A Triplex Ribozyme Expression System Based on a Single Hairpin Ribozyme

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Triplex ribozyme (RZ) configurations allow for the individual activity of *trans*-acting RZs in multiple expression cassettes (multiplex), thereby increasing target cleavage relative to conventionally expressed RZs. Although hairpin RZs have been advantageously compared to hammerhead RZs, their longer size and structural features complicated triplex design. We present a triplex expression system based on a single hairpin RZ with *trans*-cleavage capability and simple engineering. The system was tested *in vitro* using *cis*- and *trans*-cleavage kinetic assays against a known target RNA from HPV-16 E6/E7 mRNA. Single and multiplex triplex RZ constructs were more efficient in cleaving the target than tandem-cloned hairpin RZs, suggesting that the release of individual RZs enhanced *trans*-cleavage kinetics. Multiplex systems constructed with two different hairpin RZs resulted in better *trans*-cleavage compared to standard double-RZ constructs. In addition, the triplex RZ performed *cis*- and *trans*-cleavage in cervical cancer cells. The use of triplex configurations with multiplex RZs permit differential targeting of the same or different RNA, thus improving potential use against unstable targets. This prototype will provide the basis for the development of future RZ-based therapies and technologies.

Introduction

RIBOZYMES (RZs) HAVE ATTRACTED ATTENTION because of their potential use for gene silencing (Benítez-Hess and Alvarez-Salas, 2006). The hairpin RZ is the 50-nt catalytic core of three plant satellite RNAs, which include the negative strand of the satellite RNA from the tobacco ringspot virus (sTRSV), chicory yellow mottle virus type I (sCYMV1), and arabis mosaic virus (sArMV) (Haseloff and Gerlach, 1989; Hampel, 1998). The overall structure of the hairpin RZ is composed of four helices and five loops for the RZ-substrate complex within two structural domains. Domain A contains helices 1 and 2 formed between the RZ and the substrate RNA. Domain B is formed by helices 3 and 4 within the RZ and maintains the catalytic capacity of the RZ (Yu and Burke, 1997). Target cleavage occurs by Watson-Crick hybridization through helices 1 and 2 followed by a molecular docking and a *trans*-esterification reaction using the 2'-hydroxy group at the scissile linkage primary nucleophile generating cleavage products with 5'-hydroxy and 2',3'-cyclophosphate termini. Minimal amounts of Mg²⁺ are required for correct positioning relative to the target and there is no apparent dependence on other cofactors for cleavage (Berzal-Herranz et al., 1993; Walter and Burke, 1998).

The evolution of RZ expression systems has led to the engineering of multiple expression (multiplex) configurations harboring several *trans*-acting RZs within a single transcript. However, the mere tandem cloning of several *trans*-acting RZs leads to cleavage efficiencies similar to those obtained by single RZs due to the lack of independent activity of each RZ. To avoid this, the RZ expression system must allow the trimming of the *trans*-acting RZs from the nascent transcript to permit independent action of each RZ thus increasing overall efficiency (Taira et al., 1990). These so-called shotgun systems can be designed as a triplex configuration using two *cis*-cleaving RZs flanking each *trans*-cleaving RZ, resulting in their release and independent activity (Ohkawa et al., 1993).

In this study, a triplex system (pRz434bis) based on a single hairpin RZ resulted in higher *trans*-cleavage activity than conventionally expressed RZs. This system uses a single hairpin RZ to self-release from the nascent transcript while keeping full *trans*-cleavage kinetics for the target RNA. Moreover, the use of single RZs in a triplex configuration to perform trimming and *trans*-cleavage activities resulted in minimal loss of R434 cleavage *trans*-activity, suggesting that such configuration may be used

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to express multiple RZs. Double-RZ triplex systems successfully self-released and cleaved the target RNA with better kinetics than tandem-cloned RZ. Cell culture testing showed that the single-RZ triplex system withheld *cis*- and *trans*-cleavage activities in the intracellular environment. Therefore, we propose the use of multitarget (multiplex) constructs based on single-RZ triplex configuration for better *in vivo* HPV-16 E6/E7 mRNA degradation. In addition, the model can be adapted for use with other targets besides human papillomavirus.

