Characterization of a mobilization-competent simian immunodeficiency virus (SIV) vector containing a ribozyme against SIV polymerase

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Exploitation of the intracellular virus machinery within infected cells to drive an anti-viral gene therapy vector may prove to be a feasible alternative to reducing viral loads or overall virus infectivity while propagating the spread of a therapeutic vector. Using a simian immunodeficiency virus (SIV)-based system, it was shown that the pre-existing retroviral biological machinery within SIV-infected cells can drive the expression of an anti-SIV pol ribozyme and mobilize the vector to transduce neighbouring cells. The anti-SIV pol ribozyme vector was derived from the SIV backbone and contained the 5′- and 3′LTR including transactivation-response, Ψ and Rev-responsive elements, thus requiring Tat and Rev and therefore limiting expression to SIV-infected cells. The data presented here show an early reduction in SIV p27 levels in the presence of the anti-SIV pol ribozyme, as well as successful mobilization (vector RNA constituted ~17% of the total virus pool) and spread of the vector containing this ribozyme. These findings provide direct evidence that mobilization of an anti-retroviral SIV gene therapy vector is feasible in the SIV/macaque model.

INTRODUCTION

Current anti-retroviral combination drug therapy (highly active anti-retroviral therapy, HAART) reduces morbidity and mortality in human immunodeficiency virus type 1 (HIV-1)-infected individuals (reviewed by Gazzard, 1999; Shafer & Vuitton, 1999). However, the toxicity of anti-retroviral drugs, compliance with the life-long regimen and the evolution of antiretroviral resistance in the face of drug pressure illustrate the limitations of this approach (Shafer & Vuitton, 1999). Alternative strategies to inhibit virus replication, either alone or in combination with those currently practised, are desirable (Mautino & Morgan, 2002).

One adjunctive strategy explored here involves the use of a ribozyme. Ribozymes are catalytic RNA molecules that can be engineered to cleave specifically and effectively destroy a given target RNA (Cech, 1987), presenting an attractive method for reducing viral load in HIV-1 infection (reviewed by Rossi, 2000). A limiting step in the current use of ribozymes, however, is their delivery to virus-infected cells. Due to safety concerns, emphasis on therapeutic gene delivery has relied on the development of replication-defective, recombinant retroviral vectors (Barinaga, 1994; Miller & Wolgamot, 1997). Successful gene transfer to human T cells, stem cells, dendritic cells and bone marrow has been achieved, with expression of the marker gene ranging from weeks to 36 months (Bauer et al., 1997; Leavitt et al., 1994; Mangeot et al., 2002; Yu et al., 1995). The delivery of gene therapy vectors and subsequent gene transfer have involved direct transduction of the target cells with the desired vector (Buchschacher & Wong-Staal, 2000). This method has not proved practical as it involves ex vivo transductions, with the infusion of the transduced cells back into the infected individual.

An alternative strategy for a gene therapy vector delivery system involves revising the current paradigm and using conditional-replicating or mobilizable vectors, with the cells already infected for vector propagation. However, the use of such vectors could be expected to be limited by any anti-viral genes within the vector (Klimatcheva et al., 2001). None the less, it has previously been shown that the packaging of vector RNA creates competition for encapsidation of viral RNA, reducing the amount of wild-type virions roughly sixfold, leading to reduced particle...
infectivity and mobilization of vector to target cells (Corbeau & Wong-Staal, 1998; Evans & Garcia, 2000). Moreover, when the vectors express ribozymes targeting HIV-1 RNA, a significantly higher competitive advantage for the vector RNA packaging into virions over the wild-type viral RNA is observed (Dropulic & Pitha, 1996).

The use of vectors that are replication-defective unless co-infected with cells with replication-competent virus may provide an avenue of antiretroviral vector delivery to viral reservoirs that could be tested in the simian immunodeficiency virus (SIV)/rhesus macaque model. The formation of such replication-defective particles has been shown to be contingent not only on a viable packaging cell line, but also on the 5’- and 3’LTR from HIV or SIV (HIV Tat can transactivate vectors containing the SIV LTR; Arya, 1988), the Ψ packaging signal and the Rev-responsive element (RRE) site (Aronoff & Linial, 1991; Garzino-Demo & Arya, 1995; Geigenmüller & Linial, 1996; Joshi et al., 1997; Parolin et al., 1996). A mobilizable vector that expresses anti-viral genes, such as a ribozyme, represents a shift in objective from protecting uninfected cells to potentially reducing overall virus propagation in already infected cells (Klimatcheva et al., 2001; Mautino et al., 2001; Mautino, 2002).

