Design of extended short hairpin RNAs for HIV-1 inhibition

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ABSTRACT

RNA interference (RNAi) targeted towards viral mRNAs is widely used to block virus replication in mammalian cells. The specific antiviral RNAi response can be induced via transfection of synthetic small interfering RNAs (siRNAs) or via intracellular expression of short hairpin RNAs (shRNAs). For HIV-1, both approaches resulted in profound inhibition of virus replication. However, the therapeutic use of a single siRNA/shRNA appears limited due to the rapid emergence of RNAi-resistant escape viruses. These variants contain deletions or point mutations within the target sequence that abolish the antiviral effect. To avoid escape from RNAi, the virus should be simultaneously targeted with multiple shRNAs. Alternatively, long hairpin RNAs can be used from which multiple effective siRNAs may be produced. In this study, we constructed extended shRNAs (e-shRNAs) that encode two effective siRNAs against conserved HIV-1 sequences. Activity assays and RNA processing analyses indicate that the positioning of the two siRNAs within the hairpin stem is critical for the generation of two functional siRNAs. E-shRNAs that are efficiently processed into two effective siRNAs showed better inhibition of virus production than the poorly processed e-shRNAs, without inducing the interferon response. These results provide building principles for the design of multi-siRNA hairpin constructs.

INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved mechanism in which double stranded RNA (dsRNA) induces sequence-specific gene silencing (1). RNAi is initiated by the RNase III-like enzyme Dicer that processes dsRNAs into 21–25 bp dsRNAs called small interfering RNAs (siRNAs) (2–4). Subsequently, the siRNA is incorporated into the RNA-induced silencing complex (RISC), which uses one strand of the siRNA as a guide to target the complementary mRNA for cleavage (4). In mammalian cells, RNAi can be induced via transfection of synthetic siRNAs (5) or DNA vectors for intracellular expression of short hairpin RNAs (shRNAs) (6).

RNAi induction via transfection of siRNAs or via stable expression of shRNAs has been shown to be highly effective in inhibiting HIV-1 (7–11). However, HIV-1 can escape from RNAi by introduction of nucleotide substitutions or deletions in the siRNA target sequence (12–14). In addition, HIV-1 can also escape from RNAi-mediated inhibition through mutations that alter the local RNA secondary structure (14). To reduce the chance of escape, the virus should be targeted simultaneously with multiple highly efficient siRNAs. We recently identified a large set of potent shRNA inhibitors against different highly conserved HIV-1 sequences (15), which can be used to target multiple sites. There are several ways to express multiple effective siRNAs. One possibility is the insertion of multiple shRNA-expression cassettes in a viral vector. However, repeats of the same regulatory sequences, e.g. the H1 polymerase III promoter, may cause genetic instability and reduced titer of the vector system (Ter Brake and Berkhout, submitted for publication). Ideally, the expression of multiple antiviral shRNAs should be coordinated by putting them in a single transcript. Another possibility is to use long hairpin RNAs (lhRNAs), from which multiple siRNAs can be produced. Several reports described efficient RNAi induction by lhRNAs against human deficiency virus 1 (16,17), hepatitis C virus (18,19) and hepatitis B virus (20). Importantly, intracellular lhRNA expression does not seem to induce non-specific type 1 interferon (IFN) responses in cells, which may occur when dsRNA longer than 30 bp is introduced in mammalian cells (21,22). Induction of a type I IFN response will lead to non-sequence specific degradation of mRNAs. However, recent studies showed that smaller duplexes can also

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activate the IFN response (23,24), which is dose-dependent.

To learn how to design effective extended hairpin RNAs (e-shRNAs), from which several effective siRNAs can be produced, we constructed a series of antiviral e-shRNA constructs and examined their silencing activities on luciferase reporters and HIV-1. We determined the minimal hairpin stem length to produce two active siRNAs. Furthermore, we demonstrated that siRNA activity correlates with proper processing of the e-shRNAs. Importantly, we showed that extended hairpin RNA transcripts are highly efficient in inhibiting HIV-1 production, without induction of the IFN response in cells. These results provide building principles for the design of multi-siRNA hairpin constructs for durable inhibition of escape-prone RNA viruses.

MATERIAL AND METHODS

DNA constructs

Hairpin RNA constructs were made by annealing of complementary oligonucleotides and inserting them into the BglII and HindIII site of the pSUPER vector (6). The sequences of the oligonucleotides used are listed in Supplementary Table 1. siRNA sequences for constructing the hairpin construct targeting the luciferase gene (GL3) were described previously (5,25). Luciferase reporters containing 50 nt of the HIV-1 gag, pol and nef gene in the 3′ UTR were made using annealed oligonucleotides, which were ligated between the EcoRI and PstI sites of the firefly luciferase vector pGL3 (Promega). The same procedure was used for construction of the luciferase reporter with a 19 nt gag target. The plasmid encoding the HIV-1 isolate LAI (26) was used to produce virus in transfected 293T cells.

For amplification of plasmids, *Escherichia coli* GT116 cells were transformed with the constructs by electroporation with Gene Pulser II, 25 μF, 200 Ω and 2.5 kV. The bacteria were grown in Luria Broth medium containing 100 μg/ml ampicillin by shaking at 37°C or on LB agar plates containing 100 μg/ml ampicillin at 37°C. Plasmids were isolated using the Qiagen Maxiprep or Midiprep kit (Qiagen) and the sequences were verified using the BigDye Terminator Cycle Sequencing kit (ABI, Foster City, CA, USA). For sequencing of hairpin RNA constructs, a sample denaturation temperature of 98°C was used and 1 M of Betaine was added in the reaction (27). The Mfold program (28) was used to determine the secondary structure of RNA transcripts.

Cell culture and transfection

The human embryonic kidney (HEK) cell line 293T was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 U/ml streptomycin and minimal essential medium non-essential amino acids at 37°C and 5% CO_2. For luciferase assays, HIV-1 inhibition assays and the interferon assay, 293T cells were seeded in 24-well plates at a density of 1.5×10^5 cells per well in 1 ml of DMEM with 10% FCS without antibiotics one day prior transfection. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. For small RNA analysis, 1.6×10^6 293T cells were seeded in T25 flasks. The next day, the cells were transfected with 5 μg of the hairpin RNA constructs using Lipofectamine 2000 reagent (Invitrogen) as suggested by the manufacturer.

Luciferase assays

For luciferase assays, cells were co-transfected with 100 ng of firefly luciferase expression plasmid (pGL3; Promega, Madison, WI, USA), 1 ng of renilla luciferase expression plasmid (pRL-CMV) and different amounts of hairpin RNA expression constructs. pBluescript (pBS) was added to the transfection mixture to ensure equal DNA amounts. Transfected cells were lysed at 48 h post-transfection in 150 μl 1× passive lysis buffer (Promega) by gentle rocking for 15 min at room temperature. The cell lysates were centrifuged for 5 min at 4000 rpm at 4°C and 10 μl of the supernatant was used to measure firefly and renilla luciferase activities with the Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity was calculated as the ratio between the firefly and renilla luciferase activities and corrected for between-session variation (29).

