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Maintaining Inhibition: siRNA Double Expression Vectors Against Coxsackieviral RNAs

Steffen Schubert[1], Hans-Peter Grunert[2], Heinz Zeichhardt[2], Denise Werk[1] Volker A. Erdmann[1] and Jens Kurreck[1]*

[1]*Institute for Chemistry (Biochemistry), Free University Berlin, Thielallee 63, D-14195 Berlin, Germany*

[2]*Department of Virology Institute for Infectious Diseases Medicine, Charité-University Medicine Berlin, Campus Benjamin Franklin Hindenburgdamm 27, D-12203 Berlin, Germany*

**Corresponding author*

The potential of RNA interference (RNAi) to inhibit virus propagation has been well established in recent years. In several studies, however, emergence of viral escape mutants after prolonged exposure to RNAi has been observed, raising a major hurdle for a possible therapeutic application of this strategy. Here, we report the design and characterisation of a vector that allows the simultaneous expression of two short hairpin RNAs (shRNAs), thereby maintaining high silencing activity even against a viral RNA bearing mutations in one of the target sites. Two short interfering RNAs (siRNAs) against the 3D-RNA dependent RNA polymerase of coxsackievirus B3 were identified that displayed efficient inhibition of virus propagation in HeLa cells and reduced the virus titre by up to 90%. We generated two expression vectors encoding these newly identified siRNAs and evaluated their silencing efficiency against the target gene in a reporter assay. Viral escape was then simulated by introducing a point mutation into either of the target sites. This substitution led to complete abrogation of silencing by the respective vector. To bypass this blockade of silencing, an siRNA double expression vector (SiDEx) was constructed to achieve simultaneous expression of both siRNAs from one plasmid. The silencing efficiency of both siRNAs generated by SiDEx was comparable to that of the individual mono-expression vectors. In contrast to the conventional expression vectors, SiDEx displayed substantial gene regulation also of the mutated target RNA. As our approach of expressing various shRNAs from one vector is based on a simple and universally applicable cloning strategy, SiDEx may be a helpful tool to achieve sustained silencing of viruses, ultimately reducing the risk of emergence of viable mutants. An additional application of SiDEx vectors will be the simultaneous knock-down of two targeted genes for functional studies.

Keywords: shRNA; RNAi; viral escape; enterovirus; coxsackievirus

Introduction

RNA interference (RNAi) is a process of post-transcriptional gene silencing induced by double-stranded RNA. In mammalian cells, the introduction of double-stranded small interfering RNAs (siRNA) 21 nucleotides in length can be used to inhibit the expression of a target gene in a sequence-specific manner.[1] Originally, chemically presynthesised siRNAs were used for silencing, whereas more recently, vectors have been developed that express short hairpin RNAs (shRNA) that are processed intracellularly, giving rise to siRNAs.[2] The siRNA is then incorporated into a protein complex referred to as the RNAi-induced silencing complex (RISC) that harbours helicase and nuclease activity. The anti-sense strand of the siRNA guides RISC to the target RNA, which is eventually cleaved by an endo-nuclease.[3–5]

The use of these vectors makes possible long-term silencing of the target gene by the selection of stably transfected cells.[6] Furthermore, shRNA

Abbreviations used: RNAi, RNA interference; siRNA, short interfering RNA; shRNA, short hairpin RNA; RISC, RNAi-induced silencing complex; GFP, green fluorescent protein; RdRP, RNA-dependent RNA polymerase; CBV-3, coxsackievirus B3; SiDEx, siRNA double expression vector.

E-mail address of the corresponding author: jkurreck@chemie.fu-berlin.de

expression cassettes can be incorporated into viral vectors that can be employed for gene therapeutic approaches. Gene silencing by RNAi has been shown to be a highly efficient process both *in vitro* and *in vivo*.[7–9] Consequently, it has already found widespread application in target validation for drug research, and its potential use for therapeutic purposes is under debate.[10–13]

One focus of research is the development of RNAi approaches to treat viral infections.[14] This strategy has been demonstrated to be effective for the inhibition of replication of several different viruses in cell culture, including human immunodeficiency virus type 1, hepatitis C virus, and influenza virus A. In *in vivo* studies, the replication of hepatitis B virus has been repressed successfully in mice.[15,16] RNAi seems to be particularly suited to target cytoplasmic RNA viruses, since viral genomes of DNA viruses and integrated proviral DNAs of retroviruses are not susceptible to siRNAs.

Picornaviruses are a family of (+)-stranded RNA viruses comprising some major pathogens of clinical and agricultural relevance including rhinoviruses, poliovirus, coxsackieviruses and foot-and mouth disease virus.[17] It is therefore surprising that only few studies have been published that describe RNAi approaches against members of this family.[18,19] To our knowledge, no siRNA targeting coxsackieviruses has been published, in spite of the fact that these viruses are known to cause severe diseases with high mortality rates, like meningitis and myocarditis.[20]

Long-term silencing of virus replication by RNAi has been reported to result in the emergence of escape mutants. This problem is particularly relevant for RNA viruses that exhibit increased genetic variation due to an error-prone replication machinery.[18,21,22] In the studies cited, siRNAs against human immunodeficiency virus type I and poliovirus, respectively, were found to inhibit virus replication initially, but upon prolonged incubation virus titres increased again. Sequence analysis of the progeny viruses revealed either a point mutation in the siRNA target region or extensive rearrangements to be responsible for virus escape from silencing.

