Ribozymes, genomics and therapeutics

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Genome-sequencing projects are proceeding at a rapid pace and determining the function of open reading frames is the next great challenge. Ribozymes with site-specific cleaving activity could aid greatly in this process. High-throughput screening methods to identify optimal target sites for ribozyme cleavage will provide tools for functional genomics as well as therapeutic reagents.

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Introduction

The versatility of RNA as a form of information storage and retrieval is well known. In pioneering work, Cech and Altman and colleagues [1,2] demonstrated that RNA can also function as an enzyme, which has created opportunities for engineering RNA enzymes (ribozymes) as surrogate genetic tools and for therapeutic applications. Engineered ribozymes have several useful attributes, including site-specific cleavage of the target RNA (and hence functional inactivation), multiple turnover, and the ability to be exogenously delivered or endogenously expressed from an appropriate transcriptional cassette [3–5]. Already, ribozymes have proven useful as surrogate genetic tools and they are now being examined as therapeutic agents. With the rapid progress in sequence analyses of genomes, ribozymes are becoming increasingly important as tools for studying gene function and identifying potential new therapeutic targets for the treatment of disease. To take full advantage of ribozymes as genetic tools and therapeutic agents, new approaches for high-throughput functional screening of ribozymes along with methods for enhancing intracellular activity are sought.

Ribozyme usage and catalytic motifs

Interest in ribozyme technology among the scientific community is steadily increasing, as is evidenced by surveying the National Library of Medicine database of publications in which ribozyme is in the title or is a key word. During the last decade there have been over 1800 articles focusing on ribozymes, beginning with three listed publications in 1989 and increasing steadily every year to 335 in 1997. The increasing interest in catalytic RNAs is also reflected in the use of ribozymes in several human clinical trials. This article will focus upon the simplest catalytic RNA found in nature, the hammerhead ribozyme [6,7], but at least five other ribozymes have been demonstrated to function as site-specific RNA cleaving reagents: the hairpin, group I intron, hepatitis delta, RNAse P and Neurospora VS RNAs.

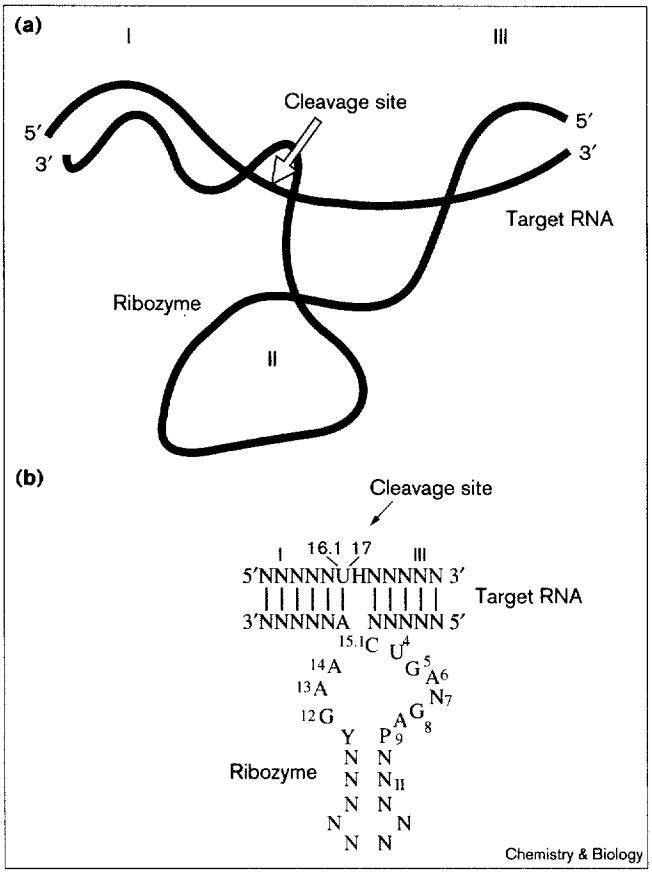
The hammerhead ribozyme has a catalytic core consisting of 11 conserved nucleotides flanked by three duplexes (Figure 1). Extensive *in vitro* biochemical analyses of the hammerhead ribozyme reaction mechanism have been complemented by the acquisition of X-ray crystallographic structures [8–10]. The structural studies have verified the importance of the conserved catalytic core nucleotides, and have provided new information that could be useful for the design of *trans*-acting ribozymes.