HIV-1 inhibition assay

To determine inhibition of virus production, 293T cells were co-transfected with 250 ng of the HIV-1 pLAI, 1 ng pRL-CMV and 2.5, 10 or 25 ng of the hairpin RNA constructs. pBS was added to the transfection mixture to ensure equal DNA amounts. The cells were grown at 37°C and harvested 48 h after transfection. Virus production was monitored by determining the CA-p24 level in the culture supernatant by ELISA as described previously (30). Subsequently, the cells were lysed as described above and 10 μl of the supernatant was used to measure the renilla luciferase activities using the Renilla Luciferase Assay System (Promega). The relative CA-p24 production was calculated as the ratio between the CA-p24 level and the renilla luciferase activity and corrected for between-session variation (29).

siRNA detection by northern blotting

Small RNAs were purified from the transfected 293T cells at 72 h post-transfection using the mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. For northern blot analyses, 3 μg of small RNAs was resolved on urea 15% denaturing polyacrylamide gels (Invitrogen). For a size reference, we used the decade RNA molecular weight marker (Ambion, Austin, TX), which was radioactively labeled and pre-treated as suggested by the manufacturer. Prior to loading, the samples were heated for 5 min at 95°C and immediately placed on ice. After electrophoresis, the gels were stained with 2 μg/ml ethidium bromide for 20 min and destained with milliQ water for 10 min. Then the tRNA bands were visualized under UV light to check for equal sample loading. Subsequently, the gels were electrotransferred to

positively charged nylon membranes (Boehringer Mannheim, GmbH, Mannheim, Germany). The RNA was crosslinked to the membrane under ultraviolet light at 254 nm wavelength (1200 μJ × 100). Labeled LNA oligonucleotides that are complementary to the antisense strand of the siRNAs pol and nef were used as probes. Probes were 5′ end labeled with the kinaseMax kit (Ambion) in the presence of 1 μl of [γ-^{32}]PATP (0.37 MBq/μl, Amersham Biosciences) and purified using the Sephadex G-25 spin columns (Amersham Biosciences) according to manufacturer's protocol. We used LNA oligonucleotides 5′ ACAGGAGCAGATGATACAG 3′ (pol probe) and 5′ GGGACTGGAAGGGCTAATT 3′ (nef probe). Underlined are the positions that contain locked nucleic acids. Hybridizations were performed at 42°C in 10 ml ULTRAhyb hybridization buffer (Ambion) in the presence of the labeled probe, as suggested by the manufacturer. After hybridization, the membranes were washed twice for 5 min at 42°C in 2 × SSC/0.1% SDS and signals were detected by autoradiography using a phosphorimager (Amersham Biosciences).

Interferon assay using reverse transcriptase-polymerase chain reaction (RT-PCR)

To determine the induction of the IFN system by the e-shRNA constructs, 293T cells were transfected with 25 ng of e-shRNAs using Lipofectamine 2000 reagent (Invitrogen). Transfection of 2 μg *in vitro* transcribed dsRNA was used as a positive control for IFN-β induction as described previously (16). Total RNA was isolated from cells 24 h post-transfection using RNeasy mini kit (Invitrogen) according to the manufacturer's protocol. Genomic DNA was removed by DNase treatment using the TURBO DNA-free™ kit (Ambion). First strand cDNA was reverse transcribed using 1 μg of total RNA, Thermoscript™ reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen).

PCR amplification was performed on 2 μl RT product with IFN-β, OAS, MxA, ISG56 and beta actin specific primers. The following primer combinations were used: IFN-β, f: 5′-GATTCATCTAGCACTGGCTGG-3′; r: 5′-CTTCAGGTAATGCAGAATCC-3′ (186 bp product) (31), OAS, f: 5′-TCAGAAGAGAAGCCAACGTGA-3′; r: 5′-CGGAGACAGCGAGGGTAAAT-3′ (399 bp product) (32), MxA, f: 5′-AGTATGGTGTCGACATACC GGA-3′; r: 5′-GAGTCTGGTAAACAGCCGAATG-3′ (145 bp product) (33), ISG56, f: 5′-CTTGAGCCTCCT TGGGTTCG-3′; r: 5′-GCTGATATCTGGGTGCCTA AGG-3′ (137 bp product) (33) and β-actin f: 5′-GACT ACCTCATGAAGATCCTCAC-3′; r: 5′-ATTGCCAA TGGTGATGACCTG-3′ (197 bp product). For PCR amplification, the Reddymix™ Master Mix (Abgene) was used in a 50 μl reaction. The PCR program was as follows: 95°C for 3 min, 30 cycles of 30 s at 95°C, 30 s at 57°C, 45 s at 72°C and a final extension for 8 min at 72°C.

The PCR products were analyzed on a 1.5% agarose gel. The SmartLadder (Eurogentec) was used as a size marker.

RESULTS

The position of an effective siRNA within an extended hairpin determines its activity

In an initial attempt to learn the rules for constructing multiple siRNAs in a single, extended hairpin, we tested the importance of the siRNA position within the hairpin stem. We made a first set of three 39 bp hairpin RNA constructs that are expressed from the H1 polymerase III promoter. These RNA hairpins have identical 5 nt loop sequences and top 2 bp of the pSUPER system (6) and end with a 2 nt UU overhang (Figure 1A). We positioned a highly effective anti-gag siRNA in the base (39B), center (39C) or top (39T) of the hairpin stem and compared these with the original 21 bp shGag inhibitor (Figure 1A). The guide/antisense strand of the gag siRNA is boxed in Figure 1A. This 19 nt sequence is flanked in the 39 bp hairpins by contiguous antisense HIV-1 gag sequences.

To determine the inhibitory activities of these hairpin RNAs, two luciferase reporter constructs were generated. One reporter contains a broad 50 nt gag target sequence that should reveal the activity of any siRNA processed from the extended hairpins (Figure 1B, left). The other reporter contains the minimal 19 nt gag target sequence that only scores the activity of the precise siRNA against gag (Figure 1B, right). We co-transfected 293T cells with the luciferase reporter constructs and different amounts of the hairpin RNA constructs. Plasmids encoding renilla luciferase were included to correct for transfection variation and to monitor for cell viability that may be affected by off-target effects of the encoded siRNAs. Firefly and renilla luciferase expression was measured 48 h post-transfection and the ratio was used as an indicator for target inhibition. Luciferase expression in the absence of inhibitor was set at 100%. Hairpins 39B and 39T were equally active on the 50 nt gag reporter, but hairpin 39C showed significantly less inhibition (Figure 1C, left). All extended hairpin RNAs clearly showed less inhibition than shGag. When these constructs were tested on the luciferase reporter with the exact 19 nt gag target, we observed the same ranking order of activity, but the differences are more pronounced (Figure 1C, right). Construct 39B is most efficient, 39T is marginally active and 39C did not show any inhibitory effect. These findings suggest that processing of the 39 bp hairpins starts at the base of the hairpin RNA transcript, as suggested by the marginal inhibitory activity of 39T on the 19 nt gag reporter. 39T does have some activity on the broad 50 nt gag reporter because it also detects the siRNAs produced from the base of 39T. In case of 39C, the centrally located 19 nt siRNA is probably destroyed because Dicer cleavage will occur near the center of the extended hairpin. Again, some 39C activity is recovered on the 50 nt gag reporter, which likely represents siRNAs processed from the base of the hairpin.

