Silencing of HIV-1 with RNAi

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Chapter One

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General Introduction

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General introduction

The retrovirus HIV-1

Human immunodeficiency virus type I (HIV-1) was identified as the causative agent of the acquired immunodeficiency syndrome (AIDS) in 1983 (1). At present almost 40 million people worldwide are infected with HIV-1. In 2006 alone, four million persons were newly infected and nearly three million persons died from AIDS (http://www.unaids.org). HIV-1 belongs to the *lentivirus* genus, a subfamily of the *Retroviridae* (2). Uniquely, retroviruses are diploid, carrying two copies of the positive stranded RNA genome in each virion. Upon entering a host cell, the RNA genome is reverse transcribed into double stranded DNA that is subsequently integrated into the host cell genome.

Genome organization

The proviral DNA genome (Figure 1A) is approximately 9.8 kb in length and contains nine genes (3,4). The Gag open reading frame encodes the Gag polyprotein precursor, which is proteolytically processed into the structural proteins matrix (MA), capsid (CA) and nucleocapsid (NC). The viral protease (PR), reverse transcriptase (RT) and integrase (IN) enzymes are produced by posttranslational cleavage of the Gag-Pol polyprotein precursor, which is encoded by the Pol open reading frame. The Env open reading frame encodes the surface (SU-gp120) and transmembrane (TM-gp41) subunits of the Envelope glycoprotein (5). The transcriptional activator protein Tat (6) is encoded by the tat gene and the rev gene encodes the other essential regulatory protein Rev. The Rev protein is required for the nuclear export of unspliced and singly spliced transcripts (7). In addition, the HIV-1 genome encodes the four accessory proteins Vif, Vpr, Vpu and Nef. These proteins are not strictly required for virus replication in some cell culture systems, but are important for viral replication, efficient virus spread and pathogenesis *in vivo* (8,9).

The HIV-1 coding region is flanked by non-coding domains termed the long terminal repeats (LTRs), which are subdivided in the U3 (unique 3′), R (repeat) and U5 (unique 5′) domains. These regions contain the transcriptional promoter and a number of structure/sequence motifs that are important for viral replication (10,11). Viral transcription starts at the first residue of R in the 5′ LTR and the transcript is polyadenylated at the last residue of R in the 3′ LTR.

Particle structure

In the mature virus particle (Figure 1B), the viral RNA genome is directly associated with the NC protein (12). This RNA-protein complex together with the viral enzymes PR, RT and IN reside in a cone-shaped core formed by CA protein.

The MA protein forms a shell surrounding this core (5). The outer surface of the viral particle is composed of a bilayered lipid envelope, derived from the host cell membrane, in which gp41 is anchored. The gp120 protein on the outside of the virus is non-covalently linked with the transmembrane gp41 protein (13,14).

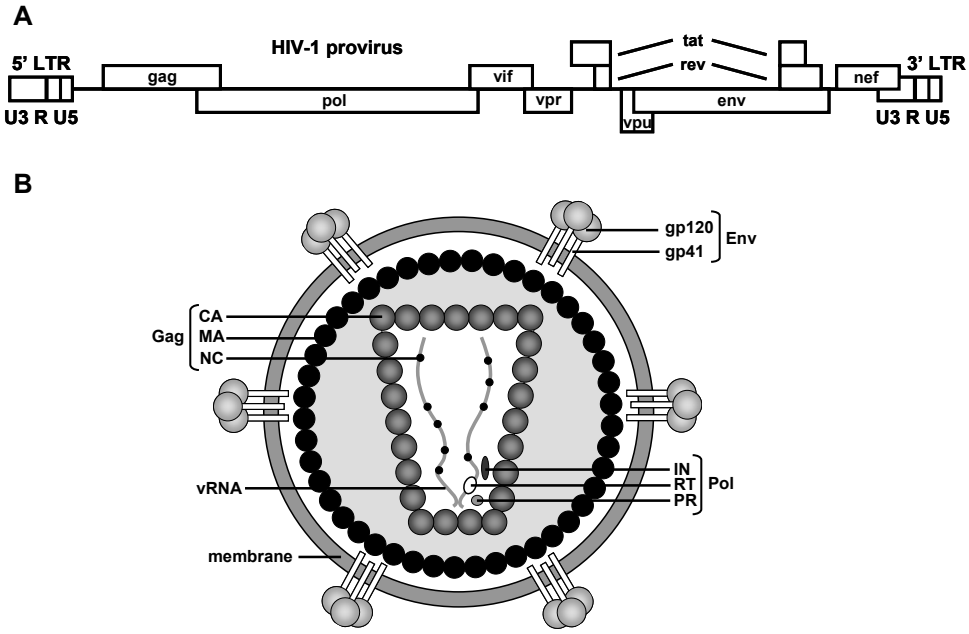

Figure 1. Organization of the HIV-1 genome **(A)** and an HIV-1 particle **(B)**. See text for further details.

HIV-1 replication cycle

HIV-1 replicates in CD4-positive cells (Figure 2), including T lymphocytes, monocytes and macrophages (15). By infecting cells of the immune system, HIV-1 triggers a gradual collapse of the human defense system, resulting in opportunistic infections, various cancers and ultimately death (16,17). The HIV-1 virion interacts with the host cell through binding between its envelope gp120 protein and the cellular CD4 receptor (18). This binding induces a conformational change in gp120 that facilitates binding to the CXCR4 or CCR5 coreceptor (19,20). These interactions induce a conformational change in gp41 that triggers fusion of the viral and cellular membranes (21,22). The viral core is released into the cytoplasm where it is partially uncoated to form the pre-integration complex with numerous host factors (23). Within the viral core, the RNA genome is converted into double stranded DNA by the viral RT enzyme (24). The core is translocated to the cell nucleus where the viral DNA is inserted into the host genome by the IN enzyme (12). The

integrated proviral DNA serves as a template for the production of spliced viral mRNAs and full-length progeny RNA genomes. The translated structural proteins and two full-length RNA strands are assembled at the cell membrane to form a viral particle, which is released from the cell by budding. After maturation of these progeny virions, the new infectious HIV-1 particles can infect new host cells (13,25,26).

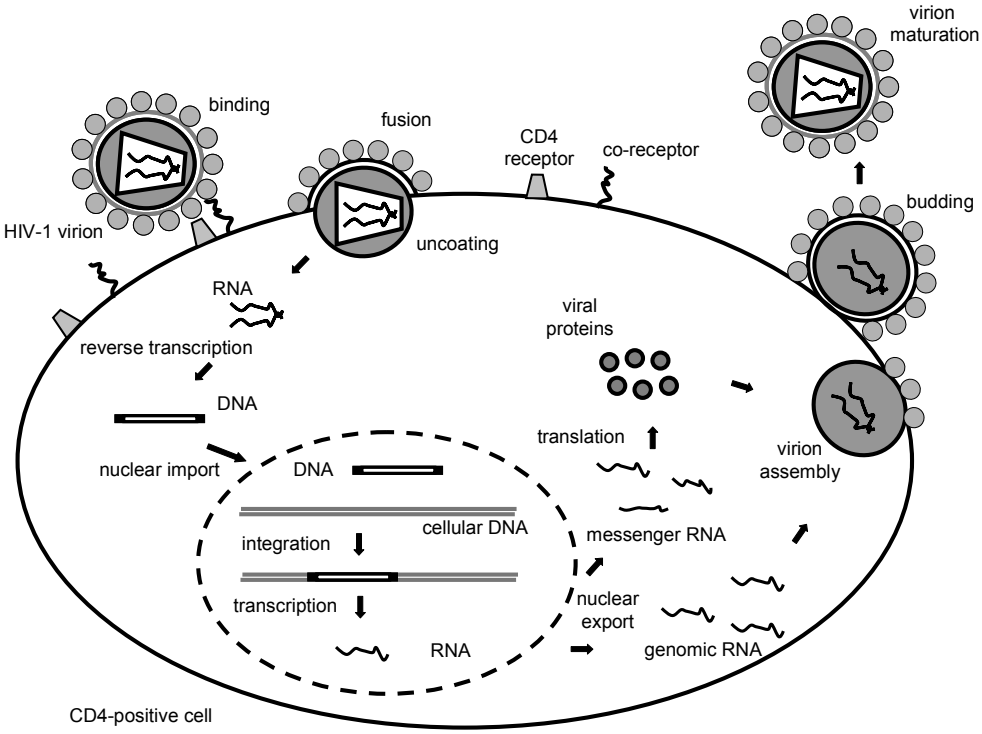

Figure 2: Schematic of the HIV-1 replication cycle. See text for further details.

Counteracting HIV-1

Replication of HIV-1 can be blocked by a variety of antiretroviral drugs. Given the high rates of viral replication and the frequency of mutations occurring during each replication cycle, drug-resistant viral strains appear under the selective pressure of antiretroviral therapy (27). By treating patients with a combination of antiretroviral drugs, termed highly active antiretroviral therapy (HAART), the HIV-1 infection can be controlled effectively (28,29). However, current drug regimens are expensive, not curative and have a significant degree of toxicity for the patient. Moreover, drug-resistant HIV-1 strains are observed more frequently in the epidemic (30,31). Therefore, novel therapeutic approaches against HIV-1

infection need to be explored. An upcoming approach is the use of gene therapy (32,33) to deliver antiviral genes that interfere with HIV-1 replication (34,35). Antivirals relying on base pairing with complementary HIV-1 sequences, such as ribozymes and antisense molecules are in development (36,37). Recently, the discovery of RNA interference (RNAi) (38) offers a new method to potently inhibit HIV-1 replication in a sequence-specific manner (39).

RNAi

The first observation of gene silencing phenomena was made in plants. To alter the pigmentation of petunia, exogenous transgenes were introduced. However, the purple color of the flowers was not deepened, instead some flowers lost their color completely (40,41). These first observations of gene silencing, termed co-suppression, are in retrospect related to silencing mechanisms such as virus-induced gene silencing (42) and are now collectively referred to as RNA silencing or RNA interference (RNAi). RNAi was first discovered in worms when introduction of double stranded RNA (dsRNA) homologous to an endogenous gene resulted in a loss-of-function phenotype of the corresponding gene (38,43). This sequence-specific post-transcriptional gene silencing by dsRNA is conserved in a range of organisms (44-47).

Mechanism of RNAi

Our current understanding of the mechanisms underlying RNAi is a combined result of biochemical and genetic approaches in several experimental systems (reviewed in (48-50)). The pathway of RNAi (Figure 3) proceeds via a two-step mechanism. In the first step, the dsRNA silencing trigger is recognized and processed in the cytoplasm by Dicer (51,52), an RNase III family nuclease, into small interfering RNA (siRNA). These double stranded siRNAs are 21 to 24 nucleotides (nt) in length and have characteristic 3'-dinucleotide overhangs. In the second step, one strand of the siRNA duplex, named the guide strand, is incorporated into the RNA-induced silencing complex (RISC). The other strand, the passenger strand, is cleaved, released and degraded (53-55). The multiprotein RISC is then guided to messenger RNAs (mRNAs) that have a high sequence complementarity to the siRNA strand, leading to cleavage and degradation of the transcripts (56-59).

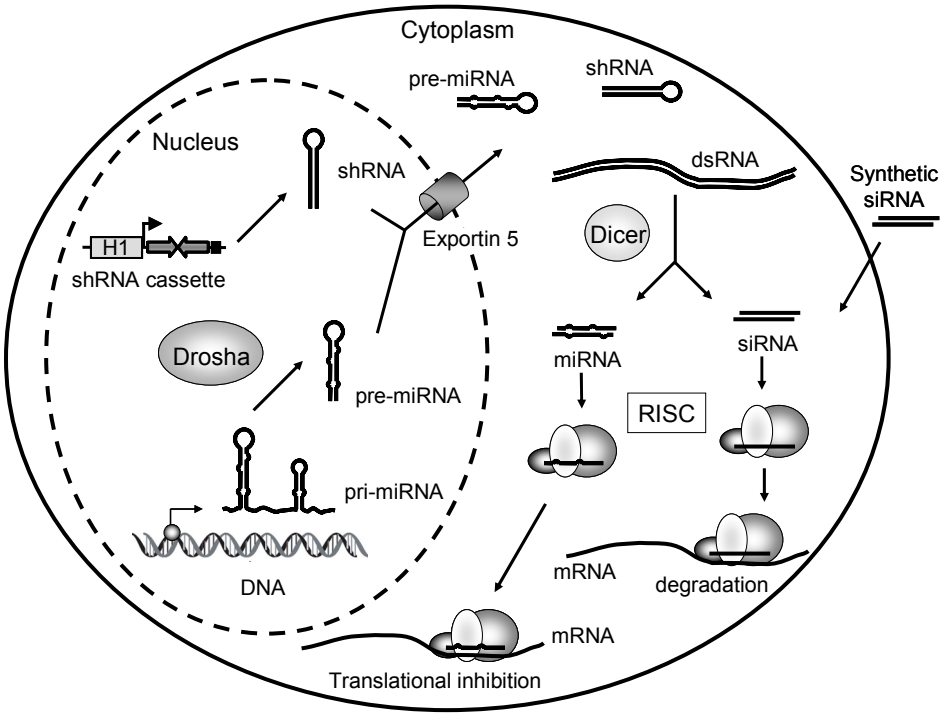

Figure 3: Schematic of the RNAi pathway. See text for further details.

Dicer

Dicer is a multidomain protein (Figure 4A and 4C) containing a DexH RNA helicase/ATPase domain, the DUF283 and PAZ signatures, two neighboring RNase III-like domains and a dsRNA binding domain (dsRBD) (reviewed in (60)). Cleavage of dsRNA by Dicer is mediated by a single processing center formed through intramolecular dimerization of the two RNase III domains, and generates products with 2 nt 3′ overhangs (61). Dicer preferentially cleaves dsRNA at their termini and the distance between the PAZ and RNase III domains sets the length of the siRNAs produced (61,62). Other characteristic features of the produced siRNAs are a 3′ hydroxyl and a 5′ phosphate, of which the latter is essential for the RNAi mechanism in flies and mammals (59,63,64).

Recently, the HIV-1 transactivating response (TAR) RNA-binding protein TRBP (65) has been identified as a dsRBD protein partner of human Dicer (66,67). TRBP-binding to Dicer is not only required for efficient processing of endogenous miRNAs, but is also required for optimal RNA silencing mediated by exogenously introduced siRNAs (66,67). It appears that TRBP is responsible for the recruitment of the RISC component Ago2 to the siRNA bound by Dicer.

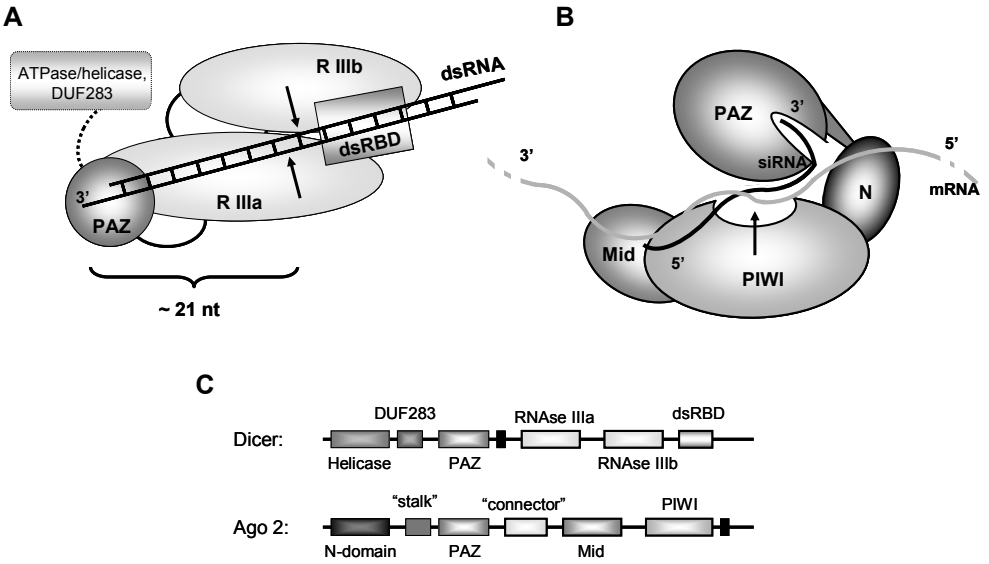

Figure 4: Schematic of **(A)** Dicer, **(B)** Ago2 and **(C)** their domain structures. See text for further details.

RISC

RISC is a multicomponent nuclease that cleaves messenger RNA that is complementary to the incorporated small RNA strand (52). Although the protein composition of RISC is not completely known, it always includes the Argonaute protein Ago2 (Figure 4B and 4C). Characteristic signatures of all eukaryotic Argonaute proteins are the PAZ and PIWI domains. Structural studies indicate that the PAZ domain specifically recognizes and binds the 3′ protruding end of small RNAs (68-72). The PIWI domain folds a structure that is very similar to the RNase H enzyme and provides a binding pocket for the 5′ end of small RNAs (73,74). Ago2 mediates cleavage of the target mRNA at the site 10 nt upstream of the 5′-most residue of the siRNA/target mRNA duplex (75-78).

MicroRNAs

Shortly after Dicer was discovered, it was found that this enzyme is also part of the production machinery of a class of small regulatory RNAs (79-82), the microRNAs (miRNAs) (83-85). Cellular miRNAs are synthesized as part of longer primary RNA transcripts (pri-miRNAs) that are usually several kilobases long and that contain local hairpin structures (86-89) (Figure 3). A nuclear microprocessor complex, consisting minimally of the endonuclease Drosha and a cofactor termed DGCR8, cleaves the pri-miRNA to release the stem-loop miRNA precursor (pre-miRNAs) (90-92). Drosha is a relatively large protein that contains two tandem

RNase III domains and a dsRBD that are crucial for catalysis (93,94). The primary determinant for Drosha cleavage is the tertiary structure of pri-miRNAs and both the double stranded stem structure around the cleavage site and the large terminal loop (consisting of >10 nucleotides) are important (90,95,96). After nuclear processing by Drosha, the pre-miRNA is exported to the cytoplasm by the nuclear transport receptor Exportin-5 (97-99). In the cytoplasm, the pre-miRNAs are processed by Dicer into miRNA duplexes (87,88).

Although miRNAs differ from siRNAs in their biogenesis, their functions are similar. miRNAs can cause translational inhibition when bound to partially complementary sites that are clustered in the 3' untranslated region of the target mRNAs (100-103). The targeted mRNAs are thought to be sequestered into ribosome-free cytoplasmic compartments called P-bodies (processing bodies) (104,105). However, when an miRNA encounters an RNA with (near) complete complementarity, it can guide cleavage and subsequent target degradation (106-108). Conversely, the extent of complementarity between an siRNA guiding strand and the target mRNA determines whether mRNA silencing is achieved via site-specific cleavage or through an miRNA-like mechanism of translational repression (108,109).

Biological function

The degree of evolutionary conservation of the RNAi mechanism suggests an important biological function. In both plants and animals, one key function of the pathway is to maintain the integrity of the genome by suppressing the mobilization of transposons and the accumulation of repetitive DNA in the germline (110-113). In plants, the RNAi machinery serves as an adaptive, antiviral defense system, which is transmitted systemically in response to viral invasion (114,115). Many plant viruses have evolved defenses to counteract RNAi (116,117). Recently, evidence has been found that vaccinia virus and human influenza A, B, and C viruses also encode a protein capable of inhibiting silencing in *Drosophila* and in plants (118-120). It has also been suggested that the HIV-1 Tat protein is a suppressor of RNAi (121). These reports support the notion that RNAi may have an antiviral function in animals as well.

Additionally, it has been discovered that the miRNA pathway plays an important role in the regulation of gene expression of diverse pathways including cell proliferation, haematopoietic cell differentiation, apoptosis, control of developmental timing and organ development (122,123). In fact, miRNAs may influence as much as one-third of the human genes (124,125).

Inducing RNAi

In mammalian cells, dsRNAs larger than 30 base pairs (bp) can trigger the interferon pathway, which promotes sequence non-specific degradation of mRNA

through the activation of RNAseL, as well as a global inhibition of mRNA translation following activation of protein kinase R (126,127). The ultimate outcome of this set of dsRNA-triggered responses is cell death via apoptosis (128). Therefore, an important discovery in the RNAi field was the demonstration that synthetic short RNA duplexes (21-23 bp), mimicking Dicer-generated siRNAs, could also trigger RNAi (129) (Figure 3).

The use of synthetic siRNAs to suppress gene expression has been successfully applied in mammalian cell lines, primary cells and embryonic stem cells (129-132). The basic structure requirements of effective siRNAs were defined and it became clear that siRNA silencing acts in a strictly sequence specific manner (133-136). Subsequently, criteria and guidelines for siRNA design have been developed (135,137-139) and computational methods to predict functional siRNAs are continuously updated (140-142). Not all siRNA duplexes matching the design criteria are potent inhibitors and thus their efficacy has to be assessed experimentally (143,144).

Short hairpin RNAs

A second important development was the finding that siRNAs could be generated from a gene construct that expresses a short hairpin RNA (shRNA). This shRNA contains a 19-29-bp stem and a small loop, and is usually synthesized by a polymerase (pol) III promoter such as U6 or H1 (145,146) (Figure 3). The transcription initiation site of a pol III promoter is well defined and transcription termination occurs at a stretch of four consecutive T residues in the DNA template (147). Well defined ends of a shRNA are critical for nuclear export by Exportin-5 (148) and for Dicer-mediated processing to generate a mature siRNA with the characteristic 2-nt 3' overhang (145,146). Because of the structural similarities, an shRNA might be seen as an artificial pre-miRNA. When shRNAs are transcribed from pol II promoters (149,150) as pri-miRNA-like transcripts, these also include the flanking sequences and are processed in the cell like an endogenous pri-miRNA.

Such plasmid vectors are potent experimental tools for inducing gene silencing in mammalian cells (134,151,152). To overcome limitations in terms of transfection efficiency, particularly in primary cells (151), researchers have developed adenoviral (153), retroviral (154) and lentiviral (155) vector systems for shRNA delivery.

RNAi as therapeutic

The success of RNAi in silencing gene expression in mammalian cells triggered the development of RNAi-based therapies against a wide variety of diseases, including cancer, infectious and autoimmune diseases. The advantage of RNAi-mediated gene suppression is its sequence specificity, efficiency (silencing can reach > 90%)

and versatility (any mRNA as target). There are also some serious challenges such as efficient delivery, safety issues and efficacy. In spite of these issues, RNAi shows potential as a powerful strategy for intracellular immunization against human pathogenic viruses such as HIV-1.

Scope of this thesis

After the discovery of RNAi, researchers addressed the hypothesis that RNAi can be used to selectively block viral gene expression, and thus viral replication, in human cells. Initial experiments showed that HIV-1 replication can be potently inhibited by transient transfection of siRNAs targeting either viral RNA sequences or host genes essential for HIV-1 entry or replication (39,156-159). In this thesis, we describe potent RNAi-mediated inhibition of HIV-1, but also diverse viral escape routes. In particular, we zoom in on the effect of target RNA structure on the RNAi efficiency.

We stably introduced vectors expressing shRNAs directed against various HIV-1 sequences into a human T-cell line through retroviral transduction (**Chapter 2**). The target sequences selected were highly conserved among virus isolates and highly accessible as determined with antisense DNA oligonucleotide arrays. We observed efficient inhibition of HIV-1 replication with only one out of eight siRNA constructs. However, the inhibition obtained with this single shRNA was very potent, reproducible and long-lasting. Nevertheless, HIV-1 escape variants emerged in prolonged cultures and we examined the acquired mutations in these RNAi-resistant viruses.

In **Chapter 3**, the RNAi-resistant HIV-1 variants were analyzed in more detail. We studied the sequence changes in nine escape viruses. We determined the level of RNAi resistance and whether the ability of the siRNA to bind the mutated target RNA correlates with the RNAi efficiency. However, this residual stability of the siRNA/target-RNA duplex does not explain the RNAi resistance of all mutants and we investigated the effect of target RNA structure on RNAi efficiency as an alternative resistance mechanism. These data provide insight into the impact of target RNA structure on RNAi efficiency and document a different route of viral escape from RNAi-mediated inhibition.

To gain further insight in the impact of target RNA structure on RNAi, we performed a systematic mutational analysis with a normally efficient target sequence (**Chapter 4**). This target was placed in a perfect RNA hairpin within a reporter construct, which was totally insensitive to RNAi. We gradually destabilized this RNA hairpin and analyzed the effect on siRNA binding and RNAi efficiency. Additionally, the impact of exposing certain positions of the target sequence within the RNA structure were studied. We discuss the possible implications with respect to target selection and lentiviral-mediated delivery of shRNAs.

In **Chapter 5**, we describe the modification of a conditionally replicating HIV-1 variant to become an inducible vector for the efficient delivery of anti-HIV shRNAs to cells that are susceptible to HIV-1 infection. This RNAi vector will spread efficiently in the presence of inducer and its subsequent withdrawal should result in cells with a transcriptionally silent integrated provirus, but with an active shRNA expression cassette. We studied the ability of these cells to resist an HIV-1 challenge and discuss the possible use of such a vector in a therapeutic setting.

There have been conflicting results on whether RNAi-mediated inhibition can target the RNA genome that is present within infecting HIV-1 particles. Efficient targeting of the incoming RNA genome would be very beneficial in an RNAi-based therapeutic setting. Not only will the replication of HIV-1 be inhibited, but the establishment of the provirus will be prevented. We have addressed this issue in different experimental settings in **Chapter 6** using HIV-1-based lentiviral transduction as a quantitative model for HIV-1 infection.

In **Chapter 7**, we discuss the variety of strategies used by viruses to avoid or overcome an antiviral RNAi attack.

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Chapter Two

Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition

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Abstract

Short-term assays have suggested that RNA interference (RNAi) may be a powerful new method for intracellular immunization against human immunodeficiency virus type 1 (HIV-1) infection. However, RNAi has not yet been shown to protect cells against HIV-1 in long-term virus replication assays. We stably introduced vectors expressing small interfering RNAs (siRNAs) directed against the HIV-1 genome into human T-cells by retroviral transduction. We report here that an siRNA directed against the viral Nef gene (siRNA-Nef) confers resistance to HIV-1 replication. This block in replication is not absolute, and HIV-1 escape variants that were no longer inhibited by siRNA-Nef appeared after several weeks of culture. These RNAi-resistant viruses contained nucleotide substitutions or deletions in the Nef gene that modified or deleted the siRNA-Nef target sequence. These results demonstrate that efficient inhibition of HIV-1 replication through RNAi is possible in stably transduced cells. Therefore, RNAi could become a realistic gene therapy approach with which to overcome the devastating effect of HIV-1 on the immune system. However, as is known for antiviral drug therapy against HIV-1, antiviral approaches involving RNAi should be used in a combined fashion to prevent the emergence of resistant viruses.

The introduction of small interfering RNA (siRNA) into mammalian cells can activate RNA interference (RNAi), resulting in sequence-specific degradation of the targeted RNA. RNAi may be a powerful new method for intracellular immunization against human immunodeficiency virus type 1 (HIV-1) infection. It has been demonstrated in short-term assays that HIV-1 replication can be inhibited by siRNAs directed against viral or cellular targets (1-9). To demonstrate that RNAi can protect cells against HIV-1 in long-term virus replication assays and to study the evolution of the inhibited virus, we stably introduced vectors expressing siRNAs directed against eight HIV-1 targets into human T-cells (Figure 1A). We selected target sequences in the infectious HIV_{LAI} molecular clone that are highly conserved among virus isolates (10), which warrants a widespread application of the potential RNAi therapy. Furthermore, we selected targets within the structured HIV-1 RNA genome that are highly accessible as determined with antisense DNA oligonucleotide arrays (11,12). There is recent evidence that the efficacy of siRNAs is similarly influenced by secondary structure in the target transcript (13,14). These two selection criteria forced us in some cases to accept a sub-optimal design of the siRNA molecule. The selected targets include essential replication signals such as the primer-binding site (PBS-A and PBS-B) and the polypurine tract (PPT), a segment in the overlap between the Tat and Rev genes (TatRevA and TatRevB), and part of the 5′ untranslated leader region (UTR-A and UTR-B). Furthermore, we selected an siRNA targeting the viral Nef gene (siRNA-Nef) that was previously demonstrated to provide efficient silencing in a transient-transfection system (2).

The pRETRO-SUPER vector (15) expresses the siRNAs from the human H1 polymerase III promoter as a small hairpin. These vectors were stably transduced into the human T-cell line SupT1. This cell line allows the rapid and massive spread of HIV_{LAI}, as is apparent for the control SupT1 cells transduced with the empty pRETRO-SUPER vector (Figure 1B). SupT1 T-cells expressing a specific siRNA were infected with equal amounts of HIV_{LAI}. Fast virus replication was observed in all SupT1 cultures except those with cells expressing the siRNA against Nef (Figure 1C). The replication of HIV_{LAI} was profoundly reduced in cells expressing siRNA-Nef compared with that in the control cells transduced with the empty vector. We did not observe any growth retardation of the siRNA-Nef cells, indicating that the decrease in HIV-1 replication in these cells is not due to a nonspecific cell toxicity problem.