Here, we report the design of siRNAs directed against the RNA-dependent RNA polymerase (RdRP) gene of coxsackievirus B3 (CBV-3) that efficiently reduce viral reproduction in HeLa cells. Transcription of shRNAs of the same sequence under control of a human U6 promoter inhibited expression of a reporter fused to the viral gene. Even one artificially introduced mutation in the centre of the target site of one of the siRNAs, however, completely blocked silencing of gene expression by the corresponding siRNA expression vector. We therefore developed an siRNA double expression vector termed SiDEx that encodes two distinct siRNAs. This vector maintained silencing activity also against the mutated viral RNA. SiDEx vectors thus seem to be highly suitable tools to achieve long-term silencing of viral gene expression and eventually to prevent the enrichment of escape mutants.

Results

The object of our work was the development of an efficient RNA interference strategy against coxsackievirus B3, a major myocardial pathogen. To select suitable molecules for this approach, we initially evaluated knockdown efficiencies of a number of chemically synthesized siRNAs targeted against a region on the viral mRNA that codes for the 3D-RNA dependent RNA polymerase (RdRP), a protein essential for virus replication. A convenient activity assay for siRNAs was established by cloning the RdRP-cDNA of CBV-3 (nucleotides 5911–7296) into an expression vector immediately trailing a green fluorescent protein (GFP) coding sequence and a TGA translational stop codon (Figure 1(a)). The viral sequence was thus turned into an artificial 3′-untranslated region of the GFP mRNA. Transfection of the resulting target vector into Cos-7 cells led to strong GFP fluorescence. Successful targeting of the viral sequence by siRNAs diminished GFP expression, since cleavage of the chimeric mRNA induced by siRNAs results in immediate degradation of the complete mRNA molecule.

Two siRNAs directed against nucleotides 6315–6333 (siRNA2) and 6736–6754 (siRNA4), respectively, were found to cause strong specific inhibition

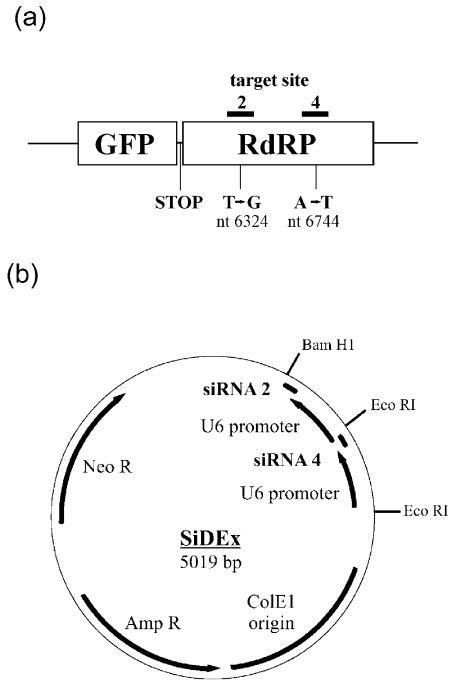


Figure 1. (a) Set-up of the target mRNA. The gene of interest (CBV-3 3D-RdRP, nucleotides 5911–7296) is fused to GFP as a reporter, separated by a translational stop codon. Target sites for pSiR2 and pSiR4 are indicated, as are the sites of point mutations in the mutated RdRP. (b) Design of the double expression vector, SiDEx.

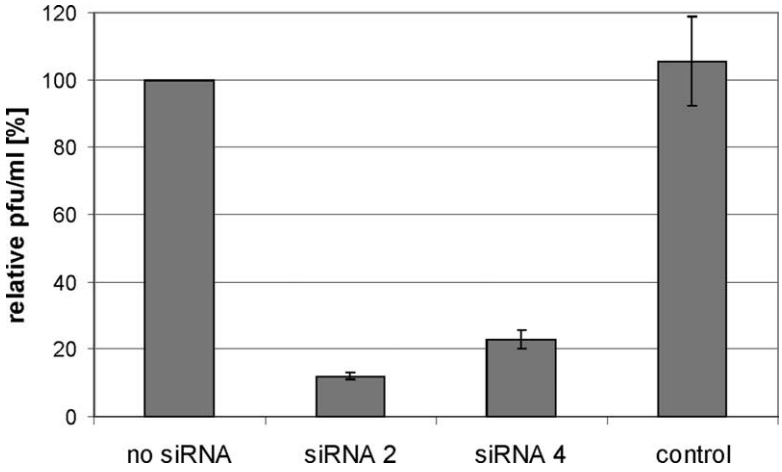


Figure 2. Relative reduction of viral titre by 100 nM of the indicated siRNAs. Titre of coxsackievirus B3 is expressed as plaque-forming units (p.f.u.)/ml on HeLa cells. Plaque reduction reveals inhibition of virus reproduction induced by siRNA.