Design of e-shRNAs encoding two functional siRNAs

To study whether efficient inhibition of two genes can be obtained by a single e-shRNA, we designed extended hairpin constructs encoding two highly effective
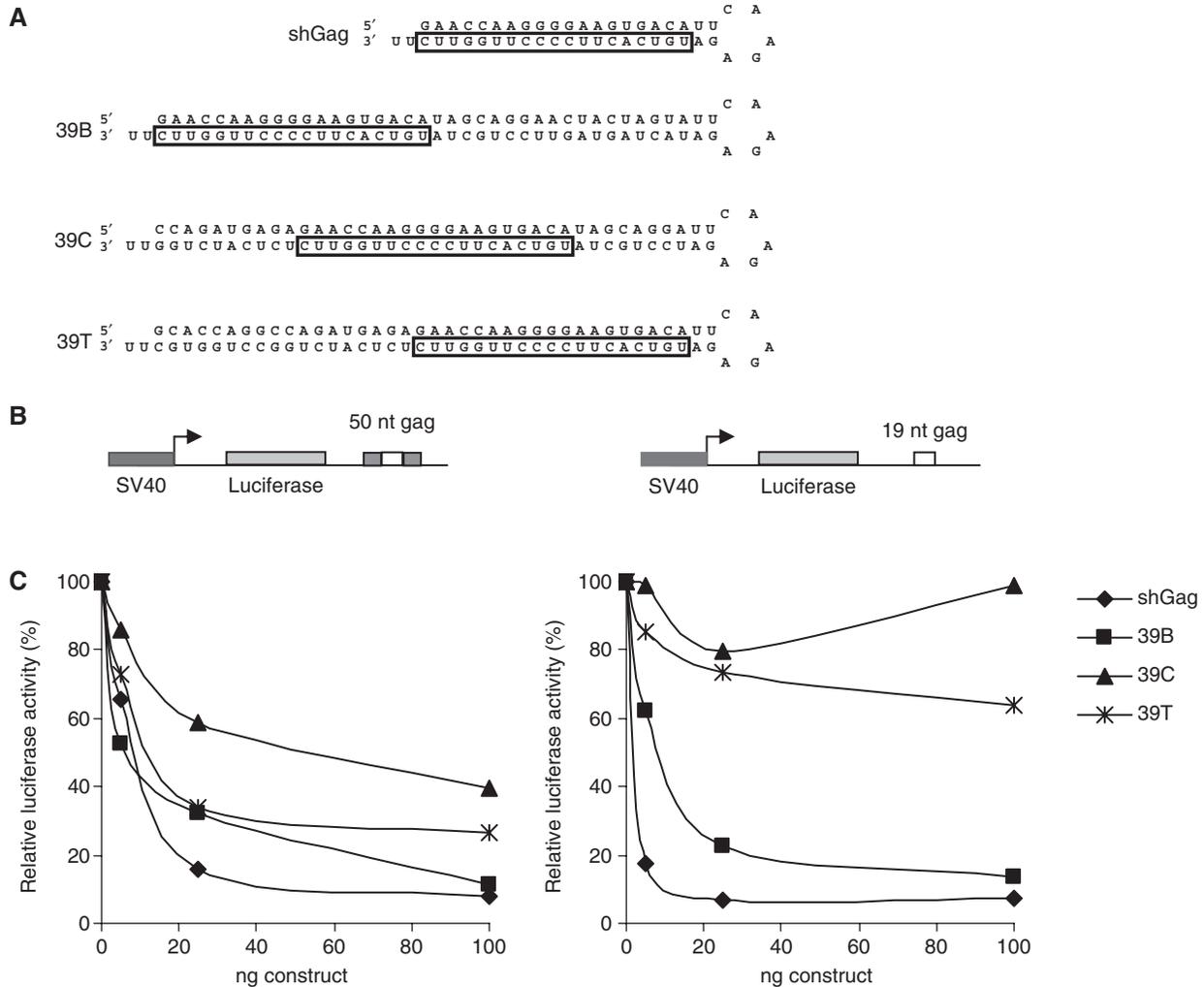
Figure 1. Silencing activities of extended hairpin RNAs depend on the position of the active siRNA in the stem. (**A**) Schematic of the 39 bp extended hairpin RNAs and the 21 bp original shGag. Extended hairpins against gag were constructed with the effective gag siRNA (antisense strand boxed) at the base (39B), center (39C) and top (39T). (**B**) Luciferase reporters containing a broad 50 nt or a precise 19 nt gag target. (**C**) Reporter constructs (100 ng) and the control renilla luciferase expression plasmid (1 ng) were used to co-transfect 293T cells together with different amounts of 39 bp extended hairpin RNA constructs and the original shGag. Firefly and renilla luciferase activity was measured 2 days post-transfection and the ratio was used to calculate the relative luciferase activity. Luciferase activity in the absence of inhibitor was set at 100%. The mean values obtained in three independent experiments are shown.

siRNAs: one against HIV-1 pol gene (15) and the other against the nef gene (34). Two versions were made: pol-nef and nef-pol e-shRNAs (Figure 2A). We extended the original short hairpin constructs and placed a second siRNA on top of the first siRNA, resulting in a hairpin of 40 bp (Figure 2A). Our previous findings suggest that processing of 40 bp hairpins by Dicer will start at the base of the hairpin. However, the exact cleavage site of Dicer is unknown, therefore we made four additional constructs with increasing length (41–44 bp) by insertion of 1–4 bp linkers between the two siRNAs (Figure 2A). The linker sequences were obtained by extending the sense strand of the first siRNA with HIV-1 derived sequences. This design allowed us to study the silencing activities of hairpins with increasing stem length.

