

Target-Induced SOFA-HDV Ribozyme

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Abstract

Small *cis*-acting ribozymes have been converted into trans-acting ribozymes possessing the ability to cleave RNA substrates. The Hepatitis Delta Virus (HDV) ribozyme is one of the rare examples of these that is derived from an RNA species that is found in human cells. Consequently, it possesses the natural ability to function in the presence of human proteins in addition to an outstanding stability in human cells, two significant advantages in its use. The development of an additional specific *on/off* adaptor (SOFA) has led to the production of a new generation of HDV ribozymes with improved specificities that provide a tool with significant potential for future development in the fields of both functional genomics and gene therapy. SOFA-HDV ribozyme-based gene inactivation systems have been reported in both prokaryotic and eukaryotic cells. Here, a step-by-step approach for the efficient design of highly specific SOFA-HDV ribozymes with a minimum investment of time and effort is described.

Key words: Ribozyme, Gene inactivation, Functional genomics, Gene therapy

1. Introduction

Ribozymes (RNA enzyme, Rz), are commonly found as self-cleaving RNA motifs that are essential in the life cycles of infectious RNA that replicate via a rolling circle mechanism. These self-cleaving RNA motifs include both the *hammerhead* and *hairpin* structures that are found in the viroid and viroid-like satellite RNAs infecting plants. A self-cleaving ribozyme is also retrieved in the hepatitis delta virus (HDV) that infects several eukaryotes including humans. All of these *cis*-acting RNA motifs have been converted into trans-acting ribozymes possessing the ability to specifically recognize and subsequently catalyze the cleavage of an RNA target. As a consequence, they have become attractive tools in the development of gene inactivation systems (for reviews see refs. (1–4)).

For a long time, the only example of a ribozyme derived from an RNA species naturally found in human cells (i.e. infected hepatocytes) was the HDV ribozyme (5, 6). Its evolution in human cells confers to it several unique properties for its use as a potential tool, including the natural ability to function both in the presence of human proteins and at the physiological magnesium concentration (i.e. ~1 mM magnesium). Due to its outstanding stability in human cells, the HDV ribozyme is an interesting potential candidate for the development of a gene-knockdown system (7). However, the potential development of such a gene inactivation system based on the HDV ribozyme has been relatively neglected because it suffers from a lack of substrate specificity when used as a molecular tool (8). Specifically, its substrate specificity depends on the formation of stem I that includes only 7 bp (see Fig. 1, recognition domain, RD), while a total of 13–14 bp has been estimated to be required in order to ensure the targeting of a unique RNA species from the human transcriptome (9). In order to overcome this hurdle, a module named SOFA (Specific *On/off* Adaptor) was engineered for the HDV ribozyme (see Fig. 1) (10). The SOFA module switches the cleavage activity from the *off* to the *on* state solely in the presence of its cognate target. Initially, the recognition domain site forms a short duplex with an inserted sequence element (the blocker, Bl), thereby locking the ribozyme in an inactive conformation (*off*) by increasing the energetic barrier for non-specific base pairing interactions and thus reducing the potential for off-target cleavages. A second inserted sequence element (the biosensor, Bs) extends the base pairing with the target in order to favour the binding of the genuine target, and the formation of this duplex concomitantly results in the disruption of the short duplex involving the blocker and the recognition domain, switching the ribozyme into an active conformation (*on*). In other words, the blocker acts as a safety lock for the ribozyme in which the key is the recognition of the target RNA by the biosensor. The combined work of the biosensor and the blocker has been shown to increase the substrate specificity of the ribozyme's cleavage by several orders of magnitude when compared with the wild-type version (10, 11). Finally, the last component of the SOFA module is a stabilizer stem that brings both the 5' and 3' strand ends together. This additional stem has no effect on the cleavage activity of the SOFA-HDV ribozyme, but data suggests that it is important for its molecular stability in cellulo (7, 10). Importantly, experiments performed in *Lactococcus lactis* confirmed that SOFA-HDV ribozyme retained the property of enzyme turnover, meaning that one molecule of ribozyme can successively cleave several target molecules (12). To our knowledge, the SOFA-HDV ribozyme constitutes the first example of a ribozyme bearing a target-dependent module that is activated by its RNA target, an arrangement which greatly diminishes non-specific effects. This new approach provides a specific