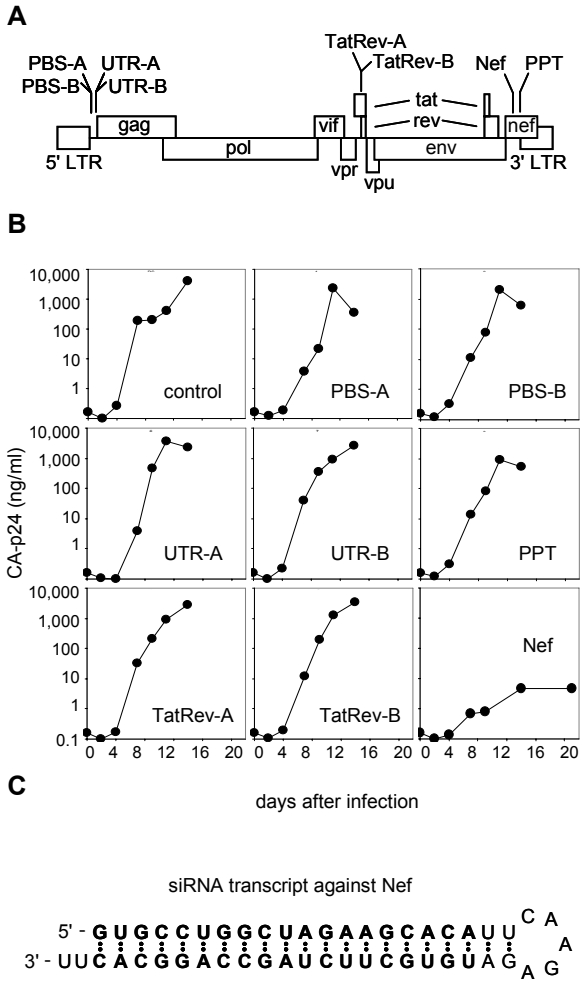

Figure 1. siRNA targeting of HIV-1. **(A)** SupT1 cells were stably transduced with the empty pRETRO-SUPER retroviral vector (15,16) (control) or with vectors expressing siRNAs against eight different target sequences in the HIV-genome: PBS-A, GTGGCGCCCGAACAGGGACTT; PBS-B, GGCGCCCGAACAGGGA-CTT; PPT, GGGGGACTGGAAGGG-CTA; TatRev-A, CCTTAGGCATCT-CCTATG; TatRev-B, CCTATGGCA-GGAAGAAGCG; UTR-A, GCGGAG-GCTAGAAGGAGAG; UTR-B, GG-CTAGAAGGAGAGAGATG; Nef, GT-GCCTGGCTAGAAGCACA.
(B) Cells were infected with HIV_{LAI} (800 pg CA-p24 in a 5-ml culture), and virus replication was monitored by determining the CA-p24 level in the culture supernatant.
(C) Predicted structure of siRNA-Nef transcript. The sequence targeting the Nef gene is shown in bold.

A second independently prepared batch of siRNA-Nef transduced cells showed the same resistance phenotype against both low and intermediate doses of HIV_{LAI} (Figure 2A, top and middle panels, respectively). However, the block in viral replication was not absolute, and a high dose of input virus allowed a low level of replication (Figure 2A, bottom panels). We demonstrated efficient replication of HIV_{LAI} on a new batch of control cells transduced with the empty vector (Figure 2A, right panels). As a further control for the specificity of the observed inhibition, we used an HIV-1 variant in which the Nef gene is replaced by the rtTA gene (HIV_{rtTA}) (17,18). HIV_{rtTA} lacks the Nef target sequence and replicated efficiently in both control cells and the cells that express the siRNA against Nef (Figure 2A). These results demonstrate the sequence-specific inhibition

of HIV-1 replication by RNAi targeting of Nef gene sequences that are present in the unspliced viral RNA genome and all spliced subgenomic HIV-1 transcripts.

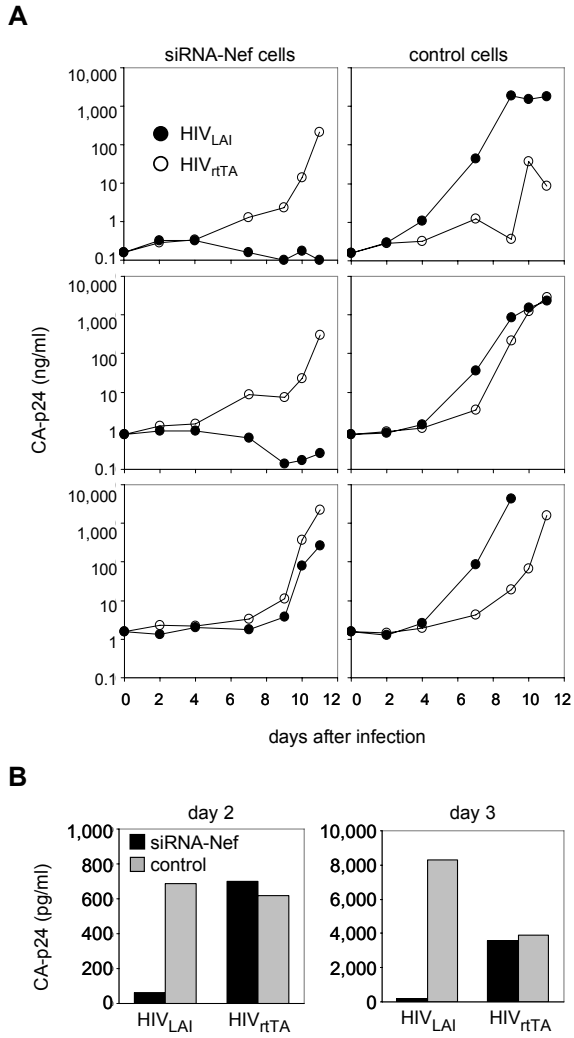

Figure 2. Sequence-specific inhibition of HIV-1 replication.
(A) SupT1 cells stably transduced with the siRNA-Nef vector (left panels) or the empty vector (right panels) were infected with wild-type HIV$_{LAI}$ (closed circles) or HIV$_{rtTA}$ with the Nef gene deleted (open circles). The virus input levels were 800 pg of CA-p24 (top panels), 4000 pg of CA-p24 (middle panels) and 8000 pg of CA-p24 (bottom panels) in 5-ml cultures. Virus spread was monitored by determining the CA-p24 level in the culture supernatant.
(B) siRNA-Nef and control cells were transfected with 10 µg DNA encoding wild-type HIV$_{LAI}$ or HIV$_{rtTA}$ with the Nef gene deleted as described previously (19). Virus production in the culture supernatant at 2 and 3 days after transfection was measured by a CA-p24 enzyme-linked immunosorbent assay.

SiRNA-mediated gene silencing may inhibit virus replication at a number of stages of the HIV-1 replication cycle. It is most likely that the siRNA-Nef is able to target the newly synthesized viral transcripts in HIV-infected cells. To directly test this possibility, we transfected the set of SupT1 cells with the proviral HIV$_{LAI}$ DNA and measured virus production after 2 and 3 days (Figure 2B). The cells that actively produce siRNA-Nef showed a >10-fold reduction in virus production compared with that in the control cells. Furthermore, this inhibition of gene

expression was specific for wild-type HIV-1, because no such effect was observed for the HIV_{rtTA} variant with the Nef gene deleted.

Only one out of eight siRNA constructs tested can effectively control a spreading HIV-1 infection. This siRNA against Nef was also effective when transfected as an oligonucleotide in a transient-transfection assay (2). The other siRNAs tested did not inhibit HIV_{LAI}, even though the sequences perfectly matched the viral transcript. Presently, we do not know why siRNA-Nef is such an effective inhibitor of HIV-1 replication. One reason may be that this siRNA targets both the unspliced and all spliced forms of HIV-1 RNA. However, the inactive siRNA-PBS and siRNA-PPT constructs also have this property. Thus, there may be additional features of the siRNA (e.g., expression level and intracellular location) or the target RNA (e.g., masking by viral and/or cellular proteins within the cell or virion particle) that determine the overall efficiency of inhibition. We have already mentioned that the selection of highly conserved and highly accessible target sites within the HIV-1 RNA genome had a negative impact on the design of some siRNAs. More detailed studies are needed to resolve some of these issues.

We cultured the siRNA-Nef transduced cells for more than 8 months and frequently assayed HIV_{LAI} and HIV_{rtTA} replication. The cells maintained a constant level of resistance to HIV_{LAI} replication, demonstrating the stable expression of the siRNA-Nef gene. The nearly complete resistance of these T-cells to HIV-1 replication allowed the selection of HIV-1 escape mutants that are no longer sensitive for RNAi against the Nef target sequence. To select an escape virus, we massively infected the nonpermissive cells that express siRNA-Nef, and a relatively fast replicating variant was obtained after 23 days. The targeted Nef gene was PCR-amplified (Figure 3A) at each passage, and DNA gel analysis of the PCR product indicated the loss of viral sequences around day 23 (Figure 3B). Sequence analysis revealed a deletion of 106 bp within the Nef gene that removes the siRNA-Nef target sequence (Figure 3C). The deletion of the target sequence provides a simple explanation for the observed resistance phenotype. To verify this explanation, we used equal amounts of the evolved HIV_{LAI} (LAI^{R1}) and the wild-type virus (LAI) to infect control SupT1 cells and cells that express siRNA-Nef. Whereas LAI is selectively inhibited in the siRNA-Nef-expressing cells (Figure 3D, left panel), no such effect is apparent for the resistant LAI^{R1} variant (Figure 3D, right panel).

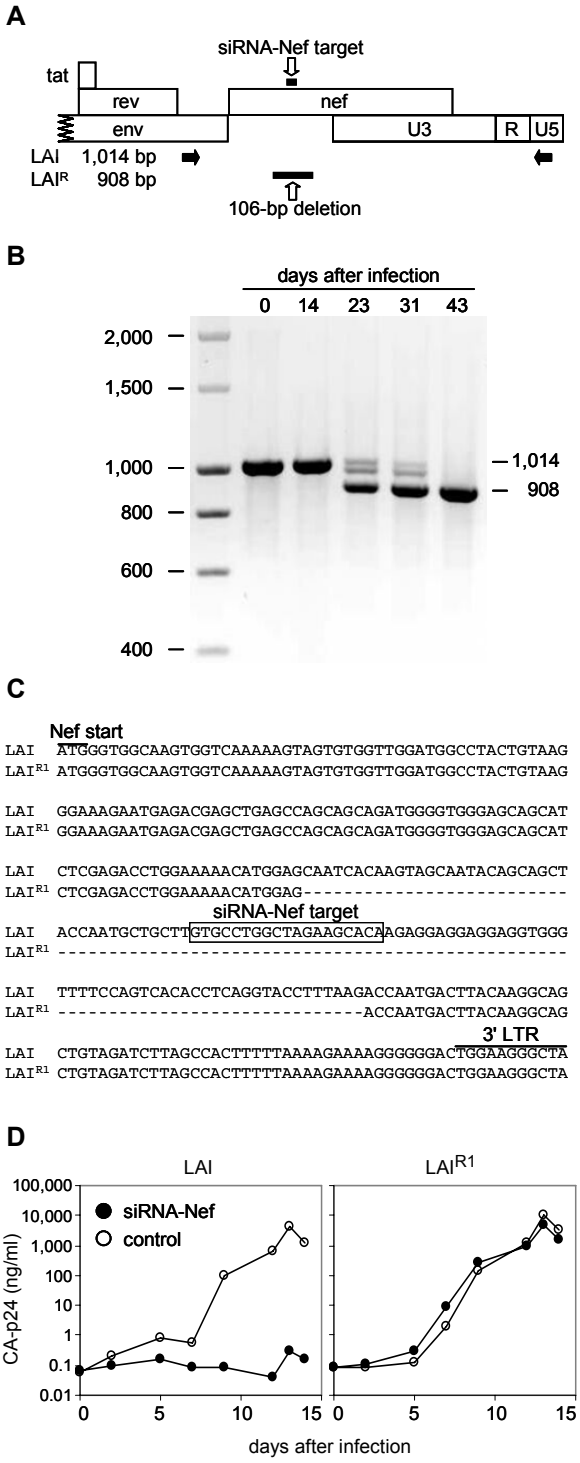

Figure 3. HIV_{LAI} develops resistance against siRNA-Nef by deleting the Nef-target sequence. SupT1 cells expressing siRNA-Nef were massively infected with HIV_{LAI} to overcome the siRNA-mediated inhibition of replication. The virus was passaged repeatedly onto fresh SupT1-siRNA-Nef cells. Cells were initially infected with a high virus dose that could be reduced gradually.

(A) Schematic of the 3' end of the HIV_{LAI} proviral genome. Indicated are the positions of the siRNA-Nef-targeted sequence, the PCR primers (black arrows), and the 106-bp deletion observed upon prolonged virus culture.

(B) At each passage, the proviral DNA present in infected cells was PCR amplified with primers that amplify the complete Nef gene as 1,014-bp fragment. At 23 days after infection, a fast-replicating variant containing a 106-bp deletion in the Nef gene was observed. At 43 days after infection, this siRNA-Nef-resistant virus dominated the virus population.

(C) Sequence of the Nef gene in the HIV_{LAI} virus and in the evolved siRNA-Nef resistant virus (LAI^{R1}) with the 106-bp deletion encompassing the siRNA-Nef target sequence.

(D) Infection of SupT1 control cells and the siRNA-Nef-expressing cells with wild-type LAI (left panel) or the evolved LAI^{R1} variant harvested at day 43 (right panel). We used equal virus input levels (400 pg of CA-p24 in a 5-ml culture).

We selected six additional resistant HIV-1 variants in independent cultures of HIV_{LAI} on siRNA-Nef-expressing cells (Figure 4). In four of these escape variants, the siRNA-Nef target sequence was completely or partially deleted (variants LAI^{R2}, LAI^{R4}, LAI^{R5} and LAI^{R7} in Figures 4A and 4B). These deletions did not affect genes other than the Nef gene and did not affect important regulatory elements such as the 3' polypurine tract or long terminal repeat sequences. For two variants (LAI^{R3} and LAI^{R6}), we observed nucleotide substitutions within the siRNA-Nef target sequence. These results show that resistance to RNAi can result from both the deletion and the mutation of the siRNA-targeted sequence. In one of the cultures (LAI^{R3}), the RNAi-resistant virus contained one nucleotide substitution after 27 days of culture, and a second mutation was acquired after 62 days. These result suggests that one nucleotide mismatch between the siRNA and its target sequence provides only partial RNAi resistance.

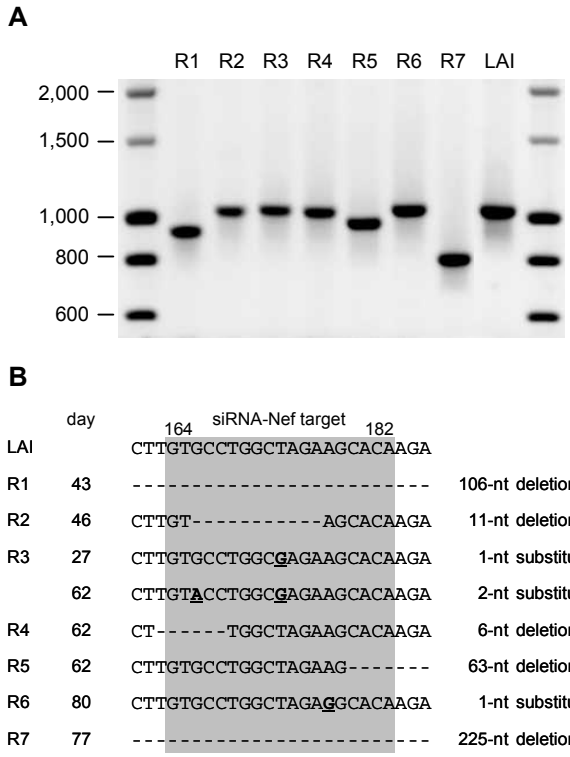

Figure 4. RNAi-resistant HIV-1 variants. HIV_{LAI} variants resistant to siRNA-Nef were selected in independent cultures and analyzed as described in the legend to Figure 3.
(A) PCR amplification of the Nef gene on the indicated day (LAI, input HIV_{LAI} virus; R1, LAI^{R1} escape virus shown in Figure 3 at day 43; R2 to R7, independent HIV_{LAI} escape variants).
(B) Nef target sequence in the evolved RNAi-resistant viruses. In LAI^{R5}, nucleotides 179 to 241 of the Nef gene are deleted. In LAI^{R7}, we observed the deletion of nucleotides 44 to 268 and a T269A substitution.

Antiviral therapy based on siRNA has been proposed previously for the rapidly replicating and highly cytolytic poliovirus, and this study also reported an escape virus with a point mutation in the middle of the targeted sequence (20). This RNAi-resistant poliovirus variant was likely present in the initial virus

population (20). In contrast, the HIV-1 experiments in the present study were initiated with a molecularly cloned viral genome. Furthermore, we observed diverse deletions and substitutions in the resistant viruses. Therefore, we can conclude that the selected RNAi-resistant HIV-1 variants emerged *de novo*. The combined analysis of siRNA escape viruses in both studies confirms that the antiviral effect is potent and sequence specific.

LAI^{R6} is the only RNAi-resistant HIV-1 variant with a silent codon change and thus produces the wild-type Nef protein. Nonsilent nucleotide substitutions in LAI^{R3} result in two amino acid changes (A56T and L58R). The LAI^{R1} and LAI^{R2} variants have a deletion that results in a frameshift and large C-terminal truncation of the Nef protein. The in-frame deletions in LAI^{R4}, LAI^{R5} and LAI^{R7} result in the absence of 2, 31 and 75 amino acids in the Nef protein, respectively. The complete inactivation of the accessory Nef gene has a relatively minor impact on the replication fitness of HIV-1 *in vitro* but significantly attenuates replication *in vivo* (21). It therefore seems less likely that HIV-1 will acquire resistance *in vivo* by deletion of the Nef sequence. The virus may instead evolve resistance by accumulating silent point mutations in the Nef target sequence. Also, when targeting essential viral genes, the virus may become resistant as a result of silent mutations. The targeting of cellular genes could therefore be advantageous. Obvious cellular targets that have an immediate impact on HIV-1 replication are the CD4 receptor and the CCR5/CXCR4 coreceptors. As was recently demonstrated, siRNAs specific for these receptors do indeed inhibit HIV-1 replication (1,4,6). Although suppression of CD4 and CXCR4 may be restricted by their normal role in the immune system and cell migration, respectively, CCR5 seems dispensable for normal life (22). Unfortunately, not all HIV strains require CCR5, and inhibition of CCR5 may result in the selection of HIV-1 variants that use CXCR4 as coreceptor. We therefore propose that HIV-1 should be targeted by multiple siRNAs against essential HIV-1 elements, either genes or regulatory sequences. Although the targeting of a single HIV-1 sequence can result in strong inhibition of viral replication, it is likely followed by viral escape. Analogous to the current clinical use of combinations of antiviral drugs that target the reverse transcriptase and protease enzymes, antiviral approaches using RNAi should also be administered in a combined fashion to prevent viral escape.

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Chapter Three

HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome

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Abstract

HIV-1 replication can be efficiently inhibited by intracellular expression of an siRNA targeting the viral RNA. However, HIV-1 escape variants emerged after prolonged culturing. These RNAi-resistant viruses contain nucleotide substitutions or deletions in or near the targeted sequence. We observed an inverse correlation between the level of resistance and the stability of the siRNA/target-RNA duplex. However, two escape variants showed a higher level of resistance than expected based on the duplex stability. We demonstrate that these mutations induce alternative folding of the RNA such that the target sequence is occluded from binding to the siRNA, resulting in reduced RNAi efficiency. HIV-1 can thus escape from RNAi-mediated inhibition not only through nucleotide substitutions or deletions in the siRNA target sequence, but also through mutations that alter the local RNA secondary structure. The results highlight the enormous genetic flexibility of HIV-1 and provide detailed molecular insight into the sequence specificity of RNAi and the impact of target RNA secondary structure.

Introduction

Double stranded RNA (dsRNA) can induce RNA interference (RNAi) in cells, resulting in sequence-specific degradation of the homologous single-stranded RNA (1,2). RNAi is an evolutionary conserved process that may provide the host with a mechanism directed against transposable elements (3) and infecting viruses (4-6). The dsRNA trigger is processed by a ribonuclease (Dicer) into the effector molecules, ~22nt double stranded RNAs termed short interfering RNAs (siRNAs) (7,8). One strand of the siRNA duplex is incorporated into the RNA-induced silencing complex (RISC), which subsequently binds and cleaves complementary RNA sequences (9,10). The efficiency of target RNA cleavage is affected by the stability of the siRNA/target-RNA duplex, which depends on the sequence complementarity between the siRNA and its target RNA (11), the nucleotide composition of the duplex and the precise position of nucleotide mismatches (12,13). Moreover, it has been suggested that RNAi efficiency is affected by the accessibility of the target RNA, which may be influenced by protein binding (12) and the formation of RNA secondary structure (14-18).

Introduction of siRNAs into cells has proven to be a powerful tool to suppress gene expression. Transfection of synthetic siRNAs into cells results in transient inhibition of the targeted gene (19). Long-term gene suppression can be achieved by the introduction of vectors that stably express short hairpin RNAs (shRNAs) that are processed into siRNAs by Dicer (20,21).

RNAi may be a powerful new method for intracellular immunization against human immunodeficiency virus type 1 (HIV-1). It has been demonstrated in short-term assays that HIV-1 replication can be inhibited by synthetic siRNAs targeting either viral RNA sequences or cellular mRNAs encoding protein co-factors that support HIV-1 replication (22-27). Recently, we demonstrated long-term inhibition of HIV-1 replication in human T-cells that stably express siRNAs directed against the viral Nef gene (28). However, viral escape variants that were no longer inhibited by siRNA-Nef emerged. The siRNA-Nef target sequence in these RNAi-resistant viruses was either partially or completely deleted, or modified by nucleotide substitutions, which demonstrates the exquisite sequence specificity of the RNAi mechanism. In this study, we analyzed the sequence changes in nine escape viruses and performed experiments to study the resistance mechanism. We observed a strong correlation between the stability of the siRNA/target-RNA duplex and the level of RNAi resistance. In addition, two viruses were found to escape from RNAi through mutations that induce an alternative secondary structure of the target RNA. These results demonstrate that occlusion of an siRNA-target sequence by RNA secondary structure reduces RNAi efficiency. Moreover, our results highlight the extreme versatility of HIV-1 and its evolutionary capacity to escape from RNAi-mediated antiviral therapy.

Materials and Methods

Cells and viruses

SupT1 T-cells transduced with pRetro-SUPER expressing siRNA-Nef were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 units/ml streptomycin at 37°C and 5% CO_2. SupT1 cells ($2.5x10^5$ cells in 1 ml medium) were infected with wild-type or mutant HIV-1 LAI (1 ng of CA-p24), and viral replication was monitored by determining the CA-p24 level in the supernatant by ELISA.

C33A cervix carcinoma cells were grown as a monolayer in DMEM supplemented with 10% FCS and minimal essential medium nonessential amino acids at 37°C and 5% CO_2. C33A cells were transfected by the calcium phosphate method. Briefly, cells were grown in 3 ml of culture medium in 10 cm^2 wells to 60% confluency. A mix of 5 μg wild-type or mutant HIV-1 LAI DNA in 110 μl water, 125 μl 50 mM HEPES (pH 7.1), 250 mM NaCl, 1.5 mM Na_2HPO_4 and 15 μl 2M $CaCl_2$, was incubated at room temperature for 20 min and added to the culture medium. The culture medium was changed after 16 h and viruses were harvested 3 days posttransfection.

DNA constructs

The full-length molecular HIV-1 clone LAI (29) was used to produce wild-type and mutant viruses. Nucleotide numbers presented here refer to the position on the genomic HIV-1 RNA transcript, with +1 being the capped G residue. The mutant proviral DNA sequences were PCR-amplified from cellular DNA with the 5′ Env primer tTA1-AD (+8269 to +8289) and the 3′ U5 primer CN1 (+9253 to +9283). The PCR fragments were digested with XhoI and BspEI and cloned into the plasmid Blue-3′LTR (30). The XhoI-BglI fragments (1709 bp) of these plasmids were cloned into the wild-type LAI clone, resulting in the full-length mutant clones R1-R9.

The firefly luciferase expression vector pGL3 control (Promega) was used to construct the wild-type and mutant reporter-Nef target plasmids (pGL3-Nef). An approximately 250 bp Nef fragment (+8448 to +8698) was PCR amplified from the full-length molecular clones with the primers EW1 (5′-ACGTCTAGAATTCTG-AGACGAGCTGAGCCAGCA-3′) and EW3 (5′-GACTCTAGACTGCAGGAGTGA-ATTAGCCCTTCCA-3′). The PCR product was digested with XbaI and cloned into the XbaI site located downstream of the luciferase gene in pGL3 control. The forward orientation of the insert was checked by sequence analysis.

To construct the m1-m4 mutants, base changes were introduced into pGL3-Nef by mutagenesis PCR (31). Mutagenic (m) primers EWmut1 (5'-ACAGC-AGCTACCAATCCTGCTTGTGC-3', mismatching nucleotide underlined; m1), EWmut2 (5'-CACAAGTAGGAATACAGCAGCTACCAATCCTGCTTGTGC-3'; m2), EWmut3 (5'-CACAAGTAGTAATACAGCAG-3'; m3 and m4), and the general primers EW1 (Primer 1), EW2 (5'-TGAGGCCCGGTACCTGAGGTGTGACT-3'; primer 2), and EW3 (Primer 3) were used with the wild-type pGL3-Nef (m1, m2, and m4) or the R8 pGL3-Nef (m3) template. Briefly, PCR reactions were performed with primer M plus primer 3, and with primer 1 plus primer 2. The PCR products were purified, mixed, and PCR amplified with primers 1 and 3 as described (31). The PCR fragments were digested with XbaI and cloned in the corresponding site of pGL3 control. All mutations were verified by sequence analysis.

The pRetro-SUPER-shNef vector, which expresses siRNA-Nef under control of the H1 RNA polymerase III promoter was digested with EcoRI and XhoI, and the 314-bp expression cassette was ligated into the EcoRI/XhoI sites of pBluescriptII (KS^+) (Stratagene) to produce pBS-siRNA-Nef. Plasmid pRL-CMV (Promega) expresses renilla luciferase under control of the CMV promoter.

Luciferase assay

C33A cells were grown in 1 ml culture medium in 2 cm^2 wells to 60% confluence and transfected by the calcium phosphate method. A 100 ng wild-type or mutant pGL3-Nef was mixed with 0.5 ng pRL-CMV, 0.5-500 ng pBS-siRNA-Nef and completed with pBluescriptII to 1 μg of DNA in 15 μl water. The DNA was mixed with 25 μl of 2x HBS and 10 μl of 0.6M $CaCl_2$, incubated at room temperature for

20 min and added to the culture medium. The culture medium was refreshed after 16 h. After another 24 h, the cells were lysed in 150 µl of Passive Lysis Buffer (PLB) (Promega) by shaking for 20 min at room temperature. The cell lysate was centrifuged and 10 µl of the supernatant was used to measure firefly and renilla luciferase activities with the Dual-luciferase Reporter Assay System (Promega). The renilla luciferase expression in transfected cells allowed us to correct for variation in transfection efficiency.

In silico RNA analysis

The stability of the duplex of siRNA-Nef (UGUGCUUCUAGCCAGGCAC) and wild-type or mutant Nef target sequences (+8448 to +8698) was predicted with the Hybridization Mfold program (32,33) at http://www.bioinfo.rpi.edu/applications/mfold. The RNA structure of the wild-type and mutant target sequences (+8524 to +8579) was predicted with the RNA Mfold program (32,33). Similar results were obtained with larger RNA fragments (+8448 to +8698; data not shown).

RNA structure probing

Wild-type LAI and R8 proviral plasmids were used as template for PCR amplification with primers EWr1 (5′-AATTTAATACGACTCACTATAGGGGTG-GGAGCAGCATCTCGAG-3′; T7 RNA-polymerase promoter underlined) and EWr2 (5′-TGAATTAGCCCTTCCAGTCC-3′). The resulting PCR product contains a T7 RNA-polymerase promoter upstream of the HIV-1 nucleotides (+8474 to +8694). DNA products were purified with a PCR purification kit (Qiagen). RNA transcripts were produced by *in vitro* transcription with the Megashortscript T7 transcription kit (Ambion), and transcripts were purified on a NucAway™ spin column (Ambion). RNA concentrations were determined by spectrophotometry.