To evaluate the suppressive activities of these hairpin RNA constructs, we co-transfected 293T cells with the inhibitors and luciferase reporter constructs containing either the 50 nt pol target (Luc-pol) or the 50 nt nef target (Luc-nef) (Figure 2B). The renilla luciferase expression plasmid was included to control for transfection variation. Firefly and renilla luciferase expression levels were measured 48 h post-transfection and the ratio in the presence of pBS was set at 100%. The irrelevant hairpin RNA construct 39C (against HIV-1 gag) was used as a negative control. The highest inhibitory activity was observed for the siRNAs present at the base of the hairpin. All pol-nef hairpins inhibit the Luc-pol reporter (Figure 2C, upper left) and all nef-pol hairpins inhibit the Luc-nef reporter efficiently (Figure 2C, lower right). The activity of the e-shRNAs is comparable to that of the original shRNAs, although the pol-nef constructs are slightly less active than shPol (Figure 2C, upper left). Poor activity was scored for the top siRNAs in the original
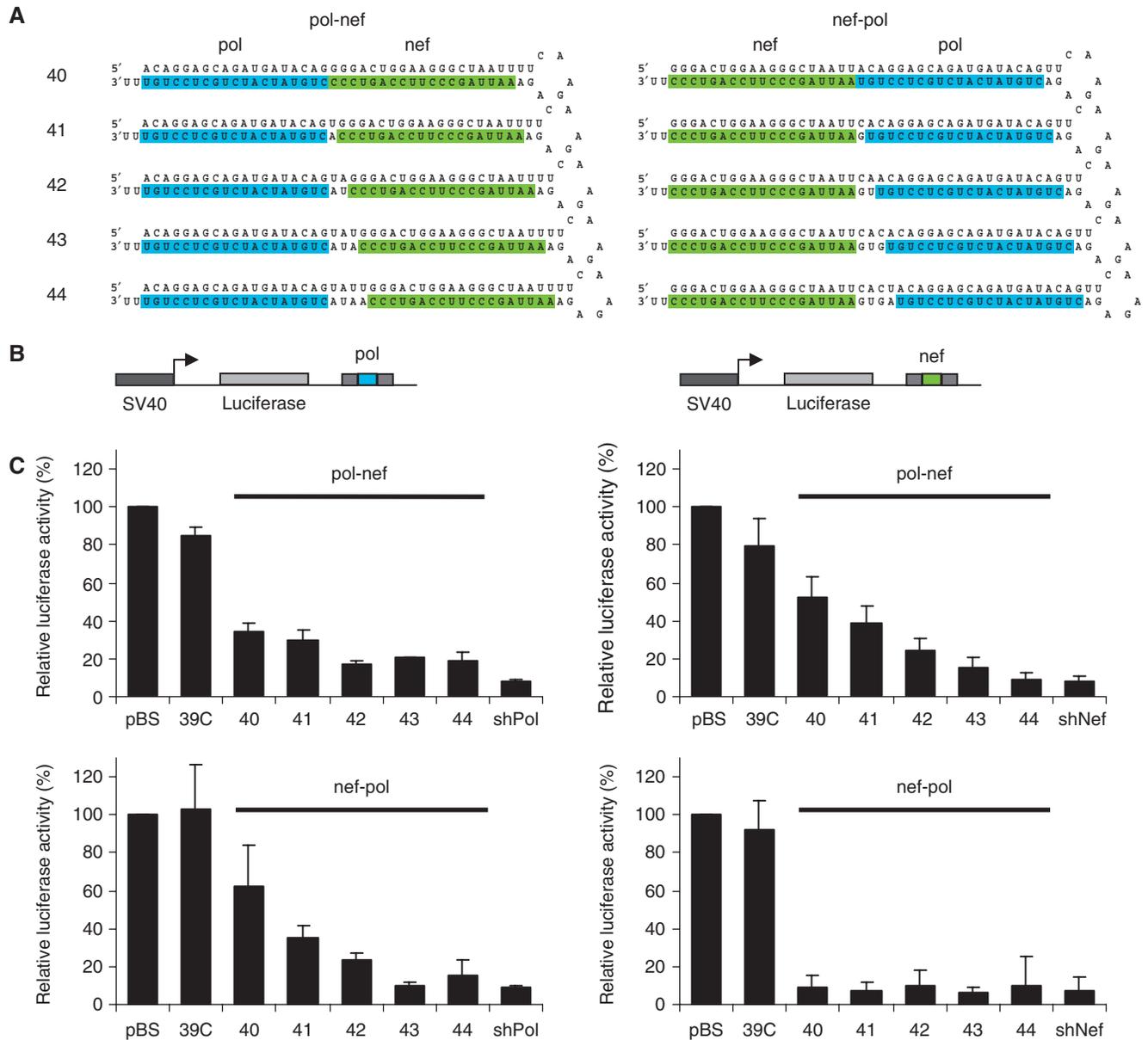
Figure 2. Optimization of a bifunctional extended shRNA. (**A**) Schematic of the e-shRNAs pol-nef and nef-pol of 40 bp and the length variants of 41–44 bp. The antisense strand of the siRNAs against pol and nef are marked in blue and green, respectively. (**B**) Luciferase reporter constructs containing 50 nt HIV-1 pol (Luc-pol) or nef (Luc-nef) sequences, with the 19 nt target in the center. (**C**) 293T cells were co-transfected with 100 ng of the Luc-pol (left) or Luc-nef (right) reporter, 1 ng of the renilla luciferase plasmid and 10 ng of the e-shRNAs Relative luciferase activities were determined from the firefly and the control renilla luciferase expression ratios. Luciferase expression in the presence of pBS was set at 100%. The 39C construct against HIV-1 gag was used as a negative control. The original shPol and shNef were included as positive controls. Averages and standard deviations represent three independent transfections.

40 bp context, that is nef in the pol-nef construct (Figure 2C, upper right) and pol in the nef-pol construct (Figure 2C, lower left). However, addition of spacer residues improved the activity of these top siRNAs. The nef siRNA from the pol-nef 43 construct showed optimal activity that approaches the activity of shNef. Further size increases (44 bp) do not significantly enhance the inhibitory potential. The pol siRNA from the nef-pol constructs also showed optimal activity when the hairpin stem length is 43 bp. Importantly, increased activity of the top siRNA is not at the expense of the siRNA at

the base of the hairpin. Thus, a hairpin stem of at least 43 bp is required to produce two functional siRNAs from a single hairpin RNA. Of note, renilla luciferase expression of the transfection control was not affected by the extended shRNAs, indicating that the extended hairpins do not cause cytotoxicity in 293T cells.

