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Local RNA Target Structure Influences siRNA Efficacy: Systematic Analysis of Intentionally Designed Binding Regions

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Contradictory reports in the literature have emphasised either the sequence of small interfering RNAs (siRNA) or the structure of their target molecules to be the major determinant of the efficiency of RNA interference (RNAi) approaches. In the present study, we analyse systematically the contributions of these parameters to siRNA activity by using deliberately designed mRNA constructs. The siRNA target sites were included in well-defined structural elements rendering them either highly accessible or completely involved in stable base-pairing. Furthermore, complementary sequence elements and various hairpins with different stem lengths and designs were used as target sites. Only one of the strands of the siRNA duplex was found to be capable of silencing *via* its respective target site, indicating that thermodynamic characteristics intrinsic to the siRNA strands are a basic determinant of siRNA activity. A significant obstruction of gene silencing by the same siRNA, however, was observed to be caused by structural features of the substrate RNA. Bioinformatic analysis of the mRNA structures suggests a direct correlation between the extent of gene-knockdown and the local free energy in the target region. Our findings indicate that, although a favourable siRNA sequence is a necessary prerequisite for efficient RNAi, complex target structures may limit the applicability even of carefully chosen siRNAs.

Keywords: RNA interference; small interfering RNA; siRNA; siRNA efficacy; RNA structure

Introduction

RNA interference (RNAi) denotes the sequence-specific cleavage of a target RNA induced by double-stranded RNA (dsRNA). The phenomenon was first discovered in the nematode *Caenorhabditis elegans*,[1] and was rapidly adopted as a powerful means to inhibit gene expression in various species. Its application in mammals, however, was initially foiled due to the fact that long dsRNAs activate the interferon response in higher eukaryotes, resulting in non-specific reduction of mRNA levels. A solution to this problem came with the finding that 21–23 bp long dsRNA fragments with 3′ overhangs of two nucleotides efficiently trigger sequence-specific mRNA degradation.[2] These so-called small interfering RNAs (siRNAs) are suited to inhibit the expression of targeted genes in mammalian cells without inducing the interferon response.[3] RNAi has since been used increasingly for functional genomics and is thought to be a promising new tool for therapeutic applications.[4–9]

The primary difficulty at the outset of an RNAi approach to silence a specific gene is the identification of efficient siRNAs. Initially, some basic criteria were defined to this end regarding GC

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Abbreviations used: RNAi, RNA interference; dsRNA, double-stranded RNA; siRNA, small interfering RNA; GFP, green fluorescent protein; VR1, rat vanilloid receptor subtype 1; TRP, transient receptor potential; UTR, untranslated region.

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content and position of the targeted sequence in the context of the RNA.[10] A drastic variation of silencing efficiency, however, was observed for different siRNAs compliant with these guidelines and targeting the same RNA.[11–14] Significant progress was achieved more recently, when the assembly of the RNAi enzyme complex was described as being dependent on thermodynamic characteristics of the siRNA.[11,15] The relative stability of both ends of the duplex was observed to have profound effects on the extent to which the individual strands enter the RNAi pathway. In addition, certain sequence motives at defined positions of the siRNA were reported to influence its potency.[16,17] On the basis of these findings, sophisticated algorithms have been developed to increase the success rate of siRNA design.[16–18]

The studies cited above focus exclusively on features of the siRNA, implying that a favourable thermodynamic organization of the duplex is a sufficient prerequisite for strong silencing activity. Other researchers, however, have observed that the inhibition of gene expression by siRNAs is dependent on the structure of the target RNA.[12,14,19] Luo and Chang, for example, found the number of hydrogen bonds formed between the target region and the rest of the mRNA to be a useful parameter, correlating negatively to silencing efficiency.[20] These contradictory findings prompted us to examine the contributions of siRNA sequence and target accessibility to silencing activity of a highly potent siRNA individually.

Here, we report that gene silencing by a potent siRNA is diminished drastically when target nucleotides are incorporated into various hairpin structures. The target sequence of a highly effective siRNA duplex described previously was included systematically in various intentionally designed structural environments upstream of the start codon of the green fluorescent protein (GFP).[21] Computer analysis of secondary structures resulted in a linear correlation between the local free energy of the target region and silencing of the reporter gene by the siRNA. In addition, we find one strand of the siRNA duplex to be clearly superior for the inhibition of gene expression, demonstrating that thermodynamic parameters of siRNAs are of fundamental importance in RNA interference. Our findings were transferred to an siRNA unable to silence its full-length target mRNA.[22] Interestingly, this duplex was highly active when acting on an isolated target sequence lacking higher-order structure, thus confirming our finding that features of the target molecule and characteristics inherent to the sequence of the siRNA itself can both be limiting factors of silencing efficiency. Our data correspond to the study by Overhoff *et al.* (see the accompanying article),[23] in which the influence of target structure on siRNA efficiency is established by screening a larger number of different siRNAs against natural targets and relating their efficacy to predicted target structures.