Wild-type and R8 RNA (20 pmol) were denatured in 60 µl water at 85°C for 3 min followed by snap cooling on ice. After addition of 20 µl 4x MO buffer (final concentration: 125 mM KAc, 2.5 mM MgAc, 25 mM Hepes, pH 7.0) and incubation for 30 min at 37°C, the transcripts were incubated with 5 mM lead(II) acetate at room temperature. Samples (15 µl) were taken at 0, 5, 15, 25 min, and cleavage was stopped by adding 3 µl of 1M EDTA. RNA products were purified over a NucAwayTM spin column (Ambion). Oligonucleotide EWr2 (5′-TGAATTAG-CCCTTCCAGTCC-3′; +8675 to +8694) was 5′ end labeled with the kinaseMax kit (Ambion) in the presence of 1 µl of [γ-^{32}P]ATP (0.37 MBq/µl, Amersham Biosciences). Three picomoles of ^{32}P-labeled oligonucleotide EWr2 was annealed to 3 pmol of the lead(II)-treated RNA by incubation at 85°C for 3 min followed by slow cooling to 60°C. The primer was extended at 60°C for 1 h using the Thermoscript reverse transcriptase (Invitrogen). After adding 20 µl gel-loading buffer II (Ambion), the samples were heated to 95°C and 10 µl was analyzed on a

denaturing 6% acrylamide gel. A sequence ladder of the wild-type Nef region was produced with the ^{32}P-labeled oligonucleotide EWr2, the pGL3-Nef plasmid as template and the thermo sequenase cycle sequencing kit (USB).

Electrophoretic mobility shift assay (EMSA)

The siRNA-Nef antisense oligonucleotide UGUGCUUCUAGCCAGGCAC (Eurogentec) was 5′ end labeled with the kinaseMax kit (Ambion) in the presence of 1 µl of [γ-^{32}P]ATP (0.37 MBq/µl, Amersham Biosciences). Wild-type and R8 RNA were denatured in 30 µl water at 85°C for 3 min followed by snap cooling on ice. After addition of 10 µl 4x MO buffer (final concentration: 125 mM KAc, 2.5 mM MgAc, 25 mM Hepes, pH 7.0), the RNA was renatured at 37°C for 30 min. The transcripts were diluted in 1x MO buffer to a final concentration varying from 0 to 7.5 µM in MO buffer. The 5′-labeled oligonucleotide was added (2.6 nM) and the samples (20 µl) were incubated for 30 min at room temperature. After adding 4 µl non-denaturing loading buffer (50% glycerol with bromophenol blue), the sample was analyzed on a non-denaturing 4% acrylamide gel. Electrophoresis was performed at 150 V at room temperature, and the gel was subsequently dried. Quantification of the free and bound oligonucleotide was performed with a Phosphor Imager (Molecular Dynamics).

Results

Selection of RNAi-resistant HIV-1 variants

We previously demonstrated potent inhibition of HIV-1 replication in the SupT1 T-cell line by stable expression of an siRNA directed against the viral Nef gene (28). This target sequence is located near the 3′ end of the viral genome (Figure 1A), and is present in both the unspliced genomic RNA and all spliced subgenomic RNAs. This replication block is apparently not absolute as HIV-1 escape variants appeared after several weeks of culture. We previously reported the acquisition of either a nucleotide substitution or a deletion in the Nef target sequence in such escape viruses. We now selected additional escape variants and analyzed a total of nine cultures by RT-PCR of the Nef segment and subsequent sequence analysis (Figure 1B). A single nucleotide substitution was observed within the 19-nt target sequence in three escape viruses (R3, R6, R9). The R3 virus had acquired an additional nucleotide substitution at a later time (sample R3′). This finding suggests that the single R3 mutation did not provide complete RNAi resistance. Partial or complete deletion of the Nef target was observed in five cultures (R1, R2, R4, R5, R7). These changes obviously affect the Nef open reading frame, resulting in the synthesis of a Nef protein with an internal deletion or a truncated protein due to a frameshift mutation. Since the Nef protein is not essential for HIV-1 replication in T-cell lines,

these mutations will have no major impact on viral replication in our culture system. Most surprisingly, the R8 escape virus has no mutation in the siRNA-Nef target sequence, but does instead have a single nucleotide substitution 7 nt upstream of the target sequence.

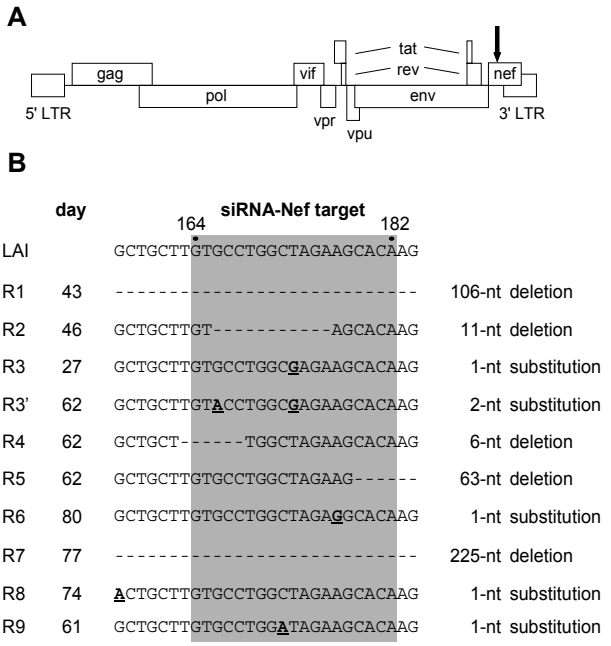

Figure 1. HIV-1 escape variants that resist siRNA-Nef inhibition. **(A)** Schematic representation of the HIV-1 LAI proviral genome. The position of the siRNA-Nef target sequence is indicated with an arrow. This sequence is present in both the unspliced and all spliced forms of HIV-1 RNA.
(B) HIV-1 LAI variants resistant to siRNA-Nef were selected in nine independent cultures. The Nef target sequence (gray box indicates nucleotides 164-182 of the Nef gene; nucleotides 8553-8571 in the LAI RNA genome) and flanking sequences are shown for the wild-type (LAI) and the evolved RNAi-resistant viruses (R1-R9). The day at which the escape variants were sequenced is indicated. Deletions are shown as dashes, substitutions are underlined and in bold. In the R1 virus, nucleotides 125-230 of the Nef gene are deleted. In the R5 virus, nucleotides 179-241 are deleted. In the R7 virus, we observed deletion of nucleotides 44-268 and a T269A substitution.

To verify that the observed mutations in or near the target sequence mediated the RNAi-resistant phenotype, we introduced the mutant Nef sequences in the HIV-1 LAI molecular clone. SupT1 cells that stably express siRNA-Nef were infected with wild-type or mutant HIV-1 virus. Wild-type HIV-1 is potently inhibited by the siRNA-Nef and did not initiate a spreading infection (Figure 2). In contrast, all mutant viruses replicate, demonstrating that the observed mutations in the Nef gene confer resistance against siRNA-Nef. The different escape variants do seem to replicate with different efficiencies. For instance, R3' is more fit than the R3 virus, suggesting that the additional mutation in the target sequence provides a higher level of resistance against siRNA-Nef. However, we decided not to focus on the differences in replication because it is a complex phenotype made up of several factors (level of RNAi resistance, impact of Nef protein modification, potential removal of RNA replication signals etc.).

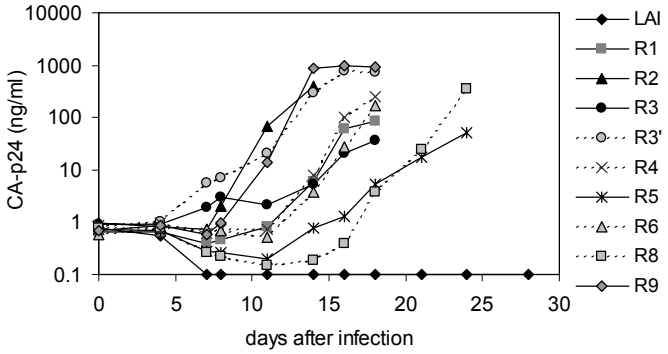

Figure 2. Nef mutations provide resistance against siRNA-Nef.
The Nef mutations shown in Figure 1B were cloned in the HIV-1 LAI molecular clone. Virus stocks were produced in transiently transfected C33A cells and used to infect SupT1 cells stably expressing siRNA-Nef. Virus replication was monitored by determining the level of CA-p24 in the culture supernatant. Similar results were obtained in independent infection experiments. The R7 variant was excluded from further analysis since it contained a complete deletion of the siRNA-Nef target sequence very similar to the R1 variant. The wild-type LAI virus that does not replicate on these SupT1-siRNA-Nef cells replicates efficiently on control SupT1 cells [results not shown, see also (28)].

The siRNA/target-RNA duplex stability influences the level of RNAi resistance

To accurately quantify the level of RNAi resistance, we made reporter gene constructs in which the wild-type or mutant Nef target sequence was placed downstream of the luciferase reporter gene (Figure 3A, pGL3-Nef). These constructs were cotransfected with an increasing amount of an siRNA-Nef expressing plasmid (pBS-siRNA-Nef) into the human cervix carcinoma cell line C33A, and luciferase production was measured after 48 h (Figure 3B). Reporter gene expression of the construct with the wild-type Nef sequence was significantly reduced by cotransfection with 0.5 ng pBS-siRNA-Nef (40% residual expression), and nearly complete inhibition (~10% residual expression) was obtained with higher amounts of siRNA-Nef. Expression of the reporter gene construct in which the target sequence was completely deleted (R1) was not inhibited by siRNA-Nef, demonstrating complete resistance. The constructs with a partial deletion of the target sequence showed complete (R2, 11-nt deletion) or partial resistance against siRNA-Nef (R4 and R5, with a deletion of 5 and 4 nt in the target sequence, respectively). Expression of the latter constructs was inhibited marginally at low amounts of pBS-siRNA-Nef, and high siRNA-Nef levels resulted in partial inhibition (40-60% residual expression). The constructs with a single nucleotide substitution showed either partial resistance (R3 and R9) or nearly complete resistance (R6). The acquisition of a second mutation in the R3 virus (R3') only marginally increased the level of resistance in this assay system. The construct with the mutation upstream of the target sequence (R8) also demonstrates partial resistance against siRNA-Nef in this assay.

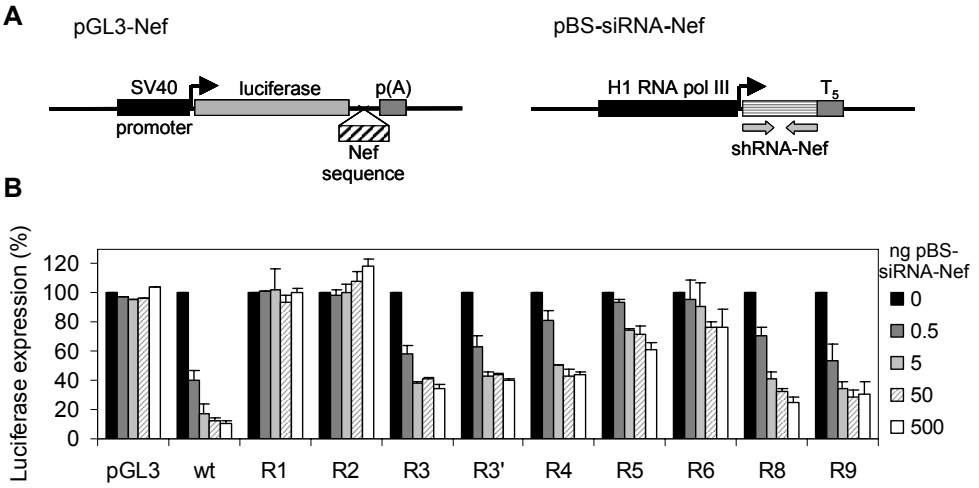

Figure 3. Quantification of the level of RNAi resistance. **(A)** A 250-bp Nef fragment (nucleotides 8949-9199 in the LAI RNA genome) that encompasses the target and flanking regions was cloned downstream of the firefly luciferase gene in the pGL3 reporter plasmid. These reporter gene constructs were cotransfected into C33A cells with the siRNA-Nef expressing plasmid pBS-siRNA-Nef. **(B)** Luciferase expression observed after transfection of the reporter constructs with increasing amounts of pBS-siRNA-Nef. The level of expression observed in the absence of siRNA-Nef was set at 100% for each reporter construct. This level did not vary significantly for the different constructs. The mean values obtained in three independent experiments are shown (± standard error).

Since RNAi is dependent on the sequence-specific base pairing of siRNA to the target RNA sequence, reduced binding due to mismatches may explain the resistance-phenotype of all escape variants, except for the R8 variant with a nucleotide substitution outside the target sequence. We therefore calculated the predicted thermodynamic stability (ΔG) of the siRNA/target-RNA interaction for the wild-type and mutant targets and plotted this value against the measured level of resistance (Figure 4). The siRNA-Nef forms a perfectly base paired duplex with the wild-type target (ΔG = –26.1 kcal/mole), consistent with efficient inhibition by siRNA-Nef (18% resistance). Both the substitution and deletion mutants show reduced siRNA/target-RNA duplex stability that correlates with increased resistance against siRNA-Nef (Figure 4). However, two escape variants (R6 and R8) do not follow this general pattern and show an exceptionally high level of resistance. The nucleotide substitution in R8 is located upstream of the target sequence. The siRNA/target-RNA duplex stability is thus not affected for this mutant, yet it is fairly resistant against RNAi. The R6 mutant contains an A-to-G substitution in the target sequence that has only a minor effect on the siRNA/target-RNA duplex because an U-A base pair is replaced by an U-G base pair. Whereas this substitution only marginally affects the stability of the duplex (ΔG = -25.7 kcal/mole), the R6 mutant shows nearly complete resistance.

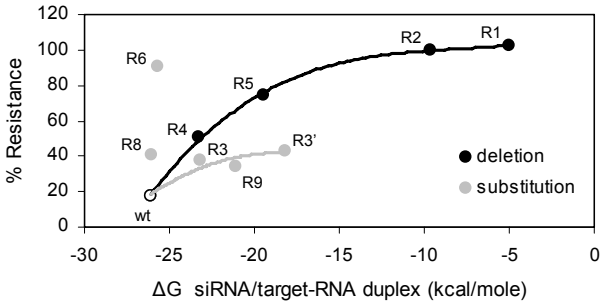

Figure 4. RNAi resistance due to reduced stability of the siRNA/target-RNA duplex. The thermodynamic stability (ΔG) of the siRNA-Nef/target-RNA duplex was calculated with the Hybridization Mfold program and plotted against the level of resistance as observed in Figure 3B with 5 ng of pBS-siRNA-Nef. The high level of resistance of the mutants R8 and R6 does not correlate with their predicted duplex stability.

Changes in target RNA structure can cause RNAi resistance

Binding of siRNA-Nef to its target sequence may also be affected by the accessibility of the target sequence, and thus by local RNA structure. Since RNAi resistance of the R6 and R8 mutants could not be explained by an altered stability of the siRNA/target-RNA duplex, we examined the effect of these mutations on the local RNA structure. We first used the Mfold program (32,33) to predict the secondary structure of the wild-type, R6 and R8 Nef target RNA. The energetically most favorable RNA structure of the wild-type Nef region (Figure 5A; ΔG = -17.9 kcal/mole) is an extended hairpin (S hairpin) that partially overlaps with the siRNA target sequence (marked in gray), but the 3′ half of the target sequence is predicted to be single stranded. The G-to-A substitution upstream of the target sequence in R8 destabilizes this hairpin structure (ΔG = -13.0 kcal/mole), and the RNA is likely to fold an alternative, more stable conformation with two smaller hairpins (Figure 5A; ΔG = -15.0 kcal/mole). In this alternative conformation, the second hairpin (R hairpin) encompasses the complete target sequence of which both the 5′ and 3′ ends are occluded by base-pairing. This new hairpin configuration of the target sequence may prevent the binding of siRNA-Nef.

This scenario is confirmed by inspection of the R6 escape variant. In fact, the R6 mutation in the target sequence does not reduce the stability of the wild-type S hairpin, but it profoundly stabilizes the alternative R hairpin (Figure 5B; ΔG = -21.3 kcal/mole). The A-to-G substitution in the target sequence allows the formation of two additional base pairs in this hairpin, thereby reducing the loop size from 10 to 6 nt. Thus, the exceptionally high level of resistance of R6 is probably due to the occlusion of the target sequence in a stable hairpin structure, although a contribution of the slightly reduced stability of the siRNA/target-RNA duplex due to the formation of the G-U base pair cannot be excluded.

In Figure 5C, the stability of the RNAi-sensitive (S) and the RNAi-resistant (R) conformations for the wild-type (wt), R6 and R8 RNAs are plotted. The wild-type can fold both RNA structures, but the S conformation is favored because it is

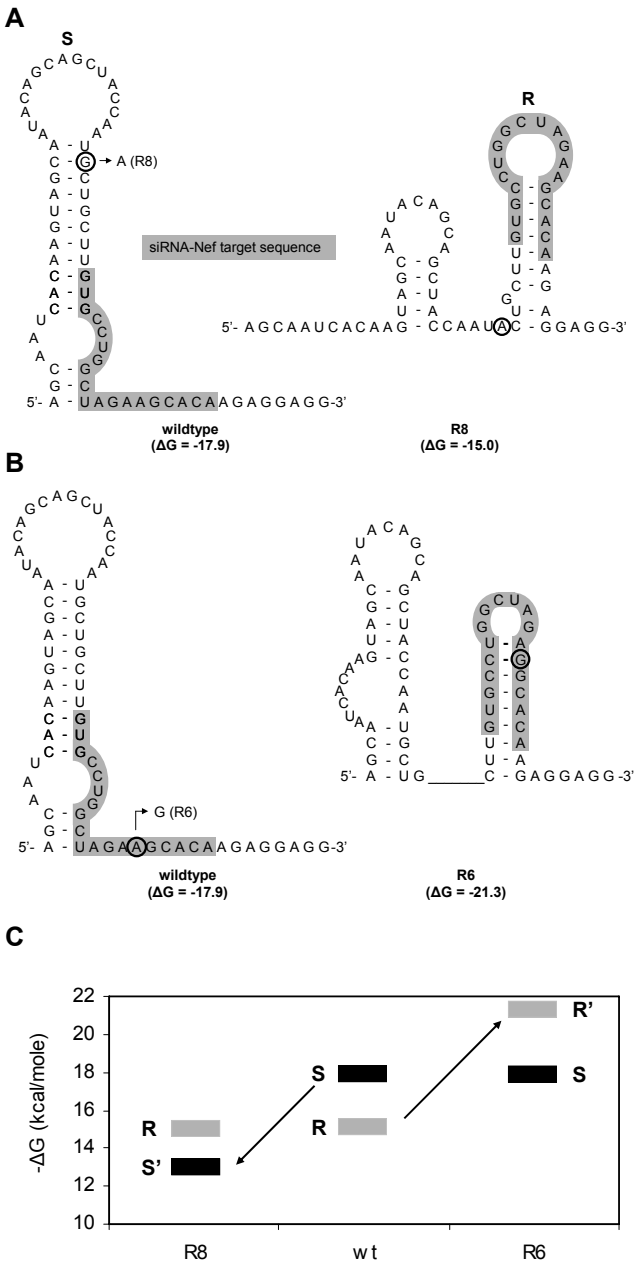

Figure 5. Local target RNA structure can cause resistance against RNAi.
(A) The predicted RNA structures (Mfold program) of the wild-type and R8 Nef sequences. The siRNA target sequence is highlighted by a gray box. The G-to-A substitution 7 nt upstream of the target sequence in R8 is encircled. This mutation disrupts the preferred sensitive (S) hairpin (ΔG = -17.9 → ΔG = -13.0 kcal/mole), resulting in folding of a more stable alternative structure with the resistant (R) hairpin and a small upstream stem-loop (ΔG = -15.0 kcal/mole).
(B) RNA structures of the wild-type and R6 Nef sequences. The A-to-G substitution at nucleotide 14 of the target sequence in R6 is indicated. This mutation does not affect the S hairpin, but profoundly stabilizes the alternative R hairpin through formation of two additional base pairs (ΔG = -17.9 → ΔG = -21.3 kcal/mole).
(C) The thermodynamic stability (ΔG) of the RNAi-sensitive (S) and the RNAi-resistant (R) structure are indicated for the wild-type (wt) and mutant (R6 and R8) Nef sequences. In wild-type RNA, the S structure (ΔG = -17.9 kcal/mole) is more stable than the R structure (ΔG = -15.1 kcal/mole). In mutant R8, the sensitive structure is destabilized (S'; ΔG = -13.0 kcal/mole) and energetically less favorable than the resistant structure (ΔG = -15.0 kcal/mole). Stabilization of the R hairpin in mutant R6 makes this conformation (R'; ΔG = -21.3 kcal/mole) more favorable than the sensitive conformation (S; ΔG = -17.9 kcal/mole).

more stable than the R conformation. The S conformation is destabilized (S′) in mutant R8, which thus becomes energetically less favorable than the R conformation. Stabilization of the R hairpin in mutant R6 makes this alternative conformation (R′) more favorable than the wild-type S structure.

The S and R RNA structure equilibrium dictates the level of RNAi resistance

We designed additional mutants to confirm that the presence or absence of the wild-type S hairpin determines RNAi sensitivity. All new mutants affect the -26/-7 base pair of the S hairpin that was opened in the R8 escape variant, and these nucleotide changes are thus positioned outside the actual Nef target sequence (Figure 6A). The RNA Mfold program was used to estimate the thermodynamic stability of the S and R conformations and RNAi sensitivity was determined in the luciferase-assay system (Figure 6B). The G-to-C substitution at position -7 in mutant m1 resembles the R8 mutation and similarly opens the S hairpin. Like the R8 mutant, this m1 mutant shows partial resistance against siRNA-Nef (Figure 6B). However, the m1 mutation also stabilizes the alternative R conformation (m1: ΔG^R = -19.4 kcal/mole) by the formation of two additional base pairs in the R hairpin (-7/+5 and -8/+6). This explains the higher level of resistance of m1 in comparison with R8 in which only hairpin S is destabilized. This resistant phenotype of m1 is reversed almost completely in the double mutant m2 by the introduction of a compensatory base change at position -26 (C-to-G), which restores base-pairing and preferential formation of the S hairpin. However, the m2 mutant is slightly more resistant than the wild-type. This can be explained by the relatively higher stability of the R hairpin in mutant m2 due to the -7 change. The -26 mutation in m2 also affects the stability of the small upstream hairpin of the R conformation. The ΔG values plotted in Figure 6A do include all these effects.

In the double mutant m3, we restored base pairing in the original escape variant R8 (which has a G-to-A at position -7) by introducing a compensatory mutation (C-to-U at position -26). Accordingly, this compensation reversed the RNAi-resistant phenotype of R8. The corresponding single mutant m4, which contains only the C-to-U mutation at position -26, maintains base pairing capacity in the S hairpin with a U-G base pair. Indeed, this mutant remains RNAi sensitive. The -26 mutation in m3 and m4 also affects the stability of the smaller hairpin in the R conformation, which is included in the ΔG^R calculations. These combined results strongly suggest that it is not the identity of the -26/-7 base pair per se, but rather the stabilization of the S hairpin structure that is critical for RNAi sensitivity.

We calculated the differences in ΔG of the S and R conformations ($\Delta\Delta G$), which reflects the S-R structure equilibrium, incorporating all mutational effects on the two conformations (Figure 6A). A negative $\Delta\Delta G$ value indicates that the RNA preferentially folds the S hairpin structure, whereas a positive value reflects a preference for the R hairpin conformation. Preference for the S hairpin correlates with RNAi sensitivity and mutations that shift the equilibrium towards the R hairpin conformation result in increased resistance against siRNA-Nef (Figure 6C).

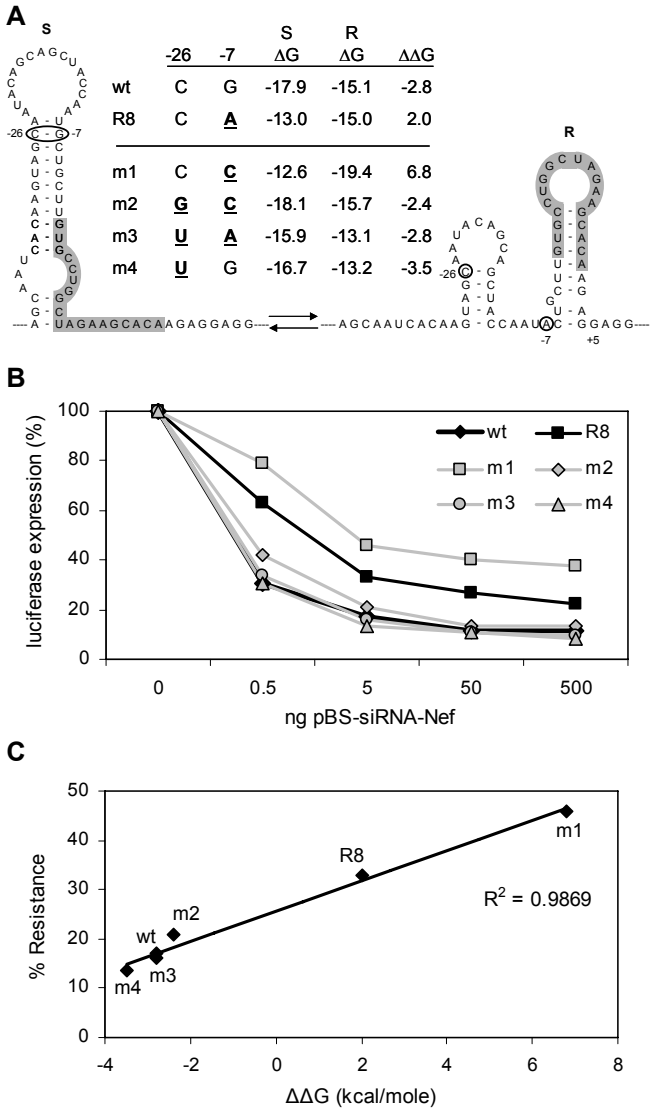

Figure 6. Stability of the S hairpin determines the level of resistance.
(A) Mutant pGL3-Nef reporter constructs with nucleotide substitutions upstream of the siRNA-Nef target sequence were constructed (mutation at position -7 and/or -26 in bold and underlined). The stability (ΔG) of the S and R structures was predicted with the Mfold program. The ΔΔG, providing a measure of the S and R structure equilibrium, was calculated ($\Delta\Delta G = \Delta G^S - \Delta G^R$).
(B) Luciferase expression after transfection of the reporter constructs with increasing amounts of pBS-siRNA-Nef. The level of expression in the absence of siRNA-Nef was set at 100% for each reporter construct.
(C) The ΔΔG of the structures is plotted against the level of resistance as observed in Figure 6B with 5 ng of pBS-siRNA-Nef.

RNA structure probing

To demonstrate that the R8 mutation does indeed affect the local RNA structure, we performed RNA structure probing experiments on the Nef region using lead(II), which is known to preferentially cleave single stranded RNA (Figure 7A). The cleavage pattern of the wild-type RNA is consistent with the proposed RNA secondary structure model (Figure 7B, left). For instance, we observed high reactivity in the loop and single stranded regions and low reactivity in the base-paired stem. The 3′ end of the siRNA-Nef target sequence in the wild-type RNA is

highly sensitive to lead, indicating that this region is single stranded. The cleavage pattern of the R8 mutant is different and confirms the proposed alternative folding (Figure 7B, right). In contrast to the wild-type RNA, the 3′ end of the siRNA-Nef target sequence is resistant to lead-induced cleavage, indicating that this region is base-paired as in hairpin R. The R8 RNA shows increased lead-sensitivity near the center of the target sequence, which corresponds with the exposed loop of the R hairpin. Domains further upstream and downstream on the wild-type and R8 RNA show a very similar lead-cleavage pattern, indicating that the change in RNA secondary structure occurs locally. These findings confirm that the R8 mutation results in alternative RNA folding that affects the presentation of the target sequence.

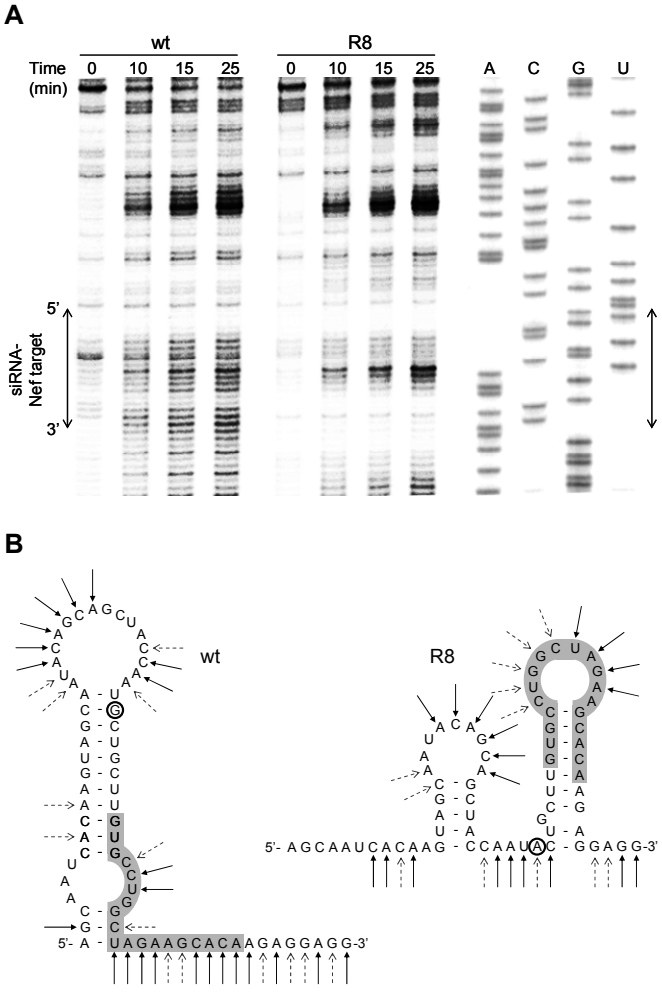

Figure 7. RNA structure probing of the wild-type (wt) and R8 Nef sequence.
(A) Wild-type and R8 RNA were treated with 5mM lead(II) acetate for different times as indicated above the lanes. Sites of lead-induced cleavage were detected by primer extension on the RNA. The wild-type sequence was analyzed in parallel to assess cleavage positions, and the position of the siRNA-Nef target sequence is indicated with an arrow.
(B) The lead-induced cleavage sites are represented on the secondary structure models of wild-type and R8 RNA. Strong and weak cleavages are indicated by solid and dotted arrows, respectively.

