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Review

Hammerhead ribozyme design and application

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Abstract. The emerging knowledge about RNA-based enzymes has already had great impact on our concept of evolutionary history, making the 'RNA world' more likely. It may well have an equally important impact on the diagnostic and therapeutic practices of human and veterinary medicine in the next decade. We are not quite there yet. This review addresses the design and application of hammerhead ribozymes, two aspects of a conserved and most commonly studied and used enzymatically active entity among the RNA enzymes. The emerging picture is one of great diversity. There is at this stage no general cell model nor a clearly preferable ribozyme structure. Each and every cell line (and tissue) may be unique in that they vary with respect to structural requirements for optimal uptake, activity and stability of ribozymes. We may have seen only the tip of the iceberg when it comes to RNA-based enzymes and their roles in biology and medicine.

Key words. Hammerhead; ribozyme; ribozyme stability; ribozyme design; flanking arms; oligonucleotide facilitators; target selection; cellular delivery.

Introduction

The hammerhead is one of several structural motifs of ribozymes which were originally detected in RNA molecules of several plant viroids and viruses. These RNAs can mediate self-cleavage in the course of their replicative cycle, which involves production of unit-length molecules from concatemeric RNA produced by rolling circle replication [1–4]. Self-cleavage by these RNAs was found to require a minimum sequence of only ~50 nucleotides which could be modelled into a secondary structure resembling a hammerhead [3, 4]. This model included three putative helical stems (I–III), of which two flank the susceptible phosphodiester bond, and two single-stranded regions which are highly conserved among different self-cleaving RNAs and implicated in catalysis (fig. 1). In addition to the single-stranded regions, a GUC sequence in stem III immediately preceding the susceptible phosphodiester bond was also found to be highly conserved among self-cleaving RNAs.

The hammerhead structure described by Symons and co-workers cleaved in cis. Cleavage in trans where the basic hammerhead structure was separated into two sequences at loops I and II was, however, soon described [5]. Separately synthesized catalytic and substrate parts could be reannealed through their two complementary sequences and still be active. The cleaved substrate strand was derived from the minus strand of the self-cleaving avocado sunblotch virus (ASBV) RNA. Haseloff and Gerlach [6] subsequently demonstrated in trans cleavage of a completely un-related substrate strand by separating the catalytic and substrate parts of the basic hammer-

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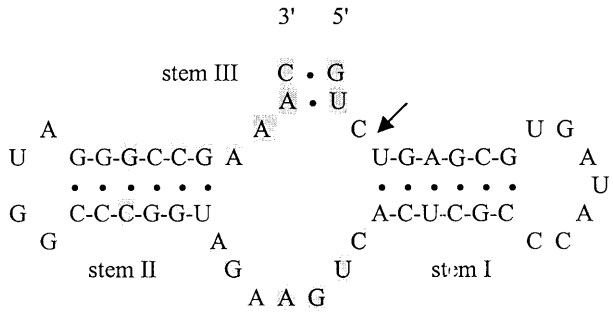

Figure 1. The hammerhead secondary structure of the minimal sequence required for self-cleavage by vLTSV RNA [4]. Cleavage site is indicated by the arrow. Shaded nucleotides were conserved among nine proposed hammerhead structures of self-cleaving RNAs [3].

head motif at helices I and III, creating a so-called I/III hammerhead. The resulting catalytic sequence contained most of the conserved sequences of the hammerhead, while the only sequence requirement of the substrate part was the presence of a GUC cleavage triplet (fig. 2). The limited substrate sequence requirements of the Haseloff and Gerlach model meant that ribozymes acting in trans could potentially be targeted against any RNA sequence containing a GUC triplet. In contrast, the substrate strand of the I/II trans-acting ribozyme of Uhlenbeck had additional sequence requirements. The Haseloff and Gerlach model has become the paradigm for the design of in trans-acting hammerhead ribozymes.

A target-specific ribozyme is obtained by designing the stem I and III sequences after the target RNA according to simple Watson-Crick base-pairing rules. Under optimal conditions ribozymes will bind to and cleave only RNAs which have sequences perfectly complementary to the stem I and stem III sequences flanking the GUC triplet. Thus, in theory, only a single RNA species should be cut and degraded if the proper considerations are made in designing the ribozyme. This fact made the hammerhead ribozyme a potentially very useful drug which could specifically reduce the expression of a gene product by targeting an appropriate sequence of the corresponding messenger RNA (mRNA), as noted by Haseloff and Gerlach [6]. The 10 years that have passed since the first report of hammerhead-mediated in trans cleavage have witnessed a steady accumulation of knowledge about how these molecules work. The three-dimensional (3D) crystal structure has been solved [8–10], which together with other experimental data [11–18] has cast light on the reaction mechanism. The kinetics of the hammerhead reaction have also been

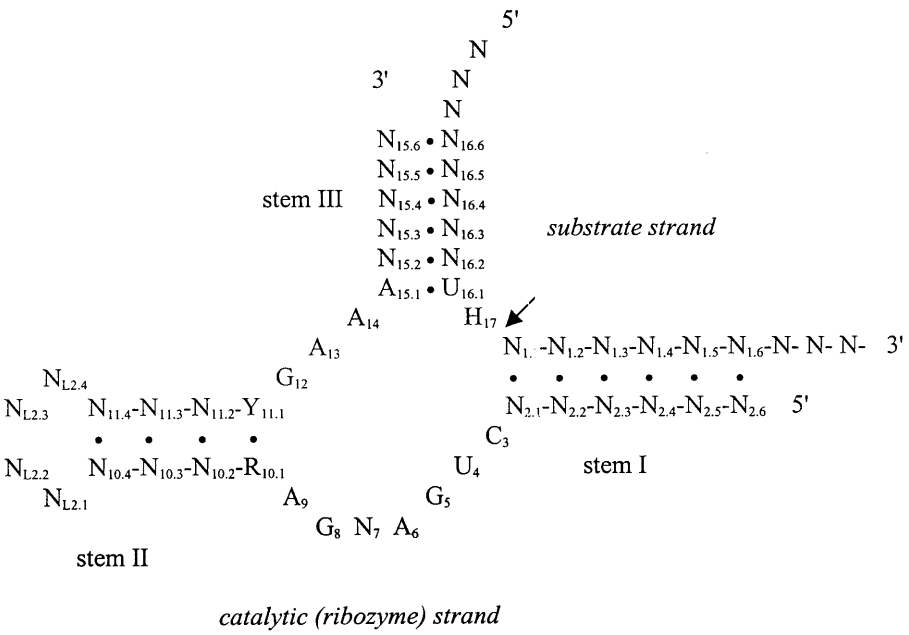

Figure 2. Haseloff and Gerlach [6] model I/III trans-acting hammerhead ribozyme. Nonconserved positions are indicated by N. H indicates any nucleotide except G, while R and Y indicate purine and pyrimidine nucleosides, respectively. The residues are numbered according to the nomenclature of Hertel et al. [7].

thoroughly described [19, 20]. The 3D structure, mechanism and kinetics of the hammerhead ribozyme are not, however, the subject of this review, and the interested reader should consult the original papers (v.s.) and several excellent reviews [21–23]. The present review will attempt to cover aspects of design and delivery of ribozymes for cell culture and in vivo application.

Structural requirements for hammerhead cleavage

The cleavage triplet

The strict requirement for a GUC cleavage triplet in the substrate was soon relaxed as mutagenesis data accumulated. Haseloff and Gerlach [6] found a preference for G in the first position of the triplet and for C in the third position, but cleavage was also observed to occur to different extents with any other base in these positions except for G in position 3. Poor cleavage after a G was also observed by others [24]. Thus the requirement was relaxed to the general NUH rule, where N is any base and H is any base except G. The poor cleavage after G is most likely due to its 2-amino group, as substituting the guanosine with inosine (which differs from guanosine only by the lack of the 2-amino group) results in a significant increase in cleavage rates [25a]. Nuclear magnetic resonance (NMR) studies have further demonstrated that the rG at the cleavage site forms a Watson-Crick base pair with C3 in the conserved core of the hammerhead [25b]. The authors suggest that this interaction with rG substrates inhibits the cleavage reaction by stabilizing an inactive conformation of the hammerhead molecule. Systematic permutation of the NUH sequence in a study by Zoumadakis et al. [26] indicated a strong preference for C as the third nucleotide and for A or G as the first nucleotide, in good agreement with the conclusions of Haseloff and Gerlach. The best triplets in terms of cleavage rates were found to be AUC, GUC and UUC, whereas the lowest cleavage rate constants were observed for UUU, CUU and CUA. The best triplet (AUC) had a 40-fold higher cleavage rate constant than the poorest triplet (UUU). Results from several studies of cleavage triplet efficiency [6, 24, 26–29] largely concur as to the preferred nucleotides at the various positions, the consensus being a purine at the first position and cytosine at the third. Ranking of cleavage efficiencies for the individual triplets, however, varies significantly. This may be due to differences in the sequence context of the NUH motif. Chemical modification of the ribozyme may also influence the relative cleavage efficiency of the triplets. Most studies concluded, however, that GUC worked best or at least very well in most cases. It has therefore been the target triplet of choice. Very recently, the specificity of the preferred hammerhead ribozyme cleavage triplet was changed from NUH to NCH by replacing a base-pairing nucleotide (A15.1) in the ribozyme sequence with inosine monophosphate [30]. The NCH-specific hammerhead ribozymes had cleavage rates which were at least as high as cleavage rates measured for NUH-specific ribozymes. The authors observed a high preference for a purine in the first position of the cleavage triplet. In the third position the relative reactivity was $A > C \gg U$. This order of reactivity agrees well with that observed by Zoumadakis et al. [26] for NUH-specific ribozymes. Cleavage after the normally prohibited G in the third position of the cleavage triplet has recently also been accomplished by an in vitro selected ribozyme with secondary structure similar to the native hammerhead [31]. This ribozyme, having an altered core and stem-loop II sequence, exhibited specificity for a purine in the third position, and tolerance for A in the second position (cleavage occurred after AAG). Thus, it seems that the NUH rule of specificity can be extended to include other cleavable triplets by introduction of specific mutations in the conserved sequence of the native hammerhead.

The conserved hammerhead core

In the single-stranded regions of the hammerhead ribozyme structure, the sequence is highly conserved among several naturally occurring self-cleaving RNAs at all positions except U7 (see fig. 2 for numbering). Although U at position 7 is most common, incorporation of any other normally occurring base has only minor effects on activity [27]. Other conserved positions include the innermost base pair of stem II (G10.1-C11.1) and residue A15.1 in stem III (which pairs with the conserved U16.1 of the cleavage triplet) (fig. 2). The latter is actually semiconserved, as a G can be tolerated at this position due to the stability of the G-U wobble base-pair. Results from several in vitro selection experiments have established that the phylogenetically conserved structure is also optimal for activity, as sequences that comprise the core of naturally occurring hammerhead dominate the population of selected ribozymes [32, 33]. Although all positions in the core of the hammerhead except N7 are conserved among self-cleaving RNAs, not all are essential for cleavage activity. Mutagenesis studies and incorporation of base analogues have established several highly important or essential positions and their essential functional groups. The importance of the 2′-hydroxyl groups of all the residues in the conserved core has been investigated, as well as the exo- and endocyclic nitrogens of the purines, and the O^6 keto group of the guanines [34–41]. The 2′-hydroxyl groups of G5, G8 and A9 in the ribozyme and of U16.1 and N17 in the substrate (a 2′-hydroxyl at this position acts as the nucleophile in the reaction and

is therefore essential) have been found to be important for full catalytic activity (reviewed in ref. 42). Besides the role as nucleophile in the cleavage reaction, the hydroxyl groups affect the structure of the catalytic core by engaging in hydrogen bonding and by influencing sugar puckering, which may affect folding. Other important functional groups include N^7 of A6, and the exocyclic amino groups of G5 and G12. The amino group of the latter is critical, and its removal results in almost complete abolishment of activity [38]. Crystal structure data suggest that some of these functional groups are involved in hydrogen bonds that stabilize the catalytic core. For several of the functional groups, however, no such associations can be inferred from the crystal structures. In these cases, the functional groups may be involved in critical associations in the transition state. Such a role has been suggested for the endocyclic N^3 nitrogen of residues A13 and A15.1 [41].

