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ORIGINAL ARTICLE

Inhibition of human immunodeficiency virus type 1 by RNA interference using long-hairpin RNA

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Inhibition of virus replication by means of RNA interference has been reported for several important human pathogens, including human immunodeficiency virus type 1 (HIV-1). RNA interference against these pathogens has been accomplished by introduction of virus-specific synthetic small interfering RNAs (siRNAs) or DNA constructs encoding short-hairpin RNAs (shRNAs). Their use as therapeutic antiviral against HIV-1 is limited, because of the emergence of viral escape mutants. In order to solve this durability problem, we tested DNA constructs encoding virus-specific long-hairpin RNAs (lhRNAs) for their ability to inhibit HIV-1 production. Expression of lhRNAs in mammalian cells may result in the synthesis of many siRNAs targeting different viral sequences, thus providing more potent inhibition and reducing the chance of viral escape. The lhRNA constructs were compared with in vitro *diced double-stranded RNA and a DNA construct encoding an effective* nef*-specific shRNA for their ability to inhibit HIV-1 production in cells. Our results show that DNA constructs encoding virus-specific lhRNAs are capable of inhibiting HIV-1 production in a sequence-specific manner, without inducing the class I interferon genes.*
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Introduction

RNA silencing or RNA interference (RNAi) is an evolutionary conserved sequence-specific post-transcriptional gene regulation mechanism that plays an important role in cell differentiation and development.[1–3] In addition, RNAi serves as a defence mechanism against invading viruses and transposons.[4–6] RNA interference is triggered by double-stranded RNA (dsRNA) molecules, which are processed in the cytoplasm by the dsRNA-specific endonuclease Dicer into 19–24 nucleotides (nt) small interfering RNAs (siRNAs) or micro-RNAs (miRNAs).[7] These si/miRNAs are incorporated into the multiprotein RNA-induced silencing complex (RISC) that guides the recognition and ultimately the cleavage or translational repression of complementary single-stranded RNA, such as messenger RNA or viral genomic RNA.[8–10]

RNA interference has been employed to inhibit the replication of a wide range of viruses, including the human immunodeficiency virus type 1 (HIV-1), hepatitis C virus (HCV), hepatitis B virus (HBV), dengue virus, poliovirus, influenza virus A, coronaviruses, herpesviruses and picornaviruses.[11] Human immunodeficiency virus type 1 virions contain a single-stranded RNA genome that is a putative RNAi target. After entry into a host cell the genomic RNA is reverse transcribed into dsDNA, which is integrated into the host chromosomal DNA. Newly synthesized unspliced genome-length and spliced subgenomic viral RNAs are possible targets for RNAi in the cytoplasm. It has recently been reported that HIV-1 encodes a suppressor of RNAi, the Tat protein, indicating that HIV-1 replication is controlled by RNAi in human cells.[12] Due to its sequence specificity, RNAi is a potentially powerful and selective method for intracellular immunization against HIV-1 infection. RNA interference-mediated suppression of HIV-1 replication has been accomplished by synthetic siRNAs in a transient manner[13–16] and by short-hairpin RNA (shRNA)-expression vectors in stably transfected cells.[17–19] Despite potent inhibition, the use of siRNA/shRNA as a therapeutic antiviral is limited, because of the rapid emergence of HIV-1 escape mutants.[19–21] Minor sequence changes in the target sequence, sometimes even a single point mutation, are sufficient to abrogate the inhibition of virus replication. Strategies to reduce the chance of viral escape include the simultaneous use of multiple siRNAs[22,23] or the use of long-hairpin RNA (lhRNA, a single-hairpin molecule) or long dsRNA (two complementary molecules that form a duplex).[16,24] Another possibility is the use of miRNA-based approaches, which do not require perfect sequence complementarity.[25,26]

Several reports describe efficient RNAi induction by lhRNA and long dsRNA as *in vitro* generated transcripts that are transfected into cells or as gene constructs that produce the transcripts intracellularly. Transfection of pre-implantation mouse embryo cells, undifferentiated

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embryonic stem cells and embryonic carcinoma cells with *in vitro* synthesized long dsRNA confers specific gene silencing.[27,28] However, exposure of non-embryonic mammalian cells to dsRNAs longer than 30 basepairs (bp) leads to rapid induction of a specific set of cytokines, including the class I interferons (IFNs).[29] During natural virus infections, the IFN response is activated by virus-produced dsRNAs, and acts as an innate defense mechanism. Viruses counter this response by encoding IFN antagonists, which are also responsible for the fact that antiviral IFN therapy is often not successful.[30,31] So far, virus-encoded RNAi suppressor factors, like the HIV-1 Tat protein, do not appear to be able to suppress induced antiviral RNAi. Strong induction of RNAi by intracellular expression of virus-specific dsRNAs is likely to outcompete the inhibiting effects of RNAi suppressors.

Efficient RNAi-mediated gene silencing has been shown in mammalian cells by endogenously expressed long dsRNAs.[28,32,33] In Chinese hamster ovary (CHO) cells, a DNA construct encoding a 700 bp long dsRNA specifically inhibits luciferase expression in a sequence-specific manner.[34] Complete and specific gene silencing was achieved in different mammalian cell types by expression of 500, 800, or even 1000 bp long dsRNAs.[32,35–37] Interestingly, intact dsRNA could not be detected in these cells, suggesting that it is rapidly processed by Dicer in the cytoplasm. Recently, Ski knockdown mice have been produced using a DNA construct encoding long dsRNA-specific for the murine Ski gene.[38] These results suggest that dsRNA is tolerated in mammalian cells, most likely because it is rapidly processed by the RNAi machinery.

Several antiviral approaches using extended dsRNA have been reported in plant and insect cells lacking the innate antiviral IFN response. Although plants and insects lack the IFN response, they also have potent innate antiviral responses, comparable to those in mammals.[39] Transient expression of DNA constructs encoding virus-specific dsRNA in plant protoplasts or insect cells partially protects the cells from infection by the homologous virus.[40,41] Stable expression of such constructs in plant or insect cells renders the cells completely resistant or immune to infection.[42,43] *In vitro* made long dsRNAs have been used to inhibit HIV-1 production under certain conditions without induction of the IFN response.[16,24] We have previously demonstrated potent inhibition of HIV-1 replication in T cells that stably express an shRNA targeted to viral *nef* gene sequences.[19] To test whether endogenously expressed lhRNA and long dsRNA can inhibit HIV-1 at least as potently as sh-*nef*, we constructed and tested a series of DNA constructs.

Results

RNA interference targets in the human immunodeficiency virus type 1 genome

It has previously been demonstrated that HIV-1 replication can be inhibited by siRNAs and shRNAs directed against viral targets.[11,14,15,18,19,44] Most of the active siRNAs against HIV-1 are targeted to the early regulatory *tat*, *rev* and *nef* genes.[19,20,45,46] Interference with an early stage of the HIV-1 replication cycle may be beneficial. For this reason, the DNA constructs encoding lhRNAs (a single-hairpin molecule) and long dsRNAs (two complementary molecules that form a duplex) were designed to target *tat*, *rev* and *nef* sequences as indicated in Figure 1.

