Small Ribozymes

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The course of history is strewn with revolutionary discoveries, which have changed the scientific views or dogmas of the period and stimulated subsequent research. An example is the landmark discovery, in the laboratories of Altman and Cech in the early 1980s, of RNA molecules with the ability to catalyze phosphodiester bond cleavage in the absence of proteins (Kruger et al. 1982; Guerrier-Takada et al. 1983). These findings gave the first indication that in addition to carrying genetic information, RNA molecules were also capable of catalyzing chemical reactions. RNA may therefore hold the key to understanding the origin of life, and the present-day cooperation between DNA, RNA, and proteins may have evolved out of a purely RNA world (Gesteland and Atkins 1993). This notion is consistent with the large variety of ribonucleotides that have been identified in RNA (Limbach et al. 1994) which are thought to be leftovers from the more archaic activities of RNA molecules. Since the initial publications of ribozyme activity, other naturally occurring ribozyme motifs have been discovered. In addition, the advent of in vitro selection has expanded the number of chemical reactions, thought previously to be performed by proteins, that can be catalyzed by RNA molecules (Gold et al. 1995). This technique has rapidly expanded our knowledge of RNA motifs capable of supporting catalytic activity and has led to an explosion of data regarding the structural and functional diversity of RNA.

In addition to fulfilling a scientific curiosity, the findings thus far also indicate that RNA holds promise for various practical applications such as diagnostics or therapeutics. In particular, ribozymes have the potential to become an important therapeutic in the fight against genetic and viral diseases by virtue of their ability to intercept and cleave messenger RNA. This aspect of ribozymes is discussed briefly later in this chapter.

Traditionally, ribozymes have been grouped by size. Large ribozymes such as the group I introns, group II introns, and RNase P are discussed by Harris et al. (this volume). Our focus is on the small ribozymes that have common terminal products after the cleavage reaction, namely a 5′-hydroxyl group and a 2′,3′-cyclic phosphodiester. The small size of

these ribozymes, in particular hammerhead and hairpin ribozymes, has put them within easy reach of chemical synthesis, which has facilitated extensive studies on their structure-function relationships. Therefore, the emphasis of this review is on the hammerhead and hairpin ribozymes, followed by a brief discussion on the hepatitis delta and *Neurospora* VS ribozymes. We discuss purely the structural studies aimed toward ascertaining the role that various nucleotides and/or functional groups play in catalysis and also the experiments that have marked the road leading to the tertiary structures of ribozymes (for recent reviews, see Eckstein and Lilley 1996).

THE HAMMERHEAD RIBOZYME

Sequence and Structural Requirements

The hammerhead ribozyme was first discovered in the plus strand of the avocado sunblotch virus (Hutchins et al. 1986) and was originally termed a "hammerhead" because of its two-dimensional representation (Fig. 1) (for recent reviews, see Symons 1992; Cech and Uhlenbeck 1994; Doudna 1994; Sczakiel 1995; Sigurdsson and Eckstein 1995; Tuschl et al. 1995; Uhlenbeck 1995; Heidenreich and Eckstein 1997). The secondary structure of the hammerhead ribozyme consists of a core of single-stranded nucleotides flanked by three base-paired arms. This stable secondary structure arrangement, elucidated from phylogenetic comparison of self-cleaving plant virus satellite RNAs, is supported by thermodynamic nearest-neighbor calculations (Freier et al. 1986; Christofferssen et al. 1994) and nuclear magnetic resonance (NMR) measurements (Odai et al. 1990; Pease and Wemmer 1990; Heus and Pardi 1991). An extensive mutagenesis study has also supported this secondary structure, since the mutation of any of the core nucleotides results in at least a 50-fold reduction in activity. The only exception is U7, which can be replaced by A, G, or C with only a 5-fold reduction in activity. Additionally, functional group modifications in the single-stranded regions invariably result in a reduction in catalytic activity (for recent reviews, see Bratty et al. 1993; Grasby et al. 1995).

In the naturally occurring hammerhead ribozymes, cleavage after a GUC triplet sequence is the most predominant. Two exceptions to this are found in the GUA cleavage triplet of the satellite RNA of the lucerne transient streak virus (Forster and Symons 1987) and the AUA cleavage triplet of the satellite RNA of barley yellow dwarf virus (sBYDV) (Miller et al. 1991). The sBYDV RNA differs from the consensus sequence by the presence of an additional A/C base mismatch in the central core

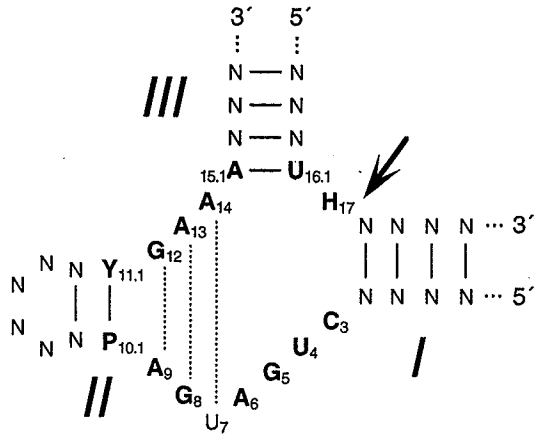

Figure 1 Secondary structure of the hammerhead ribozyme. Numbering system is according to Hertel et al. (1992). Roman numerals indicate the numbers of the helices. Arrow shows the site of cleavage. Essential and important residues are shown in boldface. Solid lines indicate base-pairing. Broken lines indicate additional base-pairing according to X-ray analysis (see text). H = A, C, or U; N = any nucleotide, except in helices where there is a requirement for base-pairing; P = A or G and Y = U or C.

preceding helix II; however, there is evidence that it is not required for efficient cleavage of AUA triplets (Nakamaye and Eckstein 1994; Shimayama et al. 1995b). Numerous systematic studies have been carried out to establish the sequence requirements of the cleavage triplet, and the results indicate that any triplet sequences of the NUH type (N = any nucleotide; H = A, U, or C) can be cleaved, although the rates are variable (Forster and Symons 1987; Koizumi et al. 1988; Ruffner et al. 1990; Perriman et al. 1992; Shimayama et al. 1995b; Zoumadakis and Tabler 1995).

Mechanism, Metal Ions, and Kinetics

Stereochemistry of Substrate Cleavage

The hammerhead ribozyme catalyzes a phosphodiester transesterification reaction to yield two fragments, one containing a 5′-hydroxyl and the other a 2′,3′-cyclic phosphate terminus (Uhlenbeck 1987). Analysis of the configuration of the reaction products, from substrates containing ei-

ther an S_P or an R_P phosphorothioate linkage (a phosphodiester where a non-bridging oxygen has been replaced by sulfur) at the cleavage site indicated inversion of configuration at the phosphorus center (van Tol et al. 1990; Koizumi and Ohtsuka 1991; Slim and Gait 1991). The stereochemistry of the product is consistent with in-line attack of the 2′-hydroxyl group in an S_N2-type transesterification reaction (Fig. 2) similar to the first step of RNA hydrolysis by pancreatic ribonuclease (RNase A) (Usher et al. 1972; Saenger et al. 1974). The requirement for a 2′-hydroxyl at the cleavage site has been demonstrated by the inability of substrates containing either a 2′-deoxynucleotide (Perreault et al. 1990) or a 2′-aminonucleotide (Pieken et al. 1991) at the cleavage site to undergo cleavage.

