Generation of a retroviral vector that expresses an anti-HIV-1 *tat* hammerhead ribozyme

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Ribozymes have emerged as promising therapeutics for HIV and have been shown to bind and cleave target RNA's in a sequence-specific manner. The HIV-1 regulatory protein *tat* plays an essential role in the upregulation of viral transcription and elongation of viral transcripts, and has therefore been identified as a target for ribozyme studies. To further study the use of these reagents, we have designed a number of hammerhead ribozymes targeted to specific sequences within the HIV-1 NL43 *tat* sequence. One of these, Tat5910 and its non-catalytic control Tat5910Δ, were cloned into the retroviral vector pSuper.retro.neo+GFP (pSRNG) for cell culture studies. Recombinant retrovirus particles were generated by transient transfection of 293T cells using a two-plasmid system consisting of the helper plasmid, pPAM3, and pSRNG5910Δ. Identical experiments were carried out using two Mo-MLV retroviral vectors, pLNCE, and pLNCLZRz. The resulting recombinant virus was used to transduce NIH-3T3 cells, and virus titer (virus particles/ml) was determined from the number of Green Fluorescent Protein (GFP)- or β-galactosidase-positive cells. Although producer cells transfected with pSRNG5910Δ expressed GFP, the transfection efficieny was low. This resulted in levels of transduced NIH-3T3 cells that were too low to obtain a reliable titer measurement. Attempts to optimize titer by increasing the number of transfected producer 293T cells was successful using pLNCE and pLNCLZRz. Positive GFP or β-galactosidase expression in NIH-3T3 cells transduced with LNCE and LNCLzRz recombinant virus indicated that our two-plasmid virus production system was successful. Extremely low titer along with lower cellular expression of GFP suggests that the pSRNG5910 vectors may be better suited to virus production using a stable producer cell line such as PA317

Introduction

Human Immunodeficiency Virus (HIV-1) infection results in the gradual loss of T_H cells, decreased immune competence, and increased susceptibility to various opportunistic infections, including *Pneumocystis jirovecii* pneumonia, tuberculosis, and cytomegalovirus. Infection by these opportunistic infections, in addition to a T_H lymphocyte count below 200/mm^3, define the Acquired Immune Deficiency Syndrome (AIDS) (1).

Traditional HIV treatments have sought to reduce viral load by utilizing drugs that prohibit effective viral replication (2). For example, Triple Combination Therapy (TCT), which involves one protease inhibitor and two reverse transcriptase (RT) inhibitors, has been shown to effectively reduce viral load (2). Nevertheless, resistance to protease and RT inhibitors have already developed, requiring a continued search for therapies that can inhibit other HIV gene functions (3). For HIV, these functions are generally associated with the six accessory genes that control infection and replication: *tat*, *rev*, *nef*,*vif*, *vpr*, and *vpu* (4).

Tat increases viral transcription from the HIV-1 Long Terminal Repeat (LTR) by binding to the *cis* acting RNA enhancer element, the transactivation response region (TAR), which is transcribed from the R (Repeat) region of the 5' LTR (5). Once transcribed, TAR forms a hairpin loop consisting of a base-paired stem, a three nucleotide non-base-paired bulge, and a six-nucleotide Guanine-rich loop (2) that is present at the 5' end of all viral transcripts. The Tat/TAR interaction recruits numerous cellular transcriptional coactivators to TAR, including P-TEFb (Positive Transcription Elongation Factor b), an RNA polymerase II C-terminal kinase, resulting in the phosphorylation of the C-Terminal Domain (CTD) of RNA Polymerase II (6). The phosphorylation of the CTD increases RNA polymerase II processivity, thereby inducing elongation of viral transcripts (7). Tat is absolutely required for HIV replication and is, therefore, an important target for novel anti-HIV reagents.