Inhibition of human immunodeficiency virus type 1 by in vitro *transcribed ds-*nef2 *RNA and its diced product*

We initially tested whether *in vitro* transcribed and annealed *nef2* dsRNA and its *in vitro* diced product si-*nef2*, a mixture of *nef*-specific siRNAs, can inhibit HIV-1. *Nef2* dsRNA of 300 bp was diced *in vitro* to create si-*nef2* RNAs of approximately 21 bp (Figure 2a). We cotransfected 500 ng of the HIV-1 molecular clone pLAI with and without 10 ng inhibitory RNA in human embryonic kidney (HEK) 293T cells. DNA of pRL expressing Renilla luciferase was included in the transfection mixtures to monitor cell viability and possible non-specific effects, for example, due to IFN induction by dsRNA. Virus production was measured by CA-p24 enzyme-linked immunosorbent assay (ELISA) in the culture supernatant 3 days after transfection. The amount of virus production without an inhibitory RNA, generally in the 50–250 ng/ml CA-p24 range, was set at 100%. *nef2* dsRNA induced a significant decrease in CA-p24 production, but even more pronounced level of inhibition was obtained with diced si-*nef2* (Figure 2b). This can be explained by the fact that si-*nef2* bypasses the intracellular dicing step, which may be a limiting factor in the RNAi pathway.

One of the hallmarks of the RNAi is its sequence specificity. Therefore, we tested if *nef2* dsRNA and its *in vitro* diced product si-*nef2* would inhibit pGL3-Nef reporter, in which 250 nt from the *nef2* target sequence was placed downstream of the luciferase reporter gene.[21] *nef2* dsRNA induced a decrease in luciferase expression, but an even more pronounced level of inhibition was

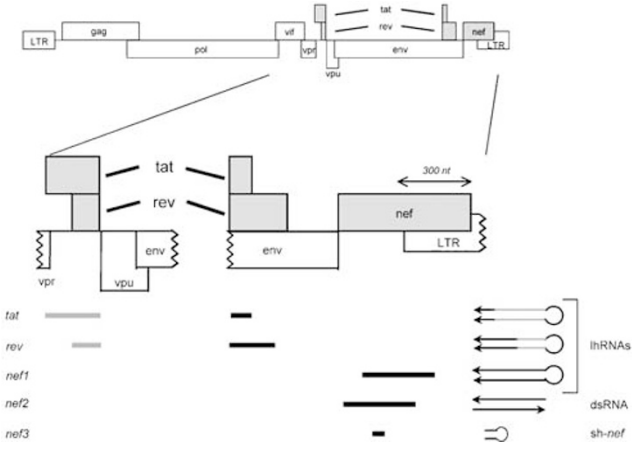

Figure 1 Scheme of the human immunodeficiency virus type 1 (HIV-1) pLAI proviral genome and target sequences used for the design of long-hairpin RNAs (lhRNAs). The target sequences are indicated as bars below the HIV-1 coding regions. lhRNA (300 basepairs (bp)) *tat* fuses *tat* exon 1 (gray bar, 5422–5626) and *tat* exon 2 (black bar, 7972–8017) sequences, *rev* fuses *rev* exon 1 (gray bar, 5562–5626) and *rev* exon 2 (black bar, 7972–8206) and *nef1* contains *nef*-LTR sequences (8519–8818). Double-stranded RNA *nef2* is a duplex of two separate, complementary sense and antisense *nef* sequences (8416–8695). The positive control sh-*nef* is a 21-bp hairpin consisting of *nef* sequences (8552–8571).[19]

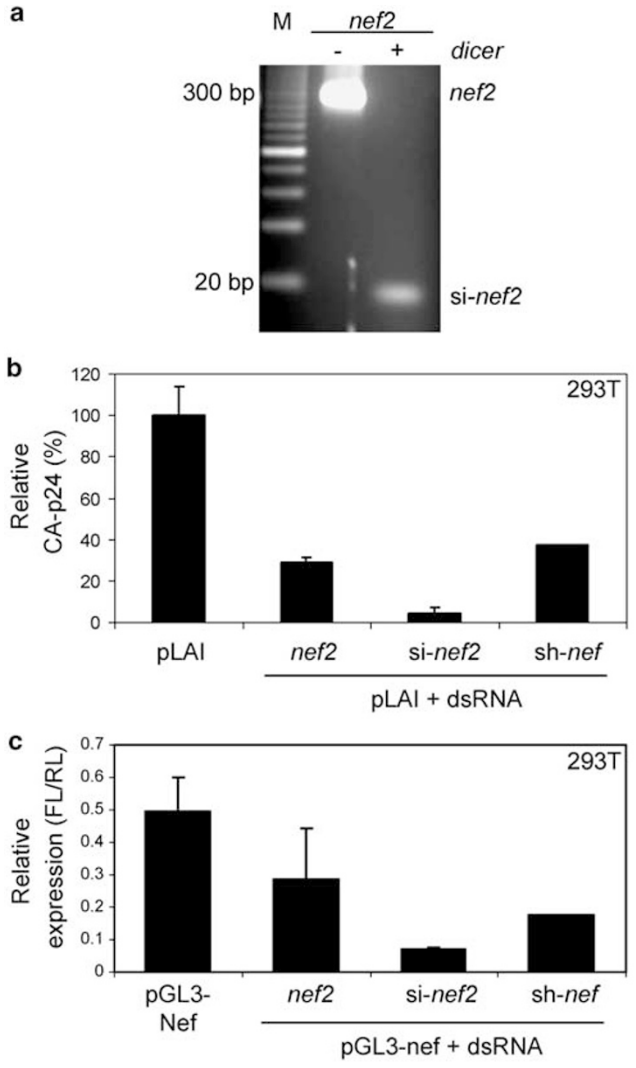

Figure 2 Inhibition of human immunodeficiency virus type 1 (HIV-1) by *in vitro* transcribed *nef2* double-stranded RNA (dsRNA), *in vitro* diced si-*nef2* and sh-*nef* RNA. (**a**) 2% MetaPhor agarose gel stained with ethidium bromide, showing the 300 basepairs (bp) *nef2* dsRNA and the diced 19 bp si-*nef2* products; M, 20 bp marker. (**b**) Cotransfection of human embryonic kidney (HEK) 293T cells with 10 ng of the indicated RNA, 500 ng pLAI and 2.5 ng pRL as an internal control. Transfections were performed with Lipofectamine 2000 and 1.5×10^5 cells. Virus production was measured in the culture supernatant 2 days after transfection. CA-p24 values are given as percentage of the pLAI production without inhibitory RNA. Standard error bars represent the means of four independent experiments. (**c**) Cotransfection of HEK 293T cells with 10 ng of the indicated dsRNA, 100 ng pGL3-Nef and 2.5 ng pRL as an internal control. Transfections were performed as described above. After 72 h cells were lysed and firefly and renilla luciferase expression was measured.

obtained with diced si-*nef2* (Figure 2c). The pRL expression was not influenced (results not shown). In fact, *in vitro* diced si-*nef2* is a much more effective inhibitor than the *in vitro* synthesized short-hairpin inhibitor sh-*nef* RNA, which was included as a positive control. We next tested the inhibitory potential of *nef2* dsRNA in the 1–1000 ng range. At amounts above 10 ng, non-specific decrease of *Renilla reniformis* luciferase (RL) expression was observed, which is most likely due to IFN induction (results not shown, see also Figure 8). The high level of inhibition obtained with low amounts of dsRNA convinced us to design and test a series of DNA expression plasmids encoding long HIV-1-specific dsRNAs.