Role of Divalent Metal Ions in Catalysis

Ribozymes are a novel class of metalloenzymes that require the presence of a divalent metal ion, usually Mg^{++}, in order to catalyze the chemical

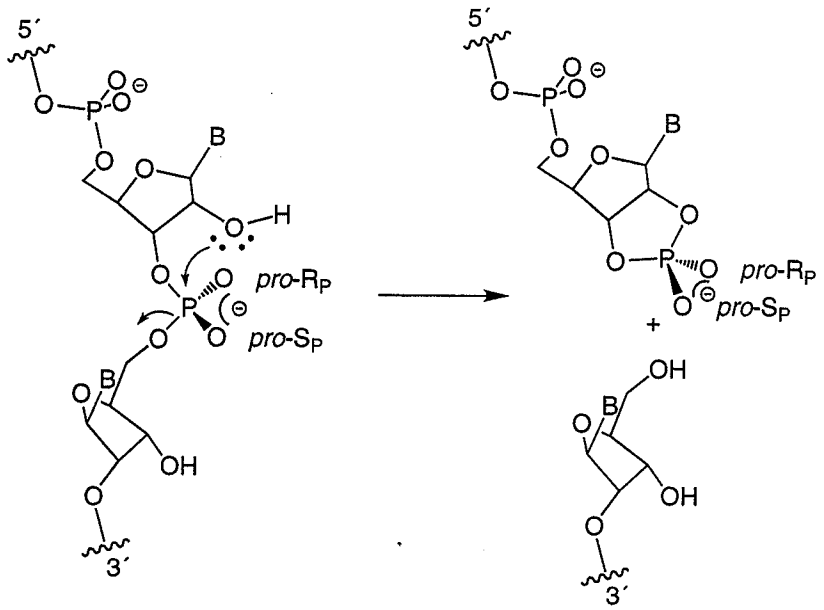

Figure 2 A generic cleavage mechanism for the ribozymes discussed here. B stands for a nucleoside base. *pro*-R_P and *pro*-S_P indicate the stereochemical assignment of the non-bridging oxygens according to the Chan-Ingold-Prelog system.

reaction (Pyle 1993). Metal ions can bind to RNA through coordination either to phosphates, sugar 2′-hydroxyls, or the nucleobase, and although a large variety of metal ions are capable of supporting phosphodiester bond cleavage, optimal activity is in 10 mM Mg^{++} (Dahm and Uhlenbeck 1991). Metal ion coordination can be probed using phosphorothioates, which have little affinity for "hard" metals, preferring to coordinate to "soft" metals (Pearson 1966; Pecoraro et al. 1984). Stereospecific phosphorothioate incorporation has indicated that a divalent metal ion is directly coordinated to the *pro*-R_P oxygen of the scissile phosphate in the transition state of the hammerhead ribozyme cleavage reaction (Dahm and Uhlenbeck 1991; Koizumi and Ohtsuka 1991; Slim and Gait 1991).

Despite these data on metal-ion binding, there is still a dearth of knowledge regarding the precise involvement of metal ions in the cleavage mechanism. Alternative counterions such as spermine or Na^+ can assume the structural role of Mg^{++}, helping to fold the RNA into the active conformation. However, the fact that Mg^{++} cannot be fully replaced demonstrates its requirement for catalytic activity (Dahm and Uhlenbeck 1991). On the basis of pH-rate profiles, obtained for various metal-ion-catalyzed hammerhead ribozyme cleavage reactions, it has been proposed that the corresponding metal-ion-hydroxo complex is responsible for the proton abstraction of the attacking 2′-hydroxyl group (Dahm et al. 1993). Alternatively, it has also been proposed that the 2′-hydroxyl is activated by direct coordination of Mg^{++} (Sawata et al. 1995). In any case, this activated oxo anion then attacks the phosphorus atom to form a pentacoordinate intermediate, which breaks up to yield the cleavage products.

There is considerable debate over whether the 5′-leaving group, from the pentacoordinate intermediate, is stabilized through metal ion coordination or is protonated by water or a hydrated Mg^{++} ion. Recent studies have addressed the possible metal ion coordination to the leaving group in a more direct manner, using the strategy of Piccirilli et al. (1993), by the incorporation of a 5′-bridging-phosphorothioate into the cleavage site of a DNA substrate strand of the hammerhead ribozyme (Kuimelis and McLaughlin 1995). In the presence of various divalent metal ions, no acceleration of substrate cleavage was observed, relative to the unmodified 5′-oxo substrate, and this was attributed to the lack of metal ion coordination to the leaving group in the transition state. However, such metal ion coordination would only have been observed if cleavage of the P-S bond was rate-limiting. Therefore, a two-metal-ion mechanism for the hammerhead ribozymes, which has been proposed on

the basis of the lack of a kinetic isotope for reactions performed in deuterated water (Sawata et al. 1995), cannot be rigorously ruled out by these experiments.

Kinetic Description

In nature, the hammerhead ribozyme exclusively cleaves its target RNA in an *in cis* (or intramolecular) fashion. However, to fully assess its kinetic properties, the hammerhead domain has also been engineered to cleave *in trans* (or intermolecularly), enabling both multiple and single turnover conditions to be applied (Fig. 1) (Haseloff and Gerlach 1988).

Hammerhead ribozyme cleavage takes place after successful association of the substrate to the ribozyme's hybridizing arms to form stems I and III. Substrate association is dependent on the length and sequence of the substrate and the number of complementary base pairs it can form with the ribozyme. In the case of a fully complementary 17-nucleotide-long substrate, the rate of association of 2×10^7 M^{-1} min^{-1} was determined (Hertel et al. 1994) and is similar to that observed for RNA duplex formation. However, the rate of association of ribozymes to long substrates, such as mRNA greater than 1000 nucleotides in length, can be extremely slow due to the propensity of the RNA to form stable self-structures (Heidenreich and Eckstein 1992). However, this situation can be ameliorated in the presence of RNA-binding proteins (Herschlag 1995).

The rate of substrate and product dissociation is well described by the stability of RNA duplexes. The use of recognition sequences greater than 7 bp in each arm reduces substrate and product dissociation rates, and single-turnover conditions are required for efficient cleavage (Hertel et al. 1994). A ribozyme with 8 bp in each arm had a k_{cat} of only 0.008 min^{-1}, which reflects the rate of product dissociation. In contrast, a ribozyme with 5 bp in each arm had a k_{cat} value almost three orders of magnitude higher at 1.4 min^{-1}, which reflects the rate of the chemical step (Fedor and Uhlenbeck 1992; Hertel and Uhlenbeck 1995). An additional consequence of long recognition sequences is the toleration of mismatches in the ribozyme-substrate complex, which can result in a loss of specificity in ribozyme cleavage. Mismatches in the innermost base pair of helix I and any of the 4 innermost base pairs of helix III greatly reduce the rate of the chemical cleavage step. However, distal mismatches have no such discriminatory effect (Herschlag 1991; Werner and Uhlenbeck 1995).

Ribozymes containing all DNA nucleotides in the hybridizing arms have been demonstrated to have 10- to 20-fold higher values of k_{cat}, although the catalytic efficiency is always compromised due to the much higher values of K_M (Sawata et al. 1993; Hendry and McCall 1995). An extraordinarily high k_{cat} value of 100 min^{-1} was reached using 800 mM Mg^{++} ions rather than the more commonly used conditions of 10 mM (Shimayama et al. 1995a).

Three-dimensional Structure

X-ray Crystallography

The structure of the hammerhead ribozyme has been highlighted in several recent reviews (Cech and Uhlenbeck 1994; Doudna 1994; Pardi 1994; Sczakiel 1995; Tuschl et al. 1995; Thomson et al. 1996). The two crystal structures of the hammerhead ribozyme (Pley et al. 1994; Scott et al. 1995) are in very good agreement with each other, differing only slightly around the cleavage site and in the region C_3, U_4, G_5, and A_6, perhaps due to the different substrate strands used to form helices I and III. Whereas Pley et al. used an all-DNA substrate, Scott et al. used an all-RNA substrate containing a 2′-O-methyl cytidine at the cleavage site. Helices II and III are collinear and are connected via noncanonical G_{12}/A_9, A_{13}/G_8, and A_{14}/U_7 mismatch base pairs (Fig. 1). Helix I is connected to helix II through the continual right-handed helical path of nucleotides C_3, U_4, G_5, and A_6, which adopt a uridine turn motif to form the only single-stranded region of the molecule. Helices I and III are linked via $U_{16.1}$ and C_{17}, and the phosphate connecting these two residues is considerably splayed out in both structures. Evidence of metal-ion binding near the cleavage site was only indirectly observed through the presence of a solvent cluster (Scott et al. 1995). An additional metal-ion-binding site was also observed in both crystal structures, which coordinated the metal ion between the N7 of $G_{10.1}$ and a phosphate oxygen of A_9, and has been proposed to be purely structural in nature due to its distance from the cleavage site.

Despite consistencies between the functional group modification data and the role of the various functional groups as observed from the crystal structures, many discrepancies are apparent and have been discussed previously (Tuschl et al. 1995). A problem in comparing the data from functional group modification experiments and the X-ray crystallography is that the former provides information on the transition state structure whereas the latter yields data on the ground state. This could clearly be a plausible reason since, from the crystal structure analyses, conforma-

tional changes are necessary to align the 2′-hydroxyl for the required in-line attack at the phosphorus.