The data presented here suggest that an SIV-based vector containing an anti-SIV pol ribozyme has an antiretroviral activity that is the result of the ribozyme cutting transcripts containing the SIV pol gene encoding the reverse transcriptase (RT). The vector tested here was able to exploit the intracellular viral machinery of the SIV-infected cells and propagate in a fashion similar to the wild-type virus. Such a mobilization-competent anti-SIV vector relies on the viral proteins expressed within the virus-infected cells to drive the expression of the anti-SIV pol ribozyme, as well as to propagate and package the vector. Importantly, data presented here are, to our knowledge, the first characterization of a mobilization-competent SIV-based vector containing a ribozyme targeting the wild-type virus.

**METHODS**

**Vectors.** The vectors used in this study were derived from SIVmac239A3, a two-plasmid system; the plasmid 5’p53Δvpr contains the 5’ half of SIV (9411 bp) and 3’p239-3’ΔnefA3U3 contains the 3’ half (6789 bp) (Desrosiers et al., 1998; Gibbs et al., 1994; Regier & Desrosiers, 1990). To use the Srl site, which lies 5’ of the deleted nef, for the cloning of the anti-SIV pol ribozyme, the Srl site in the flanking genomic DNA was first removed. Briefly, the 3’LTR (minus flanking genomic DNA) was PCR-amplified from 3’p239-3’ΔnefA3U3 with the primers 5’ Srl1(5’-CCCCAGAGGATTAGACAAAGGCTTGAGCTACACT-3’) and 3’ EcoRl (5’-GGGAGACCTTCGTAGGTTTTCTCCTG-3’) under the following conditions: 1 cycle of 95°C for 8 min; 30 cycles of 95°C for 45 s, 55°C for 45 s and 72°C for 45 s; followed by 1 cycle of 72°C for 10 min. Unless otherwise stated, all PCRs were performed in a total volume of 50 μl, comprising 5 μl 10× PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 7.5, 25 mM MgCl2), 0.8 μl dNTP mix (1.25 mM solution), 2 μl each primer (20 pmol μl−1) for a total of 40 pmol per reaction, 0.25 μl Taq DNA polymerase (5 U μl−1), 31.75 μl sterile DNase-free water and 1 μl of sample. The PCR-amplified 3’LTR was Srl and EcoRl digested along with the similarly digested 3’ p239-3’ΔnefA3U3, which was 0.8% agarose gel purified to remove the 3’LTR and then ligated with the resultant PCR-amplified 3’LTR to produce 3’p239-3’ΔnefA3U3st.

The 5’LTR was amplified by PCR with the primers 5’ LTR-Xhol (5’-GCGGATCCGCGCAGCCAACTTTACAAGGCTTTCGACT-3’) and 3’ LTR-Sphl (5’-GCGGTCGAGTCCTACCTCTCTTATTACCTCCCTCTCCTCCTGGA-3’), using the 5’p33Δvpr plasmid containing the 5’ half of SIV as the template (Desrosiers et al., 1998; Gibbs et al., 1994; Regier & Desrosiers, 1990). One microgram of the PCR-amplified 5’LTR was Xhol/Sphl digested. The 3’p239-3’ΔnefA3U3st plasmid was digested with Xhol (10–60 U μl−1) and Sphl (1–4 μg μl−1) (Stratagene) and ligated to the similarly treated 5’LTR Plasmid. To confirm that the 5’LTR was correctly cloned into the vector, sequencing was carried out using the primers 3’ LTR-Xhol and 3’ SIV env (5’-GGGCTATACAGACTGCTTCAATTCCACCATGTTCACT-3’).