Materials and Methods

Cell culture

The HPV-negative C33-A (ATCC HTB-31) and the HPV-16 positive SiHa (ATCC HTB-35) cervical tumor lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corp., Carlsbad, CA) enriched with 5% fetal bovine serum (Invitrogen). Transfections were performed using 10 μ g of total plasmid DNA with Lipofectin reagent (Invitrogen). Cells were harvested for RNA extraction 48-hour posttransfection.

Oligodeoxynucleotides and plasmids

Plasmid pBtV5-434 contains the R434 RZ flanked by a mutant tRNA^{Val} and a tetraloop sequence cloned into the *Bam*HI/*Xho*I and *Mlu*I/*Sac*I sites of pBtV1-434 plasmid, respectively, that renders R434 resistant to intracellular processing (Alvarez-Salas et al., 1998). Triplex RZ expression plasmid pRz434bis contains the pBtV5-434 expression cassette (mutant tRNA^{Val} plus R434) and the 5'-CACCGACGGCTTGCCTCGGTG-3' tetraloop sequence (Zhang and Burke, 2005) flanked by the *cis*-cleavage domains TL (5'-AATTCAAACAGAGAAGTCAACCATGGT ACCTCTGACAGTCCTGTTTA-3') and TR (5'-CGCGTG ACAGTCCTGTTTCC TCCAAACAGAGAAGTCAACCAGA GCT-3') (Fig. 1). Plasmid pRz434TR contains a mutation within the cleavage site 5'-AGUC-3' of the TR domain pRz434bis that inhibits cleavage. The pRz434ibis plasmid contains the inactive RZ R434i (with a triple nucleotide substitution A₂₄A₂₅A₂₆→G₂₄C₂₅U₂₆ in the catalytic domain of R434) in a triplex configuration. This construct maintains the target specificity of R434 but does not permit cleavage activity (Alvarez-Salas et al., 1998). The duplex constructs pDR434 and pDR434bis contain two tandem copies of R434 and Rz434bis expression cassettes, respectively, cloned in the pBSKS⁻ vector (Stratagene, La Jolla, CA). The pDTR419-434 plasmid contains tandem copies of the R419 and R434 RZs.

RZ activity in cultured cells was tested using the pH1-R434bis, pH1-R434-TR, and pH1-Rz434ibis or pH1-Rz-Scramble plasmids containing the *Bam*HI-*Sac*I fragments from pRz434bis, pRz434ibis, and pRz434TR plasmids, respectively, cloned in the eukaryotic expression vector pSilencerTM 3.0-H1 (Ambion Inc., Austin, TX). For specificity control, plasmid pRz-Scramble expressed a RZ with scrambled helices 1 and 2 verified for no complementarity against GenBank using the BLAST routine. Plasmid pCR3.1-GFP containing the GFP gene cloned in the pCR3.1 vector (Clontech, Mountain View,

CA) was used as a reporter gene for cell sorting after transfection (Benitez-Hess et al., 2004). All plasmids were sequenced prior to *in vitro* transcription experiments.

In vitro transcription

Template plasmids were linearized with *Sac*I and purified by phenol–chloroform–isoamyl alcohol (25:24:1) extraction. One microgram of the linearized plasmid DNA was incubated with the RiboProbeTM T3 *in vitro* transcription system (Promega Inc., Madison, WI) in the presence of [α -³²P]UTP (3000 Ci/mmol), in accordance with the manufacturer's instructions. Labeled transcripts were loaded on to preparative 6% polyacrylamide 7 M urea denaturing gels and electrophoresed at 250 V. Gels were exposed to radiographic films, and full transcripts were excised and eluted from the gels in 350 μ L E buffer (1 mM EDTA, 0.5 M ammonium acetate, 0.1% SDS, 20 U RNaseA inhibitor) overnight at 4°C.

RZ self-digestion assays

Labeled transcripts from triplex cassettes were purified through preparative denaturing gels and incubated in RZ buffer (10 mM Tris-HCl, pH 7.0, 7 mM MgCl₂, 2 mM spermidine) at 37°C. Cleavage products were separated through 6% polyacrylamide 7 M urea denaturing gels. Dried gels were exposed to radiographic films and quantified using a Typhoon 8600 fluorographic scanner (Amersham Biosciences).

