Development of Ribozyme-Based Gene-Inactivations; The Example of the Hepatitis Delta Virus Ribozyme

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Abstract: The development of gene-inactivation systems is an active and important field for both functional genomics and gene therapy. Towards this end, ribozymes (i.e. RNA enzymes), that specifically recognize and subsequently catalyze the cleavage of other target RNA molecules, are attractive molecular tools. Ribozymes represent an interesting alternative to the RNA interference (RNAi) approach for gene inactivation, especially given the fact that RNAi seems to trigger an immunological response and has demonstrated off-target effects. However, the design and optimization of a ribozyme-based gene-inactivation system is not a straightforward procedure. Several aspects need to be considered in the experimental design in order to provide a suitable suppression system. In this review we present the advances in this domain made available from work using the hepatitis delta virus (HDV) ribozyme as a *cis*-acting RNA motif in molecular biology, as well as a *trans*-acting molecular scissor for the development of a gene-inactivation system. This HDV ribozyme technology possesses several unique features that are all related to the fact that it is the only catalytic cleaving RNA motif that has been discovered in humans.

Keywords: HDV ribozyme, RNA interference, antisense, ribozyme, deoxyribozyme, RNase P, functional genomic, gene therapy.

TARGETING THE RNA

There are many potential approaches for the development of an effective gene therapy for a specific pathological disease. The critical initial question is at which level of the genetic information, DNA or RNA, are the interventions required? Even if it is possible to readily manipulate DNA coding sequences with the abundance of chemical and molecular tools currently available, numerous limits still exist, not to mention the "uncontainable" ethics problem associated with the idea of modifying the genetic patrimony [Kimmelman 2005]. Therefore, the development of gene-inactivation systems based on the targeting of RNA intermediates appears to be the most logical approach. Moreover, targeting RNA considerably simplifies the problem of sequence specificity. The study of the human genome has revealed that more than 90% of it is not transcribed into stable RNA coding intermediates. Consequently, it should be easier to target RNA molecules that adequately represent a cell's phenotype than to its corresponding DNA counterparts.

There are two general approaches in the targeting of RNA molecules. Both depend on the nature of the recognition motif on the substrate. First, there is recognition of a specific structural motif found in the RNA. Facilitating advancements in X-ray crystallography, nuclear magnetic resonance and the fluorescence spectroscopy of RNA molecules have led to the elucidation of the three-dimensional structures and conformations of several RNA species. In these cases, the rational design of inhibitors appears to be appealing and would involve targetting specific motifs of these RNA molecules [Yen *et al.* 2006]. However, redundancy exists in the RNA motifs used to build most RNA species, consequently it is difficult to develop a specific inhibitor directed exclusively against a given species [Goffeau *et al.* 1996]. Second, there is another mode of sequence recognition found in nature based on the formation of Watson-Crick base pairs. Since these types of interactions are predictable and modifiable as required, they offer the advantage of ensuring the specificity and selection of any RNA species. Following this rational, several approaches, collectively known as oligonucleotide-based drugs, have been developed over the last 25 years (Fig. **1**).

Each of these approaches have their own distinct mechanism of action and their own advantages and disadvantages. Various aspects of these different approaches were recently extensively reviewed [Bagheri and Kashani-Sabet 2004, Bartolome *et al.* 2004, Crooke 2004, Schubert and Kurreck 2004, Fadl 2005, Aiggner 2006, Behlke 2006]. Here, we concisely present their mode of action. Antisense oligonucleotides (ASONs) consist of complementary sequences that interact with their targets, causing either the inhibition of translation or the triggering of ribonuclease H which then cleaves the RNA strand form a RNA-DNA heteroduplex [Crooke 2004, Fadl 2005]. Since antisense oligonucleotides are deprived of catalytic activity, they have been the subject of profound chemical modifications aimed at increasing their nuclease resistance and their thermodynamic stability with

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the target in an intracellular environment. Gene silencing action associated with antisense oligonucleotides emerge mainly when they inhibit translation. Consequently, ASONs do not modify their specific target, nor do they exhibit any turnover. Their potential for the knockdown of gene expression was demonstrated in cell culture, animal models and in clinical trials. In fact, the first antisense oligonucleotide, Fomivirsen, that targets CMV retinitis in AIDS patients, is now commercially available [Fadl 2005].

The ability of ribozymes to specifically recognize, and subsequently catalyze the cleavage of, a RNA target make them attractive tools for the development of gene-inactivation systems [Bagheri *et al.* 2004, Schubert and Kurreck 2004]. The resulting cleavage products are then hydrolyzed by cellular endonucleases and exonucleases, thereby eliminating these unstable RNA molecules (Fig. **1**). The ribozymes, including hammerhead, hairpin and hepatitis delta virus (HDV) ribozymes, are found as self-cleaving RNA motifs essential to the rolling circle replication mechanism of infectious RNA species. In the laboratory these RNA strands have been separated in order to isolate catalytic RNA molecules that can successively cleave several RNA molecules. As a result, they potentially offer the advantage of modifying their target and possessing a turnover. Their activity has been shown to be effective with a wide repertoire of targets, and has led to many preclinical and clinical trials from phases I, II, and III [Bagheri *et al.* 2004, Schubert and Kurreck 2004, Fadl 2005, Behlke 2006]. For example, several studies have shown their ability to target various regions in the HIV genome, including the 5'-leader region, the gag and tat genes as well as the Ψ packaging site [for a review see Sun *et al.* 2000]. However, clinical trials performed with hammerhead and hairpin ribozymes were unsuccessful, which consequently diminished the interest in ribozyme development as a tool for gene-inactivation systems.

Similarly, some deoxyribozymes (also known as DNazymes), which were synthetically engineered, share the same advantages and disadvantages as ribozymes [Zhang *et al.* 1999, Sun *et al.* 2000]. Such nucleic acid enzymes have not yet been found in nature; however, this is not an indication of a fundamental inability of DNA to perform catalysis. Deoxyribozymes were obtained via *in vitro* selection, and, being DNA, appeared to be relatively stable in comparison to RNA. They cannot be expressed exogenously, which limits their use for gene knockdown. On the positive side, their chemical synthesis is cheap and efficient, with the product subsequently being transfected into cultured cells (Fig. **1**). Several demonstrations of their activity targeting mRNAs have been reported. One of the best examples is the 10-23 deoxyribozyme that has been shown to have potent antiviral activity against HIV by either directly targeting HIV-1 RNA [Zhang *et al.* 1999] or by preventing virus entry by down regulation of the CCR5 core receptor [Goila and Banerjea 1998].