of gene expression in cotransfection experiments even at subnanomolar concentrations (data not shown). These siRNAs were subsequently employed in a viral plaque reduction assay in HeLa cells. In this assay, inhibition of virus reproduction in the presence of siRNAs is expressed by a reduced number of virus-induced plaques in comparison to virus-infected cells in the absence of inhibitor. At 100 nM, siRNA2 reduced the virus titre by 90%, while siRNA4 displayed approximately 80% inhibition (Figure 2).

In order to obtain long-term silencing of the targeted gene, cDNA constructs yielding shRNAs of these highly effective siRNAs were designed and cloned into separate pSilencer vectors (Ambion Inc.). The resulting molecules, designated pSiR2 and pSiR4, respectively, showed efficient knock-down of the RdRP-GFP fusion construct over a broad range of concentrations (Figure 3), making both agents promising tools for possible antiviral applications. Interestingly, siRNA2 was found to be more efficient in the virus plaque reduction assay (Figure 2), whereas the vector encoding siRNA4 displayed stronger silencing of the viral gene in the reporter assay.

Antiviral strategies, however, are often counteracted by the high rate of mutation that is encountered with many viruses. We wanted to investigate to what extent a mutation in one of the target sites would be detrimental to our knock-down-strategy. We substituted thymidine at position 6324, the central nucleotide of the pSiR2 target site, by a guanosine residue (see Figure 1(a)). Figure 4 shows that this single mutation alone was indeed enough to abrogate target gene inhibition by pSiR2 completely. As expected, the action of pSiR4 on the target gene remained unaffected.

This finding underlines the susceptibility of RNAi approaches to viral escape, since a single central point mutation may be sufficient to render an siRNA inactive. In conventional antiviral combination therapy, this risk is addressed and alleviated by the simultaneous employment of several efficient drugs. An analogous reasoning prompted us to devise a single tool expressing two efficient shRNAs at the same time to achieve knock-down of a target gene. A mutation in one target site could then be compensated by the unaltered efficiency of the other shRNA.

We applied a simple cloning strategy: an EcoRI restriction site was introduced into pSiR4, the "donor" vector, downstream of the sequence coding for the shRNA. This additional feature allowed us to excise the complete expression cassette

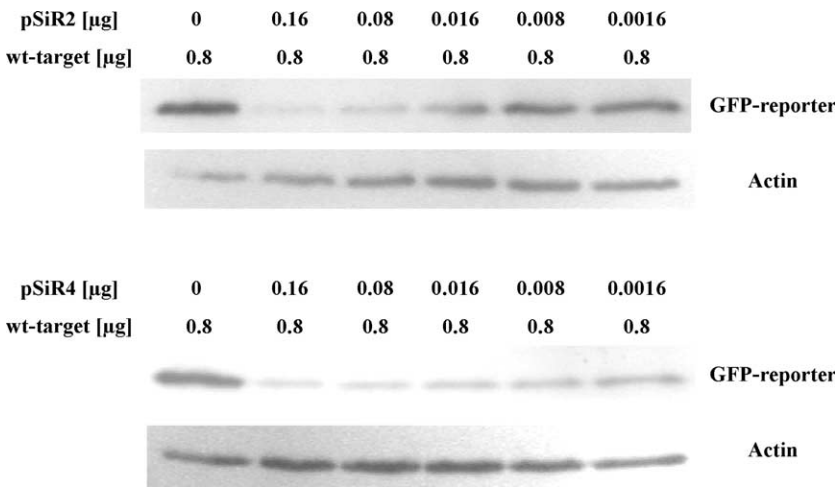


Figure 3. Western blots showing down-regulation of GFP reporter by vectors pSiR2 (upper panel) and pSiR4 (lower panel). GFP was expressed from 0.8 µg of a GFP-wild-type RdRP fusion plasmid (wt-target). The indicated amount of pSilencer vector was employed. Actin is included as a loading control.