The stem-loop II

The lengths of stem II and the associated loop (L2.1–L2.4) are not conserved and do not seem to have any essential function. The stem and loop have therefore been targeted in attempts to reduce the size of hammerhead ribozymes. Results from several studies indicate that the whole stem/loop structure is partly dispensable [43–49]. The loop can be replaced by nonnucleotide linkers [45, 46], and stem II can be shortened from four to two base pairs [44], without significantly affecting activity. Further reduction of stem II to a single base-paired stem, however, results in reduced activity [44]. Inversion of the G10.1–C11.1 pair at the base of stem II also leads to loss of activity, even with a 4-bp stem. The reason for this is suggested by the observation that deletion of the 2-amino group of G10.1 results in drastically reduced activity which could be partially restored by increasing the Mg^{2+} concentration [37], indicating the involvement of the functional group in binding of an important Mg^{2+} ion. Replacing the whole stem and loop with a few nucleotides that cannot form Watson-Crick (W-C) base pairs results in minimized ribozymes (minizymes) which may retain significant cleavage activity. Minizymes normally cleave their short substrates much less efficiently (around a 100-fold reduction of cleavage rates has been described) than do full-length ribozymes [44, 47, 48]. Minizymes have been reported, however, to cleave long RNAs more efficiently than normal ribozymes [48]. In vivo, a minizyme was as efficient as the full-sized ribozyme in inhibiting target expression [49]. Deletions at stem II may actually increase activity of ribozymes under certain suboptimal conditions, such as when the ribozyme has very long flanking arms and/or when the Mg^{2+} concentration is low [50, 51], as is expected to be the case in vivo. Part of the reason for improved cleavage by minizymes on a long substrate may be their apparently tighter substrate binding [48]. It has also been suggested that deletion of stem II may increase activity by disruption of otherwise inhibitory interactions between residues in stem II and stem I, when the latter is long [52]. The lack of a preformed helix II may also reduce potentially inhibitory interactions with proteins in vivo. The activity of the minizyme may be dependent on the length, sequence and chemical composition (DNA or RNA) of the stem II linker, and possibly also the composition of the flanking arms [48]. Optimal activity of minizymes seems to be achieved by replacing the stem-loop II with four or five nucleotides, preferably all deoxyribonucleotides [43, 48, 53]. The preferred sequence of these linkers is not known, although all-purine or all-pyrimidine sequences might be preferable to avoid the possibility of W-C base pairings. This may not be a problem with short linkers, as the linker sequence 5′-CGUG-3′ was found to be heavily enriched in the selected pool in an in vitro selection experiment, and also showed the highest activity [51]. Minizymes with only a 2nt G-C linker have been shown to be active on their long RNA substrates [53]. Activity required the formation of a dimeric structure with a common 2-bp stem II and two catalytic cores capable of simultaneously cleaving two target molecules. Cleavage of two separate targets on the same molecule was also accomplished through heterodimerization of two separate minizymes. The stability of the dimeric structure depended on both the Mg^{2+} concentration and the number of G-C pairs in the stem [53]. The ability to dimerize was essential to the activity of these minizymes, as a minizyme with a nonnucleotide triterpenoid linker lacking in the ability to dimerize exhibited no activity [54].

Increasing the stability of hammerhead ribozymes

An inherent flaw of pure RNA hammerhead ribozymes is their very limited stability in biological fluids due to degradation by RNases. Many attempts at improving ribozyme stability have therefore been made by introducing different modifications at catalytically nonessential positions. The instability of RNA compared with DNA is due to the former having a 2′-OH group, which is utilized by RNAses in their course of action. One strategy for increasing stability is therefore to make various 2′ modifications at critical positions in the hammerhead. By judicious choice of type of modification and which residues to modify, substantial increases in stability can be achieved without compromising catalytic activity. One of the primary sites of hammerhead ribozyme degradation in biological fluids has been found to be internal pyrimidine nucleotides [55]. The

pyrimidines U4 and U7 in the single-stranded region have therefore been most often targeted for 2′ modification. Many different 2′ modifications confer resistance towards RNase degradation, including 2′-deoxy, 2′-fluoro, 2′-amino, 2′-O-Me, 2′-O-allyl and 2′-C-allyl [55–61]. Introduction of 2′-amino modifications of U4 and U7 increases stability without compromising the catalytic activity, possibly due to the hydrogen-donating capacity which is common to the amino and the hydroxyl group [59]. Modification at U7 may even result in increased catalytic activity, in addition to increased stability. Several base substitutions at position 7 are reported to increase the rate of the chemical cleavage step, with the largest increase (12-fold) observed for a pyridin-4-one base [62]. The effects on activity and stability of combinations of different 2′ modifications (O-Me, araOH, NH_2, C-allyl, $=CH_2$, $=CF_2$) at U4 and U7 have been investigated and several correlations noted [60]. An OH/O-Me modification (U4/U7) resulted in a ribozyme with wild-type activity which was unstable in serum. The $=CH_2$ and $=CF_2$ modifications, which distort ribose ring puckering, resulted in ribozymes with reduced activity. A very high activity of an NH_2/NH_2 modified ribozyme (second only to the OH/O-Me modified ribozyme) was in agreement with a proposed involvement of these groups in a hydrogen-bonding network. However, similar activities were also observed for several 2′-C-allylated ribozymes, which may indicate that hydrophobic interactions in this region are also of importance. The two ribozymes with the best combination of activity and stability were NH_2/NH_2 and C-allyl/O-Me.

Modification at the 2′ positions of only the pyrimidines U4 and U7 is not sufficient to maximally stabilize hammerhead ribozymes. It has been suggested that all pyrimidine nucleotides should be replaced by their 2′-modified analogues in order to achieve stability in serum [59]. Presumably, stability would be increased further by also targeting purine nucleotides for 2′ modification. Extensive 2′ modification at all positions, however, would result in loss of activity, since many of the 2′-OH groups are important or essential for activity [56]. The minimum amount of unmodified ribonucleotides necessary for activity has been determined [57, 58]. Paolella et al. [57] demonstrated that the presence of ribonucleotides at only positions U4, G5, A6, G8, G12 and A15.1 was sufficient for efficient cleavage. The remaining positions could be 2′-modified (with O-Me or O-allyl) with little or no effect on activity. Of these six positions, modification seemed to be less detrimental for activity at positions U4 and A6, as only a partial loss of activity was observed. In a similar but less rigorous study (G12 was not included among the positions tested), Yang et al. [58] found that a 2′-O-methylated ribozyme with ribonucleotides only at positions 5, 6, 8, 15.1 and 15.2 had an activity comparable to that of the nonmodified wild-type. Allowing for differences in experimental design, there was good accordance between the results of the two studies. The consensus seems to be that ribonucleotides are needed only at positions 5, 6, 8, 12 and 15.1. Such ribozymes where all but the above-mentioned residues are 2′-O-Me-modified have more recently been shown to have catalytic activities comparable to that of wild-type ribozymes [60]. The stability of these ribozymes in serum was increased several thousandfold (half-lives increased from less than 0.1 min for the wild-type to 4 to 8 h for differently modified ribozymes). Similarly designed ribozymes with either 2′-O-allyl [63] or 2′-O-Me [64] modifications have also been used successfully in vivo.

Maximizing stability of ribozymes also required incorporation of structures that protected against degradation by exonucleases. A modified ribozyme incorporating four 3′-terminal phosphorothioates in addition to 2′ modification of all pyrimidines was shown to be stable in undiluted fetal calf serum (FCS) for at least 24 h [59]. The unmodified ribozyme, on the other hand, was completely degraded within less than 2 min in FCS. The incorporation of the four terminal phosphorothioates at the 3′ end, increased the stability significantly compared with a ribozyme which was only 2′-modified at the pyrimidines, indicating that most of the degradation of the latter ribozyme was due to 3′-exonuclease activity. This is in accordance with results from another study which demonstrated that ribozyme decay consisted of two phases, where the faster phase was delayed by incorporation of a hairpin structure at the 3′ end, thus apparently due to 3′-exonuclease activity [65]. The stability and efficacy of ribozymes with 2′ modifications at pyrimidines and phosphorothioate linkages at both ends have been investigated in suspensions of nuclei [61]. These ribozymes were significantly more stable and exhibited severalfold higher cleavage efficiency than their unmodified counterparts. The efficacy of the ribozymes was generally found to correlate well with their stability. The increased resistance against nuclease degradation was mainly due to the terminal phosphorothioate linkages. In this system 2′ modification at pyrimidine residues did not further enhance the efficiency. In a similar study the optimal ribozyme configuration was found to include the incorporation of four phosphorothioate linkages at the 5′ end [66]. Incorporating phosphorothioates only at the 3′ end resulted in ribozymes which exhibited almost no efficacy. The ribozymes with optimal effect were additionally 2′-O-Me-modified and had any of a variety of 2′ sugar or base modifications at positions U4 and U7. The combined results of these studies suggest that in addition to internal 2′ modification, stabilizing structures should be incorporated at both ends of the ribozyme, as both 5′-

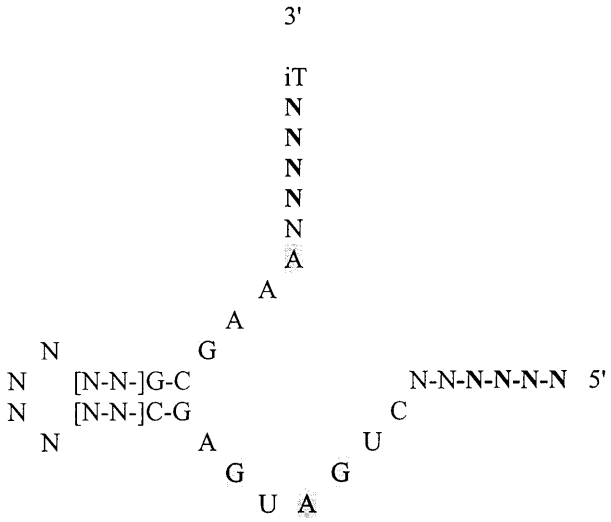

Figure 3. A maximally stabilized modified hammerhead ribozyme. All nucleotides are 2′-modified (O-methyl or O-allyl) except the shaded positions, which are ribonucleotides. Up to four phosphorothioate links are incorporated at both ends (bold nucleotides), in addition to an inverted T at the 3′ end. In the positions marked N, 2′-modified ribonucleotides may be substituted with deoxynucleotides. To reduce the size, two base pairs (indicated in brackets) may be deleted from stem II.

and 3′-exonucleases seem to be involved in degradation (fig. 3). Internal 2′ modifications may not be necessary for optimal stability in all cases, and the delivery mode of the ribozymes may influence the choice of modifications necessary for optimal stability [61].

Protection against 3′-exonuclease activity can also be accomplished by the addition of a hairpin structure at the 3′ end [65, 67a]. Alternatively, an inverted T (a thymidine in a 3′-3′ link) can be incorporated at the 3′ end, serving the same purpose as a hairpin structure and greatly reducing the size of the ribozyme [60]. Another strategy for reducing degradation of ribozymes by exonucleases is to replace their catalytically nonessential hybridizing arms with DNA. This modification may, however, in addition to increasing stability also affect turnover and specificity of ribozymes for their substrate, as will be discussed below.

Activity and specificity of ribozymes

Effect of flanking arms

Both the turnover and specificity of a ribozyme will depend on the length and sequence of its flanking arms. Optimal turnover requires that product dissociation not be slow. For short (<20 nt) substrates, the optimal length of flanking arms seems to be in the range of six to seven nucleotides [66]. For long substrates with extensive secondary structure the optimal flanking arms may be longer, but also in this case increasing the arms beyond a certain length reduces the rate substantially. Goodchild et al. [50] observed a 10-fold increase in cleavage rates upon reducing the lengths of the flanking arms of a ribozyme targeted against human immunodeficiency virus-1 (HIV-1) RNA from 20 to 12 nt. Screening a library of ribozymes with $13 + 13$ randomized arms, Lieber et al. [68] found that the most active ribozymes in vivo had a consecutive complementary stretch with the substrate of only 15 nt (7 and 8 nt on either arm). Thus the optimal length of hybridizing flanking sequences for cleavage of long structured substrates may be in the same range (roughly 6–8) as that for cleavage of short substrates under certain conditions. Reduced cleavage rates of ribozymes with long flanking arms may be due to impaired folding into the active conformation caused by the helices forming the ribozyme-substrate complex [67b]. Cleavage rates of such hammerheads were found to be increased by the inclusion of the folding-promoting polycation spermidine during preannealing, and were also enhanced by very high concentrations of magnesium [67b]. In the study of Lieber et al., the most active ribozymes had additional nonhybridizing extraneous sequences which did not seem to adversely affect the activity. Such sequences may in fact increase the intracellular efficiency of ribozymes if they fold up in stem-loop structures that increase stability, although there is evidence that nonhybridizing sequences may limit activity when they become too long [69a].