**Anti-SIV pol ribozyme.** The design of the SIV-specific anti-pol hammerhead ribozyme (anti-SIV-RT) followed previously established protocols in ribozyme design (Akhtar et al., 1995; Amarzguioui & Prydz, 1998; James & Gibson, 1998; Marschall et al., 1994; Regier & Desrosiers, 1990) (Fig. 1d). The anti-SIV-RT ribozyme was synthesized by PCR amplification using the ribozyme template 5’-AAGGTTCTTTCTGTAGTCCGTAGGAGACAAACTGTTAAACTA-3’ with the primers 5’-Sense RT (5’-GGCGAGCTCAGATGTTCTCTGATTGATGTC-3’) and 3’-Antisense RT (5’-GGGGAGACCTTTGAGCTGGTGGGAGAAGAGG-3’) and either the anti-SIV-RT 5’-Sense or 3’-Antisense RT ribozyme primers. Vectors containing the anti-SIV-RT ribozyme in sense (p4T) and antisense (pIT) orientations were selected (Fig. 1b and c, respectively).

**Characterization of mobilized vector from SIV-infected cultures.** CEMx174 cells (5 × 10⁶) were infected with SIVmac251 (m.o.i. of 6 for 2 h), washed with PBS and added to 1 × 10⁷ CEMx174 cells to create a culture containing 0.5% infected cells. Twenty-four hours later, the 0.5% infected cultures were DEAE-dextran transfected (Milman & Herzberg, 1981; Naidu et al., 1988) in duplicate with 5.0 μg of the respective vectors (Fig. 1a–c). Mock cultures were not transfected with vector. These cultures were washed with PBS and split 24 h post-transfection into quadruplicate wells and incubated at 37°C, 5% CO₂ and the medium changed every 3–4 days.

Virions from the SIVmac251-infected vector-transfected culture were isolated from filter-sterilized (0.45 μM) supernatants by centrifugation (SS34 rotor, Sorvall RC5 centrifuge, 20 000 g at 4°C for 2 h). RNA was extracted from the pellets using QiaAmp viral extraction kit and quantitated by spectrophotometer (Pharmacia Biotech, Gene Quant II). One microgram RNA-seq-free DNase I-treated (4 h at 37°C) viral RNA in 10 μl RNase-free water served as the template for the RT reaction. The 20 μl RT reaction contained 2 μl SIV primer cocktail with primers specific for pol (5’-GAATACACACCGCTCCGAGAAC-3’), the 3’LTR (5’-CTCCACCTCTATTTCTACCTCTCTTCGTGATAAAGACACG-3’) and the 5’ SIV env (20 pmol μl−1 of each primer), 0.5 μl AMV RT (5 U μl−1), 1 μl 2×5 mM dNTPs, 4 μl RT buffer (100 mM Tris/HCl, pH 9.0 at 25°C, 500 mM KCl, 15 mM
MgCl₂, 1% Triton X-100, 0.5 μl RNaseOUT (20–40 U μl⁻¹), 1 μl DTT, 1 μl RNase-free water and 10 μl RNA. The viral RNA was added to this and reverse transcription was carried out at 42°C, then inactivated for 10 min at 95°C. The presence of vector cDNA was determined by PCR using the 5’ SIV env primer and either the 5’-Sense RT (as a 3’ primer) or the 3’-Antisense RT ribozyme-specific primer, depending on the orientation of the ribozyme within the vector. The products were sequenced for final confirmation. Supernatants shown to contain the anti-SIV-RT ribozyme vectors packaged by wild-type SIVmac251 were filter-sterilized (0.2 μm) and 100 μl was serially passaged in 1 × 10⁶ uninfected CEMx174 cells. Viral RNA from the supernatants of the serially passaged CEMx174 cultures was also RT-PCR amplified and sequenced to confirm the presence of the anti-SIV-RT ribozyme.