Results

It was the aim of our current study to analyse the contributions of intrinsic thermodynamic features of siRNAs and structural characteristics of their target molecules to the efficacy of RNAi approaches in detail. We examined these parameters using an siRNA, named VsiRNA1, that has been employed recently to silence the expression of the rat vanilloid receptor subtype 1 (VR1, TRPV1).[21] This transient receptor potential (TRP) channel is believed to function as a molecular integrator of various noxious stimuli.[24] Efficient inhibition of the expression of the full-length TRPV1 fused to GFP as a reporter in the subnanomolar range is shown in Figure 1(a).

To determine whether this remarkably high activity could be ascribed mainly to factors intrinsic to the siRNA sequence, or whether it was due to favourable characteristics of the target molecule, we took the 21mer target sequence out of the complex sequence context of the 2600 bases long TRPV1 mRNA and placed it upstream of a GFP reporter. This setting allowed us to investigate silencing irrespective of higher-order target structures. In addition, it permitted the deliberate introduction of defined structure elements into the target region. The resulting vector, termed VR1straight, showed strong fluorescence on transfection into Cos-7 cells. As expected, cotransfection of this plasmid with VsiRNA1 led to a substantial decrease in GFP expression, illustrated by the extensive down-regulation of the target gene at subnanomolar concentrations of the siRNA (Figure 1(b)).

This reporter system was employed first to determine the importance of sequence characteristics of VsiRNA1 for protein knockdown. To do so, the silencing activity of the corresponding sense strand of the siRNA duplex was probed for comparison. Both strands are equivalent in structure and, being complementary, they display the same affinity towards their respective target sequences. Therefore, the only variation was in the sequence of the siRNA guide strand, and differences in activity could be attributed solely to this aspect. The genuine target site in the reporter vector was exchanged for its complementary sequence, constituting a potential area of attack for the sense strand of VsiRNA1. Figure 1(c) depicts the design of the target RNA. As can be seen in the blot, silencing by the sense strand of VsiRNA1 was poor compared to the corresponding antisense strand. We conclude that characteristics of the primary sequence of the antisense strand of VsiRNA1 are of critical importance for its observed high activity.

The question remained, is a favourable siRNA sequence a sufficient prerequisite for successful silencing, or does target structure also play a role? As an example of an adverse target conformation, we generated an artificial stem-loop structure, so that all 21 nucleotides of the VsiRNA1 target sequence were engaged in base-pairing interactions