The use of asymmetrical flanking arms may in some cases result in increased turnover. Hendry et al. [52] investigated the cleavage rates of a series of ribozymes with asymmetric flanking arms where helices I and III consisted of 5 and 10 nt, respectively (or vice versa). In two independent systems the asymmetric ribozymes with the shorter helix I cleaved their substrates up to 130-fold more rapidly than asymmetric ribozymes with the shorter helix III. This effect was observed with all-RNA and DNA-armed ribozymes. This large difference in activity was unexpected under conditions when the chemical cleavage step should be rate-limiting. To explain this effect, the authors proposed that the ribozyme-substrate complex was required to undergo a conformational change involving the hybridizing arms in order for cleavage to occur, resulting in the generation of a rapid equilibrium between an active and an inactive conformation. The preference for a short helix I was explained by the presence of interactions between distant residues in stem I (past position 2.5) and residues in the stem and loop II which stabilize the inactive conformation. The presence of such interactions is corroborated by X-ray crystallography. Increasing the length of helix I beyond 5 bp reduces the

cleavage rate [69b]. It was suggested that the two innermost base pairs of helix I might have a significant effect on cleavage rates. The highest rates were obtained with the $U_{1.1}$-$A_{2.1}$ and $A_{1.2}$-$U_{2.2}$ base pairs. The length of the helix I arm has been reduced even further while simultaneously increasing the length of stem III. A stem I of only 3 nt was sufficient for full catalytic activity of a ribozyme with a very long stem III arm of 280 nt [70]. A conventional ribozyme with 8 + 8 nt arms, however, failed to cleave the target RNA in vitro. Due to long arms and consequent slow dissociation, each ribozyme molecule can only cleave once. Several researchers have reported the requirement for high molar excess of expressed ribozymes for sufficient efficacy [71, 72]. This suggests that single turnover may occur in vivo also with ribozymes with shorter flanking arms, possibly due to limited accessibility of the target. In this case, ribozymes with very long flanking arms (catalytic antisense RNA) may, at least in some cases, be superior to ribozymes with short flanking arms. The question then is if the catalytic antisense RNA is more efficient than regular antisense RNA when both are in molar excess. One would not expect any significant difference if the antisense RNA completely inactivates every target it encounters. It has been reported, however, that a hammerhead ribozyme with very long flanking arms was four- to sevenfold more efficient in suppressing HIV-1 expression in vivo than pure antisense RNA or an inactivated version of the ribozyme [73]. It has been suggested that a single-strand displacement activity of endogenous RNA-binding proteins may account for this surprising finding [74], and consequently that turnover may be possible also for hammerhead ribozymes with very long flanking arms. The optimum antisense arm length for efficacy of catalytic antisense RNAs can be determined specifically for each target by in vitro selection of fast-hybridizing sequences [75]. In vitro hybridization data may, however, not be entirely predictive for the in vivo situation, as efficient hybridization in vitro has been observed with much shorter flanking arms than were required for in vivo efficacy [76]. A systematic study of the in vivo efficacy of anti-HIV-1 ribozymes with varying antisense arms suggested that a minimum of 30 nt on each arm was required for optimum efficacy, although it is unclear whether the inhibition of replication was due to ribozyme or antisense effect [77].

The specificity of the hammerhead reaction is influenced by many of the same factors as turnover, including the size of the flanking arms. When the flanking arms are short, increasing their lengths will enhance discrimination between the specific substrate and other substrates. Increasing the lengths of the flanking arms beyond a certain optimal length will, however, reduce specificity, as the hammerhead will bind with comparable strength to targets with a few mismatched base pairs [78]. The reduced specificity obtained with ribozymes having long flanking arms is a result of the very slow substrate dissociation, which does not allow equilibration of the hammerhead with its 'correct' potential substrate. Thus the ribozyme is unable to take advantage of the difference in affinity to distinguish properly between the specific and nonspecific substrates. Aside from reducing the size of the flanking arms, specificity may be increased by choosing an A + U-rich recognition sequence, as the weaker binding energy of the A-U base pair compared with the G-C base pair is expected to increase dissociation rates [78]. The specificity of cleavage of short substrates has been investigated by Hertel et al. [79], using an 8 + 8 ribozyme (HH16) to cleave a 17-nt substrate. Under standard conditions, the ribozyme was unable to discriminate between its full-length substrate and several 3′-truncated substrates. However, HH16 did exhibit high specificity towards certain 3′-truncated versions of altered substrates that either also contained a single base mismatch or were shortened at the 5′ end (by 3 nt). Two important conclusions from this study are that helix III seems to be more important for specificity than helix I, and that complete specificity is only observed with relatively short complementary sequences between ribozyme and substrate. The limit was 10–12 nt of hybridizing sequences in the above study, but may be higher in vivo, where the conditions are such that the cleavage reaction is expected to be significantly slower than in vitro, and thus allow more time for substrate dissociation and equilibration. The effect of base mismatches in the substrate recognition helices on binding and catalysis has also been investigated by Werner et al. [80]. A mismatch in the innermost base of helix I affected the chemical cleavage step, while more distal mismatches had no such effect. In contrast, mismatches in any of the four innermost base pairs of helix III affected the chemical cleavage step. The deleterious effect on cleavage of mismatches close to the cleavage triplet can be utilized to differentiate between related substrates having the same cleavage triplet. A ribozyme designed to cleave GUC↓A efficiently cleaved the HIV-1 SF2 RNA, but not the RNA of the closely related strain IIIB having a G instead of an A following the cleavage triplet [81]. This specificity was also observed in cells, as ribozyme-expressing cells were significantly protected against infection by HIV-1SF2 but exhibited no protection against HIV-1IIIB infection.

Both the sequence and chemical composition of the flanking arms can greatly affect cleavage rates irrespective of flanking arm length. A variation of cleavage rates by more than 70-fold under similar reaction conditions was observed for four self-cleaving hammerhead ribozymes with the same core sequences but differing

13-nt substrate sequences [82]. These differences in catalytic activity were almost entirely an effect of changes in K_m, which varied 60-fold for the ribozymes. Several researchers have reported increased activity of DNA-armed ribozymes [83–87]. While catalytic activities for all-RNA hammerhead ribozymes are often in the region of 1 min^{-1}, k_{cat} values as high as 27 min^{-1} have been measured for DNA-armed ribozymes [86]. A chimeric ribozyme with 24 bases of HIV-1 Gag complementary DNA had a sixfold greater k_{cat} value than the all-RNA counterpart, and this increased activity appeared to be a direct result of enhanced product dissociation [84]. While several cases of increased rate of the chemical cleavage step by DNA-armed ribozymes compared with their all-RNA counterparts have been reported [84, 85], a more recent paper concluded that the presence of DNA in the hybridizing arms had little effect on the overall cleavage rate when the chemical cleavage step was rate-limiting [88]. A sequence-dependent effect on cleavage rate may exist, thus possibly explaining that DNA-flanking arms increase the overall rate in some but not all cases when the chemical cleavage rate is rate-limiting. It has also been suggested that cleavage rate enhancement with DNA-armed ribozymes may be due to slight changes in structure [89]. Such changes may, for instance, involve a conformational change from an inactive to an active conformation [88]. K_m values are generally increased for DNA-armed ribozymes, due to the lower stability of DNA-RNA helices compared with RNA-RNA helices. The increase in K_m value is in most cases higher than the increase in k_{cat}, so that catalytic efficiencies of DNA-armed ribozymes are generally lower than for their all-RNA ribozyme counterparts.

The DNA arms of mixed DNA-RNA ribozymes make the bound RNA substrate prone to degradation by RNaseH as well as by the pure hammerhead ribozyme cleavage activity. The DNA-RNA hybrid in the hammerhead-RNA complex is a substrate for RNaseH (present in high amounts in the nucleus), which site-specifically degrades the RNA-strand of the hybrid by a combination of endonuclease and exonuclease activities [90–92]. Complete replacement of the nucleotides in the ribozyme stems with their DNA analogues thus induces a potent RNaseH activity in the nucleus [61]. This RNaseH-mediated cleavage may be faster than the ribozyme-mediated cleavage and thus result in more efficient cleavage. However, this RNaseH-mediated cleavage may also reduce specificity, as it does not require perfect alignment of stems I and III [93]. A contiguous base-paired stretch of only 5 nt is sufficient for RNaseH-mediated cleavage [94]. Such DNA-armed ribozymes might be powerful agents for the modulation of gene expression due to their capability to both induce nuclear RNaseH-activity and cleave directly in the cytoplasm. However, extra care should be taken in choosing the target site in the RNA and in designing the flanking arms in order to prevent nonspecific cleavage.

Effect of oligonucleotide facilitators

Dissociation of the ribozyme-substrate complex may be faster than cleavage to products for long substrates [95], in contrast to what has been reported for short substrates [20]. Thus, most substrate molecules may need to interact with a ribozyme more than once before cleavage can occur. If the complex could somehow be stabilized so that cleavage is faster than substrate dissociation, the result would be an enhancement of cleavage activity. One way to accomplish this is by using 'facilitator' oligonucleotides. In long structured substrates facilitators have the potential to preform the substrate for the ribozyme attack, increasing the rate of association and subsequent cleavage [95, 96a]. The addition of oligonucleotide facilitators may also result in multiple turnover activity on long substrates under conditions when the ribozyme alone acts only as a single-turnover catalyst [96b]. The mechanism of action for the facilitators probably involves cooperative binding between the facilitator and ribozyme, which would tend to increase cleavage rates only when binding of substrate is rate-limiting. This is corroborated by the observation that facilitators have relatively little effect under single-turnover conditions when the Mg^{2+} concentration is high [97, 98]. Even under single-turnover conditions, however, facilitators are efficient at low (1 mM) Mg^{2+} concentrations, when cleavage rate constants are significantly reduced [98]. The helical stability of the flanking arms is increased by six orders of magnitude upon increasing the length of the arms from $5 + 5$ to $10 + 10$ [98]. Since facilitators are most efficient when substrate binding is rate-limiting, their activity would be expected to be highly dependent on the arms of the ribozymes. Under multiple-turnover conditions, Nesbitt et al. [98] observed that a $6 + 6$ ribozyme resulted in the highest activity in the presence of a 13-mer facilitator. A facilitator also increased the cleavage activity of a $5 + 5$ ribozyme considerably, while a slight reduction in activity was observed for a $10 + 10$ ribozyme. Presumably, the substrate dissociation rate was reduced sufficiently upon increasing the hybridizing arms from 5 to 10 nt to make some other step than substrate binding (most likely product dissociation) rate-limiting.