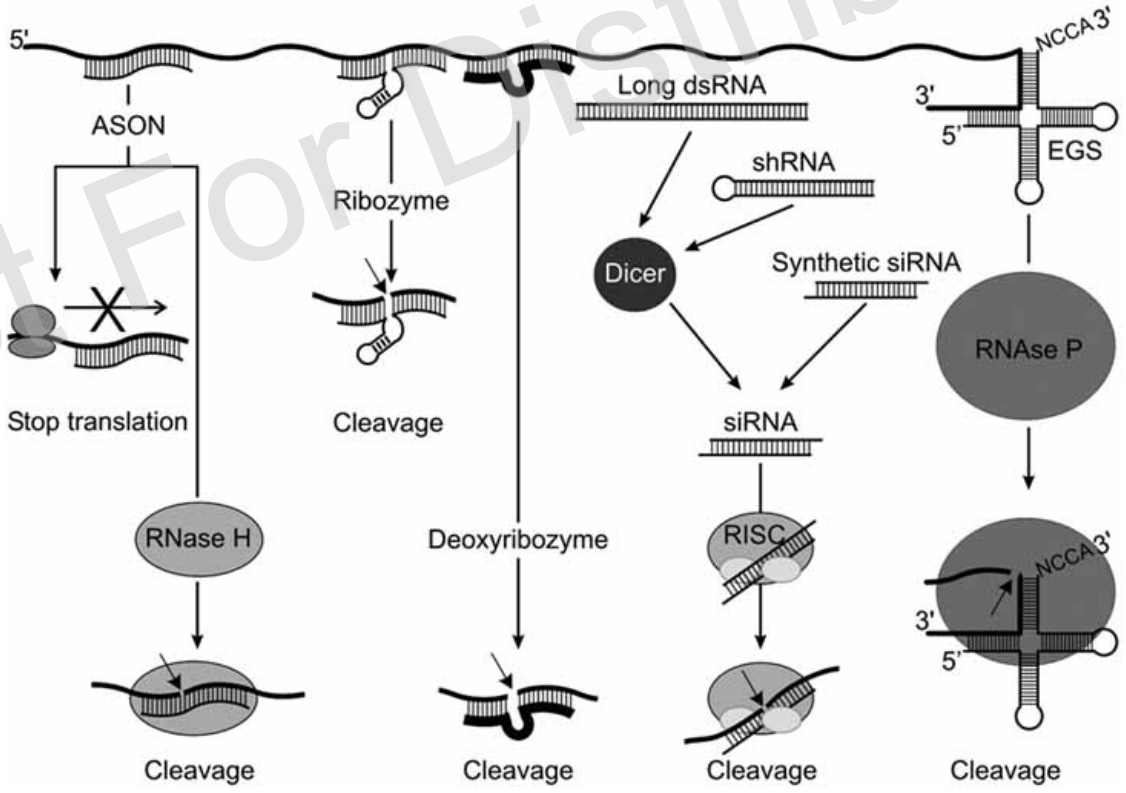

Fig. (1). Schematic mechanisms of the activities of several oligonucleotide-based drugs.
Antisense oligonucleotides (ASON) either block RNA translation, or induce cellular ribonuclease H (RNase H). Ribozymes (thin line) and deoxyribozymes (thick line) directly bind to and subsequently cleave the target RNA. Small interfering RNAs (siRNAs; or alternatively long dsRNA or shRNA), are incorporated into RNA-induced complexes (RISCs) that trigger the degradation of the complementary RNA. Ribonuclease P (RNase P), in the presence of an external guide sequence (EGS), catalyzes the cleavage of the target RNA. The EGS determines the cleavage site.

The ribonuclease P (RNase P), which is an ubiquitous enzyme, acts as an endonuclease and generates the mature 5'-end of tRNA precursors. In bacteria, RNase P exists as a ribonucleoprotein complex in which the RNA component alone possesses the catalytic activity and acts *in trans* upon multiple substrates. In fact, RNase P can be considered as the only true naturally occurring *trans*-cleaving RNA enzyme known to date. RNase P can be used in gene inactivation through the presence of an external guide sequence (EGS) which, upon hybridization with its specific target region, provides a complex that resembles the upper portion of natural tRNA [Guerrier-Takada and Altman 2000] (Fig. **1**). However, in higher eukaryotic cells like human cells, the EGS-target complex has to mimic the entire tRNA, an assembly requirement that can only be fulfilled by either incorporating, *in vivo*, a synthetic EGS exogenously, or by expressing it intracellularly. Alternatively, the EGS can be covalently linked to the RNA component of *Escherichia coli* RNase P, providing a sequence-specific ribozyme, and has been used as a molecular scissor in various applications [Liu and Altman 1995, Trang *et al.* 2000].

RNA interference (RNAi), which is active in organisms from fungi to human, is the latest approach used for gene-inactivation (Fig. **1**). This approach can be initiated by the introduction of a double-stranded RNA (dsRNA) that causes the silencing of its corresponding gene. Once introduced into a cell, dsRNA are processed by an RNaseIII like protein called Dicer, which results in the generation of 21-23 base pair short interfering RNA (siRNA) possessing two overhanging nucleotides at their 3' end [Bernstein *et al.* 2001]. The cleaved products are subsequently incorporated into the RNA-induced silencing complex (RISC) [Hammond *et al.* 2000]. The delivery of siRNA mimicking Dicer cleavage substrates results in the sequence-specific silencing of the corresponding endogenous gene [Elbashir *et al.* 2001]. RNAi can also be induced by endogenous expression of short hairpin RNAs (shRNAs; see Fig. **1**) [Brummelkamp *et al.* 2002]. shRNAs are structurally related to a highly conserved class of small RNAs, known as microRNAs (miRNAs), that mediate RNAi through a translational inhibition mechanism involving imperfect complementation at the 3' end of the targeted genes [He and Hannon 2004]. miRNAs are transcribed as precursors that are processed by the RNaseIII protein Drosha [Lee *et al.* 2003]. Thereafter, these miRNAs are further processed by Dicer into mature miRNAs, and are selectively incorporated into a RISC-like silencing complex [Mourelatos *et al.* 2002].

RNAi has been extensively used in mammalian cells to study cellular activity on many levels: the functional roles of individual genes, especially in disease [Dillon *et al.* 2004]; *in vivo* pathway dissection studies [Siripurapu *et al.* 2005]; gene expression knockdown in various cellular processes, including endocytosis [Huang *et al.* 2004]; signal transduction [Debes *et al.* 2002]; apoptosis [Kartasheva *et al.* 2002]; and, in the study of genes relevant to neurodegenerative disorder [Alberi *et al.* 2004]. Furthermore, RNAi has been used to treat cancer [Deininger *et al.* 2005] and both infectious [DeFilippis *et al.* 2003] and respiratory diseases [Shao *et al.* 2004]. Despite the gene silencing potential of siRNA and shRNA, specificity and efficiency issues remain to be solved. Nucleic acid base pairing is highly specific, mismatches at one position in a short sequence are often sufficient to completely prevent hybridization under physiological conditions. Indeed, a single mismatch can disrupt siRNA action against its target gene [Amarzguioui *et al.* 2003]. Unfortunately, traditional homology and cross hybridization analysis is insufficient, and siRNA can cause widespread changes in the expression levels of apparently unrelated genes [Jackson *et al.* 2003, Persengiev *et al.* 2004]. The most significant unexpected side effect in the application of siRNA is its ability to trigger a type-1 interferon response [Stark *et al.* 1998]. These effects can be either sequence or cell type specific, and therefore are not always encountered. For example, members of the Tool-like receptor family recognize dsRNA and trigger the IFN pathway [Tosi 2005]. Furthermore, other molecules that recognize dsRNA and can trigger the IFN pathway response include dsRNA dependent protein kinase (PKR), 2',5'-oligoadenylate synthetase (OAS) and retinoic acid-inducible gene I (RIG-I) [Stark *et al.* 1998, Yoneyama *et al.* 2004].