Stable target RNA structure blocks siRNA binding

To demonstrate directly that the alternative structure formed by R8 RNA does occlude the Nef target sequence from binding to the complementary siRNA-Nef strand, we performed binding assays. A 19-nt RNA oligonucleotide corresponding to the antisense strand of the siRNA-Nef was radioactively labeled, incubated at room temperature with increasing amounts of wild-type and R8 target RNA, and analyzed in an electrophoretic mobility shift assay (Figure 8A). Binding of this siRNA-Nef oligonucleotide to the target RNA results in a profound shift on a non-denaturing gel. The free siRNA-Nef and the siRNA-Nef/target-RNA duplex bands were quantitated to calculate the percentage of binding (Figure 8B). The siRNA-Nef bound less efficiently to R8 RNA than to wild-type RNA. Whereas almost all siRNA is shifted at high levels of wild-type RNA, the same amount of R8 RNA is not able to shift more than 39% of the siRNA-Nef. Because RNA structures should be temperature sensitive, we also performed assays at elevated temperatures. Indeed, the binding defect of the R8 mutant was diminished under such conditions (results not shown). Furthermore, kinetic experiments with an excess of siRNA-Nef showed that binding to wild-type RNA is significantly faster than to R8 RNA (3- to 4-fold difference at room temperature, results not shown). Since these two RNA

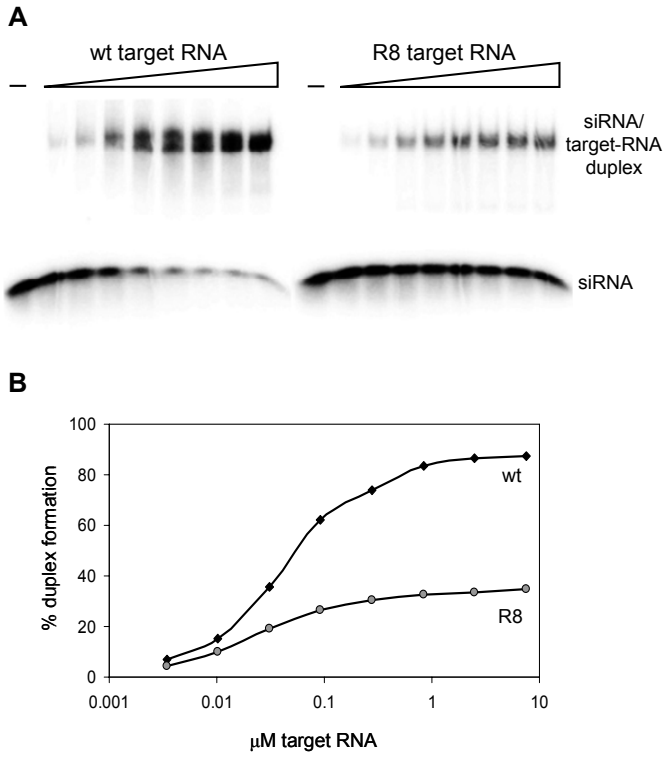

Figure 8. Target RNA structure can inhibit siRNA binding.
(A) Radioactively labeled oligonucleotide simulating the antisense strand of siRNA-Nef was incubated with an increasing amount of wild-type or R8 RNA. The siRNA/target-RNA duplex formation was analyzed by EMSA.
(B) Free and bound siRNA-Nef oligonucleotide was quantified to calculate the level of duplex formation (bound siRNA / free + bound siRNA).

templates do not differ in the actual target sequence, these results indicate that the alternative structure of R8 does occlude the target sequence from interacting with siRNA-Nef.

Discussion

HIV-1 replication can be inhibited efficiently through RNAi by targeting the viral Nef sequence. However, RNAi-resistant viruses emerged that contained nucleotide substitutions or deletions in or near the siRNA-Nef target sequence. We recloned these mutant Nef sequences in the HIV-1 genome and demonstrate that these mutations do indeed confer resistance against siRNA-Nef. We used a reporter-target-gene construct to accurately quantitate the level of resistance. For most mutants, we observed an inverse correlation between the level of resistance and the stability of the siRNA/target-RNA duplex. Two mutants did not follow this trend and demonstrated an unexpected high level of resistance. We demonstrate that these mutations induce alternative folding of the RNA that occludes the target sequence from binding to siRNA-Nef. These results demonstrate that the efficiency of RNAi-mediated inhibition depends on the efficiency of siRNA-binding to the target RNA. This interaction can be diminished by nucleotide substitutions or deletions in the target sequence that cause mismatches with the siRNA, or by mutations that induce an RNA structure in which the target sequence is occluded.

It has previously been reported that a mismatch between the target and siRNA can affect RNAi to a variable degree, depending on the position of the mismatch in the duplex (13,34). Kinetic analyses suggested that different regions of the siRNA play distinct roles regarding target recognition, cleavage and product release (35). The 5′ end of the siRNA contributes to initial target RNA binding, and mutations in either the central or the 3′ region of the target affect RNAi efficiency most dramatically (13,34). Most nucleotide substitutions that we observed in the RNAi-resistant HIV-1 variants are located in the center of the Nef target sequence, consistent with this notion. Mutations in the 5′ region of the target RNA have a milder effect, which may explain the small difference in resistance of the early R3 and late R3′ variants. The partial deletion mutants R4 and R5 differ significantly in their levels of resistance against siRNA-Nef (respectively 50% and 75% with 5 ng of siRNA-Nef-expressing plasmid). Focusing on the siRNA-Nef target sequence, R4 has a 5-nt deletion at the 5′ side and R5 a 4-nt deletion at the 3′ side. The fact that R5 is more resistant than R4 is also consistent with the notion that mismatches in the 3′ region in the target RNA are less well tolerated by the RNAi machinery.

Several studies have suggested an inhibitory effect of target RNA structure on RNAi efficiency (14-18). However these studies mainly compared the efficiency

of different siRNA and target sequences, such that only correlations with target RNA structure could be suggested. Furthermore, these studies did not experimentally verify the proposed RNA structures. We assayed the efficiency of a single target sequence when present in a different structural context. We confirmed these alternative RNA secondary structures in probing experiments. We demonstrate that occlusion of the target sequence by RNA structure inhibits binding of the siRNA, and thus reduces RNAi efficiency. Interestingly, the wild-type RNAi-sensitive hairpin S occludes only the 5′ side of the target sequence, whereas both the 5′ and 3′ sides are base paired in the alternative RNAi-resistant structure. This result indicates that an accessible 3′ target end is sufficient for target recognition, which is consistent with the idea that this 3′ region is mainly responsible for target recognition and binding (35).

It has previously been suggested that RNA hairpins are poor targets for RNAi because the corresponding antisense siRNAs will also have the ability to fold a stem-loop structure, which may hamper their activity (17). Although the 19-nt siRNA-Nef can indeed form a hairpin (6-bp stem, 6-nt loop: ΔG= - 5.1 kcal/mole), we know that this siRNA-Nef is very effective in silencing of the wild-type virus, indicating that this siRNA structure is not inhibitory.

Gene therapy to impose HIV-1 specific RNAi could become a realistic approach to potently inhibit virus replication (36-38). The siRNA directed against the viral Nef gene demonstrated a potent and sequence-specific antiviral effect. However, due to the high mutation rate of HIV-1, the virus can escape from RNAi-mediated inhibition (28,39). The fact that RNAi-resistant HIV-1 variants can emerge not only through deletions or substitutions in the siRNA target sequence, but also through mutations that alter the local RNA structure, demonstrates the extreme flexibility of the HIV-1 virus. Some escape viruses have lost the ability to encode a functional Nef protein. Whereas complete inactivation of the accessory Nef function has a relatively minor impact on the HIV-1 replication fitness *in vitro*, it significantly attenuates replication *in vivo* (40,41). It is therefore less likely that HIV-1 will acquire resistance *in vivo* by deletion or frameshifting of the Nef open reading frame. Nevertheless, the virus may still evolve resistance by accumulating silent point mutations. It is therefore important to identify additional targets in highly conserved sequences in essential HIV-1 genes. There may be other constraints in the viral genome, e.g. overlapping open reading frames, which could be used to design potent siRNAs from which escape is very difficult. Furthermore, targeting of a sequence located in or nearby a functionally essential secondary RNA structure motif could be advantageous. Escape mutations (even silent codon changes) may disrupt such replication signals and are thus less likely to emerge. Additionally, the transcripts of cellular cofactors can be targeted, but the knockdown of such functions should not affect viability of the host cell. Ideally,

HIV-1 should be targeted by multiple siRNAs against essential HIV-1 sequences (42). Similar to current antiviral drug combination therapies, this should prevent the emergence of escape mutants that are resistant to RNAi-mediated inhibition.

Acknowledgements

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Chapter Four

A systematic analysis of the effect of target RNA structure on RNA interference

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Abstract

RNAi efficiency is influenced by local RNA structure of the target sequence. We studied this structure-based resistance in detail by targeting a perfect RNA hairpin and subsequently destabilized its tight structure by mutation, thereby gradually exposing the target sequence. Although the tightest RNA hairpins were completely resistant to RNAi, we observed an inverse correlation between the overall target hairpin stability and RNAi efficiency within a specific thermodynamic stability (ΔG) range. Increased RNAi efficiency was shown to be caused by improved binding of the siRNA to the destabilized target RNA hairpins. The mutational effects vary for different target regions. We find an accessible target 3′ end to be most important for RNAi-mediated inhibition. However, these 3′ end effects cannot be reproduced in siRNA-target RNA binding studies *in vitro*, indicating the important role of RISC components in the *in vivo* RNAi reaction. The results provide a more detailed insight into the impact of target RNA structure on RNAi and we discuss several possible implications. With respect to lentiviral-mediated delivery of shRNA expression cassettes, we present a ΔG window to destabilize the shRNA inserts for vector improvement, while avoiding RNAi-mediated self-targeting during lentiviral vector production.

Introduction

RNA interference (RNAi) is induced by double stranded RNA (dsRNA) and results in gene silencing through sequence-specific degradation of the target RNA (1). RNAi provides plants and animals a defense mechanism against viruses (2-4) and retrotransposons (5,6). The ribonuclease Dicer processes the long dsRNA replication intermediates into small interfering RNAs (siRNAs) of ~22 nucleotides (nt) (7-9). These siRNAs are incorporated into the RNA-induced silencing complex (RISC) that finds complementary RNA sequences, resulting in cleavage of the target RNA (10,11). The central catalytic component of RISC is an Argonaute protein, which contains the signature domains PAZ and PIWI responsible for binding the siRNA strand (12). Transfection of siRNAs into cells or intracellular expression of short hairpin RNAs (shRNAs), which are processed into siRNA duplexes by Dicer, are powerful tools to suppress gene expression (13-15).

RNAi can also be used as a therapeutic strategy against human pathogenic viruses such as HIV-1 (16). HIV-1 replication can be inhibited transiently by transfection of synthetic siRNAs targeting viral RNA sequences or cellular cofactors (17-20). Furthermore, long-term inhibition of HIV-1 replication has been demonstrated in transduced cell lines stably expressing antiviral siRNAs or

shRNAs (21-26). However, HIV-1 escape variants with nucleotide substitutions or deletions in the siRNA target sequence do emerge after prolonged culturing (24,27,28). The emergence of RNAi-resistant variants may be blocked by a combination-shRNA therapy, which simultaneously targets multiple conserved viral RNA sequences (26,29).

We demonstrated that HIV-1 can also become resistant against RNAi by placing the target sequence in a stable RNA structure, which prevents binding of the siRNA (28). We also suggested that such structure-based target occlusion occurs in the RNA genomes of lentiviral vectors with a shRNA-cassette (ter Brake, in press). By inserting these cassettes, the target sequence will automatically be present in the vector genome, and self-targeting by the shRNA should reduce the lentiviral production level. However, since the target sequence in the genome is also located in this perfect shRNA hairpin, it is protected against RNAi, ensuring a normal vector titer. Indeed, when the target in the lentiviral genome is unstructured, the titer is significantly reduced by the shRNA (30).

The inhibitory effect of target RNA structure on RNAi efficiency has been described in several studies (31-33). These studies compared the efficiency of different siRNAs on a fixed target, and found a correlation between target availability and RNAi efficiency. Schubert *et al*. suggested that the local free energy of base pairing in the target region determines RNAi efficiency (34). Ideally, one should test this concept by a mutational analysis of one target instead of comparing different siRNAs with intrinsically different RNAi efficacies. In this scenario, mutations that affect the RNA structure should not affect the target sequence itself, such that the same siRNA inhibitor can be used. In this study, we set out to determine the exact hairpin stability at which RNAi suppression occurs by systematically destabilizing a 21-base pair (bp) hairpin structure that occludes the complete target sequence. We monitored the effects on siRNA binding *in vitro* and RNAi efficiency *in vivo*. The 3′ end of the mRNA target sequence is initially recognized by bases 2-5 of the antisense/guide strand siRNA, therefore named the "seed" sequence (35,36). Thus, one may expect a more prominent effect of an accessible target 3′ end, which primed us to address positional effects when destabilizing the target hairpin. The results demonstrate a clear correlation between the overall stability of the target hairpin and RNAi efficiency, but positional effects were also apparent.

Materials and Methods

Plasmid constructs

The luciferase plasmids pGL3-wt, pGL3-T1 to pGL3-T7 (Figure 1B) and pGL3-A to pGL3-G (Figure 3A) were constructed by annealing of forward (fwd) and reverse

(rev) oligonucleotides (Supplementary data, table 1), and ligation into the EcoRI and PstI sites of the firefly luciferase expression vector pGL3-Nef (28).

The pSUPER-shPol vector (26) encodes an effective shRNA against a conserved 19-nt HIV-1 region (Pol1; ACAGGAGCAGAUGAUACAG) under the control of an H1 polymerase III promoter (14). The plasmid pRL-CMV (Promega) expresses renilla luciferase under control of the CMV promoter.

Cell culture and luciferase assays

C33A cervix carcinoma cells were grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% FCS, minimal essential medium nonessential amino acids, 100 units/ml penicillin, and 100 units/ml streptomycin at 37°C and 5% CO_2. C33A cells were grown in 1 ml culture medium in 2 cm^2 wells to 60% confluence and transfected by the calcium phosphate method. The pGL3-variant (100 ng) was mixed with 0.5 ng pRL-CMV, 0.1-100 ng pSUPER-shPol and pBluescriptII (KS^+) (Stratagene) to have 1 μg of DNA in 15 μl water. The DNA was mixed with 25 μl of 2x HBS and 10 μl of 0.6M $CaCl_2$, incubated at room temperature for 20 min and added to the culture medium. The culture medium was refreshed after 16 h, and cells were lysed after another 24 h. Firefly and renilla luciferase activities were measured with the Dual-luciferase Reporter Assay System (Promega) as described previously (28).

In vitro transcription and electrophoretic mobility shift assay (EMSA)

The pGL3-variant plasmids were used as template for PCR amplification with primers EWr8 (5'-TCC*TAATACGACTCACTATAGG*TTCCCCACAGGAGCAGA-TGA-3'; T7 RNA-polymerase promoter in italics) and EWr9 (5'-GACTCTAGAC-TGCAGAAAAC-3'). The resulting PCR product contains a T7 RNA-polymerase promoter upstream of the hairpin (hairpin nt underlined). DNA products were purified from agarose gel using QiaexII Gel extraction kit (Qiagen). RNA transcripts were produced by *in vitro* transcription with the Megashortscript T7 transcription kit (Ambion), and transcripts were checked for integrity and isolated from an 8% acrylamide gel. RNA concentrations were determined by spectrophotometry.

The siRNA-Pol antisense/guide oligonucleotide CUGUAUCAUCUGCUC-CUGU (Eurogentec) was 5' end labeled with the kinaseMax kit (Ambion) and 1 μl [γ-^{32}P]ATP (0.37 MBq/μl, Amersham Biosciences). The target hairpin RNAs were denatured in 30 μl water at 85°C for 3 min followed by snap cooling on ice. After addition of 10 μl 4x MO buffer (final concentration: 125 mM KAc, 2.5 mM MgAc, 25 mM Hepes, pH 7.0), the RNA was renatured at 37°C for 30 min. The transcripts were diluted in 1x MO buffer to a final concentration varying from 0 to 1.0 μM in MO buffer. Unlabeled tRNA (1 μg) was added as competitor to each reaction to minimize aspecific RNA interactions. The 5'-labeled oligonucleotide (1.0 nM) was

added and the samples (20 µl) were incubated for 30 min at 37°C. After adding 4 µl non-denaturing loading buffer (50% glycerol with bromophenol blue), the sample was analyzed on a non-denaturing 4% acrylamide gel. Electrophoresis was performed at 150 V at room temperature and the gel was subsequently dried. Quantification of the free and bound oligonucleotide was performed with a Phosphor Imager (Molecular Dynamics).

In silico RNA analysis

The structure and stability of the target hairpins cloned into the pGL3-variants (total 54nt; CCCC-hairpin-UUU) was predicted with the RNA Mfold program (37,38) at http://www.bioinfo.rpi.edu/applications/mfold. The presence of the predicted hairpin structures in the context of the luciferase reporter construct was verified by Mfold.

Results

Target hairpin destabilization triggers RNAi

We investigated the effect of target RNA structure on RNAi efficiency. As a model system we used a very potent shRNA inhibitor that is directed against the Pol gene of HIV-1 (Figure 1A; left) and that has been tested extensively against HIV-1 and appropriate reporter genes (26). Such a luciferase reporter with the HIV-1 Pol target sequence in the 3′ UTR is shown in Figure 1A (right). Next, we made the target inaccessible by inclusion in a perfect hairpin of ΔG = -36.6 kcal/mole (Figure 1B; wild-type (wt), the target sequence is marked in gray). In fact, this hairpin structure is identical to the shRNA itself. The top 2 base pairs and the 5-nt loop are standard in the optimized pSUPER system (14). We systematically destabilized this target hairpin in mutants T1-T7 by introducing nt substitutions in the descending strand of the stem (encircled in Figure 1B), thus leaving the target sequence intact. The mutations were chosen such that the predicted thermodynamic stability (ΔG) decreases gradually. We first destabilized the hairpin by replacing stable G-C by weak G-U base pairs (mutants T1-T3), followed by more gross destabilizations, e.g. by introducing mismatches (mutants T4-T7). The ΔG value was reduced in a step-wise manner to -7.2 kcal/mole for mutant T7.

To accurately quantify the RNAi efficiency against these differentially structured targets, we placed them downstream of the luciferase reporter gene (Figure 1A; right). These constructs were cotransfected into cells with increasing amounts of the shRNA-Pol expression vector and luciferase expression was measured after 48 h (Figure 1C). Expression of the reporter construct with the target sequence embedded in the wt hairpin was completely resistant against shRNA-Pol. The same expression pattern was observed for the T1 construct, but T2

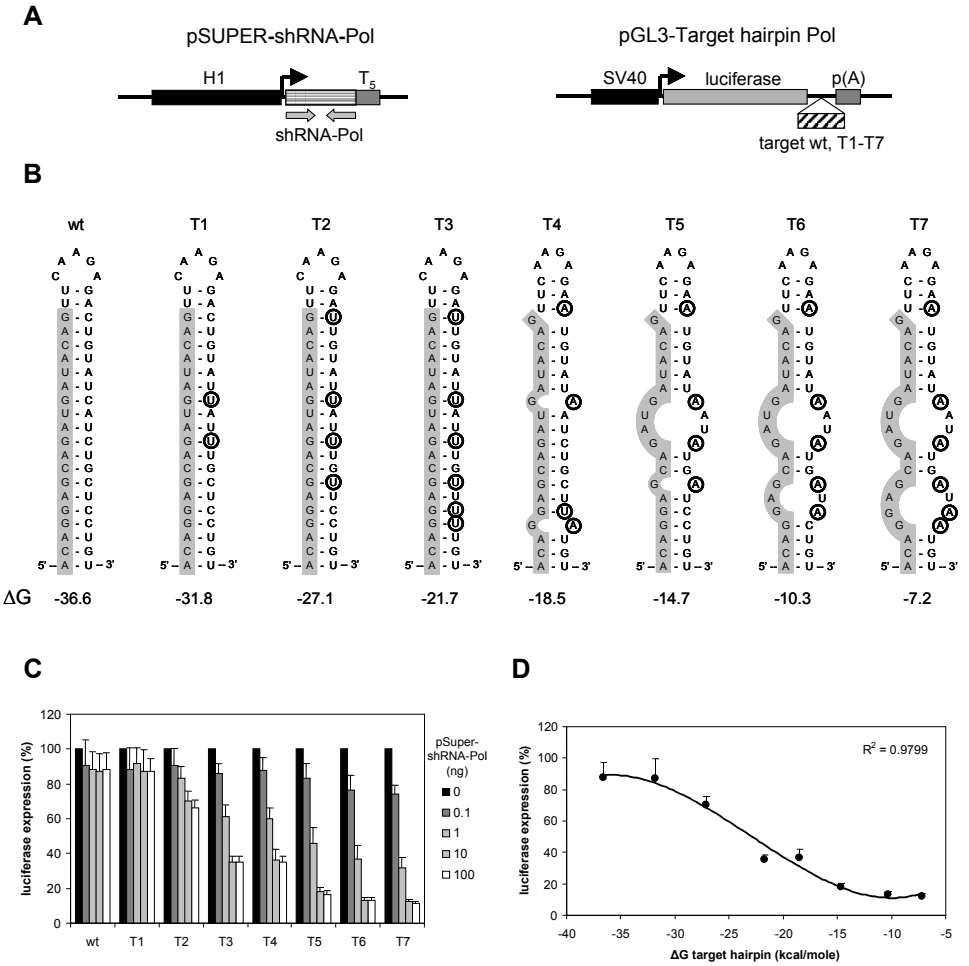

Figure 1. Target RNA structure influences RNAi efficiency. **(A)** The HIV-based target sequences (53 nt) were cloned downstream of the firefly luciferase gene in the pGL3 reporter plasmid. These reporter constructs were cotransfected into C33A cells with the shRNA expressing plasmid pSUPER-shRNA-Pol. The respective H1 (polymerase III) and SV40 (polymerase II) promoter units are indicated by a black box, the arrow marks the transcription initiation site. **(B)** The predicted RNA structures (Mfold program) of the wild-type (wt) shPol and mutated hairpins (T1-T7). The 19-nt target sequence is highlighted as a gray box and the mutated nucleotides are encircled. The thermodynamic stability (ΔG in kcal/mole) of the target hairpins is indicated. **(C)** Luciferase expression upon transfection of the reporter constructs with increasing amounts of pSUPER-shRNA-Pol. The firefly luciferase activity was normalized to that of the renilla luciferase to correct for variation in transfection efficiency. The level of expression observed in the absence of shRNA-Pol was set at 100% for each reporter construct. This level did not vary significantly for the different constructs. The mean values of 6 independent experiments are shown (± standard deviation). **(D)** The thermodynamic stability of the target hairpins is plotted against the level of luciferase expression as observed in Figure 1C with 10 ng pSUPER-shRNA-Pol.

already showed some susceptibility for RNAi-mediated inhibition with higher amounts of shRNA-Pol, with a maximal inhibition of 34% (66% residual luciferase expression). The next reporter constructs (T3,T4) showed a significant drop in luciferase expression (64% inhibition). Inhibition of the remaining destabilized target hairpins (T5-T7) was very effective, showing more than 80% inhibition. This is similar to the maximal inhibition level that can be obtained with this potent shRNA inhibitor against a reporter with the 19-nt target sequence in an unstructured setting ((26) and results not shown).

We plotted the measured level of luciferase expression against the predicted stability of the target hairpins (Figure 1D). The results suggest an inverse linear correlation between RNAi susceptibility and target hairpin stability in the -30/-15 kcal/mole range. The curve shows two plateaus. A reduction in hairpin stability from -36 to -30 kcal/mole does not significantly induce RNAi-mediated inhibition (less than 20% inhibition), and further destabilization above -15 kcal/mole shows no significant improvement of the already maximal inhibition of approximately 86%.

Target hairpin destabilization triggers siRNA binding

To demonstrate that the increased RNAi efficiency on destabilized target hairpins is due to more efficient binding of the siRNA, we performed *in vitro* binding experiments by means of electrophoretic mobility shift assays (EMSA). For this, we used short T7 transcripts with the complete hairpin and a 19-nt RNA oligonucleotide, which corresponds to the antisense/guide strand of the siRNA-Pol (complementary to boxed sequence in Figure 1B). The siRNA was radioactively labeled and incubated with increasing amounts of target transcript (wt, T1-T7), and subsequently analyzed on a non-denaturing acrylamide gel (Figure 2A). Binding of siRNA to the target RNA leads to duplex formation that results in a band shift on the gel. Unbound siRNA and the siRNA/target-RNA duplex were quantified to calculate the percentage of binding (Figure 2B).

We repeated the binding experiment multiple times with 0.2 μM target RNA because efficient binding can be observed, yet most variants stay within the linear range of the binding assay. We plotted these binding percentages against the predicted stability of the target hairpins (Figure 2C). A general trend can be observed that is the opposite of the graph in Figure 1D: reduced hairpin stability results in more efficient binding of the siRNA to the target RNA. Thus, a decrease in the stability of the target hairpin increases RNAi efficiency due to more efficient binding of the siRNA. The largest improvement in RNA-RNA interaction and RNAi efficiency is observed for mutant T3 in comparison with T2, indicating that a threshold stability is passed by going from ΔG = -27.1 to -21.7 kcal/mole.

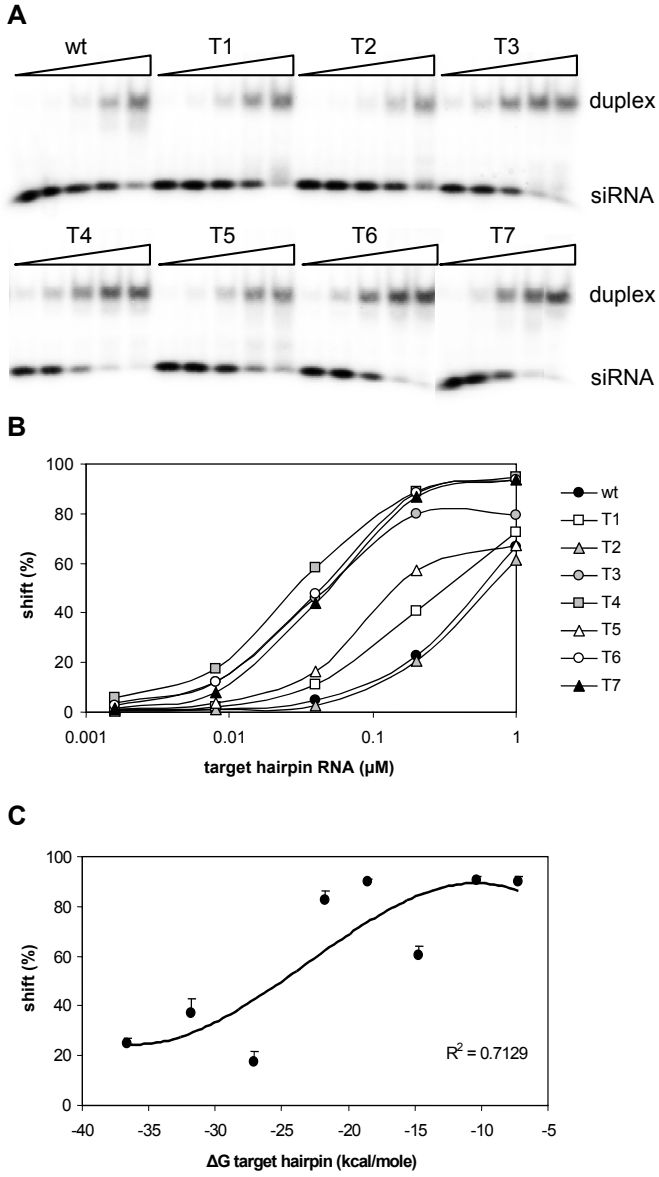

Figure 2. Target RNA structure influences siRNA binding.
(A) Radioactively labeled oligonucleotide simulating the siRNA antisense strand (processed from shRNA-Pol) was incubated with increasing amounts of hairpin target RNA variants (wt, T1-T7). SiRNA/target-RNA duplex formation was analyzed by EMSA.
(B) Free and bound siRNA oligonucleotide was quantified to calculate the level of duplex formation (bound siRNA/free + bound siRNA).
(C) The thermodynamic stability of the target hairpins is plotted against the level of duplex formation with 0.2 µM target RNA.