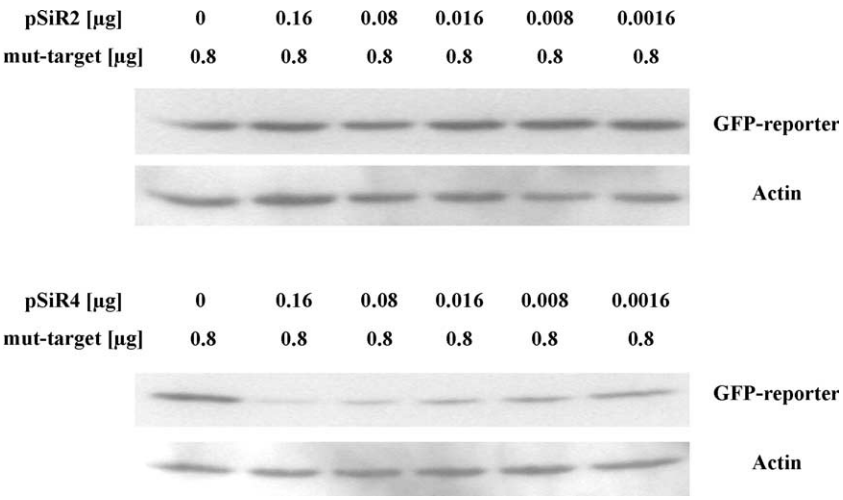


Figure 4. Western blots showing down-regulation of GFP reporter expressed from a GFP-RdRP fusion plasmid containing a point mutation in target site 2 (mut-target). Regulation by pSiR2 (upper panel) and pSiR4 (lower panel) is shown; 0.8 µg of target vector and the indicated amounts of pSilencer were employed. Actin is included as a loading control.

consisting of U6 promoter and shRNA coding sequence *via* EcoRI restriction, and to insert this fragment at the unique EcoRI restriction site in pSiR2, the "acceptor". The resulting vector contained the two expression cassettes for the shRNAs in tandem formation (Figure 1(b)). The new molecule was termed siRNA double expression vector, SiDEx.

In order to establish the functionality of the shRNAs expressed from the novel vector, we designed a set-up that allowed us to monitor silencing by each shRNA individually and to compare their efficiency to that seen with pSiR2 and pSiR4. The 19mer target sequence for either shRNA was placed directly upstream of a GFP-coding sequence, so that the decrease in GFP expression could again be used as a marker for RNA interference. Both pSiR2 and pSiR4 showed efficient regulation of GFP expression *via* their respective target sequences (Figure 5(a), black and white columns, respectively). Conversely, pSiR2 gave no regulation of GFP fused to target site 4 and *vice versa* (data not shown). Importantly, the double expression vector SiDEx caused GFP knockdown with both target sequences. As can be seen in the quantification of target gene expression in Figure 5(a) for target sites 2 (upper diagram) and 4 (lower diagram), target regulation by SiDEx (grey columns) was virtually indistinguishable from that by the respective pSilencer vectors over a range of at least three orders of magnitude. We infer from these findings that SiDEx generates functional shRNAs that are not obstructed in the capacity to silence their respective target.

After having shown that both shRNAs of the SiDEx vector were highly efficient, we employed SiDEx to regulate the expression of wild-type full-length GFP-RdRP. As expected, target protein expression was diminished extensively when SiDEx was employed at molar ratios of target vector to SiDEx of 1 : 0.2 to 1 : 0.001 (Figure 5(b)). The observed knockdown induced by SiDEx closely resembles the inhibition seen with each of the two pSilencer vectors (Figure 3), but it was not found to be significantly stronger or extending to lower concentrations of vector.

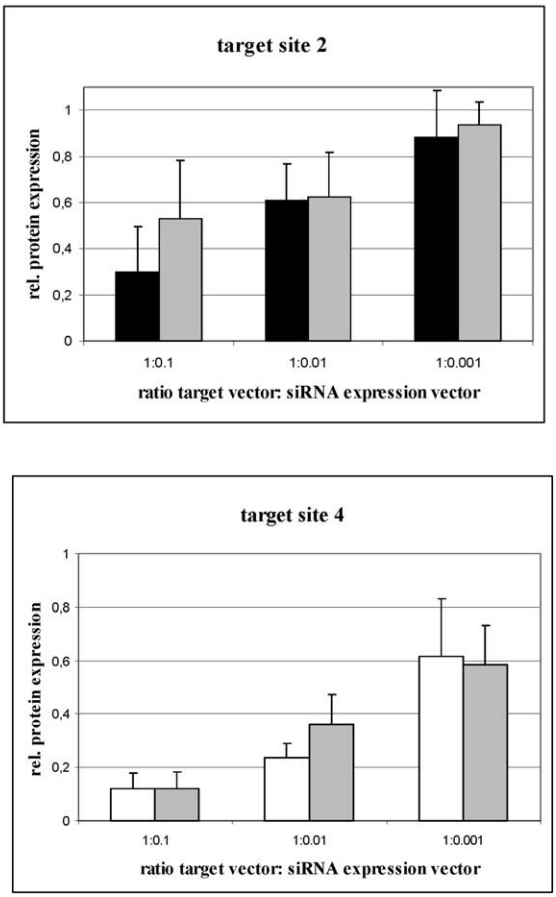
The specific advantage of the novel double expression vector, however, was anticipated to be its sustained activity against an mRNA with a mutation in one of the target sites that renders the respective single shRNA ineffective. We thus employed the target vector described above, containing a T to G point mutation at nucleotide 6324, in the centre of the target site of pSiR2.