Considering the above facts regarding the therapeutic adaptation of antisense oligonucleotides in an intracellular environment, we believe that it is time to re-evaluate the potential of ribozymes in this regard and to evaluate whether or not they can be swiftly adapted to human cells.

HDV RIBOZYME IS A SUITABLE CATALYTIC RNA

HDV ribozyme catalyzes the transesterification of a phosphodiester backbone, yielding reaction products containing a 5′-hydroxyl and a 2′,3′-cyclic phosphate. Conversion of the HDV ribozyme's catalytic capacity into a *trans*–acting system has been achieved by separating the self-catalytic sequence of the *cis*-acting catalytic motif into a substrate recognizing *trans*-acting enzyme (Fig. **2**). These modifications promptly facilitated the use of HDV as a gene inactivation tool in ongoing research [for reviews see Shih and Been 2002, Bergeron *et al.* 2003, Puerta-Fernandez *et al.* 2003, Been 2006]. In solution, structural studies and structure/function assays of HDV ribozyme revealed the presence of a double-pseudoknot secondary structure (Fig. **2**) [for examples see Perrotta and Been 1991, Tanner *et al.* 1994, Bravo *et al.* 1996, Lee *et al.* 1996, Nishikawa *et al.* 1997, Nishikawa *et al.* 1999]. Notable features of the HDV ribozyme's structure are the presence of two stems (P1 and P2, which form a pseudoknot in the *cis* version), two stem-loops (P3-L3 and P4-L4) and three single-stranded junctions (J1/2, J1/4, and J4/2). The J1/4 junction and the L3 loop are single-stranded in the initial stages of folding, and are subsequently involved in the formation of the P1.1 pseudoknot [Wadkins *et al.* 1999, Nishikawa and Nishikawa 2000, Deschênes *et al.* 2003]. The P1 stem enables substrate binding to the ribozyme in the *trans*-acting version. Substrate specificity is based primarily on eleven consecutives nucleotides (see Fig. **2**). The nucleotides in positions –4 to –1 contribute as external determinants that differentiate an accurate substrate from a bad one [Deschênes *et al.* 2000], while those in positions +1 to +7 are internal determinants that base-pair with the ribozyme. The first base pair of the P1 stem must be a GU wobble, while the subsequent six base pairs can be any Watson-Crick pairing [Perrotta and Been 1991]. Finally, the interaction at the level of the P1 stem has been shown to be more complex than simple Watson-Crick base pairing, sug-

gesting the development of tertiary contacts between the P1 domain of the ribozyme-substrate complex and the catalytic core of the ribozyme [Ananvoranich and Perreault 1998, Ananvoranich *et al*. 1999].

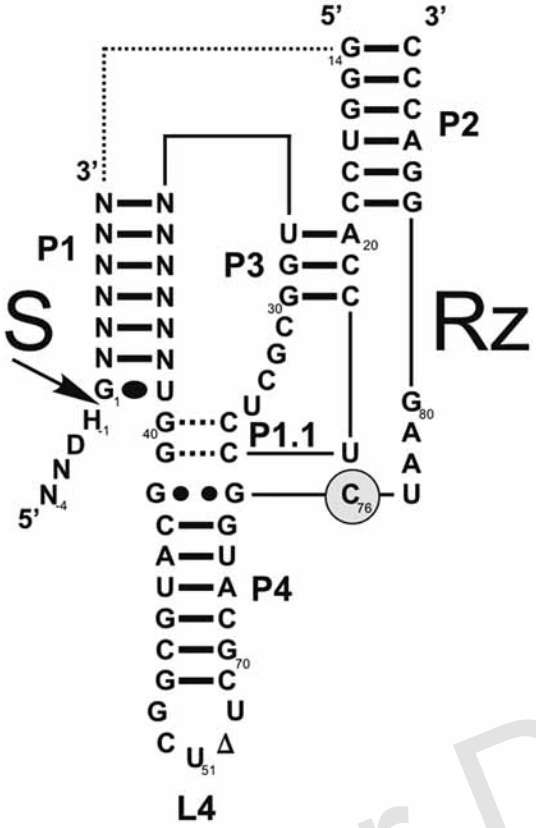

Fig. (2). Nucleotide sequences and secondary structure of the HDV ribozyme derived from the hepatitis delta virus antigenomic strand.

The numbering system is that of Shih and Been [2002]. The dotted line between the substrate (S) and the ribozyme (Rz) indicates the strand removed in order to generate the *trans*-acting catalytic RNA from the self-cleaving RNA strand (*cis*-acting). The Δ in the L4 loop indicates the location of the P4 deletion (as compared to the natural variants). The wobble base pair at the bottom of the P1 stem is indicated by a single large dot, while the homopurine base pair at the top of the P4 stem is represented by two large dots. The base pairs of the P1.1 pseudoknot are illustrated by the dotted lines. The catalytic cytosine (C_{76}) is circled. The arrow indicates the cleavage site. The identity of the nucleotides in positions -4 to -1 relative to the scissile phosphate influences the level of cleavage observed. The minimal requirement at these positions was determined to be $_{-4}$NNDH$_{-1}$, where H indicates U, A or G and D indicates U, C or A. More specifically, the order of reactivity for these positions was established to be: position -1 A > C >U // G; -position -2 A > G > U // C; position -3 U = C = A > G; and, position -4 C = U > G > A [Deschênes *et al.* 2000]. The closer the nucleotide in question is to the cleavage site, the more important this reactivity index.

The essential nucleotides from the single-stranded region (e.g. C_{76}) are all in close proximity to the cleavage site, generating a solvent-inaccessible core [Rosenstein and Been, 1996]. Several approaches, including p*Ka* measurements, proton inventory and kinetic solvent isotope effect, were used to characterize both the proton transfer and the protonation from the catalytic cytosine (C_{76}) [Nakano *et al.* 2000, Luptak *et al.* 2001, Nakano and Bevilacqua 2001, Shih and Been 2001, Oyelere and Strobel 2002, Tingley *et al.* 2003, Doudna and Lorsch 2005, Fedor and Williamson 2005]. Indeed, the HDV ribozyme was the first RNA for which a general acid-base catalysis was proposed, more specifically the C_{76} behaving as a histidine [Das and Piccirilli 2005, Been 2006]. Most importantly, the extrapolated rate constant for the chemical step of the HDV ribozyme's reaction may approach to the cleavage level of RNase A, suggesting that the general acid-base catalysis of HDV ribozyme has the potential of providing enormous cleavage rate enhancements [Nakano *et al*. 2000].

