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SURVEY AND SUMMARY

Recent developments in the hammerhead ribozyme field

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ABSTRACT

Developments in the hammerhead ribozyme field during the last two years are reviewed here. New results on the specificity of this ribozyme, the mechanism of its action and on the question of metal ion involvement in the cleavage reaction are discussed. To demonstrate the potential of ribozyme technology examples of the application of this ribozyme for the inhibition of gene expression in cell culture, in animals, as well as in plant models are presented. Particular emphasis is given to critical steps in the approach, including RNA site selection, delivery, vector development and cassette construction.

INTRODUCTION

The field of catalytic RNA, or ribozymes, continues to develop at an impressive pace. The large amount of material which has accumulated in the field over the years makes it impossible to cover the field exhaustively in this review. Instead we will focus only on developments over the last two years and will concentrate on the hammerhead, the subject of several recent reviews (1–4). This ribozyme belongs to the class of so-called 'small ribozymes' which have also been reviewed recently (5). Another member of this group, the hairpin ribozyme, has been discussed by Walter and Burke (6). A comprehensive account of different aspects of ribozymes in general is the subject of a book (7).

Two aspects which attract attention in studies of the hammerhead and other ribozymes are structure–function relationships and applications for the inhibition of gene expression. Both will be discussed here.

STRUCTURE–FUNCTION RELATIONSHIP

The hammerhead ribozyme can cleave any RNA as long as it contains any of the cleavable triplets 5′-NUH-3′, where U is conserved, N is any nucleotide and H can be C, U or A, but not G (8; Fig. 1). One assumption is that the inability to cleave 3′ of G is due to an unfavourable interaction with C3 in the core (9). NMR studies have confirmed that this base pair exists and that it stabilizes the ground state ribozyme–substrate complex (10). However, an additional contribution to the lack of cleavage comes from destabilizing the transition state. Various pyrimidine nucleoside analogues have been used as H (11). Although all such modifications had no effect on binding of the substrates to the ribozyme, cleavage occurred more slowly than when H was cytidine. This indicates that functional groups at the cleavage site are important for transition state formation. Contrary to published results, the conserved U in the NUH triplet can be changed to A, by inverting the A15.1-U16.1 base pair to U15.1-A16.1. Cleavage rates 3′ of GAC were at least 2–10% compared with those of the conventional GUC triplet and depended somewhat on the sequence context (12). No loss of activity is encountered if the central U in NUH is changed to C and the complementary nucleotide A15.1 changed to I (13). The rationale for this base change was that the I-C pair retains the single H bond seen between the A-U pair in the X-ray structure (14). These observations suggest an extension of the NUH rule to that of NHH, where H can be any nucleotide except G. Besides the implications for the basis of cleavage specificity, these new results extend the repertoir of cleavage sites for hammerhead ribozyme application to the inhibition of gene expression.

The use of pyrimidine nucleoside analogues at position 7, always considered the only variant position in the core, also gave rise to a surprise observation. Pyridin-4-one at this position increased the cleavage rate up to 12-fold, another result unexpected from the X-ray structure (15). The explanation for this result is that this analogue might improve stacking as a consequence of its sugar pucker, thereby affecting the positioning of domain 2 and the catalytic site.

It had been presumed that sequences up- or downstream from the NUH triplet had little or no effect on cleavage. Surprisingly, when two U-A base pairs are present 3′ of the cleavage site, the cleavage rate is enhanced 10-fold (16). A 3- to 4-fold rate enhancement has also been observed when the sequence was changed from GGUC to UCGA (17). It was suggested that the sequence in stem I is responsible for the angle between helices I and III and thus contributes to formation of the active conformer.

A purine-specific hammerhead-like ribozyme, cleaving 3′ of G and A, has been identified by *in vitro* selection (18). The selected ribozyme has two fewer core nucleotides but nevertheless shows great similarity to the hammerhead ribozyme in a two-dimensional representation based on limited nuclease digestion (Fig. 2). However, there are differences between the two. The most striking difference is seen in loop II, which cannot be replaced by a polyethylene linker in the selected ribozyme. A detailed

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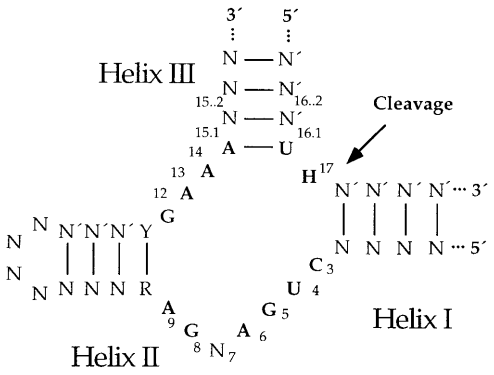

Figure 1. Two-dimensional structure of the hammerhead ribozyme. N, any nucleotide; N′, nucleotide complementary to N; H, any nucleotide but G; Y, pyrimidine nucleotide; R, purine nucleotide complementary to Y.