Figure 6(a) illustrates strong silencing of the artificially mutated gene by SiDEx. Quantification of the knockdown as compared to that seen with pSiR2 and pSiR4 yielded virtually the same silencing efficiency for SiDEx and pSiR4, which is directed against the unmutated site (Figure 6(c)). As an additional verification of the general applicability of the SiDEx vector, we generated an RdRP-GFP fusion gene bearing a point mutation in the centre of target site 4. While pSiR2 silenced the mutated gene to an extent similar to that for the wild-type RdRP, the mutation abolished silencing by pSiR4. Again, SiDEx compensated this loss of activity and reduced the level of reporter to the same level as did pSiR2 (Figure 6(b)). We conclude from our findings that our strategy to devise a double expression vector for efficient shRNAs is an easy way to overcome reduced silencing due to mutations in a target molecule that may find numerous applications, especially in antiviral treatments.

Discussion

Coxsackievirus B3 is a major myocardial pathogen. To our knowledge, this study is the first report of siRNAs capable of interfering with coxsackievirus reproduction in cell culture experiments. Two independent siRNAs targeting the 3D-RNA dependent RNA polymerase are shown to reduce virus titre by 80% and 90%, respectively. In a previous publication, Yuan *et al.* reported inhibition of CBV-3 by an antisense

(a)



(b)

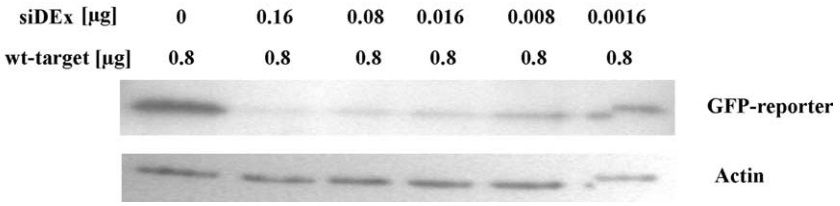


Figure 5. (a) Quantification of specific gene-knockdown by pSiR2 (black columns), pSiR4 (white columns), and SiDEx (grey columns) acting on target sites 2 (upper diagram) and 4 (lower diagram). (b) Western blot showing down-regulation of GFP reporter by SiDEx. GFP was expressed from 0.8 µg of a GFP-RdRP wild-type fusion vector. The indicated amount of SiDEx was used. Actin is included as a loading control.

oligonucleotide.[23] The phosphorothioate was employed at a concentration of 10 µM and reduced virus titre by 0.5 $\log_{10}$. With the siRNAs presented in this study, a 100-fold lower concentration was sufficient to achieve a reduction of one order of magnitude. Even though experimental set-ups differ significantly between the two studies, for example different cell lines were employed, the results presented here serve to demonstrate the enormous potential of the RNAi approach.

To construct tools for sustained inhibition of virus propagation, oligonucleotides encoding the siRNAs as hairpin constructs were subsequently cloned into pSilencer 2.1-U6 neo. The resulting vectors, designated pSiR2 and pSiR4, silenced the RdRP-GFP fusion construct in co-transfection experiments even at large excess of target plasmid. They are therefore likely to be suitable also for long-term inhibition of the target.[6]

Viral escape as a result of long-term exposure to antiviral RNAi has been observed in several studies recently.[18,21,22] Two strategies have been proposed to overcome this limitation of RNAi applicability: (i) Targeting of conserved sequences is thought to lower the frequency of the generation of viable mutants. It should be mentioned, however, that this strategy is better suited for antiviral agents that act at the protein level than for anti-RNA approaches,

