The EMBO Journal vol.11 no.4 pp.1525 - 1530, 1992

Expression of a chimeric ribozyme gene results in endonucleolytic cleavage of target mRNA and a concomitant reduction of gene expression *in vivo*

Peter Steinecke[1], Thomas Herget[1,3] and Peter H.Schreier[1,2,4]

[1]Max-Planck-Institut für Züchtungsforschung, Abteilung J.Schell, Carl-von-Linne-Weg 10, W-5000 Köln 30 and [2]Bayer AG, PF-E Institut für Biotechnologie, Geb. 6240, W-5090 Leverkusen, FRG

[3]Present address: Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

[4]Corresponding author

Communicated by J.Schell

The subclass of catalytic RNAs termed ribozymes cleave specific target RNA sequences *in vitro*. Only circumstantial evidence supports the idea that ribozymes may also act *in vivo*. In this study, ribozymes with a hammerhead motif directed against a target sequence within the mRNA of the neomycin phosphotransferase gene (*npt*) were embedded into a functional chimeric gene. Two genes, one containing the ribozyme and the other producing the target, were cotransfected into plant protoplasts. Following *in vivo* expression, a predefined cleavage product of the target mRNA was detected by ribonuclease protection. Expression of both the ribozyme gene and the target gene was driven by the CaMV 35S promoter. Concomitant with the endonucleolytic cleavage of the target mRNA, a complete reduction of NPT activity was observed. An A to G substitution within the ribozyme domain completely inactivates ribozyme-mediated hydrolysis but still shows a reduction in NPT activity, albeit less pronounced. Therefore, the reduction of NPT activity produced by the active ribozyme is best explained by both hydrolytic cleavage and an antisense effect. However, the mutant ribozyme-target complex was more stable than the wildtype ribozyme-target complex. This may result in an overestimation of the antisense effect contributing to the overall reduction of gene expression.

Key words: gene regulation/plant cells/ribozyme/transient expression

Introduction

Until the discovery of the first autocatalytic RNA, reactions in living cells were thought to be mediated exclusively by proteins. Besides *Tetrahymena* preribosomal RNA, which cuts and splices itself to remove intron sequences (Cech and Bass, 1986), another class of catalytic RNAs, called ribozymes, is capable of *trans*-acting specifically with target RNAs *in vitro* (Zaug *et al.*, 1986; Uhlenbeck, 1987; Haseloff and Gerlach, 1988). The basic structure of designed ribozymes must contain a monomolecular consensus structure, called the 'hammerhead' motif (Forster and Symons, 1987), which appears to be essential as a replication intermediate in the life cycle of some viroids, virusoids and linear satellite RNAs (Symons, 1989). These RNA intermediates are concatameric transcripts which originate by a rolling circle replication (Branch and Robertson, 1984) and undergo autocatalytic intramolecular cleavage into unit sized progeny. Self-catalysed RNA cleavage reactions share a requirement for divalent metal ions and neutral or basic pH. Cleavage occurs 3′ to the GUX triplet where X can be C, U or A (Haseloff and Gerlach, 1988; Koizumi *et al.*, 1988) and results in the production of RNA with termini possessing 5′-hydroxyl and 2′,3′-cyclic phosphate groups (Buzayan *et al.*, 1986; Prody *et al.*, 1986).