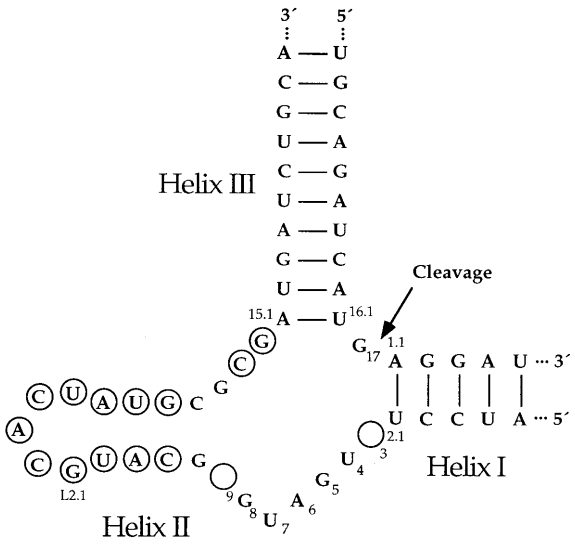

Figure 2. Two-dimensional structure of an AUG-cleaving hammerhead-like ribozyme. Circles, positions different from those in the hammerhead ribozyme. Empty circles, no nucleotide in the AUG-cleaving ribozyme.

mutational analysis identified G2.1 in loop II as a conserved nucleotide (N.K.Vaish and F.Eckstein, unpublished).

The three-dimensional structure of the hammerhead ribozyme has been elucidated by X-ray crystallography (19,20). A recent FRET study followed the ion-induced folding of the ribozyme (21). A two-step folding as a function of Mg^{2+} could be discerned where the first corresponds to the coaxial stacking of helices II and III, the second to the formation of the catalytic domain. The overall folding is in agreement with the X-ray structure. Time-resolved, transient electric birefringence measurements are consistent with a model in which the active centre has similar conformations before and after cleavage (17). Thus, there is good evidence for the ground state structure of the ribozyme but many details of the mechanism and the specificity of the hammerhead ribozyme reaction are still unresolved. There is general agreement that conformational changes have to occur to reach the transition state. Their characterization requires studies in which the dynamics of the system are coupled to structure and are difficult to obtain. However, attempts to follow the reaction pathway by X-ray crystallography has provided evidence for a remarkable, and long sought after, conformational change at the cleavage site. Use of a 5′-C-methylated ribose 3′ of the cleavage site permits kinetic trapping of the usually transient conformation positioning the 2′-OH group of the nucleophile, the phosphorus and 5′-O of the leaving group in an in-line fashion as required from stereochemical studies (22). Tb(III) inhibits the Mg^{2+}-dependent ribozyme reaction by binding to a site adjacent to G5 in the core, distant from the cleavage site (23). It is argued that the Mg^{2+} at this site acts as a critical switch in the conformational change leading to the transition state. G5 is crucial for ion-induced folding of the ribozyme, which is in good agreement with earlier uranyl-induced photocleavage experiments (24). Another metal ion-binding site, first suggested by X-ray crystallography, has been functionally confirmed using the phosphorothioate diastereomers at A9 and Cd^{2+}. Even though this position is 20 Å away from the cleavage site, it is also invoked as participating in a necessary conformational change (25).

The phosphorothioate interference analysis has been extended to include phosphorothioates with the Sp configuration, which are not accessible by employing the usual enzymatic transcription reaction (26). The authors chemically synthesized a mixture of the two diastereomers and compared their activity and iodine cleavage patterns with those of the all Rp isomers obtained by transcription. They confirmed earlier results with the Rp isomers (27) and additionally identified the Sp configuration at A6 and U16.1 at the cleavage site as inhibitory. They also concluded that metal ion coordination to the phosphate of A9 is likely by rescue of the inhibitory effect of the Rp isomer in the presence of Mg^{2+} by using thiophilic Cd^{2+}.