Specificity of a functional e-shRNA

To address the hairpin length requirement from another perspective, we made two additional mutants in the
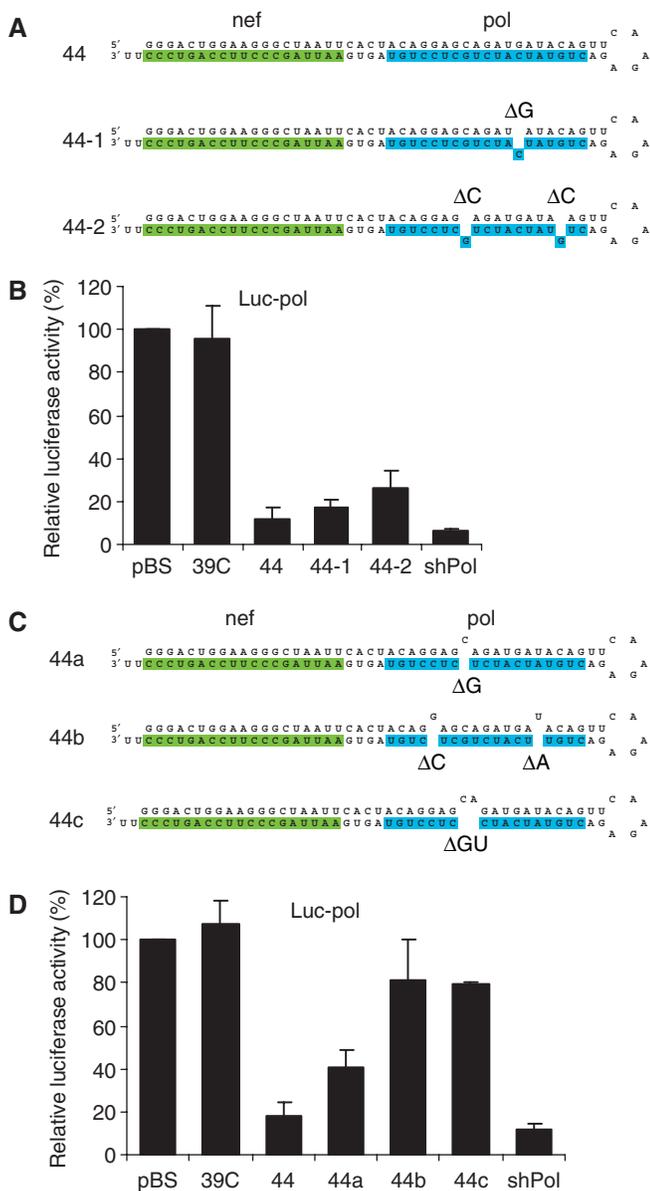
Figure 3. Variants of nef-pol 44 to test the specificity of the e-shRNA design. (**A**) The original nef-pol 44 hairpin and two variants that contain either a 1 or 2 nt deletion (44-1/44-2) in the passenger/sense strand. These variants have a reduced stem length of 1 bp (43 bp) or 2 bp (42 bp). (**B**) 293T cells were co-transfected with 10 ng of the indicated hairpin constructs, 100 ng of the luciferase reporter and 1 ng of the renilla luciferase plasmid. Two days post-transfection, knock-down efficiencies were determined using the firefly and renilla luciferase expression ratios. (**C**) Three additional mutant hairpins were made that contain either one (44a) or two (44b and 44c) deletions in the guide/antisense strand of the hairpin. (**D**) 293T cells were co-transfected with 10 ng of 44a, 44b and 44c, 100 ng of the Luc-pol reporter and 1 ng of the renilla luciferase plasmid. Two days post-transfection, firefly and renilla luciferase expression was measured and the ratio was used to determine the relative luciferase activity. Luciferase expression in the presence of pBS was set at 100%. Averages and standard deviations represent three independent transfections.

nef-pol 44 context. We removed either one or two nucleotides (Figure 3A, mutant 44-1 and 44-2, respectively) from the passenger/sense pol strand, thus effectively reducing the number of basepairs by one or two in the

hairpin stem without affecting the guide/antisense strand. Anti-gag construct 39C was used as a negative control and we set the luciferase expression in the presence of pBS at 100%. All three constructs showed efficient silencing of the nef reporter by the siRNAs at the base of the hairpins (data not shown). However, Luc-pol silencing is sensitive to the hairpin length. Removal of one nucleotide marginally reduced Luc-pol silencing activity and removal of two nucleotides showed a significant reduction (Figure 3B). These findings confirm the results obtained with the original constructs in that the hairpin stem length is critical for e-shRNA activity.

To rule out that the silencing activity observed for the extended shRNAs is due to aspecific effects, e.g. IFN induction by the extended hairpins, we made additional nef-pol 44 variants. If the observed inhibition is due to RNAi, it should be sequence-specific. We made three mutants containing one or two nucleotides deletions in the guide/antisense pol strand of nef-pol 44 (Figure 3C, mutant 44a, 44b and 44c). Anti-gag construct 39C was again used as a negative control and the luciferase expression with pBS was set at 100%. We observed a gradual reduction in the ability of the deletion mutants to silence Luc-pol expression (Figure 3D), but no effect on Luc-nef silencing was observed (data not shown). These combined results indicate that the e-shRNA produces two functional siRNAs that induce RNAi in a sequence-specific manner.