RZ trans-cleavage assays

Isolated unprocessed RZ RNA was incubated with radiolabeled target RNA containing HPV-16 nt 410–445 in RZ buffer at 37°C, as described previously (Alvarez-Salas et al., 1998). Cleavage products were separated through denaturing polyacrylamide gel electrophoresis. For circular templates, plasmid DNA was incubated directly with the RiboProbe T3 system for 1 hour before the addition of labeled target RNA. Dried gels were exposed to radiographic films and quantified using a Typhoon 8600 fluorographic scanner (Amersham Biosciences). Cleavage rates were calculated from initial velocity of percentage of cleavage vs. time plots, as the average of at least three independent experiments.

RNase protection assays (RPAs)

For RZ probing, 25 μ g of total RNA was hybridized with a ³²P-labeled antisense RNA probe produced from T7-transcribed pRz434bis plasmid and processed with the Direct ProtectTM Lysate RPA kit according to the manufacturer's instructions (Ambion). For β -actin probing, 15 μ g of total RNA was used. Protected RNA fragments were separated through denaturing 7 M urea 6% polyacrylamide gels. Dried gels were exposed to radiographic films.

Cell sorting

Transfected cells were sorted using a FACScalibur flow-cytometer (BD Biosciences, Chicago, IL) with a band-pass

filter at 585/42 nm for GFP fluorescence. Excitation was performed with a 488-nm argon laser.

RT-PCR assays

First strand cDNA synthesis was carried out with 500 ng of total RNA using SuperScriptTM One-Step RT-PCR system (Invitrogen) at 50°C for 30 minutes. Resulting cDNA was PCR-amplified by 30 cycles of denaturation step at 92°C for 1 minute, hybridization step at 45°C for 45 seconds, and polymerization step at 72°C for 1 minute. For HPV-16, we used the E6/E7 mRNA-specific set of primers E6U (5'-CAGCAATACAACAAACCG-3') nt 371–388 and E7L (5'-TAGATTATGGTTTCTGAGAAC-3') hybridizing within the E7 gene nt 862–841 (Alvarez-Salas et al., 1998). As internal control, the β -actin gene was probed with the oligonucleotide set 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' and 5'-CTAGAAGCATTTCGGGTGGACGATGGAGGG-3' using the PCR conditions in accordance with the manufacturer's instructions (Stratagene). Amplicons were resolved in native agarose electrophoresis gels.

Results

Self-processing of the triplex RZ system

The pRz434bis triplex system was designed containing only one hairpin RZ (R434) tasked with the dual role of trimming and *trans*-cleavage. Specific *cis*-cleavage was directed by hairpin RZs lacking the catalytic domains flanking the tRNA^{Val}-R434 cassette (TL and TR). This design would likely divert some R434 activity from *trans*-cleavage and produce a slower kinetics, but greatly reduced the size of the triplex cassette leading to simpler secondary structures and easier cloning of multiple units (Fig. 1).

Initial *in vitro* experiments using the pRz434bis template showed that increased amounts of free R434 end-product correlated with a decrease in full-length and intermediary transcripts suggesting self-release. As expected, some full and intermediary transcripts remained after 2-hour digestion indicating slow *cis*-cleavage kinetics probably produced by the double cleavage necessary for complete R434 release (Fig. 2A). To further confirm self-release, transcripts from circular (ccc) pRz434bis templates were used in *cis*-cleavage reactions. In this study, full-length pRz434bis transcripts, intermediaries, and the end-product TR are linked to long transcripts and thus the corresponding bands would be absent from the electrophoregrams. As with transcripts from linear templates, bands corresponding to TL-R434, R434, and TL were clearly visible after 15-minute incubation, thus confirming self-release from long transcripts (Fig. 2A). Therefore, a single-RZ system can be used as a base for a triplex expression cassette based on hairpin RZs.

Specificity of self-release activity from pRz434bis was tested by introducing a mutation within the 5'-GUC-3' cleavage site of the TR domain (pRz434TR). This resulted in total inhibition of R434 cleavage in TR and thus no isolated R434 was produced although the presence of fragments R434-TR and TL suggested that R434 was still active. A triple mutation within the catalytic domain of R434 (pRz434ibis) completely inhibited self-processing further confirming that

pRz434bis transcript undergoes *cis*-cleavage on both flanks by using the catalytic domain of R434 (Fig. 2B).