Parameters affecting ribozyme function

Ribozyme testing *in vitro* has revealed a number of important considerations for enhancing ribozyme function. The factors that should be considered include the lengths of the base-pairing regions, the structure of the RNA in the region of the cleavage site, the nucleotide(s) at and surrounding the targeted site of cleavage, the effects of various modifications in the ribozyme primary sequence, the effects of various backbone modifications on ribozyme turnover and stability, and the role of cellular proteins [3–5,11–15]. From such studies, a set of general rules has been established concerning the hammerhead ribozyme reaction. The reaction is comprised of a series of sequential steps: binding of the ribozyme to the target, cleavage, and release of the cleavage products [16] (Figure 2). Simple principles govern the rate of each step. The rates of target binding and the release of each cleavage product follow the general rules of nucleic-acid hybridization. The cleavage rate (about 1 per minute) is relatively independent of the primary sequence of the binding arms of the ribozymes, provided the ribozyme forms stable base pairs surrounding the site of cleavage. For optimal ribozyme activity *in vitro*, the lengths of the substrate binding arms should allow sufficiently stable ribozyme–substrate base pairing to facilitate cleavage, yet allow rapid dissociation of the cleaved products. Exhaustive mutational analyses of the hammerhead ribozyme and its cleavage targets have revealed that cleavage can occur after any NUH triplet (where N = any nucleotide and H = A, C or U). The kinetics of the reaction can vary significantly (up to one or more orders of magnitude) with different triplet-flanking sequence combinations [12,17] so the choice of an appropriate ribozyme cleavage site is the first and most critical step in hammerhead ribozyme design.

In contrast to the rather extensive knowledge of the rules governing effective ribozyme function *in vitro*, we have

Figure 1



The hammerhead ribozyme. **(a)** A representation of the X-ray derived structure from Pley *et al.* [8]. **(b)** A generic hammerhead ribozyme paired with a substrate RNA. N, any nucleotide; Y, pyrimidine; P, purine; H = A,C or U. Roman numerals I, II and III refer to the helical stems of the ribozyme substrate (I,III) and stem–loop II of the ribozyme.

only a limited set of rules for predicting ribozyme efficacy in an intracellular or *in vivo* environment. Despite extensive studies of the parameters that are most likely to influence the intracellular functioning of ribozymes (expression levels, intracellular co-localization with the target RNA, stability of the ribozyme and protein interactions [18–21]), predicting *in vivo* efficacy remains enigmatic, and successful ribozyme-mediated down-regulation of target RNAs relies mainly on empirical testing. The most effective strategies for achieving ribozyme function *in vivo* involve mechanisms that maximize the ability of a ribozyme to base pair with and cleave the target RNA. For *trans*-acting ribozymes, base pairing to the target RNAs is the rate-limiting step *in vivo* [18,19,22].

Many studies have employed computer-assisted RNA folding to identify sites in RNA that are potentially open and accessible to base pairing with a ribozyme. The use of such programs does not, however, guarantee successful ribozyme functioning within the complex environment of a cell because RNAs are continually associated with proteins from the time of synthesis until they are degraded. Some of these proteins can facilitate ribozyme pairing by acting as chaperones for RNA interactions, as well as ribozyme and target mRNAs subcellular localization [14,15,23]. Despite the absence of a well-defined set of rules for targeting and expression, several experiments demonstrating ribozyme efficacy *in vivo* have been conducted in model organisms including *Drosophila* [24], mice [25,26] and Zebrafish [27]. These experiments provide proof of principle that effective ribozyme use *in vivo* is a possibility for any target. Once well-defined sets of rules for ribozyme targeting, expression and intracellular localization are developed, it should be possible to use ribozymes effectively to downregulate any RNA target.