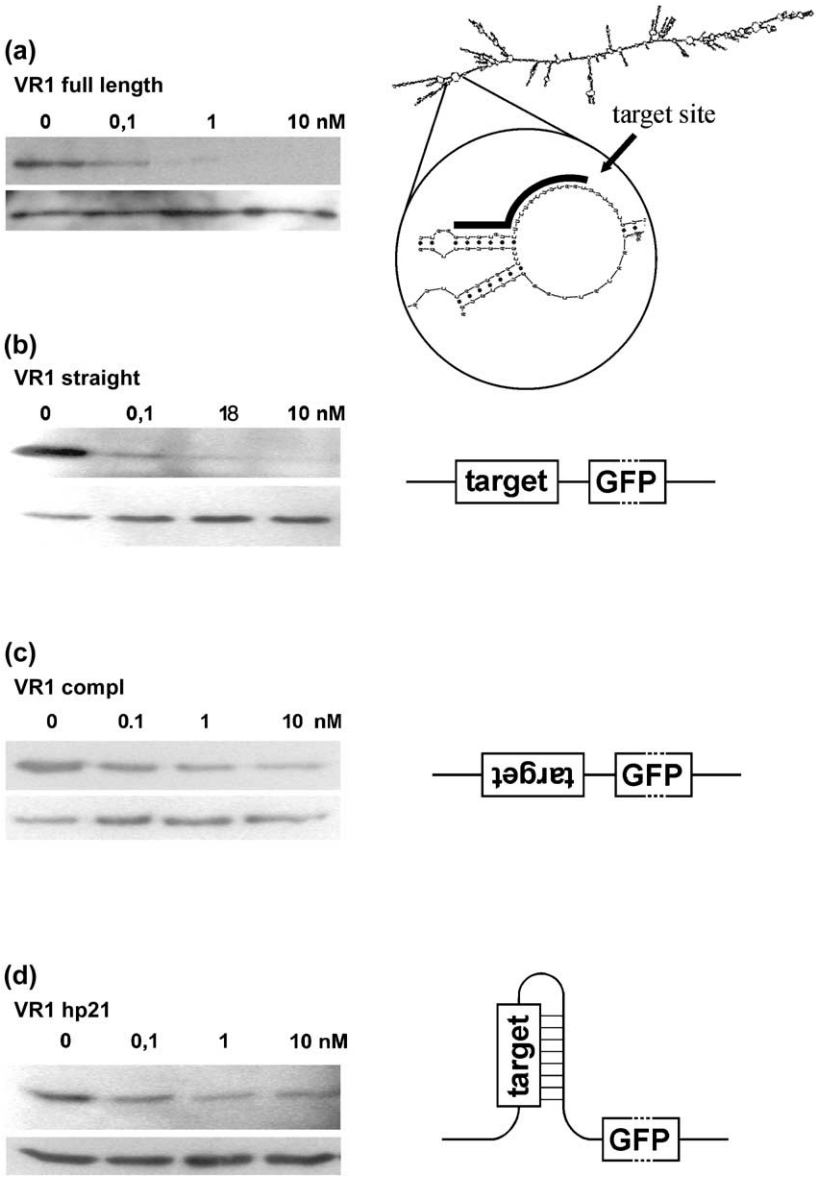

Figure 1. Influence of target sequence and structural context on silencing efficiency. An siRNA specific for the mRNA of the vanilloid receptor 1 was used to silence various target constructs upstream of GFP. Expression of the reporter gene was detected by Western blotting; actin is included as a loading control. All results were verified by independent experiments. The set-up of different target RNAs is depicted to the right: VR1-full-length, the 2600 bases long VR1 mRNA upstream of GFP; VR1straight, the isolated 21mer target sequence of VsiRNA1 upstream of GFP; VR1compl, complementary sequence as target for the sense strand of the siRNA; VR1hp21, all 21 nucleotides of the target sequence incorporated in a hairpin structure upstream of GFP. Concentrations of the siRNA are indicated.

(Figure 1(d)). Western blot analysis showed that this hairpin design severely compromised gene silencing by VsiRNA1 compared to the exposed sequence.

Quantitative evaluation of a series of experiments yielded the diagram shown in Figure 2. Silencing of the full-length RNA of VR1 and the fully accessible sequence (black and white columns, respectively) reached about 50% at subnanomolar concentrations of siRNA and proceeded almost to completion in the low nanomolar range. Exchanging the target sequence for its respective complementary sequence or introducing an unfavourable secondary structure (light grey and dark grey columns, respectively), diminished the silencing activity of VsiRNA1. Even at a concentration of siRNA of 10 nM, gene expression was reduced by only approximately one-half.

The high level of efficiency of VsiRNA1 is in spite of the fact that the sequence is not entirely in

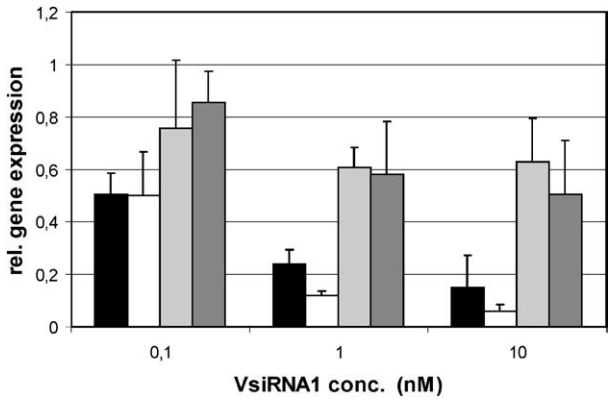

Figure 2. Quantification of reporter gene knockdown by VsiRNA1 acting on different target constructs as described in the legend to Figure 1. Black columns, VR1-full-length; white columns, VR1straight; light grey columns, VR1compl; dark grey columns, VR1hp21.

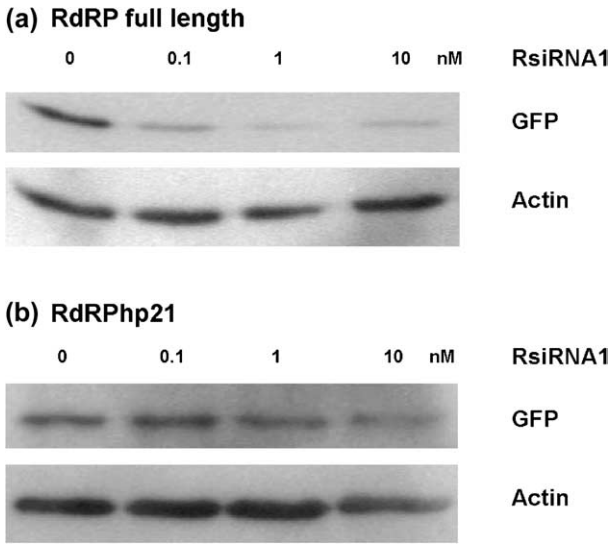

Figure 3. Silencing of a coxsackievirus gene by RNA interference. The full-length RNA of the RdRP or the siRNA target sequence incorporated into a stable hairpin structure were fused upstream of GFP. Reporter gene expression was analyzed by immunoblotting, actin is included as a loading control. (a) Silencing of full-length RdRP; (b), expression of GFP with the siRNA target sequence in a stable hairpin upstream of the translational start.

accordance with the criteria for siRNA design established more recently.[16,17] It might therefore be argued that the limited capacity to compete with stable internal structures of the target RNA was due to suboptimal design of the siRNA. This consideration prompted us to verify the influence of higher-order target structure with an siRNA designed to fit all criteria known to date. RsiRNA1 is an siRNA directed against the RNA-dependent RNA polymerase (RdRP) of coxsackievirus B3. It was designed by Dharmacon Inc. (Lafayette, CO) according to recently published requirements. Indeed, RsiRNA1 was found to silence the gene of interest fused to a reporter gene with great efficiency (Figure 3(a)). To assess the effects of a stable RNA structure on silencing by this siRNA, we again base-paired all target nucleotides of RsiRNA1 in a defined stem-loop configuration upstream of a GFP reporter. In agreement with our results using VsiRNA1, a significant obstruction of RNA interference was induced by the protected target sequence (Figure 3(b)).