Accessibility of the 3' end of the target sequence is beneficial for RNAi

We globally determined the stability at which hairpin structures become inhibitory to the RNAi machinery. However, not all domains within the 19-nt target sequence may contribute equally to siRNA binding and the RNAi mechanism. For instance, it has previously been suggested that the 3' end of the target sequence is initially recognized by the siRNA within RISC (36). To test this, we made a second set of

luciferase-target constructs (Figure 3A, mutants A-G). By introducing clustered mutations in the target hairpin, we destabilized either the 3′ end, the center or the 5′ end of the target sequence. Modest G-U changes were introduced in mutants A (3′), B (center) and C (5′). More gross destabilizing mutations were introduced in mutants D (3′), E (center) and F (5′). However, it is apparent that the two mutations in D have a more modest effect on the ΔG value because a realignment of the sequences trigger an alternative folding of the top of the hairpin. We therefore constructed the additional mutant G with three mutations to obtain a hairpin with a destabilized 3′ target end that is comparable in ΔG to hairpins E and F. Target hairpins A through G were cloned in the luciferase reporter and cotransfected into cells with increasing amounts of the shRNA-Pol expression vector to quantify the RNAi efficiency (results not shown).

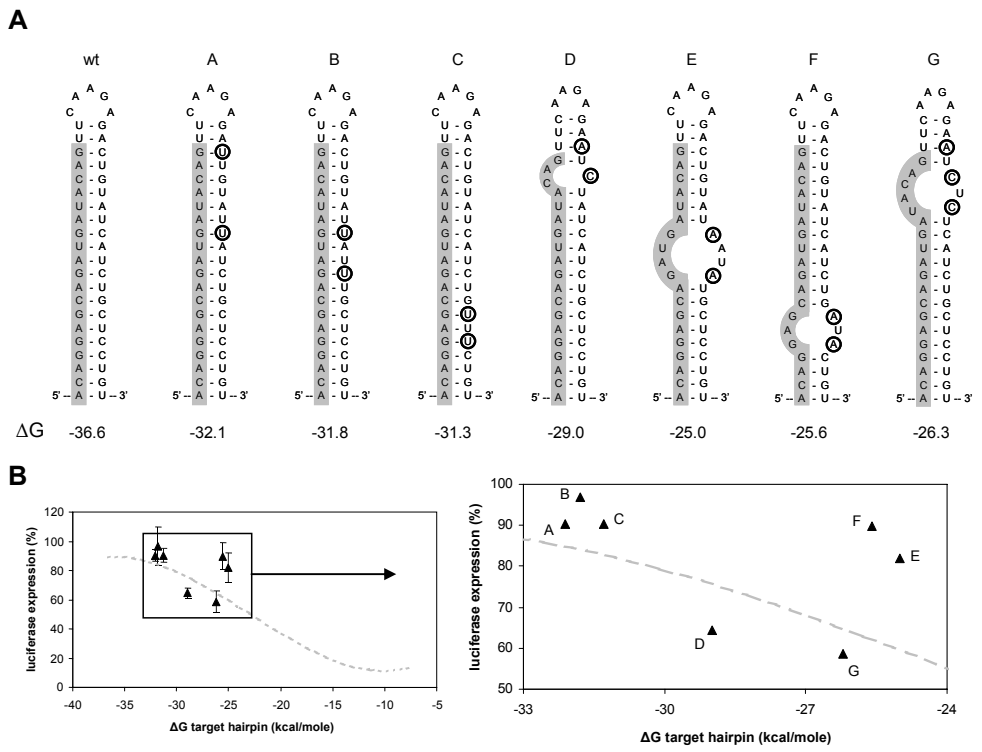

Figure 3. Position-specific destabilization of target hairpin triggers RNAi differentially. **(A)** Predicted RNA structures of the wt and mutant hairpins A-G. The target sequence is highlighted in the gray box and the mutated nucleotides are encircled. The thermodynamic stability (ΔG in kcal/mole) of the target hairpins is provided for each structure. **(B)** Luciferase expression observed after transfection of the reporter constructs with 10 ng pSUPER-shRNA-Pol is plotted against the thermodynamic stability of the target hairpins. The mean values of 5 independent experiments are shown (± standard deviation). The gray dotted line represents the trend line observed for the initial set of mutants in Figure 1D. The right graph zooms in on a smaller ΔG segment.

The luciferase values obtained with 10 ng shRNA-Pol were plotted against the predicted hairpin stability (Figure 3B, left) and we zoom in on a smaller ΔG range (Figure 3B; right graph). The target hairpins A, B and C follow the general trend that we described previously (gray dotted trend line). Independent of where the hairpin is destabilized, the introduction of G-U base pairs is a too modest manipulation to trigger RNAi activity. The target hairpins E, F and G have more dramatic changes that reduce the overall hairpin stability to -25/-26 kcal/mole, which should become susceptible to RNAi according to the previous results. However, mutants F (5′) and E (center) remain largely insensitive, but mutant G with a free 3′ end shows increased RNAi sensitivity when compared to the trend line. Even the D mutant with a more modest destabilization of the target 3′ end shows reasonable RNAi activity and clearly drops below the trend line. These results confirm the importance of initial recognition of the 3′ target end, which explains the deviations from the general trend.

In vitro siRNA-target RNA binding does not accurately mimic the RNAi mechanism

We performed *in vitro* binding experiments to study the A-G mutants for their ability to bind the siRNA. The radioactively labeled siRNA was incubated with increasing amounts of the target transcripts A-G and analyzed on gel (Figure 4A). The shifts representing the siRNA/target-RNA duplex and the free siRNA bands were quantified to calculate the percentage of binding (Figure 4B).

The percentage of binding with 0.2 μM target RNA was plotted against the predicted stabilities of the target hairpins (Figure 4C). Remarkably, these *in vitro* binding results differ significantly from the *in vivo* RNAi results. The target hairpins D and G (both 3′), which are efficiently targeted by RNAi in the luciferase assay (Figure 3B), are inefficient in siRNA-binding. In contrast, the target hairpins F (5′) and G (center) showed a slightly increased binding efficiency, although these construct were relatively more RNAi-resistant in the luciferase assay. These results may reflect the oversimplification of the *in vitro* binding assay and point to a contribution of the RISC/siRNA complex in the recognition and binding of the target sequence *in vivo*.

Discussion

RNAi efficiency is influenced by the local RNA structure of the targeted sequence. We investigated this phenomenon in detail by placement of the target sequence in a perfect hairpin structure (ΔG = -36.6 kcal/mole), which indeed resisted RNAi. Subsequently we destabilized this tight target structure resulting in a gradual exposure of the target sequence. Destabilization of the hairpin structure has little

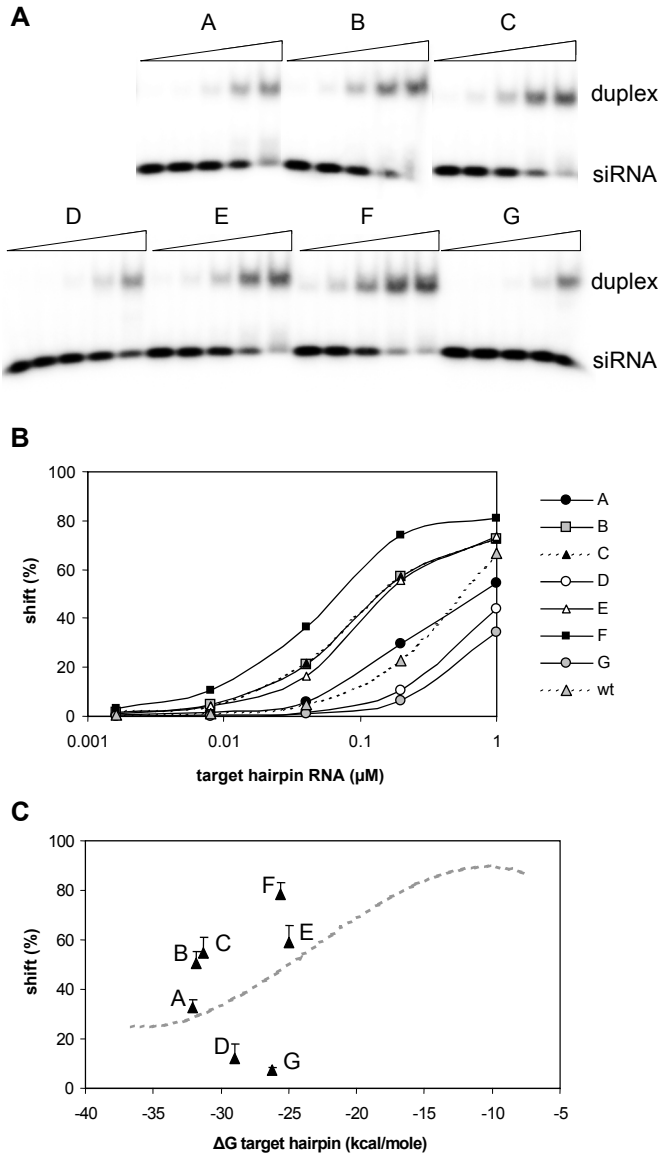

Figure 4. *In vitro* siRNA binding does not prefer an accessible 3' end of the target.
(A) Radioactively labeled oligonucleotide simulating the siRNA antisense strand (processed from shRNA-Pol) was incubated with increasing amounts of target variants A-G. SiRNA/target-RNA duplex formation was analyzed by EMSA.
(B) Free and bound siRNA oligonucleotide was quantified to calculate the level of duplex formation (bound siRNA/free + bound siRNA).
(C) The thermodynamic stability of the target hairpins is plotted against the level of duplex formation with 0.2 μM target RNA. The gray dotted line represents the trend line observed in Figure 2C in the experiments with the initial set of mutants.

effect on RNAi activity until a threshold is reached (ΔG = ± -30 kcal/mole). Beyond this threshold we demonstrate an inverse correlation between hairpin stability and RNAi-mediated inhibition. Maximal RNAi efficiency was observed with hairpins of ΔG ≥ -15 kcal/mole. *In vitro* binding experiments suggested that the increase of RNAi-mediated inhibition is due to efficient siRNA binding to the destabilized target RNA hairpins.

When we introduced position-specific mutations in the target hairpin, we observed RNAi efficiencies that deviate from this trend. Hairpins with an opened 5′ end or central part of the target sequence show less RNAi activity than predicted based on their overall stability. In contrast, hairpins with an opened 3′ end are more susceptible to RNAi than expected. These results are consistent with the current notion that the 3′ region of the target is initially recognized and bound by the RISC/siRNA complex (36). This model is supported by structural data on RISC bound to the siRNA strand. The 3′ end of the siRNA is recognized and bound in a pocket by the PAZ domain of the Argonaute protein (39). The 5′ end of the siRNA is anchored at the PIWI domain of Argonaute and these 5′ nucleotides are readily accessible for base pairing to complementary 3′ nucleotides of the target RNA (40,41). The importance of the target 3′ end was also revealed in experiments that selected for viruses that resist RNAi-mediated inhibition. We described a unique HIV-1 escape variant that acquired a mutation outside the 19-nt target, which forces the RNA into an alternative structure that occludes the 3′ end of the target (28).

Besides the *in vivo* RNAi measurements, we also tested the different RNA targets for their ability to interact with the siRNA *in vitro*. The overall ΔG effect of stable target hairpins is confirmed in this simplified *in vitro* setting, demonstrating that RNAi resistance is due to the inability of the siRNA to interact with the base paired stem of the hairpin. We realize that the siRNA does not act by itself *in vivo* as it is part of RISC, of which the Argonaute protein may melt local structure in the target RNA. In fact, we observed an interesting discrepancy between the *in vivo* and *in vitro* results for the 5′/center/3′-destabilized hairpins. We observed that an accessible 3′ target is key for RNAi activity, but this effect was not seen *in vitro*. This result underscores the important contribution of RISC, in particular the PIWI domain of Argonaute, in the siRNA-target RNA annealing step.

Thus, target RNA structure is an important factor when selecting a suitable target sequence, as it can have a negative effect on RNAi efficiency. For instance, it has been shown that the TAR hairpin of the HIV-1 genome is an unsuitable target because of its tight structure (24,33,42). On the other hand, it is obvious that an accessible sequence does not automatically make a good siRNA target (24), as the matching siRNA may not meet the criteria of an effective siRNA (43). It has been proposed to include a calculation of the amount of hydrogen bonds within the target sequence as a parameter for efficient target sequences (32). We now provide the general ΔG threshold at which RNA structure becomes inaccessible, and we differentiate between different target positions. When designing antiviral siRNAs one may also consider ways to obstruct viral escape via folding of an alternative target RNA structure (28). The local RNA region should be screened for the absence of alternative folding that occludes the 3′ end of the target and that can be

selected by one or two mutations. If not available, the genetic threshold for structure-based escape might prove too high, even for a fast evolving virus like HIV-1.

RNA structure-mediated resistance against RNAi is in fact beneficial when expressing highly structured shRNAs or miRNAs in cells. For instance, the incorporation of shRNA-expression cassettes in a lentiviral vector is potentially problematic, because the shRNA will target the viral RNA genome during vector production, thus reducing the titer. Such self-targeting has not been reported (44,45), we think because the target is not accessible as part of the perfectly base paired shRNA hairpin. The apparent absence of such self-targeting is particularly important for the development of multi-shRNA lentiviral vectors without titer reduction. However, placing many tight RNA structures in the vector genome may negatively influence the titer by other means. For instance, reverse transcription is very sensitive to excessively stable RNA structure (46) and RNA polymerase II transcription may pause at sites where the RNA products folds stable hairpin structures (47). We did indeed observe that four shRNA cassettes reduce the lentiviral vector titer (Ter Brake, unpublished results). Destabilizing the introduced shRNAs may avoid such vector problems, and provide additional benefits for cloning and sequencing of inverted repeat sequences (48). In our target model system, we mutated the antisense strand of the shRNA hairpin, leaving the sense target sequence intact. In the case of a true shRNA-expression cassette, modifications will be made in the sense (target) strand to leave the guide/antisense siRNA strand unaltered. The obvious advantage will be reduced complementarity between the target and the siRNA inhibitor. The impact of such mutations on self-targeting is likely to depend on the position and type of mismatches that are introduced (49,50). It is therefore impossible to make general rules for shRNA design and destabilization as each hairpin RNA structure will have its unique characteristics as target and effector in the RNAi mechanism. Here we provide a ΔG window for shRNA destabilization without activating RNAi self-targeting. Positional effects should be considered, and hairpins may be destabilized to ΔG = -25 kcal/mole as long as the target 3' end remains base paired.

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Supplementary Table 1: The oligonucleotides, forward (fwd) and reverse (rev), used for cloning the luciferase target hairpin constructs.

Name	Sequence 5'→3'
Fwd wt	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGACTGTATCATCTGCTCCTGTTTTCTGCA
Rev wt	GAAAACAGGAGCAGATGATACAGTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd T1	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGACTGTATTATTTGCTCCTGTTTTCTGCA
Rev T1	GAAAACAGGAGCAAATAATACAGTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd T2	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGATTGTATTATTTGTTCCTGTTTTCTGCA
Rev T2	GAAAACAGGAACAAATAATACAATCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd T3	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGATTGTATTATTTGTTTTTGTTTTCTGCA
Rev T3	GAAAACAAAAACAAATAATACAATCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd T4	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGAATGTATAATCTGCTTATGTTTTCTGCA
Rev T4	GAAAACATAAGCAGATTATACATTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd T5	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGAATGTATAATATGATCCTGTTTTCTGCA
Rev T5	GAAAACAGGATCATATTATACATTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd T6	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGAATGTATAATATGATACTGTTTTCTGCA
Rev T6	GAAAACAGTATCATATTATACATTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd T7	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGAATGTATAATATGATAATGTTTTCTGCA
Rev T7	GAAAACATTATCATATTATACATTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd A	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGATTGTATTATCTGCTCCTGTTTTCTGCA
Rev A	GAAAACAGGAGCAGATAATACAATCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd B	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGACTGTATTATTTGCTCCTGTTTTCTGCA
Rev B	GAAAACAGGAGCAAATAATACAGTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd C	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGACTGTATCATCTGTTTCTGTTTTCTGCA
Rev C	GAAAACAGAAACAGATGATACAGTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd D	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGAATCTATCATCTGCTCCTGTTTTCTGCA
Rev D	GAAAACAGGAGCAGATGATAGATTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd E	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGACTGTATAATATGCTCCTGTTTTCTGCA
Rev E	GAAAACAGGAGCATATTATACAGTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd F	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGACTGTATCATCTGATACTGTTTTCTGCA
Rev F	GAAAACAGTATCAGATGATACAGTCTCTTGAACTGTATCATCTGCTCCTGTGGGG
Fwd G	AATTCCCCACAGGAGCAGATGATACAGTTCAAGAGAATCTCTCATCTGCTCCTGTTTTCTGCA
Rev G	GAAAACAGGAGCAGATGAGAGATTCTCTTGAACTGTATCATCTGCTCCTGTGGGG

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Chapter Five

A conditionally replicating HIV-based vector that stably expresses an antiviral shRNA against HIV-1 replication

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Abstract

Human pathogenic viruses can be targeted by therapeutic strategies based on RNA interference (RNAi). Whereas the administration of synthetic short interfering RNAs (siRNAs) may transiently inhibit viral replication, long-term inhibition may be achieved through stable intracellular expression of siRNAs or short hairpin RNAs (shRNAs). Both approaches face serious problems with delivery to the right cells in an infected individual. We explored the potential of a replicating HIV-based vector to deliver an antiviral shRNA-expression cassette into HIV-1 susceptible target cells to block chronic HIV-1 infection. The vector is based on a doxycycline (dox)-dependent HIV-1 variant that we previously proposed as a conditional-live HIV-1 vaccine. With dox, this virus spreads efficiently to all HIV-susceptible cells. Subsequent dox-withdrawal generates cells with a transcriptionally silent integrated provirus, but with an active shRNA-expression cassette. Because the shRNA targets viral sequences that are removed from the vector construct, there is no self-targeting, yet specific shutdown of HIV-1 replication.

Introduction

RNA interference (RNAi) can be used as a therapeutic strategy to target human pathogenic viruses. RNAi is induced by double stranded RNA, resulting in the sequence-specific degradation of homologous single stranded RNA (1,2). The effector molecules of this evolutionarily conserved mechanism, which are produced by a ribonuclease named Dicer, are short interfering RNAs (siRNAs) of ~22nt (3,4). Transfection of siRNAs into cells has proven to be a powerful tool to transiently suppress gene expression (5). Stable expression of short hairpin RNAs (shRNAs), which are processed into effective siRNAs by Dicer, can result in long-term gene suppression (6,7). We have demonstrated long-term inhibition of HIV-1 replication in human T-cells stably expressing shRNAs that target the viral Nef gene (shRNA-Nef) (8,9). Thus, RNAi shows potential as a means to achieve intracellular immunization against human immunodeficiency virus type 1 (HIV-1).

We previously described an HIV-1 variant that replicates conditionally in the presence of doxycycline (dox) (10,11). In this HIV-rtTA virus, the Tat-TAR regulatory mechanism controlling viral gene expression and replication was inactivated and functionally replaced by the Tet-on system for inducible gene expression (12). The rtTA gene encoding the new transcriptional activator replaces the accessory Nef gene and the tet operator (tetO) DNA elements were introduced in the viral LTR promoter. Dox induces a conformational change in the rtTA

protein such that it can bind to tetO and subsequently activates viral transcription. The initial HIV-rtTA construct has been significantly improved through virus evolution (13-15). Efficient dox-dependent replication has been demonstrated *in vitro* in T-cell lines and primary cells, and *ex vivo* in human lymphoid tissue (16).

The HIV-rtTA virus is under development as a conditional-live virus vaccine. In this study, we have modified the HIV-rtTA virus such that it becomes a replicating vector for the efficient delivery of anti-HIV shRNAs to those cells that are susceptible to HIV-1 infection. Replication of this therapeutic virus with dox results in virus spread to HIV-susceptible cells. Subsequent dox-withdrawal generates cells containing a silent integrated provirus with a constitutively active shRNA-expression cassette. The HIV-rtTA-encoded shRNA-Nef inhibitor does not target the vector itself because the rtTA gene has replaced Nef sequences, but it does efficiently inhibit HIV-1 replication in transduced cells.

Materials and Methods

Cells and viruses

SupT1 T-cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 units/ml streptomycin at 37°C and 5% CO_2. SupT1 cells were transfected with the variant HIV-rtTA molecular clones by electroporation. Briefly, 5 x 10^6 cells were washed in RPMI 1640 medium with 20% FCS and mixed with 5 μg of DNA in 250 μl of RPMI 1640 medium with 20% FCS. Cells were electroporated in 0.4 cm cuvettes at 250 V and 975 microfarads and subsequently resuspended in RPMI 1640 medium with 10% FCS and 1 μg/ml doxycycline (Sigma). Cells were split 1 to 5 twice a week.

For the virus evolution experiments, virus replication was maintained by passage of the cell-free culture supernatant of massively infected cells (as apparent from the presence of large syncytia) onto uninfected SupT1 cells. Cells and supernatant samples were isolated and stored at -80°C.

SupT1 cells transduced with the HIV-rtTA-shNef variants (2.5 x 105 cells in 1 ml medium) were infected with HIV^{wt} or HIV^{R2} (200 ng of CA-p24), and viral replication was monitored by determining the CA-p24 level in the supernatant by ELISA as described (17).

C33A cervix carcinoma cells were grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% FCS and minimal essential medium nonessential amino acids at 37°C and 5% CO_2. For the production of virus stocks, C33A cells were transfected by the calcium phosphate method. Briefly, cells were grown in 3 ml of culture medium in 10 cm^2 wells to 60% confluence. HIV^{wt} or HIV^{R2} DNA (5 μg) in 110 μl water was mixed with 125 μl 50 mM HEPES (pH 7.1)-250 mM NaCl-1.5 mM Na_2HPO_4 and 15 μl 2 M $CaCl_2$, incubated at room

temperature for 20 min and added to the culture medium. The culture medium was changed after 16 h and viruses were harvested 2 days after transfection.

Cotransfection of HIV-1 and HIV-rtTA-shNef variants was performed in C33A cells grown in 1 ml culture medium in 2 cm^2 wells at 60% confluence. A total of 1 μg of DNA was mixed in 15 μl water (500 ng HIV^{wt} or HIV^{R2}, 100 ng ΔU3-HIV-rtTA-shNef variants, 400 ng pBluescriptII (KS^+; Stratagene)). The DNA was mixed with 25 μl of 2x HBS and 10 μl of 0.6M $CaCl_2$, incubated at room temperature for 20 min and added to the culture medium. The culture medium was changed after 16 h and virus was harvested 3 days after transfection. Virus was quantitated by CA-p24 ELISA.

DNA constructs

The pRetro-SUPER-shNef vector (8,18), which expresses shRNA-Nef under control of the H1 RNA polymerase III promoter, and the empty pRetro-SUPER-vector were digested with EcoRI and XhoI and the polymerase III expression cassette (310 and 249 bp, respectively). The strands were completed with Klenow DNA polymerase in the presence of dNTPs and cloned into the EcoRV site of pBlue 3'LTRext-ΔU3-$rtTA_{F86Y\ A209T}$-2ΔtetO (15), which had been digested with EcoRV and subsequently treated with shrimp alkaline phosphatase to prevent self-ligation. Sequence analysis was performed by big dye terminator cycle sequencing to identify shRNA-Nef and control inserts (empty polymerase III units) in both orientations (F= forward and R= reverse). The BamHI-BglI fragment of these shuttle vectors was exchanged with the BamHI-BglI fragment in HIV-rtTA, resulting in the HIV-rtTA-F-shNef and HIV-rtTA-R-shNef therapeutic vectors, and the empty controles HIV-rtTA-F and HIV-rtTA-R. The HIV-rtTA version used in this study, HIV-$rtTA_{F86Y\ A209T}$-2ΔtetO, carries the inactivating Y26A mutation in the Tat gene, five nucleotide substitutions in the TAR (trans-acting response region) hairpin motif, the improved $rtTA_{F86Y\ A209T}$ gene (15) in place of the Nef gene and the optimized 2ΔtetO promotor configuration (13,14) in the 5' and 3' LTR. In the ΔU3-HIV-rtTA constructs the 5' U3 sequences from position -333 up to -159 (with position +1 representing the transcription initiation site (19)) were deleted through substitution of the 5' LTR-tetO promoter region of the HIV-rtTA-variants with the corresponding fragment of HIV-rtTA-dNF construct in which the NFκB sites and upstream U3 sequences had been deleted (Δ-333/-159; Das *et al*. manuscript in preparation).

The full-length molecular HIV-1 clone LAI (20) was used to produce the wild-type HIV-1 virus (HIV^{wt}). Construction of the HIV^{R2} molecular clone with a 11-nt deletion in the shRNA-Nef target sequence and the firefly luciferase expression vectors pGL3-Nef^{wt} and pGL3-Nef^{R2}, containing an ~250-bp Nef fragment (+8448 to +8698) downstream of the luciferase gene, were described previously (9). The pBluescriptII-based (KS^+) (Stratagene) plasmid pBS-shRNA-Nef

contains the shRNA-Nef-expression cassette (9). The plasmid pRL-CMV (Promega) expresses renilla luciferase under control of the CMV promoter.

Viral RNA isolation

C33A cells (25 cm^2; 65% confluent) were transfected with 10 μg ΔU3-HIV-rtTA DNA constructs using Lipofectamine (Invitrogen). Dox (1 μg/ml) was added to the culture medium 6 h after transfection. Cells were lysed 2 days after transfection and RNAs were isolated using the *mir*Vana™ miRNA isolation kit (Ambion).

Northern blotting

Gel electrophoresis of 5 μg RNA was performed on a 15% acrylamide Novex® TBE-Urea gel (Invitrogen) at 180 V in 1x TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.3). RNA was transferred onto a positively charged nylon membrane (Boehringer Mannheim) for 2 h at 80 V and cross-linked to the membrane with a UV crosslinker (Stratagene). A 19-nt LNA-molecule (Eurogentec) with a sequence similar to the shRNA-Nef target sequence (5′ GTGCC-TGGCTAGAAGCACA 3′; locked nucleotides are underlined) was used as a probe. The probe was 5′ end labeled using the kinaseMax kit (Ambion) in the presence of 1 μl of [γ-^{32}P]ATP (0.37 MBq/μl, Amersham Biosciences) and purified over a MicroSpin™ G-25 column (GE Healthcare). Prehybridization and hybridization was done in ULTRAhyb buffer (Ambion) at 42°C for 30 min and 18 h, respectively. The membrane was washed twice at 42°C with low-stringency buffer (2x SSC, 0.2% SDS). Images were obtained using the Typhoon Trio phosphorimager (Amersham Biosciences).

Proviral DNA analysis

HIV-rtTA-infected cells were pelleted by centrifugation at 5000 rpm for 5 min and washed with phosphate-buffered saline. DNA was solubilized by resuspending the cells in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA-0.5% Tween 20, followed by incubation with 200 μg/ml of proteinase K at 56°C for 30 min and at 95°C for 10 min. Proviral DNA sequences were PCR amplified from total cellular DNA with the 5′ primer tTA-tetO-1-AD (annealing to rtTA sequences 54-30 nt upstream of the 3′ U3) and the 3′ primer CN1 (annealing to U5 sequences 124-152 nt downstream of U3).

Analysis of clones

SupT1 cells transduced with ΔU3-HIV-rtTA-F-shNef were used for limiting dilution to obtain cell clones. Serial dilutions (5-fold) were prepared in 96-well plates. Fresh medium was added every 5 days. The presence of the ΔU3-HIV-rtTA-F-shNef provirus was determined by PCR and RNAi-mediated inhibition of HIV-1 was determined in an HIV^{wt} challenge (500 ng of CA-p24).

Luciferase assay

C33A cells were grown in 1 ml culture medium in 2 cm^2 wells to 60% confluence and transfected by the calcium phosphate method. 100 ng pGL3-Nef^{wt} or pGL3-Nef^{R2} was mixed with 0.5 ng pRL-CMV, 100 ng of the ΔU3-HIV-rtTA-shNef variants and 800 ng pBluescriptII (1 μg total DNA) in 15 μl water. The DNA was mixed with 25 μl of 2x HBS and 10 μl of 0.6M $CaCl_2$, incubated at room temperature for 20 min and added to the culture medium. The culture medium was refreshed after 16 h. After another 24 h the cells were lysed in 150 μl of Passive Lysis Buffer (PLB) (Promega) by shaking for 20 min at room temperature. The cell lysate was centrifuged and 10 μl of the supernatant was used to measure firefly and renilla luciferase activities with the Dual-luciferase Reporter Assay System (Promega). The expression of renilla luciferase in transfected cells allowed us to correct for variation in transfection efficiency.