**Characterization of anti-SIV-RT ribozyme action by HPLC.**

Cellular RNA was isolated (RNase isolation kit; Qiagen) and reverse-transcribed as described above. The limiting-dilution end-point PCR (Sykes et al., 1992) was carried out under the following conditions: 1 cycle of 95°C for 8 min; 25 cycles of 95°C for 45 s, 55°C for 45 s and 72°C for 45 s; followed by 1 cycle of 72°C for 8 min. The products of the limiting-dilution PCR or RT-PCR (RT step as described previously) (12 μl) were run on a 3% agarose gel and compared with end-points of the known plasmid, either p4T or p239. The final viral or vector copy number ml⁻¹ was calculated from standard plasmid (p4T or p239) end-point copies ml⁻¹ using the equation: 

\[ C = (X*6-022*10^{3})/(660*Y*1.0*10^{6}) \]

where C = number of copies, X = concentration of DNA/cDNA (μg ml⁻¹) and Y = vector or virus base pair numbers.

**SIV p27 ELISA.** Two hundred microlitres of each transfected and SIVmac251-infected CEMx174 cell culture supernatant and SIVmac251-infected rhesus macaque sera, titred and used as standards (a gift from Joanne Higgins, University of California Davis Center for Comparative Medicine), were placed in each well of a 96-well plate coated with canine anti-SIV IgG-biotin (Lohman et al., 1991) and incubated for 1 h at 37°C. The plates were washed three times with ELISA wash buffer (0.015 M NaCl, 0.05% Tween 20 in distilled water) and incubated for 1 h at 37°C with rabbit anti-SIV-p27 serum (Lohman et al., 1991) diluted 1:20 000. The plates
were washed three times with ELISA wash buffer and incubated for 20 min with goat anti-rabbit horseradish peroxidase-labelled IgG (1:5000 dilution). The plates were washed three times in ELISA wash buffer and developed using the substrate 3,3′,5,5′-tetramethyl benzidine in the presence of H₂O₂ and citric acid, as described previously (Lohman et al., 1991). All absorbance values were derived from readings on a Bio-Rad plate reader at 450 nm with a reference wavelength of 570 nm using Microplate Manager 2.2 software (Macintosh).

RESULTS
Vectors p5T (no ribozyme), p4T (containing the ribozyme anti-SIV-RT in the sense orientation) and p1T (containing the ribozyme anti-SIV-RT in the antisense orientation) (Fig. 1a–c, respectively) containing the minimal requirements for expression and mobilization – a functional 5’- and 3’LTR, a Ψ packaging signal and the RRE site (Barinaga, 1994; Kim et al., 1998) – were transfected into a mixture of CEMx174 cells of which 0–5 % were infected with SIVmac251. This mixture of cells was used to simulate, in vitro, a spreading retroviral infection. Transfected cultures were monitored for SIV p27 expression by ELISA and for cell viability by trypan blue staining. Ribozyme-transfected cultures did not differ from controls with regard to cell viability. From day 7 to 14, the p4T-transfected (sense) SIVmac251-infected cultures produced lower levels of SIV p27 than controls. These cells expressed 83 and 86 % p27 relative to SIVmac251-infected cells transfected with p1T, 82 and 83 % p27 relative to infected cells transfected with p5T, and 84.4 and 86.4 % p27 relative to mock-transfected infected cells on days 7 and 14 post-transfection, respectively (Fig. 2a).

To assess vector mobilization, 100 μl of filter-sterilized supernatants from the above cultures from day 14 post-transfection were passaged onto fresh uninfected CEMx174 cells. While a trend towards reduced SIV p27 expression initially appeared conserved in the cultures treated with passaged supernatants, the differences were not statistically significant (p27 expression in cells transfected with p4T relative to mock-transfected cells was 69 % on day 2, 100 % on day 5, 90 % on day 11 and 86 % on day 14; Fig. 2b).
To determine whether the ribozyme-containing vector was present in the passaged supernatants, we isolated and amplified viral RNA from supernatants at day 21 post-transfection. RT-PCR with the 5' SIV env primer and either the 5' or the 3' anti-SIV-RT-specific primer showed the presence of the anti-SIV-RT ribozyme-containing vector in the culture supernatants (Fig. 3a). The competency of the vector to mobilize and subsequently transduce new cells was determined using the same PCR primers and genomic DNA, as well as supernatants from the passaged supernatant-infected CEMx174 cells. At day 2 post-serial passage, there were detectable levels of the anti-SIV-RT ribozyme in the genomic DNA of the fresh supernatant-exposed CEMx174 cells (Fig. 3b).