Trans-cleavage activity of R434 RZ expressed from triplex cassettes

Because pRz434bis produced several fragments from self-release and at least four of these fragments contained an active R434 RZ, *trans*-cleavage rates may represent the sum of RZ activities rather than those from isolated R434. This hypothesis was tested using the pRz434TR and pRz434ibis mutants in *trans*-cleavage assays using the radiolabeled HPV-16 target RNA (nt 410–445). *Trans*-cleavage by the pRz434bis transcript was superior to that of pRz434TR. However, the pRz434TR transcript retained significant *trans*-activity confirming a *trans*-cleavage role for intermediary triplex products. The inactive RZ control pRz434ibis did not present *trans*-cleavage (Fig. 3A). *Trans*-cleavage experiments using full transcripts from linear or circular pRz434bis and nontriplex pBtV5-434 plasmids showed similar overall *trans*-cleavage efficiency, indicating that the dual role of R434 in pRz434bis has little effect on R434 *trans*-cleavage performance (Fig. 3B).

The expression of double RZs was tested using circular templates from pDR434 and pDR434bis plasmids containing tandem copies of the nontriplex R434 and Rz434bis expression cassettes, respectively. As described above, circular templates entrap R434 in long transcripts and thus trimming activity was expected to be a critical factor for overall *trans*-activity. Adequate release of R434 from pDR434bis transcripts was confirmed by self-digestions assays (data not shown). Tandem-cloned RZs from pDR434 did not exhibit significant changes in cleavage activity rate relative to single RZs from pBtV5-434 (Fig. 3C, *top left*). In contrast, duplex cassette pDTR434bis transcript increased the cleavage rate of R434 relative to their single triplex cassette unit Rz434bis (Fig. 3C, *top right*). No apparent effect on cleavage specificity was noticed with either pDR434 or pDTR434bis (Fig. 3C, *bottom*). These results suggest that isolated R434 units released by trimming activity perform independently over the target.

Multiple RZ expression with a 419–434 double-RZ triplex (pDTR419-R434) was performed to test whether self-processing capabilities relied only on R434 and thus establish the multiplex potential of the design. The R419 RZ targeted to HPV-16 nt 415–429 has similar kinetic features to R434 (Alvarez-Salas et al., 1998). In fact, R419 self-released from pDTR419-434 transcript with kinetics similar to R434 in *cis*-cleavage assays (Fig. 3D, *top left*). Together, R419 and R434 RZs within pDTR419-R434 independently processed the target RNA with a kinetics similar to pDTR434bis (Fig. 3D, *top right*). However, R419 and R434 performed independent cleavage over the target as suggested by the residual fragments observed in *trans*-cleavage reactions (Fig. 3D, *bottom left*). Therefore, triplex systems based on single hairpin RZs can be readily adapted to express multiple RZs against different targets.

Triplex RZ self-processing in vivo

To test *cis*- and *trans*-cleavage activities of the single-RZ triplex within the intracellular environment, SiHa cells (cervical tumor cells containing HPV-16) were transfected

FIG. 1. Single-RZ triplex design (pRz434bis) for HPV-16 E6/E7 mRNA. (Upper panel) Secondary structure representation of full transcripts from the single-RZ triplex system (pRz434bis), showing R434 *trans*-acting RZ, mutant tRNA^{Val}, tetraloop, and trimming domains TL and TR. Arrows indicate RZ cleavage sites. The HPV-16 target sequence (nt 430–445) is shown for reference. (Lower panel) Map of pRz434bis plasmid. The relative positions of TR and TL domains are shown within boxes.

with pRz434bis, pRz434TR, pRz-Scramble, and pRz434bis plasmids cloned in the pSilencerTM 3.0-H1 expression vector. These vectors used a RNapol III promoter (human H1), which is appropriate for expressing small RNAs not bound for translation in mammalian cells (Koseki et al., 1999). Total RNA was analyzed by RPA 48-hour posttransfection using the full pRz434bis antisense transcript sequence as probe and β -actin as input control. Because only R434 was protected from endogenous ribonuclease attack with a 5' mutant tRNA^{Val} and a 3' tetraloop, it was unlikely that TL and TR fragments would survive within the ribonuclease-rich intracellular environment. However, because of the probe used we expected that the RPAs would reveal not only

the R434 status but also the cellular tRNAs which were used as additional internal controls.

