual cells, a flow cytometric assay was developed based on immunofluorescence staining for intracellular p24. The advantage of this assay is that subpopulations of cells which inhibit HIV to varying degrees can be identified within heterogeneous populations of cells. This is in contrast to assays of HIV expression and replication which rely on measuring p24 antigen concentration or RT activity in the media of cell cultures. In these latter assays, the p24 which is measured reflects the average of the entire population and fails to discriminate between subpopulations which inhibit HIV from populations which support HIV expression and replication. Simultaneous staining for CD4 in addition to intracellular p24 allows greater discrimination between p24-positive and -negative cells. In addition, measurement of CD4 staining indicates that inhibition of p24 expression is not merely due to overgrowth of the cultures by CD4-negative cells.

To evaluate the effectiveness of AAV-vector-mediated inhibition of HIV via expression of RNA decoys, selected clonal cell lines were challenged with HIV-1 and analyzed using the intra-

cellular p24 assay at progressive time points until maximal expression of intracellular p24 was observed in control cells (approximately 24 days). Fig. 6 depicts a representative FACS analysis performed 24 days following HIV-1 infection. At this time, over 90% of CEM cells expressed p24 and failed to express CD4 (Fig. 6(C)) and over 85% of A1TN transduced cells expressed p24 and failed to express CD4 (Fig. 6(D)). In contrast, p24 expression was significantly inhibited in the two cell lines transduced with the retroviral vectors DCT:RRE4 (Fig. 6(E)) and DCT:TAR3 (Fig. 6(G)) and in cell lines transduced with the AAV vectors A1TN/RRE4 (Fig. 6(F)) and A1TN/TAR3 (Fig. 6(H)).

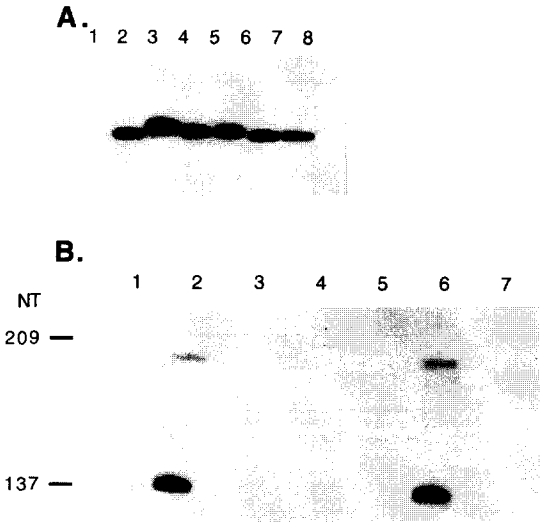

Fig. 5. Expression of RNA decoys in AAV-transduced CEM cells. (A) RT PCR analysis of RNA obtained from a reagent negative control (lane 1), CEM-SS transduced with the retroviral vector DCT:RRE4 (lane 2), plasmid AW108 positive control (lane 3), bulk CEM-SS cells transduced with A1TN/RRE4 in forward and reverse orientations (lanes 4 and 5) and A1TN/TAR3 in forward and reverse orientations (lanes 6 and 7) and nontransduced CEM-SS cells (lane 8). (B) RNA blot analysis of TAR3 RNA transcripts expressed in bulk populations of CEM-SS cells using a ^{32}P-labeled TAR-specific oligonucleotide probe. Samples include CEM-SS cell transduced with DCT:RRE4 (lane 1), DCT:TAR3 (lane 2), untransduced (lane 3), A1TN/RRE4 (lane 4, 5), A1TN/TAR3 (lane 6) and A1TN (lane 7). The 209-nucleotide band corresponds to unprocessed tRNA-TAR transcripts while the 137-nucleotide band corresponds to the liberated TAR oligonucleotide.