Importance of Stem-loop II

Stem II forms one of the three A-helical regions of the hammerhead ribozyme and in both crystal structures is connected to stem III by a $G_{12}A_9$ and $A_{13}G_8$ tandem mismatch and an $A_{14}U_7$ base pair (Pley et al. 1994; Scott et al. 1995). This mismatched region effectively joins stems II and III in a nearly continuous A-helix. The importance of stem II appears to lie in its ability to stabilize the mismatched region, ensuring that the GA double mismatches adopt the correct conformation, as discussed by Thomson et al. (1996). It has been demonstrated that the closing base pair of stem II is very important and that it should form a purine-10.1/pyrimidine-11.1 base pair for maximum activity (Ruffner et al. 1990; Tuschl and Eckstein 1993). This pattern has also been confirmed in two separate in vitro selection experiments (Long and Uhlenbeck 1994; Nakamaye and Eckstein 1994). To maintain the conserved base pair 10.1/11.1 in a stable base-paired conformation, at least one additional base pair (10.2/11.2) is required in helix II. The sequence of the loop-closing nucleotides of helix II is not important, and they can be replaced by non-nucleotidic residues (Benseler et al. 1993; Thomson et al. 1993; Beigelman et al. 1994; Fu et al. 1994; Hendry et al. 1994; Kuimelis and McLaughlin 1996). Therefore, in contrast to stems I and III which are utilized for substrate binding, in *trans*-acting ribozymes, stem II seems to serve as a clamp for correct folding of the hammerhead ribozyme.

Global Geometry

A fairly consistent three-dimensional picture of the hammerhead ribozyme has also been built up by the independent application of the biophysical techniques of fluorescence resonance energy transfer (FRET) (Tuschl et al. 1995), electrophoretic mobility (Bassi et al. 1995), and electric birefringence (Amiri and Hagerman 1994) measurements. Of these three studies, the structure obtained from the FRET measurements yielded the most information, since it defined the orientations of helices I, II, and III, enabling the central core region to be modeled (see also below), whereas the other three techniques only yielded information regarding the spatial positioning of the three helical arms.

All the above methods agree that in the presence of Mg^{++}, the overall shape of the ribozyme is characterized by a wishbone configuration, with

helices II and III being nearly collinear and helix I proximal to helix II. In the absence of Mg^{++}, both Bassi et al. (1995) and Amiri et al. (Amiri and Hagerman 1994; Gast et al. 1994) predict a collinear arrangement of helices I and II, which undergo a reorientation to the above wishbone configuration upon the addition of millimolar amounts of Mg^{++}. Additionally, FRET measurements have indicated Mg^{++}-dependent conformations (T. Tuschl and F. Eckstein, unpubl.). Conformational changes upon Mg^{++} binding have also been detected by monitoring the change in fluorescence of ribozymes containing 2-aminopurine ribonucleoside (Mengel et al. 1996). For a hammerhead containing 2-aminopurine at the cleavage site, the Mg^{++} binding constant was determined to be approximately $2 \times 10^3\ M^{-1}$, which is consistent with the value of $10^4\ M^{-1}$ from circular dichroism analysis (Koizumi and Ohtsuka 1991). Recent NMR analysis has also highlighted the importance of Mg^{++} ions for inducing correct folding of the ribozyme-substrate complex (Orita et al. 1995).

A metal-ion-binding site was observed in the base mismatch region of the central core in both crystal structures (Pley et al. 1994; Scott et al. 1995). This is consistent with the identification of three phosphates by phosphorothioate substitutions, the phosphate connecting G_8 and A_9 and the two phosphates between G_{12} and A_{14}, as being important for Mg^{++} coordination (Ruffner and Uhlenbeck 1990). An additional metal-ion-binding site was observed in the $C_3U_4G_5A_6$ uridine turn motif of the structure by Scott et al. (1995), in accordance with a metal-ion site at G_5 identified by uranyl cleavage (Bassi et al. 1995).

Interhelical Cross-linking

Despite the observed similarities between the two crystal structures and the FRET model, there is a difference in the relative orientations of helices I and II. As a consequence, in the X-ray structure derived by Pley et al. (1994), the seven-nucleotide loop connecting helices I and II is located exclusively on one face of the molecule. In contrast, this loop threads its way between the helices from one face of the ribozyme to the other in the FRET model (Tuschl et al. 1994). Exploiting these differences in the relative orientations of helices I and II between the X-ray and FRET models, site-specific cross-linking was utilized to distinguish between the models (Sigurdsson et al. 1995). Thus, two hammerhead ribozymes were prepared that were constrained between helices I and II by incorporating a 16-Å-long reversible disulfide cross-link between two 2′-amino-nucleotides, one on each of helices I and II. In one hammerhead, the distance cross-linked was ~11 Å in the crystal structure but cor-

responded to a 32-Å distance in the FRET model. In the second ribozyme, the cross-linked residue was based on a 13-Å distance observed in the FRET model and a 33-Å distance in the X-ray structure. The former cross-linked ribozyme had full activity and the latter had no measurable activity, thus supporting the structure derived from X-ray crystallography. These results additionally imply that the conformational change required for in-line attack is localized to the cleavage site and does not require a more global rearrangement of the helical arms. This cross-linking technique should be useful for probing the tertiary structures of other RNA molecules.

THE HAIRPIN RIBOZYME

Secondary Structure and Sequence Requirements

The satellite RNA of the tobacco ringspot virus undergoes a rolling cycle replication, during which the multimeric forms are autolytically cleaved to form the monomer subunits (Buzayan et al. 1986a; Prody et al. 1986). In the plus strand, a hammerhead ribozyme was found to be responsible for the autocatalytic processing. In the minus strand, processing was located around two minimal sequences: a catalytic 50-nucleotide domain, which can catalyze both cleavage and ligation (Buzayan et al. 1986a,b), and a 10- to 14-nucleotide substrate domain (Forster and Symons 1987; Hampel and Tritz 1989) and was termed the hairpin ribozyme (Feldstein et al. 1989; Hampel and Tritz 1989; Haseloff and Gerlach 1989; Hampel et al. 1990; for recent reviews, see Burke 1994; Burke et al. 1996). The resultant two-dimensional structure, based on minimum energy folding (Hampel et al. 1990), has been supported by mutagenesis experiments (Hampel and Tritz 1989; Haseloff and Gerlach 1989; Hampel et al. 1990; Joseph et al. 1993; Anderson et al. 1994; De Young et al. 1995) and limited phylogenetic comparison (Fig. 3) (Rubino et al. 1990; De Young et al. 1995). The main features of the secondary structure are four helical regions and two single-stranded loops. Helices I and II are formed upon substrate binding, and the ribozyme catalyzes the cleavage of the phosphodiester between nucleosides G_{+1} and A_{-1} in loop A.

Further evidence for the secondary structure comes from in vitro selection studies, which have been used to generate an artificial phylogenetic library (Berzal et al. 1992, 1993; Joseph et al. 1993). This study confirmed the presence of the helical and single-stranded regions. In helices I–IV any sequence was tolerated, providing that base-pairing was maintained, with the exception of the base pair flanking loop A (Berzal et al. 1993; Joseph et al. 1993). In contrast, the majority of residues in