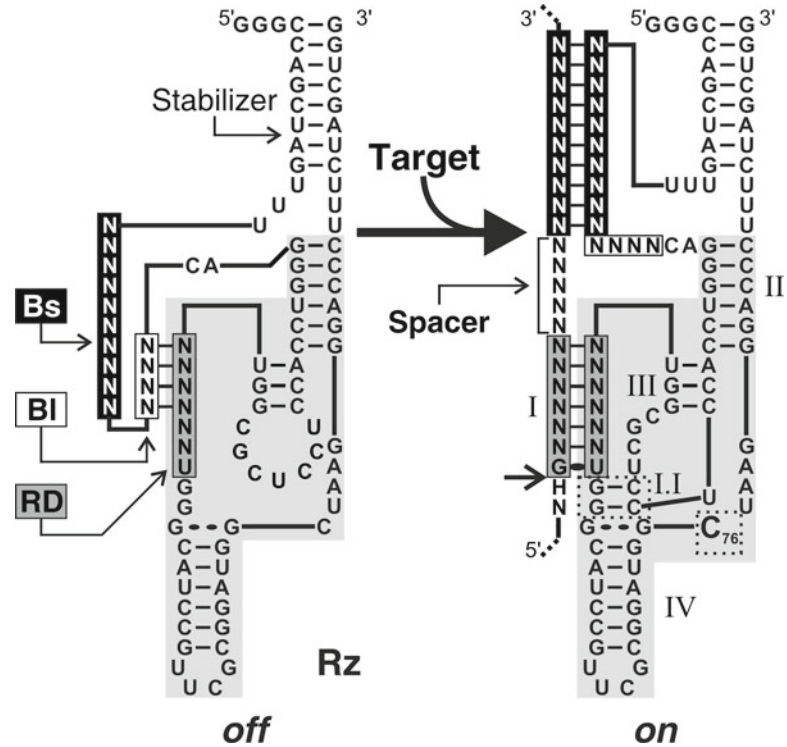


Fig. 1. Detailed representation of the SOFA-HDV ribozyme. The secondary structures of both the *off* (left) and *on* (right) conformations of the SOFA-HDV ribozyme are shown. The SOFA-HDV ribozyme (Rz) is illustrated in both cases, while the target is only shown for the *on* conformation. The hepatitis delta virus (HDV) ribozyme is highlighted in *grey* in each case. The recognition domain, the biosensor and the blocker are identified by RD (*black on grey*), Bs (*white on black*) and BI (*black on white*), respectively. The stabilizer and the spacer (the region in the target that is localized between the recognition domain and the biosensor binding site) are also indicated. The roman numerals *I, I.I, II, III* and *IV* identify the corresponding stems or stem-loops in the HDV ribozyme. The variable nucleotides (i.e. those that can be A, C, G or U) of the ribozyme are indicated by the *boxed letter N* (*black* for Bs, *white* for BI and *dark grey* for RD). All *unboxed* nucleotides are invariable and are constant from one ribozyme to the other, regardless of the target sequence. Positions where mutations can be inserted in order to produce a catalytically inactive SOFA-HDV ribozyme (I.I pseudoknot and the catalytic cytosine 76) are *boxed with dashed lines* on the *on* conformation. Finally, the *short arrow* indicates the cleavage site.

and improved tool with significant potential for application in the fields of both functional genomics and gene therapy. The development of several gene inactivation systems based on SOFA-HDV ribozyme in both prokaryotic and eukaryotic cells have been reported (10, 12–15). These studies have permitted the identification of the important features that must be considered in an optimized design process of SOFA-HDV ribozymes. Here, a simple method for designing SOFA-HDV ribozymes, highlighting the key points that need to be considered in order to improve the rate of success, is described.

2. Materials

2.1. Specificity Analysis

Ribosubstrate: <http://www.riboclub.org/ribosubstrates>.

2.2. Synthesis of DNA Template

1. SOFA-HDV RzX primer at a concentration of 100 μM :
5'-TAATACGACTCACTATAGGGCCAGCTAGTTT(N)_{10Bs}
(N)_{4Bl}CAGGGTCCACCTCCTCGCGGT(N)_{6RD}TGGGCAT
CCGTTCGCGG-3'.
2. Universal SOFA reverse primer at a concentration of 100 μM :
5'-CCAGCTAGAAAGGGTCCCTTAGCCATCCGCGAAC
GGATGCCC-3'.
3. 10 \times PCR Buffer: 200 mM Tris-HCl (pH 8.8), 100 mM KCl,
100 mM (NH₄)₂SO₄ and 1% Triton X-100.
4. 100 mM MgSO₄ stock solution.
5. dNTP stock solution: 10 mM of each dATP, dCTP, dGTP and
dTTP.
6. *Pwo* DNA polymerase (Roche Diagnostics).
7. 3 M Sodium acetate (pH 5.2).
8. 100% and 70 Ice-cold ethanol.