Low-level inhibition of human immunodeficiency virus type 1 by long-hairpin RNA expression plasmids

To make lhRNA constructs, we cloned the HIV-1 *tat*, *rev* and *nef* gene sequences as inverted repeats under the transcriptional control of the constitutive EF1α promoter (Figure 3). These vectors should produce lhRNA, a long-hairpin structure consisting of an approximately 300 bp stem and a 46 nt loop. During transcription of the inverted sequences, an RNA molecule is made, which folds back on itself to form a hairpin structure with a stem of approximately 300 bp. *In silico* RNA analysis with the Mfold program[47] confirms the folding of these extended hairpins (data not shown). We tested inhibition of HIV-1 in several mammalian cell lines (C33A, HEK 293T and Vero). Cotransfection of pLAI with the pEF1α-*tat*, pEF1α-*rev* or pEF1α-*nef1* vectors resulted in marginal inhibition of HIV-1 production in C33A and HEK 293T cells, and only the pEF1α-*tat* vector was inhibitory in Vero cells (Figure 4a).

We also designed a control pEF1α-green fluorescent protein (GFP) plasmid that expresses a similar extended lhRNA against GFP mRNA. No inhibition of virus production was observed with the control pEF1α-GFP vector, thus providing additional evidence for the specificity of lhRNA-mediated inhibition of HIV-1 production. We previously demonstrated potent inhibition of HIV-1 replication in T cells that stably express an shRNA targeted to viral *nef* gene sequences.[19] Therefore, if lhRNA inhibits HIV-1 potently, it should be at least as active as the sh-*nef* control. Unlike the results with *in vitro* synthesized *nef2* dsRNA, either diced or not, all lhRNA constructs (*tat*, *rev* and *nef1*) were much less potent inhibitors than the control sh-*nef* construct, which produces the short hairpin from a polymerase III promoter. Because pEF1α-*tat* and pEF1α-*nef1* were slightly more effective than pEF1α-*rev* in C33A and 293T cells, we focused on these lhRNAs in the subsequent experiments.

Inducible long-hairpin RNA expression

In order to avoid innate viral responses or possible other side effects and to obtain controllable expression of lhRNA, we placed the expression of the lhRNAs *tat* and *nef1* under control of inducible promoters: (i) the doxycycline (dox) inducible Tet system[48] and (ii) the Tat-inducible long-terminal repeat (LTR) promoter/enhancer of HIV-1[49] (Figure 3). The latter system seems ideally suited to restrict lhRNA expression to cells that are infected by HIV-1, thus providing a unique safety feature. Furthermore, we replaced the 46 bp spacer between the repeats by the 1000 bp EF-1α intron, since it has been shown that this improves the inhibitory potential of lhRNA-encoding DNA constructs.[50]

The Tet-On system is based on the specific, high-affinity binding of the rtTA trans-activator to the tet operator (tetO) in the presence of dox, triggering transcription of downstream genes. This system has recently been used to express synthetic miRNA precursors in mouse or human genomes, following trans-

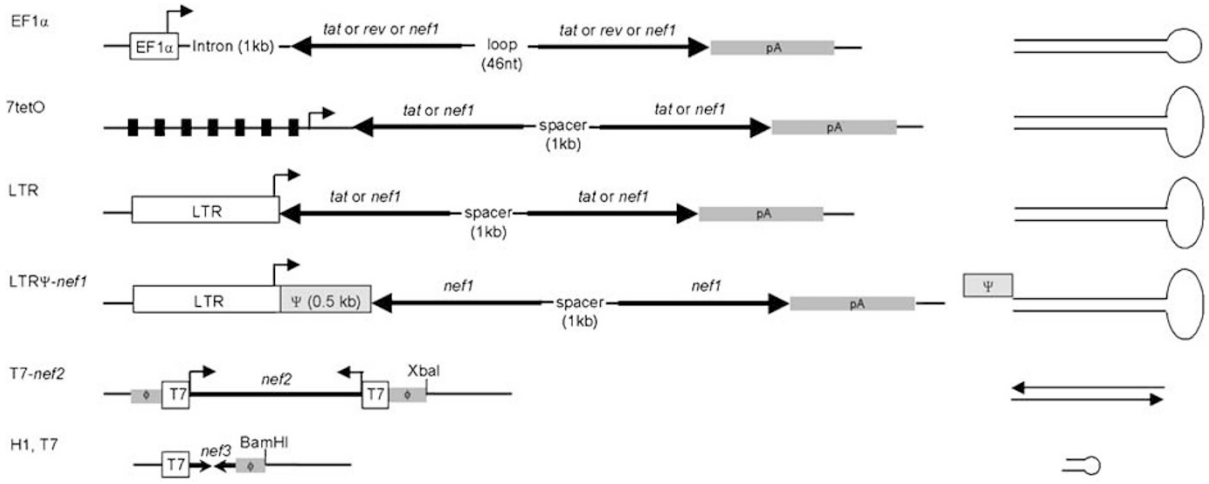

Figure 3 Expression vectors for long-hairpin RNA (lhRNA), double-stranded RNA (dsRNA) and short-hairpin RNA (shRNA). Long-hairpin RNAs were created by cloning the 300-nucleotide (nt) inverted repeats from *tat*, *rev* and *nef1* (see Figure 1) downstream of the EF1α, 7tetO or long-terminal repeat (LTR) promoters. The 1 kb EF1α intron is positioned downstream of the EF1α promoter. A schematic representation of the final hairpin structures is shown on the right. In pEF1α constructs, the two complementary RNA strands are separated by a 46 nt loop. In the p7tetO and pLTR constructs, the complementary sequences are separated by a 1 kb spacer that contains splice donor and acceptor sites. Vector pLTRΨ-*nef1* is a derivative of pLTR-*nef1* in which the human immunodeficiency virus type 1 (HIV-1) leader sequence (Ψ, 76–630, marked as a gray box) was inserted. The predicted transcript will have the Ψ domain upstream of the RNA hairpin. All transcripts contain a polyadenylation signal (pA) downstream of the hairpin sequences. Vector pT7-*nef2* has 300 basepairs (bp) long double-stranded *nef* sequences flanked by T7 promoters (T7) and terminators (ϕ) at both 5′ and 3′ ends. Two separate complementary RNA chains, potentially capable of forming dsRNA, are transcribed from the convergent promoters by T7 RNA polymerase (encoded by expression plasmid pT7-pol). Vectors pT7sh-*nef* and pH1sh-*nef* express sh-*nef* from the T7 and H1 promoters, respectively.

duction with retroviral or lentiviral vectors.[51] Vector pLAI was cotransfected with p7tetO-*tat* or p7tetO-*nef1* (Figure 3). We also included pCMV-rtTA and the control pRL, and dox was added 4 h later. p7tetO-*tat* or p7tetO-*nef1* conferred no or very poor inhibition of HIV-1 as compared to the sh-*nef* control (Figure 4b). The potency of the lhRNAs was not increased by varying the ratio of lhRNA-expression vector to pCMV-rtTA or the dox concentration. Moreover, a non-specific decrease of RL expression was observed at higher pCMV-rtTA concentrations, which is probably due to promoter squelching (results not shown).