The evolution of the HDV ribozyme, in contrast to that of the hammerhead and hairpin ribozymes in plants, suggests that it is ideal for use as a functional enzyme in an intracellular environment as it is derived from an active RNA species found in human cells (i.e. the hepatitis *delta* virus). More recently, a genome wide search for innate ribozyme entities has revealed the presence of HDV-like sequences in the human *CPEB3* gene [Salehi-Ashtiani *et al.* 2006]. As a result, it should not be recognized by the immune system as an external, invading RNA. This characteristic probably prevents the triggering of either antibody production, or of the interferon response. Interestingly, the HDV ribozyme is the only catalytic RNA known to be fully active in the presence of calcium [Wu *et al.* 1989]. In contrast to other silencing catalytic RNA species (e.g. the hammerhead and hairpin ribozymes), which were isolated from non-mammalian hosts, the HDV ribozyme has the intrinsic ability to properly function in the low ionic concentrations found in human cells. Furthermore, it has been shown that the HDV ribozyme exhibits remarkable stability *in vivo*, regardless of both the human cell line tested and the means of transfection used [Lévesque *et al.* 2002]. More precisely, some HDV ribozymes were shown to retain higher catalytic activity and to demonstrate longer half-life stability when compared with the hammerhead ribozyme (i.e. >100 hr as compared to only 10 hr, respectively). For example, 12 hours after transfection, only 3% degradation of HDV ribozyme was observed as compared to 40% degradation of hammerhead ribozyme. Analysis of the hydrolysis pattern for the hammerhead ribozyme has suggested that the presence of single-stranded regions at both the 5′ and 3′ extremities of an uncapped RNA species constitutes a serious drawback in terms of stability. Conversely in the case of HDV ribozyme, both the 5′ and 3′ ends are located within the P2 stem (Fig. **2**), which appears to increase the ribozyme's stability. Furthermore, the stems forming the secondary structure of the HDV ribozyme appear to be too short to be hydrolyzed by double strand specific ribonucleases, since all of them require a minimum of twelve base pairs as substrate [Nicholson 1999]. The HDV ribozyme's stabilty, at least in part, seems to be due to the comparatively limited flexibility of its tightly packed pseudoknot containing tertiary structure [Doherty and Doudna 2000]. This idea is well supported by the study of several thermodynamic parameters, including the demonstration that the self-cleaving sequence of HDV ribozyme has an optimal temperature of 65°C and retains activity up to as high as 80°C in buffers containing either 5M urea or 18M formamide. This

suggests the presence of a catalytic core that is highly stabilized by a solid network of tertiary interactions, which is inaccessible to single-stranded specific endonucleases. More importantly, the higher stability of HDV ribozyme is an indication that no further chemical modification would be required for *in vivo* activity. It has been shown that chemical modifications in hammerhead ribozyme impair's its catalytic ability [Sioud *et al.* 1994, Rossi 2000]. Clearly, the HDV ribozyme benefits from its adaptation to the human cell environment; hence, it can be considered as an ideal candidate for the development of gene-inactivation systems.

USE OF THE SELF-CLEAVING HDV RNA MOTIF AS A TOOL IN MOLECULAR BIOLOGY

The first attempt to take advantage of the HDV ribozyme's ability was its use as a molecular scissor targeting various RNA species both *in vitro* and *in vivo*. Large amount of desired RNA transcripts can be synthesized using various versions of the universal methodology of *in vitro* transcription, a commonly used technique in molecular biology [Milligan and Uhlenbeck 1989]. Briefly, the gene of interest is inserted into a plasmid vector carrying a bacteriophage promoter (SP6, T3 or T7) upstream of a multiple cloning site. These vectors are then linearized by restriction enzyme digestion downstream of the inserted sequence, and subsequently used for run-off transcription with an appropriate RNA polymerase. Alternatively, run-off transcription templates can be obtained by PCR amplification using overlapping oligodeoxyribonucleotides corresponding to the desired RNA sequence and harboring the 5' end of the promoter sequence. This technique is very useful for providing large quantities of transcripts; however, it does not meet the requirements for protocols where homogeneity is of the utmost importance [Schürer *et al.* 2002]. It is well known that RNA polymerases exhibit the tendency to fall off before reaching the very end of the DNA template (i.e. premature termination), and that they can add non-encoded nucleotides to the 3' end of the nascent nucleic acid, resulting in transcripts that are relatively heterogeneous at their 3' ends [Draper *et al.* 1988, Kholod *et al.* 1998, Schürer *et al.* 2002]. Moreover, the cloning strategy may add extra sequences that often result in the attenuation of the RNA's catalytic potential. This is probably due to either the ribozyme's ability to recognize its cleavage site, or to misfolding of the catalytic unit. For example, the activity of *trans*-acting ribozymes was shown to be impaired by the presence of additional sequences at both the 5' and 3' termini of the catalytic core sequence [for examples see Bertrand *et al.* 1994, Ruiz *et al.* 1997].

In order to overcome this hurdle, hammerhead or hairpin *cis*-acting catalytic sequences were inserted at the 3' end of DNA templates. Since the transcriptional buffer already contains magnesium (2.5-25 mM), these sequences can self-cleave producing transcripts that are homogenous at their 3' ends [e.g. Bussière *et al.* 2003]. However, both catalytic motifs have sequence requirements located at the 5' end of the self-cleaving site, therefore, the applicability of these cassettes is restricted to certain RNA sequences. Conversely, HDV ribozyme is the only known catalytic RNA motif that has no specific prerequisites for the sequence located upstream to the cleavage site. More specifically, in the case of *trans*-acting HDV ribozyme the nucleotides located at positions –1 to –4 were shown to be important in substrate specificity [Deschênes *et al.* 2000]. However, the impact of these nucleotides was only of minor importance in determining the substrate specificity of *cis*-acting HDV ribozymes. The only absolute requirement for HDV self-cleavage is the presence of a guanosine residue in position +1, thus making it ideal for positioning at the 3' end of the target RNA. Therefore, *cis*-acting HDV ribozymes were considered very suitable for the development of cassettes for the production of RNA species with homogenous 3' ends (Fig. **3A**). For example, mitochondrial $tRNA^{Phe}$ and VA RNA transcripts derived from adenovirus were synthesized *in vitro* in large quantities using *cis*-acting HDV ribozyme cassettes and were shown to produce a single band upon denaturing polyacrylamide gel electrophoresis [Schürer *et al.* 2002, Walker *et al.* 2003]. Alternatively, the original transcripts can be extended up to seven nucleotides by run-off transcription followed by removal of the extended region through the action of a *trans*-acting HDV ribozyme recognizing extended nucleotide signature (Fig. **3B**). This strategy was used in the production of yeast $tRNA^{Phe}$, where HDV ribozyme was employed to cleave off additional stretches with high efficiency [Wichlacz *et al.* 2004].