2.3. In Vitro Transcription

1. NTP stock solution: 25 mM of each ATP, CTP, GTP and
UTP.
2. 5 \times Transcription buffer: 400 mM HEPES-KOH (pH 7.5),
120 mM MgCl₂, 10 mM spermidine and 200 mM DTT.
3. Pyrophosphatase (1 U/ μL , Roche Diagnostics) diluted 1:100
(V:V) in a dilution buffer containing 50% glycerol, 20 mM
Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM DTT, 1 mM EDTA,
100 $\mu\text{g}/\text{mL}$ BSA and 0.03% NP40.
4. RNaseOUT (40 U/ μL , Invitrogen) diluted 1:2 (V:V) in a
buffer containing 50% glycerol, 20 mM Tris-HCl (pH 8.0),
50 mM KCl, 0.5 mM EDTA and 8 mM DTT.
5. Purified T7 RNA polymerase (5 $\mu\text{g}/\mu\text{L}$).
6. RQ1 RNase-free DNase (1 U/ μL , Promega).
7. Phenol and chloroform.
8. Loading buffer: 98% formamide, 10 mM EDTA, 0.025%
xylene cyanol and 0.025% bromophenol blue.
9. Elution buffer: 500 mM ammonium acetate, 1 mM EDTA and
0.1% SDS.

2.4. Target Labelling

1. 10 \times Antarctic Phosphatase Reaction Buffer (New England
Biolabs).

2. Antarctic Phosphatase (5 U/ μ L, New England Biolabs).
3. 10 \times T4 polynucleotide kinase (PNK) (USB).
4. T4 PNK (30 U/ μ L, USB) diluted 1/10 times in T4 PNK dilution buffer (USB).
5. [γ -³²P]ATP (6,000 Ci/mmol, PerkinElmer).
6. Glycogen (20 mg/mL, Roche Diagnostics).

2.5. Cleavage Assays

1. 500 mM Tris-HCl (pH 7.5).
2. 100 mM MgCl₂.

2.6. Denaturing Polyacrylamide Gel Electrophoresis

1. Denaturing gels: 8 M urea and 19:1 ratio of acrylamide/bisacrylamide in 1 \times TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM EDTA).

2.7. Autoradiography and RNA Quantification

1. Hyperfilm MP films (GE Healthcare).
2. Phosphoscreen (GE Healthcare).
3. ImageQuant software (GE Healthcare).

3. Methods

The step-by-step protocol illustrated in Fig. 2 provides for a fast and simple selection of SOFA-HDV ribozymes directed against any target of choice. This protocol is composed of three main modules that are then subdivided into various steps. Briefly, the modules include, successively, the identification of the potential targeting sites, the design of the SOFA-HDV ribozymes and, finally, the selection of the most prominent ones for further development.

3.1. Target Module

The first step is solely related to the selected RNA target. It includes all of the prerequisite analyses and considerations that must be taken into account before moving on with the designing of SOFA-HDV ribozymes. This step can be subdivided into two parts: the documentation of the target and the identification of potential sites to be targeted.

3.1.1. Documentation of the Target

Initially, it is paramount to both gather all available information on the target and to establish all considerations that might influence the targeting of a given RNA species. For example, the targeting of a viral RNA with the aim of controlling the propagation of this pathogen may benefit from a sequence analysis in order to identify any stretches of highly conserved nucleotides that could then be used for ribozyme binding. This consideration increases the chances of targeting a wide range of viral genotypes. In addition, targeting