As indicated by the RNase(+) and RNase(–) controls, the experimental conditions for the RPA allowed total degradation of the nonhybridized RNA probe (Fig. 4A). RNA from nontreated (NT) and empty pSilencerTM 3.0-H1 vector (Vector) control transfections showed no RZ-specific fragments but presented low molecular weight bands corresponding to cellular tRNAs. All RZ-expressing transfections had very low levels of full transcripts (TL-R434-TR), suggesting rapid endogenous nuclease degradation or self-processing. Intracellular decay of RZ-containing transcripts was evident in all treatments as shown by fragments common to

FIG. 2. Catalytic release of R434 from pRz434bis. (A) Labeled TL-R434-TR transcripts from *SacI*-linearized pRz434bis template were incubated in RZ buffer for 0–120 minutes at 37°C and loaded on to analytical 6% polyacrylamide 7 M urea denaturing gels. The position of full-length, intermediary, and end-products is indicated by arrows (*left panel*). Labeled RZ transcripts produced from circular templates of pRz434bis were incubated in RZ buffer before denaturing electrophoresis as described above. Relative migration of intermediary and end-products is indicated by arrows (*right panel*). (B) R434 self-cleavage specificity. Transcripts from pRz434bis, pRz434TR (containing a mutated RZ cleavage site within TR domain), and pRz434ibis (containing an inactive RZ) were incubated in RZ buffer for up to 120 minutes. The resulting fragments were separated in polyacrylamide denaturing gels. The position of the resulting fragments is indicated by arrows.

FIG. 3. *Trans*-cleavage analysis of triplex R434 cleavage. **(A)** *In vitro* transcribed RNA from *Sac*I-linearized templates pRz434bis (squares), pRz434TR (triangles), and the inactive mutant pRz434ibis (circles) were incubated with the labeled HPV-16 415–445 target RNA. Cleavage activity was plotted as the percentage of residual target relative to the input. **(B)** Triplex configuration does not affect R434 *trans*-cleavage. The labeled HPV-16 target RNA (nt 415–445) was incubated with equimolar quantities from linear (*left panel*) or circular (*ccc*) (*right panel*) templates pBtV5-434 (triangles) or pRz434bis (circles). **(C)** *Trans*-cleavage of pRz434bis duplex cassettes. (*Top panel*) RZ *trans*-cleavage kinetics produced by triplex cassettes with one copy and two copies of R434 in standard cassettes pBtV5-R434 (black triangles), pDR434 (white triangles), or triplex cassettes pRz434bis (black squares) and pDTR434 (white squares). (*Bottom panel*) *Trans*-cleavage of HPV-16 415–445 target RNA by pBtV5-434, pDR434, pRz434bis, and pDTR434bis cassettes. Arrows indicate the position of the residual fragments. **(D)** *Cis*- and *trans*-cleavage of pDTR419-R434 double-RZ cassette. (*Top left*) Labeled transcripts from pRz434bis and the multiplex construct pDTR419-R434 (triangles), containing RZs R419 and R434 were incubated in RZ buffer as stated in Figure 2 legend. The bands corresponding to released R419 and R434 are indicated by arrows. (*Bottom left*) Nonlabeled transcripts from pRz434bis, pDTR434, and pDTR419-R434 were incubated with the labeled HPV-16 target as stated above. The residual fragments of the target were resolved by denaturing gel electrophoresis and are indicated by arrows. (*Top right*) The *trans*-cleavage kinetics of pRz434bis (black squares), pDTR434 (white squares), and pDTR419-R434 (triangles) are presented in the graph. All the graphs represent the mean and standard error of three independent experiments.