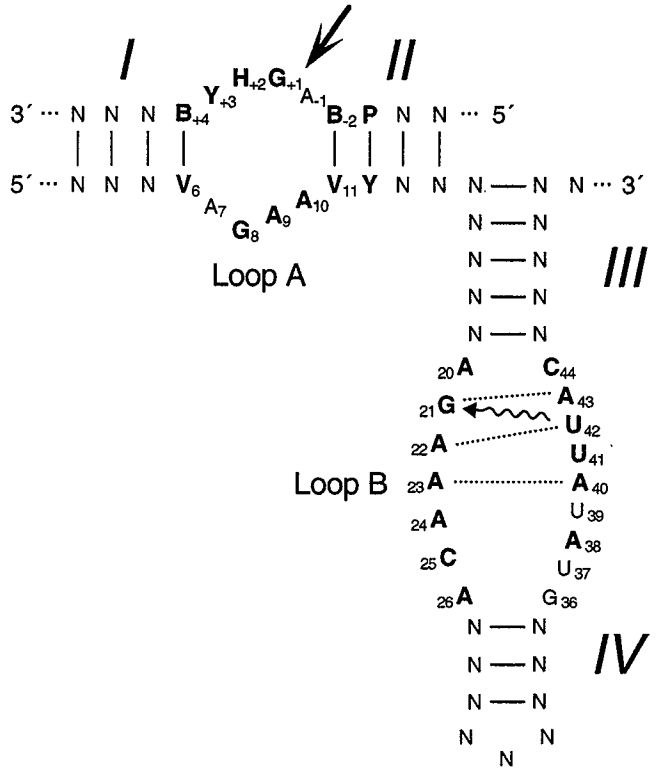

Figure 3 Secondary structure of the hairpin ribozyme. Roman numerals indicate the numbers of the helices. Straight arrow shows the site of cleavage. Essential and important residues are shown in boldface. Solid lines indicate base-pairing. Broken lines indicate proposed base-pairing scheme in the UV-sensitive structure, and the squiggled arrow shows which residues are efficiently UV-cross-linked (Butcher and Burke 1994a). B = U, C, or G; H = A, C, or U; N = any nucleotide, except in helices where there is a requirement for base-pairing; P = A or G; V = A, C, or G; and Y = U or C.

loops A and B were vital for efficient catalysis (Fig. 3) (Berzal et al. 1992, 1993; Anderson et al. 1994; Schmidt et al. 1996).

Functional Group Rrequirements

For the substrate part of loop A, only G_{+1} has been identified as an essential nucleotide where all mutations of this residue reduce the catalytic efficiency by at least a factor of 10^5. Mutations of the other residues in

this strand do not reduce the cleavage efficiency by more than 10- to 20-fold (Chowrira et al. 1991; Berzal et al. 1992; Anderson et al. 1994). The structural features of the G_{+1} base that are important for catalysis have been probed by replacing it with inosine or 2-aminopurine: The former yielded an inactive ribozyme and the latter a fully active one (Chowrira et al. 1991). This strongly implies that the 2-amino group of G_{+1} is directly involved in catalysis. Introduction of O^6-methyl guanosine at this site causes a dramatic loss in activity (Grasby et al. 1995). It has been postulated that this effect might have been caused by steric interference of the methyl group with a putative magnesium-binding site at N7 of this residue, which is plausible in the tight confines of a catalytic center (Grasby et al. 1995).

The functional group requirements of the essential purine residues in the ribozyme have been systematically studied by substituting them with purine (P), inosine, O^6-methyl guanosine, N7-deaza A (^{7}cA), and N7-deaza G (Grasby et al. 1995). All of these analogs caused considerable decrease in the rate of reaction when substituted for G_8-G_{11} in loop A, and the effect was mainly due to a change in k_{cat} rather than K_M. The reductions in rate caused by each of these residues were correlated with the loss of a hydrogen bond in the catalytically active complex. In loop B, the only tolerated mutations were $A_{24}{}^7$cA, A_{40}P, $A_{40}{}^7$cA, and A_{43}P. For mutant ribozymes, the effects of Mg^{++} concentration on Mg^{++} binding and the rate of cleavage were interpreted as evidence for Mg^{++} binding to the N7 atom of G_{+1} and A_{43} in the ground state and to A_9 in the transition state (Grasby et al. 1995). Taken together, these results indicate that most of the functional groups of the essential purine nucleosides are involved in hydrogen bonding or coordination to metal ions, reminiscent of the hammerhead ribozyme.

The role of the base and sugar of the nucleosides A_{20}, G_{21}, and U_{37}-C_{44} in loop B has been probed by substitutions with abasic residues and propyl linkers (Schmidt et al. 1996). The only position where both substitutions were tolerated was U_{39}, suggesting that it only acts as a spacer, although the U_{39}C mutation has been found to be a suppressor for G_{21} mutations through in vitro selection (Berzal et al. 1993). All other propyl substitutions resulted in considerable rate reductions (20- to 1000-fold), whereas substitutions at U_{37} and C_{44} resulted in a modest decrease (<10-fold) when substituted with abasic residues (Schmidt et al. 1996).

The importance of the 2′-hydroxyl groups within the ribozyme-substrate complex for catalysis has been systematically studied (Chowrira and Burke 1991; Chowrira et al. 1993b; Grasby et al. 1995).

The only hydroxyl group shown to be important in the substrate is on A_{-1}, the nucleophilic hydroxyl that participates in the cleavage of the scissile bond (Chowrira and Burke 1991). Incorporation of either 2′-deoxy- or 2′-O-methyl-ribonucleotides at A_{10}, G_{11}, A_{24}, and C_{25} resulted in a drastic reduction in rate (Chowrira et al. 1993b). For G_{11} and A_{24}, activity was restored at high concentrations of Mg^{++}, which suggests the involvement of the 2′-hydroxyl groups in metal-ion coordination-dependent tertiary interactions. For all of these mutations, the effect was found to reside in k_{cat} rather than in K_M, indicating interference with a catalytic function of the ribozyme-substrate complex rather than with substrate binding. Hairpin ribozymes incorporating 2′-deoxyribonucleotides at positions U_{37}, A_{38}, and U_{41} in loop B demonstrated a modest reduction in activity (Schmidt et al. 1996). In addition to furthering our knowledge of possible structural roles that the 2′-hydroxyl groups play, these studies will also contribute toward the development of nuclease-resistant ribozymes, similar to that already established for the hammerhead ribozyme (see below).

Mechanism, Metal Ions, and Kinetic Description

Mechanism and Stereochemistry

As with the hammerhead ribozyme, the hairpin-promoted cleavage of RNA occurs via a nucleophilic attack on the scissile phosphate by the adjacent 2′-hydroxyl group, generating a 5′-hydroxyl group and a 2′-3′ cyclic phosphate (Fig. 2) (Buzayan et al. 1986a). The hairpin ribozyme has been shown to have an appreciable rate of ligation, the reverse of the cleavage reaction, which requires two oligoribonucleotides, one possessing a 5′-terminal guanosine and the other a terminal 2′,3′-cyclic adenosine monophosphate (Buzayan et al. 1986a,b; Berzal et al. 1992; Feldstein and Bruening 1993; Komatsu et al. 1993; Hegg and Fedor 1995). A phosphorothioate with the R_p configuration has been introduced at the cleavage site to probe the stereochemistry of cleavage and is consistent with in-line attack of the 2′-hydroxyl group on the scissile phosphate (Fig. 2) (van Tol et al. 1990).

For the hammerhead ribozyme, the rate-determining step in the chemical reaction is considered to be the abstraction of the 2′-hydroxyl proton, since rates increase linearly with pH (Dahm et al. 1993; Hertel and Uhlenbeck 1995). In contrast, the rate of cleavage for the hairpin ribozyme is relatively unaffected between pH 5.5 and pH 8 (Hampel and Tritz 1989) and thus implies a different rate-determining step. This could be an indication that the two ribozymes have different elemental steps in

their mechanisms. Further support can be derived from phosphorothioate experiments, which suggested that a catalytically essential metal ion was coordinated to the pro-R_p oxygen at the scissile phosphate during cleavage by the hammerhead, but not the hairpin, ribozyme (van Tol et al. 1990). However, it is possible that both ribozymes have the same elemental mechanistic steps, because the hairpin could, for example, have a conformational change as the rate-determining step in its mechanism.

Divalent Metal Ions Promote Ribozyme Folding and Catalysis

As with other ribozyme motifs, the hairpin ribozyme requires divalent metal ions in order to cleave the substrate efficiently (Hampel and Tritz 1989; Chowrira et al. 1993a). A number of ions have been tested, but only Mg^{++}, Sr^{++}, and Ca^{++} have the ability to support activity, with Mg^{++} being the most efficient (Chowrira et al. 1993a). Interestingly, Na^+ and K^+ inhibit the Mg^{++}-dependent reaction, presumably due to their displacement of essential Mg^{++} ions from sites within the ribozyme substrate complex. As with the hammerhead ribozyme, the Mg^{++} ion coordination sites on the phosphates have been probed by the introduction of phosphorothioates into the sugar phosphate backbone of a three-piece ribozyme-substrate complex (Chowrira and Burke 1992). Ribozymes in which all guanine, uridine, and cytidine residues had a 5′-R_p-phosphorothioate were efficient catalysts, but multiple substitution of the adenosine phosphates decreased the ribozyme's activity by a factor of 25. Chemical-modification interference experiments suggested that this effect resided mainly with residues A_7, A_9, and A_{10}. Additionally, the 2′-hydroxyl groups of G_{11} and A_{24} (Chowrira et al. 1993b), and N7 positions of G_{+1}, A_9, and A_{43} (Grasby et al. 1995), have been identified as potential Mg^{++}-binding sites. A substrate containing an R_p-phosphorothioate at the cleavage site was efficiently cleaved in the presence of Mg^{++}, indicating that the pro-R_p oxygen is not coordinated to a catalytically essential Mg^{++} ion at the cleavage site (van Tol et al. 1990), as has been suggested with the hammerhead ribozyme (van Tol et al. 1990; Dahm and Uhlenbeck 1991; Slim and Gait 1991).