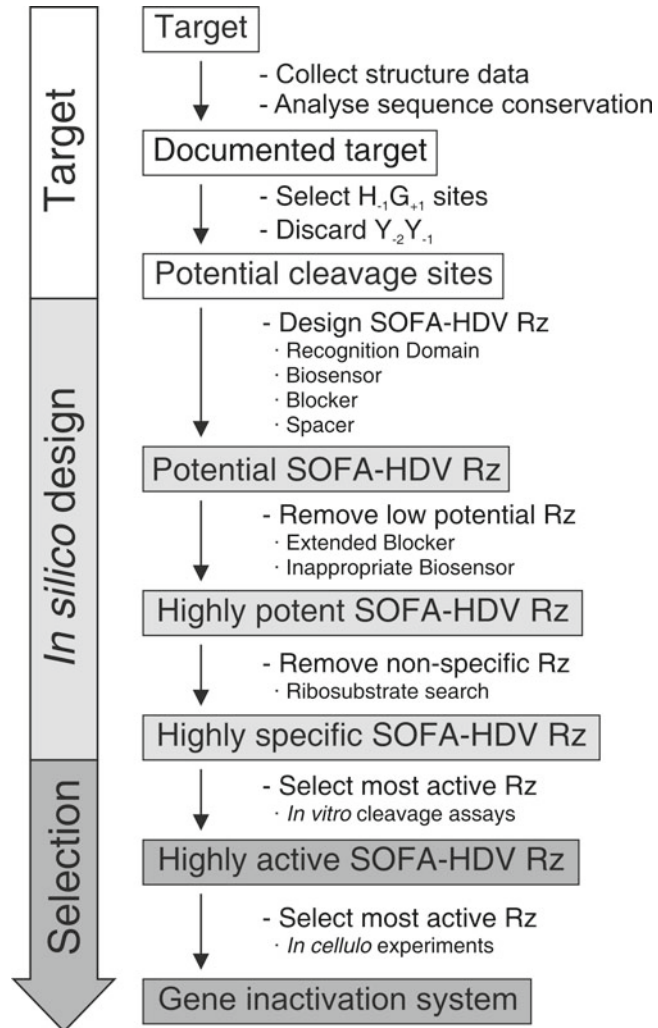


Fig. 2. Scheme of the design process for the SOFA-HDV ribozyme. The *large arrow* represents the three major, sequential, design steps. The target (*white box*), the in silico design (*light grey box*) and the selection (*dark grey box*) modules are divided into different analyses that are described in Subheading 3. The *horizontal boxes* indicate what is obtained after each procedure, while the text on the right side of each *small arrows* describes the key element in the process.

more conserved nucleotides should reduce the potential escape of any resistant mutants (16, 17). Conversely, the identification of sequences specific to a unique mRNA belonging to a family of relatively well-conserved proteins may lead to a reduction in the level of a single protein species even though several related ones are similarly expressed in a cell.

Effective cleavage at a specific site depends on its accessibility to the ribozyme. In principle, target sites located in single-stranded

regions of an RNA species possess a higher potential because they should be more accessible for ribozyme binding than those located in double-stranded regions (18–20). Within the double-stranded regions, the ribozyme might compete unfavourably with intramolecular base pairing when trying to bind the target. Therefore, any available data on the secondary and tertiary structures of the target might be of great value. In the past, bioinformatic and biochemical procedures, as well as a combination of both, have been used (18, 20). However, most of these approaches remain exhausting and may not necessarily be relevant if they are performed in test tube, as many factors influence the structure of an RNA species in vivo. Therefore, when no structural data is available, many years of work has led us to suggest limiting these investigations to only bioinformatic analysis and instead compensating by designing and testing a larger number of ribozymes in the subsequent steps. Moreover, the biosensor has been demonstrated to act as a facilitator (i.e. unwinds the secondary structure in the neighbourhood of the target site after its binding), thereby reducing the importance of the accessibility of the target sites (10).

3.1.2. Identification of Potential Sites

The resulting sequences (i.e. conserved, specific and mostly accessible) are then further analyzed in order to identify those that fulfil the essential criteria for efficient HDV ribozyme cleavage. The minimum requirement for an RNA to be cleaved by an HDV-derived ribozyme is the presence of a $H_{-1}G_{+1}$ at the cleavage site, where H can be A, C or U (8). More specifically, the first nucleotide downstream of the cleavage site (position +1) must be a guanosine in order to allow for formation of the essential G–U Wobble base pair with the ribozyme. At position –1, the presence of a guanosine residue is detrimental to cleavage. A systematic analysis of the sequences in positions –1 to –4 of a collection of small targets revealed that each of these nucleotides contributes differently to the ability of a given target RNA to be cleaved (21). Further analysis using longer RNA targets indicates that the nucleotides located in positions –1 and –2 may significantly influence the cleavage level. Clearly, it is of interest to select the more favourable ones when identifying potential sites in order to improve the chances of success (14). Specifically, the presence of two consecutive pyrimidines in these positions (i.e. –1 and –2) must be avoided as it is detrimental for the cleavage. Based upon these analyses, a list of all potential target sites is generated, and the sequences surrounding the cleavage sites from positions –2 up to at least +27, which includes all of the features important for designing a ribozyme, are extracted and used in the next step.

3.2. Ribozyme Design Module

The second module is directed towards both the design and the pre-selection of ribozymes in silico.

3.2.1. Designing SOFA-HDV HDV Ribozymes

Over the years, the sequence of the SOFA-HDV ribozyme has been optimized in order to favour a high cleavage level (10, 13). Although the backbone of this RNA molecule is largely conserved, there are three domains that are considered to be variable in terms of the designing of ribozymes: the recognition domain, the biosensor domain and the blocker domain. The recognition domain is complementary to nucleotides +1 to +7 of the target and includes the uridine residue that forms a G–U Wobble base pair (see Fig. 1). In other words, the recognition domain is determined by the cleavage site ($H_{-1}G_{+1}$). Subsequently, the sequence composing the biosensor, which interacts with the target downstream of the recognition domain, must be determined (see Fig. 3). The regions of the target interacting with both the recognition domain and