There has been an on-going debate as to whether the reaction requires one or two metal ions for cleavage and about their precise role (28,29). Previously, attempts were undertaken to identify metal ion coordination to the 5′-oxygen of the leaving nucleoside using an internucleotide-bridging 5′-phosphorothioate. The hope was that coordination with Mn^{2+} would accelerate cleavage over that obtained with Mg^{2+}, but results were not unambiguously interpretable (30,31). An elegant new approach to understand the role of Mg^{2+} in the cleavage reaction has been taken by Hermann *et al.* (32). Molecular dynamic simulations of the ribozyme crystal structure provides evidence for a μ-bridging OH^- between two Mg^{2+} close to the scissile phosphate. These simulations further show the necessary conformational change for the established in-line nucleophilic substitution leading to cleavage. Interestingly, such simulations suggest that the ammonium groups of aminoglycoside antibiotics mimic metal ions in their interaction with the ribozyme (33).

The two metal ion mechanism has gained further support from cleavage experiments using a constant concentration of Mg^{2+} while varying the concentration of La^{3+} (34). These two metal ions have greatly different pK_a values and are suggested to play two different roles. One likely coordinates to the 2′-oxygen, thereby increasing its nucleophilicity, and the other acts as a Lewis acid by binding to the 5′-oxygen on the leaving group. This is probably the strongest evidence for the two metal ion mechanism. However, this result cannot be extrapolated to the related hairpin ribozyme. Several studies demonstrated that when the ligand exchange inert $[Co(NH_3)_6]^{3+}$ complex is present as the only metal ion, in combination with a phosphorothioate substrate, the ribozyme is fully active. This shows that no direct metal ion

coordination to phosphate oxygens or metal-bound hydroxide as a general base is required for activity (35–37). This observation puts this ribozyme in a special class. Interestingly, it implies that RNA functional groups alone can act as acids and bases.

APPLICATION

Much of the excitement in the ribozyme field stems from the potential application of ribozymes in the sequence-specific inhibition of gene expression. Although simple in theory, the practicality of this application poses such challenges as mRNA site selection, delivery and cellular localization of the ribozymes. Examples of *in vitro* and *in vivo* applications are described and the associated problems discussed in several reviews (3,4,38).

Of course, ribozyme action on a given target should be specific and efficient. The criterion of specificity requires a number of controls. The systems used in the various studies are too varied to recommend a strict set of controls. However, one simple control for sequence specificity is the exchange of the ribozyme's binding arms. The inhibitory effect exerted by ribozymes can have a certain contribution from an antisense effect which can easily be determined with a catalytically inactive ribozyme. To a large extent efficiency is influenced by the sequence of the mRNA selected for ribozyme binding.

Site selection

The first step for inhibition of gene expression by a ribozyme is its binding to the mRNA. This step is akin to the antisense oligodeoxynucleotide method (AS-ODN) used for the same purpose. It is, therefore, not surprising that both approaches benefit from experience in each others area. Experience with the AS-ODN method has taught us that accessibility of the mRNA to oligonucleotide binding is rather restricted because of mRNA secondary structure. The problem was, at least in the beginning, underestimated. Even though RNA folding programs such as MFOLD are available to identify oligonucleotide-accessible sites, experimental approaches seem to give more reliable results (39). Some of these methods use an oligonucleotide library hybridized to a labelled transcript and subsequent digestion with RNase H (40). Another method consists of binding of the target RNA to an array of oligonucleotides (41). Birikh *et al*. have extended the oligonucleotide/RNase H method successfully to identify triplets suitable for ribozyme binding at RNase H-sensitive sites on a transcript of the human acetylcholinesterase gene where no such site had been found before (42). All these approaches are *in vitro* methods and use a transcript for site identification. Thus it is not certain that the same sites will be available at the mRNA in the cell, where folding could be different or proteins could obscure such sites. However, the example reported by Ho *et al*. (40) is encouraging in that an oligonuleotide targeted against such a site gave the expected results even in the animal.

There is a site selection protocol with a ribozyme library, expressed from a plasmid, which can be performed in cytoplasmic extracts. The method is very demanding experimentally (43). Sites identified by this procedure were also accessible in a transgenic mouse model, where successful interference with the expression of human growth hormone in the gastrointestinal tract and liver was obtained (44).

Delivery

There are two approaches to deliver ribozymes to cells for the successful inhibition of gene expression. One is exogenous delivery where the presynthesized ribozymes are delivered directly to cells. The other is endogenous delivery, which provides the gene encoding the ribozyme, as part of a vector, to the cells where transcription generates the ribozyme.