Targeting HIV-1 at the genomic level can be accomplished by hammerhead ribozymes, which are small catalytic RNAs that can be targeted to cleave viral mRNAs (8). These RNA enzymes target and cleave mRNA at any XUX', where X is any nucleotide, and X' is Adenine, Cytosine, or Uracil (8). Hammerhead ribozyme design, based on the Hasseloff and Gerlach model (9), consists of two flanking regions that provide target specificity by Watson-Crick base pairing and a catalytic core that cleaves at the target site. Cleavage of *tat* mRNA sequences by hammerhead ribozymes may negatively impact transcription initiation and elongation, leading to the inability of HIV to replicate within cells (10;11).

Previously, our lab designed and cloned a number of hammerhead ribozymes targeted to the *tat* genomic sequence of the HIV-1 genomic clone NL43. Each of these ribozymes and a corresponding non-catalytic control ribozyme was cloned into the shuttle vector, pPCR-Script. For initial cell culture testing, Tat5910 and its non-catalytic control Tat5910Δ, were subcloned into the retroviral vector pSuper.Retro.Neo+GFP (pSRNG). This modified Moloney Murine Leukemia virus (Mo-MLV) vector can infect non-dividing cells (e.g., hematopoietic stem cells) and is self-inactivating due to a deletion in the 3' LTR (12). Importantly this vector has been designed to express siRNAs from the RNA Polymerase III H1 promoter (13), making it a logical choice for expressing other small RNAs such as hammerhead ribozymes. For selection purposes pSRNG expresses neomycin

phosphotransferase and green fluorescent protein (GFP) from a single IRES-containing transcript controlled by the phosphoglycerokinase (PGK) promoter (13).

This paper describes cloning of an anti-HIV hammerhead ribozyme Tat5910 into the retroviral vector, pSuper.retro.neo+GFP, along with initial cell culture testing. These tests involved analysis of transgene expression and optimizing virus titer produced in a transient producer cell system. For this we utilized a two-plasmid system consisting of a retroviral vector and a helper plasmid (14).

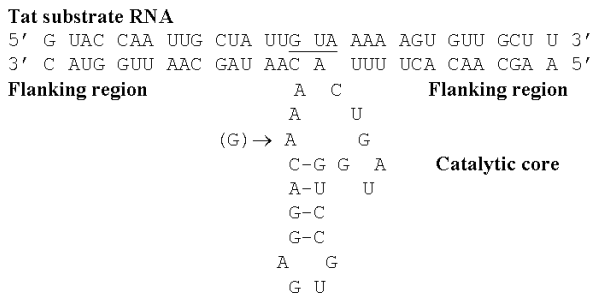

Figure 1. Hammerhead ribozyme design. Tat RNA showing the pGUA cleavage site at nucleotide 5910 is shown annealed to the ribozyme flanking regions. The catalytic core was made inactive by an A to G mutation (11).

Materials and Methods

Oligonucleotides and cloning

A hammerhead ribozyme, based on the Haseloff and Gerlach model (9) was designed to cleave a pGUA sequence at nucleotide 5910 within the HIV genomic clone NL43 (Accession number M19921) (Figure 1). The Tat5910 sense (5'-gat ccA AGC AAC ACT TTT CTG ATG AGT CCG TGA GGA CGA AAC AAT AGC AAT TGG TAC Aa- 3') and antisense sequences (5'-agc ttT GTA CCA ATT GCT ATT GTT TCG TCC TCA CGG ACT CAT CAG AAA AGT GTT GCT Tg- 3') were synthesized with BamHI and HindIII sites to facilitate cloning. Two-hundred picomoles of each oligonucleotide were combined in annealing buffer (100mM NaCl, 50mM HEPES buffer (pH 7.4)) and cooled slowly from 90 to 4˚C. The annealed oligonucleotides were ligated into the BglII and HindIII sites of the retroviral vector pSuper.retro.neo+GFP (pSRNG) according to the manufacturer's protocol (Oligoengine). A non-catalytic ribozyme targeted to the same sequence was created by an A to G mutation in the catalytic core. This oligonucleotide was synthesized and similarly cloned into pSRNG.