In order to restrict lhRNA expression to cells that are infected with HIV-1, the inverted *tat* and *nef1* repeats were cloned downstream of the Tat-inducible LTR promoter/enhancer of HIV-1, resulting in plasmids pLTR-*tat* and pLTR-*nef1* (Figure 3). A Tat-inducible Pol II promoter expressing anti-HIV shRNA has been described for inhibition of HIV-1 gene expression in mammalian cells.[52] In this setting, lhRNA or shRNA expression is activated *in trans* by the Tat protein encoded by HIV-1. To avoid self-targeting, we deleted a large part of the U3 region (up to position −179) in the LTR promoter of both LTR expression plasmids that overlaps with the *nef* coding domain (323 nt). Human embryonic kidney 293T cells were cotransfected with pLAI and pLTR-*tat* or pLTR-*nef1*. Both expression plasmids inhibit virus production by approximately 60% (Figure 5a).

Directing long-hairpin RNA along viral pathways

The poor inhibitory potency of the different lhRNA constructs could be due to expression problems, but the lhRNA may also encounter difficulties in entering or proceeding along the RNAi pathway. In order to increase the efficacy of the lhRNA molecules, we cloned the HIV-1 leader sequence (Ψ) between the LTR and *nef1* in pLTRΨ-*nef1* (Figure 3). Control plasmid pLTRasΨ-*nef1* was made with the Ψ element inserted in antisense orientation. Previously, we reported that sequences from the 5′ untranslated leader of the HIV-1 genome, such as the RNA dimerization signal, can be used to inhibit HIV expression in *trans*.[53] We presumed that Ψ should bring the antiviral RNA along viral pathways, conferring a stronger inhibitory effect. Indeed, in HEK 293T cells transfected with pLAI and pLTRΨ-*nef1*, HIV-1 production was almost completely inhibited (Figure 5a). The level of inhibition conferred by pLTRΨ-*nef*1 is comparable to that of the positive control sh-*nef*. Inhibition is specific and not due to a more general cell toxicity problem because no significant decrease in RL expression was observed (results not shown). Control plasmid pLTRasΨ-*nef1* was much less effective in inhibiting HIV-1 production, with a potency comparable to that of the original pLTR-*nef1* construct. Plasmid pLTRΨ, in which the *nef1* sequence has been deleted, failed to inhibit HIV-1 production (Figure 5b). This result indicates that the presence of the Ψ element enhances lh-*nef1*-mediated inhibition of HIV-1, but the presence of the *nef1* sequence is essential for the potent effect of pLTRΨ-*nef1*.

Transcript Ψ-*nef1* expression from the LTR promoter is induced by pLAI-encoded Tat protein. Because pLAI gene expression is strongly inhibited by pLTRΨ-*nef1*, a negative feedback loop may have been established, which leads to an underestimation of the inhibitory potential of pLTRΨ-*nef1*. We therefore added a Tat-expression plasmid (pcDNA3-Tat) in *trans* to secure pLTRΨ-*nef1* expression. Human embryonic kidney 293T cells were transfected with 100 ng pLAI and 10–100 ng pLTRΨ-*nef1* with or without pcDNA3-Tat. As shown in Figure 5c, pronounced HIV-1 inhibition was obtained with as little as 10 ng pLTRΨ-*nef1*. The presence of

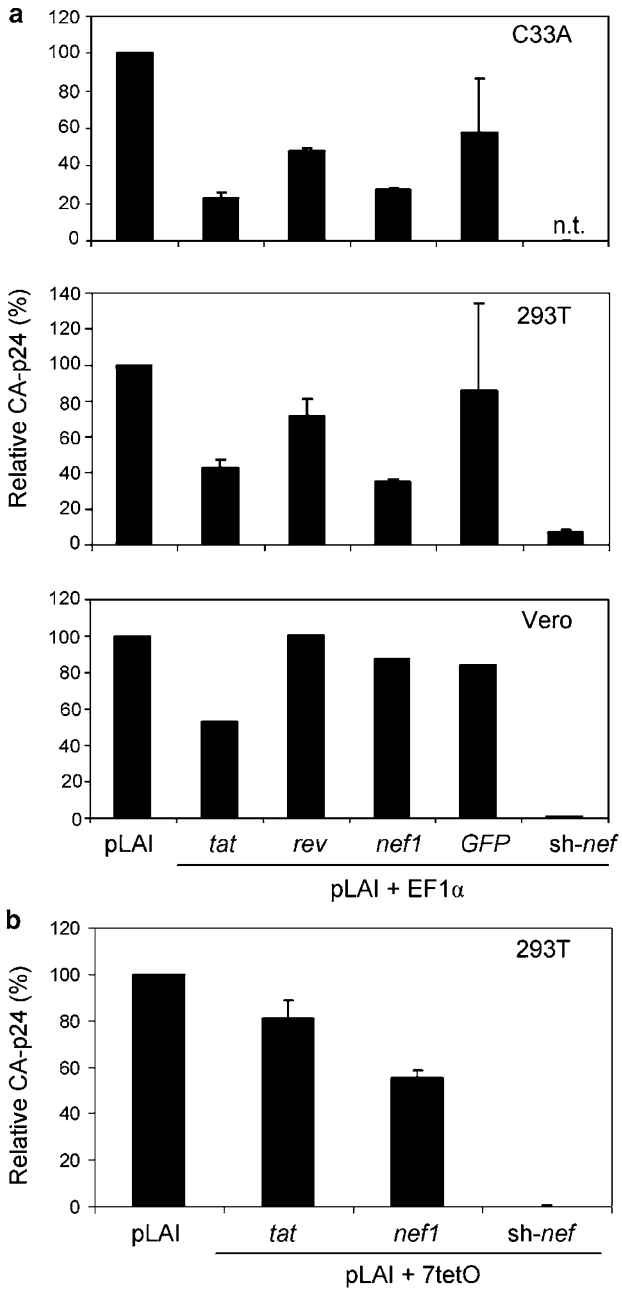

Figure 4 Marginal inhibition of human immunodeficiency virus type 1 (HIV-1) production by pEF1α- and p7tetO-driven long-hairpin RNA (lhRNA) constructs. (**a**) Cells (C33A, human embryonic kidney (HEK) 239T and Vero) were lipofectamine-transfected with 500 ng pLAI, 500 ng inhibitory construct, 3 ng pCMV-rtTA and 2.5 ng pRL. Vector pEF1α-green fluorescent protein (GFP) was used as a control expressing an irrelevant lhRNA against GFP. Vectors pH1sh-*nef* and the empty vector were used as negative and positive controls, respectively. Virus production was determined as described in the legend to Figure 2. Standard error bars represent the means of three independent experiments. The sh-*nef* control construct was not tested (n.t.) in C33A cells. (**b**) HEK 239T cells were cotransfected with 100 ng pLAI, 100 ng p7tetO-*tat*, p7tetO-*nef1* or pH1sh-*nef*, 3 ng pCMV-rtTA and 2.5 ng pRL as an internal control. Culture medium was refreshed after 16 h and 1 μg/ml doxycycline was added. Virus production was determined as described in the legend to Figure 2. Standard error bars represent the means of four independent experiments.