Since such 'hammerhead' ribozymes can be targeted to different sequences, ribozymes provide an attractive complement to antisense constructs for prevention or inhibition of undesired gene expression. Consequently, ribozymes may provide a route toward the production of phenotypic mutants. Previous studies of ribozyme action *in vivo* have demonstrated that high ribozyme/substrate ratios are required for ribozyme-mediated suppression (Cameron and Jennings, 1989). In these experiments and in other *in vivo* experiments, either the ribozymes were synthesized as short oligonucleotides *in vitro* (Saxena and Ackerman, 1990) or no presumed cleavage products have been detected (Cameron and Jennings, 1989; Cotten and Birnstiel, 1989; Sarver *et al.*, 1990; Sioud and Drlica, 1991). Thus no direct demonstration of specifically targeted cleavage of RNA by ribozymes *in trans* has been provided thus far *in vivo*. Alternatively the observed inhibition of gene expression has been interpreted as an antisense function rather than resulting from ribozyme cleavage (Saxena and Ackerman, 1990). Also no direct comparison with a catalytic inactive ribozyme has been performed *in vivo* to determine if cleavage capacity of the ribozyme is involved in the observed inhibition of gene expression (Cotten, 1990). We have embedded sequences coding for ribozymes into a functional gene driven by the CaMV 35S promoter and tested their fate using transient expression in plant protoplasts. Earlier studies demonstrated that a DNA polymerase II-transcribed gene could be used for the expression of a ribozyme (Sarver *et al.*, 1990). Transient expression assays in plant protoplasts have been successfully used for the study of antisense RNA dependent inhibition of gene expression (Ecker and Davis, 1986). We have chosen *Nicotiana tabacum* cv. Petit Havana SR1 (Maliga *et al.*, 1973) protoplasts because of their high and reproducible uptake of DNA and their viability during and after transfection. In our experiments, the protoplasts were cotransfected with separate plasmids producing the ribozyme transcripts and the mRNA for the bacterial neomycin phosphotransferase enzyme. The *npt* gene represents a well established, easily assayed reporter gene.

This report shows the endonucleolytic cleavage of a ribozyme encoded by a chimeric gene directed against a mRNA target *in vivo*. Concomitant with cleavage we observed enhanced inhibition of gene expression whereas a mutant non-catalytic ribozyme had a simple antisense

effect. The antisense effect was measured using a ribozyme construct with the conserved GAAAC sequence mutated to GAGAC, a mutation which eliminates ribozyme activity. We demonstrate that a chimeric ribozyme gene transcribed by polymerase II is suitable for producing ribozymes of endonucleolytic capacity *in vivo* and that this activity contributes significantly to reduced gene expression, to the extent of complete suppression.

Results

Construction of the ribozyme genes

We constructed two different ribozymes (Rz), one hydrolytically active ($Rz_{136\text{-}2}$) and the other inactive (Rz_{89}), containing the catalytic domain from the satellite RNA of subterranean clover mottle virus (SCMoV) (Davies *et al.*, 1990) flanked by 12 or 10 nucleotides complementary to the target *npt* mRNA, respectively (Figure 1A). The catalytic domains of the anti-*npt* ribozymes contain an extended helix II−loop region of six nucleotides whereas the wildtype loop has five nucleotides. The mutant, supposedly inactive, ribozyme was designed such that the combined hydrolytic-antisense action of the active ribozyme could be distinguished experimentally from a simple antisense effect.

An A → G transition was introduced which is known to inactivate RNA catalysis fully *in vitro* (Ruffner *et al.*, 1990; Lamb and Hay, 1990) and this construct (Rz_{89}) was used to monitor the antisense effect. To compare the influence of the A → G substitution on the intramolecular structure of Rz_{89} with that of the wildtype, the secondary structures of both ribozyme gene products were analysed by computer simulation with the computer program LINALL (Steger *et al.*, 1984;). It was found that the intramolecular structure remains unaffected by the mutation, so that, in both cases, helix II can be formed and the flanking sequences are accessible to interact with the target mRNA sequences. However, if the bimolecular reaction is simulated, the thermodynamic stability of helices I and III (Figure 1A) of Rz_{89} with the target RNA is enhanced and results in a bimolecular complex stabilized by 7.2 kJ/mol with respect to that formed with the wildtype $Rz_{136\text{-}2}$, thus potentially increasing the antisense action of this mutant ribozyme. This must be considered if the antisense effect of the mutant is used to estimate its influence for the overall reduction of target gene expression by the wildtype ribozyme. A third ribozyme, Rz_{70}, was constructed that contained a wildtype catalytic domain but no homology to the *npt* mRNA sequence. This ribozyme was designed against a GUC sequence within the transit peptide coding region of the potato *rbcS*1 gene (Wolter *et al.*, 1988; Fritz *et al.*, 1991) and was shown to be active *in vitro* (Steger,G., Herget,Th., Steinecke,P. and Schreier,P.H., in preparation). It provides