A time course of intracellular p24 expression following HIV-1 infection in the three characterized cell lines transduced with A1TN/RRE4 as well as negative and positive control cell lines is depicted in Fig. 7(A). A similar time course for three cell lines transduced with A1TN/TAR3 is depicted in Fig. 7(B). Three out of three cell lines transduced with A1TN/TAR3 and one of three cell lines transduced with A1TN/RRE4 significantly inhibited HIV-1 compared to controls transduced with A1TN for the duration of the cultures. In all three cell lines transduced with A1TN/TAR3, inhibition of p24 expression was more pronounced than in a clonal cell line transduced with the murine retroviral vector DCT:TAR3. Inhibition of p24 was similar between A1TN/RRE4 cell line # 2 and a cell line transduced with the murine retroviral vector DCT:RRE4. This latter cell line had the highest degree of inhibition from among eight DCT:RRE4 individual transduced cell lines previously analyzed (Lee et al., 1994, and data not shown). In two out of three cell lines transduced with the control vector A1TN, a slight delay in p24-expressing cells was noted, but by day 24, all three A1TN transduced cell lines expressed p24 at levels similar to nontransduced CEM cells (Fig. 7(A) and data not shown). The third A1TN transduced control cell line expressed p24 identically to nontransduced CEM cells for the duration of the cultures. To confirm that inhibition of viral production was also occurring in addition to inhibition of intracellular p24 production, measurements of extracellular p24 were performed on the cell lines demonstrating optimal inhibition of intracellular p24 (Fig. 8). Consistent with the intracellular p24 analysis, AAV encoded TAR and RRE decoys significantly inhibited production of extracellular p24. Between days 20 and 24, there was an increase in intracellular and extracellular p24 production in all cultures where HIV was originally inhibited, indicating that inhibition was transient.

To confirm the effectiveness of AAV transduced RNA decoys in preventing spread of HIV, cells were obtained 24 days following infection