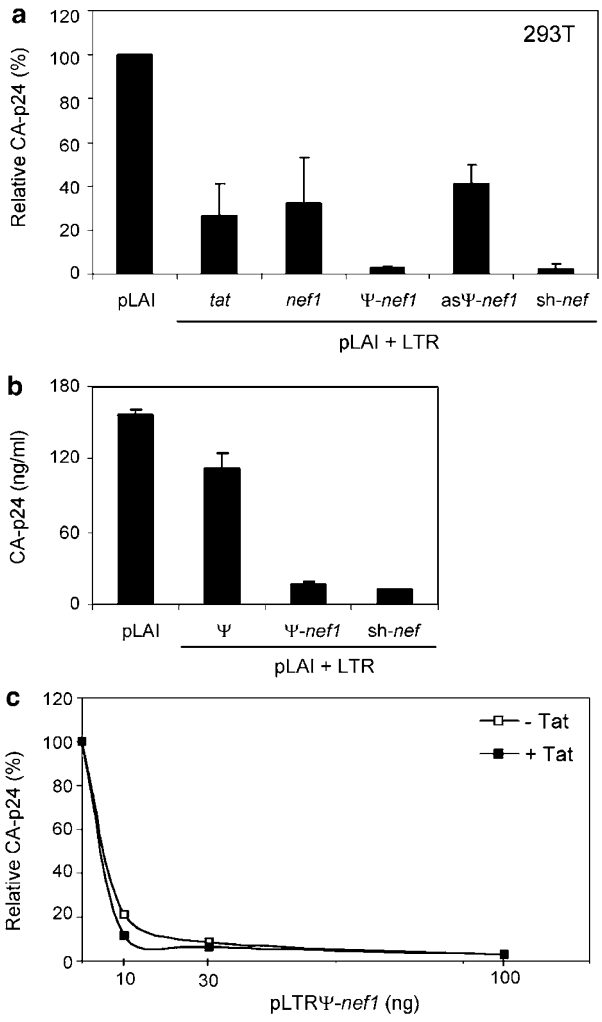

Figure 5 Antiviral long-hairpin RNA (lhRNA) production from the human immunodeficiency virus (HIV)-inducible long-terminal repeat (LTR) promoter. (**a**) Inhibition of HIV-1 production by lhRNA expressed from the Tat-inducible HIV-1 LTR. Human embryonic kidney (HEK) 239T cells were cotransfected with 100 ng of pLAI and 100 ng pLTR-*tat*, pLTR-*nef1*, pLTRΨ-*nef1* or pLTRasΨ-*nef1*. Equal amounts of a pH1sh-*nef* expression vector and the empty vector were added as positive and negative controls, respectively. (**b**) Sequence-specific inhibition of HIV-1 production by pLTRΨ-*nef1*. HEK 239T cells were cotransfected with 100 ng of pLAI and 10 ng pLTRΨ, pLTRΨ-*nef1* or pH1sh-*nef*. On the *y*-axis, the CA-p24 values (ng/ml) are presented. (**c**) Effect of the trans-activator protein Tat on the inhibitory effect of pLTRΨ-*nef1*. pLAI (100 ng) was cotransfected with 0–10–30–100 ng pLTRΨ-*nef1* with or without 20 ng pcDNA3-Tat. Virus production was determined as described in the legend to Figure 2. Standard error bars represent the means of five independent experiments.

additional Tat did not significantly improve the inhibition conferred by pLTRΨ-*nef1*, indicating that the background LTR promoter activity produces sufficient amounts of the inhibitory transcript.

We next wanted to test if the potent pLTRΨ-*nef1* construct was able to inhibit HIV-1 variants that escaped from the sh-*nef* inhibitor. We described previously a series of viral escape variants with mutations or deletions in the targeted *nef* sequence.[19] We selected mutant R1 with a 106 nt deletion that includes the complete sh-*nef* target sequence and mutant R3′ with two point muta-

Table 1 lhRNA inhibits shRNA-escape viruses

Virus	*nef3 target*	*sh-nef inhibition*[a] *(%)*	*lh-nef1 inhibition*[b] *(%)*
pLAI (WT)	GTGCCTGGCTAGAAGCACA	94	92
R1[c]	-------------------	0	83
R3′[d]	GT**A**CCTGGC**G**AGAAGCACA	28	88

Abbreviations: HEK, human embryonic kidney; HIV-1, human immunodeficiency virus type 1.
lhRNA, long-hairpin RNA; shRNA, short-hairpin RNA; WT, wild type.
[a]HEK 293T cells were cotransfected with 200 ng HIV-1 molecular clone pLAI (WT, R1 or R3′) and 40 ng pH1sh-*nef* plasmid.
[b]HEK 293T cells were cotransfected with 200 ng HIV-1 molecular clone pLAI (WT, R1 or R3′) and 40 ng pLTRΨ-*nef1* plasmid.
[c]Deletion of complete target sequence.
[d]Bold and underlined residues cause viral escape.

tions in the target (Table 1). Human embryonic kidney 293T cells were cotransfected with 200 ng HIV-1 molecular clone pLAI (wild-type, R1 or R3′) and 40 ng pLTRΨ-*nef1* or pH1sh-*nef* plasmid. Only the wild-type pLAI construct was equally inhibited by the pLTRΨ-*nef1* and sh-*nef* constructs. The deletion mutant R1 is completely resistant to sh-*nef*, but was potently inhibited by pLTRΨ-*nef1*. Mutant R3′ partially escapes from sh-*nef*, but is potently inhibited by pLTRΨ-*nef1*.

*Inhibition of human immunodeficiency virus type 1 by cytoplasmically expressed ds-*nef2

RNA interference is mainly a cytoplasmic process,[54] and putative problems in nuclear export of lhRNAs may thus hamper their inhibitory activity. In order to test whether there is a difference between the RNAi-inducing capacity of dsRNAs produced in the nucleus or cytoplasm of cells, we expressed dsRNA targeted to the *nef*-coding domain (*nef2* in Figure 1) using the cytoplasmic T7 RNA polymerase system. To generate pT7-*nef2*, we cloned a 300 bp *nef*-specific DNA fragment between convergent T7 promoters and terminators (Figure 3). In addition, the control expression plasmid pT7sh-*nef* was constructed. Cytoplasmic transcription of pT7-*nef2* and pT7sh-*nef* is performed by T7-polymerase encoded by pT7-pol. Expression of the firefly luciferase (FL) reporter from the T7 promoter (pT7-luc) was measured as a positive control in all transfection experiments with pT7-pol (Figure 6a). Human embryonic kidney 293T cells were transfected with pLAI and pT7-*nef2* or pT7sh-*nef*, with and without pT7-pol. Virus production was measured in the culture supernatant 3 days after transfection by CA-p24 ELISA. In the absence of T7-polymerase, pT7-*nef2* and pT7sh-*nef* confer a low level of inhibition on virus production probably due to leaky RNA expression from the plasmids (Figure 6b). With T7-polymerase, pT7-*nef2* and pT7sh-*nef* confer up to 90% inhibition of HIV-1 production (Figure 6b). This level of inhibition is comparable to that obtained with the sh-*nef* control expression plasmid. *Renilla* luciferase expression was not affected (Figure 6b, gray bars). To demonstrate that the effect of pT7-*nef2* is sequence-specific, we cotransfected it with the pGL3-Nef reporter (Figure 6c). In the presence of T7-polymerase, pT7-*nef2* potently inhibited luciferase expression.