Results

Construction of the therapeutic HIV-rtTA-shRNA-Nef virus

We previously described the incorporation of the Tet-on system in the HIV-1 genome (10). The shRNA-Nef-expression cassette under control of a polymerase III promoter was inserted in the 3′ U3 region of this HIV-rtTA construct (Figure 1).

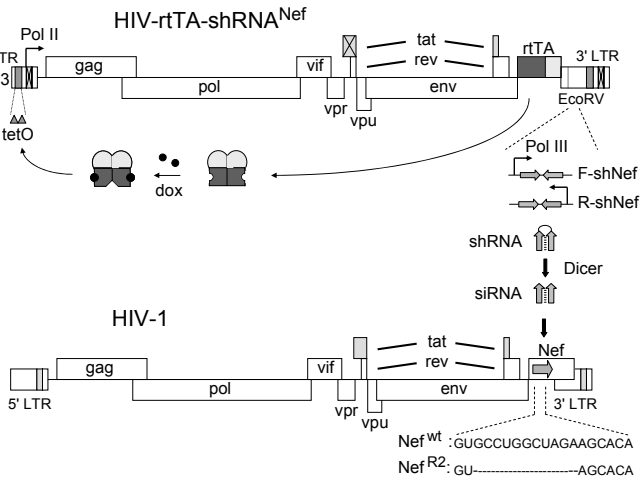

Figure 1. Construction of the therapeutic HIV-rtTA-shRNA-Nef virus.
Schematic of the HIV-rtTA genome (top) and wild-type HIV-1 LAI (bottom). In HIV-rtTA, we inserted the rtTA gene in place of the Nef gene and tetO binding sites in the LTR promoter. Upon administration of doxycycline (dox, ●) rtTA can bind to tetO and activate transcription of the viral genome, thus inducing viral gene expression and replication. The polymerase III promoter-driven shRNA-Nef-expression cassette is inserted into the 3' LTR (EcoRV site) in a forward (F) or reverse (R) orientation. Upon virus replication, the cassette is inherited in both LTRs. The shRNAs are processed by Dicer into siRNAs, which target the Nef^{wt} sequence in wild-type HIV-1 RNA. ShRNA-Nef does not self-target the Nef-minus HIV-rtTA genome. The mutant Nef^{R2} sequence was selected in an shRNA-Nef-resistant HIV-1 variant and contains an 11-nt deletion in the target sequence (9). In the ΔU3-HIV-rtTA variants, the 5' U3 sequences upstream of the tetO elements were deleted.

Upon transduction of cells, the 3′ U3 domain will be copied in the 5′ LTR, thus yielding a provirus with two shRNA-Nef-expression cassettes. We constructed HIV-rtTA variants with the cassette in forward (F-shNef) or reverse (R-shNef) orientation and two corresponding controls with an empty F or R cassette. The target sequence of shRNA-Nef is absent in HIV-rtTA-shNef but present in the Nef gene of wild-type HIV-1 variants (Figure 1).

A unique feature of this shRNA-Nef-expression vector is that it replicates in a dox-dependent manner and that it can spread to all HIV-susceptible cells. We first tested the replication capacity of the four HIV-rtTA vectors in the SupT1 T-cell line. All constructs replicated efficiently (Figure 2A) and similarly to the parental HIV-rtTA virus (15). Most importantly, replication is strictly dox-dependent. The viruses were cultured for a longer period by passage on fresh SupT1 cells. The U3 region of integrated proviruses was PCR amplified at each passage and the products were analyzed by agarose gel electrophoresis. All variants show loss of sequences after multiple passages (HIV-rtTA-R-shNef is shown as an example in Figure 2B, left panel). Sequence analysis revealed the exact deletion of the shRNA-Nef-expression cassette (Δ310 bp), which resulted in restoration of the original U3 sequence. A similar deletion and reversion to the original HIV-rtTA was observed

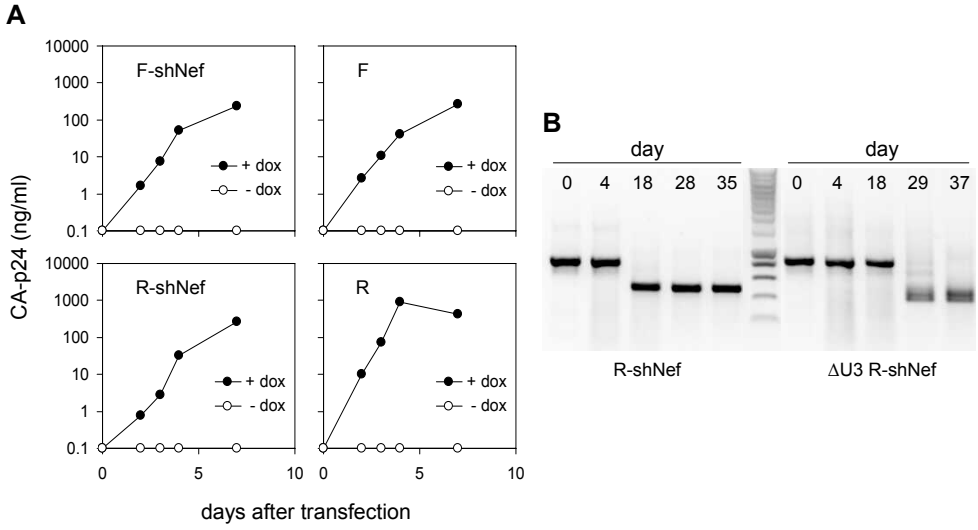

Figure 2. Replication and genetic stability of HIV-rtTA-shRNA-Nef. **(A)** The different HIV-rtTA variants (5 µg) were transfected into SupT1 T-cells. Cells were cultured with (1 µg/ml) or without dox and virus replication was monitored by determining the level of CA-p24 in the culture supernatant. No replication was scored in all cultures without dox. **(B)** Infected cells were collected at several passages. The complete shRNA-Nef-expression cassette was PCR amplified from integrated proviral DNA as an 847-bp fragment. The PCR products were analyzed by gel electrophoresis. Sequence analysis of the PCR amplified fragments demonstrated that at day 18 the HIV-rtTA-R-shNef virus contained a 310-bp deletion, which exactly removes the complete polymerase III cassette. The ΔU3 HIV-rtTA-R-shNef virus contained a deletion of either 371 bp or 423 bp at day 29.

with the other variants (results not shown). The wild-type U3 sequences are likely inherited from the 5′ LTR through an unusual recombination event during reverse transcription (21). To prevent this route, we minimized the 5′ U3 region by deletion of non-essential promoter sequences in all viral constructs (ΔU3-HIV-rtTA-shNef, see also Figure 1). These ΔU3-variants are indeed more stable during replication in SupT1 cells, but deletions occurred eventually (Figure 2B, right panel). Sequence analysis of the ΔU3 R-shNef progeny revealed deletions larger than the shRNA-Nef-expression cassette (Δ371 bp and Δ423 bp). All ΔU3-variants eventually deleted a variable part of the shRNA-Nef-expression cassette and/or the flanking U3 sequences (results not shown). Thus, although we could not completely block deletion of the shRNA-Nef-expression cassette during virus evolution, we could improve the genetic stability of the HIV-rtTA variants by blocking the dominant deletion route in which the wild-type 5′ U3 sequence was copied. We therefore considered these ΔU3-HIV-rtTA variants for their capacity to deliver the antiviral shRNA-Nef to T-cell cultures.

Expression of shRNA-Nef by the HIV-rtTA vectors suppresses target genes

We tested the expression of shRNA-Nef from these ΔU3-HIV-rtTA constructs by Northern blot analysis of transfected C33A cells. Upon expression, the shRNA molecules will be processed into siRNA molecules by Dicer. We indeed detected 19-nt siNef effector molecules when the cells were transfected with F-shNef or R-shNef, but not when transfected with the control F or R construct (Figure 3A). The C33A cells do not support HIV-1 replication because they lack the receptors for viral entry. This allows us to determine the effect of dox addition and induced polymerase II transcription on polymerase III transcription. The siNef level was slightly reduced when transcription from the proviral LTR promoter was activated with dox (Figure 3A). This observation indicates that polymerase II-driven viral transcription can interfere with polymerase III-driven shRNA-Nef expression. Most importantly, these results demonstrate that the antiviral shRNA can be efficiently expressed from the proviruses with the shRNA-Nef cassette in the forward or reverse orientation (F-shNef and R-shNef, respectively).

To demonstrate that the shRNA-Nef expressed from the viral constructs is functional, we tested the capacity of these constructs to inhibit the expression of target genes. First, we cotransfected the ΔU3-HIV-rtTA constructs in a 1 to 5 ratio with the HIV-1 LAI molecular clone into cells and measured virus CA-p24 production in the supernatant. In the absence of dox, the ΔU3-HIV-rtTA LTR promoter is silent and only HIV-1 LAI will produce CA-p24. Even in the presence of dox, the ΔU3-HIV-rtTA constructs will only slightly contribute to the total CA-p24 production due to the 1:5 ratio in the transfection mix. HIV-1 production was inhibited more than 80% upon co-transfection with F-shNef in comparison with the empty F control (Figure 3B; left panel). Similar results were obtained with R-shNef

versus the R control (Figure 3B; right panel). We observed a similar level of shRNA-Nef-mediated inhibition in the presence of dox, indicating that the slightly reduced siNef level observed upon activation of viral polymerase II-driven transcription (Figure 3A) is sufficient to inhibit HIV-1 production.

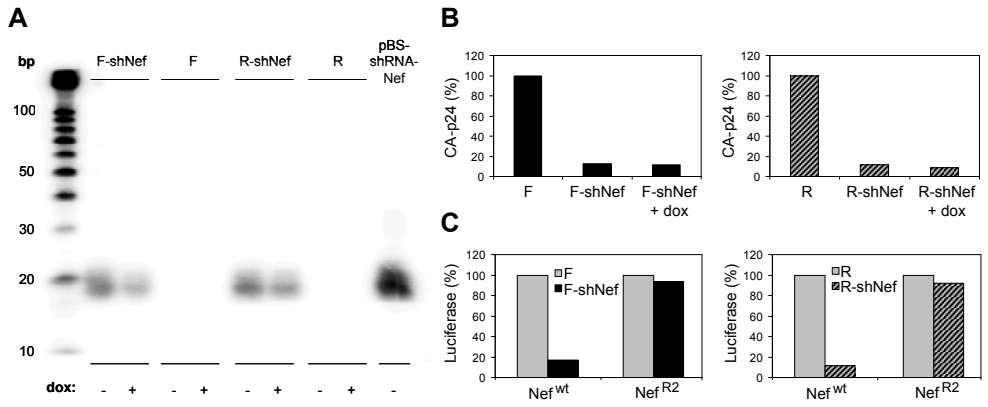

Figure 3. Expression of shRNA-Nef and efficient suppression of target genes. **(A)** Northern blot analysis of shRNA-Nef expression in C33A cells transfected with ΔU3 HIV-rtTA constructs, cultured with or without dox. The expressed shRNAs are efficiently processed into the 19-nt siNef effector molecules. C33A cells transfected with a pBluescript plasmid carrying the shRNA-Nef-expression cassette (pBS-shRNA-Nef; (9)) are included as a positive control. **(B)** C33A cells were cotransfected with HIV-1 LAI (500 ng) and the indicated ΔU3-HIV-rtTA-shNef or control variant (100 ng). When indicated, cells were cultured in the presence of dox. Virus production in the culture supernatant was measured at day two by CA-p24 ELISA. The level of HIV-1 production with the control F and R constructs was set at 100%. **(C)** The ΔU3-HIV-rtTA-shNef constructs were cotransfected into C33A cells with reporter plasmids containing either the intact shRNA-Nef target (Nef^{wt}) or a shRNA-Nef-resistant version (Nef^{R2}) downstream of the luciferase gene. The level of luciferase expression measured with controls F and R was set at 100%.

As a second test, we cotransfected the ΔU3-HIV-rtTA constructs with a firefly luciferase reporter construct with a downstream Nef target sequence (9). Both F-shNef and R-shNef potently inhibit luciferase expression when compared with the controls F and R (Figure 3C). We also tested a luciferase-reporter construct with a mutant Nef sequence in which the target sequence was partially deleted (Nef^{R2} in Figure 1). This mutant Nef sequence was selected in an HIV-1 variant that escaped from shRNA-Nef-mediated RNAi in long-term evolution studies (8,9). This luciferase-Nef^{R2} reporter is not inhibited by F-shNef or R-shNef, demonstrating the strict sequence-specificity of this RNAi-mediated silencing.

Protecting cells against HIV-1 replication

We next tested whether ΔU3-HIV-rtTA-shNef viruses can be used as a replicating vector for delivery of shRNA-Nef to cells that are susceptible to HIV-1 infection. This therapeutic strategy is illustrated in Figure 4. Dox-dependent replication of

the therapeutic virus results in spread to the HIV-1 target cells. Withdrawal of dox results in transduced cells with a silent integrated provirus, but with an active expression cassette for shRNA-Nef, which may protect these cells against subsequent HIV-1 replication.

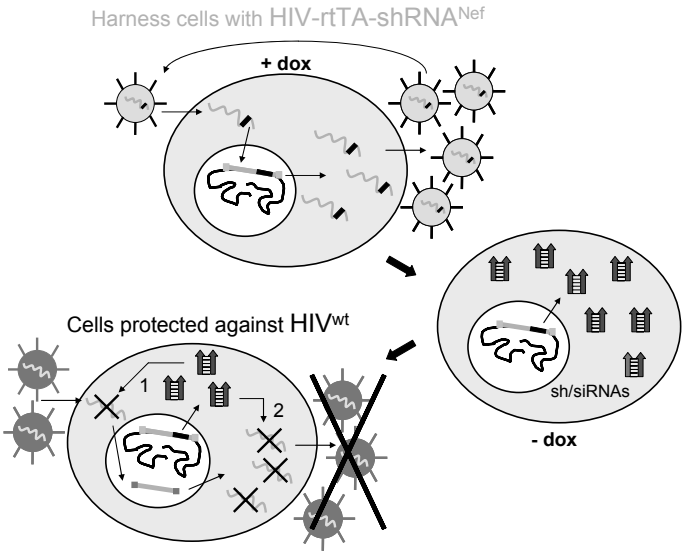

Figure 4. Protecting HIV-1 susceptible cells with HIV-rtTA-shNef. A spreading infection of HIV-rtTA-shNef in all susceptible cells is triggered by transient dox-treatment. Dox-withdrawal prohibits HIV-rtTA-shNef replication and silences the integrated provirus. However, shRNA-Nef is constitutively expressed from the polymerase III expression cassette. This shRNA-Nef may target the incoming RNA genome (22) (route 1) and/or the de novo made RNA transcripts (route 2) and thus prevent HIV-1 replication.

To test this strategy, we started a spreading infection of the ΔU3-HIV-rtTA F-shNef on SupT1 cells in the presence of dox (Figure 5A). As a control, we similarly established a spreading infection with the ΔU3-HIV-rtTA F control virus (Figure 5B). When HIV-induced cytopathic effects indicated a high infection rate (day 5), cells were washed and cultured without dox. The cells were challenged at day 38 with wild-type HIV-1 LAI virus (HIV^{wt}) or the shRNA-Nef-resistant HIV^{R2} variant (9), in which the shRNA-Nef target sequence was partially deleted (Figure 1). Whereas the F-transduced control cells supported replication of both HIV^{wt} and HIV^{R2}, the F-shNef-transduced cells only supported replication of the RNAi-resistant HIV^{R2} variant and resisted HIV^{wt} replication (Figure 5A/B). These results demonstrate that HIV^{wt} is efficiently inhibited through shRNA-Nef-induced RNAi, and not through unrelated events such as superinfection-interference (23). To verify the stable presence of proviruses with an intact polymerase III expression cassette in ΔU3-HIV-rtTA infected cells, we PCR amplified the viral U3 region and analyzed the products by agarose gel electrophoresis (Figure 5C). Both the F- and F-shNef-infected cells stably maintain the pol III expression cassette during multiple cell passages. Consistent with this result, we obtained similar HIV-1 inhibition results when the cultures were challenged at day 95 (results not shown).

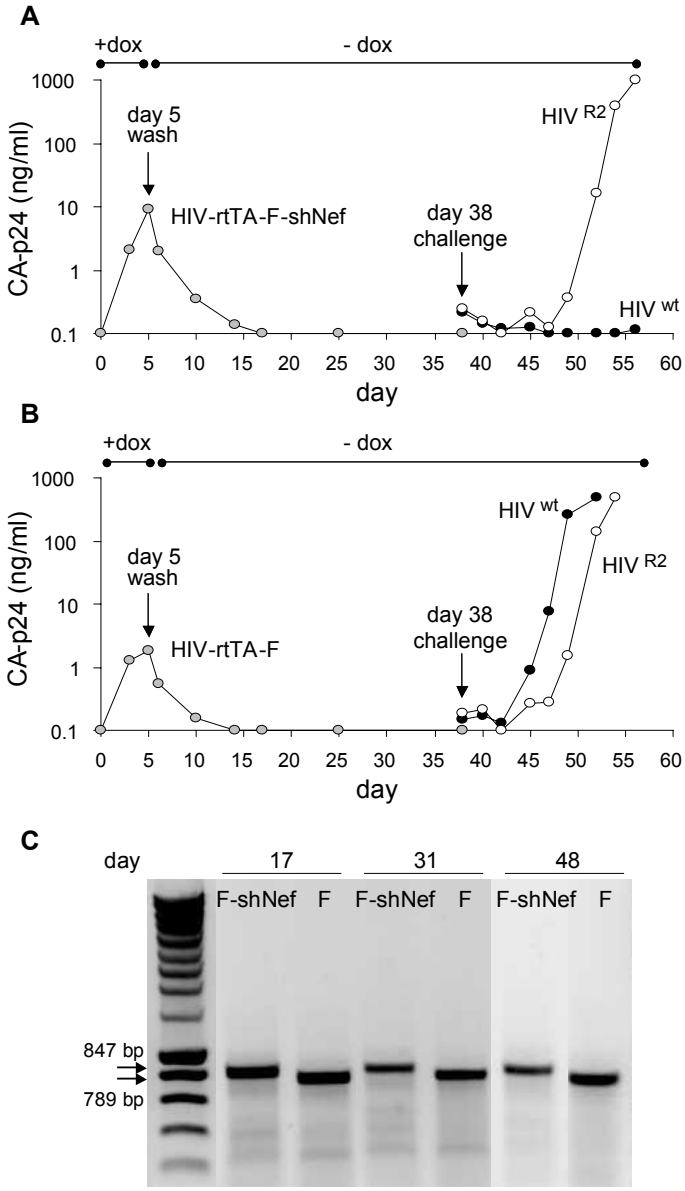

Figure 5. Harnessing SupT1 cells against HIV-1 infection.
(A, B) ΔU3 HIV-rtTA-F-shRNA-Nef (panel a) and the F control (panel b) constructs (5 µg) were transfected into SupT1 T-cells. The cells were cultured in the presence of 1 µg/ml dox to initiate a spreading infection. Dox was washed away at day 5 and the cells were cultured for over a month without dox. The cultures were challenged at day 38 with HIV^{wt} or HIV^{R2} (200 pg of CA-p24 per ml) and virus replication was monitored by determining the level of CA-p24 in the culture supernatant.
(C) Integrated proviral DNA was PCR amplified at day 17, 31 and 48 with primers that amplify the complete F-shRNA-Nef-cassette as 847-bp fragment and the F empty control cassette as 789-bp fragment. The PCR-amplification product was analyzed by agarose gel electrophoresis.

Although these experiments demonstrate that F-shNef infection of cells does inhibit replication of HIV^{wt}, this inhibition is not absolute, as a low level of replication was apparent when a high HIV^{wt} virus input was used (results not shown). This incomplete protection may have resulted from an incomplete spread of the therapeutic virus. To obtain pure F-shNef containing cells, we performed limiting dilution of the F-shNef-infected cells and selected 38 cell clones. These

clones were tested by PCR for the presence of the shRNA-Nef-expression cassette and challenged with HIV^{wt}. Clones positive for F-shNef (left half of circle is gray in Figure 6A) are expected to resist HIV^{wt} replication (right half of circle is white in Figure 6A) and cell clones lacking the F-shNef provirus (left half is white in Figure 6A) are expected to be susceptible to HIV^{wt} replication (right half is black in Figure 6A). This limiting dilution experiment reveals that approximately 63% of the cells (24/38) were infected with F-shNef (Figure 6B). Apparently, this level of spread is

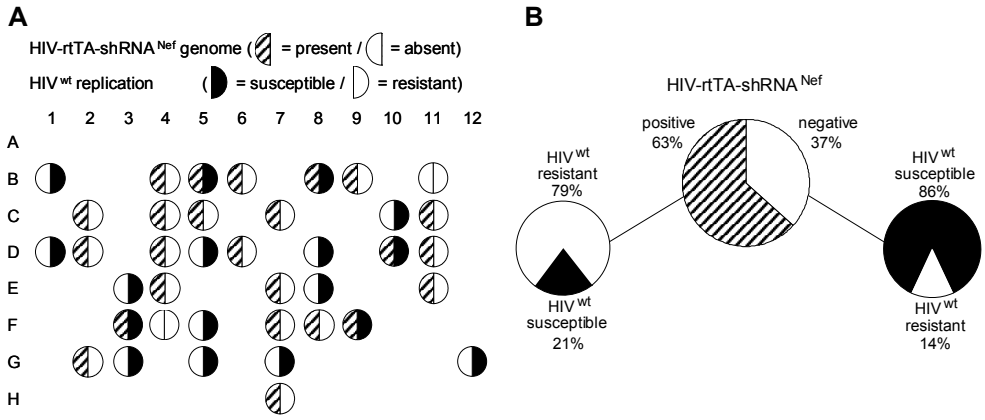

Figure 6. HIV-rtTA-shRNA-Nef containing cell clones resist HIV-1 replication. **(A)** SupT1 cells with a silent ΔU3 HIV-rtTA-F-shNef provirus (obtained from the experiment shown in Figure 5A, isolated at day 60) were serially diluted to obtain clones. A total of 38 clones were PCR-screened for the presence or absence of ΔU3-HIV-rtTA-F-shNef provirus, indicated in the left half of the circle (gray, present; white, absent). The clones were challenged with HIV^{wt} virus (750 pg CA-p24 per ml) and virus replication was monitored, indicated in the right half of the circle (black, replication; white, no replication). **(B)** Pie chart summary. ΔU3-HIV-rtTA-F-shNef positive cell clones (middle chart, gray) are predominantly HIV^{wt} resistant (left chart, white). ΔU3-HIV-rtTA-F-shNef negative cells (middle chart, white) are mainly susceptible to HIV-1 infection (right chart, black).

sufficient to obtain a high degree of protection against HIV^{wt} infection (Figure 5A), but it also explains the breakthrough replication with high input challenge virus. Of the 24 shRNA-Nef-positive clones, 79% (19 cultures) were protected against HIV^{wt} replication. The other five shRNA-Nef-positive clones were susceptible to HIV^{wt}. We tried to induce HIV-rtTA-F-shNef replication in these cells by dox administration, but failed to reactivate the integrated provirus in four of the five clones. This might indicate transcriptional silencing of the integrated therapeutic provirus, which may affect both the polymerase II LTR promoter and the polymerase III shRNA-Nef-cassette and thus explain the susceptibility of these cells for HIV^{wt}. The majority (86%; 12/14) of the shRNA-Nef-negative control cells are susceptible to HIV^{wt} replication (Figure 6B). The two clonal cultures that do not support HIV^{wt} replication may spontaneously have lost an essential cofactor such

as the CD4 receptor. Similar results were obtained when the viral challenge was performed on the clones four weeks later (results not shown). These results confirm the stable presence and shRNA expression of the therapeutic provirus.

Discussion

We have used the conditional-live HIV-rtTA virus to create a replicating vector for the efficient delivery of shRNA-Nef to cells that are susceptible to HIV-1 infection. The shRNA-Nef targets the wild-type HIV-1 RNA genome but not the viral vector. The dox-dependent replication of these therapeutic viruses allows one to control its spread. Upon dox withdrawal the HIV-rtTA-shNef-infected cells stably maintain the silent provirus and the constitutively active shRNA-Nef-expression cassette harnesses these cells against subsequent infection with the wild-type virus.

We previously demonstrated that shRNA-Nef-resistant escape variants can be selected with a variety of mutations in the viral target sequence (9). The use of combination-shRNA therapy in which multiple conserved viral RNA sequences are targeted by multiple shRNAs at the same time, may block the emergence of RNAi resistant variants (24). The introduction of multiple shRNA-expression cassettes into the HIV-rtTA genome may pose several problems. First, we targeted Nef sequences because this gene is deleted in the HIV-rtTA vector. There are a few additional options to target HIV-1 sequences that are absent or no longer required in HIV-rtTA, e.g. in the U3 domain of the LTR promoter or in Tat. When targets are chosen that are also encoded in the HIV-rtTA genome, one could try to modify these vector sequences, e.g. by silent codon changes. A second problem may be imposed by the presence of multiple similar polymerase III units in a single retroviral vector. Recombination on repeat sequence may occur during reverse transcription (13), thus seriously affecting the genetic stability of these viruses. However, the use of different polymerase III promoters may avoid such problems (ter Brake and Berkhout, submitted).

The HIV-rtTA virus is under development as a novel approach towards an HIV vaccine. Live attenuated viruses have shown promise as vaccine candidates that elicit both a humoral and cellular immune response (25). However, attenuated HIV-1 causes a persistent infection, maintains a high genetic instability and can thus evolve into a pathogenic HIV-1 variant (26). HIV-rtTA replication can be restricted by transient dox administration to the extent needed for induction of the immune system. As a result, chronic virus replication and restoration of virulence are prevented. Safety of this drug-controlled virus can be improved further by addition of other restrictions, e.g. T20-dependence (27,28). Addition of the therapeutic shNA-Nef-expression cassette, which maintains fully active when

virus replication is stopped, may prevent HIV-1 infection of those cells that are not efficiently protected by the elicited immune response. Similarly, the HIV-rtTA-shNef vector may possibly be used to protect HIV-1 target cells in HIV-1 infected individuals. Transient replication of the therapeutic virus with dox would result in a population of cells that can no longer be infected by the patient's virus. This strategy seems particularly suitable for patients infected with a multi-drug resistant virus that can no longer be suppressed by the current antivirals.

Several safety concerns remain for the use of this therapeutic virus in humans. For instance, this virus will insert at a random position in the human genome and may trigger deregulation of neighboring genes. Another problem may relate to off-target effects of the anti-HIV shRNA, but one could use inducible systems, preferably a polymerase promoter II unit that is under control of the HIV-1 Tat protein (29) such that the shRNA is expressed exclusively in virus-infected cells. The proposed therapy could provide a unique combination of vaccination with a conditional-live virus and intracellular immunization by means of RNAi.

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Chapter Six

The virion-associated incoming HIV-1 RNA genome is not targeted by RNA interference

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Abstract

Background: RNA interference (RNAi) has proven to be a powerful tool to suppress gene expression and can be used as a therapeutic strategy against human pathogenic viruses such as human immunodeficiency virus type 1 (HIV-1). Theoretically, RNAi-mediated inhibition can occur at two points in the replication cycle, upon viral entry before reverse transcription of the RNA genome, and on the newly transcribed viral RNA transcripts. There have been conflicting results on whether RNAi can target the RNA genome of infecting HIV-1 particles. We have addressed this issue with HIV-1-based lentiviral vectors.
Results: We determined the transduction efficiency of a lentiviral vector, as measured by GFP-expressing cells, which reflects the number of successful integration events in a cell line stably expressing shRNA-Nef. We did not observe a difference in the transduction efficiency comparing lentiviral vectors with or without the Nef target sequence in their genome. The results were similar with particles pseudotyped with either the VSV-G or HIV-1 envelope. Additionally, no reduced transduction efficiencies were observed with multiple other shRNAs targeting the vector genome or with synthetic siNef when transiently transfected prior to transduction.
Conclusions: Our findings indicate that the incoming HIV-1 RNA genome is not targeted by RNAi, probably due to inaccessibility to the RNAi machinery. Thus, therapeutic RNAi strategies aimed at preventing proviral integration should be targeting cellular receptors or cofactors involved in pre-integration events.

Introduction

Double stranded RNA (dsRNA) can induce RNA interference (RNAi) in cells, resulting in sequence-specific degradation of the targeted mRNA (1,2). Short interfering RNAs (siRNAs) of ~22nt are the effector molecules of this evolutionarily conserved mechanism and are produced by a ribonuclease named Dicer (3,4). One strand of the siRNA duplex is incorporated into the RNA-induced silencing complex (RISC), which binds to and cleaves complementary RNA sequences (5,6). RNAi has proven to be a powerful tool to suppress gene expression. Transfection of siRNA into cells results in transient inhibition of the target gene (7). Stable gene suppression can be achieved by the introduction of vectors that express siRNAs or short hairpin RNAs (shRNAs) that are processed into siRNAs by Dicer (8,9).