Mg^{++} ion-dependent structural changes in the hairpin ribozyme have been highlighted by the differences in the chemical modification of nucleobases of the ribozyme, particularly in the essential residues of loop B, before and after the addition of Mg^{++} (Butcher and Burke 1994b). In addition, the Mg^{++} concentration required for efficient cleavage can be reduced by the addition of spermine, which suggests that some of the Mg^{++} required for cleavage is involved in stabilizing tertiary interactions, presumably by reducing the electrostatic repulsion of the negatively charged sugar-phosphate backbones (Chowrira et al. 1993a). Moreover, Mn^{++} and Co^{++} can only promote cleavage in the presence of spermine, indicating that they can participate in the cleavage reaction but not in folding of the ribozyme into an active structure. Surprisingly, spermidine supports hairpin ribozyme cleavage in the presence of the metal-ion chelators EDTA and EGTA, although at a much slower rate, and this may be the only example of a ribozyme that functions without divalent metal ions. Taken together, these results indicate that, as with the hammerhead ribozyme, metal ions are not only involved in catalysis, but also promote the folding of the hairpin into its active conformation.

Kinetic Description

The individual rate constants for the hairpin have been derived from a combination of steady-state and pre-steady-state kinetic measurements (Hegg and Fedor 1995). The rate of the chemical cleavage step in the hairpin-mediated RNA cleavage has been found to be comparable to that of the hammerhead ribozyme (0.3 and 1 min^{-1}, respectively) under the same conditions (Fedor and Uhlenbeck 1990, 1992; Hertel et al. 1994; Hegg and Fedor 1995). In contrast to the hammerhead, the hairpin has a rate constant for ligation which is an order of magnitude higher than the rate constant for cleavage (Hegg and Fedor 1995). Although both ribozymes were discovered as part of the rolling-circle replication machinery, clearly such large differences in these chemical steps point toward separate evolutionary histories and previous different in vivo functions. Hairpin-mediated ligation is the first example of a spontaneous, nonenzymatic ligation reaction of a naturally occurring RNA sequence between a 2′,3′-cyclic phosphate and a 5′-hydroxyl group (Buzayan et al. 1986b; Hegg and Fedor 1995). However, in the excess of substrate, cleavage is the predominant reaction pathway.

The rate of the substrate binding (1×10^7 M^{-1} min^{-1}) is similar to that for other ribozymes, and the substrate dissociation rate was found to be much slower than the rate of substrate cleavage (Hegg and Fedor 1995). Comparison of the affinities of the cleavage products for the ribozyme, to that calculated from empirically determined free-energy parameters for simple RNA duplexes (Freier et al. 1986; Jaeger et al. 1989), revealed that the complex between the 3′-cleavage product and the ribozyme bound more tightly than expected for a simple duplex by –2.5 kcal/mole.

This can be attributed to base-pairing across loop A as suggested previously (Butcher and Burke 1994b), although this increased binding could be due to other tertiary interactions. On the other hand, the 5′-cleavage product and the ribozyme had a similar stability to that predicted.

Toward a Three-dimensional Structure of the Hairpin Ribozyme

Structures of the Loops

Using a substrate containing only 2′-deoxyribonucleosides, where G_{+1} was replaced with 2′-deoxy-6-thioinosine, photoinduced cross-links were formed at multiple positions to residues on the opposite strand, and this was interpreted as evidence for a flexible loop A (Vitorino dos Santos et al. 1993). A deoxy substrate containing a single ribonucleoside at the cleavage site had previously been shown to cleave, although the rate of cleavage was several orders of magnitude lower (Chowrira and Burke 1991). As previously mentioned, there is evidence that suggests base-pairing across the loop, and the involvement of G_8 has been specifically suggested, based on results from chemical modification with and without the substrate (Butcher and Burke 1994b).

There is strong evidence for interstrand interactions in loop B. Specifically, a UV-sensitive domain has been identified within loop B in which cross-links were formed between G_{21} and U_{42} (previously indicated as U_{41}; J. Burke, pers. comm.; see also Butcher and Burke 1994a). These cross-links were formed independently of either Mg^{++} ions, the substrate or the substrate-binding strand, strongly implying that they are not necessary for inducing folding of loop B. Although the cross-linked ribozymes were able to catalyze cleavage of the substrate, the rate was reduced about 100-fold, suggesting that some structural distortions occur upon cross-linking. On the basis of sequence homology with other UV-sensitive structures (Branch et al. 1985; Szewczak et al. 1993; Wimberly et al. 1993) and chemical modification experiments (Butcher and Burke 1994b), a model for loop B has been suggested, where noncanonical base pairs are formed across the loop (Fig. 3) (Butcher and Burke 1994a). NMR studies on other UV-sensitive structures indicate that efficient photo-cross-linking can be explained by unusual cross-strand stacking between residues. As a result, functional groups project into accessible grooves, which are potential sites for tertiary interactions with loop A. The validity of this model has been questioned, since replacement of either A_{40} or A_{43} with purine, which deletes one hydrogen bond from the

base pairs in the aforementioned model, causes only a 2-fold reduction in the catalytic efficiency (Schmidt et al. 1996). This argument is based on the assumption that the deletion of one hydrogen bond from this hydrogen-bonding network is enough to disrupt the structure. An alternative model has been proposed (Schmidt et al. 1996), and it is clear that more experiments will have to be performed to shed further light on this matter. In the lower part of loop B, the resistance of A_{26} and G_{36} to chemical modification is consistent with base-pairing between these two nucleosides or involvement in other tertiary contacts (Butcher and Burke 1994b).

Global Shape of the Hairpin Ribozyme

In the absence of information on the tertiary structure of the hairpin ribozyme from either X-ray or NMR analysis, other methods have been used to probe interdomain interactions. Linkers of varying length were used to connect the 5′-end of the substrate and the 3′-end of the ribozyme to ascertain whether helices II and III were coaxial or if there was a requirement for a bend to facilitate tertiary contacts between the two domains (Feldstein and Bruening 1993; Komatsu et al. 1994, 1995). Using cytidines as the linker unit, it was found that the catalytic efficiency was reduced for molecules having less than five residues in the linker, and all activity was abolished with only one residue (Feldstein and Bruening 1993). Similar results were obtained using 1,3-propanediol phosphate linkers (Komatsu et al. 1994, 1995). This suggests that instead of the more energetically favorable coaxial arrangement of helices II and III (Walter and Turner 1994; Walter et al. 1994), there is a sharp bend at the junction for facilitating tertiary interactions.

Further evidence for the interaction of the two domains comes from experiments where helix II and helix III were separated (Butcher et al. 1995). Incubation of the substrate with the substrate-binding strand did not result in any enhancement of substrate cleavage. However, partial activity was observed when the complex was incubated with the domain containing loop B. The catalytic efficiency was reduced by four orders of magnitude, primarily due to changes in K_M, indicating rather weak interdomain interactions.

Ribozyme-protein Interactions

Recently, a binding site for the bacteriophage R17 coat protein was engineered into helix IV of the hairpin ribozyme as a model for studying

ribozyme-protein interactions (Sargueil et al. 1995). The modified ribozyme showed a slight increase in catalytic activity when compared to the unmodified ribozyme, presumably due to a more efficient folding of loop B into an active conformation, as indicated by increased yield of UV-induced cross-links. The catalytic efficiency was not altered by binding of the coat protein, which was shown to be bound to the ribozyme during the catalytic cycle. In addition to providing a system to study ribozyme-protein interactions, these findings suggest a method to effect ribozyme intracellular localization for therapeutical applications.