Processing of the e-shRNA hairpins

We next asked whether the differences in silencing activity of the hairpin RNA constructs are due to differences in RNA expression, stability or processing into functional siRNAs. The latter possibility seems most likely as the siRNA at the base of the hairpin is always active, unlike the top siRNA. To study this, we transfected 293T cells with the 40–44 variants of the pol-nef and nef-pol hairpin constructs and examined siRNA production by northern blotting. To ensure equal loading of the purified small RNA samples on the polyacrylamide gel, we stained the gel with ethidium bromide to visualize tRNA species and other small RNA molecules. For detection of siRNAs, we used 5′ end-labeled 19 nt LNA probes complementary to the antisense strand of the siRNAs nef and pol (Figure 4A). The two individual shRNA constructs were used as positive control, showing the mature, fully processed siRNAs. In pol-nef e-shRNA transfected cells, we readily detected pol siRNAs of ~22 nt that are derived from the base of the hairpin (40–44), but nef siRNA processing from the top of the hairpin increases with spacer length (Figure 4B). A similar picture was observed for the nef-pol constructs (Figure 4C). Here, the nef probe detects an approximately equal amount of the siRNA at the base of the hairpin, but significantly more of the top siRNA is processed from constructs 42, and especially 43 and 44. These results correlate with the luciferase data in which we observed stable activity of the siRNA at the base of the hairpin, but increased activity of the top siRNA when a 43 bp stem length is reached (Figure 2C). Thus, the increased activity is due to more efficient processing of
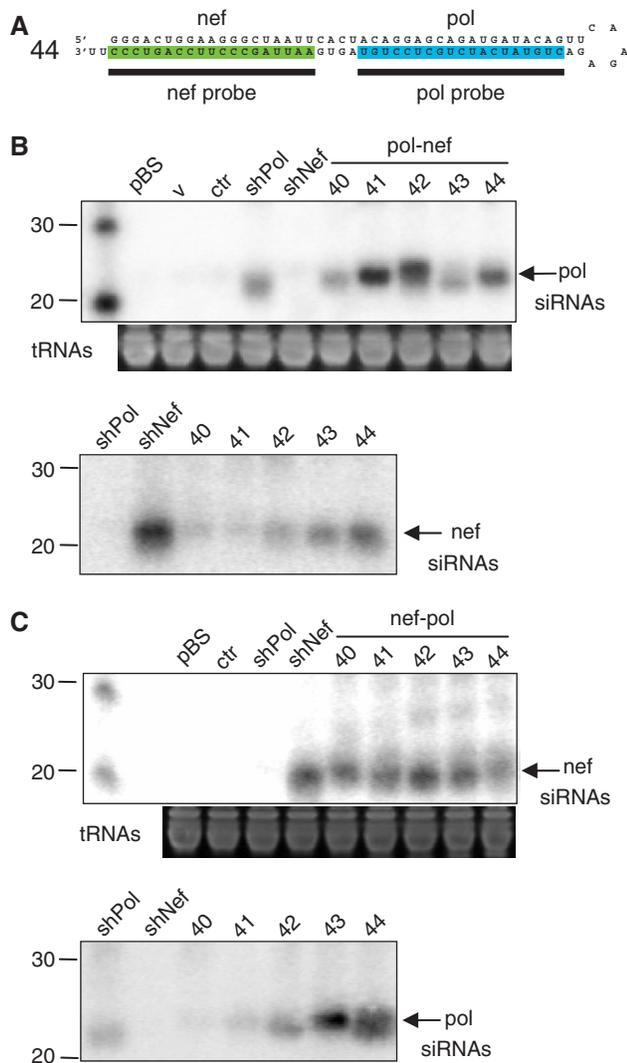
Figure 4. Northern blot analysis of the e-shRNAs derived siRNAs. (**A**) The nef-pol 44 hairpin with the probes to detect siRNAs against pol and nef. (**B**) Small RNAs were purified from 293T cells transfected with the pol-nef e-shRNA variants 40–44 for detection of siRNAs pol and nef. pBluescript (pBS), the empty vector (v) and the irrelevant 39C hairpin against gag (ctr) were used as negative controls. (**C**) Detection of siRNAs nef and pol derived from nef-pol e-shRNAs transfected 293T cells. pBS and the irrelevant 39C hairpin against gag (ctr) were used as negative controls. Ethidium bromide staining of tRNAs serves as sample loading control. Ethidium bromide stainings were shown for the upper blot of Figure 4B and C. Stainings were performed for all northern blots and a similar picture was observed.

the top siRNA. Precursor hairpin RNAs were not detected in these experiments, suggesting that the expressed hairpins are efficiently processed by the cellular RNAi machinery. Interestingly, we did not detect the top part of the hairpin of constructs 40 and 41 that are not efficiently processed into siRNAs. These results indicate that this part of the e-shRNA is rapidly degraded because it is not processed and taken up by the RNAi machinery.

Efficient HIV-1 inhibition by e-shRNAs

Next, we tested whether these e-shRNAs are indeed efficient inhibitors of HIV-1. The target sites for the

encoded pol and nef siRNAs are indicated in the HIV-1 genome (Figure 5A). To quantify the antiviral effects of these hairpins, we co-transfected the HIV-1 molecular clone pLAI with an increasing amount of the pol-nef and nef-pol construct series (40–44) in 293T cells. HIV-1 production was measured by determining CA-p24 levels in the culture supernatant 2 days post-transfection. CA-p24 levels were normalized to the renilla luciferase activities of the co-transfected control plasmid. Virus production in the presence of the empty vector was set at 100%. We observed optimal inhibition of HIV-1 production by the pol-nef 42 and 43 and nef-pol 43 constructs (Figure 5B). These results correlate with the luciferase inhibition data in Figure 2C and the processing results in Figure 4B and C.

As an additional control for the sequence-specificity of the antiviral e-shRNAs, we constructed two control hairpin constructs of 43 bp. One targets two sites within the luciferase reporter gene (hLuc) and the other contains the scrambled sequence of the 43 e-shRNA variant (hSCR). Neither hLuc nor hSCR inhibited HIV-1 production, indicating that virus suppression by the e-shRNAs is fully sequence-specific (Figure 5C).
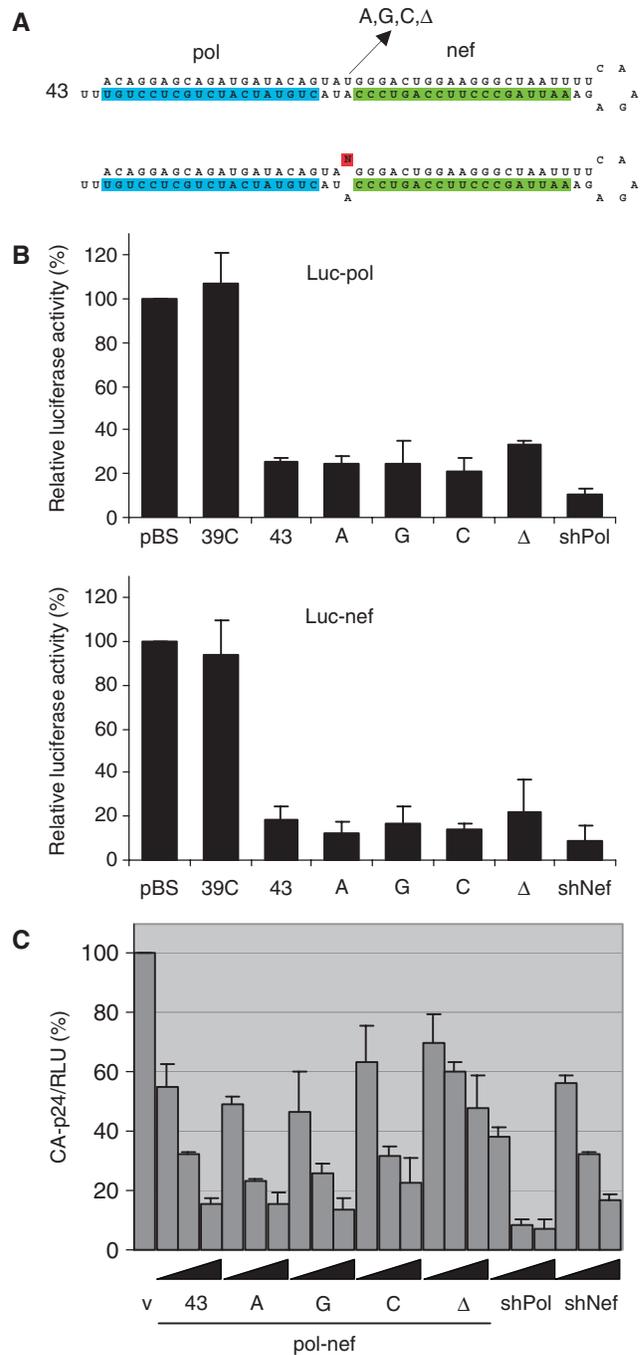
Towards user-friendly e-shRNAs

There are several technical problems associated with the construction of hairpin RNA expression constructs (35). First, it is difficult to sequence constructs containing lengthy inverted repeats. Second, these constructs are unstable in *E. coli*, resulting in a high mutation rate in the hairpin region. We experienced similar problems with the e-shRNA constructs. We were able to sequence the constructs using an optimized protocol (98°C sample denaturation and addition of 1M Betaine) (27). We attempted to modify the hairpins to allow easy cloning and sequencing, obviously without loss of their silencing activity. Therefore, we designed variants of the pol-nef 43 hairpin that are less stable, without affecting the guide/antisense strand of the hairpin RNA. We mutated or deleted the third nucleotide of the linker in the passenger/sense strand, which results in hairpins with a destabilizing mismatch in the central stem regions (Figure 6A). Indeed, these modifications allow easier sequencing and improve the genetic stability of the DNA construct (data not shown). A regular sequencing protocol can now be used to sequence these constructs.