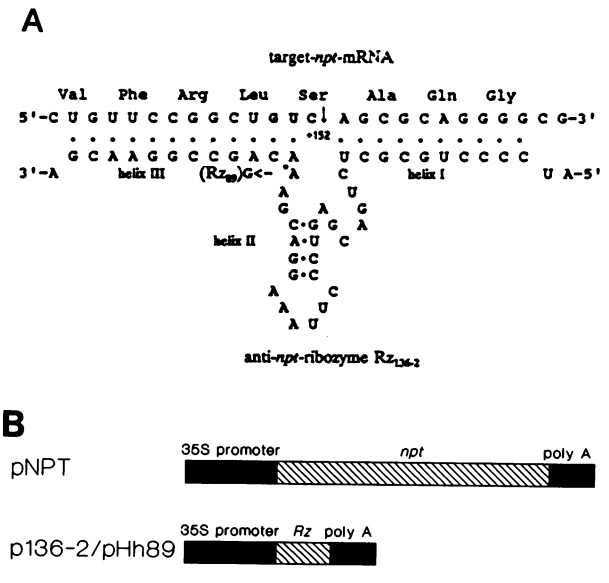

Fig. 1. Ribozyme genes and target gene used in this study. (**A**) Hammerhead structure from the (+)-strand of the subterranean clover mottle virus (Davies *et al.*, 1990) derived anti-*npt* ribozyme domain of $Rz_{136\text{-}2}$ as encoded in plasmid p136-2, associated with the target sequence of the *npt* mRNA. The nucleotide which is substituted in $Rz_{136\text{-}2}$ by mutagenesis to abolish catalytic activity is marked with an asterisk and resulted in the non-catalytic ribozyme Rz_{89} which is encoded in pHh89. The location of the cleavage site after the GUC target sequence within the *npt* mRNA is indicated by an arrow and the number +152 refers to the nucleotide position with respect to transcription initiation. (**B**) The *npt* gene and the ribozyme genes are represented in a schematic diagram. Plasmid pNPT contains the *npt* gene. Plasmids p136-2 and pHh89 contain the chimeric ribozyme genes, where Rz denotes the ribozyme in the catalytic ($Rz_{136\text{-}2}$) or non-catalytic (Rz_{89}) form with the flanking sequences. Abbreviations: poly(A), signal for poly(A) addition and termination of transcription.