C

D

FIG. 3. (Contd.)

all lanes. Cells transfected with pH1-Rz-Scramble, which has no specificity for the *cis*-cleavage target sites, and the pH1-R434ibis control with the inactive mutant derivative Rz434i also showed other fragments due to intracellular decay, but no fragment corresponding in size to R434 (Fig. 4A). On the other hand, cells transfected with pH1-R434bis presented a fragment corresponding to the isolated R434 RZ, suggesting that the complete intracellular release of isolated R434 RZ was only in fully active triplex constructs (Fig. 4A). No effect was observed in the endogenous tRNA and β -actin internal controls (Fig. 4A). Similar results were observed with the HPV-16-negative cervical carcinoma cell line C33-A (data not shown). Therefore, these results show that the Rz434bis cassette can self-release R434 within the intracellular environment.

Effect of triplex RZ expression on HPV-16 E6/E7 transcript

Previous testing of R434 *in vivo trans*-cleavage in HPV-16⁺ cells showed that this RZ was able to reduce HPV-16 E6/E7 mRNA levels to an extent similar to the inactive R434i RZ, suggesting an antisense rather than a catalytic effect (Alvarez-Salas et al., 2003). Once that *cis*-cleavage was shown *in vivo*, it was clear that the catalytic abilities of R434 were present within the cellular environment. Therefore, it was then necessary to establish the *in vivo trans*-cleavage capability of triplex-expressed R434 by measuring the HPV-16 E6/E7 mRNA levels on cervical carcinoma cells. SiHa cells were transfected with pSilencerTM 3.0-H1 (Vector), the specificity control pH1-Rz-Scramble, pH1-R434bis, or the inactive RZ control pH1-R434ibis. Transfected cells were sorted through the fluorescence produced by cotransfection with pCR3.1-GFP plasmid to reduce background HPV-16 E6/E7 mRNA from untransfected cells. SiHa cells transfected only with the pCR3.1-GFP plasmid were used in this case as a nontreated control (NT). End-point RT-PCR analysis of total RNA extracted from pH1-R434bis-transfected cells showed a noticeable decrease in HPV-16 E6/E7 mRNA levels relative to the controls (NT, vector, scrambled, and inactive Rz), as indicated by the relative intensity of the 492-bp amplicon (Fig. 4B,

upper panel). Interestingly, the pH1-R434ibis construct did not affect HPV-16 E6/E7 mRNA levels as expected. This is likely because this construct lacked self-release capacity, thus entrapping the R434i antisense moiety in long transcripts that obstruct hybridization. No differences were observed in the β -actin input control mRNA levels (Fig. 4B, lower panel). These results suggest that triplex-expressed RZs retained *trans*-cleavage capabilities *in vivo*.

Discussion

The concept of multiple RZs for therapeutic purposes has been firmly established (Alvarez-Salas and DiPaolo, 2007; Asif-Ullah et al., 2007). Although current delivery systems allow successful intracellular expression of single RZs, more recent approaches allow the delivery of multiplex RZs directed against several target RNA sites (Ramezani et al., 1997; Ruiz et al., 1997). Although multiplex expression has been successfully tested with hammerhead RZs, no attempt has been reported to express multiple hairpin RZs. In the present study, we report a novel triplex RZ expression system based on a single hairpin RZ.

The hairpin RZ architecture complicates a triplex design because hybridization with the target is made adjacent to the catalytic domain as opposed to the target hybridization arms flanking the catalytic domain in hammerhead RZs. Early triplex systems based on three hammerhead RZs units resulted in the efficient *in vivo* inhibition of HIV-1 (Yuyama et al., 1994) and down-regulation of the retinoblastoma gene (Benedict et al., 1998). Nevertheless, the complex engineering of multiplex triplex constructs based in triple-RZ units may result in recombination due to the sequence similarities between the multiple *cis*-cleaving and *trans*-cleaving RZs (Altschuler et al., 1992).

It has been shown that the modular structure of the hairpin RZ permits the physical separation of A and B domains. Thus, the catalytic domain B can independently recognize single or multiple domain A regardless of their relative position (Shin et al., 1996). Therefore, the same hairpin RZ can be used for both trimming and therapeutic duties. Initial experiments using two