Exogenous delivery, at first glance, looks simple, straightforward and attractive. Presynthesized ribozymes can be delivered directly to cells or administered to an animal. This mode of application is identical to that of the AS-ODN method. Given the amount of accumulated experience, it is not surprising that most of the delivery techniques used in the ribozyme field are adopted from the AS-ODN strategy. Exogenous delivery requires stabilization of oligonucleotides against nuclease degradation. This is particularly true for ribozymes, which are rapidly degraded by RNases. AS-ODNs are customarily protected by incorporation of phosphorothioates, but ribozymes demand modification of the 2′-OH group, which the RNases also require for cleavage. Stabilization should not compromise catalytic activity of the ribozyme, thus the choice of derivative and its placement are crucial. The most commonly used modifications at the 2′-position are the fluoro, amino, allyl and *O*-methyl derivatives, often in combination with terminal phosphorothioates or an inverted nucleotide (45). These modifications help to increase the half-life of ribozymes in serum and in nuclei suspensions from minutes to days (46–48). Unmodified hammerhead ribozymes, when entrapped in biodegradable poly(L-lactic acid) polymer matrices, also have enhanced stability in serum, changing it from seconds to >2 weeks (49).

Exogenous delivery

So far there are two examples of exogenous delivery of carrier-free nuclease-resistant ribozymes, by injection, in animal models. Ribozymes directed against the metalloproteinase stromelysin, a key mediator in arthritic diseases, were administered to a rabbit knee joint intra-articularly. The ribozymes were taken up by cells in the synovial lining and reduced interleukin 1α-induced stromelysin mRNA was observed (50).

A nuclease-resistant hammerhead ribozyme was also used to inhibit amilogenin synthesis in newborn mice. Amilogenins are proteins specific for mammalian enamel biomineralization. When carrier-free ribozymes were injected close to developing mandibular molar teeth, amilogenin synthesis was specifically inhibited for several hours, resulting in improper mineralization of tooth enamel (51). Whether these two examples warrant the statement that delivery to animals in general doesn't require a carrier is not yet clear.

There is one example of successful exogenous delivery of a ribozyme to cell culture without a carrier. A phosphorothioate-modified hammerhead ribozyme, directed against the carbamoyl phosphate synthetase II gene of *Plasmodium falciparum*, reduced malarial viability up to 55% in infected erythrocytes (52). However, this is probably a special case as infected erythrocytes are particularly prone to take up extracellular material. In addition, these cells must have low RNase activity as the type of phosphate backbone modification usually does not provide sufficient protection against nuclease degradation.

In general, poor uptake is a challenge for exogenous delivery of ribozymes to cells in culture. Significant improvement can be

achieved with the help of carriers such as cationic liposomes. There are three examples of delivery of chemically stabilized ribozymes. In the first a ribozyme, directed against a point mutation at position 763 of the N-*ras* gene, was complexed to lipofectAMINE and delivered to HeLa cells to cleave an mRNA where the lucifease gene was under the control of the N-*ras* gene. A reduction of 54% in luciferase activity was observed (53). The sequence specificity of this inhibition was demonstrated by fusing the luciferase gene to the wild-type N-*ras* gene. No inhibition could be observed with the ribozyme. This very nicely demonstrates that a single nucleotide mutation was recognized by the ribozyme. However, this was a particularly fortunate situation, as the mutation introduced a GUC triplet for cleavage whereas the wild-type had a GUG triplet and thus was not expected to be cleavable. The efficiency of mismatch discrimination by the hammerhead ribozyme is a matter of debate (54,55).

In the second example, c-*myb* mRNA-targeted, chemically modified hammerhead ribozymes were delivered to a culture of rat aortic smooth muscle cells with the aid of cationic lipids. This resulted in a reduction in the level of c-*myb* which caused inhibition of serum-stimulated cell proliferation (56,57). Thus, this ribozyme shows promise for the treatment of restenosis after coronary angioplasty.

The third example describes a ribozyme, made nuclease-resistant by the exchange of all pyrimidine nucleotides for the corresponding 2′-amino derivatives, for the inhibition of protein kinase Cα expression (58). Cationic liposome delivery of this ribozyme inhibited glioma cell growth *in vitro* and a single injection into rat glioma tumours inhibited their growth.