As is hinted above, facilitators can act as both activators and inhibitors of ribozyme activity under certain conditions. The stability of the facilitator-substrate hybrid, which is a function of the facilitator length, does not seem to determine whether the facilitator acts as an activator or an inhibitor [99a]. The absolute effect of

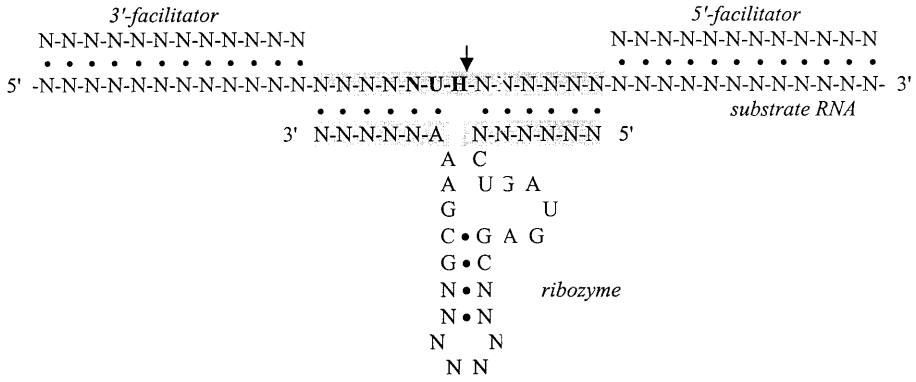

Figure 4. Placement of 12 nt 3′ and 5′ facilitators (RNA or DNA) relative to the ribozyme, as described by Jankowsky and Schwenzer [99a]. The facilitators can be either DNA or RNA.

the facilitators, however, increases with the theoretical stability of the hybrid, as RNA facilitators cause greater effects than DNA facilitators of the same sequence, and longer facilitators are more effective than shorter ones. An equimolar amount of short substrate and facilitator is optimal for maximal effect of the facilitator [99a], whereas a considerable molar excess of facilitator is required for long substrates [96a]. Facilitators can be placed adjacent to the ribozyme-binding site at either the 5′ end or the 3′ end of the ribozyme (fig. 4). Jankowsky and Schwenzer [99a] found that the position of the facilitator relative to the ribozyme had a crucial effect on its ability to enhance the ribozyme reaction. Various 3′ facilitators of differing lengths were able to activate a ribozyme at all concentrations under both multiple- and single-turnover conditions. Activation was a result of both increased cleavage rates (k_2 or k_{cat}) and a decreased substrate dissociation constant (K_d) or K_m. Decreased dissociation of the ribozyme-substrate complex was attributed to a stabilization of the complex due to stacking of the terminal bases of the ribozyme and the facilitator. Because this stacking provides a constant amount of energy, the relative facilitator effect decreases with increasing stability of the ribozyme-substrate complex. The ribozyme-product complex is also stabilized, and when this stabilization reaches a critical value, the multiple-turnover reaction is inhibited. The results suggest that the rate-limiting step in multiple-turnover reactions changes from the association step without facilitator to the cleavage step with 3′-end facilitator. The corresponding 5′-end facilitators inhibited the ribozyme reaction at all concentrations under multiple-turnover conditions, whereas their effect was dependent on both concentration (inhibitory effect at high concentrations) and length under single-turnover conditions [96a, 99a]. The rate-limiting step of the multiple-turnover reaction with 5′-end facilitators was, unlike the 3′-facilitated reaction, most likely product dissociation. The association constants for complexes of cleavage products with ribozyme with flanking arms ranging from $5 + 5$ to $10 + 10$ have been measured, and indicate that the complex of the 3′-end product with ribozyme is more stabilized than the 5′-end product by an order of magnitude [98]. This difference in the stabilities of the stem I and III helices causes the difference in the mode of action of 3′ and 5′ facilitators. The former will increase the helical stability of the weaker stem III, which presumably is not increased enough to affect the rate of product dissociation. This is dominated by the properties of stem I [98]. The observed effects of 5′ facilitators can be explained by further stabilization of stem I, making product dissociation rate-limiting.

Facilitators have also been shown to function in vivo. Incubation of cells expressing an anti-β-amyloid peptide precursor (βAPP) hammerhead ribozyme in the presence of oligodeoxynucleotides targeted immediately upstream of the ribozyme (3′ facilitator) resulted in specifically lowered βAPP compared with scrambled oligonucleotide controls [99b]. The extent of the reduction depended on the presence and proximity of the ribozyme to the oligonucleotides but not on the length of the oligonucleotides.

Effect of RNA-binding proteins

When the recognition sequence of a ribozyme is extended beyond a certain length, turnover is slowed and specificity is decreased. This limitation can, however, be overcome by certain RNA-binding proteins [100–104].

Cleavage of an RNA oligonucleotide by an 8 + 8 hammerhead ribozyme (HH16) was enhanced 10- to 20-fold upon addition of the p7 nucleocapsid (NC) protein of HIV-1 [100]. Addition of NC protein increased the rates of both substrate binding and product dissociation, possibly due to a strand dissociation/unfolding activity of the protein. Protease treatment and addition of ss-DNA 28-mer abolished this enhancement activity, as expected for an activity associated with an ss-DNA-binding protein. Furthermore, there was no enhancement effect of NC when ribozyme and substrate were preannealed under single-turnover conditions. This loss of stimulation upon preannealing is also consistent with an effect of the protein on the association step. Other DNA- or RNA-binding proteins have also been tested for enhancement activity. The gp32 protein of T4 phage and the *Escherichia coli* SSB protein could not substitute for NC, whereas the hnRNP A1 protein could imitate NC protein [101, 102]. Evidence suggests, however, that A1 and NC may work through different mechanisms and affect different steps of the hammerhead reaction [101]. The difference in the manner in which these two proteins enhance ribozyme activity is highlighted by the observation that NC but not A1 has an inhibitory effect on cleavage when the substrate-ribozyme duplexes are long (17–20 bp). Enhancement of product dissociation by both proteins required relatively short (≤ 7) ribozyme-product duplex lengths. DNA/RNA-binding proteins are not the only ones that have been shown to enhance ribozyme cleavage activity. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specifically bound and enhanced the activity of a tumour necrosis factor α (TNFα) mRNA-targeted ribozyme [104]. Binding most likely involved the Rossman fold, as it was inhibited by NAD+ and ATP. GAPDH increased the in vitro cleavage rates of ribozymes by up to 25-fold, an effect which was due to its unfolding activity. Enhancement of cleavage product dissociation was also observed for short duplexes. Thus GAPDH seems to enhance ribozyme activity and turnover by a mechanism similar to that of NC and A1 proteins.

Tsuchihashi et al. [100] found that the NC protein also enhances the specificity of the HH16 ribozyme reaction, which in the absence of NC was poor even for 3′-truncated 13-mer substrates (differing by a single mismatch). Addition of NC, however, enhanced 20-fold the ability of HH16 to discriminate between its matched and mismatched 13-mer substrates. The ability of NC to speed binding and dissociation is paramount to its enhancing effect on specificity. These effects promote equilibration of the binding of potential substrates, thereby allowing the ribozyme to take advantage of differences in binding affinity. Single turnover reactions with preannealing of substrate and ribozyme have been reported to deviate somewhat from the expected first-order reaction behaviour, especially at low concentrations [99a]. This was interpreted to be due to an equilibrium state where a fraction of the substrate molecules were not in complex with the ribozyme. This fraction may have an inaccessible secondary or tertiary structure. In this case, proteins like NC may resolve such structures and make more of the substrate accessible to the ribozyme. The protein may also resolve misfolded ribozyme-RNA complexes that are otherwise long-lived [102]. Generation of these dead-end complexes would lower the effective ribozyme concentration under multiple-turnover conditions, thereby reducing the observed turnover.

Selection of ribozyme target sites

Perhaps the single greatest problem in designing ribozymes with optimal activity on a long substrate is the selection of a suitable target site on the substrate. Long RNAs and their intracellular protein complexes have extensive secondary and tertiary structures which may severely limit the accessibility of potential ribozyme target sites [105]. In vitro catalytic efficiencies (k_{cat}/K_m) reported for several ribozymes targeted to the same long substrate differed by 400-fold under conditions where the association step was expected to be rate-limiting [106]. Chemical probing of the target RNA indicated that accessibility, measured as the relative catalytic efficiency of ribozymes on long vs. short, otherwise identical, substrates, was strongly correlated with the availability of nucleotides near the cleavage site for base pairing (nucleotides pairing with the more distal parts of stems I and III were significantly less important for accessibility). Thus target accessibility would generally be a more important determinant of intracellular cleavage than cleavage activity per se, as has been noted by others [107]. In the study of Campbell et al. [106], steric interference by the formation of higher-order structures was deemed not to contribute significantly to the variation in target accessibility. The situation may be different in vivo, where the many RNA-binding proteins which are associated with the target greatly compound the problem. These proteins may either mask potential sites or otherwise influence the structure of the RNA in its noncomplexed form. Because of these difficulties and the lack of any theory for selection of target sites, a trial-and-error method has commonly been employed for selection of cleavable sites. The regions around translation initiation and splice sites have been targeted on the informed guess that the RNA may be more accessible to hybridization by ribozymes in these regions due to the need for relatively open structures for binding of the translation machinery and

spliceosomes, respectively. The downstream coding region has, on the other hand, been considered not to be ideal for ribozyme targeting, since slow-cleaving ribozymes may be displaced by the moving translation machinery before cleavage can occur. This has recently been shown to be an important consideration in prokaryotes, for instance in the bacterium *E. coli*, where transcription and translation are coupled processes. In this system, a ribozyme targeting a plasmid-born chloramphenicol acetyltransferase (CAT) gene reduced CAT activity only when transcription was uncoupled from translation by either enhancing the transcription rate or decreasing the translation rate by using a slow ribosome mutant [108]. In the latter case administration of streptomycin, which increases the speed of translation, abolished the effect of the ribozyme on CAT activity. Although this situation is not entirely relevant to the normal application of ribozymes in eukaryotes, where the ribozyme may act in the nucleus and on unprocessed or partly processed mRNA molecules and usually has time to cleave its target before translation is initiated, it does emphasize the importance of cellular factors in modulation of ribozyme activity in vivo.

Besides the trial-and-error approach and the informed guess, both computational and experimental methods for selection of suitable target sites have been employed with varying degrees of success. One method has been based on secondary structure predictions using RNA-folding programs exemplified by MFold, SAPSSARN and ESSA [109–111]. MFold usually generates several suboptimal secondary structures which may differ significantly from each other in their folding, although they differ only slightly in energy [112]. One possible approach when using this program is to identify conserved features among the different optimal and suboptimal structures, with the assumption that these features are more likely to be part of the 'true' structure. Frequently recurring features/substructures can be employed as structural restraints for generating more accurate structures of the total RNA [113]. Ribozymes are targeted against structures that are judged to be most suitable for hybridization. Unfortunately, there is no recognized general theory for which substructures to choose for ribozyme targeting. A positive correlation between the inhibitory effect of antisense RNA and low local folding potential (high ΔG value) of the targeted region has, however, been reported [114a]. Thus, ribozymes should presumably be targeted against sequence stretches that cannot form stable structures. This can be done by considering the melting behaviour of the RNA (generating several structures at successively higher temperatures) and avoiding selecting as targets those regions that form stable structures even at high temperatures. A recent study of antisense RNA effects reported that fast RNA-RNA annealing in vitro, as well as in vivo inhibition of HIV-1 virus replication, were related to the presence of unpaired terminal nucleotides and overall flexibility of the antisense RNA, as predicted by a computer program [114b]. Computer-selected antisense RNA species exhibited inhibitory effects in cells that were 10–10,000-fold stronger than for species predicted to anneal slowly. The observed correlation between local folding potential (or flexibility) and efficacy of antisense RNA suggests that single-stranded or loop regions may be the best targets. Presumably, a ribozyme would attach more readily to an unpaired strand than to one that is already base-paired. This is corroborated by the observation that single-stranded RNA interactions, so-called kissing, induce hybridization [115]. Based on the above assumption, ribozymes have been successfully targeted to various predicted loop regions [116–118]. Sakamoto et al. [117] found that among four different ribozymes targeted to sites in the noncoding and core gene regions of hepatitis C virus RNA, the two most effective ribozymes in both in vitro and cell culture experiments were targeting regions predicted to be single stranded. Experimental in vitro selection strategies have also indicated a preference for targeting of ribozymes to single-stranded regions [68]. Targeting of the single-stranded region of loop C of animal and plant 5S ribosomal RNA (rRNA) was, however, unsuccessful [119]. The authors suggest that the targeted loop structure may not be flexible enough to allow formation of the correct hammerhead structure with the ribozyme for cleavage to occur, or that the loop may be inaccessible due to its involvement in higher-order structures. The former explanation would be in agreement with a rival theory for selection of efficient target sites which is based on the assumption that efficient binding of the ribozyme should minimally affect the overall energy of ribozyme and substrate. According to this theory, the ribozyme should bind to already base-paired regions with minimal effect on the helical structure of the binding region. Presumably, the base-paired region should not be part of a very stable substructure, so that the ribozyme can readily displace the antisense strand. A compromise of the above two hypotheses is the targeting of stem-loop regions, in which the ribozyme recognition sequence overlaps with both single-stranded and base-paired sequences in the target. In this case, the loop might represent an open entry or kissing site for nucleation of hybridization, which requires the presence of at least a short stretch of nonpaired sequences.