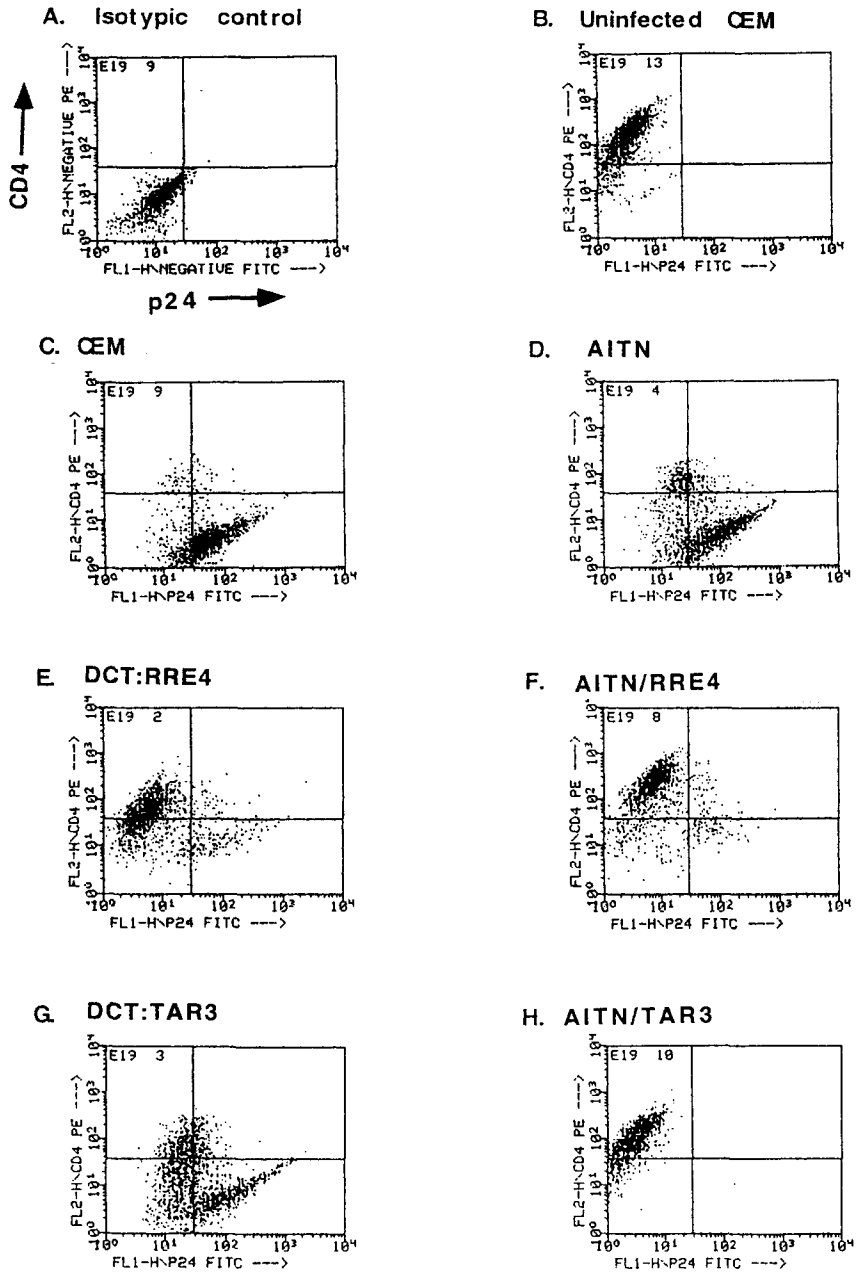

Fig. 6. Intracellular p24 expression and CD4 expression in retroviral vector and AAV vector transduced cells infected with HIV-1. Controls include uninfected CEM-SS cells stained with isotypic negative control antibodies (panel A) and uninfected CEM-SS cells stained for intracellular p24 and extracellular CD4 (panel B). Panels C–H depict cells stained for intracellular p24 extracellular CD4 24 days following infection with HIV-1. These samples include untransduced CEM-SS cells (panel C), A1TN clone # 2 (panel D), DCT:RRE4 clone # 2 (panel E), A1TN/RRE4 clone # 2 (panel F), DCT:TAR3 clone # 5 (panel G), and A1TN/TAR3 clone # 1 (panel H).

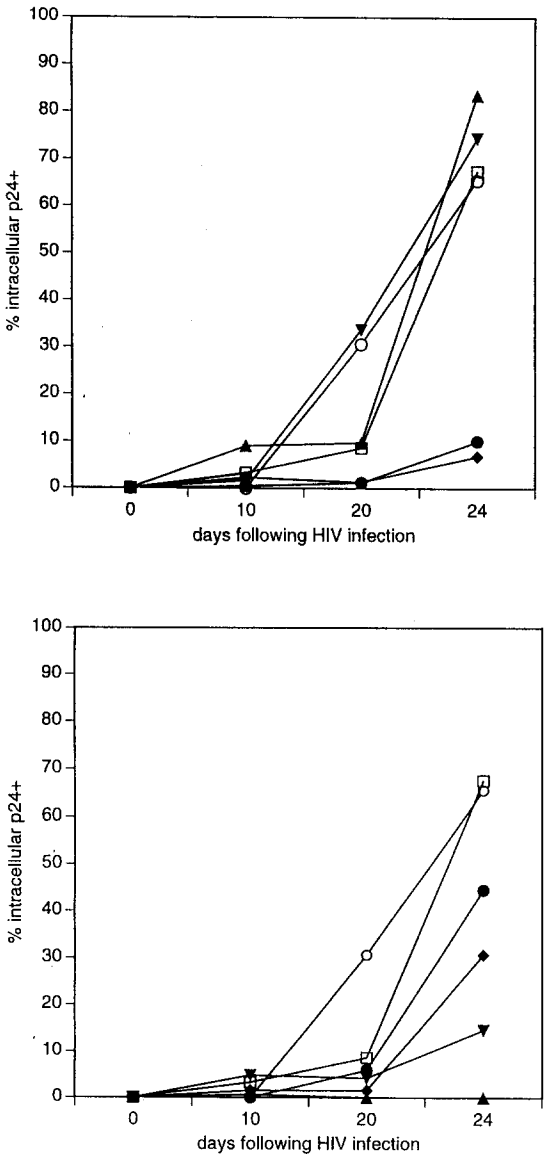

Fig. 7. Time course of intracellular p24 expression in RRE4 transduced cells infected with HIV-1. Samples include untransduced CEM-SS cells (○), A1TN clone #2 (□), DCT:RRE4 clone #2 (●), A1TN/RRE4 clone #1 (▲), A1TN/RRE4 clone #2 (◆) and A1TN/RRE4 clone #3 (▼). The proportion of intracellular p24 was determined at progressive time points following HIV-1 infection using FACS analysis as described in Fig. 6. (B) Time course of intracellular p24 expression in TAR3 transduced cells infected with HIV-1. Samples include untransduced CEM-SS cells (○), A1TN clone #2 (□), DCT:TAR3 clone #5 (●), A1TN/TAR3 clone #1 (▲), A1TN/TAR3 clone #2 (◆) and A1TN/TAR3 clone #3 (▼).

and immunofluorescence staining was performed with HIV-1-specific anti-serum (Fig. 9). Again, little or no immunofluorescence was noted in selected cell lines transduced with AAV RNA decoy vectors (Fig. 9(C) and (D)) compared to A1TN transduced control cells which exhibited extensive staining with HIV-1-specific anti-serum (Fig. 9(B)).