Cis-acting HDV ribozyme cassettes have been constructed and used for many applications in cells where the objective was to produce RNA molecules having appropriate flanking extremities. A self-cleaving HDV ribozyme was shown to successfully release a hammerhead ribozyme which could subsequently bind to and degrade, in *trans*, a herpes simplex virus mRNA [Pattnaik *et al.* 1992]. Following the same strategy, *cis*-acting HDV ribozymes were also employed for the synthesis of transcripts with homogenous 3' ends that are then involved in the release of defective particles of vesicular stomatitis virus [Chowrira *et al.* 1994], the synthesis of protein involved in the rescue of influenza A virus [Fodor *et al.* 1999], the production of complementary DNA segments (cDNA) to study genomes such as bursal disease virus [Boot *et al.* 1999], the synthesis of a full-length mumps virus [Clarke *et al.* 2000], and the production of recombinant mesogenic newcastle disease virus or recombinant fowl pox virus [Evans *et al.* 2000; Krishnamurthy *et al.* 2000], to name only a few examples.

DESIGNING HDV RIBOZYMES TARGETING *TRANS* RNA MOLECULES

The activity of ribozymes depends on many factors, including: the relative amount of active ribozyme in cells, their co-localization with target RNAs, the structural features of the target RNAs (which influence both the accessibility and the specificity of target sites), their catalytic efficiency, the interactions of target RNAs with proteins, the intracellular stability of both the target RNA and the ribozymes, the ribozyme delivery, the cellular uptake, the pharmacokinetics, the biodistribution, the *in vivo* toxicity, etc. Some of these problems are solved with the use of HDV ribozyme and require no redesigning of the catalytic RNA domains. For example, HDV ribozymes are relatively stable in cell cultures and no further chemical modification is required. Hovewer, other issues like the cellular localization remain very important and will be discussed in detail in the following section along with the progress achieved in HDV ribozyme devel-

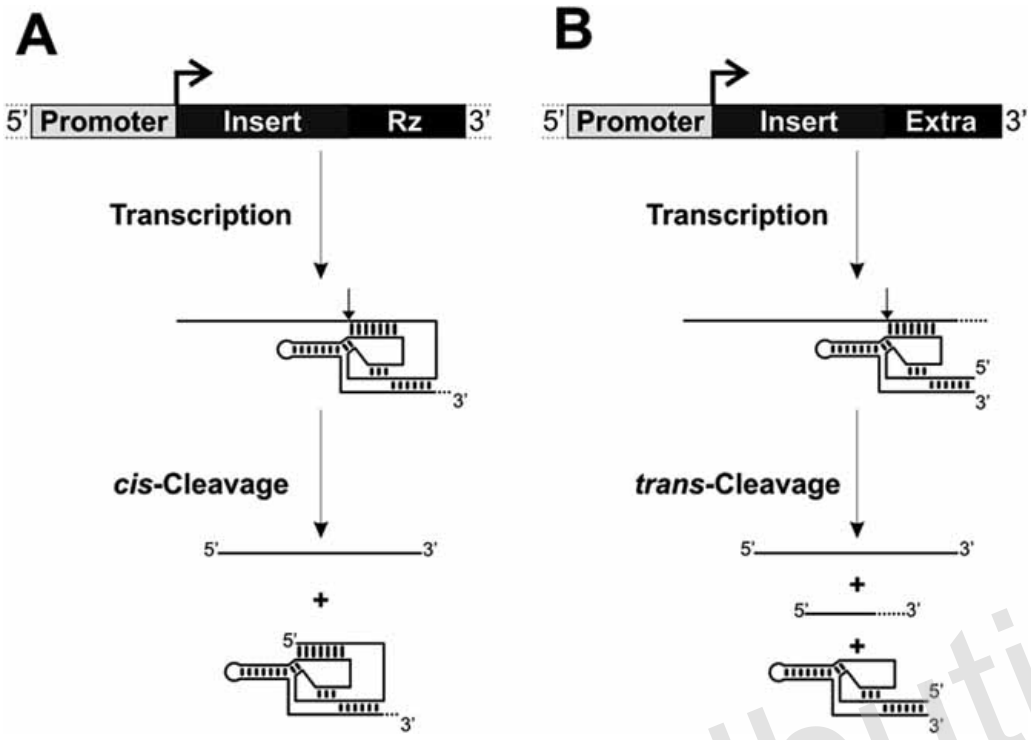

Fig. (3). Schematic representation of the use of the HDV catalytic sequence as a molecular scissor for the production of RNA transcripts with homogenous 3' termini.
The HDV catalytic motif is used either as a *cis*-acting (**A**) or a *trans*-acting (**B**) sequence.

opment as a molecular scissor targeting, in *trans*, RNA molecules.

Fruitful research on HDV ribozyme, as compared to other catalytic RNAs, has been delayed for several reasons, most notably its late discovery. However, this situation offered the advantage of using previously established improved approaches of "*ribozymology*" intended for functional genomics and gene therapy. Therefore, new experimental strategies can be designed to resolve some of the important issues relating to the activity of HDV ribozyme. The panorama of HDV ribozymes for the targeting of natural RNAs was demonstrated by the specific *in vitro* cleavage of the mRNA encoding the Hepatitis D Virus (HDV) related antigens, the pregenome RNA of Hepatitis B Virus (HBV) and of the 5'-untranslated region (UTR) of the Hepatitis C Virus (HCV) [Roy *et al.* 1999, Bergeron and Perreault 2002, Yu *et al.* 2002, Pan and Han 2003]. Successful *in vitro* targeting of HBV and HCV has clearly illustrated the operational potential of HDV ribozyme.

These studies have permitted the establishment of highly efficient procedures for designing ribozymes targeting accessible substrate regions. Target sequences located in single-stranded regions of RNA are more accessible for ribozyme binding and cleavage than those in double-stranded regions [Campbell and Cech, 1995, Bririkh *et al.* 1997]. Double-stranded RNA regions can compete with ribozyme substrate binding involving unfavourable intramolecular base pairings. Importantly, HDV ribozyme base pairing with the target RNA requires only seven consecutive nucleotides (i.e. the P1 stem; see Fig. **2**). Several methods have been reported for the identification of RNA target sites with increased accessibility, and were used for nucleic acid based drugs ity, and were used for nucleic acid based drugs including ribozymes (for examples see Matthews *et al.* 1999, Zu *et al.* 1999, Ding and Lawrence 2001, Kruger *et al.* 2001, Pan *et al.* 2001]. In the case of HDV ribozyme the approach of bioinformatic predictions coupled with biochemical assays (i.e. RNase H probing and oligonucleotide binding) were shown to be relatively unsuccessful [Bergeron and Perreault 2002; see also Bergeron *et al.* 2003 for a detailed review on the accessibility]. Conversely, combinatorial library-based approaches using either ASONs reflecting ribozyme's binding domain, or randomized ribozyme libraries, can provide a significantly larger proportion of active ribozymes, at least *in vitro* Bergeron and Perreault 2002]. As a matter of fact, target accessibility and the ability to form active ribozyme-substrate complexes both involve interdependent factors that cannot be accurately predicted on the basis of today's limited knowledge. Consequently, they can be better addressed using a combinatorial library of either oligonucleotides or ribozymes.