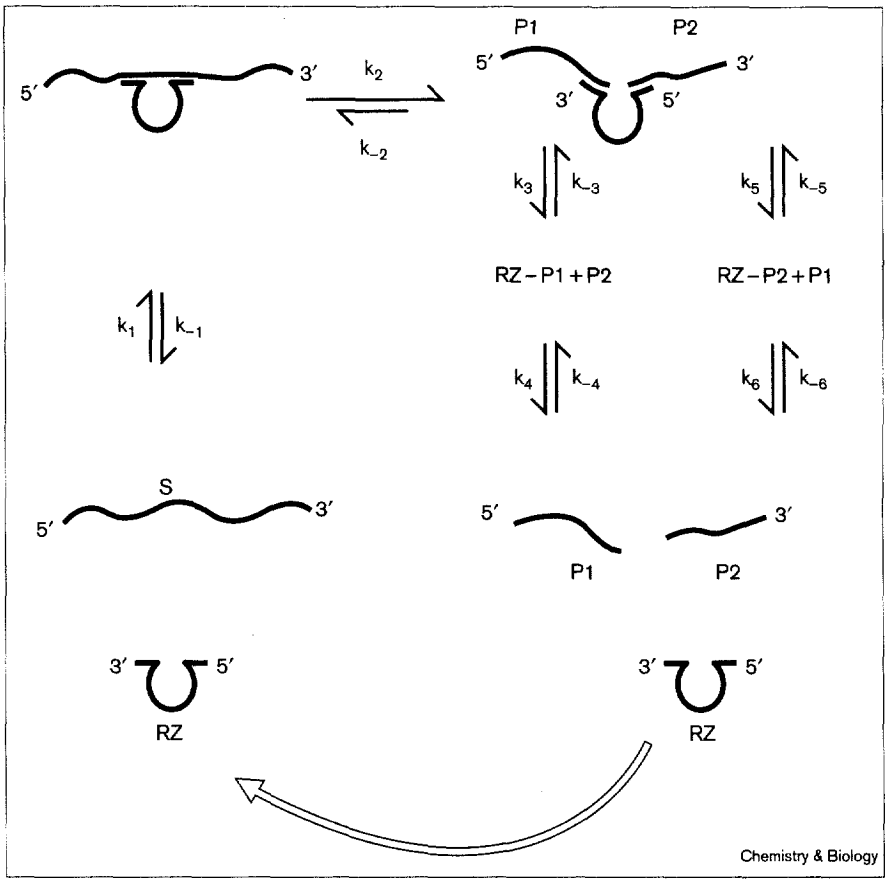
Therapeutic ribozymes

HIV infection is the first human disease for which ribozymes are being tested as potential therapeutic agents (several human clinical trials are currently in progress). The simplicity of the sequence requirements for hammerhead and hairpin ribozyme cleavage means that there are hundreds of potential cleavage sites along the length of the viral genome. Because HIV mutates rapidly, and can become resistant to single-drug therapies, multidrug and multitarget-site strategies are the most effective methods for treating HIV-1 infection. Ribozymes therefore represent a reasonable therapeutic agent for inhibiting HIV because multiple ribozymes targeted to a number of different sites in the HIV genome can be delivered simultaneously to cells.

The effective therapeutic use of ribozymes requires their effective delivery into the appropriate cells. For example, the current ribozyme trials for the treatment of HIV infection utilize viral vector-mediated gene-delivery approaches. Ribozyme genes encoded within retroviral vector backbones are used to deliver these genes into either T lymphocytes or hematopoietic stem cells. Thus far, these trials have utilized *ex vivo* transduction of cells with the viral vectors and subsequent reintroduction into patients. For other diseases, delivery to target cells might require the systemic injection of ribozyme genes or preformed ribozymes. At present, the systemic delivery of ribozymes is problematic because of technical problems in the scaled-up synthesis of ribozymes and/or viral vectors carrying ribozyme genes coupled with the lack of effective and efficient specific cell targeting. Methods for effectively delivering ribozymes and/or ribozyme genes to cells *in vivo* could impact greatly on the treatment of a variety of diseases, including cancer. Ribozymes that are highly resistant to serum and cellular nucleases have already been developed [13]. The next challenges are to develop scaled-up synthetic procedures for these backbone-modified ribozymes and new carriers to facilitate delivery and cellular uptake. For viral vector-based delivery, evolving new methods for

Figure 2

Steps in the hammerhead ribozyme reaction. Kinetic constants are indicated. The size of the arrows for the cleavage step indicates that the equilibrium is strongly in favor of cleavage. RZ, ribozyme; S, substrate; P1 and P2, cleavage products.



producing high titer viral stocks with specific cell tropism is a challenge for the future.

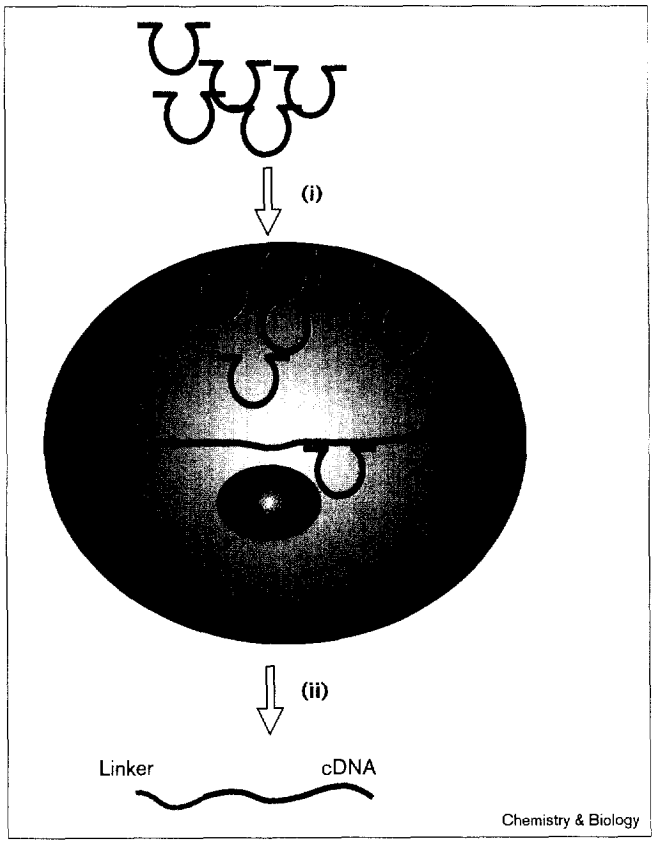
New methodologies for identifying ribozyme cleavage sites