RNAi can be used as a therapeutic strategy against human pathogenic viruses such as HIV-1 (10). Several studies have demonstrated that HIV-1 replication can be inhibited transiently by transfection of synthetic siRNAs

targeting either viral RNA sequences or cellular mRNAs encoding protein co-factors that support HIV-1 replication (11-20). Furthermore, several groups have demonstrated long-term inhibition of HIV-1 replication in transduced cell lines that stably express an antiviral siRNA or shRNA (21-28). However, HIV-1 escape variants with nucleotide substitutions or deletions in the siRNA target sequence emerge after prolonged culturing (22,24). We have also demonstrated that HIV-1 can gain resistance against RNAi through mutations that mask the target in a stable RNA secondary structure (29). The use of combination-shRNA therapy, in which multiple conserved viral RNA sequences are targeted by multiple shRNAs at the same time, may block the emergence of RNAi-resistant variants (30).

During the HIV-1 life cycle, there are two phases that could potentially be targeted by RNAi (31,32). Newly made viral transcripts, synthesized from the integrated proviral DNA, are the obvious targets. In addition, RNAi may target the virion-associated or "incoming" viral RNA genome during the initial phase of infection prior to completion of reverse transcription that converts the RNA genome into DNA. During the infection, the HIV-1 core particle traverses through the cytoplasm, where the RNAi machinery resides. If the RNA genome within the virion core is accessible to RISC, reverse transcription and subsequent proviral integration would be blocked, which is highly desirable in a therapeutic setting. There have been conflicting results on whether RNAi can target the RNA genome of infecting HIV-1 particles. Several groups have reported degradation of the incoming RNA genome in cells transfected with siRNAs (11,12,16). Recently, a study showed inhibition of HIV-1 provirus integration in cells stably expressing shRNAs at a low virus input (33). Other publications report no RNAi-mediated degradation of the RNA genome in siRNA-transfected or shRNA-producing cells (17,18,34). In the present study, we have readdressed the issue of incoming HIV-1 genome targeting using HIV-1-based lentiviral vectors in which we used transduction as a model for proviral integration. Targeting of the incoming genome did not reduce transduction efficiency, indicating that the HIV-1 RNA genome is not a target for RNAi during the initial phase of infection.

Materials and Methods

Plasmid construction

Lentiviral vector plasmids are derived from the construct pRRLcpptpgkgfppreSsin (35), which we renamed JS1. The plasmids JS1-Nef and JS1-R2 were obtained by digestion of the firefly luciferase expression vectors pGL3-Nef and pGL3-R2, containing an ~250-bp Nef fragment downstream of the luciferase gene (29), with XhoI and PstI and inserting this fragment into the corresponding sites of JS1. The other firefly reporter plasmids (pGL3-LDR9 and -Pol29 and -Nef19) were

constructed by insertion of a 50-70-nucleotide (nt) HIV-1 sequence, with the 19-nt target in the center, in the EcoRI and PstI sites of pGL3-Nef (36).

The pSUPER vector (8), which contains the H1 polymerase III promoter, was linearized with BglII and HindIII. Sense and antisense strand oligonucleotides, which encode the shRNA sequence against a conserved 19-nt HIV-1 region (LDR9; AGATGGGTGCGAGAGCGTC [798], Pol29; CAGTGCAGGGGAAAGAATA [4811] and Nef19; GGGACTGGAAGGGCTAATT [9081]) (36) or the Nef (24) sequence, were annealed and ligated into pSUPER. The number between the brackets indicates the nucleotide position in prototype HIV-1 strain HXB2. The plasmid pRL-CMV (Promega) expresses renilla luciferase under control of the CMV promoter.

Cell culture

Human embryonic kidney (HEK) 293T adherent cells were grown at 37°C and 5% CO_2 in DMEM (Gibco BRL) and SupT1 suspension cells were grown in RPMI 1640 (Gibco BRL), both supplemented with 10% Fetal Calf Serum (FCS), penicillin (100 U/m) and streptomycin (100 μg/ml). The SupT1 cells stably expressing shRNA-Nef were described previously (24).

Lentiviral vector production

293T cells were grown to 50% confluence in 2 ml culture medium in 9.4 cm^2 wells. The medium was replaced with 2 ml medium without antibiotics. Subsequently, the lentiviral vector plasmid (2.2 μg) was cotransfected with packaging plasmids pMDLg/pREV (1.45 μg), RSV-REV (0.56 μg), and pVSV-G (0.78 μg) (37,38) or the pSV7D plasmid encoding HXB2 gp160 (0.78 μg). The pSV7D Envelope gp160 plasmid was a kind gift of Dr. J. Binley (Torrey Pines Institute for Molecular Sciences, La Jolla, CA, USA). Cotransfection in 3 ml was performed with 5 μl lipofectamine-2000 and 0.5 ml Optimem (Gibco BRL). The culture medium was refreshed after 16 h. Medium containing the lentiviral vector was harvested the next day and replaced with fresh medium. This procedure was repeated after 24 h. The supernatants were mixed, cellular debris was removed by low speed centrifugation and aliquots of 0.5 ml were stored at -80°C. For lentiviral vectors produced with HIV-1 envelope, the stocks were concentrated with an Amicon Ultra concentrator, MWCO 100,000 (Millipore Corporation, Bedford, MA, USA).

Lentiviral vector transduction

Lentiviral vector stocks were titrated on 293T cells and SupT1 cells. SupT1 (1.0 x 10^5 cells in 0.5 ml medium) and 293T (1.0 x 10^5 cells in 0.5 ml medium) were subsequently transduced at various m.o.i. (from 0.01 to 1). Two days after transduction the cells were harvested, fixated in 4% paraformaldehyde and analysed by FACS for GFP expression (FACScan, BD Biosciences).

Transfection experiments

293T cells (2 cm^2; 1.0 x 10^5 cells) were seeded in 500 ml DMEM with 10% FCS without antibiotics. The next day, 1 μg pSUPER-shRNA plasmid, 125 nM siRNA or 1 μg control pBS (pBluescriptII (KS^+); Stratagene) was transfected with 1 μl lipofectamine-2000 in a reaction volume of 100 μl according to the manufacturers instructions (Invitrogen). Sixteen hours post-transfection the medium was replaced with 500 ml medium with antibiotics, and the cells were subsequently used for transduction or luciferase experiments.

For luciferase experiments, 293T cells (2 cm^2; 60% confluent) were transfected with 200 ng pGL3-constructs and 1 ng pRL using lipofectamine-2000. SupT1 cells (shRNA-Nef-expressing and control) were transfected with luciferase plasmids by electroporation. Briefly, 5 x 10^6 cells were washed in RPMI 1640 medium with 20% FCS and mixed with 5 μg pGL3-constructs and 150 ng pRL in 250 μl of RPMI 1640 medium with 20% FCS. Cells were electroporated in 0.4 cm cuvettes at 250 V and 975 μF and subsequently resuspended in RPMI 1640 medium with 10% FCS. The culture medium was refreshed after 16 h. After another 24 h, the cells were lysed in 150 ml of Passive Lysis Buffer (PLB) (Promega). Firefly and renilla luciferase activities in the lysate were measured with the Dual-luciferase Reporter Assay System (Promega).

Results

To determine the amount of incoming HIV-1 RNA in cells expressing antiviral siRNAs, the integrated HIV-1 DNA product or pre-integration DNA intermediates have been quantified (12,16-18,33,34). Instead, we use an HIV-1 based lentiviral vector system to study proviral integration in cells expressing shRNAs against the HIV-1 lentiviral vector genome. We chose the lentiviral vector system because it is ideally suited to study proviral integration since viral infection is limited to a single cycle and is easily scored with FACS analysis detecting reporter gene expression in transduced cells. JS1 is a third generation self-inactivating lentiviral vector containing a GFP reporter gene (Figure 1). Lentiviral vector particles are produced in 293T cells by cotransfection of the vector plasmid with the packaging constructs encoding Gag-Pol, Rev, and the VSV-G envelope protein (Figure 1). Transduction titers of the produced lentiviral vectors were determined. All infection experiments were subsequently carried out at relatively low multiplicity of infection (m.o.i) such that transduced cells were preferably infected by a single vector. Thus, a transduced cell represents a single successful reverse transcription and proviral integration event.

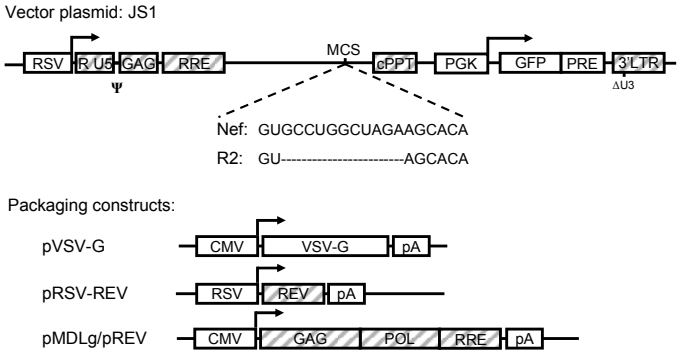

Figure 1. The lentiviral vector and packaging constructs.
The lentiviral vector JS1 is a third generation self-inactivating vector (35), which contains a GFP reporter gene expressed from the phosphoglycate kinase promoter (PGK) with the posttranscriptional regulatory element (pre) from hepatitis B virus. The vector genome is expressed from the Rous sarcoma promoter (RSV) and transcription starts with the R and U5 regions of the HIV-1 long terminal repeat (LTR), the packaging signal (ψ) and part of the gag open reading frame (gag). It contains the rev responsive element (RRE), central polypurine tract (cPPT) and the 3' LTR, which has a deletion in the U3 region (ΔU3). The HIV-1 sequences are tinted gray. Transcription of the vector genome and GFP reporter terminates at the HIV-1 polyA within the 3' LTR. The Nef target sequence (wild-type or mutant) was cloned into the multiple cloning site (MCS). The three packaging constructs encode the trans-acting proteins required for the production of infectious virus (HIV-1 sequences in gray).

We cloned an approximately 200-bp Nef fragment into the multiple cloning site (MCS) of the lentiviral vector genome (JS1-Nef). This sequence contains the target sequence for the potent shNef inhibitor that we described in earlier studies (24,29). As a control, we constructed a vector with a mutant Nef sequence (JS1-R2), lacking 11 nucleotides of the shNef target sequence, which was shown to be completely resistant to shNef attack (24,29). During lentiviral vector

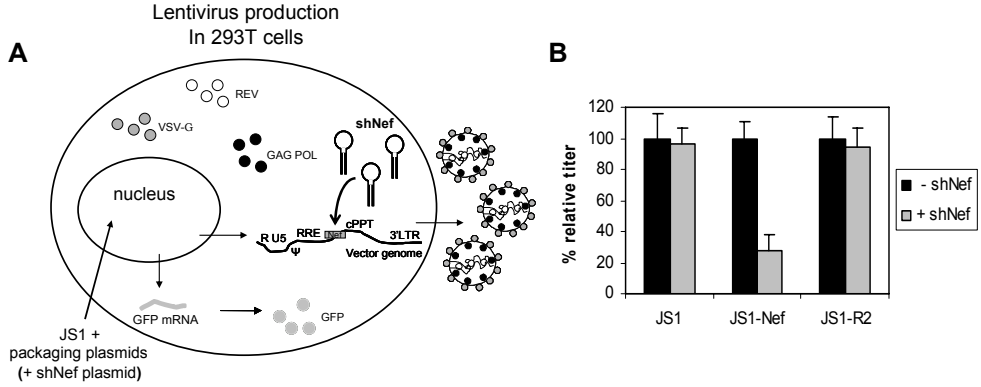

Figure 2. Sequence-specific inhibition of lentiviral production by RNAi. **(A)** Schematic of lentiviral production. When an shNef-expression plasmid is cotransfected during lentiviral vector production, the lentiviral vector RNA genome containing the Nef target (gray box) can be targeted by RNAi (dark arrow). **(B)** Lentiviral vector stocks (JS1, JS1-Nef and JS1-R2) were produced in 293T cells in the absence (- shNef) or presence (+ shNef) of an shNef-expression plasmid and were titrated on SupT1 cells. Transduced cells were analyzed by GFP-FACS. The mean values of three independent experiments are shown. The control values (- shNef) were set at 100% for each lentiviral vector.

production, the vector genome is transcribed and transported to the cytoplasm where it becomes packaged in the vector particle (Figure 2A). When the JS1-Nef lentiviral particles were produced in the presence of the shNef expression plasmid in the transfection mix, we observed a significant reduction in titer (Figure 2B). In contrast, the titer of JS1 and JS1-R2 vectors was similar to the their titer produced in the absence of shNef. This result shows that the vector genome is in principle an effective target for RNAi.

The lentiviral vectors JS1, JS1-Nef and JS1-R2 were produced and subsequently used to infect the SupT1 T-cell line that stably expresses shNef (24) and control SupT1 cells. When the incoming RNA genome is targeted by shRNA induced RNAi, the number of cells that obtain an integrated proviral DNA copy should be reduced. This will be reflected in a reduced transduction efficiency of shNef cells compared to the control SupT1 cells (Figure 3A). Two days after infection, the cells were analyzed by FACS analysis. We did not observe a significant difference in the transduction efficiency of JS1-Nef in the control cells versus shNef-expressing cells, indicating the incoming vector genome was not targeted by RNAi (Figure 3B). Results were similar for the empty vector JS1 and control vector JS1-R2 with a deletion in the shNef target sequence. The results were independent of the m.o.i., which ranged from 0.03 to 1. These combined results clearly indicate that the incoming lentiviral RNA genome is not a target for RNAi.

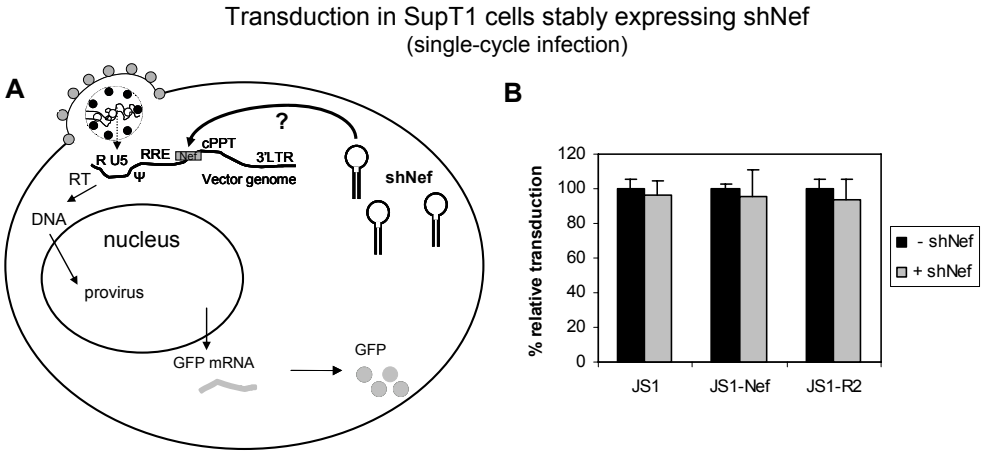

Figure 3. No sequence-specific inhibition of lentiviral transduction by RNAi. **(A)** Schematic of lentiviral transduction. When shNef is stably produced in the target cells, the question is whether the incoming vector genome with the shNef target sequence is targeted by RNAi (dark arrow with question mark). **(B)** SupT1 cells stably expressing shNef (+ shNef) or control SupT1 cells (- shNef) were transduced at an m.o.i. of 0.03, 0.3 or 1.0 with the control vector (JS1) or vectors containing a complete (JS1-Nef) or mutated (JS1-R2) shNef target sequence. Infected cells were analyzed by GFP-FACS. The control values (- shNef) were set at 100% for each lentiviral vector. The mean values of three experiments are shown.

As an additional control for the presence of a functional shNef in the shNef-expressing SupT1 cells, we transfected the luciferase reporter constructs (29) containing the complete (pGL3-Nef) or mutant (pGL3-R2) target sequence (Figure 4A). Luciferase expression of pGL3-Nef was reduced to 20% in the shNef-expressing cells compared to the control cells (Figure 4B). In contrast, luciferase expression of pGL3-R2 is similar in both cells. This confirms that SupT1 cells expressing the shNef induce sequence-specific inhibition of RNAs containing the Nef target sequence.

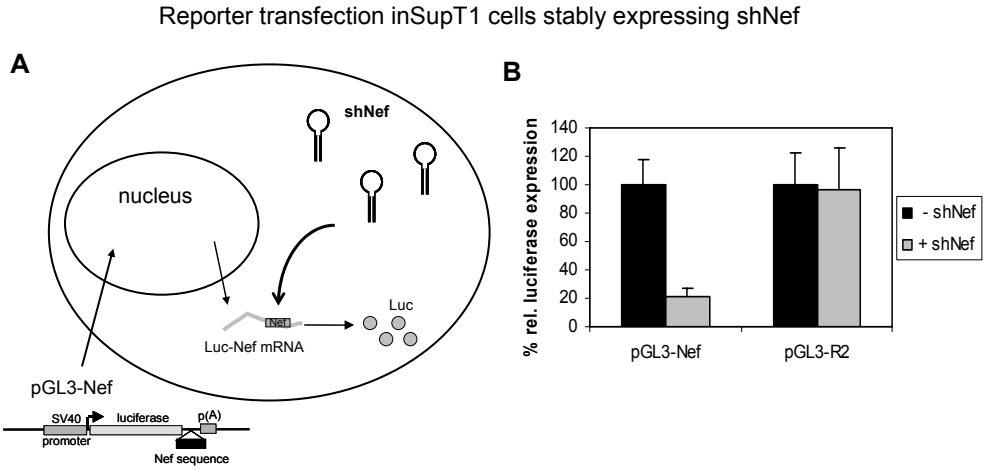

Figure 4. Sequence-specific inhibition in shNef-expressing cells. **(A)** Schematic of RNAi-mediated targeting of mRNA with the shNef target sequence (gray box) in shNef-expressing SupT1 cells. **(B)** SupT1 cells stably expressing shNef (+ shNef) or control SupT1 cells (- shNef) were transfected with luciferase reporter constructs that contain the complete shNef target sequence (pGL3-Nef) or not (pGL3-R2). The mean values obtained in two independent experiments are shown. Values measured in the control transfection (- shNef) were set at 100% for each reporter construct.

The lentiviral particles used in the experiments described above are pseudotyped with the VSV-G envelope. One could argue that VSV-G mediated entry and subsequent intracellular processes are different from wild-type HIV-1 virions that contain the HIV-1 Envelope protein. The use of VSV-G would thus explain why we do not observe targeting of the incoming genome. To exclude this possibility, we produced lentiviral vectors with an HIV-1 Envelope and repeated the experiment. Infection of SupT1 cells expressing shNef with JS1-Nef lentivirus containing HIV-1 envelope was similar to that of control SupT1 cells, which demonstrates that the mode of entry does not contribute to the absence of incoming genome targeting (Figure 5).

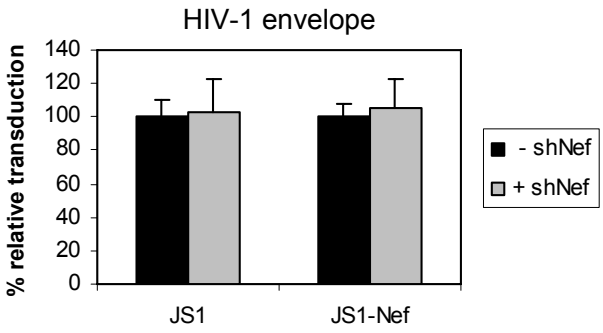

Figure 5. No inhibition of lentiviral transduction with virions containing the HIV-1 Envelope. SupT1 cells stably expressing shNef (+ shNef) or control SupT1 cells (- shNef) were transduced at an m.o.i. of 0.03, 0.2 or 0.5 with either the control (JS1) or the shNef target sequence containing (wt-Nef) lentiviral vector with an HIV-1 envelope protein. Infected cells were analyzed by GFP-FACS. The control values (- shNef) were set at 100% for each infection. The mean values of two independent experiments are shown.

The contradicting results in literature on inhibition of the incoming HIV-1 RNA genome by RNAi may be due to differences in experimental conditions. In fact, most studies used chemically synthesized siRNAs that were transfected into various cell types prior to challenge with HIV-1. We therefore tested a synthetic siRNA directed against the same shNef target. This siNef is the same as the one shown by Jacque *et al.* to affect the level of integrated provirus (12). Cells transfected with siNef or a shNef-expression plasmid reduced pGL3-luciferase Nef reporter expression, when the reporter was transfected 24 hours post siRNA or shRNA transfection (Figure 6B). In contrast, when these siRNA or shRNA-expressing cells were infected with JS1-Nef lentiviral particles, no drop in transduction efficiency was observed compared to mock (-) or pBS-transfected cells (Figure 6C). Similar results were obtained with a range of m.o.i. (results not shown). Thus, an active siRNA is also unable to inhibit the incoming RNA genome.

In literature, a variety of different targets have been used and variation in target accessibility in the context of the packaged RNA genome may explain the contradicting results. Our lab has constructed multiple potent shRNAs against conserved regions in the HIV-1 RNA genome (36). Some of these shRNAs also target the lentiviral vector genome (Figure 6A; LDR9, Pol29 and Nef19). We transfected 293T cells with the different shRNA-expression constructs and 24 hours later with the appropriate reporter constructs. Alternatively, we infected these cells after 24 hours with JS1-wtNef lentiviral vector. The 3 additional shRNAs demonstrated full inhibitory activity on the luciferase reporters (Figure 6B; right 3 panels), but lacked any activity on the incoming RNA genome (Figure 6C), with one notable exception: shNef19 is an effective inhibitor in both systems. The explanation for this exception comes from inspection of its target in the lentiviral vector genome (Figure 6A), which is actually located in the 3′ LTR region, and thus part of the GFP transcript. The observed drop in GFP-expressing cells is therefore

caused by direct RNAi-inhibition of the reporter transcript, and not by targeting of the incoming RNA genome.

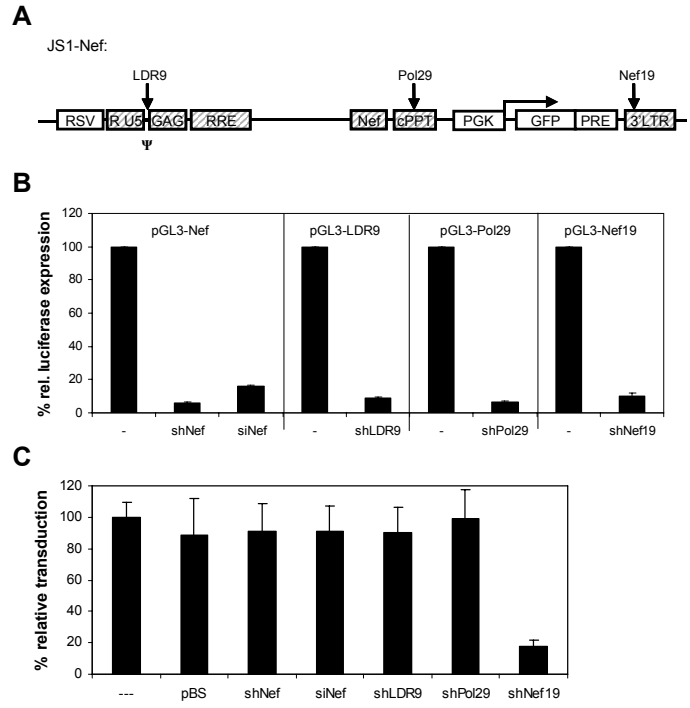

Figure 6. No inhibition of lentiviral transduction in cells transfected with different shRNA-expressing plasmids or siRNAs.
(A) Map of the JS1-Nef genome with the positions targeted by the shRNA inhibitors.
(B) 293T cells were mock transfected or transfected with siNef or plasmids expressing the indicated shRNAs. The cells were subsequently transfected with luciferase reporter constructs containing the target sequences and the luciferase expression was measured. The mean values obtained in two independent experiments are shown. The mock value (-) was set at 100% for each luciferase reporter.
(C) 293T cells were mock transfected or transfected with the control pBS, siNef or plasmids expressing the indicated shRNA. The cells were subsequently transduced with the JS1-Nef vector. Transduction efficiency was determined by GFP-FACS. The mean values obtained in two independent experiments are shown. The transduction efficiency for the control experiment (-) was set at 100%.

Discussion

We have not observed RNAi-mediated targeting of the HIV-1 RNA genome of incoming particles using our lentiviral vector transduction system. The human T-cell line that stably expresses shRNAs directed against the viral Nef gene shows effective inhibition of HIV-1 replication (24). However, we could not demonstrate an effect on the level of transduction with lentiviral particles, pseudotyped either with VSV-G or wild-type HIV-1 envelope. Similar results were obtained in a cell line transiently transfected with an shNef-expressing plasmid prior to infection. The intracellular levels of shRNAs is much higher upon transfection than in stable cell lines (results not shown), but even this increased concentration did not seem to affect the transduction efficiency. In addition, we failed to obtain an inhibitory effect on the incoming RNA genome with other shRNAs that target different parts

of the HIV-1 RNA genome or after transfection of a synthetic siRNA against Nef. All these results strongly indicate that the incoming HIV-1 RNA genome is not a target for RNAi.

The contradicting results that have been reported in literature may be due to differences in experimental conditions. It has been claimed that differences in target accessibility of different regions of the packaged RNA genome contribute to the variation in experimental results, but we detected a lack of inhibition with a range of targets, which are all highly accessible for RNAi-mediated inhibition in the context of reporter constructs. Furthermore, we demonstrated efficient targeting of the HIV-1 RNA genome in the producer cell, before it is encapsidated in the virion particle. It has been reported that the cellular environment can affect both the efficiency and the specificity of siRNAs and shRNAs (39). The use of different cell types can influence the observed RNAi effect. Additionally, the use of different promoters in shRNA-expression plasmids might also influence the potency of inhibition (40). In addition, "nude siRNAs", not associated with RISC, may be able to enter the viral core when present at high concentrations. Subsequent binding to the viral RNA genome can induce antisense-mediated inhibition of reverse transcription, but not an RNAi effect.

An explanation for the absence of targeting of the incoming viral RNA genome is inaccessibility to the RNAi machinery. After fusion of viral particles with the target cell membrane, the virion core is released into the cytoplasm. This coneshaped core consists of the capsid (CA-p24) protein containing the RNA genome and viral enzymes. This core is dissolved only partially during the infection process. Furthermore, when the reverse transcription complex (RTC) is formed, the genomic RNA is still associated with multiple proteins (nucleocapsid [NC], reverse transcriptase [RT], matrix protein [MA] and integrase [IN]). The limited knowledge about the structure of intracellular retroviral complexes prohibits a detailed discussion, but there is supportive evidence that large molecules cannot enter the core particle in which reverse transcription occurs. For instance, it was shown that tRNA molecules can enter the core particle in virus-infected cells, but with an efficiency that is 4 to 5 orders of magnitude lower than the tRNA packaging efficiency in virion-assembling cells (41). We made a similar observation with RNAi targeting the vector genome. During lentiviral vector production the RNA genome is an efficient target, resulting in reduced titers. In contrast, RNAi directed against the incoming genome could not reduce the transduction efficiency. Given the size of RISC, it is likely that this complex cannot enter the viral particle, thereby explaining our results.

Conclusions

Using lentiviral vector transduction as a model for HIV-1 infection, we have shown that the incoming HIV-1 genome cannot be targeted directly by RNAi. For effective gene therapy applications based on RNAi, it would be beneficial to target the incoming genome, thus blocking provirus establishment and in fact new infection of cells. To achieve this objective, one should target cellular receptors or cofactors that are involved in the initial phase of infection (15,42).

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Chapter Seven

General Discussion

Viral defense mechanisms against RNAi

Manuscript in preparation

Viral defense mechanisms against RNAi

The studies described in this thesis demonstrate the potential of using the RNAi pathway to silence HIV-1, but they also document spontaneous escape of the virus from artificially induced RNAi by mutation of the targeted viral genome sequence. In addition, we described that HIV-1 uses a replication strategy that evades RNAi-mediated silencing of the virion-associated "incoming" RNA genome. Interestingly, different viruses seem to use unique replication mechanisms to evade the induced or natural antiviral RNAi mechanisms. In this discussion, we review the results of mutational virus escape from induced RNAi together with natural viral escape strategies.