The usual way to make use of such computer programs is for structure prediction of the target only. However, it can be worthwhile to also consider the secondary structure of the hammerhead. Secondary structure prediction of the candidate hammerhead ribozymes can help to select those ribozymes that have hybridizing

arms that are not likely to be involved in stable intramolecular structures [120]. Such intramolecular hybridizations involving the arms are more likely to occur with their increasing length. Other computational methods for prediction of suitable ribozyme target sites consider the secondary structure of the substrate-hammerhead complex. Hypothetical cis structures of target RNA and candidate ribozymes are folded into their lowest-energy structures using a standard RNA-folding algorithm, and these are then assessed for their ability to form recognizable hammerhead structures [121]. Using this method, Denman et al. [121] showed that cis-acting RNA structures that do not fold readily into a hammerhead secondary structure had trans-acting counterparts that displayed reduced cleavage activity in vitro.

The lack of any recognized theory for evaluating which structural features are most suited for targeting by ribozymes limits the value of the theoretical approach. This was demonstrated in a recent study where the computer program MFold was used to select ribozymes targeted at or near putative single-stranded regions of the human acetylcholinesterase (AChE) mRNA [122]. These ribozymes, although exhibiting reasonable cleavage efficiency on their respective short substrates, cleaved the full-length AChE transcript with only moderate activity or not at all. In this study, ribozymes were also selected by an experimental approach involving probing of the AChE transcript with RNaseH and randomized decadeoxyoligonucleotides. The principle behind this approach is that sites accessible for hybridization will be cleaved by RNaseH (which cleaves the RNA strand of a DNA-RNA hybrid) and can thus be identified by determining the size of the radioactively labelled cleavage product. It is then assumed that regions accessible to hybridization with oligonucleotides will also be accessible to ribozymes. Five ribozymes targeted to the most accessible sites were all shown to be significantly more active at cleaving the transcript in vitro than the ribozymes selected based on the MFold program [122].

Relatively good correlation between in vitro RNaseH-mediated cleavage by oligodeoxynucleotides (ODNs) and in vitro cleavage activity of corresponding ribozymes has also been reported by others [66]. Ribozymes targeting the most accessible sites of rat c-myb RNA, as determined by a RNaseH assay, were synthesized and tested. In general, the sites that were accessible to oligonucleotide binding in the RNaseH assay were also susceptible to cleavage by ribozymes, although there were notable exceptions. Since the activities of these ribozymes were only tested in vitro, it is difficult to evaluate the relevance of these results for the in vivo situation. Some researchers have observed poor correlation between in vitro and in vivo activity of ribozymes [71, 72, 123]. The lack of strict correlation between in vitro and in vivo cleavage is consistent with a presumed modulation of ribozyme activity by cellular factors in vivo. However, good evidence exists from several other studies that the RNA structure intracellularly is in most cases comparable to that in vitro [68, 124–126]. Correlation between RNaseH-mediated cleavage in vitro and efficacy in vivo has been demonstrated for antisense oligodeoxynucleotides [125]. Accessible sites for hybridization on the human multidrug resistance-1 (MDR-1) transcript were identified by probing with a library of chemically synthesized, semi-random ODNs. Based on this analysis, 'secondary' ODNs (20 nt) were designed to hybridize to eight of the accessible sites identified. These ODNs exhibited a range of activities which seemed to correlate with their GC content; moderate to strong inhibition was obtained with ODNs having a minimum of 11 G or C residues. For randomly selected 'walking' ODNs (overlapping ODNs covering a continuous stretch of the first $\sim$200 nt of the transcript), high GC content alone was insufficient to produce a potent antisense effect if inaccessible regions were targeted. A strong correlation was obtained between in vitro and cell culture data for the walking ODNs; a similar but somewhat lower correlation was obtained with the 'secondary' ODNs. These data suggest that an in vitro RNase assay can serve as an accurate predictor of ODN efficacy in cell culture, and this should also apply to ribozymes.

Another strategy for selection of optimal target sites is by functional screening of a ribozyme library where the ribozyme-binding sequences have been randomized. In theory this is the best way to select the most efficient ribozyme for any target. This approach has a practical difficulty, however, in that each species of ribozyme molecule is present in very small amounts, which makes detection of cleavage products difficult and time-consuming. Lieber et al. [68] reported a method for functional screening of a ribozyme library in which the detection of cleavable sites following in vitro cleavage of a total cellular RNA preparation involved oligo-dT selection, reverse transcription, tailing, PCR-amplification using specific primers, cloning and sequencing. The best ribozymes were identified and their genes amplified using polymerase chain reaction (PCR). In a test of the selected ribozymes in a cellular assay, an inhibitory effect of more than 99% was observed for a ribozyme species which had a stretch complementary to the target of only 15 nt. All cleavage sites experimentally detected were located within regions predicted to be single stranded by a computer program. This lends support to the hypothesis that ribozymes should be targeted to predicted single-stranded regions.

Cellular delivery of ribozymes

The are two principal methods for delivery of ribozymes into cells: exogenous delivery of presynthesized ribozymes or endogenous expression of the ribozymes from an appropriate vector construct. Of these two delivery modes, the former probably has the most promise for eventual clinical use of ribozymes as drugs. Presynthesized ribozymes can be modified as desired to optimize both stability and activity (v.s.). Any adverse effects of its administration are not irreversible, and treatment can be discontinued at any time. In the laboratory, however, endogenous delivery is a very valuable tool which offers important advantages compared with exogenous delivery, that is the possibility of inducible or continuous expression of high levels of ribozyme. Several methods exist for both exogenous and endogenous delivery. The former includes, among others, liposome- and receptor-mediated strategies, microinjection and even passive diffusion. Ribozymes can be expressed endogenously from both plasmid-based and viral constructs, as unique entities or in fusion with other RNAs in order to increase their intracellular stability or target them to a specific intracellular compartment. A brief review of these different methods will be presented here.

Delivery of exogenous ribozymes

The size of chemically synthesized ribozymes is commonly in the region of 30 to 40 nucleotides. Simple diffusion, or pinocytosis, is very inefficient for oligonucleotides of this size. The use of hydrophobic groups such as allyl for 2′-O modification may increase the lipophilicity of the ribozyme sufficiently for adequate internalization by simple diffusion, at least in certain tissues [63]. With other cell types (Cos-1, HeLa), however, our experience is that extensive allylation of the hammerhead ribozyme does not result in significant cellular uptake by simple diffusion. Specific uptake of ribozyme by receptor-mediated endocytosis has been utilized by coupling the ribozyme to some other molecule for which there is an efficient uptake mechanism. A fibrillin-1 (FBN-1)-specific ribozyme in a complex with transferrin and polylysine was delivered into cultured dermal fibroblasts [127]. The complex was taken up by the cells and efficiently reduced both cellular FBN-1 mRNA and deposition of fibrillin in the extracellular matrix. Microinjection is another option for the exogenous delivery of premade ribozymes. One great advantage of microinjection is the ability to direct the ribozyme to a specific intracellular compartment and thus analyse the effect of localization. This method has recently been used to deliver ribozymes to both the nucleus and the cytoplasm for a comparison of the efficacy of differently constructed ribozymes in the two compartments [76]. The most commonly used and generally applicable method for exogenous delivery of ribozymes into cells is by complexation with cationic liposomes [128, 129]. An important consideration when using liposome-mediated delivery is to ensure that any observed effect is not due to toxicity of either the liposomes or the liposome-ribozyme complex. Freedland et al. [130] observed that treatment of cultured cells with ribozyme-cytofectin complexes resulted in reduction of both the level of the target protein [interleukin (IL)-6] and the number of cells. The reduction in IL-6 levels and cell numbers reflected a dose-dependent nonspecific toxic effect of the ribozyme alone (after internalization by the liposome) or of the liposome-RNA complexes, as similar results were seen following treatment with control RNA/lipid complexes but not with either ribozyme or liposome alone. Thus proper controls are imperative to ensure the specificity of any observed effect. Cationic liposomes have been employed successfully to deliver both in vitro transcribed [65, 67a, 117, 130, 132] and chemically synthesized ribozymes [66, 118, 132–136] into cultured cells of very different origin, including osteosarcomas, fibroblasts, vascular smooth muscle cells (SMCs), cervical cancer cells (HeLa) and hepatocytes. In vitro transcribed ribozymes have also been complexed with liposomes prior to intraperitoneal injection into mice [137]. Complexation of ribozymes with cationic liposomes results in rapid internalization and high stabilization of the ribozymes. In vitro transcribed, and thus unmodified, ribozymes have been detected in RNA isolates 22–48 h after transfection with lipofectin or lipofectamine reagents [66, 133, 137]. This is comparable to the lifetime in suspensions of nuclei reported for certain chemically modified ribozymes [61].

An important issue when using exogenous delivery of ribozymes is the subcellular localization of the internalized ribozymes. Cationic liposomes are thought to facilitate the cellular uptake of DNA and RNA by two alternative mechanisms: the cationic liposomes encapsulate the DNA or RNA and subsequently fuse with the cell membrane, or merely bind them on their surfaces prior to cellular uptake. Fusion with the cell membrane would be the preferred mode of action, as it would lead to the ribozyme being transported directly to the cytosol without going through the endosomes. Otherwise, the ribozyme might be trapped in the intracellular vesicles, unable to cleave its target, and ultimately be degraded in the lysosomes. DOTAP (dioleoyl trimethylammonium propane)-mediated transfection of a fluorescently labelled ribozyme resulted in a punctuated pattern of fluorescence in cultured cells which was interpreted to be due to extensive sequestration of the ribozyme in intracellular membrane vesicles, possibly endosomes [135]. No in vivo ribozyme cleavage activity

was observed, which most likely was due to lack of cocompartmentalization of the ribozyme with its target RNA. This suggests that lack of cocompartmentalization may explain the lack of in vivo activity of ribozymes which show good activity in vitro on full-length substrate. However, the effect of RNA structure differences and inhibitory RNA-binding proteins cannot be discounted. Testing the ribozyme in a cell-free coupled transcription-translation system [138a] will help clarify whether the lack of activity is due to lack of cocompartmentalization or some other cellular factors. Other potentially useful test systems are cytosolic extracts from *Xenopus laevis* [138b] or suspensions of cell nuclei [61]. The efficiency may differ with various cationic liposome reagents, and thus in case of negative results it may be a good idea to use a different reagent. Recently, a new strategy was described for efficient cytoplasmic delivery of premade ribozymes, in which either anionic or cationic liposomes were fused to haemagglutinating virus of Japan (HVJ) virus [138c]. HVJ-cationic liposomes were three to five times more efficient than HVJ-anionic complexes in promoting cellular uptake. The complexes promoted accumulation of ribozymes in the cytoplasm and accelerated transport to the nucleus. Target protein (HTLV-1 Tax) levels were reduced by about 95% by the active tax ribozyme, while an antisense effect of about 20% was observed with inactive ribozyme and antisense oligonucleotides.

For optimum efficacy the ribozyme should probably penetrate to both cytoplasm and nucleus. It has been suggested that the subcellular localization of ribozymes may significantly influence the optimum ribozyme configuration for efficacy in cells [76]. Upon microinjection into the cytoplasm, short-chain ribozymes, including those with chemical modifications, were shown to be superior to long-chain ribozymes. Efficacy in the nucleus, however, required much longer antisense arms; ribozymes with short arms were almost ineffective. Sioud et al. [66] observed a preferential localization to the nucleus of a long (94 nt) in vitro transcribed ribozyme. Transfection of shorter in vitro transcribed or chemically synthesized ribozymes has, however, resulted in predominantly cytoplasmic localization of internalized ribozymes, with no significant penetration to the nucleus [103, 133, 135]. The lack of penetration to the cell nucleus may be due to entrapment of the lipid-ribozyme complex in membrane vesicles, as mentioned above. The consensus of the studies reported so far seems to be that cationic liposome-aided exogenous delivery of relatively short chemically modified ribozymes results in accumulation in the cytoplasm and that no significant amount of ribozyme reaches the nucleus. Increasing the size of the ribozymes synthesized by in vitro transcription may aid in their transport to the nucleus.