4. Discussion

In this study, we evaluated AAV vectors encoding RNA decoys as reagents for gene therapy of HIV infection. Our observations indicate that AAV vectors encoding RNA decoys can be produced at useful titers and transduce immortalized CD4$^+$ human T-cell lines. No adverse effect of AAV vector transduction and RNA decoy expression was noted on two general aspects of cell physiology, cell growth and CD4 expression (Fig. 2). DNA blot analysis of G418 selected clonal cell lines indicated that the AAV vectors encoding RNA decoys stably integrated into chromosomal DNA without significant rearrangements (Fig. 3(B) and Fig. 4). DNA sequence analysis confi-

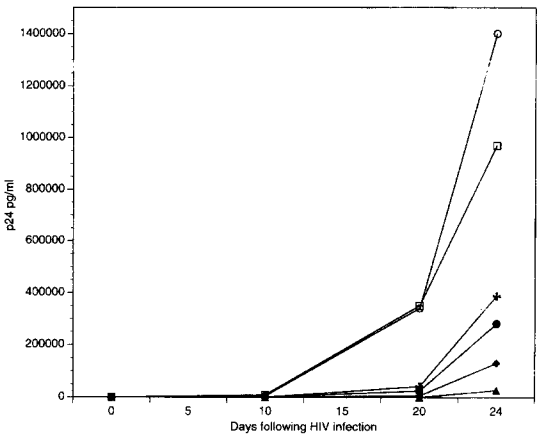

Fig. 8. Time course of extracellular p24 production in AAV vector transduced cell lines challenged with HIV-1. p24 production per ml of culture supernatant was measured at progressive time points following HIV-1 infection from untransduced CEM-SS cells (○), A1TN clone #2 (□), DCT:RRE4 clone #2 (●), DCT:TAR3 clone #5 (×), A1TN/RRE4 clone #2 (◆), and A1TN/TAR3 clone #1 (▲).