Next, we tested the silencing activities of the destabilized hairpins compared to the original pol-nef 43 construct on Luc-pol and Luc-nef reporters. In general, we observed no significant loss of RNAi inhibition on the pol and nef reporter, although a slight reduction was observed for the deletion mutant, which creates a 1 nt internal loop (Figure 6B). To test for HIV-1 inhibition, we co-transfected 293T cells with the pLAI molecular clone, the renilla expression plasmid and an increasing amount of the modified hairpin constructs. All mutant hairpins showed similar suppression compared to the parental pol-nef 43 construct, except for the mutant with the 1 nt deletion (Figure 6C). These results indicate that a single
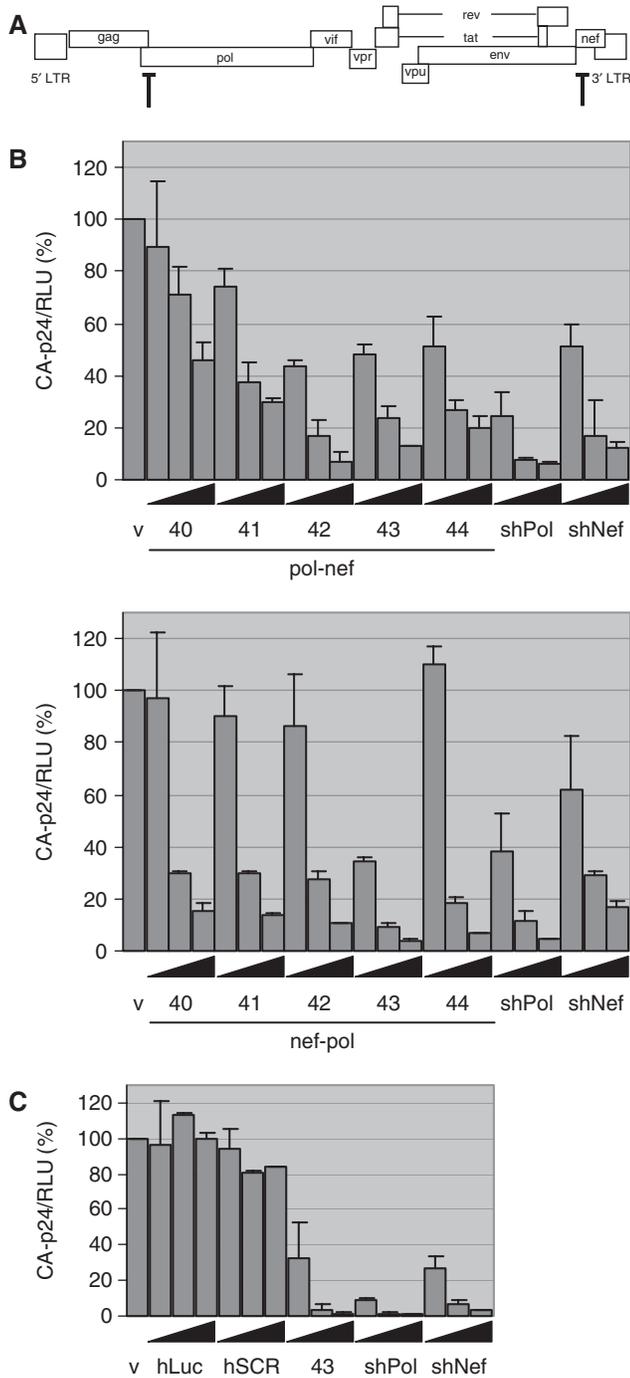
Figure 5. Inhibition of HIV-1 production by e-shRNA constructs. (**A**) The HIV-1 genome and the position of the target sequences for the e-shRNA derived siRNAs. (**B**) 293T cells were co-transfected with 250 ng of the HIV-1 pLAI, 1 ng of renilla luciferase plasmid and 2.5, 10 or 25 ng of the e-shRNA constructs. Two days post-transfection, inhibition of HIV-1 production was determined by measuring CA-p24 levels in the culture supernatant. CA-p24 levels were normalized to the renilla luciferase activities. The ratio between the CA-p24 level and the renilla luciferase activity in the presence of 25 ng of the empty vector (v) was set at 100%. (**C**) 293T cells were co-transfected with two control hairpin constructs. One encodes two siRNAs targeting the luciferase gene (hLuc) and the other encodes the scrambled sequence of the 43 bp e-shRNA (hSCR). The nef-pol 43 bp construct was used as a positive control. The level of HIV-1 inhibition was determined as described in B. Averages and standard deviations represent two independent experiments.