FIG. 4. Intracellular processing of Rz434bis triplex cassette. (A) SiHa cells were transiently cotransfected with the reporter vector pCR3.1-GFP and plasmids pSilencerTM 3.0-H1 (vector), pH1-Rz434bis, pH1-Rz434-TR, pH1-Rz-Scramble, and pH1-Rz434ibis (containing the *Bam*HI-*Sac*I fragments from pRz434bis, Rz434TR, pRz-Scramble, or pRz434ibis, respectively) and harvested 48-hour posttransfection for cell sorting through the fluorescence produced by GFP. For ribonuclease protection assays (RPAs), total RNA was purified and incubated with a labeled antisense probe for Rz434bis (upper panel) or human β -actin (lower panel). Fragments were analyzed after polyacrylamide denaturing gel electrophoresis. Arrows indicate the relative migration of Rz434bis-related fragments, intracellular decay fragments, and the β -actin input control. The curly bracket indicates the migration of endogenous tRNA cross-hybridization bands. MWM, molecular weight marker produced from *in vitro* transcribed Rz434bis transcript. NT, RNA from nontransfected cells transfected only with pCR3.1-GFP. Probe, intact antisense Rz434bis labeled probe. Control(+), RPA with unlabeled R434 transcript. RNase(+), Rz434bis antisense probe incubated in ribonuclease buffer. RNase(-), Rz434bis antisense probe incubated with ribonuclease buffer only. (B) End-point RT-PCR analysis of HPV-16 E6/E7 mRNA from triplex RZ-transfected SiHa cells. SiHa cells were cotransfected with the reporter plasmid pCR3.1-GFP and plasmids pSilencerTM 3.0-H1 (Vector), the specificity control pH1-RZ-Scramble (Scramble), pH1-R434bis, or the inactive RZ control pH1-R434ibis. Transfected cells were sorted through the GFP fluorescence to reduce background levels from untransfected cells. Total RNAs obtained after cell sorting were reverse-transcribed and amplified by PCR using E6/E7-specific primers before nondenaturing electrophoresis. RT-PCR for β -actin mRNA was used as input control. Arrows indicate the position and sizes of the expected amplicons for E6/E7 (492 bp) and β -actin (661 bp). MWM, molecular weight marker. NT, nontreated cells transfected with the pCR3.1-GFP plasmid only; Control(-), no cDNA PCR control.

trimming hairpin RZs confirmed efficient orientation-independent cleavage of target RNA in *cis*, suggesting the potential of hairpin RZs for triplex configurations (Schmidt et al., 2000).

The pRz434bis cassette presented here used a single hairpin RZ to perform both *cis*- and *trans*-cleavage duties. Such designs are based on the modular nature of hairpin RZs that can form catalytic four-way junctions with isolated domains (Walter et al., 1998). The pRz434bis design displayed behavior similar to previously reported triplex systems based on hammerhead RZs (Yuyama et al., 1994). Triplex systems with active trimming activity producing independent R434 were consistently more efficient for *trans*-cleavage than their trapped-R434 counterparts, suggesting that long transcripts affect R434 *trans*-cleavage. Nevertheless, *in vitro* assays of both systems clearly showed that considerable amounts of R434 RZ remained attached to full-length transcripts or intermediary products. This positively affected overall *trans*-activity suggesting a synergistic effect of all R434-containing fragments. Although this might be interpreted as a disadvantage, the pRz434bis design provided nuclease protection (mutant tRNA^{Val} and tetraloop) only for R434 that has been released. In fact, cell culture testing of the single-RZ triplex showed that unreleased R434 does not play a major role *in vivo*.

The use of single-RZ triplex designs such as in pRz434bis is superior to other designs because the variables affecting cleavage of only one RZ can be controlled better than in triplex designs based in three RZs where such variables multiply. In addition, the construction of multi-RZ expression systems can be simplified by tandem cloning of pRz434bis-like cassettes. The synergistic cleavage activity obtained with the triplex systems suggests a possibility of using a cassette that would be more efficient than their nontriplex counterparts. Independent activity of trimming RZs from triplex systems offers the possibility of multitargeting the same or several transcripts using different types of RZs and other therapeutic RNAs to produce a more efficient gene silencing. Such a possibility has been shown in transgenic mice with β_2 -microglobulin and HIV-1 targets (Andang et al., 2004). The present results confirm the feasibility of using hairpin RZs as trimming moieties in triplex designs. Moreover, pRz-434bis is the first triplex system using a single hairpin RZ (R434) to perform both trimming and *trans*-cleavage activities. Therefore, it will be possible to use this design to focus in other molecular targets for gene inactivation.

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