Next DHPLC was employed, based on previous methodologies (Dropulic et al., 1992), in an attempt to determine whether or not the reduced SIV p27 levels seen post-transfection were a result of anti-SIV-RT ribozyme cutting the polymerase (pol)-containing transcript of SIVmac251. Cellular RNA from transfected cell cultures was DNase I-treated and RT-PCR-amplified for SIV RNA and the respective ratios of cut versus uncut RNA products were compared by DHPLC (Fig. 4a). The p4T sense anti-SIV-RT vector-transfected cultures contained a significant increase in the ratio of cut to uncut pol transcripts from day 4 to day 9, as well as a reduction in the overall percentage of nef transcripts, a trend that was not observed in the p1T-treated samples containing the ribozyme in the antisense orientation, nor in mock-transfected or p5T-transfected culture controls (compare Fig. 4b and c). Furthermore, the increased ratio of cut to uncut pol transcripts detected by DHPLC coincided with the time when there was a noticeable reduction of SIV p27 expression (compare Fig. 2a with Fig. 4c).

To assess more thoroughly the packaging of wild-type SIV and p4T vector RNA, a limiting-dilution PCR was performed to quantify the amount of virus compared with p4T vector post-transfection to day 2 post-passage (day 16 following transfection). The number of vector copies per cell following transfection was 13.9% of the number of virus copies per cell (Table 1). Following serial passage of the transfected culture supernatants, the vector was detected at 14.2% of the detectable virus (Table 1), indicating little or no loss in mobilization during the passage of vector to new cells. At day 14 following passage, the vector remained detectable by RT-PCR in the filter-sterilized supernatants at 1.9 × 10⁶ copies (ml supernatant)⁻¹, while the virus was detectable at 1.14 × 10⁶ copies (ml supernatant)⁻¹ to yield a proportion of vector to viral RNA of 16.7% (Table 1).

**DISCUSSION**

Even in a very ‘non-optimal’ experimental setting and despite low transfection efficiencies (<0.3 vector copies per cell), these studies have described the passage of an anti-viral vector and suggest that mobilization is a feasible paradigm that needs further exploration. The antiretroviral effect observed in these studies, albeit relatively minimal, was retained with the passage of the anti-SIV vector p4T. However, there did appear to be a loss of efficiency with passage, as differences in virus replication in cultures

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**Fig. 3.** (a) Detection of anti-SIV-RT-containing vectors in viral RNA from culture supernatants by RT-PCR using 5’ SIV env and 3’-Antisense RT or 5’-Sense RT (for the reverse-orientated ribozyme) ribozyme primers at day 21 post-transfection. Lane 1, detection of p4T anti-SIV-RT sense ribozyme vector in viral RNA from p4T-transfected cell culture supernatant. The product was sequenced and shown to contain the anti-SIV-RT ribozyme within the context of the SIV-based vector. Lane 2, viral RNA from mock-transfected CEMx174 cell culture supernatant. Lane 3, viral RNA from p5T-transfected cell culture supernatant. Lane 4, viral RNA from p4T-transfected cell culture supernatant without RT enzyme added (negative control). Lane 5, amplification of p4T anti-SIV-RT sense ribozyme vector plasmid (positive control), MW, molecular mass marker φX174 Hae. (b) Detection of anti-SIV-RT ribozyme vector at day 2 post-passage in CEMx174 cellular DNA. Lane 1, amplification of the p4T anti-SIV-RT ribozyme plasmid (positive control). Lanes 2 and 6, p1T-transfected cells. Lanes 3 and 8, p5T-transfected cells. Lanes 4 and 9, mock-transfected CEMx174 cells. Lanes 5 and 7, p4T-transfected cells. This PCR product was sequenced and shown to contain the anti-SIV-RT ribozyme within the context of the SIV-based vector. The 5’ SIV env and 3’-Antisense RT primers were used in lanes 1–5, while the 5’ SIV env and 5’-Sense RT primers were used in lanes 6–9.
infected with supernatants were not significant and could not be detected upon a third passage (data not shown).