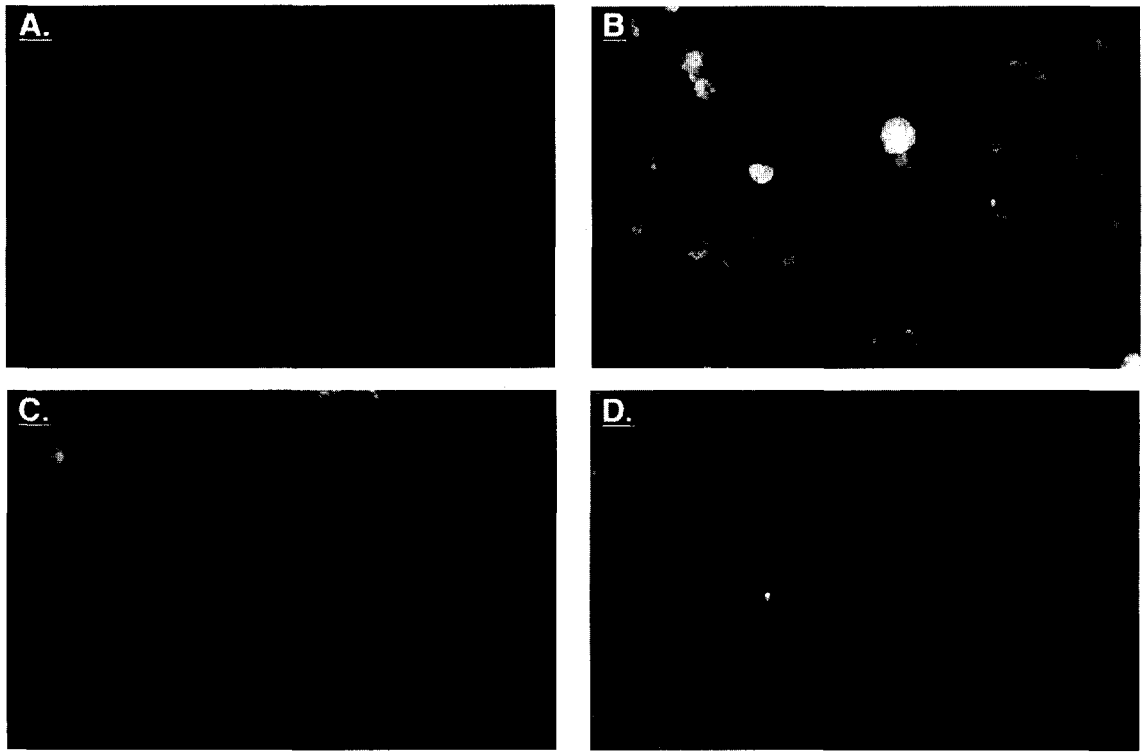

Fig. 9. Immunofluorescence staining with HIV-specific anti-serum of cells infected with HIV-1. Cells were stained 24 days following HIV-1 infection. Samples include uninfected CEM-SS (A), and 3 HIV-1-infected cell lines: A1TN clone # 2 (B), A1TN/RRE4 clone # 2 (C) and A1TN/TAR3 clone # 1 (D).

rmed that the coding sequence for the RNA decoy expression cassette remained intact despite prolonged passage of the transduced cells.

DNA blot analysis of DNA digested with the single internal cutter *Eco*R1 revealed several interesting features of AAV vector transduced cells. First, the finding that different size bands are generated in different transduced clones (see Fig. 3(C)) indicates that site-specific integration may not have occurred, confirming previous reports (Berns and Linden, 1995). In addition, in the clonal line transduced with the parental AAV vector A1TN, an intense band is seen at 2.0 kb. This size band corresponds to a fragment generated from either a monomeric nonintegrated episome, a head-to-tail concatemerized nonintegrated episome, or a multimeric integrated head-to-tail concatamer (Fig. 3(A) and (C)). The fact that this band was observed after the clonal cell line had gone through over 30 population doublings attests to the stability of this vector form. Regardless of the vector form, DNA blot analysis of DNA extracted from the same clonal cell line digested with two internal cutters did not reveal any significant bands corresponding to rearranged provirus (Fig. 3(B), lane 2). In addition to stability of the vector DNA, we observed that the AAV vectors were capable of long-term high-level expression of RNA decoys (Fig. 5(A) and (B)).

CEM-SS cell lines transduced with AAV vectors expressing RNA decoys inhibited acute infection with HIV-1 as well as cell lines transduced with retroviral vectors expressing RNA decoys. Both intracellular expression of p24 and extracellular production of p24 were significantly inhibited for extended periods of time, compared with control cell lines (Figs. 6–8). Immunofluorescence staining of HIV-1-infected transduced cells further

confirmed that inhibition of HIV-1 expression and replication had occurred (Fig. 9). Significant heterogeneity in the degree of HIV inhibition was noted between different clones of A1TN/TAR3 transduced CEM-SS cells (Fig. 7(B)). In addition, two of three A1TN/RRE4 cell lines failed to inhibit HIV-1 replication (Fig. 7(A)). This heterogeneity is not due to differences in CD4 expression between different clones as all had similar levels of surface CD4 (Fig. 2(B)). While the reason for this heterogeneity remains unclear, variation in resistance to HIV-1 between different transduced clonal lines has been observed by ourselves and others in prior experiments involving murine retroviral vector-mediated inhibition of HIV (Lee et al., 1994). We have never observed spontaneous resistance of CEM-SS cells to HIV-1 in dozens of previous experiments, indicating that the resistance of the single HIV-resistant A1TN/RRE4 transduced cell line is due to the RNA decoy. In addition, towards the ends of the cultures, breakthrough of HIV-1 production occurred. This could have been due to either production of escape mutants which are resistant to RNA decoys or insufficient potency of the RNA decoys due to inadequate expression or intrinsic limitations of the decoys. Currently, we are evaluating newer RNA decoys and combinations of RNA decoys to identify improved HIV inhibitors.

All three cell lines transduced with AITN/TAR3 inhibited HIV more effectively than a murine retroviral vector transduced line expressing the same TAR decoy (Fig. 7(B)). While the small number of clones analyzed precludes definite conclusions, it is possible that the TAR3 decoy is synergizing with some subtle effect produced by the AITN backbone. This is also suggested by the fact that a slight lag in p24 expression was observed in two of three cell lines transduced with A1TN compared to non-transduced cells (Fig. 7(A) and (B)). One possible explanation for this effect is that a portion of one or more of the *rep* proteins may be expressed from the 5′ sequence of pA1Tneo-derived vectors. This region of pA1Tneo contains a portion of the AAV *rep* coding sequence and the AAV p5 promoter. The Rep78 and Rep68 proteins transcribed from the p5 promoter appear to inhibit HIV transcriptional activation by interacting with sequences within the HIV LTR or possibly with 5′ noncoding regions of the HIV RNA (Oelze et al., 1994; Antoni et al., 1991). Since this inhibition appears to be antagonized by Tat, inhibition of Tat with TAR decoys may enhance any rep-mediated inhibition of HIV. Currently, this remains speculation, since the regions of Rep78 and Rep68 which mediate this effect are unknown and it is unknown whether A1TN actually encodes any portion of the *rep* proteins. If this observation is confirmed and it appears to be due to some portion of one of the *rep* proteins, synergism between TAR decoys and *rep* should be further evaluated for therapeutic applications.