The potential of HDV ribozyme was subsequently established in cell culture using a variety of RNA targets [Kato *et al.* 2001, Seng *et al.* 2004, Al-Anouti and Ananvoranich 2002, D'Anjou *et al.* 2004]. Each experiment was performed using ribozymes that were expressed endogenously from a plasmid. Moreover, each of the study was designed to answer specific questions related to the HDV ribozyme's potential, or to provide a novel demonstration. For example, an HDV ribozyme was designed to target the junction of a BCR-ABL chimeric mRNA that causes chronic myelogenous leukemia [Kato *et al.* 2001]. This study compared the activities of various ribozymes *in vitro* versus cell culture. *In*

vitro, the HDV ribozyme possessed an activity almost three orders of magnitude lower than that of hammerhead ribozyme, but, it still exhibited activity in cultured cells similar to that observed *in vitro* while that of hammerhead ribozyme appeared to be significantly reduced. This result supported the idea of the better adaptation of HDV ribozyme to the cellular environment. In another study, HDV ribozymes were shown to efficiently cleave the RNA transcripts encoding uracil phosphoribosyl-transferase and hypoxanthine-xanthine-guanine-phosphoribosyltransferase of the pathogen *toxoplasma gondii* [Seng *et al.* 2004]. In fact, complete gene knock-down was possible even though the ribozymes were only transiently expressed. Extension of this work also revealed that HDV ribozymes were significantly more efficient in modulating the expression of the uracil phosphoribosyl-transferase than was RNAi, which in turn was more efficient than antisense RNA [Al-Anouti and Ananvoranich 2002]. Finally, a stable human cell line expressing a specific HDV ribozyme was reported to mediate the complete knock-down of the subtilisin pro-convertase 2 (SPC2) mRNA [D'Anjou *et al.* 2004]. The absence of any SPC2 protein biological activity in the cell line was confirmed by both HPLC and radioimmunoassay. This study unambiguously demonstrated that the observed silencing was a result of the ribozyme's catalytic activity and not an antisense effect. Coupled with proteomic analysis, another natural substrate of the SPC2 was revealed, specifically secretogranine II. Clearly, these studies constitute an elegant demonstration that HDV ribozyme is now an established tool for the development of gene-inactivation systems.

A NEW GENERATION OF HDV RIBOZYMES WITH IMPROVED FIDELITY

A need for fifteen to sixteen consecutive base pairing nucleotides has been estimated to be required in order to ensure sufficient substrate specificity for the targeting of a unique mRNA in human cells [Peracchi 2004]. Unfortunately, as the HDV ribozyme's substrate specificity is determined by only eleven nucleotides, when considering only the internal (i.e. 7 base pairs forming the P1 stem) and external (i.e. the single-stranded nucleotide at positions –1 to –4) determinants (Fig. **2**), it does not meet the criteria for targeting the entire collection of all human RNAs. However, HDV ribozyme does meet the criteria when possessing a biosensor module that switches the cleavage activity from *off* to *on* solely in the presence of the appropriate substrate [Bergeron and Perreault 2005]. The original version of this module was located at the end of the P2 stem, consequently it does not interfere with the catalytic core (Fig. **4**). This specific On/ofF Adaptor (SOFA) includes three domains: a blocker, a biosensor and a stabilizer. The proof-of-concept for this riboswitch was demonstrated both *in vitro* and *in vivo* using HDV ribozymes that can cleave RNA transcripts derived from the Hepatitis B and C viruses [Bergeron and Perreault 2005]. This was the first report of a ribozyme bearing a target-dependent module that is activated by its RNA substrate, an arrangement which greatly diminishes non-specific off target effects. An original feature of SOFA is the control of the ribozyme's activity by a blocker sequence that acts as a «safety lock», thereby preventing its folding into an active structure in the absence of the appropriate substrate, even in the presence of a substrate possessing the appropriate P1 but devoid of a region complementary to the biosensor [Bergeron and Perreault 2005]. Thus, the SOFA is not only an extension of the binding domain, but it also plays an active role in the discrimination of imperfect substrates. Analysis of the cleavage activity using a large collection of substrates and SOFA-HDV ribozyme mutants has provided information on both the effects and optimal design of each domain [Bergeron and Perreault 2005, Bergeron *et al.* 2005]. For example, it was determined that: i. the optimal size of the blocker sequence is four nucleotides; ii. the optimal size of the biosensor, without affecting turnover, is ten nucleotides; iii. a single mismatch between the substrate and the biosensor domain, which is responsible for the initial binding of the substrate and thereby switches the SOFA-ribozyme *on*, is sufficient to permit the selection of an appropriate substrate; iv. the stabilizer, which joins the 5' and 3' ends of the SOFA-ribozyme, has only a structural role; v. the optimal spacer length, which on the substrate separates the binding regions of the biosensor from the ribozyme's catalytic domains, is one to seven nucleotides; vi. both the blocker and the biosensor go through conformational transitions; and, vii. the SOFA-ribozymes were highly stable after transfection in cell culture, although less stable then the original HDV ribozymes which have an estimated half-life of 75 hours (unpublished data D. Lévesque and J.P. Perreault).

It is obvious that in order to develop an effective ribozyme *in vivo*, extensive *in vitro* and intracellular studies are required. The ribozyme's ability to properly recognize, bind and cleave a specific cellular RNA target will primarily depend on its pharmaco-kinetic properties. Ribozyme kinetics are dependent on many factors including the hybridization step in an intracellular setting, the rates of product release from a cleaved target and the length of both the recognition and the hybridization regions of the ribozyme [Herschlag 1991, Ellis and Rogers 1993]. Under saturating substrate conditions, ribozymes appear to obey simple Michaels-Menton kinetics that can be expressed in terms of k_{cat}/K_m [Uhlenbeck 1987, Hertel *et al.* 1994]. These factors were considered upon the development of the SOFA module, which acts synergistically with the recognition motif of the HDV ribozyme by increasing target recognition in terms of the number of Watson-Crick base pairs [Bergeron and Perreault 2005, Bergeron *et al.* 2005]. Kinetic studies comparing SOFA-HDV ribozymes to their counterparts lacking the SOFA module demonstrated an average increase in k_{cat}/K_m values of up to 25 fold by the simple addition of the SOFA module (e.g. from $9.3x10^3$ to $2.3x10^5$ min^{-1} M^{-1}; [Bergeron and Perreault 2005]). Considering that the k_{cat}/K_M ratio is the specificity constant, such a difference means a significant improvement in terms of the substrate specificity. Moreover, it has been shown that the difference in the k_{cat}/K_M constants between a $SOFA^+$- and a $SOFA^-$-HDV ribozyme (i.e. either with or without a biosensor complementary to the substrate) varied by a factor of an average of above 2 500 fold (up to more than 15 000 fold in some cases), a degree which appeared to be sufficient to provide the required level of substrate specificity for the recognition and cleavage of a unique mRNA in a cell.