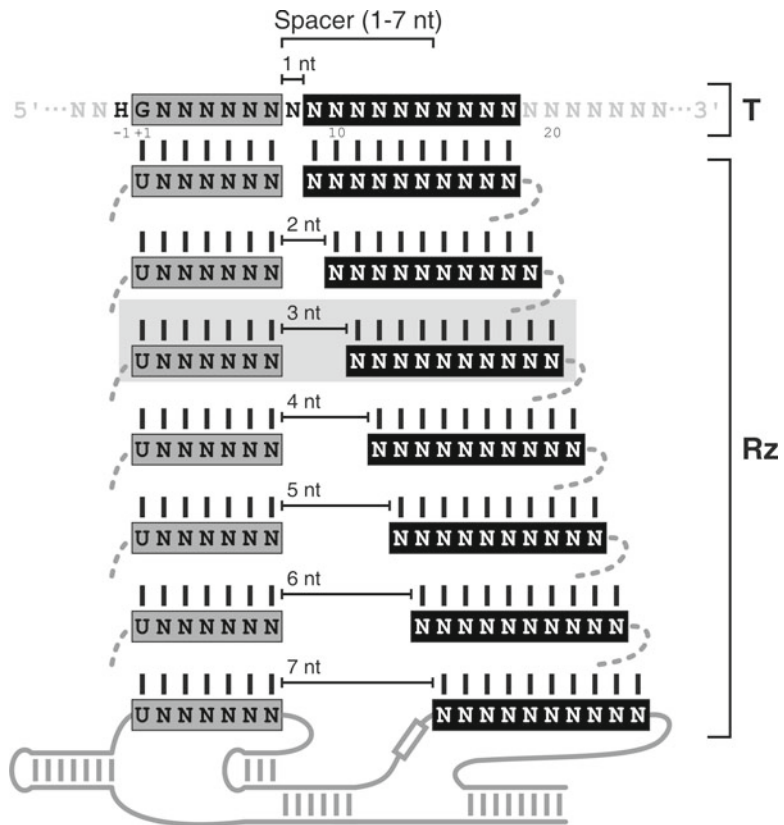


Fig. 3. Details on spacer flexibility and biosensor design. Representation of a potential cleavage site in a target (T), and of the corresponding SOFA-HDV ribozymes (Rz). The length in nucleotides (nt) of each spacer is indicated. The corresponding recognition and biosensor domains are shown below the target RNA. The optimal ribozyme with a spacer of 3 nt is highlighted in a light grey box. The sequences of the recognition domain, and of the biosensor binding sites on both the target and the ribozyme are identified using the same colour code as in Fig. 1 (i.e. the recognition domain is in black letters within grey boxes and the biosensor is in white letters in black boxes).

biosensor are separated by a spacer sequence that was included in order to avoid the stacking of both domains, as they are in close proximity to one another. This stacking, should it occur, could be detrimental to the product release (see Figs. 1 and 3). Previous experiments demonstrated that it is preferable to have a spacer of at least 1 nt, but no longer than 7. The optimal length is 3 nt, but anything between 1 and 5 nt works well (see Fig. 3) (13). Therefore, a 3 nt spacer is generally used in the initial design; and consequently, the next 10 nt of the target can be considered as being the region bound by its complementary biosensor. A length of 10 nt for the biosensor has been shown to be optimal for efficient binding without preventing product release and thus conserving the turnover property of the ribozyme. Finally, the sequence of the blocker is determined. It consists of 4 nt that are complementary to the 5' part of the ribozyme's recognition domain (see Fig. 1) (see Notes 1 and 2 for details).

3.2.2. Removal of Low-Potential SOFA-HDV Ribozymes

The various series of SOFA-HDV ribozymes developed in the past have led to the elucidation of guidelines for the pre-selection of the ribozymes possessing the greatest potential prior to the investment of any time in laboratory experiments.

The length of the blocker sequence that base pairs with the recognition domain has been shown to affect the cleavage activity ((13); unpublished data). The formation of more than 4 bp with the recognition domain may significantly reduce the cleavage activity by either preventing the switch from the *off* to the *on* conformation, or by generating a *cis*-acting ribozyme possessing the ability to self-cleave and therefore reduce the quantity of active SOFA-HDV ribozyme. The additional nucleotides that might be involved in these extra base pairs are in fact those located at the 3' end of the ribozyme's biosensor. Therefore, it is important to analyze the biosensor's sequence in terms of the possibility of finding such base pairing. When it occurs, the simplest solution is to consider slightly displacing the biosensor's binding sequence by repeating the previous step. If no solution appears to be possible, the given SOFA-HDV ribozyme must be removed from the collection.

Previous experiments have shown that significant complementarity between either the recognition domain, or the biosensor, with the sequence composing stem-loop III may also impair the cleavage activity of a given ribozyme. Specifically, whenever the possibility exists of forming at least six consecutive base pairs, or of any stability equivalent (e.g. 7 bp including a bulge), it is preferable to either remove or redesign the SOFA-HDV ribozyme in question because it will most likely be inactive (unpublished data).