To analyse the detrimental influence of shielded target nucleotides in more detail, we designed a series of mRNAs that contained the target sequence in hairpin structures of varying design and stability. First, a set of experiments was performed in which matching bases were provided for 16, ten and five nucleotides of the target sequence, respectively, to yield stems with perfect base-pairing of different lengths. Figure 4(a), shows the extent of gene inhibition by VsiRNA1, when portions of the 5′-end were left unpaired. A clear correlation was found between the number of exposed nucleotides and the efficiency of gene silencing: When all nucleotides were incorporated in a stable hairpin (black bars), silencing was impaired drastically, while exposure of 16 nucleotides resulted in efficient inhibition of the expression virtually indistinguishable from that observed with the straight target (see above). Constructs with ten and five nucleotides incorporated in a hairpin gave intermediate results. These findings furthermore underline the importance of target site accessibility for siRNA activity.

Subsequently, we wanted to determine whether a directional bias exists with respect to the end of the target site that is being exposed or shielded. Therefore, a second set of constructs was generated in which the 3′-end of the target site remained unpaired. The same picture arose from these experiments (Figure 4(b)): The strong inhibition of siRNA activity seen with a completely base-paired

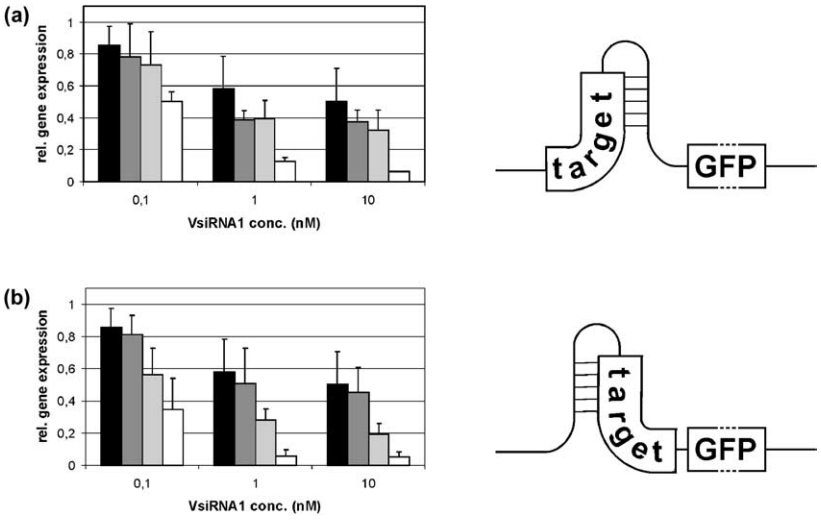

Figure 4. Silencing by VsiRNA1 acting on partially base-paired target sequences. (a) Nucleotides of the 5′-end exposed. (b) 3′ nucleotides exposed. Black columns, all nucleotides base-paired; dark grey columns, five nucleotides unpaired; light grey columns, ten nucleotides unpaired; white columns, 16 nucleotides unpaired.

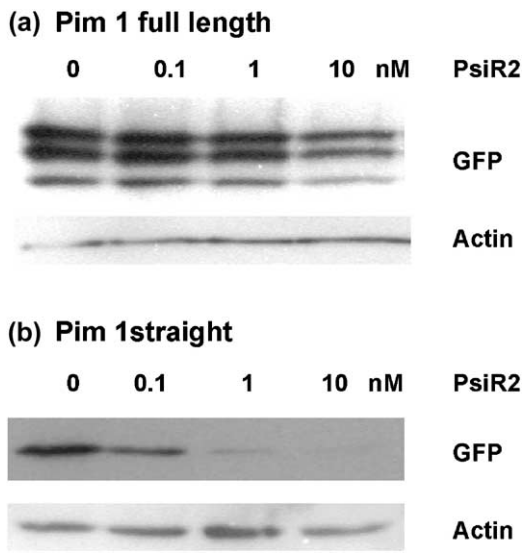

Figure 5. Silencing of rat Pim-1 kinase by RNA interference. The full-length mRNA of Pim-1 or the isolated siRNA target sequence were fused upstream of GFP. (a) Pim-1-specific siRNA acting on the full-length target; (b) knockdown of gene expression by the same siRNA with the isolated and fully accessible target sequence upstream of GFP.

target sequence is diminished gradually as a greater part of the target site becomes accessible. We therefore conclude that the silencing efficiency of an siRNA correlates with the number of accessible target nucleotides, but does not depend on the end of the target site that is being exposed.