We next tested the inhibitory potential of both pT7-*nef2* and pT7sh-*nef* plasmids in the presence of an increasing amount (5–100 ng) of pT7-polymerase. The inhibition was consistently strong even at low amounts (5 ng) of the pT7-pol plasmid (Figure 6d). When more than 100 ng pT7-pol was used in the transfection experiments, a non-specific decrease in RL reporter expression was observed. A high amount of T7-polymerase accumulating in the cytoplasm of cells may be toxic or high amounts of dsRNA accumulating in the cytoplasm of cells may induce the innate antiviral IFN response.

To measure the RNA expression levels of selected lhRNA-expression constructs, we performed a reverse transcriptase-polymerase chain reaction (RT-PCR) assay with *nef*-specific primers. All constructs expressed *nef* RNA (Figure 7). Notably, pT7-*nef2* produced low amounts of RNA even in the absence of the pT7pol inducer. This can explain the slight inhibition of gene expression obtained with pT7-*nef2* in the absence of pT7pol (Figure 6b and c). No *nef*-specific fragment was detected in mock-transfected cells or in cells transfected with poly(I:C).

Endogenously expressed long-hairpin RNA and long double-stranded RNA do not induce the interferon response

It has been reported that introduction of dsRNAs longer than 30 bp in mammalian cells induces the innate antiviral IFN response. We determined IFN-β induction by RT-PCR in HEK 293T cells upon transfection with *in vitro* made *nef2* dsRNA and DNA constructs encoding pEF1α-*nef1*, pLTR-*nef1*, pLTRΨ-*nef1*, pT7-*nef2* or pH1sh-*nef*. Transfection of 1 μg poly(I:C), a known inducer of IFN and other cytokines, was used as a positive control. The lhRNA- and sh-*nef*-expression plasmids did not induce detectable levels of IFN-β mRNA (Figure 8). Both *nef2* dsRNA and poly(I:C) controls induced high amounts of the IFN-β mRNA.

Discussion

The use of synthetic siRNAs or shRNA-expression plasmids as inducers of RNAi-based therapy against HIV-1 faces the major obstacle of the emergence of virus escape variants. Similar to the combined use of antivirals in highly active antiretroviral therapy (HAART), one could design an RNAi therapy with extended lhRNA/dsRNA. Endogenous expression of two long complementary RNAs, with the potential to form an extended dsRNA duplex, leads to specific suppression of gene expression in mammalian cells.[32,34,35,37] Interestingly, full-length dsRNAs could not be detected, suggesting that