3.2.3. Specificity Analysis

When working towards the development of a gene inactivation system, a key question to ask is how to design target-specific ribozymes that do not produce any side effects. There are several potential

sources of these side effects including, for example, the triggering of immunological responses. Moreover, in many cases, the side effects may result from the cleavage of non-desired mRNA species (i.e. off-target effects). In order to evaluate this possibility, the Ribosubstrates (<http://www.riboclub.org/ribosubstrates>) software was developed. This integrated software searches in selected cDNA databases for all potential targets of a given SOFA-HDV ribozyme (22). These potential targets include not only mRNAs having perfect matches with the catalytic RNA in question, but also for those interactions that include Wobble base pairs and/or mismatches. The results generated permit a rapid selection of the sequences suitable as targets for SOFA-HDV ribozymes. We suggest removing from the list, at a minimum, all ribozymes that would potentially recognize another target. Further selection criteria may also take into consideration the occurrence of any mismatches between the target and the ribozyme's binding domain. The presence of only one mismatch in the recognition domain is significantly more detrimental than the one occurring in the biosensor.

3.3. Ribozyme Screening Module

In this final module, an experimental design using *in vitro* cleavage assays for selecting the best candidate is presented. In addition, some important elements to be taken into consideration while using SOFA-HDV ribozyme in cellulo are discussed.

3.3.1. *In Vitro* Cleavage Assay

Preparations of DNA Templates

Most of the time, SOFA-HDV ribozymes are produced by *in vitro* transcription from PCR-generated DNA templates as described previously (14). Briefly, the PCR-based strategy includes two complementary and overlapping oligonucleotides. The sense primer, namely the SOFA-HDV RzX primer (where the X corresponds to the identification of the specific ribozyme, that is to say the cleavage site's position), is specific for each ribozyme and is composed of a T7 promoter followed by the SOFA-HDV ribozyme's sequence, stopping after loop IV (5'-TAATACGACTCACTATA GGGCCAGCTAGTTT(N)_{10Bs}(N)_{4Bl} CAGGGTCCACCTCCTCG CGGT(N)_{6RD} TGGGCATCCGTTTCGCGG-3', where N represents A, C, G or T, and Bs, Bl and RD indicate the biosensor, the blocker and the recognition domain, respectively). The reverse primer, specifically the Universal SOFA reverse primer, is universal to all ribozymes (5'-CCAGCTAGAAAGGGTCCCTTAGCCATCCGC GAACGGATGCCC-3'). The underlined nucleotides represent the overlapping sequences between the primers. Those two DNA primers are then used in a filling PCR reaction in order to produce the template for the run-off transcription. The target's DNA template is obtained either from a digested plasmid, or through a PCR strategy as described below (see Note 3).

1. Mix 2 μ L of both the SOFA-HDV RzX and the SOFA reverse primers (100 μ M each) with 10 μ L of 10 \times PCR buffer, 2 μ L of

100 mM MgSO₄, 2 μL of 10 mM dNTP, 81.5 μL of sterilized deionised water and 0.5 μL of *Pwo* DNA polymerase (2.5 U) in a final reaction volume of 100 μL.

2. Run the PCR for 12 cycles (1 cycle = 45 s at 95°C, 45 s at 55°C and 45 s at 72°C).
3. Transfer the reaction to a tube containing 10 μL of sodium acetate. Add 2.5 volumes of ice-cold 100% ethanol and centrifuge at 16,200×*g* for 25 min. Remove the supernatant and wash the pellet by adding 150 μL of 70% ethanol and centrifuge at 16,200×*g* for 5 min.
4. After removing the supernatant, quickly dry the pellet and then dissolve it in 52 μL of RNase-free water.

Preparation of RNA Ribozymes and Targets

Both the targets and the SOFA-HDV ribozymes are produced by run-off transcription as described below.