Ligation was carried out using T4 DNA Ligase (Promega) in a 4ºC overnight reaction. The ligated plasmids were transformed into JM109 cells and the resulting colonies were screened for the presence of the ribozyme. For this, miniprep DNA was screened by PCR using a plasmid-specific primer pair that framed the cloning site. The PCR reaction consisted of 100 pMol of each primer and 2X PCR Master Mix (50units/mL Taq DNA Polymerase) (Promega). Positive clones were indicated by the presence of a 482 bp fragment. Correct ribozyme insertion into the resulting plasmids, pSRNG5910 and pSRNG5910Δ, was verified by sequencing.

Cell lines and transfections

293T and NIH-3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) in a humidified 37°C incubator with 5% CO_2. All transfections were done by calcium phosphate precipitation based on the method of Berkner and Sharp (15). Briefly, 293T cells were plated to 50-70% confluency onto 60 mm dishes 24 hours prior to transfection. The cells were transiently transfected with 4 μg of plasmid DNA in 125 mM $CaCl_2$, and 2X HBS (280 mM NaCl/ 1.5 mM Na_2HPO_4/ 50 mM HEPES Buffer (pH 7.05)). The resulting precipitate was added to the culture medium and allowed to be taken up by the cells. Transfection efficiency, as measured by GFP (pLNCE and pSRNG5910) or β-galactosidase (pLNCLZRz) expression was analyzed as a function of transfection time. For this, 293T cells were allowed to remain in contact with the DNA precipitate for 4, 8, 12, or 24 hours. Transgene expression was assayed 24 hours after removal of the DNA precipitate.

GFP expression was analyzed directly using an Olympus CKX41 inverted microscope with epifluorescence. β-galactosidase expression was measured by the ability to metabolize X-gal (10) For this, cells were washed twice with PBS and fixed for 15 minutes with 0.1 M Sodium Phosphate, pH 7.0/1 mM $MgCl_2$/0.25% glutaraldehyde. Subsequently, the cells were washed three times with PBS, and stained by the addition of X-gal (PBS/4 mM $K_4Fe(CN)_6.3H_2O$/4 mM $K_3Fe(CN)_6$,/10 mM $MgCl_2$ /0.03% X-gal). The presence of blue-stained cells after 24 hours indicated positive transfection.

Recombinant Virus Production

Recombinant viruses were produced in 293T cells using a transient two-plasmid system consisting of a retroviral vector and a helper plasmid (10;14). Three retroviruses were analyzed for their ability to generate retroviruses in this system: pSRNG5910Δ, pLNCE, and pLNCLzRz. All are Mo-MLV viruses that express either GFP (pSRNG5910Δ, pLNCE) or β-galactosidase (pLNCLzRz). pPAM3 is a Mo-MLV based helper plasmid that expresses all retroviral structural genes, including an amphotropic glycoprotein (16).

To generate recombinant retrovirus particles for transduction into target cells, 0.5 x 10^6 293T cells were plated onto 60mm dishes. After 24 hours, these cells were transfected as described using 4 μg helper plasmid (pPAM3) and 4 μg retroviral vector (pSRNG5910Δ, pLNCE, or pLNCLZRz). The resulting precipitate was allowed to be taken up by the cells from 4 to 24 hours, after which time the medium was replaced. Twenty-four hours later, the virus-containing medium was harvested from the producer cells, filtered through a 0.45 μm syringe filter, and stored at -80ºC.