It is not clear why the vectors alone did not demonstrate a greater anti-viral effect, as vectors alone have been described to have anti-HIV properties due to TAR and/or RRE decoy effects as well as by competition for encapsidation of viral RNA (Corbeau & Wong-Staal, 1998; Mautino et al., 2001). This vector effect of reducing the spread of HIV-1 in cell culture has been estimated to reduce the amount of wild-type HIV-1 in particles by roughly sixfold, thus ultimately leading to reduced particle infectivity and vector mobilization to target cells (Bukovsky et al., 1999; Corbeau & Wong-Staal, 1998). However, the experiments described here only assessed p27 production in the non-ribozyme (p5T) or antisense (p1T) and sense (p4T)

Table 1. End-point dilution PCR analysis of sample genomic DNA from vector p4T-treated cultures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day 2 post-transfection (copies per cell)</th>
<th>Day 16 post-transfection/day 2 post-passage (copies per cell)</th>
<th>RT-PCR at day 14 post-passage/28 days post-transfection (copies ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p4T (5'–3'Tat)*</td>
<td>1.65</td>
<td>3.5</td>
<td>1.14 × 10⁶</td>
</tr>
<tr>
<td>p4T (5'Nef–3'RT)†</td>
<td>0.23</td>
<td>0.5</td>
<td>1.9 × 10⁵</td>
</tr>
</tbody>
</table>

*5'–3'Tat, PCR using 5' and 3' Tat primers specific for SIV. The limit of detection for this assay was 9.1 copies µl⁻¹ in a 50 µl PCR.
†5'Nef–3'RT, PCR using the 5' Nef primer, which is SIV-specific, and the 3' Antisense RT-specific primer, which is specific to p4T. The limit of detection for this assay was 1.28 copies µl⁻¹ in a 50 µl PCR.
The concept of anti-viral vector propagation has been described previously with HIV-1-based systems (Buchschacher & Wong-Staal, 1992; Bukovsky et al., 1999; Evans & Garcia, 2000; Klimatcheva et al., 2001) and was studied here with SIVmac. The data presented here provide a demonstration and quantification of a mobilization-competent anti-SIV vector that can be packaged and passed along with the wild-type virus. These data indicate that it is possible to derive an anti-SIV gene therapy vector that exploits the virus machinery of infected cells to express a ribozyme targeting the wild-type virus and not the vector, and that the vector can compete with and be spread in a fashion similar to the wild-type virus, potentially introducing the therapeutic agent to many virus reservoirs. Interestingly, the noted early reduction in SIV p27 was not retained following serial passage, possibly due to the dissimilar rate of increase in wild-type virus compared with vector or a recombination event between the vector and virus that rendered the vector-containing ribozyme defective. While recombination is a significant possibility, it has been noted that vector–virus recombination is greatest between intact Gag/Gag–Pol (Wu et al., 2001). Indeed, Gag sequences were retained in the p4T vector and the wild-type virus. However, whether the reduction of virus replication induced by p4T or a similar vector could be maintained within an in vivo setting in the presence of anti-retroviral therapy or following enhanced transduction utilizing a viral-based vector system (Mangeot et al., 2000) remains to be determined. Speculation through the interpretation of HIV-1 virus infection models has indicated that even small reductions in viral load of 2 % per replication cycle could prove significant (Coffin, 1995, 1996). More recently, molecular models of vector–virus interactions have indicated that if the vector is in ~100-fold excess of the viral mRNA, a therapeutic virus set point equivalent to HAART could be achieved (Weinberger et al., 2003). In these studies, vector p4T was shown to make up 13·9 % of the virus pool at day 2 post-serial passage and up to 17 % of the virus pool at day 14 post-serial passage. Whether these ratios could be increased by first transfecting/transducing target cells followed by virus infection or alternatively simply increasing overall transfection efficiency remains to be determined. Overall, the findings presented here indicate a need for further study of ribozyme stability and the development of vectors that are both inhibitory and capable of mobilization. These preliminary data should provide a premise to design more optimal mobilization-competent anti-SIV vectors that can be tested in the primate model.

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REFERENCES