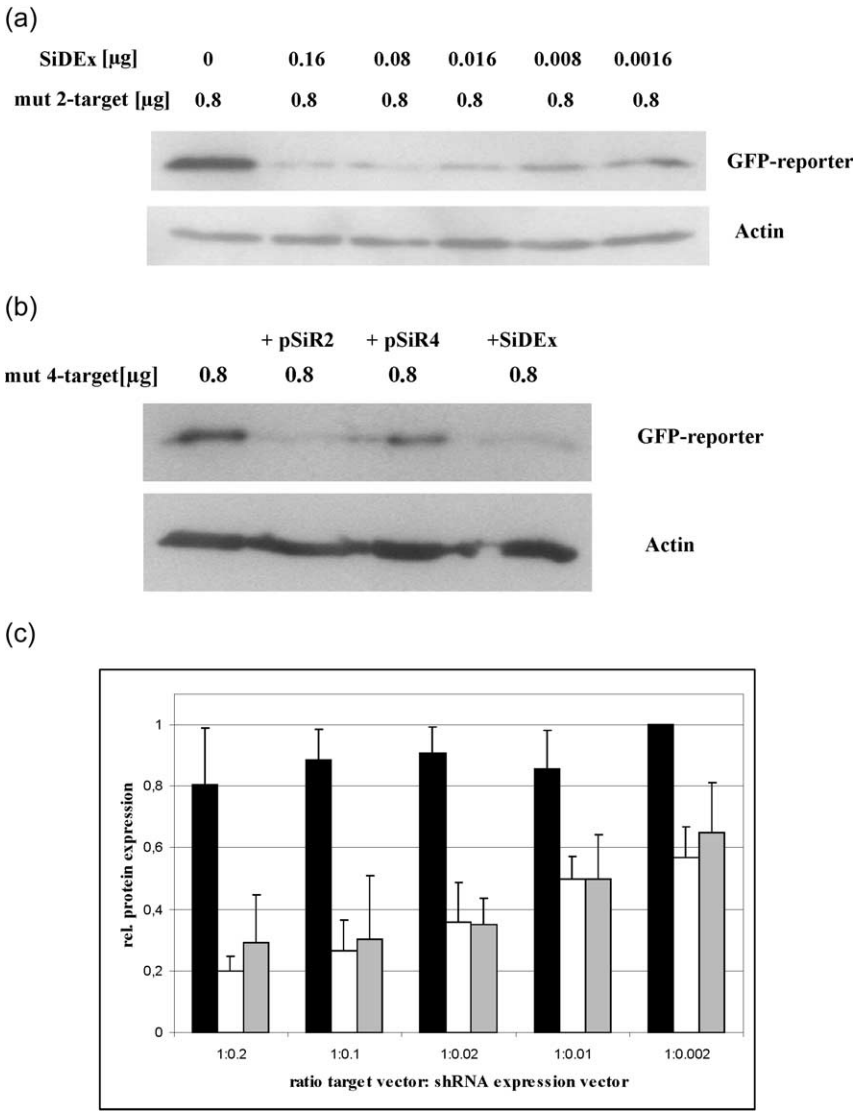


Figure 6. Down-regulation of GFP reporter expressed from a GFP-RdRP fusion plasmid containing a point mutation in one of the target sites. (a) Western blot showing specific gene-knockdown of GFP-RdRP with a mutation in target site 2 by SiDEx. Actin is included as a loading control. (b) Specific gene regulation of the GFP-RdRP fusion construct containing a point mutation in target site 4 with 0.16 µg of the indicated expression plasmids. (c) Quantification of knockdown of GFP-RdRP with a mutation in target site 2 by pSiR2 (black columns), pSiR4 (white columns), and SiDEx (grey columns).

since in the latter case viruses might escape inhibition by RNAi due to silent mutations without changes of the amino acid sequence.[18] (ii) Alternatively, the probability of generating escape mutants may be reduced by expressing two efficient shRNAs simultaneously.

To simulate the emergence of an escape mutant and to investigate the effect of base substitutions in one of our target sites, we generated artificial point mutations. Since viral escape mutants had been found to contain mutations in the centre of the siRNA target site,[18,21] a position highly sensitive to base substitutions, we decided to exchange thymidine in position 10 of the 19 nucleotides long target site 2 by guanosine. Silencing by the respective shRNA expression vector was found to be abolished. Inhibition of gene expression by the second shRNA targeting a distant region, however, remained unchanged, as expected. A corresponding result was seen when the central adenosine base in target site 4 was exchanged for thymidine. This observation suggested that the use of both shRNAs in combination would be a suitable countermeasure against target mutations deleterious for one siRNA. Consequently, we devised a cloning strategy for the construction of a silencer vector encoding both shRNAs. The resulting siRNA double expression vector was termed SiDEx.

Two promoters in close neighbourhood may cause genetic instabilities or influence expression levels, and it has been argued recently that the presence of multiple siRNAs may lead to a saturation of the proteinaceous silencing complex RISC, thereby reducing the incorporation of one or both siRNAs into the complex and decreasing their activity. To ensure that both siRNAs generated by SiDEx are functional, we developed reporter vectors that contained the isolated siRNA target sequence upstream of GFP. Expression of the reporter from plasmids encoding either target site 2 or 4 was inhibited by SiDEx with virtually the same efficiency as that observed for the two single shRNA expression vectors. These experiments made us confident that both shRNAs derived

from the SiDEx vector were fully functional, and the concerns named above do not apply to the two shRNAs used in our experiments.

Our reporter system is likely to be of use in verifying shRNA transcription from plasmids containing multiple expression cassettes, and to examine whether poor silencing activity by conventional shRNA-encoding vectors is due to weak expression. Various factors, including features of the promoter, the starter nucleotide of the transcribed RNA or the cell line, influence the efficiency of shRNA expression from vectors. To clarify a possible influence of these factors, and to establish shRNA expression in new cell lines or primary cells, cloning of the target sequence upstream of a reporter gene is a simple and convenient method to verify the functionality of shRNAs independent of complex factors like target structure, interfering proteins etc.

When the SiDEx vector expressing two shRNAs was employed against the wild-type full-length RdRP sequence, it was found to silence the gene with great potency, comparable to that of the individual single shRNA expression plasmids, but we did not observe an additive effect of the two siRNAs. This finding is in contrast to a previous report that multiple siRNAs targeting the same mRNA result in enhanced gene silencing.[24] It should be noted, however, that only a rather small additive effect was found in the study cited. Furthermore, the authors used chemically synthesized siRNA, whereas in the present study, vector-derived shRNAs were employed.