Endogenous expression of ribozymes

The vectors. Ribozymes can be expressed endogenously from expression vectors containing the ribozyme gene, an appropriate promoter for high-level expression, transcription termination sequences, and possibly other sequences to increase the stability of the transcript or facilitate selection of transformed or transduced clones in cases of stable expression. The vectors used for ribozyme expression are either plasmid-based or viral vectors. The former, generally designed for eukaryotic/mammalian protein expression, are the most diverse and commonly used. Delivery of these vectors to cells is most readily accomplished using transfection with cationic liposomes as previously described for in vitro synthesized ribozymes. Transfection of plasmid-borne ribozyme genes with cationic liposomes is the most convenient approach for the study of in vivo efficacy of a ribozyme. When stable expression of ribozyme is required, however, this method is not optimal, as transformation frequency is relatively low. Viral-based strategies for delivery of the ribozyme-expressing construct offer a promising alternative in these cases. Virus-based vectors are continuously reviewed in the context of gene therapy. The same considerations apply for the use of such vectors for delivery of ribozymes. The most widely used viral vectors in gene therapy are the retroviral (RV) vectors, which generally are derived from Moloney murine leukaemia virus (MMLV) [139–141], adenovirus (AV) vectors [142, 143] and adeno-associated virus (AAV) vectors [144, 145]. The advantages of retroviral vectors include a wide range of infectivity and stable chromosomal integration. For use as ribozyme expression vectors, the current retroviral vectors offer several convenient cloning sites for expressing the ribozyme by either polymerase II or polymerase III, as a unique transcript or as part of the viral long terminal repeat (LTR)-transcript (reviewed in ref. 146). This has resulted in the wide application of such vectors for ribozyme expression [123, 147–160]. AV and AAV vectors offer an important advantage over RV vectors in their ability to transduce nondividing cells. AAV vectors have further advantages over AV vectors in their nonpathogenicity and long-term expression due to chromosomal integration. Despite this, there are at present no reported cases of ribozyme delivery by AAV vectors, although delivery of antisense RNA genes by such vectors has been reported [161]. Adenovirus vectors have, however, been utilized to deliver ribozyme genes to both cells in culture [162–164] and to mice [165].

Expression systems. The efficacy of endogenously expressed ribozymes correlates with their expression level [158, 166–168]. Therefore, an essential feature of any ribozyme expression cassette is that it should drive the expression of a high titre of ribozyme. Expression from

common plasmid-based vectors is usually driven from the promoters of cytomegalovirus (CMV) and simian virus 40 (SV40), which are recognized by polymerase II. For high-level expression by polymerase II, other virus-derived promoters such as the LTRs of MMLV [153a, 157] and Rous sarcoma virus (RSV) [169, 170], as well as promoters of highly expressed mammalian genes such as β-actin [171] or metallothionein II [172] have also been employed for expression from either viral or plasmid vectors. Vectors for delivery of ribozymes into plant cells have included promoters of plant-specific viruses [173, 174], while heat-shock protein (hsp) promoters have been utilized for timed induction of ribozyme expression in transgenes [175, 176]. Efficient expression of most genes transcribed by polymerase II requires a polyadenylation signal, which allows the addition of a poly(A) tail. Short ribozyme genes are often cloned within the transcribed regions of other genes and transcribed as part of a multifunctional transcript [81, 117, 150, 155]. Increasing the size of the ribozyme-containing transcript with non-ribozyme vector-derived sequences at the ends presumably increases the stability of the ribozyme. However, as previously mentioned, such extraneous sequences may be inhibitory [69a]. The potential influence of such cis-inhibitory sequences may be reduced by incorporating specific ribozyme target sequences upstream and/or downstream of the ribozyme coding sequence [155, 177–181]. The insertion of such target sequences allows release by cis cleavage of a shortened ribozyme RNA lacking nonhybridizing upstream and downstream sequences. Using this strategy, ribozyme activity in vitro by an in cis cleavage product was attained when the longer vector transcript showed no activity [180]. This method has also been used to synthesize homo- and heteromultimeric self-cleaving ribozymes. An in cis-cleaving concatemer was used to target five different sites in HIV-1 RNA [179]. All the individual ribozyme units functioned independently of the others, and each specifically cleaved the HIV-1 RNA at their respective targets. This construct was able to suppress viral proliferation by up to 96% in a transient culture assay. A self-cleaving ribozyme construct targeting a site within the core region of hepatitis B virus was cloned in tandem (five copies) into in vitro expression vectors [181]. The transcribed multiunit ribozyme molecule efficiently self-cleaved to release monomeric ribozymes lacking any extraneous sequences. Furthermore, the monomers were substantially more active against their targets in vitro than monomeric non-self-cleavable ribozymes. The design of multimeric self-cleavable ribozyme constructs offers great potential advantages in both increasing the intracellular concentration of ribozyme and abolishing any cis-inhibitory effects from nonribozyme sequences. Multimeric non-self-cleavable ribozyme constructs have also been utilized.

Multitarget ribozymes designed to cleave at up to nine highly conserved regions of HIV-1 Env RNA were found to be more efficient per RNA copy than the respective monomeric ribozymes in vitro [171]. Furthermore, in contrast to monomeric ribozymes, a 400-nt nonaribozyme targeted to cleave at nine different sites within the HIV-1 env RNA substrate, retained cleavage activity when it was part of a large 3.3-kb transcript. Cotransfection of HIV-1 DNA and several multimeric ribozyme expression vectors into permissive cell lines resulted in a specific inhibition of HIV-1 replication, indicating that the multitarget ribozymes were also functional intracellularly. A variation of the multitarget HIV-1-specific ribozyme has been described in which single-target or multiple-target ribozyme genes are transcribed in fusion with decoy sequences for sequestration of transactivating proteins or binding specific secondary structure elements (like the stem-loop II of the Rev response element) on the target RNA recognized by such proteins [149, 159, 160, 182–184]. The decoys thus function by competing with either virus RNA or transactivating protein to inhibit their association. Very strong (100–1000-fold) inhibition of HIV-1 replication has been reported upon stable transduction of permissible cell lines with retroviral vectors encoding such multiple function ribozyme transcripts. The results suggest that both the decoy and ribozyme functions contribute to the inhibitory effect on viral replication, and further that ribozyme function may be facilitated by the presence of the decoy [183].

There are several strategies to achieve high-level expression of short ribozyme transcripts. One method is by utilizing the small nuclear RNAs (snRNAs) as transcription vehicles. The snRNAs are small, highly structured, lack poly(A) tails and are transcribed by either polymerase II (PolII; U1, U2 snRNA) or polymerase III (U6 snRNA) [185–186]. Their abundance and stability in the nucleus make the snRNAs well suited as vehicles of ribozyme expression. The levels of expression and subcellular localization of human U1 or U6 snRNA-chimeric ribozyme RNAs have been determined and compared with those obtained from promoters normally linked to mRNA production [187]. The snRNA-derived transcripts were localized to the nucleus and expressed at higher levels than standard PolII-transcripts. Both the U1 [107, 187–189] and U6 [187, 190, 191] snRNAs have been utilized as ribozyme expression vehicles. Cloning of a rat atrial natriuretic factor (ANF)-targeted ribozyme gene cassette between the initiation and termination sequences of the U1 snRNA gene resulted in a construct which when transfected into COS-1 cells markedly reduced ANF mRNA levels [107]. Cloning of ribozyme cassettes into stem-loop structures of U1 snRNA has likewise resulted in a dramatic reduction of expression of target RNAs which correlated with the accumulation of large amounts of U1-chimeric ribozyme in the nucleus

[188, 189]. U6 snRNA-embedded ribozymes against HIV-1 and hepatitis B virus (HBV) targets, however, had little or no effect in vivo [187, 190, 191]. An alternative to the snRNA expression systems is polymerase III-transcribed tRNA-based expression in which the ribozyme is expressed as a tRNA fusion transcript [151, 154, 174, 177, 187, 191–195]. These expression units can be delivered to cells by both plas mid-based and viral vectors. The tRNAs are small, stable molecules of known structure which are expressed at high levels, all of which are desirable properties for a ribozyme expression vehicle. Since the structure is known, the ribozyme gene cassettes can be inserted at carefully selected sites to minimize any inhibitory interactions with the extraneous tRNA-derived sequences. So far, hammerhead ribozyme cassettes have been cloned as fusions at the 3′ terminus [151, 154, 194] or embedded between the conserved A and B internal promoter boxes [177, 192, 193]. Embedding ribozymes in the tRNA does not seem to have any negative effect on their in vivo efficacy. In fact, it has been reported that a tRNA-ribozyme fusion transcript was more efficient in vivo than the nonembedded ribozyme, despite the latter cleaving its substrate more efficiently in vitro [193]. This may be due to the increased stability of the embedded ribozyme in vivo. The preferred sites within the tRNA gene for cloning the ribozyme gene have been the anticodon and intron loops. Cloning the ribozyme into the intron may have an advantage in separating the ribozyme RNA from the tRNA after maturation of the primary transcript. The tRNAs are derived from the nucleus of the organism of intended ribozyme expression, which has included human cells [151, 154], *X. laevis* oocytes [192] and tobacco plant cells [174, 193]. The matured (processed and spliced) tRNA-ribozyme chimeric transcripts are transported to the cytoplasm, where they accumulate at high levels [194, 195]. A method to increase the intracellular accumulation of a tRNA-Met_i-ribozyme transcript has been described in which the 3′ end of the transcript was modified to hybridize to its 5′ end [154]. Although tRNA-based transcripts normally accumulate in the cytoplasm, nuclear retention can be accomplished by mutating sequences which are required for the proper processing and splicing necessary for transport out of the nucleus [196, 197]. This strategy has been employed for nuclear expression of a tRNA-Met_i-ribozyme transcript [187]. The nuclear-located ribozymes were not effective, however, unlike corresponding polymerase II-expressed ribozymes located in the cytoplasm. The same lack of efficiency was observed when ribozymes were expressed from U6 snRNA-derived expression cassettes, which also resulted in nuclear localization of the chimeric ribozyme transcripts. Since higher expression levels were in fact obtained with these expression cassettes, their poor effect was attributed to lack of penetration to the cytoplasm. The poor effect of the ribozymes in this case may be due to the helix-destabilizing nuclear environment, which seems to require longer complementary stretches for efficient hybridization between ribozyme and target RNA [76]. Thus, nuclear expression of ribozymes may possibly require longer flanking arms for optimum efficacy than in the case of cytoplasmic delivery. The efficacy of ribozymes expressed with the above cassettes will of course depend on the principal compartmentalization of the target RNA. If the target spends most of its lifetime in the nucleus, expression of the ribozyme by snRNA- or modified tRNA-based cassettes would presumably give the best results. Pre-mRNA is such a candidate target. Targeting of Rev pre-mRNA of HIV-1 with a U1 snRNA-chimeric ribozyme resulted in a considerable reduction of target levels in *X. laevis* oocytes after cotransfection of ribozyme and target [188]. In cases where specific cytoplasmic expression is desirable, this can be accomplished by employing the T7-recombinant vaccinia virus system [198, 199], in which the ribozyme is transcribed from a plasmid under control of the T7 promoter by cotransfecting with a recombinant vaccinia virus carrying a gene for T7 RNA polymerase. Since the virus replicates in the cytoplasm, the ribozyme gene is transcribed directly in the cytoplasm. This system was utilized to deliver and express ribozymes targeted against α-lactalbumin mRNA [71] and HIV-1 RNA [72]. An estimated 300- to 1000-fold excess of ribozyme over target was required to achieve reduction of target mRNA levels by more than 80% with the best ribozyme species. A much lower ribozyme-to-target ratio of approximately 10:1 was reported to be required for near-complete inactivation in a case of nuclear transcription from a T7 promoter after cotransfection with a T7 RNA polymerase gene [68]. This apparent difference in efficacy may be due to the fact that the ribozyme-containing transcripts were transcribed in different cellular compartments, although they both ultimately end up in the cytoplasm. Another possible cause for the observed difference in efficacy lies in the ribozyme expression cassette. In the case of nuclear transcription, the ribozyme was embedded in a small adenoviral RNA (VAI). The stable secondary structure and protein association of this RNA may significantly increase stability of the ribozyme compared with the presumably naked RNA transcribed in the vaccinia virus system. This strategy of utilizing small virus-associated RNAs as ribozyme expression vehicles has also been employed by others [138b, 200, 202]. The adenoviral VAI RNA was used as a cytoplasmatic carrier for expressing ribozymes against HIV-1 in *X. laevis* oocytes [138b]. Ribozyme genes have also been cloned between the internal A and B promoter boxes of the fowl adenovirus type 1 (CELO) virus-associated RNA and expressed as a CELO VA RNA chimera by RNA polymerase III [200, 202]. The presence of the adenovirus RNA sequences contained in the transcript

did not seem to significantly affect ribozyme activity in vitro, while efficient inhibition of target protein activity in vivo was observed.