The presence of virus particles in the harvested medium was determined following transduction of target cells. For this, 0.25 x 10^6 NIH-3T3 cells were plated onto 35mm dishes. After 24 hours, these cells were transduced by the addition of one milliliter each of harvested medium and fresh culture medium containing 8 μg polybrene (4 μg/ml). The cells and virus particles were incubated together for 24 hours, after which the medium was replaced. After an additional 24 hours, virus titer was

determined from the number of GFP or X-gal positive cells. Virus titer was determined from the average number of positive target cells in 10-20 randomly chosen fields at 200X using bright-field or epifluorescence microscopy. This number was extrapolated to determine the total number of positive cells.

Optimizing virus titer

Transfection incubation time, referring to the length of time the transfection reagent remained on producer cells, was altered to optimize virus titer. 293T cells were incubated with DNA precipitate at four different time points: 4, 8, 12, and 24 hours. After each time point, the medium was replaced and the cells incubated for an additional 24 hours. The virus containing medium was harvested and used to transduce NIH-3T3 cells as described. GFP or β-galactosidase expression was monitored, and virus titer was determined.

Results

The HIV-1 NL43 genomic sequence was analyzed for the presence of potential hammerhead ribozyme target sites. One site, a pGUA located at nucleotide 5910 was used to design an anti-tat hammerhead ribozyme. This ribozyme and its non-catalytic control (Figure 1) were synthesized and cloned into the retroviral vector, pSuper.retro.neo+GFP. Sequencing was used to verify the ribozyme sequence in each of the resulting plasmids: pSRNG5910 and pSRNG5910Δ (Figure 2). Once cloned,

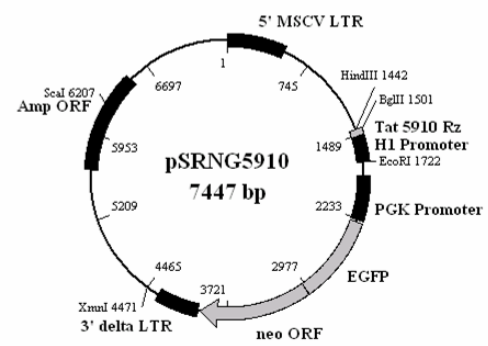

Figure 2. Retroviral vector pSRNG5910. Tat5910 oligonucleotides were synthesized and cloned into the BglII and HindIII sites of pSuper.retro.neo+GFP. The resulting vector is shown. A similar vector, pSRND5910Δ, was created by cloning the non-catalytic version of the ribozyme.

pSRNG5910Δ was tested to ensure GFP expression was not affected by the cloning process. 293T cells were transiently transfected using the calcium phosphate method and after 48 hours analyzed for transgene expression. These results indicated relatively high levels of GFP expression (Figure 3). However, GFP levels were noticeably lower than in cells transfected in parallel with pLNCE (data not shown).

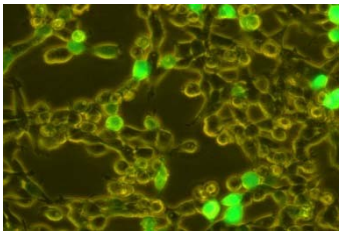

Figure 3. GFP expression. 293T cells were transfected with pSRNG5910Δ by calcium phosphate precipitation. GFP expression was monitored 48 hours later.

The ability to generate recombinant retroviral particles in a transient transfection system was next investigated using a retroviral vector (pSRNG5910Δ, pLNCE, or pLNCLZRz) and a helper plasmid (pPAM3) to produce viral particles in 293T cells. Forty-eight hours after transfection, the virus-containing medium was harvested and used to transduce NIH-3T3 cells. The producer and target cells were analyzed for either GFP or β-galactosidase expression (Figure 4) as a measure of transfection (293T cells) or transduction (NIH-3T3 cells). In these tests, virus production was observed using both pLNCE and pLNCLZRz. However virus production using the pSRNG5910D vector was not observed in this series of experiments (Figure 4).