Worldwide efforts to determine the complete nucleotide sequence of the human genome as well as the genomes of other organisms have resulted in a huge catalogue of sequence information, which will continue to increase. The goals of the human genome program are to have the project completed early in the first decade of the 21st century and the following challenge will be to determine the functions of tens of thousands of open reading frames. Among the techniques that will be utilized for this are antisense-DNA- and ribozyme-mediated inactivation of mRNAs expressed from these genes. Targeted transcript inactivation can be followed by phenotypical and more detailed physiological and biochemical analyses of cells in which the target has been destroyed. Ribozymes are especially attractive tools for targeted inactivation because judiciously chosen cleavage sites will allow discrimination between point mutations or related sequences within gene families.

To realize the full potential of ribozymes in studies of gene function as part of the human genome sequence program, it is imperative that high-throughput screening methods are developed to identify optimal ribozyme cleavage sites. As it is not possible to predict the effect cellular proteins will have on ribozyme–target interactions, approaches need to be developed that will allow ribozyme cleavage sites to be rapidly identified in mRNAs in their native, protein-associated state. The studies of Lieber and Strauss [22] and our own studies [28] have begun to address this problem by identifying sites on native mRNAs that are accessible to base pairing and hence ribozyme- and antisense-mediated destruction. The approach of Lieber and Strauss [22] uses libraries or ribozymes with random sequences in the binding arms. Successful ribozymes are sorted out via their cleaved targets using RT–PCR and subsequent cloning, which is a rather laborious process. Alternative methods should involve combining random library approaches with RNA-based, ligation-mediated PCR methods for identifying cleavage sites [29,30].

There is a need for the development of high-throughput systems for screening and identifying optimal sites for ribozyme cleavage. Ideally, these screening methods will use intact cells or cell extracts harboring target mRNAs of interest. As random approaches provide the greatest range

Figure 3



Ribozyme library screening process. The ribozyme library, made synthetically or biochemically, is added to cells or cell extracts (i). Ribozymes are allowed to find targets and cleave. The cleavage sites along the target of interest are identified by amplifying the cleaved RNAs using a specific primer complementary to the RNA of interest, and a primer added to the cDNA using a ligation step (ii) [29,30]. The cleavage sites are identified following gel electrophoresis of the amplified products as described previously [29,30]. The entire procedure could be carried out robotically.

of possibilities, they are preferred over more directed approaches. Methods need to be developed that allow rapid, unambiguous identification of ribozyme target sequences. Once such sites have been identified, specific ribozymes must be tested efficiently in the appropriate cell culture or animal model. Given that the ribozyme–target pairing interaction can be the rate-limiting step *in vivo*, thoughtful consideration must be given to the length of the pairing arms in a random library approach. An example is provided below for a ribozyme capable of forming ten base pairs with the target RNA. A semi-random sequence would identify NUH sites (N = any base, and H = A, C or U). As an example of such a pool, a library consisting of ribozymes 5′-NNNNN–catalytic core–ANNNN-3′ would contain 2×10^5 different ribozyme sequences. Screening with this pool involves de-convoluting either the successful ribozymes or the targets that are cleaved. The longer the binding arms, the larger the pool size and the more difficult the de-convolution task. The challenges of using the random or even the semi-random ribozyme approach are to incorporate a rapid throughput ribozyme delivery with the use of very sensitive assays for ribozyme or ribozyme cleavage site identification. For large-scale functional genomic studies, this process should be carried out robotically. A possible scheme for this entire process is shown in Figure 3.

Aside from the use of ribozymes in elucidating gene function, it is possible that ribozymes used for genomics studies can be developed further as therapeutic agents for the treatment of diseases associated with altered expression or mutant forms of the gene in question. Other prospects for therapeutics are perhaps more indirect. If the ribozyme approach identifies a candidate sequence for some disease process, then the product of this gene can be used as a target for small-drug therapy. The effective use of ribozyme technology in functional genomics will have an impact on many other areas of science and medicine.

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