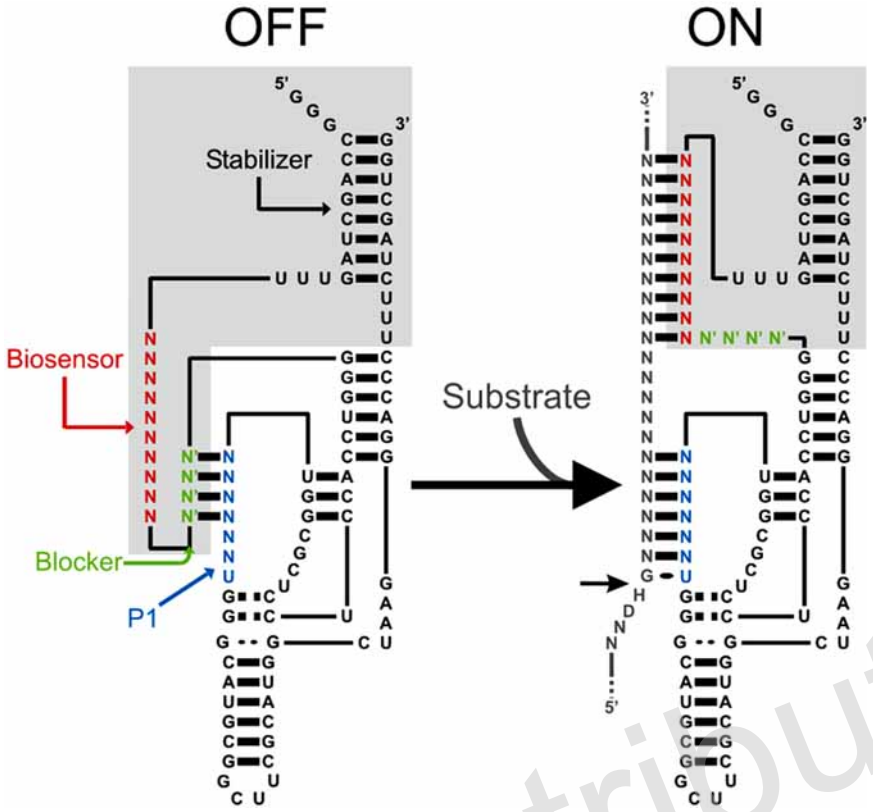

Fig. (4). The SOFA concept for improving ribozyme substrate specificity.
Secondary structure and nucleotide sequence of a SOFA-HDV ribozyme in both the "*off*" (inactive) and "*on*" (active) conformations. The gray section indicates the SOFA module. The P1 stem of the ribozyme is in blue. The biosensor and blocker are in red and green, respectively. The substrate is in gray. The arrow indicates the cleavage site.

In another set of experiments where HDV ribozymes were developed to target the abundant EFTu mRNA in the industrially important gram-positive bacterium *Lactococcus lactis*, the benefits of a riboswitch adaptor were again validated [Fiola *et al.* 2006]. These results demonstrated that the half-life of EFTu mRNA was barely reduced in the presence of classical HDV ribozymes. In contrast, the addition of a SOFA module to HDV ribozymes considerably increased the catalytic potential (approximately 10 fold), resulting in an observed reduction of up to 50% in the EFTu mRNA level. These improvements were most likely the result of a stronger formation of the ribozyme-substrate complex, less dissociation of these complexes, greater cleavage potential of the ribozyme and faster enzymatic turnover. These experiments also indicated that each intracellular molecule of SOFA-ribozyme cuts approximately 3 molecules of mRNA in 20 minutes, corresponding to a catalysis rate of ~0.05 to 0.1 targets per min^{-1}. Even if these calculated rate constants (k_{obs}) are rough estimations, it is interesting to observe that they are found to be in the same range *in vitro* under single-turnover conditions (0.10 to 0.12 min^{-1}), suggesting that they are as active in cell culture as *in vitro* [Fiola *et al.* 2006]. Importantly, this work also unambiguously demonstrated that the SOFA ribozyme exhibited turnover *in vivo*. Thus, the addition of the SOFA module to the HDV ribozyme has consistently and impressively demonstrated the ribozyme's ability to increase its catalytic potential. Clearly, this new generation of HDV ribozymes provides a highly specific and improved tool with significant potential for applications in the field of both functional genomics and gene therapy.

OTHER IMPORTANT ISSUES FOR PROGRESSING WITH RIBOZYME BASED GENE-INACTIVATION

One of the important remaining hurdles in the selection of target sequence is the design of tools that have minimal off-target effects. Ideally, it would be desirable to possess either a bioinformatic or a biochemical approach that enabled verification of whether or not a ribozyme offers the specificity required in order to target a unique mRNA species. Recently, a web-based application that permits the searching of a cDNA database for all potentially targeted substrates for a given SOFA-HDV ribozyme was developed [Lucier *et al.* 2006]. This software was also adapted for searching siRNA and hammerhead ribozyme. The search result includes mRNAs that perfectly match the specific requirements for a given ribozyme, wobble base pairs and mismatches. Therefore, this bioinformatic tool allows a rapid selection of sequences suitable as targets for RNA degradation prior initiating any laboratory experiments.

The utilization of ribozymes in cellular environment requires that they first be internalized into individual cells and then access the target mRNA. Unfortunately, the cellular uptake of ribozymes is usually inefficient due to both their charge composition and large molecular size [Miyagishi and Taira 2005]. In order to overcome this problem, ribozymes

are usually delivered with either viral or non-viral delivery systems [Wu and Ataai 2000, Lewin and Hauswirth 2001, Sullenger and Gilboa 2002]. The viral method is overwhelmed by serious problems such as toxicity, immunogenicity and is laborious to produce. Consequeltly, liposomes, charged lipids and, more recently, lipoproteins, are commonly used for safe non-viral ribozyme delivery. The integration of ribozymes into cells can be achieved using plasmid vectors encoding ribozyme genes where the ribozymes are transcribed by the host's transcription factors, since naked nucleic acids are rapidly degraded by nucleases in the cell. Vector based ribozyme delivery constitutively produces ribozymes inside target cells. This endogenous mode of delivery, and its applications, have been described in recent reviews [Glover *et al.* 2005, Christopher and Wong 2006, Dang and Leong 2006]; and has been employed for the delivery of HDV ribozymes to cells and found to be satisfactory [Lévesque, Ullah and Perreault; unpublished data].