THE HEPATITIS DELTA VIRUS RIBOZYME

The hepatitis delta virus (HDV) is a subviral human pathogen that has a circular RNA genome containing a self-cleaving domain (for recent reviews, see Been 1994; Lazinski and Taylor 1995; Tanner 1995). The HDV is an unusual animal virus in that its replication is thought to proceed by a double rolling-circle mechanism in which the anti-genomic sequence is generated by the use of RNA polymerase II from the host cell (MacNaughton et al. 1991; Fu and Taylor 1993). Both the genomic and the anti-genomic sequences promote self-cleavage, and a correlation has been found between self-cleavage in vitro and replication in vivo (MacNaughton et al. 1993).

Secondary Structure and Sequence Requirements

There is considerable sequence similarity around the cleavage sites of the genomic and the anti-genomic sequences of the HDV ribozyme, indicating structural resemblance (Rosenstein and Been 1991). The minimum sequence length required for efficient self-cleavage is 85 nucleotides (Wu et al. 1992), and internal deletions have yielded active sequences as short as 71 nucleotides (Thill et al. 1991). In contrast to the hammerhead and hairpin ribozymes, only one nucleotide is required 5′ to the cleavage site (Fig. 4) (Perrotta and Been 1990).

Different secondary-structure models have been proposed for the secondary structure of the HDV ribozyme (Branch and Robertson 1991; Perrotta and Been 1991; Smith et al. 1992; Wu et al. 1992). However, the pseudoknot model (Perrotta and Been 1991) is most consistent with mutagenesis experiments that have identified the four helical regions of the ribozyme (Fig. 4) (Perrotta and Been 1991, 1993; Been et al. 1992; Wu and Huang 1992; Wu et al. 1992, 1993; Thill et al. 1993; Tanner et al. 1994). This model places the cleavage site at the 5′ end of the substrate strand (Perrotta and Been 1991), and within this helix, only the first base pair after the cleavage site ($G_{+1}U_{37}$) is sensitive to mutations (Perrotta and Been 1991; Been et al. 1992; Wu et al. 1993). A more detailed study of this base pair revealed that only $G_{+1}U_{37}$, $U_{+1}A_{37}$, or

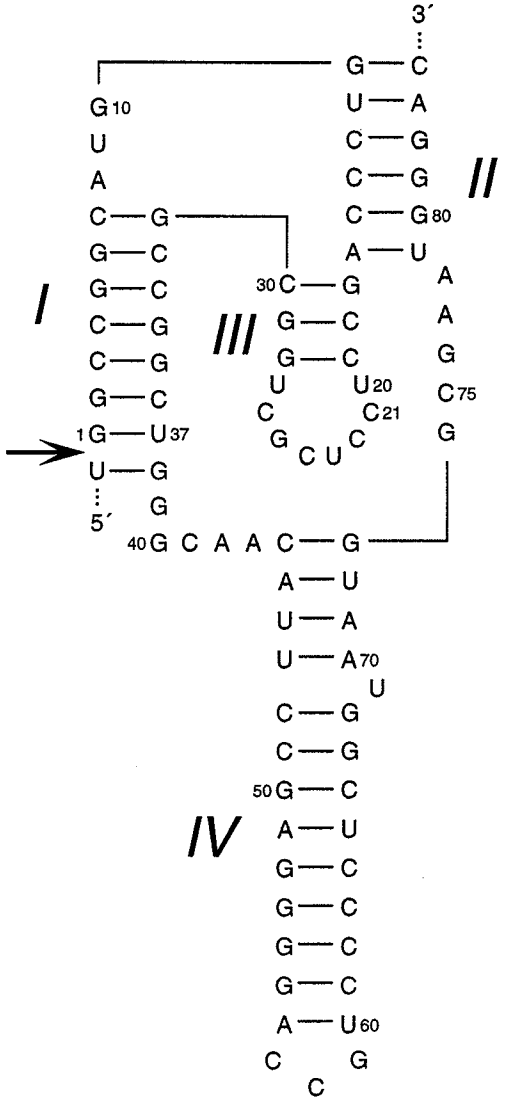

Figure 4 The pseudoknot model for the secondary structure of the hepatitis delta virus ribozyme (Perrotta and Been 1991). Roman numerals indicate the numbers of the helices. Arrow shows the site of cleavage.

$U_{+1}G_{37}$ combinations were tolerated without compromising cleavage (Wu et al. 1993). *In cis* cleavage is also dependent on the length of helix I, since ribozymes containing either 6 or 7 base pairs can support cleavage, whereas ribozymes with a 5- or 9-base-pair-long helix I could not (Wu et al. 1993).

Helix III and the 7- to 8-nucleotide hairpin loop account for over half of the conserved nucleotides necessary for catalytic activity of the HDV ribozyme (Been et al. 1992; Thill et al. 1993; Wu et al. 1993). In particular, the sequence of the two base pairs closing the loop are very important, implying that they are necessary for tertiary contacts (Been et al. 1992; Wu et al. 1993). The sequence of the loop itself is conserved between the genomic and the anti-genomic forms of the ribozyme, with the exception of the nonessential U_{27}, which is only present in the genomic sequence. Deletions of the other nucleotides in this loop were found to dramatically reduce the catalytic activity (Thill et al. 1993). Mutations of the HDV ribozyme and subsequent in vitro selection for inactive mutants have determined the base requirements of loop III as being 5′-(U/C/G)-C-N-N-(C/A/G)-(G/A/U)-N-N, where N indicates any nucleotide (Kawakami et al. 1993).

The results from both deletion studies and mutagenesis experiments have established that helices II and IV play a role in stabilization of the active structure rather than being directly involved in catalysis (Thill et al. 1991, 1993; Blumenfeld et al. 1992; Wu et al. 1992). For example, when helix IV was replaced by a 4-bp stem, closed by a tetraloop, full activity was retained as long as Watson-Crick base-pairing was maintained (Been et al. 1992). However, when the helix was replaced by a loop of two to four nucleotides, the activity was reduced by two orders of magnitude, indicating that a helical segment is essential to the structural integrity of the ribozyme (Been et al. 1992; Thill et al. 1993).

Chemical modification in the single strand connecting helices I and II does not affect catalytic activity, which indicates that it is not important (Belinsky et al. 1993), further supported by the deletion of this strand in *trans*-acting ribozymes (Been et al. 1992; Perrotta and Been 1992). On the basis of a systematic substitution of individual bases in the strand connecting helices I and IV, as well as the strand connecting helices IV and II, it was concluded that the sequence requirements for the former were 5′-G-G-(G/A/U)-N-(A/U/G)-(G/A) and for the latter 5′-(G/U)-C-N-(A/G/U)-A (Kumar et al. 1992; Suh et al. 1993b). However, the last three bases in the strand connecting helices I and IV are not present in the anti-genomic sequence, and their deletion in the genomic sequence is not detrimental to activity (Been et al. 1992).

In trans Cleavage

As for other ribozymes, the engineering of the HDV ribozyme capable of multiple turnover by the generation of *trans*-cleaving ribozymes is important for proper kinetic characterization as well as for potential application to gene therapy. Moreover, the study of the structure-function relationships of the HDV ribozyme using synthetic oligomers will be facilitated by dissecting the ribozyme into fragments of suitable size for automated chemical synthesis. For example, the previously mentioned design of a three-piece ribozyme-substrate complex for the hairpin ribozyme has greatly stimulated studies on its structure-function relationships.

Three different approaches have been taken for the design of *trans*-acting ribozymes. First, dissecting the sequence in helix IV has yielded a short ribozyme that associates with the substrate through the formation of helices II and IV (Branch and Robertson 1991; Wu et al. 1992; Perrotta and Been 1993). Second, dissecting the single-stranded region between helices I and II yielded a long ribozyme and a short substrate that associated with the ribozyme sequence through the formation of helix I (Been et al. 1992; Perrotta and Been 1992). An interesting variation of this construct was the generation of a circular, *trans*-acting ribozyme that showed enhanced stability toward cellular nucleases and may be a viable strategy in the context of application of ribozymes for gene inhibition (Puttaraju et al. 1993). Third, dissection between helices I and II and in loop III, in addition to the connection of the 5′ end of the substrate to the 3′ end of helix II, yielded a ribozyme-substrate complex in which the substrate binds through base-pairing in helices I, II, and IV (Lai et al. 1996). A three-piece ribozyme-substrate complex for the HDV ribozyme has not yet been constructed, but a suitable candidate would be through dissection between helices I and II, and of loop IV.