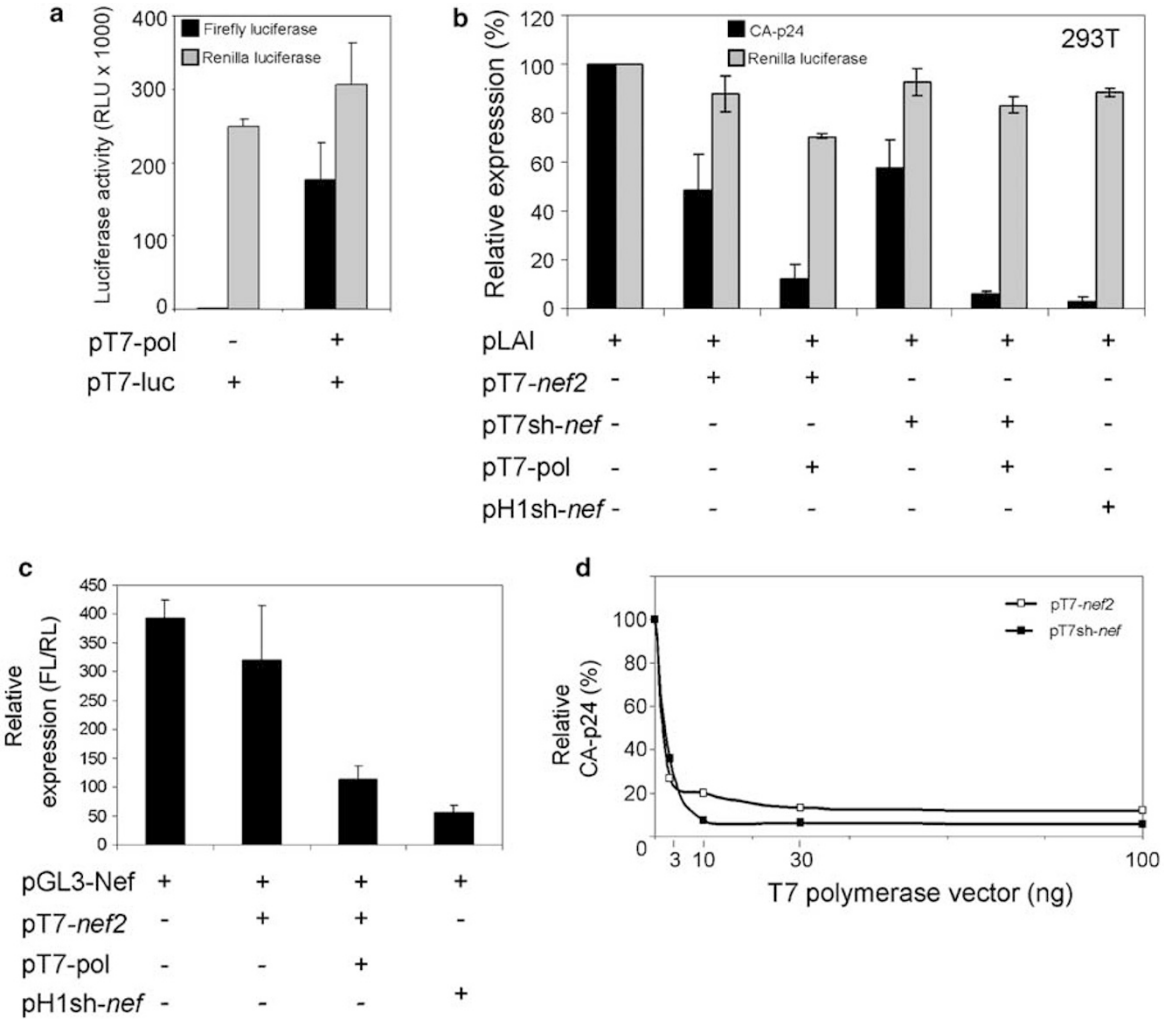

Figure 6 Inhibition of human immunodeficiency virus type 1 (HIV-1) by cytoplasmically expressed long double-stranded RNA (dsRNA). (**a**) pT7-luc (100 ng) construct was cotransfected in human embryonic kidney (HEK) 293T cells with or without 30 ng pT7-pol. At 2 days after transfection, cells were lysed and the expression of firefly luciferase was measured. (**b**) pT7-*nef*2 (100 ng) and pT7sh-*nef* vectors were linearized 3′ of the T7 termination signal with *Xba*I and *Bam*HI, respectively, and cotransfected with 100 ng pLAI, 30 ng pT7-pol and 2.5 ng pRL in HEK 293T cells. Two separate complementary RNA chains, potentially forming dsRNA, are transcribed in the cell from convergent T7 promoters. Equal amounts of a pH1sh-*nef* expression vector and the empty vector were added as positive and negative controls, respectively. Cotransfections were also performed without pT7-pol to check for non-specific effects of the T7 plasmids. Virus production was determined as described in the legend to Figure 2. Standard error bars represent the means of four independent experiments. Cells were lysed 2 days after transfection to measure *Renilla* luciferase. (**c**) Sequence-specific inhibition of the pGL3-Nef reporter, containing the 250-nucleotide (nt) *nef*2 target sequence downstream of the luciferase coding domain, by pT7-*nef*2. HEK 239T cells were cotransfected with 100 ng of pGL3-Nef, 100 ng pT7-*nef*2, 30 ng pT7-pol and 2.5 ng pRL. pH1sh-*nef* (10 ng) expression vector and the empty vector were added as positive and negative controls, respectively. (**d**) Titration of T7 polymerase. pLAI (100 ng) was cotransfected with increasing amounts (0–3–10–30–100 ng) of pT7-pol and 100 ng pT7-*nef*2 or pT7sh-*nef*.

processing by Dicer precludes their accumulation in the cytoplasm. Long dsRNAs are indeed processed *in vitro* by the RNAi machinery into multiple active siRNAs.[55,56] Several lines of evidence show that lhRNAs induce gene silencing by the RNAi mechanism. Inhibition of Dicer abrogated the gene silencing induced by lhRNA against GFP, indicating that silencing was mediated by RNAi.[32] These results suggest that long dsRNA is well tolerated in mammalian cells, most likely because it is processed rapidly by the RNAi machinery. It is also relevant to mention that mammalian cells may naturally produce dsRNA derived from repetitive and transposable elements.[57,58] A recently performed bioinformatics study revealed the presence of at least 4520 full-length transcripts, which form sense–antisense gene pairs in the human genome.[59]

We designed a set of anti-HIV-1 lhRNA/dsRNA-expression constructs and compared their ability to inhibit virus production with a very potent shRNA-based inhibitor that we described previously.[19] Ideally, a single lhRNA should provide more potent inhibition of HIV than an shRNA. An additional advantage of lhRNA is that it does not require predetermination of optimal shRNAs and HIV-1 target sequences because multiple effective siRNAs will be produced. A potential disadvantage of the use of lhRNA as therapeutic is that the multiple siRNAs are more likely to cause off-target effects.

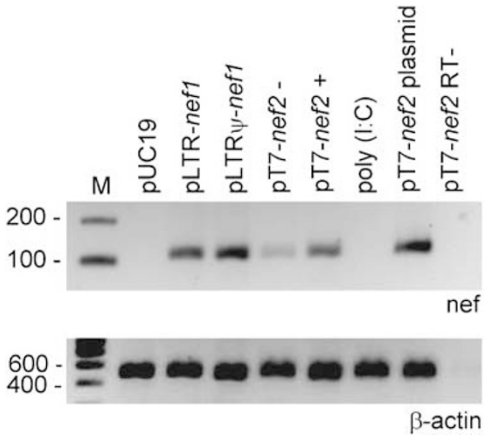

Figure 7 *In vivo* RNA expression from long-hairpin RNA (lhRNA) constructs. HEK 293T cells were transfected with 500 ng of the indicated lhRNA/double-stranded RNA (dsRNA)-expression constructs using Lipofectamine 2000. Constructs pLTR-*nef1* and pLTRΨ-*nef1* were cotransfected with 30 ng pTat. Construct pT7-*nef2* was cotransfected with 100 ng pT7-pol. The pUC19 plasmid and poly (I:C) were used as negative controls and pT7-*nef2* plasmid DNA was used as a positive control. Total RNA was isolated from the cells 48 h after transfection. The *nef* expression level was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) with primers Nef-B/X and antiU3-att, which create a 110 basepairs (bp) amplification product. *β*-Actin mRNA expression was analyzed as an internal control. pT7-*nef2* RT− is a control reaction without RT step.

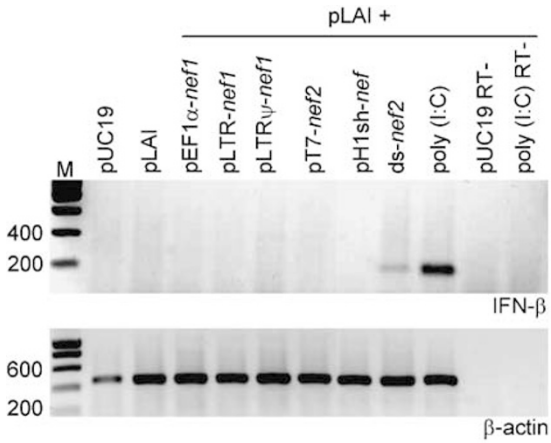

Figure 8 Long-hairpin RNA (lhRNA) constructs do not induce the interferon (IFN) response. Human embryonic kidney (HEK) 293T cells (1.5×10^5) were cotransfected with pLAI and the indicated lhRNA/double-stranded RNA (dsRNA)-expression constructs using Lipofectamine 2000. Construct pT7-*nef2* was cotransfected with pT7-pol. *Renilla* luciferase (pRL) was used as an internal control. The pUC19 plasmid was used as a negative control. *In vitro* transcribed ds-*nef2* RNA and poly (I:C) act as positive controls for IFN-*β* induction. Two separate transfections were performed, which were processed for either IFN-*β* mRNA expression or *renilla* measurement. No significant differences in *Renilla* expression were measured, except for the toxic treatment with ds-*nef2* RNA and poly(I:C) (results not shown). In addition, we measured CA-p24 in the supernatant, which showed the inhibition characteristics described earlier. Total RNA was isolated from the cells 24 h after transfection. The IFN-*β* expression level was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). *β*-Actin mRNA expression was analyzed as an internal control. PUC19 RT- and poly (I:C) RT- are control reactions without RT step.

Long-hairpin RNAs expressed from constitutive (EF1α) and inducible (7tetO, HIV-1 LTR) promoters inhibited HIV-1 production marginally, but we demonstrated potent and specific HIV-1 silencing with modified DNA constructs. The most active constructs either link the lhRNA to viral RNA sequences (Ψ) or express the long dsRNA directly in the cytoplasm, suggesting that translocation of dsRNA from the nucleus to the cytoplasm is a crucial step for these molecules to enter the cytoplasmic RNAi pathway. It is widely accepted that RNAi is a cytoplasmic process, as most protein components of the RNAi pathway, including Argonaute 2, TRBP and Dicer, assemble and function in the cytoplasm.[54,60–64] Putative problems in nuclear export of lhRNA may thus hamper its inhibitory activity. Modification of the lhRNA by addition of the HIV-1 leader sequence (Ψ) or direct cytoplasmic expression of long dsRNA by the T7 polymerase created potent inhibitors. The viral Ψ sequence may provide the transcript with a non-self signature and thereby boost RNAi. It has previously been suggested that the inhibitory effect of Ψ-containing transcripts is sequence-specific, possibly due to premature formation of RNA dimers,[53] but the exact mechanism of inhibition remains to be elucidated.