Maximum efficiency of ribozymes requires that they colocalize with their targets. Besides expressing ribozymes in specific compartments or with optimized expression vehicles, several target-specific strategies for the proper localization of the ribozyme have been employed. The efficacy of a Rev pre-mRNA ribozyme transcribed as a U1 snRNA chimera was increased by mutating the U1 snRNA vector in the region of pairing with the 5′ splice site so that it matched a splice junction of the Rev precursor and thus aided in the binding of the target [188]. Similar strategies have been employed to promote cocompartmentalization of ribozyme transcript and target and to study the effects of such cocompartmentalization. An HIV-1-specific ribozyme was coexpressed as a fusion with tRNA-Lys^3, since a specific primer of this tRNA is bound by viral proteins and copackaged with viral genomes [194]. This chimeric ribozyme, which was efficiently recognized and bound by HIV-1 reverse transcriptase in vitro, was effective in reducing viral infectivity in transient assays. Sullenger et al. [148] also utilized the viral packaging signal for cocompartmentalization of ribozyme and target, clearly demonstrating its importance in vivo. The ribozyme and its target, lacZ, were both expressed from retroviral vectors carrying a packaging signal. Thus the ribozyme transcript was expected to colocalize more closely with the lacZ-containing genomic viral RNA than with the transcribed lacZ mRNA lacking packaging signal sequences. The ribozyme was in fact found to reduce the titre of infectious virus containing the target by 90%, while no effect on translation of target mRNA was observed. This dramatic effect of cocompartmentalization of target and ribozyme suggests that coupling specific signals to the ribozymes to help them localize their appropriate targets may greatly increase their in vivo utility.

Applications of ribozymes in cell culture

The majority of the in vivo applications of ribozymes reported so far have involved the use of cultured cell lines almost exclusively of mammalian origin. Human cell lines of diverse origin and murine cell lines (the fibroblast cell line NIH3T3 in particular) dominate, but other mammalian cell lines including rat [158], porcine [132, 134] and monkey [166, 172, 203] cells have also been used. There are a few examples of ribozyme experiments in *E. coli* [108, 204–207], in yeast [208–211] and in plant cells [193, 212, 213].

HIV-1 related applications

The first HIV-1-related intracellular application involved the cleavage of HIV-1 integrase RNAs expressed from plasmids in *E. coli* [204]. Several other similar applications have followed [73, 214a]. Most applications have involved the generation of ribozyme-expressing cell lines which subsequently were subjected to infection by various HIV-1 strains. Established T-lymphocyte lines have predominantly been used for such experiments, although primary human lymphocytes and monocytes have also been transfected with ribozymes. Freshly isolated peripheral blood lymphocytes (PBLs) were resistant towards challenge with viral clones and clinical isolates following retrovirus-mediated transduction with a hammerhead ribozyme gene, while nontransduced control cells were fully permissive for HIV-1 infection [152]. The inhibition was specific, as no effect was observed on an HIV-2 clone lacking the target sequence. The continued expression of the ribozyme had no effect on the viability and proliferation of the lymphocytes. Resistance of ribozyme-transduced PBLs to subsequent viral infection has also been demonstrated by others [153, 214b]. Peripheral blood monocytes isolated from HIV-1-infected individuals displayed up to a 1000-fold inhibition of HIV-1 replication following retroviral transduction of ribozyme, demonstrating that anti-HIV-1 ribozymes may inhibit HIV-1 replication after introduction into already infected individuals [159]. Hammerhead ribozymes have been successfully targeted to many different regions in HIV-1 RNA. Conventional single-target ribozymes have been targeted to sites within the ψ-packaging sequence [124], the long terminal repeat [214a, 215], as well as the transactivating (Tat) [81, 150, 191, 214b] and reverse transcriptase (Rev) [157, 188, 191] regions. Sites within the group antigen (Gag), protease (Pro), Rev, Tat and envelope (Env) regions were targeted by Ramezani et al. [123], and the best inhibition of virus proliferation was found to be conferred by Pro- and Env-specific ribozymes. Targeting of a ribozyme to a site within the nef open reading frame resulted in the abrogation of virus production in ribozyme-expressing cells, and the apparent destruction of proviral Pol and Gag sequences within the targeted region, indicating that the ribozyme was acting on the infecting HIV-1 RNA before its integration as proviral DNA [216]. Several strategies have been employed to increase the efficacy of HIV-targeted ribozymes, including the use of multitarget [171, 179] or multifunctional ribozymes [159, 160, 183, 184]. In the latter cases, the ribozyme parts of the multifunctional transcripts have been targeted to the 5′-LTR, Tat, Rev and Rev response element (RRE) regions. The RRE has also been the

prime target for the decoy function of these multifunctional ribozymes. Homann et al. [74] obtained efficient inhibition of replication after directing a catalytic antisense RNA to the 5′-leader/Gag region. Unlike what has been found for other targets in transgenic animals (v.i.), this study indicated a higher efficacy of the catalytic antisense RNA compared with that of noncatalytic antisense RNA. This difference was attributed to dissociation of the catalytic antisense RNA facilitated by proteins with strand-dissociation (unfolding) activity. This activity may, however, be specific for certain RNAs. It is suggestive that increased efficacy of catalytic antisense RNAs has only been described for an HIV-1 RNA target, since the viral p7 NC protein, which is naturally associated with the target RNA, has been described to have the proposed unfolding activity in vitro [100–102] necessary for turnover of ribozymes with long antisense flanking arms.

Other applications related to infections

Hammerhead ribozymes have recently received some attention as potential tools to inhibit other viral infections for which there are no general vaccines or effective therapies available. Chronic hepatitis caused by the hepatitis C virus (HCV) is one such disease. Expression from an adenovirus vector of in vitro selected (from a ribozyme library) hammerhead ribozymes directed against a conserved region of the plus and minus strand of the HCV genome resulted in significant reduction of the respective HCV RNAs both in cultured cells and in primary hepatocytes obtained from infected patients [163]. In two other reported cases in which hammerhead ribozymes were targeted against the conserved 5′-noncoding region (NCR) and/or core region of the HCV RNA, up to 70 to 80% inhibition of viral RNA-dependent translation was observed in cell-free translation inhibition assays [119, 217], whereas a somewhat smaller inhibition of 50 to 60% was observed after cotransfection of target (5′-NCR in a luciferase reporter construct) and ribozyme into a hepatocyte cell line [119]. Hammerhead ribozymes have also been employed to reduce the steady-state levels of human HBV pregenomes after cotransfection with an HBV expression construct, although the antisense effect contributed substantially to this reduction in intact cells [190]. Cleavage-attributed reduction by ribozyme was obtained in Mg^{2+}-supplemented cell lysates. The different activities of ribozymes in intact cells and cell lysates were not due to lack of colocalization or viral components, but most likely cellular factors, as expressing the ribozyme in cis and reducing the viral RNA sequences in the transcript resulted in essentially the same behaviour. Other reported viral applications of hammerhead ribozymes include the inhibition of viral multiplication by targeting the polymerase gene of mouse hepatitis virus [218, 219], suppression of MMLV replication in infected NIH3T3 cells after targeting of the packaging region [124] and inhibition (70–80%) of influenza A virus superinfection in stably ribozyme-expressing COS cells [166]. Reduction of the viability of the human malaria parasite *Plasmodium falciparum* in cultures after targeting of unique regions in the carbamoylphosphate synthetase II gene is a nonviral example [220]. Adenovirus-delivered ribozymes have been targeted to the nuclear antigen 1 of Epstein-Barr virus (EBV), which is essential for the proliferation of the virus [164]. Ribozyme delivery to EBV-transformed B-lymphoblastoid cells suppressed target mRNA and protein, and significantly reduced the number of EBV genomes.

Cancer-related applications

The cancer-related applications of hammerhead ribozymes are numerous and involve targeting of diverse genes. One prominent target has been the mutated or activated oncogene H-*ras*. The wild-type gene has a mutational hot spot in codon 12 which results in the generation of mutant ras protein associated with increased tumorigenicity. The mutation in codon 12 of H-*ras* results in the generation of a new cleavable triplet, GUC, which facilitates the specific destruction of mutant H-ras mRNA by a hammerhead ribozyme in the presence of normal ras mRNA. Expression of ribozymes which specifically target the H-ras mRNA in transformed cells results in reduction of mutant H-ras expression with a concomitant partial or complete reversion of the phenotypic characteristics of these cells [158, 162, 221–225]. Expression of an anti-H-ras ribozyme in either human bladder carcinoma EJ cells [162, 222, 223] or transformed NIH3T3 cells [224] resulted in reduced growth rates of transformed cells in culture, reversion of the neoplastic phenotype in the murine cells, and suppression of tumorigenicity of both cell lines after implantation into nude mice. The reduced tumorigenicity of the ribozyme-expressing EJ cells was stable over several months and correlated with a nearly twofold increase in survival rates for the mice [222]. In both cell lines, expressing inactivated (mutated) ribozymes from mammalian expression vectors resulted in less dramatic effects on the inhibition of H-ras expression, cell growth and tumorigenicity, indicating that a significant part of the effect of the active hammerhead ribozyme was due to its cleavage activity. When expressed from an adenovirus vector for higher-efficiency transduction, an inactivated ribozyme resulted in a similar pattern of rapid tumour progression after xenograft implantation as in nontransduced control cells or cells expressing the vector alone, whereas the active ribozyme resulted in a complete abrogation of tumorigenicity [162]. The expression of the active ribozyme resulted in cell death and not merely growth suppression,