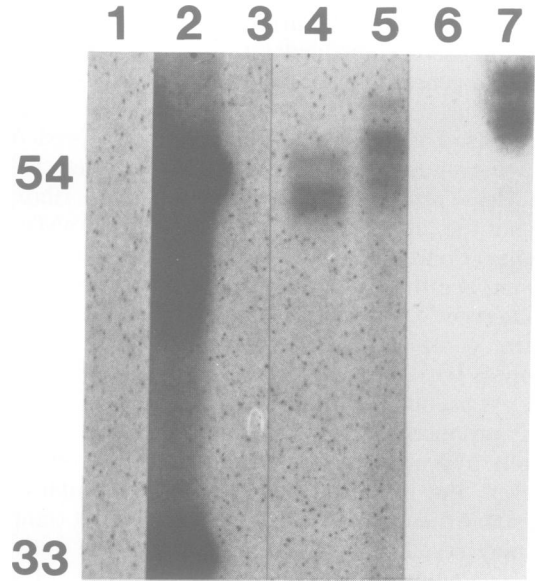

Fig. 2. Transcription of anti-*npt* ribozymes in cotransfected protoplasts. Protoplasts were isolated and transfected using 100 μg plasmid DNA per transfection with a 4-fold molar excess of the anti-*npt* ribozyme genes or control ribozyme plasmid p70 over *npt* target mRNA-expressing vector. Total RNA was isolated after 7 h and 1 μg total RNA was mapped by RNase protection assay with a 64 nucleotide [α-^{32}P]CTP-labelled antisense probe (20 000 c.p.m.) complementary over 54 nucleotides with the ribozyme $Rz_{136\text{-}2}$. For detection of Rz_{70} expression an appropriate 76 nucleotide antisense probe complementary over 58 nucleotides with the ribozyme Rz_{70} was used. Lane 1, analysis of RNA from untransfected protoplasts. Lane 2, restriction fragments of ϕ174 bacteriophage DNA digested by *TaqI* and labelled by a fill-in reaction with [α-^{32}P]dCTP and Klenow, fragments of 54 and 33 bases are indicated at the left of the figure. Lane 3, analysis of RNA from protoplasts cotransfected with pNPT and carrier plasmid. Lane 4, analysis of RNA from protoplasts cotransfected with pNPT and pHh89. Lane 5, analysis of RNA from protoplasts cotransfected with pNPT and p136-2. Lane 6, analysis of RNA from protoplasts cotransfected with pNPT and carrier plasmid. Lane 7, analysis of RNA from protoplasts cotransfected with pNPT and p70. Note that lanes 6 and 7 show the analysis of RNA assayed with an antisense probe of Rz_{70}.

a control which cannot act as an antisense molecule against *npt* mRNA or cleave the non-target RNA endonucleolytically. To ensure equivalent transcription and routing within the cell of their products, the anti-*npt* ribozymes, the control ribozyme (Rz_{70}) DNA fragment and the *npt*-coding DNA fragment were cloned into equivalent expression units employing the strong CaMV 35S promoter and its transcription termination and polyadenylation signals (Figure 1B).

Transcription of the chimeric ribozyme gene results in the cleavage of the target mRNA in vivo

To check and compare the transcriptional activity of the different chimeric ribozyme genes, we isolated RNA from protoplasts after transfection and, using an RNase protection assay, determined the relative amount of ribozyme transcripts. A 64 base antisense RNA molecule to the ribozyme gene $Rz_{136\text{-}2}$ was synthesized *in vitro* in the presence of [α-^{32}P]CTP and hybridized against total RNA extracted from protoplasts. The transcription products of the ribozyme genes Rz_{89} and $Rz_{136\text{-}2}$ form a double-stranded RNA over 54 nucleotides with the radioactively labelled probe, thus protecting it from RNase digestion. As demonstrated in Figure 2, the two ribozyme genes do not have significantly different transcriptional activities as similar amounts of RNA fragments of ~54 nucleotides are detected. Breathing, predominantly at the 3′-end of the double-stranded RNA, is probably the cause of several bands observed in Figure 2 (lanes 4 and 5) after RNase treatment. The different pattern of size distribution with the mutant anti-*npt* ribozyme (Figure 2, lane 4) is due to further destabilization of the double-stranded region at the 3′-end by the mismatch at position 37 (Freier *et al.*, 1986; Werntges *et al.*, 1986) in the hybrid between Rz_{89} and the antisense probe which is