Our finding that well-designed and efficient siRNAs can be rendered inactive by adverse target structures prompted us to look for an example of the opposite situation: We employed an siRNA duplex directed against the serine/threonine kinase Pim-1 that we had found earlier to be inactive.[22] This siRNA, termed PsiR2, did not meet the requirements for a sequence promising to be effective. Western blotting against Pim-1 is known to result in several bands, probably due to autophosporylation. More important for the question under investigation is the finding that our gene of interest fused to a reporter gene was not regulated by PsiR2 (Figure 5). When the 21mer target sequence was isolated in the manner described above and positioned upstream of GFP, however, PsiR2 displayed substantial activity, and extensive gene regulation was observed. We infer from this finding that the limitation of silencing by PsiR2 is indeed due to structural characteristics of the target molecule rather than being caused by an unfavourable siRNA sequence.

Finally, we analysed the relationship between local free energy of the target region and extent of gene silencing using computer-aided structure predictions. Secondary structures of the target-reporter mRNA were calculated with the mfold web server†.[25] Sections of the predicted folds containing the target sequence are presented in Figure 6. It is known that structure predictions have strong limitations when long RNAs are concerned, rendering only approximations of the actual folding. Nevertheless, for all constructs but one, the respective predicted structures with the lowest free energies followed the trend of shortened stem-loop motifs. The only exception is target 3′16, for which the structure with the lowest overall free energy was unrelated to all other structures. A slightly less stable structure, however, displayed the anticipated decreased stem length.

As intended, neither the straight target nor the complementary sequence result in stable structures and the targeted nucleotides are accessible to base-pairing with complementary oligonucleotides. In contrast to these constructs, the RNA molecule containing nucleotides complementary to the target sequence produces the anticipated stable hairpin. As can be seen in the second and third row of Figure 6, other target constructs were predicted to form the desired hairpins with decreasing contiguous stem lengths.

Based on these predictions, local free energies ΔG_{loc} were calculated for the structural motives in which nucleotides of the target sequence are involved for all target constructs (see also Figure 6). A high negative ΔG_{loc} value reflects a stable internal structure. Even though the computed ΔG_{loc} can be expected to give only a rough approximation of the actual situation, we found the silencing efficiency to correlate with the local energy of the target structure (Figure 7). Interestingly, even the results obtained for the unrelated targets under investigation (viral RdRP, circle, and Pim-1, triangle) fit well into the plot for VsiRNA1. The data point at -22 kcal/mol that deviates farthest from the best fit belongs to the 3′16 target construct for which the fold was found to be unrelated to the other structures (see above). Taken together, these findings support the view that silencing efficiency does not depend only on the siRNA sequence itself but is determined also by the structural environment of the target site.

Discussion

The enormous gene-silencing potential of RNA interference has promoted its employment in functional studies and offers the prospect of possible therapeutic applications. Recently, however, some concern has been raised regarding potential unspecific gene regulation by siRNAs. No off-target effects of siRNAs had been observed in initial studies,[26] but Semizarov *et al*. found an induction of stress and apoptosis genes at higher concentrations of siRNA.[27] Expression profiles after siRNA treatment revealed off-target effects in later investigations,[28] and induction of the interferon