The association of unmodified ribozymes with liposomes protects them against nuclease digestion. This mode of application has been employed in mice for i.p. injection of liposome-entrapped ribozymes directed against tumour necrosis factor-α (TNF). This treament reduced lipopolysaccharide-induced TNF protein by 70%. Active ribozymes could be recovered from peritoneal cells even 2 days post-injection (59).

In addition to liposomes, other carriers can also successfully deliver ribozymes. For example, a ribozyme was administered in the form of a ribozyme–transferrin–polylysine complex to cultured dermal fibroblasts resulting in cleavage of fibrillin-1 mRNA (60).

One of the problems associated with liposome-mediated delivery is trapping of the ribozyme in the endosome, requiring release into the intracellular space (61,62). Moreover, care must be taken in interpreting these results because non-sequence-specific ribozyme effects have been reported with liposome–ribozyme complexes (63).

Endogenous delivery

Vectors. Endogenous delivery depends on efficient transfer of the ribozyme-encoding gene into the cell. To this end the ribozyme is cloned into an expression vector which is then transfected into cells. Viral vectors are preferred and retroviral vectors are most commonly utilized for *in vivo* delivery of ribozymes because they have high transduction efficiency and they stably integrate the gene into the host cell genome. They have been widely used to deliver ribozymes for the inhibition of expression of genes linked to cancer and viral diseases such as AIDS (64–66).

Adenovirus, a class of DNA viruses, can also successfully deliver genes to the nucleus. However, these viruses provide only transient expression of the gene and generate a strong antibody response, thus the infected cells are rapidly removed and repeated application is not possible (67). However, adenoviral-aided ribozyme delivery was accomplished in transgenic mice expressing the human growth hormone in the gastrointestinal tract and in the liver. The purified vectors, carrying a ribozyme gene targeted against the human growth hormone, were infused by vein injection. A 96% reduction in hGH mRNA level for several weeks was observed (44). Epstein–Barr virus (EBV)-immortalized B lymphocytes were also treated with adenovirus-encoded ribozymes directed against the EBV nuclear antigen 1, essential for EBV genome persistence (68). Delivery of the ribozyme reduced the number of EBV genomes and nearly abolished cell proliferation in low serum. This procedure also prevented EBV infection of an EBV-negative B cell line, an encouraging result for the combat of lymphoproliferative disorders.

Adeno-associated virus is another alternative for ribozyme delivery (69). It is appealing for several reasons. It is non-pathogenic, does not require dividing cells to integrate the gene into the host genome and has a specific integration site on chromosome 19.

Recently a viral vector system that incorporates favourable aspects of both retroviral and adenoviral vectors has been devised for efficient gene delivery and long-term gene expression. However, it has not yet been explored for ribozyme delivery (70).

Cassette construction. The efficiency of ribozyme function also depends on the promoter as well as the stability and localization of the transcript. Thus, cassette design is an important part for this mode of delivery.

Several expression cassettes containing Pol II and Pol III promoter sequences from different human genes, with an AAV and a MoMuLV vector, were compared for localization and efficiency of ribozyme transcription. One ribozyme was targeted against the TAR region in an SIV growth hormone reporter plasmid, the other was an HIV-1 anti-*tat* ribozyme (69). The $tRNA^{Met}$ and U1 and U6 snRNA Pol III transcripts were nuclear and expressed at higher levels than standard Pol II-promoted transcripts, which were cytoplasmic. The $tRNA^{Met}$ transcripts had an altered 3′-terminus to prevent processing but it interfered with nuclear export. Only capped, polyadenylated cytoplasmic ribozymes, transcribed from the Pol II cassette, were functional in transiently and stably transfected cell lines. That these ribozymes were expressed at lower levels, emphasizes the importance of co-localization of ribozyme and target.

A human $tRNA^{Met}$ and a U6 snRNA-Pol III promoter have been used to express ribozymes against HIV-1 in cultured cells (71). Transcripts were protected at their 3′-end and Rev-binding RNAs blocked HIV-1 gene expression. The U6 promoter offers certain advantages over S5 RNA and tRNA Pol III promoters by virtue of the position of the promoter sequences. In the latter, their intragenic localization makes insertion of the ribozyme gene difficult, whereas in the former, the promoter sequences are upstream of the transcription start. Additionally, U6 primary transcripts are normally retained in the nucleus, in contrast to others which are exported to the cytoplasm. Whether or not this is an advantage might depend on the particular system. However, tRNA promoters are useful, as documented by a report on the inhibiton of expression of the related proteins p300 and cAMP response element-binding protein (72). This study examined the differential roles of these proteins in retinoic acid-induced