A major advantage of an lhRNA inhibitor over shRNA is the reduced chance of viral escape because a larger segment of the viral genome is targeted, as has been shown recently.[65] As an initial test of this idea, we used two HIV-1 mutants that escaped from sh-*nef* by two point mutations or complete deletion of the target sequence.[19] These escape mutants could be inhibited by the lhRNA construct pLTRΨ-*nef1*, confirming the hypothesis that lhRNA may delay or prevent the evolution of HIV-1 escape variants.

We showed that endogenously expressed lhRNA and dsRNA do not activate the innate antiviral response. A similar result has recently been described in literature.[33,66] Exposure of cells to a 50 bp *in vitro* synthesized dsRNA induces the production of class I IFNs, but not when such molecules are expressed in the cell from a DNA construct with the U6 promoter. Modification of lhRNAs expressed from a Pol III promoter by inclusion of multiple G:U wobbles induced RNAi without any non-specific effects.[66] In fact, most reports on IFN induction by long dsRNAs in mammalian cells are based on transfection of cells with *in vitro* synthesized dsRNAs.[40,67] Apparently, endogenously produced dsRNA is less active than exogenous dsRNA in inducing the IFN response. This finding may have a major impact on the further development of RNAi-based antiviral strategies.

RNA interference-based gene therapy against HIV-1 seems to be a viable option, either as mono-therapy or combined with traditional drug therapy.[22,68] The outgrowth of RNAi-resistant virus mutants presents a major obstacle for all sequence-specific inhibitory strategies. The simultaneous targeting of multiple conserved targets by lhRNA may confer increased robustness to future RNAi therapies. We will focus on optimization of the DNA constructs encoding HIV-1-specific lhRNAs, for example, the type of promoter used and the structure of the lhRNA. We will also test stable expression of these constructs in HIV-susceptible cells. These cell lines will be extensively tested for the emergence of RNAi-resistant virus variants.

Materials and methods

DNA constructs and RNA transcripts

The full-length HIV-1 molecular clone pLAI[69] was used to produce wild-type virus and to study inhibition by lhRNAs directed against the *tat*, *rev* or *nef* sequences. A detailed description of the construction of all lhRNA-expression constructs is available as Supplementary information. Plasmid pcDNA3-T7pol, expressing bacteriophage T7 polymerase (pT7-pol, a kind gift of Dr Jean-Marc Jacque, University of Massachusetts Medical School, Worcester, MA, USA), pGL3-Nef and pH1sh-*nef* have been described previously.[14,19]

The Mfold program[47] (http://www.bioinfo.rpi.edu/applications/mfold) was used to check the correct folding of extended hairpins.

In vitro *transcription and dicing of ds-*nef2 *RNA*

The ds-*nef*2 RNA was *in vitro* transcribed with the Megashortscript T7 transcription kit (Ambion, Austin, TX, USA) from the *nef*2 PCR template that contains convergent T7 promoters and terminators. The sh-*nef* RNAi inducer[19] was transcribed *in vitro* from the *Bam*HI-linearized pT7sh-*nef* vector. ds-*nef*2 RNA (1 μg) was diced *in vitro* at 37°C for 18–20 h using recombinant Dicer enzyme (Stratagene, Cedar Creek, TX, USA). The full-length ds-*nef*2 RNA, the cleaved si-*nef*2 (a mix of siRNAs derived from ds-*nef*2) and sh-*nef* were purified through MicroSpin G-25 column (Amersham Biosciences, Piscataway, NJ, USA). To remove undigested dsRNA from the dicing reaction, the si-*nef*2 was purified further on a Microcon YM-100 column (Millipore, Billerica, MA, USA). si-*nef*2 RNA was analyzed on 2% MetaPhor (BMA, Sanver Tech, The Netherlands) alongside a 20 bp DNA marker (Gensura, San Diego, CA, USA).

Cell culture and transfections

Human embryonic kidney 293T, C33A cervix carcinoma and African green monkey kidney Vero cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) (Hybond, Escondido, CA, USA), minimal essential medium with non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C and 5% CO_2. At 1 day before transfection, cells were trypsinized, resuspended in DMEM and seeded in 24-well plates at a density of 1.5×10^5 cells per well. Cells were cotransfected with 100–500 ng pLAI and 1–1000 ng *in vitro* transcribed ds-*nef*2 RNA and *in vitro* diced si-*nef*2 or 10–500 ng lhRNA (*tat*, *rev*, *nef1*) expression constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Virus production was determined by measuring the CA-p24 levels in the culture supernatant by ELISA (Abbott, Abbott Park, IL, USA) as described previously.[70] For firefly luciferase measurements, cells were cotransfected with 100 ng pGL3-Nef and 10 ng *in vitro* transcribed ds-*nef*2 RNA and *in vitro* diced si-*nef*2 or 100 ng pT7-*nef*2 and 100 ng pT7-pol. In all experiments, vector pRL (2.5 ng) (Promega, Madison, WI, USA), expressing RL under the control of the CMV promoter, was added to the transfection mix as a control for variation in transfection efficiency and cell viability. Equal amounts of the pH1sh-*nef* expression vector or the empty vector were added as positive and negative controls, respectively. Vectors pT7-luc and pT7-pol were cotransfected in equimolar amounts (100 ng) and FL reporter expression was measured. Briefly, cells were lysed 48–72 h after transfection in 150 μl 1 × passive lysis buffer (Promega) by shaking for 30 min at room temperature. The cell lysate was centrifuged for 5 min at 4000 r.p.m. and firefly and renilla luciferase expression was measured in 10 μl supernatant with the dual-luciferase reporter assay system or *Renilla* luciferase assay system (Promega).

Interferon assay

Induction of the IFN system was measured by a sensitive RT-PCR on the IFN-β mRNA.[71] RNA was isolated from HEK 293T cells with the mirVana miRNA isolation kit (Ambion) 24 h after transfection with the long dsRNA constructs. First-strand cDNA was reverse transcribed from approximately 1 μg RNA with random hexamer primers (Invitrogen) using the MMLV-RT enzyme (Invitrogen) according to the manufacturer's instructions. Approximately 200 ng cDNA was PCR amplified with primers IFN-βF and IFN-βR using standard conditions. Amplification of the β-actin gene was used as an internal control. Transfection of 1 μg poly(I:C) (Amersham Pharmacia Biotech, Piscataway, NJ, USA), a synthetic inosine/cytosine polymer that mimics viral dsRNA, was used as a positive control for IFN-β induction.

RNA of transfected cells was subjected to PCR with *nef*-specific primers Nef-B/X and AntiU3-att, yielding a 110 bp fragment (Supplementary Table 1).

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)