In summary, AAV vectors encoding RNA decoys derived from pA1Tneo are capable of stably transducing isolated cell lines of CEM-SS cells without obvious adverse effects. The AAV vector coding sequences for the RNA decoys appear stable with prolonged cell passage and AAV vectors appear to be as effective at inhibiting HIV-1 as comparable retroviral vectors. While these observations provide a foundation for considering using AAV vectors encoding RNA decoys for gene therapy of HIV, a number of significant issues remain to be addressed before proposing clinical applications of these vectors. These issues include defining the efficiency of gene transfer of RNA decoy-encoding AAV vectors in primary T-lymphocytes and hematopoietic stem cells and determining whether these vectors can protect bulk, polyclonal populations of primary cells from HIV infection. In addition, it will be important to determine whether RNA decoy-encoding AAV vectors can stably transduce nonproliferating $CD4^+$ T-cells and hematopoietic stem cells and whether these vectors undergo site-specific integration. We are currently carrying out experiments with RNA decoy-encoding AAV vectors designed to address these complicated concerns and to further define the advantages and disadvantages of these vectors relative to retroviral vectors for use in HIV gene therapy.

Acknowledgements

C.S. is supported by the NIH (1-KO8-AI01121-01A1, RFA93-AI-14), and the Pediatric AIDS Foundation. E.G. is supported by the NIH (R01-AI-93-13). We gratefully acknowledge the excellent assistance of the Duke University Comprehensive Cancer Center Flow Cytometry Core Facility.

References

Adeniyi-Jones, S., Romeo, P. and Zasloff, M. (1984) Generation of long read-through transcripts in vivo and in vitro by deletion of 3′ termination and processing sequences in the human $tRNA_i^{met}$ gene. Nucleic Acids Res. 12, 1101.

Antoni, B.A., Rabson, A.B., Miller, I.L., Trempe, J.P., Chejanovsky, N. and Carter, B.J. (1991) Adeno-associated virus Rep protein inhibits human immunodeficiency virus type 1 production in human cells. J. Virol. 65, 396.

Baltimore, D. (1988) Gene therapy. Intracellular immunization [news]. Nature 335, 395.

Berns, K.I. and Linden, R.M. (1995) The cryptic life style of adeno-associated virus. Bioessays 7, 237.

Bevec, D., Volc-Platzer, B., Zimmermann, K., Dobrovnik, M., Hauber, J., Veres, G. and Bohnlein, E. (1994) Constitutive expression of chimeric Neo-Rev response element transcripts suppresses HIV-1 replication in human $CD4^+$ T lymphocytes. Hum. Gene Ther. 5, 193.

Chatterjee, S., Johnson, P.R. and Wong, K.K.J. (1992) Dual-target inhibition of HIV-1 in vitro by means of an adeno-associated virus antisense vector. Science 258, 1485.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156.

Cullen, B.R. (1991) Regulation of HIV-1 gene expression. FASEB J. 5, 2361.

Dumenil, D., Jacquemin-Sablon, H., Neel, H., Frendal, E. and Dautry, F. (1989) Mock retroviral infection alters the developmental potential of murine bone marrow stem cells. Mol. Cell Biol. 9, 4541.

Gilboa, E. and Smith, C. (1994) Gene therapy for infectious diseases, the AIDS model. Trends Genet. 10, 139.

Goodman, S., Xiao, X., Donahue, R., Moulton, A., Miller, J., Walsh, C., Young, N., Samulski, R. and Nienhuis, A. (1994) Recombinant AAV mediated gene transfer into hematopoietic progenitor cells. Blood 84, 1492.

Kotin, R.M., Linden, R.M. and Berns, K.I. (1992) Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. EMBO J. 11, 5071.