1. To the 52 μL of DNA template from above, add 24 μL of 25 mM NTP, 20 μL of 5× transcription buffer, 1 μL of diluted pyrophosphatase (0.01 U), 1 μL of diluted RNaseOUT (20 U) and 2 μL of purified T7 RNA polymerase (10 μg) in a final reaction volume of 100 μL.
2. Incubate at 37°C for 2 h.
3. To each reaction, add 3 μL of DNase RQ1 (3 U) and incubate for 20 min at 37°C.
4. Add 0.5 volumes of both phenol and chloroform, vortex and centrifuge at 16,200×*g* for 10 min. Transfer the upper aqueous phase to a new tube containing 10 μL of sodium acetate (3 M, pH 5.2). Add 2.5 volumes of ice-cold 100% ethanol, vortex and centrifuge at 16,200×*g* for 25 min. Discard the supernatant and wash the pellet by adding 150 μL of 70% ethanol followed by centrifugation at 16,200×*g* for 5 min.
5. After discarding the supernatant, quickly dry the pellet.
6. Dissolve the pellet in 40 μL of RNase-free water. Add 80 μL of loading buffer and fractionate the RNA on a standard 8 M urea denaturing polyacrylamide gel (see Note 4). Visualize the RNA by UV shadowing (see Note 5) and excise the band. Transfer the band to a 1.5-mL tube and add 500 μL of elution buffer. Elute the RNA over night at 4°C on a rotating shaker.
7. Transfer the eluate to a fresh 2-mL tube containing 50 μL of sodium acetate (3 M, pH 5.2). Add 2.5 volumes of ice-cold 100% ethanol and centrifuge at 16,200×*g* for 25 min. Discard the supernatant and wash the pellet by adding 250 μL of 70% ethanol followed by centrifugation at 16,200×*g* for 5 min.
8. Discard the supernatant and quickly dry the pellet.
9. Dissolve the pellet in RNase-free water and quantify the RNA by absorbance at 260 nm.

RNA Target Labelling

Target RNA could either be transcribed in the presence of radiolabeled nucleotides ($[\alpha\text{-}^{32}\text{P}]\text{UTP}$), or be labelled at either the 5'- or the 3'-end in the presence of either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\alpha\text{-}^{32}\text{P}]\text{Cp}$, respectively, and then be further purified by denaturing PAGE [10, 14, 23]. Generally, the 5'-end labelling protocol described below is used (see also Note 6).

1. Mix 5 μL of 5 μM in vitro transcribed target (25 pmol) with 3 μL of RNase-free water, 1 μL of 10 \times Antarctic Phosphatase Reaction Buffer and 1 μL of Antarctic Phosphatase (5 U).
2. Incubate the 10 μL dephosphorylation reaction at 37°C for 30 min. To stop the reaction, incubate the reaction at 65°C for 7 min, then store it on ice for 5 min.
3. Transfer 2 μL (5 pmol of dephosphorylated RNA) of the previous reaction to a new tube containing 14 μL of RNase-free water and 2 μL of 10 \times T4 PNK Reaction Buffer. Add 1 μL of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 1 μL of diluted T4 PNK (3 U).
4. Incubate the 20 μL reaction at 37°C for 1 h. Add 30 μL of loading buffer and gel purify the 5'-end radiolabeled RNA as described in subheading "Preparation of RNA Ribozymes and Targets". The RNA is visualized by autoradiography, cut out of the gel and eluted overnight with 500 μL of elution buffer at 4°C on a rotating shaker.
5. Add 1 μL of glycogen to the eluate followed by 50 μL of sodium acetate (3 M, pH 5.2) and 2.5 volumes of 100% ethanol. Centrifuge at 16,200 $\times g$ for 25 min and discard the supernatant. Wash the pellet with 150 μL of 70% ethanol followed by centrifugation at 16,200 $\times g$ for 5 min and finally dissolve it in 500 μL of RNase-free water for a final concentration of less than 10 nM.

Cleavage Assays

Usually the cleavage reactions are carried out under single turnover conditions in which the ribozyme concentration greatly exceeds that of the target ($[\text{SOFA-HDV Rz}] \gg [\text{S}]$) (10, 14). The following detailed procedure describes a 10 μL end-point cleavage reaction.

1. Mix 6 μL of RNase-free water with 1 μL of both 500 mM Tris-HCl (pH 7.5) and 100 mM MgCl_2 prior to the addition of 1 μL of radiolabeled target as mentioned in subheading "RNA Target Labelling" (see Note 7).
2. Start the reaction by adding 1 μL of 1 μM of SOFA-HDV ribozyme and then incubating at 37°C for 2 h.
3. Stop the reaction by adding 20 μL of loading buffer.
4. The completed reactions are fractionated on an 8 M urea denaturing PAGE gel of the appropriate concentration, and are then exposed to a Phosphoscreen.

5. The activity of each SOFA-HDV ribozyme is expressed as a percentage of the cleaved products over the total quantity of target ($100 \times [\text{cleaved}/\text{cleaved} + \text{uncleaved}]$) as determined using the ImageQuant (Molecular Dynamics) software.

Based on our experience, SOFA-HDV ribozymes that exhibit cleavage percentages over 75% under the conditions described above are considered as being active, and possess the greatest potential for further experiments. Conversely, SOFA-HDV ribozymes that exhibit cleavage activities below 20% are not suitable for targeting experiments. All of the SOFA-HDV ribozymes that exhibit median levels of cleavage activity could give good results in cellulo, and may therefore be considered depending on the desired number of ribozymes to test.