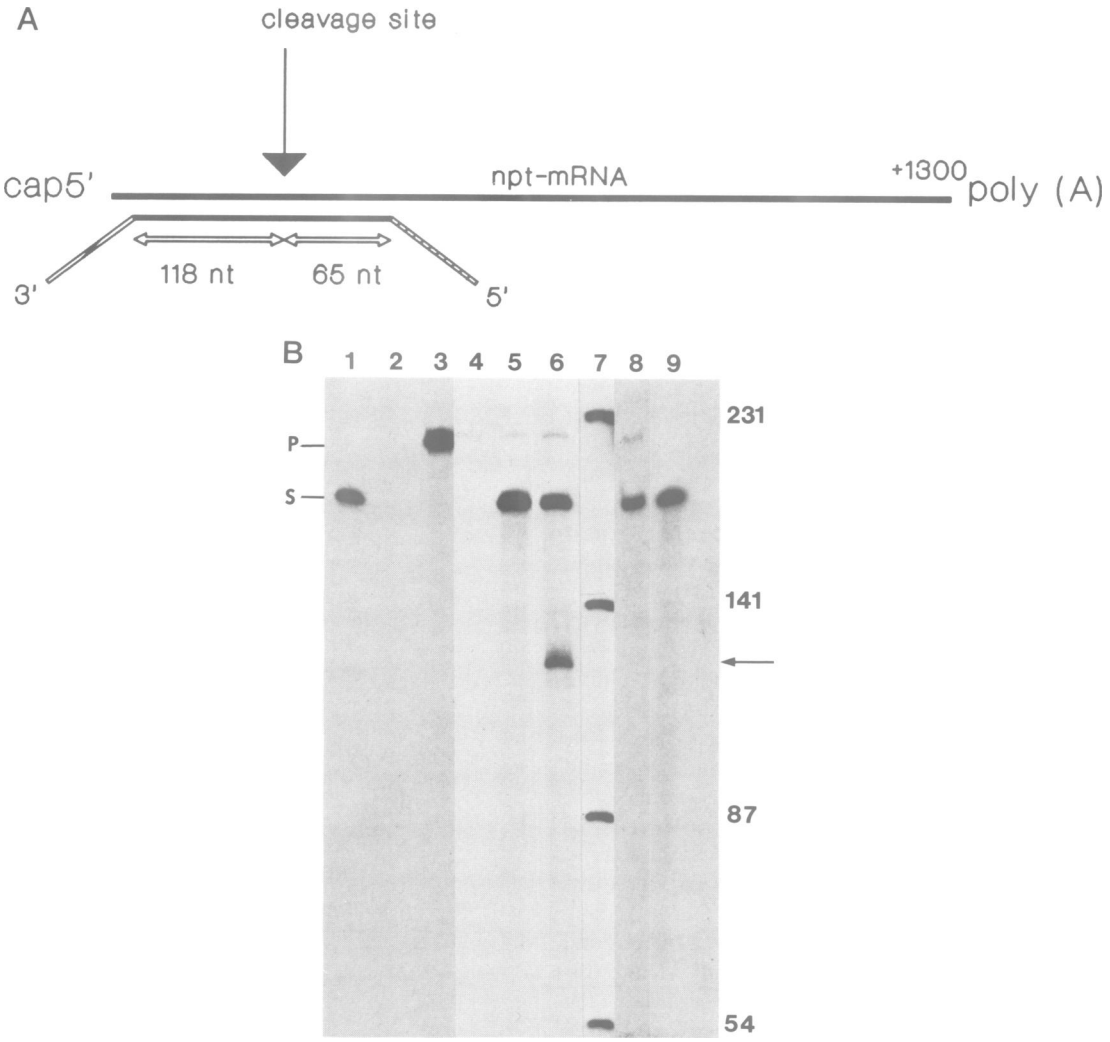

Fig. 3. Ribozyme-mediated cleavage of *npt* mRNA after cotransfection of the chimeric ribozyme genes with an *npt* gene. (**A**) The relative position of the cleavage site within the *npt* mRNA and the area expected to hybridize to the ^{32}P-labelled *npt* antisense probe with the presumed cleavage products is indicated. (**B**) RNase protection of the ribozyme treated *npt* mRNA. Protoplasts were isolated and transfected as described in Figure 2. Total RNA was isolated after 7 h and 5 μg were used for mapping by the RNase protection assay with a 217 nucleotide ^{32}P-labelled antisense probe (100 000 c.p.m.) complementary with 118 nucleotides 5′ and 65 nucleotides 3′ to the GUC cleavage site in the *npt* mRNA. Lane 1, analysis of *in vitro* transcribed *npt* RNA. Lane 2, [α-^{32}P]CTP-labelled *npt* antisense probe digested with RNase. Lane 3, undigested antisense probe. Lane 4, analysis of RNA from untransfected protoplasts. Lane 5, analysis of RNA from protoplasts cotransfected with pNPT and pHh89. Lane 6, analysis of RNA from protoplasts cotransfected with pNPT and p136-2. Lane 7, ϕ174 bacteriophage DNA as in Figure 2; here fragments of 231, 141, 87 and 54 bases are indicated on the right side of the figure. Lane 8, analysis of RNA from protoplasts cotransfected with pNPT and p70. Lane 9, analysis of RNA from protoplasts transfected with pNPT without ribozyme genes. The middle band indicated by an arrow on the right corresponds to the 5′-cleavage fragment of the *npt* mRNA. The migration of the untreated ^{32}P-labelled *npt* antisense probe (P) and the full length *npt* antisense probe (S) protected from RNase digestion are indicated on the left of the figure.