Mechanistic Considerations and Metal Ion Requirements

As for the other ribozymes in this family, the HDV-mediated RNA cleavage has been shown to yield a 5′-hydroxyl group and a 2′,3′-cyclic phosphate (Sharmeen et al. 1988; Wu et al. 1989). These findings, in addition to the fact that a deletion of the 2′-hydroxyl group adjacent to the cleavage site abolished activity (Perrotta and Been 1992), are consistent with the generic mechanism depicted in Figure 2. Analogous to the hairpin ribozyme, the rate of cleavage does not change much between pH 5.0 and pH 9.1 (Wu et al. 1989).

The HDV ribozyme requires divalent metal ions for efficient cleavage

(Sharmeen et al. 1988; Wu and Lai 1989; Wu et al. 1989; Suh et al. 1993a). However, it is different from other known ribozymes in that its catalysis is as efficient in the presence of Ca^{++} as it is with Mg^{++} (Suh et al. 1993a). Moreover, the HDV ribozyme requires a much lower concentration of metal ions for cleavage than, for example, the hammerhead or the hairpin ribozymes; it requires only 0.5–1 mM Mg^{++} for efficient catalysis (Wu and Lai 1989). Mn^{++} and Sr^{++} are also able to support efficient cleavage, but Cd^{++}, Ba^{++}, Co^{++}, Pb^{++}, and Zn^{++} to a much lesser extent (Suh et al. 1993a). The well-known effect of spermidine with regard to lowering the metal ion concentration required for efficient cleavage has also been observed with the HDV ribozyme (Suh et al. 1993a).

Incorporation of phosphorothioates at all positions of the HDV ribozyme have identified several pro-R_P oxygens that are important for catalysis, in particular at positions 1, 21, and the cleavage site (Jeoung et al. 1994). Substitution of all the adenosines with the N7-deaza analog in the ribozyme yielded an active ribozyme that required a slightly higher Mg^{++} concentration for full activity (Wieczorek et al. 1994). This can be interpreted as evidence for the involvement of some N7 atoms in the folding of the HDV ribozyme.

The genomic (Wu and Lai 1989) and the anti-genomic (Sharmeen et al. 1989) sequences of the HDV ribozyme have been demonstrated to promote ligation in vitro. However, the ligation reaction requires an RNA sequence that is not a part of the minimal ribozyme sequence. In addition, this ligation yields an equimolar mixture of 2′,5′- and 3′,5′-phosphodiester linkages, as indicated by the inability of reverse transcriptase to read through the newly formed linkage for half of the mixture (Sharmeen et al. 1989). Thus, it is likely that ligation occurs because the 5′ and 3′ ends of the cleavage reaction are brought into proximity by a template, or a guide sequence, rather than being the reversal of the cleavage reaction. The equilibrium between cleavage and ligation has been shown to be sensitive to the presence of Mg^{++} (Wu and Lai 1989). When a fully cleaved HDV transcript was treated with EDTA in a slight molar excess over Mg^{++}, a quarter of the total RNA was converted to the full-length sequence.

A remarkable property of the HDV ribozyme is its ability to efficiently perform self-cleavage in the presence of high concentrations of denaturants, which in some instances even enhances cleavage (Perrotta and Been 1990; Rosenstein and Been 1990; Smith and Dinter 1991). For example, the anti-genomic ribozyme motif fully cleaves in the presence of 20 M formamide or 10 M urea (Smith and Dinter 1991; Smith et al. 1992). These data suggest that the active form of the ribozyme has an extremely stable structure, and this is supported by the ability of the ribozyme to efficiently cleave at temperatures up to 80°C (Rosenstein and Been 1990; Wu and Lai 1990; Smith and Dinter 1991).

A Model of the Tertiary Structure

A three-dimensional model of the hepatitis delta ribozyme has been proposed, mainly based on mutagenesis experiments and molecular modeling (Tanner et al. 1994). Helices I and IV as well as helices II and III adopt a near-collinear arrangement, and the relative placement of these two domains positions a part of loop III and the single strand between helices II and IV close to the cleavage site. More specifically, the conserved nucleotides C_{75}, C_{21}, and U_{20} are placed close to the cleavage bond. The spatial arrangement of both helical domains and specific residues provides an experimentally testable model and should stimulate structural studies on the HDV ribozyme. The application of chemical and biophysical techniques discussed previously for the hammerhead ribozyme (Amiri and Hagerman 1994; Tuschl et al. 1994; Bassi et al. 1995; Sigurdsson et al. 1995), as well as those utilized in the larger ribozymes (Murphy et al. 1994), will provide further insights into the structure-function relationships of the HDV ribozyme. However, full understanding of this structure may await the determination of its X-ray structure.

THE *NEUROSPORA* VS RIBOZYME

An abundant noncoding single-stranded RNA present in mitochondria of certain natural isolates of *Neurospora*, termed VS RNA, is the most recent addition to the family of ribozymes that yield products containing a 5′-hydroxyl group and a 2′,3′-cyclic phosphate (Saville and Collins 1990; Guo et al. 1993). VS RNA is transcribed from a VS plasmid DNA, using a *Neurospora* RNA polymerase, and has been shown to replicate as a satellite of the associated Varkud plasmid (Kennell et al. 1995). After self-cleavage of the RNA to form monomers, a reverse transcriptase encoded by the Varkud plasmid synthesizes the minus-strand cDNA from which the VS plasmid is formed after second-strand synthesis and circularization (Kennell et al. 1995).

Of the 881 nucleotides present in the VS RNA, only 154 are required for efficient cleavage (Guo et al. 1993). This ribozyme, like the HDV ribozyme, requires only one nucleotide at the 5′ end for efficient

cleavage, and its identity is not important (Guo et al. 1993). Like the hairpin ribozyme, the VS ribozyme has the ability to ligate a cleaved substrate (Saville and Collins 1991).

Secondary Structure

Recently, a secondary-structure model for the VS ribozyme has been proposed (Fig. 5) (Beattie et al. 1995). A family of secondary structures was generated using an RNA folding program, and only one structure was consistent with chemical modification studies. This structure contains six essential helical regions in which there are only minor sequence requirements. Most of the nucleotides present between helices were susceptible to chemical modification under semi-denaturing conditions (without Mg^{++}). However, in the presence of Mg^{++} many of these single-stranded nucleotides became protected, suggesting their involvement in tertiary interactions.

Cleavage by the VS ribozyme *in trans* has a most unusual feature (Guo and Collins 1995). Whereas most *in trans*-cleaving ribozymes recognize their substrate through standard Watson-Crick base-pairing, the VS ribozyme relies on tertiary interactions for binding, as the minimal substrate contains residues 620–639 and folds into a stable hairpin-loop. In contrast to the hairpin ribozyme where the tertiary, interdomain interactions are weak (K_M = 270 μM) (Butcher et al. 1995), the K_M for the *trans*-cleaving VS ribozyme is 0.13 μM, which is indicative of strong binding (Guo and Collins 1995). This *trans*-cleaving VS ribozyme had a k_{cat} of 0.7 min^{-1}, but it has not been determined if chemical cleavage is the rate-determining step in the mechanism.

Metal Ions and Organic Cations Affect Cleavage Rates

As with the other ribozymes in this family, the VS ribozyme requires Mg^{++} ions for efficient cleavage, although Mn^{++} and Ca^{++} can also support the reaction at a slower rate (Saville and Collins 1991; Collins and Olive 1993; Guo and Collins 1995). Addition of spermidine or monovalent ions stimulates the reaction but cannot replace Mg^{++}, reminiscent of the other ribozymes in this family (Collins and Olive 1993; Guo and Collins 1995).