as viable cells in culture could not be identified 5 days post-infection. The more dramatic effects of the same anti-H-ras ribozyme when expressed from an adenovirus vector compared with expression from a mammalian expression vector are most likely due to the higher transduction efficiency obtained with the former vectors. Efficient reduction of mRNA (up to 100%) and protein (70%) levels, as well as reduced tumorigenicity in nude mice, have also been accomplished by retroviral delivery and tRNA-promoter-dependent expression of anti-H-ras ribozyme in transformed NIH3T3 cells and rat colon epithelial cells [158]. Regression of tumours in BALB/c mice caused by H-*ras*-transformed NIH3T3 cells was obtained by directly injecting ribozyme DNA into the tumours [226]. A recent paper on the use of anti-ras ribozymes to reverse the malignant phenotype of the EJ cell line confirmed the previously observed reduced tumour take and prolonged survival of inoculated nude mice [225], but the effects were not as pronounced as previously described. This was probably due to loss of ribozyme expression, which was demonstrated in 60% of the inoculated mice. Transfection of FEM human melanoma cells containing an H-ras mutation with an anti-ras ribozyme indicated that both proliferation and differentiation of the cells were affected [227]. Ribozymes against the nuclear protooncogenes c-*fos* and c-*myc* also affected cell proliferation, but to a lesser degree than the anti-ras ribozyme. Targeting of the c-*fos* gene by ribozymes has resulted in increased sensitivity to the chemotherapeutic agent cisplatin after expression in resistant cells [228–230]. The reduction in fos expression was accompanied by reductions in the mRNA levels of various enzymes involved in DNA synthesis and repair, as well as an increased expression of c-myc. These results suggest the utility of ribozymes as a general tool in the analysis of gene expression. Overexpression of fos has also been implicated in multidrug resistance (MDR). Transfecting cells exhibiting the MDR phenotype with an anti-fos ribozyme resulted in altered morphology and restored drug sensitivity of transformed cells, with a concomitantly decreased expression of the c-*fos*, *mdr-1*, c-*jun* and *p53* genes [231]. The important role of c-fos in establishing drug resistance was highlighted by the observation that targeting the *mdr-1* gene resulted in a slower reversal of the MDR phenotype than that induced by the anti-fos ribozyme [231]. The MDR gene *mdr*-1, which encodes a membrane transport protein, P-glycoprotein, that serves as a drug-efflux pump to reduce intracellular drug accumulation, has been targeted in various drug-resistant cell lines by endogenously expressed ribozymes [167, 232, 233]. The reductions in both mdr-1 mRNA levels and P-glycoprotein expression in these cases correlated with reversal of the MDR phenotype and restored sensitivity to chemotherapeutic drugs. Various other cancer-related genes have also been targeted by hammerhead ribozyme genes. Cai et al. [156] demonstrated that destruction of mutant p53 pre-mRNA in a human lung cancer cell line by a ribozyme targeted to cleave at codon 187 near the intron5-exon6 boundary reduced mutant p53 mRNA and protein levels, and significantly suppressed growth of transduced cells in culture. Inhibition of the growth of leukaemic cells in culture was accomplished by specific targeting of the *aml1/mtg8* fusion gene with ribozymes directed to the fusion site [234], while expression of a hammerhead ribozyme against bcl-2 mRNA induced apoptosis and restored the ability to respond to secondary apoptotic agents in low- and high-bcl-2 expressing prostate cancer cell lines, respectively [235]. Ribozymes have been utilized as tools to investigate the functions of various cancer-related genes. The involvement of v-*myc* in the induction of apoptosis was demonstrated by the observation that a myelomonocytic cell line expressing a v-*myc*-specific ribozyme was not subject to hormone-induced apoptosis, while the parental cell line overexpressing v-*myc* underwent apoptosis under growth-inhibitory conditions [236]. Similarly, capl/mts-1 [237], osteopontin [238], matrix metalloproteinase 9 [239] and integrin $\alpha 6$ [240] have been implicated in the metastatic process by reduction of their tendency to metastasis in ribozyme-expressing cell lines, whereas ribozyme targeting of the growth factor pleiotrophin in high-expressing melanoma cells indicated a direct role of the gene product in tumour growth [241, 242a].

Hammerhead ribozymes also reduce the expression of the vascular endothelial growth factor (VEGF), which is upregulated in glioblastoma angiogenesis [242b].

In vivo applications of ribozymes

The examples of in vivo application of hammerhead ribozymes are limited. There are several reports on the suppression of tumour growth in nude mice after implantation of cancer cells or transplantation of tumours (see section on cancer application). In all these cases, however, the ribozyme is delivered to cultured cells in vitro, and expression of ribozyme is limited to the implanted or transplanted cells. Regression of tumours in mice has also been accomplished by directly injecting ribozyme DNA (anti-H-ras) into tumours induced by H-*ras*-transformed NIH3T3 cells [226]. Injection of chemically modified ribozymes into whole animals has been successfully employed to suppress the expression of various targets [63, 64, 137]. Lyngstadaas et al. [63] achieved a 'knockout' of amelogenin, a major translation product in developing teeth of newborn mice, after a single injection of the chemically modified ribozyme.

Synthesis of amelogenins was inhibited by at least 90% up to 48 h after injection of 50 μg of ribozyme, and did not recover completely until 96 h post-injection. This reduction in amelogenin, which plays a crucial role in enamel mineralization, resulted in abnormal development with severe hypomineralization of the enamel in ribozyme-treated mice, as demonstrated by scanning electron microscopy. The effect of the injected ribozyme was specific, as mutated ribozyme and antisense oligonucleotide controls had only limited and similar inhibitory effects (synthesis of amelogenin was back to control levels after 24 h), whereas a scrambled oligonucleotide had no effect at all. The ribozyme in the above case was extensively O-allylated except in five residues. An O-methylated chemically synthesized ribozyme was similarly used to decrease the levels of stromelysin in rabbit synovium following injection into the knee joint [64]. Stromelysin is a matrix metalloproteinase which is involved in development of arthritis, and injection of antistromelysin ribozymes was thus perceived to be of possible therapeutic value for the treatment of this disease. The ribozyme was administred 24 h prior to stimulation of stromelysin synthesis by human interleukin-1α (IL-1α) and was taken up by cells in the synovial lining following intraarticular administration. Due to the increased stability introduced by the chemical modifications, 80–90% of the administered ribozyme was intact 24 h post-injection (at the time of stimulation). At a dose of 100 μg per knee joint, a 60% reduction of stromelysin mRNA levels compared with controls was observed for the active ribozyme, while there was no significant inhibition by an inactive ribozyme species, suggesting a cleavage-mediated mechanism of inhibition for the active ribozyme. The results from both of the above studies suggest that chemically synthesized ribozymes can be effective in vivo and that their efficacy is not merely due to antisense inhibition. Intraperitoneal injection in mice of in vitro transcribed anti-TNFα ribozymes in complex with cationic liposomes has been employed to reduce TNFα levels following stimulation by lipopolysaccaride (LPS) [137]. Up to 50% reduction of TNFα levels in peritoneal lavage supernatant was achieved following a single injection of 10 nmol (15 μg) of ribozyme. The reduction in TNFα levels was accompanied by a reduction in the levels of interferon γ (IFNγ) (but not of IL-10 levels), possibly due to involvement of TNFα in the secretion of IFNγ. To test for in vivo stability of the complexed but nonmodified ribozymes, total RNA was isolated from ribozyme-treated cells and assayed for the presence of ribozyme cleavage activity using a short synthetic RNA substrate. Significant cleavage activity was recovered from the cells after 2 days.

The continuous presence of a ribozyme in vivo can be achieved by its endogenous expression as opposed to exogenous administration. The latter method can only result in time-limited effects. Delivery of a ribozyme gene by viral vectors, however, may result in continuous expression of the ribozyme for months or perhaps even years. So far, there has been only one report of viral-mediated delivery of ribozymes to whole animals. Lieber et al. [165] used an adenoviral vector to deliver an anti-human growth hormone (hGH) ribozyme expression cassette to transgenic mice that produced hGH from the gastrointestinal tract and liver. The ribozyme was expressed in vivo and reduced hepatic hGH mRNA levels by up to 96% over a period of several weeks, demonstrating the feasibility of delivering functional ribozyme genes to somatic cells by viral vectors. Although the reports of somatic ribozyme gene delivery to animals are limited, several ribozyme-transgenic animal models have been reported. An animal model for maturity-onset diabetes of the young (MODY) associated with a heterozygous mutation of pancreatic β-cell glucokinase (GK) has been reported in which transgenic mice expressing a catalytic antisense RNA were generated [243]. Two independent lineages with islet GK activities of only about 30% of normal exhibited impaired glucose-stimulated secretion of insulin. The generation of transgenic mice expressing an anti-β 2-microglobulin ribozyme was reported by Larsson et al. [244]. Expression of ribozyme in various organs was accompanied by up to 90% reduction (in lung) of target mRNA in individual mice, and 20–80% reduction in different lines. Finally, L'Huillier et al. [168] have reported the development of mice which were double-transgenic, expressing both the ribozyme and a transgene of the target, bovine α-lactalbumin. Transgenic mice carrying a ribozyme gene (targeted to the 3′ UTR of bovine α-lac) under the control of the mouse mammary tumour virus (MMTV)-LTR were generated and cross-bred with high expressors of a bovine α-lac transgene. Expression of high levels of the ribozyme resulted in the reduction of the levels of target mRNA by 20 to 50% compared with nonribozyme transgenic littermate controls in three independent lines. The suppressive effect of ribozyme expression was generally lower than in previous reports [243, 244], despite the higher level of ribozyme expression (detectable by Northern blots as opposed to reverse transcriptase (RT)-PCR). This was most likely due to the higher abundance of the target in this case. Ribozyme-mediated reduction in bovine α-lac mRNA was specific, as no effect on the level of endogenous α-lac mRNA, which had a 67% (16 out of 24 nucleotides) complementarity to the flanking arms of the ribozyme, was observed. Reduction of protein paralleled that observed for mRNA and was positively correlated with the level of ribozyme expression.

Using *Drosophila melanogaster*, Zhao et al. [175] generated flies which were transgenic for a ribozyme against the critical developmental regulatory gene *fushi tarazu* (*ftz*). The *ftz* gene and other regulatory genes are good candidates for ribozyme-mediated knockouts due to

their generally low expression levels and dosage-dependent effects. The ribozyme gene was under the control of a heat-inducible (hsp) promoter to allow for timed induction of expression in order to distinguish between the eff ects on the two different putative roles of the target gene. It was demonstrated that expression of the ribozyme in the blastoderm disrupted the seven-stripe pattern of the embryo, thus confirming the *ftz* gene as a pair-rule gene. The involvement of the *ftz* gene in neurogenesis was similarly confirmed by induction of the ribozyme in the early phase of formation of the central nervous system. Ribozymes were used in another case to attenuate the *white* gene expression and suppress eye pigmentation in transgenic *Drosophila* expressing catalytic antisense RNA [176, 245]. Expression of ribozyme in vivo was demonstrated by the ability of total RNA extracts to catalyse specific in vitro cleavage of target RNA. Different strains of *Drosophila* were generated carrying one, two or three copies of the ribozyme gene. The steady-state expression level of the ribozyme in these different strains increased with the gene copy number, and was also positively correlated with the suppressive effect on eye pigmentation [176].

Catalytic antisense RNAs carrying one or more hammerhead motifs, as well as regular antisense RNAs, have been used to generate transgenic plants [173, 174, 246, 247]. Transgenic tomato plants carrying a catalytic antisense RNA incorporating three hammerhead structures were found to be only moderately effective in reducing the accumulation of citrus exocortis viroid (CEVd) following inoculation [246]. The pure antisense gene exhibited the same efficacy in suppressing viroid replication. Other researchers have also reported similar effects of catalytic and regular antisense RNAs in transgenic tobacco plants, despite the presence of multiple hammerhead domains in the catalytic RNAs [174, 247]. This suggests that in these cases the catalytic antisense RNAs act primarily through an antisense mechanism and that cleavage does not contribute significantly to their efficacy. This may explain the relatively poor efficiency observed with such constructs by some researchers. Only about 15% of transgenic lines expressing either type of antisense RNA against the tobacco mosaic virus genome exhibited some level of protection against virus infection [247]. In transgenic tobacco plants expressing a traditional hammerhead ribozyme, however, the expression of a reporter transgene was significantly reduced in all progeny [173]. The level of ribozyme expression was furthermore similar to that of the target, which makes it likely that suppression of expression was due to cleavage. High resistance against potato spindle tuber viroid (PSTVd) was achieved in transgenic potatoes expressing a hammerhead ribozyme against the PSTVd minus-strand RNA [248]. Nearly 70% of independent lines expressing the active ribozyme were completely free of PSTVd following challenge with the viroid; the remaining lines showed weaker levels of resistance. This resistance could be attributed almost entirely to ribozyme-mediated cleavage and not to antisense effects, as only one of 60 independent lines expressing a mutated ribozyme exhibited any resistance towards viroid challenge. Thus the consensus so far seems to be that in plants traditional ribozymes with short hybridizing flanking arms are under optimal conditions superior to catalytic antisense RNA in vivo. Due to the long hybridizing arms of the latter, turnover is not expected to occur, and thus each ribozyme molecule can only inactivate one target RNA molecule. Such catalytic antisense RNAs would presumably need to be expressed at a large molar excess over target, whereas effective suppression of target expression has been suggested to take place at significantly lower levels of ribozyme. Thus it is likely that ribozymes with short hybridizing arms will in general be more efficient in vivo than regular or catalytic antisense RNAs, provided, of course, that an optimal target site is selected. If poorly accessible sites are targeted, the short arms of hammerhead ribozymes will not allow for efficient hybridization with the target, and constructs acting essentially through an antisense mechanism will in all likelihood be more efficient in vivo. This again stresses the critical importance of target site selection in the design and application of ribozymes for in vivo efficacy.

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