After the introduction of ribozyme-expression vectors into cells, the success in ribozyme-based technology is highly dependent on their trafficking and intracellular localization (cytoplasm versus nucleus). This can be especially important in the event where the targeted RNA is sequestered within an intracellular compartment. Moreover, obtaining optimal expression levels of the ribozyme in the desired sub-cellular environment has also proven to be challenging [Kato *et al.* 2001]. These issues predominantly rely on the nature of the expression cassette's promoter driving localized ribozyme expression [Bertrand *et al.* 1997, Peracchi 2004]. The expression of ribozymes by DNA expression cassettes is now mostly directed by promoters for RNA polymerase (pol) II or III that govern their intracellular expression.

In the early studies of ribozyme expression systems, RNA polymerase II (pol II) promoters were used [Smith *et al.* 1997, Thompson 1999]. Pol II promoters allow tissue-specific and regulated expression levels of their encoded genes. Although not as potent as pol III promoters, pol II expressed products mainly localize in the cytoplasm. Ribozymes under suitable regulation have demonstrated enhanced efficiency when co-localized with their target mRNA in the cytoplasm rather than in the nucleus [Bertrand *et al.* 1997, Good *et al.* 1997, Kato *et al.* 2001]. Moreover, use of some pol II promoters that are tissue specific may provide a strategic advantage in terms of limiting the potential number of off-target effects. Technically, the introduction of pol II-regulated ribozyme can present some challenges. In some cases, low ribozyme efficiency can be attributed to the properties of pol II-based transcription, including the addition of an extended poly(A) tail and of post-transcription regions at both the 5' and 3' extremities. These additional flanking sequences can impede the proper folding of the catalytic unit, and can also encumber specific substrate recognition and binding [e.g. see Ruiz *et al.* 1997]. These challenges can be circumvented by several strategies. For example, *cis-trans-cis* multi-ribozyme cassette systems have been used [Bussière *et al.* 2003]. In this case, ribozymes are embedded between two self-cleaving catalytic RNA motifs (i.e. *cis*-acting hammerhead ribozyme ~ *trans*-acting HDV ribozyme ~ *cis*-acting hammerhead ribozyme [Fiola *et al.* 2006]). After transcription, the hammerhead sequences self-cleave to release the HDV ribozyme flanked with the desired 5' and 3' termini sequences required for effective ribozyme folding and targeting of the desired substrate.

An alternative to these obstacles is the use of other promoters mediated by RNA polymerase III, an enzyme which is normally responsible for the transcription of small, highly abundant RNA molecules within the cell [Geiduschek and Tocchini-Valentini 1988]. Pol III specific promoters includes tRNA, U1, U6 snRNA and human H1 (i.e. the gene coding the RNA component of the human RNase P) [Bertrand *et al.* 1997, Good *et al.* 1997]. In contrast to pol II promoters, pol III regulation drives ubiquitous ribozyme expression levels in all tissues without regard for either the control of transcription levels or tissue specificity. In addition, fewer extra sequences are required for transcription. The tRNA type of pol III promoters are embedded within their structural genes, thereby generating chimeric ribozymes bound to a 5'-tRNA [Kuwabara *et al.* 1999, Kuwabara *et al.* 2000]. In a recent study, pol III expression systems based on the human $tRNA^{Val}$ promoter were used for the expression of HDV ribozymes in cells [D'anjou *et al.* 2004]. In this study the use of a modified $tRNA^{Val}$ system permitted the complete knockdown of the subtilase-like pro-protein convertase-2 (see above). In designing terms, the development of an appropriate tRNA expression system results in a portion of tRNA becoming incorporated into the ribozyme. Initially, the nascent transcribed tRNAs are processed at both the 3' and the 5' ends, then the mature tRNAs are exported from the nucleus to the cytoplasm. In order to avoid processing at 3' end of the tRNA, it was modified so as to block the release of the HDV ribozyme from the $tRNA^{Val}$ portion. This results in the transcript having an extended sequence including HDV ribozyme sequence at the 3' end of the $tRNA^{Val}$, and is referred to as $tRNA^{Val}$-attached HDV ribozyme. *In vitro* assays demonstrated that for most of the chimeric constructs the presence of the tRNA did not affect the ribozyme cleavage activity (M. Lévesque and J.P. Perreault; unpublished data).

Finally, there is the possibility of using naked HDV ribozyme, as an RNA molecule, for transient cell transfection [Lévesque *et al.* 2002]. This strategy enables the researcher to properly control both the nature and the quantity of RNA ribozyme, not to mention ensuring that it possesses the desired extremities. However, in some cases, RNA transfections using cationic lipid delivery systems have shown lower catalytic activity [Yang and Huang 1997]. This could be due to the permanent cationic lipid binding to the ribozyme, a situation which attenuates substrate specificity and results in the suppression of catalytic efficiency as shown by *in vitro* cleavage assays. Moreover, the transfection of RNA ribozymes results in only a transient presence of exogenous material that will be diluted by cell division. As a result, ribozyme effectiveness will be limited by transfection efficiency, and the generation of stably expressing ribozyme cell clones will be impossible.

CONCLUDING REMARKS

The discovery of RNAi mechanism provided a powerful molecular biology approach with which to silence various RNA substrates. However, several recent reports have illustrated the limited specificity and off-target effects associated with the RNAi mechanism, hindering their therapeutic de-

velopment [for examples see Jackson *et al.* 2003, Haley and Zamore, 2004, Akashi *et al.* 2005, Birmingham *et al.* 2005]. Keeping these important prerequisites for the development of gene therapy in mind, ribozymes can be considered as an interesting alternative for this purpose. More specifically, the net advantage that evolution has provided the HDV ribozyme is that it is well adapted to the human cell environment, thus making them a suitable silencing tool for future development. Hepatocytes are natural host for the replication of hepatitis virus (HDV), consequently they can serve as the best physiological platform for HDV ribozyme efficiency. Strategies are already being designed for HDV ribozymes, and have already been applied for the inhibition of viral activity, cancer growth and dominant inherited genetic diseases. The efficiency of the HDV ribozyme as a gene inactivation system depends greatly on its specificity, enzymatic ability, expression level and subcellular localization. The choice of a expression system will also rely on the nature and properties of the targeted substrate. Often, desired effects are difficult to achieve and generally require optimization and the use of different ribozyme expression systems. In this regard the preliminary work performed using HDV ribozyme is very encouraging. The future progress towards the direction of clinical applications which would be the critical step for the true evaluation of the real potential of this catalytic RNA.

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