Figure 6. Towards user-friendly e-shRNA variants. (**A**) A mutation or deletion was introduced in the passenger/sense strand of the pol-nef 43 hairpin, which creates a mismatch in the central stem region. (**B**) 293T cells were co-transfected with 10 ng of the modified e-shRNAs, 100 ng of the Luc-pol or Luc-nef and 1 ng of the renilla luciferase plasmid. Two days post-transfection relative luciferase activities were determined using the firefly and renilla luciferase expression ratios. Luciferase expression in the presence of pBS was set at 100%. Averages and standard deviations represent three independent transfections. (**C**) Inhibition of HIV-1 production by the modified e-shRNAs was determined by co-transfection of 293T cells with 2.5, 10 or 25 ng of the hairpins, 250 ng of the HIV-1 pLAI and 1 ng of the renilla luciferase plasmid. CA-p24 levels were measured from the culture supernatant 2 days post-transfection. The ratio between the CA-p24 and the renilla luciferase activity in the presence of 25 ng of the empty vector (v) was set at 100%. Averages and standard deviations represent two independent experiments.
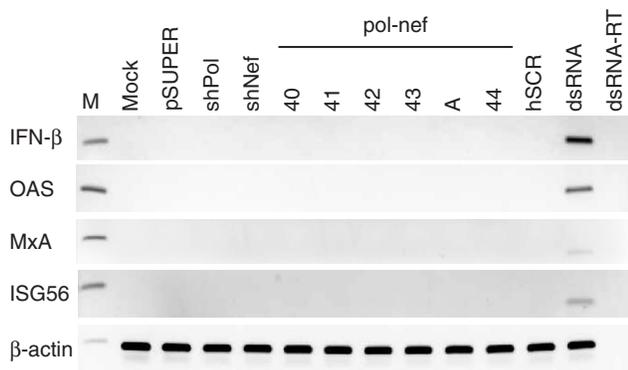
Figure 7. E-shRNAs do not induce an interferon response. Total RNA was purified from 293T cells transfected with the pol-nef e-shRNA variants 40–44, the 43 bp A mutant, the original shRNAs and a 43 bp hairpin encoding the scrambled sequence of the 43 bp e-shRNA (hSCR). The IFN-β, OAS, MxA and ISG56 expression levels were determined by RT–PCR. β-actin mRNA expression serves as an internal control. Negative controls include the mock (H$_2$O) transfection and the PCR reaction on RNA extracts derived from dsRNA transfected cells that were not subjected to reverse transcription. *In vitro* transcribed dsRNA (300 bp nef sequences) act as positive control for IFN-β induction. The 200 bp lane of the SmartLadder (Eurogentec) is shown as a size reference. For the OAS amplicon product, the 400 bp lane of the SmartLadder is shown.

nucleotide substitution, causing a mismatch in the center of the e-shRNA, is well-tolerated.

E-shRNAs do not induce the IFN response

Because the IFN response can be induced by long dsRNAs, we performed extensive RT–PCR analyses for markers of the IFN response on e-shRNA transfected 293T cells. The expression of the pol-nef e-shRNAs and the 43 bp A mutant (Figure 6A) does not induce the expression of IFN-β, OAS, MxA and ISG56 mRNAs (Figure 7). As a positive control, we used *in vitro* transcribed long dsRNAs of 300 bp. The expression of long dsRNA resulted in an IFN response in the transfected cells, showing that 293T cells are able to activate an innate IFN response.

DISCUSSION

HIV-1 replication can be efficiently inhibited via induction of RNAi using shRNA expressing constructs. However, HIV-1 can easily escape from RNAi by introducing mutations or deletions in the target sequence. For effective inhibition of HIV-1 replication, multiple shRNAs should be used simultaneously. In addition, highly conserved sequences within the HIV-1 RNA genome should be targeted to further reduce the chance of escape. The use of e-shRNA may be a promising approach to target HIV-1 at multiple sites, but little is known about the design of effective lhRNAs. In this study, we attempted to explore some principles for the design of e-shRNAs that encode two active siRNAs. We first constructed extended hairpin RNAs of 39 bp and varied the position of a potent siRNA in the stem region. We observed that positioning of the siRNA at the base of the hairpin stem yields optimal

RNAi activity. This observation is consistent with the proposed model for Dicer action, in which it counts approximately 22 nt from the 3′ end of the stem region to determine the position of cleavage (36,37). Consistent with this mechanism, Siolas *et al*. (37) showed that synthetic 29-mer shRNAs with 3′ overhangs were processed *in vitro* and *in vivo* from the 3′ end into discrete products of 21 and 22 nt.

Next, we combined two effective siRNAs to build extended hairpin RNAs of 40–44 bp. We demonstrate that a minimal length of 43 bp is needed to generate two effective siRNAs. In HIV-1 inhibition assays, we observed an optimal activity when the pol-nef hairpin is 42 or 43 bp. The pol-nef e-shRNA transcripts end with a U and the pol III promoter terminates after the second U, yielding a transcript with a 1 nt instead of a 2 nt 3′ overhang. This may be the cause for the 1 nt difference in length requirement in the HIV-1 inhibition assays. Indeed, hairpins with 1 nt or 2 nt overhangs have different Dicer cleavage sites *in vitro*, generating siRNA products of different sizes (36). We showed that RNAi activity observed in luciferase assays correlates with the *in vivo* processing data. Importantly, by removal of nucleotides in the guide/antisense strand of the hairpin RNA, the RNAi activity of the e-shRNAs is significantly impaired, indicating that the inhibition is sequence-specific. We demonstrated that these e-shRNAs profoundly inhibit HIV-1 replication. Furthermore, we designed user-friendly e-shRNA variants by introducing destabilizing mutations to avoid cloning/sequencing problems due to the lengthy inverted repeat sequences, obviously without loss of inhibitory capacity. We found similar knockdown efficiencies for the modified hairpins compared to the original e-shRNA.

Several studies have demonstrated that multiple siRNA species can be detected when a lhRNA is expressed in cells (18,20). However, these studies did not carefully examine the minimal length requirement of the hairpin to produce multiple siRNAs. Here, we show that the siRNA at the base of the hairpin is always active in an e-shRNA, but activation of the top siRNA requires a minimal stem length of 43 bp. Importantly, the e-shRNA constructs effectively inhibit two viral genes simultaneously, which should prevent or delay viral escape.

Previous reports show that inclusion of multiple mutations within the sense strand, thus creating G-U basepairs in the hairpin stem, can improve the stability of the plasmid in *E. coli* (18,35). Interestingly, it has also been shown that introduction of G-U bp can avoid the interferon response (18,19) and improve the suppressive activity of the hairpin (35). We also observed improved stability of a plasmid expressing a modified hairpin with only a single point mutation in the sense strand that destabilized the hairpin. Importantly, inclusion of this point mutation does not reduce the RNAi activity of these hairpins.

We showed that expression of the e-shRNAs does not trigger the innate antiviral IFN response. Notably, cytotoxicity can also be induced by overexpression of shRNAs, which leads to competition for and oversaturation of endogenous cellular RNAi components (38).

This emphasizes the need to use minimal amounts of shRNA expressing vectors and carefully designed shRNAs. Therefore, we think it is more effective to precisely stack potent siRNAs (43 bp) instead of using an extended consecutive sequence. The latter design may produce moderately active siRNAs that may saturate the RNAi pathway. Based on our results we suggest that the use of e-shRNAs with a specific hairpin length to ensure proper processing may be beneficial in silencing RNA viruses in a multi-targeting approach. Furthermore, based on our data and that of others (18–20,35), we would advise to introduce multiple point mutations in the sense strand of the hairpin to avoid the interferon response, enhance the stability of the plasmid in *E. coli* and to facilitate sequencing.

In conclusion, our results provide building principles for the design of multi-siRNA producing hairpins or e-shRNAs. We demonstrate that the hairpin stem length is critical for proper processing and optimal activity. In addition, expression of the e-shRNAs does not result in activation of the IFN response. Our e-shRNA constructs may be an initial step towards the design of further extended multi-siRNA transcripts. This study stresses the importance of a careful design of such hairpin RNA molecules.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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