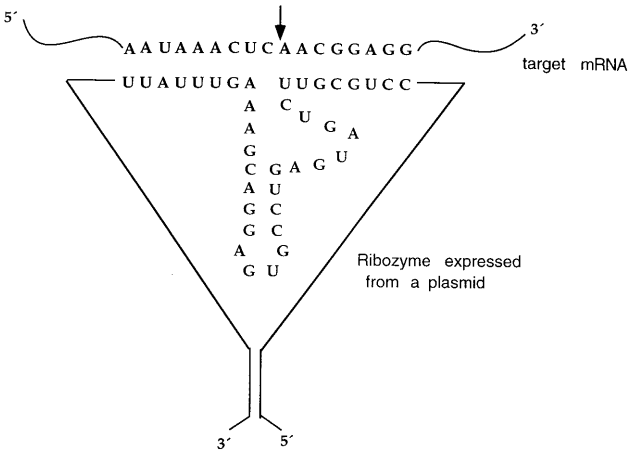

Figure 3. Schematic representation of annealing target mRNA and ribozyme for the inhibition of the no-tail gene function in zebrafish (75). Arrow, cleavage site.

processes in carcinoma F9 cells and represents an example of gene function analysis.

Animal models

The efficiency of a hammerhead ribozyme against bovine α-lactalbumin mRNA was tested in a double transgenic mouse system. The mice were generated by cross-breeding transgenic mice carrying an anti-bovine α-lactalbumin ribozyme gene with those carrying the bovine α-lactalbumin gene. Heterozygous expression of the ribozyme resulted in a reduction in the target mRNA of 50 and 78% compared with that in the non-ribozyme transgenic littermates (73). Protein levels were correlated with expression of the ribozyme. The specificity of ribozyme action was demonstrated by the lack of an effect on the production of the endogenous mouse lactalbumin.

A transgenic mouse experiment compared the inhibition of chloramphenicol acetyltransferase expression by a full-length antisense construct and one in which three ribozyme sequences had been incorporated. Inhibition of protein expression by 90 and 87%, respectively, was observed, indicating that the ribozymes did not enhance the effectiveness of the antisense RNA (74). However, this might not be the most suitable system for such a comparison as the antisense inhibition was already almost complete and any additional effect would have been difficult to observe.

Elegant gene function analysis has been carried out with ribozymes in zebrafish. The recessive dominant no-tail gene was effectively inhibited using a transient ribozyme expression system injected into fertilized eggs (Fig. 3; 75). The resulting phenotype was identical to that of a known defective mutation in the same gene. Expression of the ribozyme for successful down-regulation was required in the cytoplasm and not in the nucleus. This is an important point as the co-localization of ribozyme and target is a prerequisite for the successful action of the ribozyme and, *a priori*, it is not certain whether this is best achieved in the nucleus or the cytoplasm.

Plant models

Transgenic plants also offer a potential system for studying ribozyme effects and preparing pathogenic virus-resistant crops. A transgenic potato plant expressing a hammerhead ribozyme against the potato spindle tuber viroid RNA showed high resistance against its replication. Moreover, plant progeny stably inherited resistance against this viroid (76). Transgenic tobacco plants expressing hammerhead ribozymes also showed high resistance against cucumber mosaic virus (77). Similarly, the tobacco mosaic virus (TMV) was targeted in transgenic plants with a ribozyme containing three catalytic domains directed at three different sites (78). Homozygous progeny of some lines were highly resistant to the virus. Interestingly, resistance was not overcome by a TMV strain which was altered at the target sites, which suggests that the resistance was primarily conferred by an antisense effect.

CONCLUSIONS AND PERSPECTIVES

Numerous examples which demonstrate that ribozymes can interfere with gene expression in a sequence-specific manner, *in vitro* as well as *in vivo*, have now been reported. Thus, ribozymes have the potential to be developed as drugs or as tools for the elucidation of gene function. As therapeutics, ribozymes could be administered either by successive external application akin to chemotherapy or by endogenous transcription in a gene therapy fashion. A somewhat less ambitious use of ribozymes is in gene function analysis (79). However, for any application, additional research to better understand many of the individual steps a ribozyme has to take to reach and interact with its target in an efficient and specific manner is required. Some of these challenges have been discussed here but others, such as the importance of the lifetime of the target mRNA and cell specificity, await further review.

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