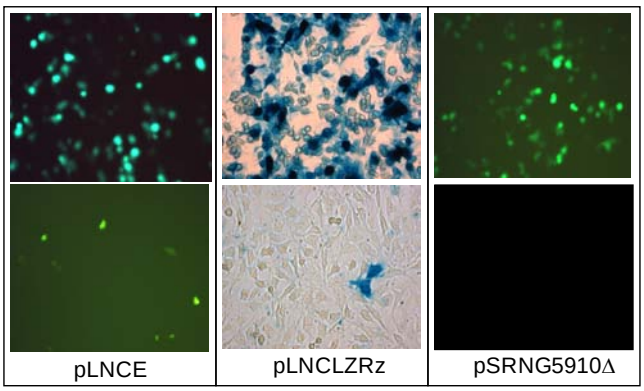

Figure 4. Production of recombinant retrovirus. 293T cells were transiently transfected with pPAM3 and one of three retroviral vectors. Transfection efficiency was determined by the relative number of GFP- or X-gal positive cells (Upper panels). Virus containing medium was harvested and used to transduce NIH-3T3 cells. Virus-mediated expression was only evident in pLNCE and pLNCLZRz transduced cells (Lower panels). GFP-positive cells are green. X-gal positive cells are blue.

To optimize virus titer, the length of transfection incubation period, as measured by the time the DNA precipitate was incubated with the 293T cells, was altered. For this, the transfection reagent was left on the 293T cells for 4, 8, 12, or 24 hours. The precipitate was removed by a change of medium and 24 hours later, virus was harvested in the culture medium. At this time the 293T producer cells were analyzed for GFP or β-galactosidase expression to make sure the transfection was successful. Regardless of the transfection incubation period, all 293T transfection experiments yielded positive GFP or β-galactosidase expression. For the retroviral vector pSRNG5910Δ, higher GFP expression was observed in the 12 hour incubation period compared to the four and eight hour periods. However, no visible differences were evident in GFP expression among the 12 and 24 hour incubation periods (data not shown).

The ultimate purpose of these transfection studies was to determine if virus production was increased by allowing the 293T producer cells to remain in contact with the transfection reagents for longer periods of time. To determine the virus titer produced in these experiments, NIH-3T3 cells were incubated with virus-containing medium harvested from the producer cells. With regards to virus production, extremely low levels of GFP expression was observed in the NIH-3T3 cells transduced with the harvested medium from producer cells transfected with pPAM3 and pSRNG5910Δ. This was true of all incubation periods, and resulted in an inability to determine a reliable titer for this vector (Figure 4). On the other hand, with the exception of the eight hour incubation period, recombinant virus was produced in target cells transduced with pLNCE or pLNCLZRz, as measured by the number of GFP or X-gal positive cells (Figure 5). Analysis of virus titer generated by pLNCLZRz transfections for all incubation periods indicated an increase over time. The maximum titer obtained with this vector was measured at the 24 hour time point as 1 x 10^4 virus particles per milliliter (Figure 5). The virus titer produced using pLNCE indicated a large increase at the 12 hour time point; however, the titer at the 24 hour time point was approximately 50% less (Figure 5). At all time points tested, the pSRNG5910D vector failed to produce a measurable titer.

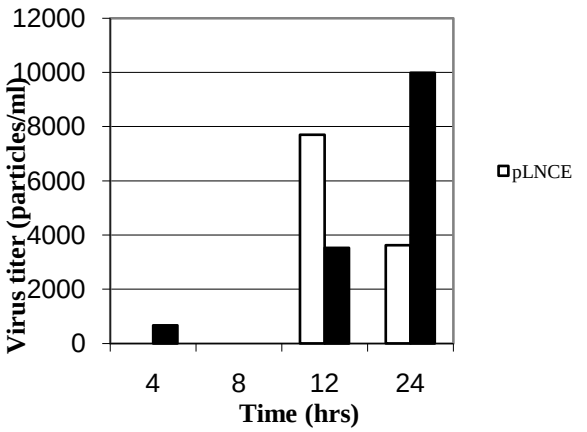

Figure 5. Optimization of virus titer. The number of virus particles per milliliter of harvested culture medium was determined for pLNCE and pLNCLZRz transduced NIH-3T3 cells. Virus titer was optimized by increasing the transfection incubation tome from 4 to 24 hours. Virus titer was found to be directly related to this increase.