The rationale of our efforts to develop a double siRNA expression vector, however, was not to increase its potency, but to sustain silencing activity against a target RNA even when point mutations in one of the siRNA target regions arise. Using the artificially mutated RdRPs as models for escape mutations, we show that SiDEx indeed maintains substantial activity in the presence of a mutation that is detrimental to silencing by the respective conventional shRNA expressing vector. The extent of silencing of the mutated RdRPs by SiDEx was comparable to the inhibition of gene expression by the respective single shRNA expression vector acting on the unmutated sequence. We therefore conclude that this approach is likely to be suitable to obstruct the emergence of escape mutants.

The next step will be the generation of stably transfected cells to investigate whether the SiDEx vector is superior in preventing enrichment of mutated viruses as compared to a conventional single shRNA expression vector. In particular, it will be interesting to analyse whether the degree of knockdown obtained by the siRNAs is sufficient to inhibit viral replication to completion. If necessary, however, it would be easy to extend the strategy described here to generate a vector that expresses three or more siRNAs in analogy to the triple combinations therapy known as highly active antiretroviral therapy (HAART) of AIDS patients.

In summary, we describe the generation of siRNAs that efficiently inhibit reproduction of CBV-3, a virus that, to the best of our knowledge, has not been targeted with the RNAi approach. We report a simple and easily applicable cloning strategy for a vector harbouring two shRNA expression cassettes, and we suggest a straightforward way of assessing functionality and efficacy of the shRNAs. The advantages of double expression vectors are demonstrated using a model system simulating the emergence of mutated viruses. The strategy to employ single vectors generating multiple shRNAs, however, is not restricted to antiviral approaches, but may be applied to knock down two or more targets at the same time in functional analyses.

Materials and Methods

siRNAs and oligonucleotides

siRNAs were designed using the Dharmacon criteria (Dharmacon, Lafayette, CO, USA) as a SMARTpool package. siRNA duplexes with symmetrical 3′-UU overhangs were purchased from MWG Biotech, Ebersberg, Germany. The target sequences of siRNA2 and siRNA4 were CUA AGG ACC UAA CAA AGU U (nucleotides 6315–6333 of coxsackievirus B3 genome, GenBank accession no. M33854), and GUA CAG GGA UAA ACA UUA C (nucleotides 6736–6754), respectively. A negative control siRNA was purchased from Qiagen, Hilden, Germany with the target sequence AAU UCU CCG AAC GUG UCA CGU, which has no matches either in the viral or the human genome. DNA oligonucleotides for PCR and cloning procedures were obtained from IBA, Göttingen, Germany and TIB MOLBIOL, Berlin, Germany.

Virus infection and plaque reduction assay

HeLa cells were cultured in six-well cell culture plates (Costar) as confluent monolayers at a density of 1.2×10^6 cells/well and incubated in Eagle's MEM (minimal essential medium) with 5% (v/v) fetal calf serum at 37 °C under 5% (v/v) CO_2. After 24 hours, cells were transfected with the respective siRNA (100 nM) or an unrelated control siRNA as described below. Four hours later, cells were infected with coxsackievirus B3 at a multiplicity of infection of 50 plaque-forming units (p.f.u.)/well for 30 minutes according to Dulbecco,[25] with modifications as described.[26] Subsequently, cells were overlaid with agar containing Eagle's MEM. Three days later, cells were stained with 0.025% (w/v) neutral red. Virus titre (p.f.u./ml) was determined by plaque counting. Values given are means and standard deviation of two independent experiments, each performed in duplicate.

Cloning and site-directed mutagenesis of CBV-3 RdRP

cDNA of the RNA-dependent RNA polymerase of CBV-3 was obtained by reverse transcription and amplification of viral RNA using the PCR primers TGA AGG TGA AAT AGA ATT TAT TG and AAA GGA GTC CAA CCA CTT CC, corresponding to bases 5911–5931 and 7297–7277 of the CBV-3 sequence, respectively. The

underlined bases represent an added translational stop codon. Subsequently, the cDNA was cloned into a pcDNA3.1/NT-GFP-TOPO vector (Invitrogen, Karlsruhe, Germany), yielding a GFP-Stop-RdRP construct.

For the generation of point mutations in the RdRP cDNA, a fragment of the vector described above was subcloned into pUC19 to obtain a smaller vector. Substitutions were introduced using one of two primers bearing a base substitution with the QuikChange site-directed mutagenesis kit from Stratagene, La Jolla, CA, USA, according to the manufacturer's instructions. The sequences of the sense primers with the respective point mutation were: Mutprimer 2, corresponding to bases 6301–6352 of CBV-3, CCT CTC TAA GAA GAC TAA GGA CCG AAC AAA GTT AAA GGA ATG, with the thymidine to guanosine substitution underlined; Mutprimer 4, corresponding to bases 6727–6760, CCA TCA CCT GTA CAG GGT TAA ACA TTA CTT TGT G, with the adenosine to thymidine substitution underlined.