3.3.2. *In Cellulo/In Vivo* Assays

The next step in the establishment of a fully functional gene inactivation system is to test the selected SOFA-HDV ribozymes either in cellulo or in vivo. One important point to mention is that each case needs to be analyzed individually in order to design a suitable experiment. Depending on both the target and the cellular model, two major aspects should be considered: the appropriate controls and the expression system.

First, two types of controls are recommended in all experiments: a catalytically inactive ribozyme and an irrelevant, active ribozyme. The catalytically inactive ribozyme differentiates between any possible antisense effects and the catalytic cleavage of the target. Such a ribozyme can be produced by mutating either the catalytic cytosine to a guanosine (C76G), and/or the residues of the two GC base pairs that form the I.I pseudoknot to four uridines (see Fig. 1). The resulting SOFA-HDV ribozyme has the ability to bind the target, but does not exhibit cleavage. The other control is an active SOFA-HDV ribozyme that is irrelevant to the target, that is to say the sequence of the recognition domain and/or the biosensor are not complementary to the target. This control allows the measurement of the overall impact of the presence of a SOFA-HDV ribozyme in the cell without cleavage of the target. This control ribozyme should be analyzed with Ribosubstrate to confirm its irrelevancy to not only the target, but also to any important cellular genes. It is crucial that this ribozyme has a minimal impact on the overall life of the cell.

Finally, the selection of the expression system is crucial for the establishment of a gene inactivation system. According to the model of choice, either DNA or RNA transfection, as well as transduction with lentivirus encoding the ribozymes, could be used in in cellulo experiments. When expressing SOFA-HDV ribozymes from a DNA template, different options are available. First, the type of promoter has a significant impact on the results. A tissue-specific RNA polymerase II promoter will favour expression in a

specific cell type, while an RNA polymerase III promoter should result in a more efficient transcription that yields a greater amount of SOFA-HDV ribozyme. The cellular localization of the target entails a specific transport of the ribozyme inside the cells. The direct use of any polymerase III promoter without any additional sequence is a good way to produce SOFA-HDV ribozymes that are restricted to the cell's nucleus. For cytoplasmic export of the ribozyme, a 5' capped and 3' polyadenylated mRNA-like expression driven by the RNA polymerase II is a good option. It is also possible to use hybrid ribozymes consisting of a fusion between SOFA-HDV ribozyme and an RNA motif that permits an active transport into the cytoplasm. A good example of this is the use of tRNA^{Val} as a leader sequence for SOFA-HDV ribozyme (14). Data from our laboratory suggest that hY RNA are also good candidates for driving the cellular localization of ribozymes (J. Perreault and J.P. Perreault, unpublished data) (see Note 8). Finally, a cocktail of ribozymes targeting two or three different sites may also be used in conjunction to knockdown of a gene during in cellulo assays.

In summary, no universal protocol governs the details of an in cellulo experiment with a ribozyme. Each unique context possesses its own constraints that define the optimal strategy.

4. Notes

1. The biosensor can be elongated up to 12 nt in order to increase the binding affinity without too significant effect on the ribozyme's turnover.
2. The stabilizer can be changed if undesired interactions can occur between the target and the stabilizer. As this part of the SOFA module only plays a structural role, the only limitation is to avoid base pairing with other parts of both the ribozyme and the target.
3. The target's DNA template must include a T7 RNA polymerase promoter upstream of the targeted sequence. If the template is a linearized plasmid, about 5 µg of DNA should be used in a 100 µL run-off transcription reaction.
4. For SOFA-HDV ribozyme purification, in general 8% PAGE is used and the electrophoresis is halted when the xylene cyanol reaches the bottom of the gel. For targets, the concentration of the gel needs to be adapted to their length.
5. In order to visualize RNA using UV shadowing, simply put the gel (covered with laboratory plastic wrap) on a TLC plate (silica gel, Whatman) and expose it to 254-nm UV light.
6. End labelling gives results that are easier to quantify as only two different products need to be quantified on the gel, and

the calculation is therefore more accurate. 5'-end labelling is usually favoured; however, when the target cannot be efficiently labelled in 5', 3'-end labelling involving the ligation of [α - 32 P]Cp is performed. Alternatively, internal labelling by direct incorporation of [α - 32 P]UTP during the transcription can be performed.

7. When testing a series of SOFA-HDV ribozymes targeting one RNA, a master mix of water, radiolabeled target, Tris-HCl and MgCl₂ is prepared in order to favour greater uniformity between the reactions.
8. The use of an RNA polymerase III promoter for the expression of SOFA-HDV ribozymes limits in the variety of sequences that can be used. All ribozymes that contain four or more consecutive uridines should be discarded because this number of uridines forms a transcription termination signal. The three uridines of the linker that are located between the stabilizer and the biosensor increase the possibility of creating a transcription terminator. In this particular case, the linker could be mutated in order to avoid having four consecutive uridines present.

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