† http://www.bioinfo.rpi.edu/applications/mfold

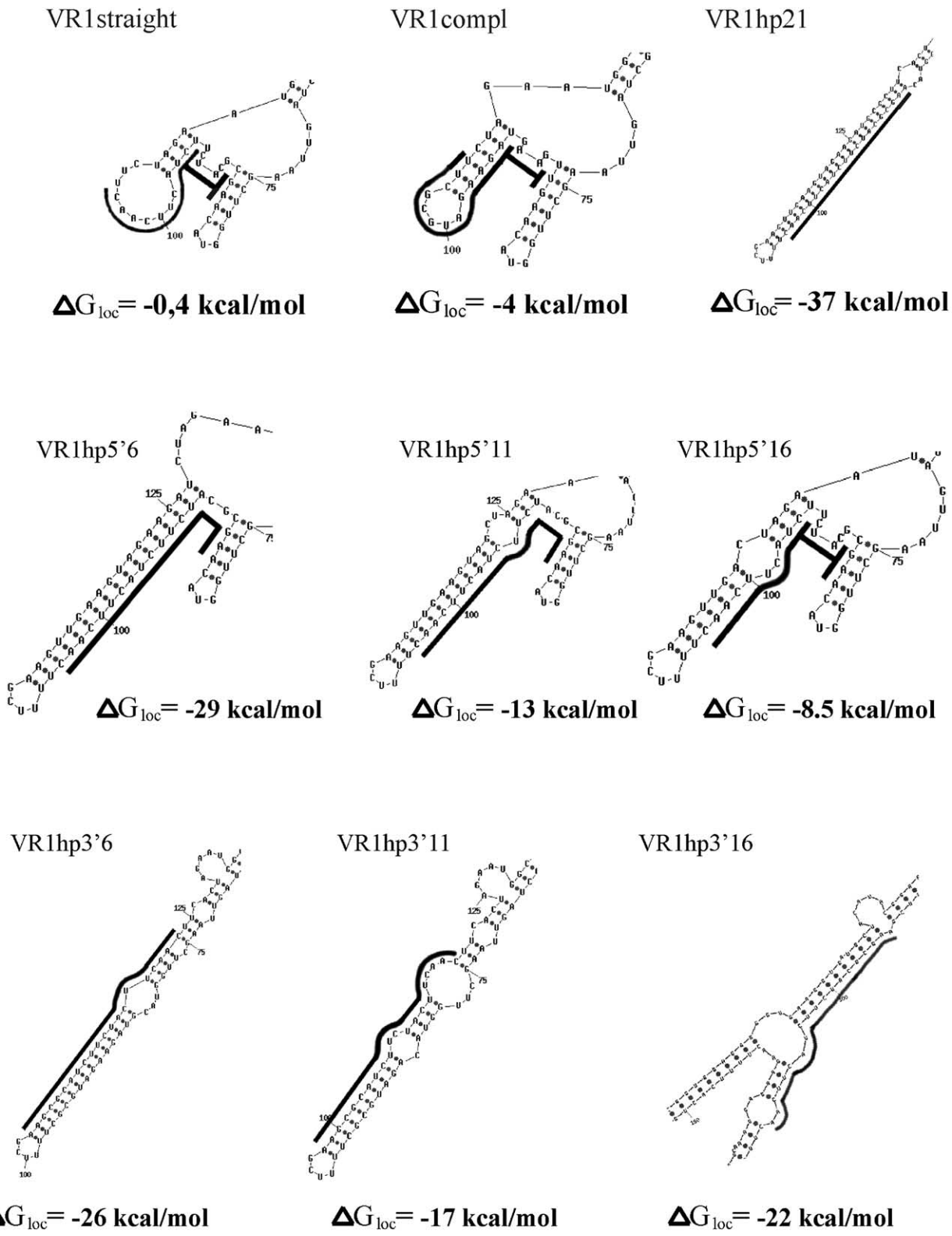

Figure 6. Predicted structures of target-reporter constructs under investigation. The target sequence of VsiRNA1 was embedded in various sequence environments. Two-dimensional foldings of the resulting constructs were calculated using the mfold web server (http://www.bioinfo.rpi.edu/applications/mfold).[25] Relevant regions of structures with the lowest free energy are shown. The position of the target sequence is indicated, and local free energy of motives in which nucleotides of the target sequence are involved is given.

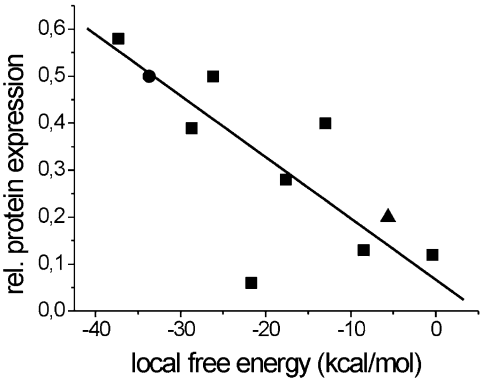

Figure 7. Dependency of reporter gene expression on local free energy at the target site. Squares, VsiRNA1 acting on different target structures; circle, RsiRNA1 against a fully base-paired target sequence; triangle, PsiR2 against an accessible target site.

response by short hairpin RNAs or siRNAs has been reported.[29,30] Moreover, siRNAs were shown to inhibit translation of partially complementary target sequences in a microRNA-like manner.[31,32] It has been argued recently that most siRNA sequences published to date are partially complementary to several human genes and might therefore cause unintended gene silencing.[33]

These findings illustrate why it is essential to identify highly efficient siRNAs, capable of inducing extensive gene knockdown even at low concentrations, to minimise unpredictable side-effects. The selection of efficient siRNAs today relies mainly on the analysis of sequence elements that mediate efficient incorporation into the proteinaceous silencing complex RISC.[11,15] In a number of studies, however, a marked impact of mRNA structure on silencing efficacy has been reported.[12,14,19] The significance of target accessibility has nevertheless not been evaluated systematically.

In the accompanying study,[23] Overhoff *et al.* correlate silencing efficiencies of a larger number of siRNAs directed against natural targets with predicted structures of target molecules. Using a different and complementary approach to investigate the requirements for efficient siRNAs, sets of well defined target regions, i.e. unpaired nucleotides, stable hairpins or stem-loop constructs of various designs were employed in the present study. First, silencing of these constructs by an siRNA that we had previously found to inhibit the expression of the vanilloid receptor 1 (TRP V1) was investigated.[21] This siRNA displayed a very high level of efficiency ($IC_{50} \sim 60$ pM) although it does not obey all sequence criteria formulated more recently. As expected, the siRNA also inhibited the expression of a reporter gene containing the isolated target sequence upstream of the translational start. Interestingly, silencing of the complementary sequence, which should be targeted by the sense strand of the siRNA, was drastically less pronounced. According to our computer-based structural models of the target-reporter RNA, neither the original target site nor its complementary sequence is involved in stable base-pairing. The observed lack of silencing of the complementary target site is thus unlikely to be due to the formation of unexpected secondary structures between the target site and the mRNA of the reporter gene. Rather, our findings are in line with reports that describe a strong strand bias determined by thermodynamic properties of the siRNA strands.[11,15] In the accompanying study by Overhoff *et al.*, however, data are presented showing that even an siRNA duplex with unfavourable thermodynamic stability may be an efficient silencing agent.[23]

In addition to the influence of intrinsic properties of the siRNA sequence, we detected a strong effect of target RNA structure on silencing efficiency. The target sequence was incorporated into hairpin structures whose stability was varied by decreasing the length of the stem. When the entire target sequence was sequestered intentionally in a stable stem-loop structure, silencing efficiency was diminished drastically, a finding that is in agreement with similar experiments reported in the literature.[12,34] The blockade of siRNA activity was relieved gradually when fewer nucleotides were involved in base-pairing.