Lebkowski, J.S., McNally, M.M., Okarma, T.B. and Lerch, L.B. (1988) Adeno-assoiated virus: a vector system for efficient introduction and integration of DNA into a variety of mammalian cell types. Mol. Cell Biol. 8, 3988.

Lee, T., Sullenger, B., Gallardo, H., Ungers, G. and Gilboa, E. (1992) Overexpression of RRE-derived sequences inhibits HIV-1 replication in CEM cells. N. Biol. 4, 66.

Lee, S.W., Gallardo, H., Gilboa, E. and Smith, C. (1994) Inhibition of HIV-1 in human T-Cells by a potent RRE decoy comprised of the 13 nucleotide long minimal Rev binding domain. J. Virol. 68, 8254.

Lee, S.W., Smith, C., Gallardo, H. and Gilboa, E. (1995) Inhibition of HIV by highly expressed trans-activating response RNA decoys. Gene Ther. 2, 377.

Lisziewicz, J., Rappaport, J. and Dhar, R. (1991) Tat regulated production of multimerized TAR RNA inhibits HIV-1 gene expression. N. Biol. 3, 82.

Miller, A.D. (1992) Retroviral vectors. Curr. Top. Microbiol. Immunol. 158, 1.

Miller, D. and Adam, M. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol. Cell Biol. 10, 4239.

Muzyczka, N. (1992) Use of adeno-associated virus as a general transduction vector for mammalian cells. Curr. Top. Microbiol. Immunol. 158, 97.

Oelze. I., Rittner, K. and Sczakiel, G. (1994) Adeno-associated virus type 2 *rep* gene-mediated inhibition of basal gene expression of human immunodeficiency virus type 1 involves its negative regulatory functions. J. Virol. 68, 1229.

Samulski, R.J., Zhu, X., Xizo, X., Brook, J.D., Housman, D.E., Epstein, N. and Hunter, L.A. (1992) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J. 10, 3941.

Smith, C., Sullenger, B., Gallardo, H.F., Ungers, G.E., Terry, L. and Gilboa, E. (1991) Resistance to HIV in human peripheral blood $CD4^+$ lymphocytes genetically altered via a retroviral vector expressing TAR decoys. Blood 78 (Suppl.), 1126.

Sullenger, B., Gallardo, H., Ungers, G. and Gilboa, E. (1990) Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. Cell 63, 601.

Sullenger, B., Lee, T., Smith, C., Ungers, G. and Gilboa, E. (1990) Expression of chimeric tRNA antisense transcripts renders NIH 3T3 cells highly resistant to Moloney murine leukemia virus replication. Mol. Cell Biol. 10, 6152.

Sullenger, B., Gallardo, H., Ungers, G. and Gilboa, E. (1991) Analysis of trans-acting response decoy RNA mediated inhibition of human immunodeficiency virus type 1 transactivation. J. Virol. 65, 6811.

Tiley, L., Malim, M., Tewary, H., Stockley, P. and Cullen, B. (1992) Identification of a high affinity RNA-binding site for the human immunodeficiency virus type 1 Rev protein. Proc. Natl. Acad. Sci. USA 89, 758.

van Beusechem, V.W., Kukler, A., Heidt, P.J. and Valerio, D. (1992) Long-term expression of human adenosine deaminase in rhesus monkeys transplanted with retrovirus-infected bone-marrow cells. Proc. Natl. Acad. Sci. USA 89, 7640.

Wigler, M., Sweet, R., Sire, G.K., Wold, B., Lacy, E., Maniatis, T., Silverstein, S. and Apel, I.L. (1979) Transformation of mammalian cells with genes from prokaryotes and eukaryotes. Cell 16, 777.

Winocour, E., Puzis, L., Etkin, S., Koch, T., Danovitch, B., Mendelson, E., Shaulian, E., Karby, S. and Lavi, S. (1992) Modulation of the cellular phenotype by integrated adeno-associated virus. Virology 190, 316.

Yu, M., Poeschla, E. and Wong-Staal, F. (1994) Progress towards gene therapy for HIV infections. Gene Ther. 1, 13.

Zhou, S.Z., Cooper, S., Kang, L.Y., Ruggieri, L., Heimfeld, S., Srivastava, A. and Broxmeyer, H.E. (1994) Adeno-associated virus 2-mediated high efficiency gene transfer into immature and mature subsets of hematopoietic progenitor cells in human umbilical cord blood. J. Exp. Med. 179, 1867.