Generation of target site-GFP fusion constructs

Sense and antisense oligodeoxynucleotides were designed corresponding to the 19mer siRNA target sites and containing overhangs for KpnI and XbaI restriction sites. The sequences used were: target site 2 sense, GAC TAA GGA CCT AAC AAA GTT; target site 2 antisense, CTA GAA CTT TGT TAG GTC CTT AGT CGT AC; target site 4 sense, CTG TAC AGG GAT AAA CAT TAC; target site 4 antisense, CTA GGT AAT GTT TAT CCC TGT ACA GGT AC. 100 pmol of the respective strands were annealed by heating to 95 °C for four minutes in annealing buffer (100 mM potassium acetate, 30 mM Hepes-KOH (pH 7.4), 2 mM magnesium acetate) and subsequent cooling to room temperature. The annealed cDNA duplexes were then phosphorylated using phage T4 polynucleotide kinase (Promega, WI, USA) and ligated into a pcDNA3.1/CT-GFP TOPO vector (Invitrogen) that had been cut with XbaI and KpnI, and dephosphorylated prior to ligation.

Generation of pSilencer and SiDEx expressing shRNAs

DNA oligonucleotides encoding hairpin siRNAs were designed according to the requirements for cloning into pSilencer 2.1-U6 neo expression vector (Ambion, Huntingdon, UK). To facilitate the generation of a double expression vector, an additional EcoRI site was inserted downstream of the RNA polymerase terminator of shcDNA4. The oligodeoxynucleotides used were: shcDNA2 sense, GAT CCC GCT AAG GAC CTA ACA AAG TTT TCA AGA GAA ACT TTG TTA GGT CCT TAG TTT TTT GGA AA; shcDNA2 antisense, AGC TTT TCC AAA AAA CTA AGG ACC TAA CAA AGT TTC TCT TGA AAA CTT TGT TAG GTC CTT AGC GG; shcDNA4 sense, GAT CCC GTA CAG GGA TAA ACA TTA CTT CAA GAG AGT AAT GTT TAT CCC TGT ACT TTT TTG GAA GAA TTC A; shcDNA4 antisense, AGC TTG AAT TCT TCC AAA AAA GTA CAG GGA TAA ACA TTA CTC TCT TGA AGT AAT GTT TAT CCC TGT ACG G. Annealing and phosphorylation of the oligonucleotides were carried out as described above. Ligation into pSilencer 2.1-U6 neo was achieved using phage T4 ligase (Promega, Madison, WI, USA), yielding pSiR2 and pSiR4.

For the generation of the double expression vector SiDEx, EcoRI digests of pSiR4, the "donor", and pSiR2, the "acceptor" plasmid, were carried out. The fragment excised from pSiR4, containing approximately 400 base-pairs comprising the U6 promoter and shcDNA4, was cloned into pSiR2, yielding SiDEx.

Cell culture and transfection experiments

COS-7 cells (African green monkey kidney fibroblasts) were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (PAA laboratories, Coelbe, Germany) supplemented with 10% fetal bovine serum (PAA), 100 µg/ml of penicillin, and 100 µg/ml of streptomycin (Invitrogen). The day before transfection, cells were detached by treatment with trypsin, resuspended in medium without antibiotics and plated in 24-well plates at a density of 0.7×10^5 cells per well in a volume of 500 µl. Transfection and cotransfection experiments using 0.8 µg of the respective target-plasmid and the appropriate amount of shRNA expression vector were carried out using 2.5 µl of Lipofectamine 2000 (Invitrogen) per well, following the manufacturer's instructions.

Detection of protein knockdown by immunoblotting

At 24 hours after transfection, cells were lysed in 24-well plates with lysis buffer (125 mM Tris–HCl (pH 6.8), 4% (w/v) SDS, 1.4 M β-mercaptoethanol, 25% (v/v) glycerol, and 0.05% (w/v) bromphenol blue). After boiling the lysate at 95 °C for five minutes, proteins were separated on a 12.5% (w/v) polyacrylamide gel. Transfer of proteins to PVDF membranes (Amersham, Freiburg, Germany) was performed with a semi-dry blotter (Peqlab, Erlangen, Germany). For immunostaining, membranes were incubated with rabbit GFP antiserum (Invitrogen), diluted 1 : 5000 (w/w) in dry milk. Secondary antibodies conjugated with alkaline phosphatase (Chemicon, Hampshire, UK) were used at a dilution of 1 : 5000. Detection by chemiluminescence was achieved using CDP-Star (Roche, Mannheim, Germany). To confirm equal loading of the samples, membranes were stripped and reprobed with a monoclonal mouse antibody specific for actin (Chemicon, 1 : 5000 (w/w) dilution). Quantification of the blots was carried out with Quantity One software (Biorad, München, Germany). Values given are means ± standard deviation of three independent experiments.

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