Conclusions

Previous studies have shown that hammerhead ribozymes targeted to HIV-1 accessory genes such as *tat* can down regulate virus replication. Many of these studies have used retroviral vectors to express ribozymes as part of an RNA Polymerase II transcript (10;11). While effective, these transcripts are often large and likely produce secondary structures that may interfere with efficient ribozyme cleavage. The pSUPER family of vectors were specifically created to express siRNAs from the RNA Polymerase I H1 promoter and should represent a good choice to express hammerhead ribozymes. Additionally, the pSUPER retroviral vectors are self-inactivating and express neomycin phosphotransferase and GFP from the PGK promoter.

The primary goal of this project was to clone a hammerhead ribozyme targeted to HIV-1 *tat* into pSRNG and to characterize the resulting vector prior to its use in anti-HIV experiments. A second goal was to determine the optimal conditions to produce recombinant retrovirus particles in a transient two-plasmid system. The resulting plasmids, pSRNG5910 and pSRNG5910Δ were generated for this purpose. Initial tests indicated that both retroviral vectors expressed GFP when transiently transfected into 293T cells. However, in all cases the non-pSUPER retrovirus, pLNCE, generated greater GFP expression than either pSRNG5910 or pSRNG5910Δ in transfected 293T cells. Although it does not explain the overall lower transfection efficiency of pSRNG5910Δ, it is likely that pLNCE generated greater levels of GFP expression due to transcriptional control of the immediate early CMV promoter. In comparison, GFP expression in the pSUPER vector is controlled by the comparatively weaker PGK promoter. In fact, a number of cells transfected with pSRNG5910Δ appeared to be very weakly positive for GFP expression, although they were not counted as GFP-positive for these studies. The other retrovial vector used in these experiments, pLNCLZRz also yielded higher numbers of transfected cells, and like pLNCE, this plasmid possesses a CMV promoter that controls expression of β-galactosidase.

The use of these plasmids as retroviral vectors was next studied by comparing the ability of pSRNG5910Δ to produce virus with the retroviral vectors pLNCE and pLNCLZRz. In these studies, pSRNG5910Δ failed to produce measurable virus. Although a very small number of GFP-positive target cells were identified, the number was too small to determine a reliable titer. Subsequently, the length of transfection incubation period was altered in an attempt to optimize virus titer. Transfection with the helper plasmid, pPAM3, and either pLNCE or pLNCLZRz produced measurable virus titers, up to a high of 10^4 particles per milliliter. Again, pSRNG5910Δ failed to produce measurable virus titers in these experiments. Overall; however, these result did indicate that our two-plasmid system successfully generated recombinant virus.

It is quite possible that the two different ways virus expression was measured and used to determine titer played a role in the discrepancies seen in the virus titer optimization experiments (Figure 5). Target cells transduced with recombinant virus produced using the pLNCLZRz vector resulted in the ability to metabolize X-gal.; therefore staining produced blue cells that were easy to identify and count using brightfield microscopy. There is a higher degree of uncertainty involved in scoring GFP-positive cells produced from transduction with recombinant virus from pLNCE and pSRNG5910Δ. It may be that when using these vectors, a more reliable way of determining titer would be to generate neomycin resistant colonies.

Ultimately, because pSRNG5910Δ failed to generate measurable levels of recombinant virus, optimization studies will be impractical until a method for producing virus with this vector can be developed. Although transient systems may be successfully used to generate virus using pSRNG-based vectors, future studies will need to increase transfection efficiency. One such method may be through the use of lipid-based transfection

procedures. Alternatively, it may be necessary to generate a stable producer line for efficient virus production.

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