The relationship between target structure and inhibition of gene silencing was analysed further by performing secondary structure predictions and calculating local free energies for the structural motives in which nucleotides of the target sequence were involved. As expected, the RNA that contains bases complementary to all nucleotides of the target sequence is predicted to fold into a stable stem-loop structure with a very low free energy (Figure 6). Target motifs designed to form hairpins with lower stability were indeed predicted to result in shorter stems and were consequently calculated to be energetically less stable. The straight target as well as the target with the complementary sequence was found to form structures with the lowest level of stability. We find a strong tendency of reporter gene expression to increase with stability of secondary structure motifs in the target region, which results in increasing absolute values of ΔG_{loc} (Figure 7). The observed deviations from this correlation may indicate that additional processes (e.g. unwinding by helicases) might influence the mechanism of RNAi.

In another study, Kawasaki and co-workers observed strong variation of siRNA efficiency when they tested several siRNAs against puromycin resistance and the H-*ras* mRNA, respectively.[35] Analysing mfold structures of their target RNAs, they did not observe any special features that allowed the prediction of efficient siRNA target sites. In contrast to the intentionally designed RNA motifs used in the present study, however, natural mRNAs consist of mixed secondary structures, including short helices, bulges and loops.

Interruption of two short helices by several bulged-out nucleotides may well be sufficient to render a target site accessible for siRNAs, as is case for the shorter hairpin constructs we used in our study.

Binding of siRNA-containing RISC to its target sequence may initiate at either the 5′ or the 3′-end. In order to investigate a possible directional bias, we conducted experiments with stem-loop structures of varying stability, in which nucleotides from either end of the target sequence were predicted to be accessible. Silencing activity of the siRNA was observed to be dependent solely on the number of nucleotides incorporated in hairpin structures, regardless of which end of the target sequence was unpaired.

Since the siRNA used in all experiments described above was designed only on the basis of criteria defined in the early stages of siRNA application,[3] we wanted to confirm our findings with an siRNA selected with recently published algorithms.[16,17] The second siRNA used had a higher G/C content at the 5′-end of the sense strand as compared to the 3′-end, it contained guanosine at position 1, adenosine at positions 3 and 19, and uridine at position 10. This well-designed siRNA inhibited the expression of its target with great efficiency. Interestingly, we again observed a severe decrease of silencing activity when the target sequence was included in a stable hairpin structure. In further experiments, we found that a seemingly inactive siRNA targeting the full-length mRNA of the rat Pim-1 kinase silenced the expression of a reporter gene downstream of the isolated target sequence with great efficiency. These findings validated the view that even carefully chosen siRNAs with high intrinsic silencing potency may, nonetheless, be incapable of inhibiting their natural target RNAs when target sites are sequestered in stable internal structures.

Our analysis of the parameters that influence the potency of siRNAs may be of practical relevance for the design of siRNAs. For example, we and others did not succeed in generating efficient siRNAs against the 5′ untranslated region (UTR) of RNA viruses.[36–39] This region is a very attractive target region because of its high degree of conservation. The 5′-UTR contains the internal ribosome entry site (IRES), which is known to consist of stable stem-loop structures. Even in cases in which virus replication could be repressed by targeting the 5′-UTR, inhibition was weak compared to approaches in which the coding region of the viral RNA was targeted.[40] (See Haasnoot *et al.* for a comparative survey.[41]) We speculate that the complex higher-order structure of the RNA makes targeting of this region especially challenging. Careful screening is required to identify efficient cleavage sites. It may therefore be advisable in many cases to avoid targeting RNA regions known to fold into extensive stable internal structures.

Additional methods have been developed to overcome the problem of siRNA efficiency. A mixture of siRNAs generated by cleavage of long double-stranded RNA by Dicer has been demonstrated to be superior to the use of single siRNAs,[35] but there is risk of off-target regulation by this method that has not been clarified fully. Furthermore, Siolas *et al.*[42] and Kim and colleagues[43] found only recently that longer short hairpin RNAs of 29 nucleotides and siRNAs of 27 nucleotides, respectively, are more potent than the conventionally used 21mer siRNAs. The longer molecules are Dicer substrates that are loaded more efficiently into RISC. The 27mer siRNAs have been shown to be more efficient, and to be suitable to target sites that are refractory to silencing by 21mer siRNAs.[43]

RNA interference is a promising new tool for functional genomics and therapeutic applications. Some reports published recently advised that