Induced antiviral RNAi

The success of inducible RNAi in mammalian cells triggered the development of RNAi-based strategies to selectively block viral gene expression, and thus viral replication. Since the first report of RNAi-mediated inhibition of the human pathogen respiratory syncytial virus (RSV) in 2001 (1), many other viruses have been efficiently targeted by RNAi (2). These include important human pathogens such as HIV-1 (3-7), hepatitis C virus (HCV) (8,9), hepatitis B virus (HBV) (10,11), dengue virus (12,13), severe acute respiratory syndrome (SARS) virus (14,15), poliovirus (16), influenza A virus (17), rhinovirus (18), herpes simplex virus type-1 (19), cytomegalovirus (20), Epstein Barr virus (EBV) (21,22), enterovirus (23), papillomavirus (24), hepatitis delta virus (25), Japanese encephalitis virus (26), West Nile virus (26) and JC virus (27,28). We have stably introduced vectors expressing siRNAs directed against various HIV-1 sequences into a human T cell line through retroviral transduction. HIV-1 replication was efficiently inhibited in cells transduced with an siRNA targeting the Nef gene (Chapter 2 and (29)).

Viral escape from induced RNAi

RNAi-based antiviral therapies are especially promising for the treatment of acute virus infections with synthetic siRNAs. For persistent virus infections, a gene therapy is likely needed to provide a constant supply of intracellularly synthesized siRNAs. Furthermore, a prolonged interaction of viruses with the induced RNAi machinery will to trigger the selection of escape variants through mutation of the target sequence (30-34). As we show in chapter 2 and 3 (29,35), the induced RNAi block of HIV-1 replication is not absolute and escape variants can evolve after several weeks of culture. These RNAi-resistant variants contain either a nucleotide substitution or deletion within the siRNA target sequence. Several other groups have also reported HIV-1 escape from RNAi-mediated inhibition (30,36-38). The majority of the selected viral escape mutants have a single point mutation within the target sequence. This suggests that a single substitution in the targeted

sequence is sufficient to overcome the antiviral activity of siRNAs, although more mutations may be required for full resistance. These results do confirm the exquisite sequence specificity of RNAi. Additionally, viral escape through a single mutation also indicates that the HIV-1 genome is not subjected to translational inhibition via the miRNA pathway of the RNAi machinery, which operates on partially mismatched targets. A switch from the siRNA to the miRNA pathway would in fact have been very beneficial in a therapy setting because a single mutation would not suffice for viral escape. However, the miRNA pathway is probably not activated because it requires multiple mismatched target sequences within the 3′ untranslated region of the mRNA (39).

We also described that over time a second point mutation is acquired in the presence of anti-HIV-1 siRNAs (29,35). This suggests that a single mismatch provides only partial RNAi resistance. Several studies reported that mismatches between the target and siRNA affect RNAi to a variable degree, depending on the position of the introduced mismatch (40-42). Although it has been suggested that the impact of specific mismatches may vary for individual siRNA-mRNA duplexes (36), mutations in either the central or the 3′ region of the target sequence affect RNAi efficiency most dramatically (40,41). These findings were corroborated in more elaborate studies on the RNAi mechanism. Kinetic analyses of siRNA/RISC complex formation showed that different regions of the siRNA play distinct roles regarding target recognition, cleavage and product release (43). The 5′ end sequences of the siRNA were found to contribute largely to the energy for binding to the target RNA, forming base pairs with the seed area of the target to initiate the double stranded (ds) helix that is required for RISC-mediated cleavage (44,45). Recent structural studies show that the 3′ end sequence of the siRNA is bound by the PAZ domain of Ago2. The PIWI domain provides a binding pocket for the 5′ end of the siRNA, which is thereby efficiently presented to the target mRNA (46,47). It has also been reported that escape viruses preferentially select mismatches in the 3′ region of the target RNA (48). These results are in agreement with models in which the 3′ region or seed area of the target RNA is critical for initial siRNA binding and RNAi activity (45,49-51).

Interestingly, we identified an RNAi-resistant HIV-1 variant with an acquired mutation upstream of the siRNA target sequence. This mutation results in an alternative local RNA structure in which the target sequence, in particular its 3′ end, is occluded from siRNA/RISC binding (Chapter 3 and (35)). Our results highlight the impact of target RNA structure on RNAi and indicate an alternative way for viruses to evade RNAi-mediated inhibition. For instance, the 3′ and 5′ untranslated regions in the human rhinovirus RNA genome are most conserved among viral isolates and therefore attractive targets for induced antiviral RNAi (18). However, targeting of these regions was ineffective, probably due to the

stable RNA structure of these genome ends, but RNA-protein interactions may also negatively influence the RNAi sensitivity (52-54). Similar results were obtained when Gitlin *et al*. targeted the well conserved and highly structured 5′ noncoding region of the poliovirus RNA genome (33).

RNAi as a natural antiviral defense mechanism?

The importance of RNAi as an innate antiviral defense response has been clearly demonstrated in plants, worms, and flies (55-58). Many plant viruses have evolved to encode proteins that actively interfere with distinct steps of the RNAi machinery to enable high virus production and efficient viral spread (55,59-61). Whether RNAi also functions as an antiviral defense mechanism in mammalian cells, in addition to the potent interferon system and other components of the innate immune system, is still debated (62,63). However, it has become clear that there is a tight interplay between mammalian viruses and the host cell RNAi machinery (64). Notable is the finding that a cellular miRNA, miR-122, positively affects the replication of HCV through a yet unknown mechanism (65). Various herpesviruses, including EBV, encode miRNAs that are thought to target specific cellular genes (66-70). Simian virus 40 (SV40) encodes miRNAs that regulate viral gene expression, thus reducing recognition of infected cells by the immune system (71).

It has been reported that the host miRNA miR-32 restricts replication of the primate foamy virus type 1 (PFV-1) retrovirus in human cells (72). This inhibition has been suggested to be circumvented by the viral Tas protein that generally suppresses miRNA-mediated events (72). Recently, it has been shown that the influenza virus NS1 and vaccinia virus E3L proteins, inhibitors of the IFN-induced protein kinase R (PKR) (73,74), can also inhibit RNAi pathways in plants and *Drosophila* cells (75-78). This inhibition may be ascribed to the unspecific binding of dsRNA, which has been shown to block RNAi in plants (79). The HIV-1 Tat protein has also been shown to have RNAi suppression activity (62). The abundantly expressed adenovirus transcripts VA-I and VA-II were shown to exhibit RNAi suppression activity by acting as decoy substrates for Exportin 5, Dicer and RISC (80,81).

Whether or not RNAi is used to defend mammals against viruses, several mammalian viruses use replication strategies that interfere with RNAi, be it on purpose or accidentally. In the next section, we will review some of these viral "tactics".

Viral evasion of natural RNAi

The replication strategy of viruses has a large influence on their susceptibility to RNAi-mediated inhibition. RNA replication intermediates that are located in the cytoplasm are theoretically efficient targets for RISC, while nuclear RNA molecules

may be protected from the RNAi machinery. The replication cycle of negative strand RNA viruses generally takes place in the cytoplasm. However, influenza virus requires cellular RNA synthesis to allow capping of the viral mRNAs, which are therefore synthesized in the nucleus (82). It was indeed reported that the genomic RNA of influenza virus is resistant to RNAi, whereas the cytoplasmic mRNA molecules are efficiently targeted (83). The circular genomic and antigenomic RNA of hepatitis delta virus, a virus that requires HBV as a helper virus for its replication, are RNAi-resistant (84). This resistance was ascribed to inaccessibility based on their nuclear localization. Some of the genomic RNA may be cytoplasmic and inaccessible to RNAi attack by its viroid-like structure (74% base pairing) or through binding of a host RNA-binding protein (84).

Reoviruses are dsRNA viruses that replicate in the cytoplasm. After cellular entry, the virions are partially digested in endosomes and become transcriptionally active (85). Replication and assembly of reoviruses occurs within viral inclusions that form in the cytoplasm of infected cells (86). It has been shown that the dsRNA genome residing in these inclusions is protected from RNAi (87). However, the newly synthesized mRNAs leave the viral inclusions to be translated in the cytoplasm, where they can be targeted for degradation by the introduction of specific siRNAs, resulting in inhibition of virus replication (87).

Flaviviruses have a positive-stranded RNA genome, which traffics from the cytoplasm to the endoplasmic reticulum (ER) where translation and replication takes place (88). The virus induces proliferation and reorganization of the ER membrane and these membranous structures are associated with replicating viral RNA (89-91). It has been reported that pretreatment of cells with antiviral siRNAs can reduce West Nile virus replication. However, siRNA application after virus infection did not affect virus replication (92). This suggests that the viral RNA is protected from the RNAi mechanism by a cellular membrane and that effective targeting may be restricted to the early phase of infection when the incoming genome travels from the cytoplasm to the ER (92).

Through association of their RNA genome with proteins, negative-strand RNA viruses seem to have a relatively simple mechanism to protect their cytoplasmic genomes against RNAi (93). Even in the initial experiments with RNAi-mediated inhibition of RSV, it was observed that the genomic RNA is not targeted (1). Recent studies have solved the detailed structure of such nucleoprotein complexes for rabies virus and vesicular stomatitis virus (94,95). The nucleoproteins oligomerize along the viral RNA to form a ring structure in which the RNA is sequestered and thereby protected, among others against RISC with antiviral siRNAs.

The RNA genome of retroviral particles is a theoretical target for RNAi before it is reverse transcribed. The retroviral particle travels through the

cytoplasm before it enters the nucleus for DNA provirus formation, thus providing a certain time window for RISC attack. However, the viral nucleocapsid particle of the Rous sarcoma virus was found to shield the incoming viral RNA from RNAi-mediated degradation (96). There have been contradicting reports whether the "incoming" HIV-1 RNA genome can be targeted by RNAi (3,4,96-100). We have addressed this issue using HIV-based lentiviral transduction as a quantitative model for HIV-1 infection, but found no targeting in any of our experimental set-ups (Chapter 6 and (101)). We argued that the cytoplasmic core particle is likely to be inaccessible to RISC, but complete coverage of the viral RNA genome by nucleocapsid protein may also help to resist RNAi attack. The ability of HIV-1 to evade RNAi-mediated targeting of the incoming genome will frustrate therapeutic actions to prevent the establishment of an integrated DNA provirus. In other words, all therapeutic effects will be due to targeting of newly synthesized viral transcripts and inhibition of the production of new virus particles.

Viruses can escape induced RNAi-mediated inhibition by evolving an alternative RNA structure that shields the target sequence (35). This indicates that viruses may use a highly structured RNA as a means to evade RNAi, as has been reported for viroids (102,103). These pathogens consist of small (200-400 nt), noncoding, single stranded circular RNA molecules, with very pronounced secondary structure (104). Most of the viroid RNA genome forms intramolecular base pairs, thus forming a quasi-rod-shaped structure that plays an important role in the viral replication strategy. Although most viroids replicate in the nucleus, they must travel through the cytoplasm to achieve cell-to-cell spread. Therefore, viroids may have evolved their structure-based replication strategy to become inaccessible to RISC and resistant to RNAi silencing (102). As another viral twist to this story, the ds nature of the viroid RNA genome has been proposed to be recognized by the cellular RNAi machinery to produce virus-encoded siRNAs that can target host mRNAs (105), and this virus action may in fact cause disease symptoms (102).

All together, there can be many obstacles for efficient RNAi-mediated inhibition of viral RNAs. This includes the expression of virally encoded suppressor proteins or RNAs, but also more passive hiding strategies. Whether or not these viral strategies have evolved in response to an antiviral RNAi response during natural infection, these strategies must be considered when developing an antiviral RNAi-based therapy. For this, we need to combine recent knowledge on the RNAi pathway with existing, though incomplete knowledge of viral replication mechanisms.

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Summary

Samenvatting

Summary

At present, almost 40 million people worldwide are infected with human immunodeficiency virus type 1 (HIV-1). Due to the availability of a variety of antiretroviral drugs, HIV-1 infection can be controlled effectively in most patients. However, novel therapeutic approaches are being explored since current drug regiments are expensive, not curative, sometimes toxic in the long run, and drug-resistant HIV-1 strains are emerging and spreading in the epidemic. An alternative approach is the use of gene therapy to deliver antiviral genes that interfere with viral replication and thus render the cells resistant to infection. The recent discovery of RNA interference (RNAi), a potent sequence-specific gene regulation mechanism triggered by double stranded RNA, offers a new method to inhibit viral replication. RNAi shows potential as a therapeutic strategy for intracellular immunization against human pathogenic viruses. In this thesis, we describe efficient inhibition of HIV-1 with RNAi and the subsequent viral evasion mechanisms that nullify efficient targeting of the virus.

It has been shown previously that HIV-1 replication can be potently inhibited by transient transfection of small interfering RNAs (siRNAs) targeting either viral RNA sequences or host mRNAs that encode proteins essential for HIV-1 entry or replication. To investigate the long-term inhibition of HIV-1 with RNAi, we stably introduced vectors expressing short hairpin RNAs (shRNAs) directed against various HIV-1 sequences into a human T-cell line through retroviral transduction (**Chapter 2**). The transduced cells were challenged with HIV-1 and virus replication was monitored. HIV-1 replication was efficiently inhibited for more than 8 months in cells stably transduced with an siRNA targeting the Nef gene (siRNA-Nef). However, the block in virus replication is not absolute as escape variants emerged in cultures that were started with a high virus input. Sequence analysis revealed that the RNAi-resistant mutants had acquired nucleotide substitutions or deletions within the siRNA-Nef target sequence of 19 nucleotides. The combined results demonstrate the potency and sequence specificity of RNAi as a gene therapy approach to control a spreading HIV-1 infection, but also the need for modified strategies to prevent viral escape.

We analyzed the spontaneously selected RNAi-resistant HIV-1 variants in more detail (**Chapter 3**). To accurately quantify the level of RNAi resistance, we used reporter constructs containing the siRNA-Nef target sequence. We found an inverse correlation between the level of resistance and the residual stability of the siRNA/target-RNA duplex. Thus, the efficiency of an RNAi reaction depends on the ability of the siRNA to bind the RNA target sequence, for which sequence complementarity is the major contributor. Additionally, we described an intriguing RNAi-resistant HIV-1 variant with a single point mutation outside the 19-

nucleotide target sequence. Further analyses indicated that the acquired mutation alters the secondary RNA structure of the target sequence in such a way that it becomes occluded from binding to the siRNA, thereby reducing the RNAi efficiency. These data provide insight into the impact of target RNA structure on RNAi efficiency and show that HIV-1 can escape from RNAi-mediated inhibition not only through mutations within the siRNA target sequence, but also through mutations -in the target or the flanking residues- that alter the local RNA structure. When selecting a suitable target sequence, its accessibility is thus an important additional factor to take into account.

Several groups have suggested an inhibitory effect of target RNA structure on RNAi efficiency, but the structure-based resistance phenomenon in the HIV-1 variant provides very strong evidence for such an effect. In an additional study (**Chapter 4**), we aimed to obtain a more detailed insight into the impact of target RNA structure on RNAi. We placed an otherwise efficient RNAi target in a perfect hairpin structure, and gradually reduced the stability of the RNA structure, obviously without mutating the actual target sequence. Reporter constructs with the differently structured targets were cotransfected with the siRNA inhibitor. We demonstrated that a target sequence that is located in a perfect hairpin structure is completely resistant to RNAi. Destabilization of the structure improved the susceptibility to RNAi above a thermodynamic stability (ΔG) of approximately -30 kcal/mole. The inverse correlation between target RNA structure and RNAi efficiency is due to improved binding of the siRNA. We next opened different portions of the hairpin structure. We found that an accessible 3' end of the target sequence is most important for RNAi-mediated inhibition, probably due to the contribution of this seed region to the initial siRNA annealing. This *in vivo* effect is not observed in *in vitro* binding studies without RISC, suggesting an active contribution of this protein complex in target site selection. In general, the ΔG rules obtained in this study can be used to optimize expression strategies, e.g. destabilizing shRNA structures, while avoiding RNAi-mediated targeting in lentiviral vectors.

In **Chapter 5**, we experimented with an alternative strategy to deliver anti-HIV shRNAs to HIV-1-susceptible cells. We modified a conditionally replicating HIV-1 variant, which is being developed as a conditional-live virus vaccine, to become an inducible vector for the efficient delivery of anti-HIV shRNAs. This therapeutic virus with an shRNA-expression cassette replicates efficiently in the presence of doxycycline (dox) and the virus spreads to HIV-1-susceptible cells. Subsequent withdrawal of dox results in cells with a transcriptionally silent, but integrated provirus with an active shRNA-expression cassette. We have demonstrated that these cells are resistant to a subsequent HIV-1 challenge. This strategy assures delivery of the anti-HIV shRNAs to exactly those cells that are

susceptible to the pathogenic wild-type virus. This unique antiviral strategy provides a combination of vaccination with a conditional-live virus and intracellular immunization through RNAi.

The virion nucleocapsid of Rous sarcoma virus, another retrovirus, was found to shield the viral RNA genome of virion particles from RNAi-mediated degradation in newly infected cells. However, there are contradicting reports whether the "incoming" HIV-1 genome can be targeted by RNAi. We addressed this issue using HIV-1-based lentiviral transduction as a quantitative model for HIV-1 infection. We measured absolutely no reduction in transduction efficiency when the viral genome was targeted with either stably expressed shRNAs or a variety of transiently transfected shRNA-expression plasmids or synthetic siRNAs (**Chapter 6**). These results indicate that the incoming HIV-1 RNA genome is not targeted by RNAi, probably because it is inaccessible to RISC in the virion nucleocapsid. The ability of HIV-1 to evade RNAi-mediated targeting of the incoming genome is of obvious therapeutic relevance, as it means that proviruses will be established in protected cells.

In **Chapter 7**, we review the array of possible viral escape routes from the RNAi pathway. Obvious strategies are the expression of viral proteins or RNA molecules that suppress the RNAi machinery. The mutational escape from induced RNAi is evident from the experiments presented in this thesis. In addition, there are several viral replication strategies that lead to escape from RNAi, whether intentional or not. For instance, viral RNA genomes can be protected against RNAi in the cytoplasm by extensive secondary RNA structure or the binding of proteins. Other viral tactics include replication in the nucleus away from RISC, or within vesicles in the cytoplasm.

The development of a gene therapy approach using RNAi against HIV-1 is still attractive. Mutational escape from RNAi, due to the extreme mutation rate of this virus, may be circumvented by simultaneous targeting of conserved sequences at multiple locations in the HIV-1 genome. This combination-siRNA therapy will not only boost the efficiency of inhibition, but significantly raise the genetic threshold for viral escape. Since we have shown that the incoming viral genome is not targeted, one could also target cellular receptors or cofactors that are critically involved in the initial phase of HIV-1 infection.

Samenvatting

Momenteel zijn bijna 40 miljoen mensen wereldwijd geïnfecteerd met het humaan immunodeficiëntie virus (HIV), de veroorzaker van AIDS (Acquired Immune Deficiency Syndrome). Door de ontwikkeling en beschikbaarheid van verschillende antiretrovirale middelen kan een HIV infectie in de meeste patiënten effectief onder controle worden gehouden. De huidige medicijncocktails zijn echter kostbaar en soms op de lange termijn toxisch voor de patiënt. Verder zijn de middelen niet genezend en verschijnen er steeds vaker varianten in de virus populatie die resistent zijn tegen de huidige therapieën. Daarom wordt er nog veel onderzoek gedaan naar nieuwe therapeutische strategieën. Eén van de alternatieve benaderingen is het gebruik van gentherapie om antivirale genen in de cel af te leveren de die virusreplicatie remmen en zodoende cellen resistent maken tegen infectie. De recente ontdekking van RNA interferentie (RNAi), een zeer potent sequentiespecifiek genregulatie mechanisme dat geactiveerd wordt door dubbelstrengs RNA, biedt een nieuwe methode om virusreplicatie te remmen. RNAi toont potentie als therapeutische strategie voor intracellulaire immunisering tegen humane pathogene virussen. In dit proefschrift beschrijven wij de efficiënte remming van HIV door RNAi en de daaropvolgende virale mechanismen om aan de geïnduceerde remming te ontkomen.

In **Hoofdstuk 1** wordt de opbouw en structuur van een HIV deeltje en de HIV replicatiecyclus beschreven. Vervolgens worden het RNAi mechanisme en de componenten ervan geïntroduceerd. De meest belangrijke hiervan zijn de effectieve moleculen van het RNAi mechanisme, genaamd "small interfering RNAs" (siRNAs) en het "RNA-induced silencing complex" (RISC). De laatste is een eiwitcomplex waarin een siRNA wordt opgenomen waarna het complex op zoek gaat naar het bijpassende mRNA in een cel. Wanneer dit mRNA gevonden en gebonden is, wordt het geknipt en dus geïnactiveerd, waardoor het mRNA niet meer kan zorgen voor eiwitproductie.

De siRNAs kunnen door middel van transfectie efficiënt in cellen gebracht worden. Hun aanwezigheid in de cel is dan van tijdelijke aard. Men heeft eerder aangetoond dat de replicatie van HIV sterk geremd kan worden door siRNAs gericht tegen virale RNA sequenties. Om remming van HIV door middel van RNAi op lange termijn te onderzoeken hebben we vectoren, die anti-HIV siRNAs tot expressie brengen, stabiel in een humane T cellijn geïntroduceerd met behulp van retrovirale transductie (**Hoofdstuk 2**). De getransduceerde cellen werden blootgesteld aan HIV, waarna de virusreplicatie werd gecontroleerd. HIV replicatie werd langer dan 8 maanden efficiënt geremd in cellen die stabiel getransduceerd waren met een siRNA gericht tegen het virale Nef gen (siRNA-Nef). Deze remming van virusreplicatie is echter niet absoluut, aangezien

ontsnappingsvarianten geselecteerd werden in cultures die gestart waren met een hoge virus input. Sequentieanalyse onthulde dat de RNAi-resistente mutanten ofwel nucleotide (nt) substituties, ofwel deleties binnen 19-nt de siRNA-Nef targetsequentie hadden verworven. Deze resultaten demonstreren de potentie en sequentiespecificiteit van RNAi als gentherapie strategie om een HIV infectie te remmen, maar ook de behoefte aan aanpassingen in deze benadering om virale ontsnapping te verhinderen.

We hebben de spontaan geselecteerde RNAi-resistente HIV varianten in meer detail onderzocht (**Hoofdstuk 3**). Om de mate van de RNAi resistentie nauwkeurig te kwantificeren hebben we reporter constructen gebruikt die de siRNA-Nef targetsequentie bevatten. We vonden een omgekeerde correlatie tussen het niveau van de resistentie en de sterkte van de binding tussen de siRNA en het target RNA. De efficiëntie van een RNAi reactie hangt dus af van de capaciteit van de siRNA om de RNA targetsequentie te binden, wat voor een groot deel bepaald wordt door de sequentie complementariteit. We beschrijven bovendien een interessante RNAi-resistente HIV variant met een enkele puntmutatie buiten de 19-nt targetsequentie. Uitgebreide analyses wezen erop dat de verworven mutatie de secundaire structuur van de targetsequentie zodanig verandert dat het afgeschermd wordt voor binding aan de siRNA, waardoor de RNAi efficiëntie verminderd wordt. Deze resultaten geven informatie over het effect van target RNA structuur op RNAi efficiëntie. Ze tonen aan dat HIV niet alleen aan RNAi-remming kan ontsnappen door mutaties binnen de targetsequentie, maar ook door mutaties in de target, of daarbuiten, die de locale RNA structuur veranderen. Bij het selecteren van een geschikte targetsequentie is de toegankelijkheid van het RNA dus een belangrijke factor om rekening mee te houden.

Verschillende researchgroepen hebben een remmend effect van de target RNA structuur op RNAi efficiëntie geopperd en de op structuur gebaseerde resistentie van de HIV variant levert zeer sterk bewijs voor een dergelijk effect. In een volgende studie (**Hoofdstuk 4**), hebben wij geprobeerd een meer gedetailleerd inzicht te krijgen op de invloed van de target RNA structuur op RNAi efficiëntie. We plaatsten hiervoor een normaliter efficiënte RNAi target sequentie in een perfecte haarspeldstructuur en verminderden geleidelijk de stabiliteit van deze RNA structuur, zonder de targetsequentie zelf te muteren. Reporter constructen met de verschillende gestructureerde targets werden samen met de remmende siRNA in cellen getransfecteerd. Wij laten zien dat een targetsequentie, die aanwezig is in een perfecte haarspeldstructuur, volledig resistent is tegen RNAi. Destabilisatie van de structuur verbeterde de gevoeligheid voor RNAi boven een thermodynamische stabiliteit (ΔG) van ongeveer -30 kcal/mol. De omgekeerde correlatie tussen de target RNA structuur en RNAi efficiëntie is toe te schrijven aan een betere binding aan de siRNA. Vervolgens hebben we de target

haarspeldstructuur op gerichte posities gedestabiliseerd. Wij zagen dat een toegankelijk 3′ einde van de targetsequentie het meest belangrijk is voor het RNAi mechanisme, waarschijnlijk door de bijdrage van deze targetregio aan de initiële binding van de siRNA. Dit *in vivo* effect werd niet waargenomen in de *in vitro* bindingsstudies zonder de aanwezigheid van RISC, wat een actieve bijdrage van dit eiwitcomplex tijdens target binding suggereert. In het algemeen kunnen de hier verkregen ΔG regels gebruikt worden om de expressie van haarspelden in lentivirale vectoren te optimaliseren d.m.v. destabilisatie van de RNA structuur, terwijl de haarspeld zelf geen efficiënte target wordt voor inhibitie door RNAi.

In **Hoofdstuk 5** laten we experimenten zien met een alternatieve strategie om anti-HIV siRNAs in cellen te brengen die vatbaar zijn voor HIV infectie. We modificeerden een conditioneel replicerende HIV variant, die in ontwikkeling is als een conditioneel-levend virusvaccin, om een induceerbare vector te krijgen voor het effectief leveren van anti-HIV siRNAs. Dit therapeutische virus met een siRNA expressiecassette repliceert efficiënt in de aanwezigheid van doxycycline (dox) en verspreidt zich naar HIV-vatbare cellen. Het vervolgens ontnemen van dox resulteert in cellen met een geïntegreerd, maar transcriptioneel inactief provirus met een actieve siRNA expressiecassette. Wij hebben aangetoond dat deze cellen resistent zijn tegen blootstelling aan HIV. Deze strategie verzekert levering van anti-HIV siRNAs aan precies die cellen die vatbaar zijn voor het originele pathogene HIV virus. Deze unieke antivirale strategie verschaft een combinatie van vaccinatie met een conditioneel-levend virus en intracellulaire immunisatie door RNAi.

Het nucleocapside van het Rous sarcoma virus, een ander retrovirus, bleek het virale RNA genoom van virusdeeltjes in geïnfecteerde cellen te beschermen tegen degradatie door RNAi. Of hetzelfde opgaat voor het "binnenkomende" HIV genoom bestaan tegengestelde verslagen. Wij hebben deze kwestie onderzocht d.m.v. lentivirale transductie als kwantitatief model voor HIV infectie. Wij hebben absoluut geen vermindering van transductie-efficiëntie gemeten in cellen die ofwel stabiel siRNAs tot expressie brachten, ofwel transiënt getransfecteerd waren met siRNAs gericht tegen het virale genoom (**Hoofdstuk 6**). Deze resultaten wijzen erop dat RNAi remming zich niet richt tegen het binnenkomende HIV RNA genoom, waarschijnlijk omdat het omhullende nucleocapside ontoegankelijk is voor RISC. De capaciteit van HIV om RNAi remming gericht tegen het RNA genoom te ontwijken is therapeutisch zeer relevant, aangezien het betekent dat provirussen zich toch kunnen vestigen in het genoom van RNAi-beschermde cellen.

In **Hoofdstuk 7** bespreken we een reeks van mogelijke virale manieren om aan het RNAi mechanisme te ontsnappen. Voorbeelden van duidelijke strategieën zijn de expressie van virale eiwitten of RNA moleculen die de RNAi mechaniek

kunnen onderdrukken. Het ontsnappen aan geïnduceerde RNAi remming door middel van mutaties is ook duidelijk, zoals gevonden in de experimenten beschreven in dit proefschrift. Bovendien zijn er verscheidene virale replicatie-strategieën die, al dan niet opzettelijk, het ontwijken van RNAi remming tot gevolg hebben. Zo kunnen virale RNA genomen in het cytoplasma beschermd worden tegen RNAi door een extensieve secundaire RNA structuur of door binding van eiwitten. Andere virale tactieken zijn virusreplicatie in de celkern of in vesicles in het cytoplasma, zodat het virale RNA afgeschermd wordt voor RISC.

De ontwikkeling van een gentherapie strategie met RNAi gericht tegen HIV blijft aantrekkelijk. Ontsnapping van het virus aan RNAi door mutaties, vanwege de extreme mutagene eigenschap van HIV, kan wellicht omzeild worden door gelijktijdig siRNAs gericht tegen meerdere geconserveerde sequenties, verspreid over het HIV genoom, te gebruiken. Dergelijke combinatie siRNA-therapie zal niet alleen de efficiëntie van de RNAi remming, maar ook de genetische drempel voor virale ontsnapping verhogen. Aangezien wij hebben aangetoond dat het binnenkomende virale RNA genoom ongevoelig is voor RNAi, zou men wellicht siRNA gericht tegen cellulaire receptoren of co-factoren kunnen gebruiken die noodzakelijk zijn voor de initiële fase van HIV infectie.