Various antibiotics have been shown to interact specifically with RNA or RNA-protein complexes (Schroeder and von Ahsen 1996), as exemplified by the inhibition of hammerhead-mediated RNA cleavage by neomycin (Clouet-d'Orval et al. 1995; Stage et al. 1995). The effects

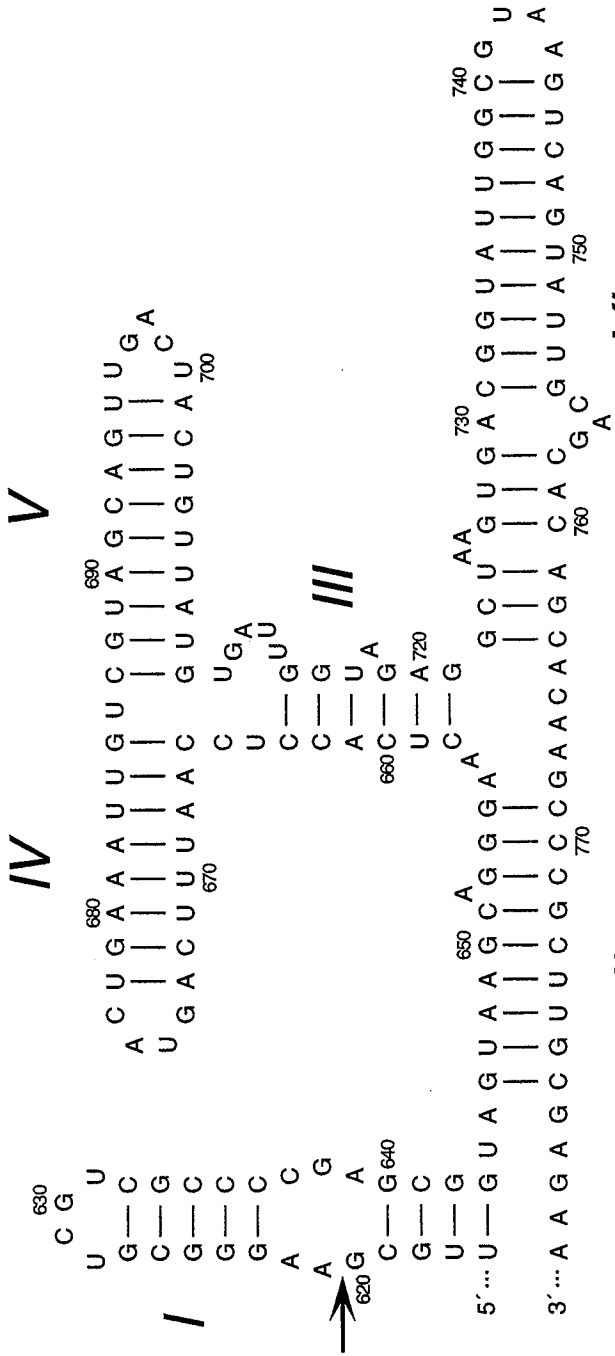

Figure 5 Secondary structure of the *Neurospora* VS ribozyme (Beattie et al. 1995). Roman numerals indicate the numbers of the helices. Arrow shows the site of cleavage.

of various antibiotics on the rate of VS-mediated RNA cleavage have been systematically studied (Olive et al. 1995). Viomycin, which has been shown to inhibit the action of other RNA or RNA-protein complexes (Liou and Tanaka 1976; Wank et al. 1994), was unexpectedly demonstrated to stimulate cleavage by the VS ribozyme (Olive et al. 1995). Viomycin is a cyclic, basic peptide that belongs to the family of tuberactinomycin antibiotics, and its interactions with the VS ribozyme were shown to be more complex than simple electrostatic interactions, as spermidine and other organic cations had a much smaller effect. Viomycin was shown to promote aggregation of the RNA, and this effect was postulated to be the origin of the rate enhancement because viomycin stimulated cleavage *in trans* for a mutant ribozyme that was unable to support cleavage *in cis*.

Mechanism and Tertiary Interactions

Limited mechanistic studies have been performed with the VS ribozyme. Analogous to the hairpin and HDV ribozymes, the rate of cleavage does not change much between pH 5.5 and pH 8.9, implying that abstraction of the proton from the 2′-hydroxyl at the cleavage site is not the rate-determining step in the mechanism (Collins and Olive 1993; Guo and Collins 1995). Unlike the HDV ribozyme, the VS ribozyme is inactivated in the presence of low concentrations of denaturants (Collins and Olive 1993).

The aforementioned studies on the susceptibility of nucleotides to chemical modification with and without Mg^{++} ions have identified nucleotides that are probably involved in tertiary interactions in the active ribozyme complex (Beattie et al. 1995). Very little information is available on interhelical tertiary interactions; however, mutational studies indicate that stem-loops of helices I and V interact by base-pairing (Olive et al. 1995).

APPLICATION OF RIBOZYMES FOR THE INHIBITION OF GENE EXPRESSION

The interest in ribozymes not only rests on their fascination as RNA catalysts and the associated mechanistic questions, but also stems from their potential application for the inhibition of gene expression. This development of ribozymes as therapeutics has stimulated the ribozyme field enormously and should therefore be discussed here briefly, even though it does not relate directly to structure-function relationships (for

recent reviews, see Marschall et al. 1994; Christoffersen and Marr 1995; Kiehntopf et al. 1995; Rossi 1995; Eckstein and Lilley 1996; Heidenreich and Eckstein 1997).

The use of ribozymes for this purpose has so far been limited to the hammerhead and the hairpin ribozymes. This application is attractive due to the fact that the ribozymes offer the opportunity to sequence-specifically cleave an mRNA and thereby prevent the expression of the corresponding gene. The sequence specificity is achieved by preparing a ribozyme that is sequence-complementary in its sequence to the target mRNA in the regions responsible for formation of the ribozyme-substrate complex. The rates of cleavage of long substrates such as mRNAs or transcripts are considerably slower than that expected from the cleavage of synthetic, short substrates (Heidenreich et al. 1994). This is interpreted as a slow step in binding of the ribozyme because of secondary structure of the mRNAs. Thus, the more accessible a region in the target RNA is, the more efficient the cleavage should be, although it remains difficult to identify such regions. However, in vitro determinations of cleavage rates might not be comparable to the in vivo rate, as it has been shown that certain RNA-binding proteins can accelerate cleavage by facilitating substrate binding and product release (Heidenreich et al. 1995; Herschlag 1995).

In principle, two methods of ribozyme delivery exist, endogenous and exogenous delivery. In the first, a plasmid or retroviral vector is constructed that contains the gene for the ribozyme behind a promoter. These constructs are brought into the cell by either transfection or transduction. In the latter, the ribozyme must be taken up by cells and is prepared either by chemical synthesis or transcription outside the cell and is usually introduced with the aid of a carrier such as liposomes. Using exogenous delivery, the ribozyme is transported to the endosome, where it cannot easily escape into the cytoplasm (Stein and Cheng 1993). More development is required to improve this uptake process.

Ribozymes are readily hydrolyzed by serum RNases; stabilization against such degradation is essential when exogenous delivery is used. An increase in stabilization from several minutes to several days can be obtained, without dramatic loss of catalytic efficiency, by modification of the 2′ positions of the pyrimidine nucleosides by 2′-fluoro-, 2′-amino- and 2′-O-alkyl derivatives (Pieken et al. 1991; Paolella et al. 1992; Bratty et al. 1993; Heidenreich et al. 1994; Beigelman et al. 1995; Heidenreich and Eckstein 1997).

Even after successful entry into the cell, however, ribozyme and target RNA have to be able to interact with each other for cleavage to oc-

cur. This requires physical proximity, which could be prevented by compartmentalization. This has been demonstrated in an experiment with two retroviral vectors, one carrying the gene for the target RNA, the other the ribozyme directed against this sequence (Sullenger and Cech 1993). The result indicates that these two RNAs did not interact in the cytoplasm but only during packaging of the virus.

Sequence specificity of the ribozyme-mediated cleavage should be guaranteed by the complementarity of ribozyme substrate binding arms to the target sequence. However, when targeting closely related genes, such as oncogenes, which differ only by one mutation from the proto-oncogene, a high degree of specificity is required. Tight binding between ribozyme and target RNA will reduce the discrimination between wild type and mutant as the time for cleavage will be short in relation to dissociation of uncleaved substrate (Herschlag 1991). Thus, decreasing affinity will increase discrimination, although a certain length has to be maintained for sufficient binding.

A considerable number of successful applications of ribozymes for the inhibition of gene expression have been reported and are discussed in the reviews mentioned above. There is no doubt that this method has considerable potential but must be further developed before it becomes a routine method.

CONCLUSIONS

Ribozymes represent a fascinating area of research for studying the structure-function relationship and catalytic power of RNA. Although we have learned that RNA can fold into many secondary and tertiary structures, the paucity of functional groups, in contrast to proteins, is still difficult to reconcile with the concept of a catalyst. Thus, considerable challenges for the researcher lie ahead in this area. The interest in these molecules as potential therapeutics will continue to stimulate the field.

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