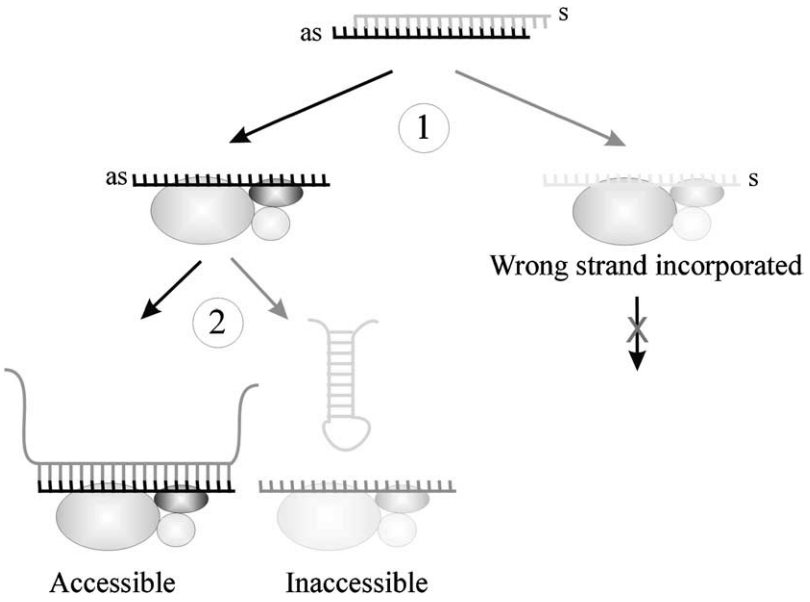

Figure 8. Efficiency of an siRNA is determined at two points of the RNAi pathway. (1) A strand bias exists that is defined by intrinsic thermodynamic properties of the siRNA duplex. Strands are incorporated depending on their A/T contents at the ends. The strand with a higher A/T content at its 3′ end is less suitable for gene silencing. (2) A highly ordered structure may have a detrimental influence during the annealing of the ribonucleoparticle siRNA/RISC to its target mRNA. Therefore, even carefully designed siRNAs with the intended antisense strand being favoured for incorporation into RISC may fail to silence gene expression.

siRNAs be employed at low concentrations in order to prevent unspecific effects, thus underlining the necessity to select highly efficient siRNAs. We systematically analysed parameters influencing siRNA activity by using a set of deliberately designed, well-defined target sites. The results obtained are in keeping with a model of RNAi in which efficiency is determined at two points of the multi-step process, as illustrated in Figure 8: (i) asymmetric strand incorporation into RISC, controlled by thermodynamic properties of the siRNA, is fundamental to successful RNAi experiments; whereas (ii) site recognition depending on local secondary structures of the target RNA further modulates the efficacy of silencing. The activity even of well-designed siRNAs may be compromised severely when directed against a highly structured RNA. We thus conclude that it is necessary to take into account both the intrinsic siRNA sequence and the structure of the target RNA, in order to achieve efficient and specific silencing.

Materials and Methods

siRNAs and oligonucleotides

siRNAs consisting of a 19 base-pair duplex with overhangs of two nucleotides were purchased from Dharmacon (Lafayette, CO, USA) or IBA GmbH (Göttingen, Germany). Target sequences of the siRNAs were: VsiRNA1, GCG CAU CUU CUA CUU CAA C, RsiRNA1, GUA CAA AAC UUU CCA CCU A, PsiR2, GUG GUC CUG CUG AAG AAG G. Oligonucleotides for PCR and cloning procedures were obtained from IBA GmbH and TIB MOLBIOL, Berlin, Germany.

Cell culture and transfection experiments

COS-7 cells (African green monkey kidney fibroblasts) were grown in Dulbecco's modified Eagle's medium (PAA laboratories, Coelbe, Germany), with 10% (v/v) fetal bovine serum (PAA), 100 µg/ml of penicillin, and 100 µg/ml of streptomycin (Invitrogen, Karlsruhe, Germany) added. The day before transfection, cells were trypsinised, resuspended in medium without antibiotics and plated in 24-well plates at a density of 0.7×10^5 cells/well in a volume of 500 µl. Transfection and cotransfection experiments were carried out using 2.5 µl of Lipofectamine 2000 (Invitrogen) per well, following the manufacturer's instructions: 0.8 µg of the respective target plasmid and the appropriate amount of siRNA were used.

Detection of protein knockdown by immunoblotting

At 24 hours after transfection, cells were lysed with lysis buffer (125 mM Tris–HCl (pH 6.8), 4% (w/v) SDS, 1.4 M β-mercaptoethanol, 25% (v/v) glycerol, 0.05% (w/v) bromphenol blue). The lysate was boiled at 95 °C for five minutes, and 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 in a dilution of 1 : 5000 (w/w). 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.

Target plasmids

Plasmids expressing Pim1-GFP and VR1-GFP fusion proteins were obtained as described.[21,22,44] cDNA of the RNA-dependent RNA polymerase of coxsackievirus B3 (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).

Sense and antisense oligodeoxynucleotides containing overhangs for KpnI and XbaI restriction sites and corresponding to the 19mer siRNA target sites were designed to yield defined secondary structures after transcription. The respective strands (100 pmol) were annealed by heating to 95 °C for four minutes in annealing buffer (30 mM Hepes–KOH (pH 7.4), 100 mM potassium acetate, 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.

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