an exact complement of $Rz_{136\text{-}2}$. The transcriptional activity of the chimeric ribozyme gene Rz_{70} was determined accordingly; as expected it results in similar amounts of RNA (Figure 2, lane 7). As a next step the endonucleolytic capabilities of active ribozyme ($Rz_{136\text{-}2}$) and mutant (Rz_{89}) were compared. For this, we cotransfected protoplasts with the *npt* gene and a 4-fold molar excess of plasmids containing the ribozyme gene. Production and cleavage of *npt* mRNA was analysed after 7 h by RNase protection with an antisense probe covering the anticipated site of interaction and cleavage by the anti-*npt* ribozyme. As demonstrated in Figure 3, the expected 5′-cleavage product of 118 nucleotides is detected due to the action of ribozyme $Rz_{136\text{-}2}$ (lane 6), whereas the target RNA in control transfections (no ribozyme, lane 9 and Rz_{70}, lane 8) or coexpressed with the mutated ribozyme Rz_{89} (lane 5) remains unaffected. Even after a prolonged exposure, no comparable signal becomes visible. The 3′-cleavage product could not be detected either by RNase protection or by primer extension analysis (data not shown). Since only the 5′-cleavage product contains a cap structure, it should be much better protected against 5′ → 3′ exonuclease activity (Proudfoot, 1989; Brawerman, 1990) than the unprotected 5′-end of the 3′-cleavage product. A cytoplasmic 3′-exonuclease is not considered since there is no evidence for its occurrence in eukaryotes (Brawerman 1990).

Reduced expression of npt in cotransfected protoplasts

In order to test the influence of the different ribozymes on the overall expression of the *npt* gene, the enzymatic activity of the protein product was monitored in different experiments. Increased levels of ribozyme genes caused a complete reduction of NPT activity for $Rz_{136\text{-}2}$. As shown in Figure 4, the extent of reduction was proportional to the amount of plasmid DNA containing the ribozyme genes. When equal amounts of ribozyme and target gene-containing plasmid were co-transfected, $Rz_{136\text{-}2}$ reduces NPT activity by an average of 24% whereas the mutant, non-catalytic ribozyme Rz_{89} reduces the activity by only 9% (Figure 4A); in the case of a 10-fold molar excess of plasmid containing ribozyme genes, NPT activity is reduced by an average of 58% (active) and 30% (mutant) respectively (Figure 4B). A 100-fold molar excess of ribozyme-containing plasmid completely abolishes NPT activity with the active ribozyme whereas in the case of mutant ribozyme, 46% remains (Figure 4C). The ribozyme Rz_{70} leaves NPT activity unaffected (Figure 4C); this is not unexpected since this ribozyme cannot act as an antisense molecule due to the lack of flanking sequences complementary to the *npt* mRNA and as a consequence also does not hydrolytically cleave this target RNA. Furthermore, this control demonstrates that the observed reduction of gene expression in the case of $Rz_{136\text{-}2}$ and Rz_{89} is not the result of competition for cellular transcription factors. Thus the transient expression assays show that the reduction in *npt* gene expression can be observed with a catalytic active and non-catalytic ribozyme. Crucially, however, the reduction is at least twice as high in the first instance.

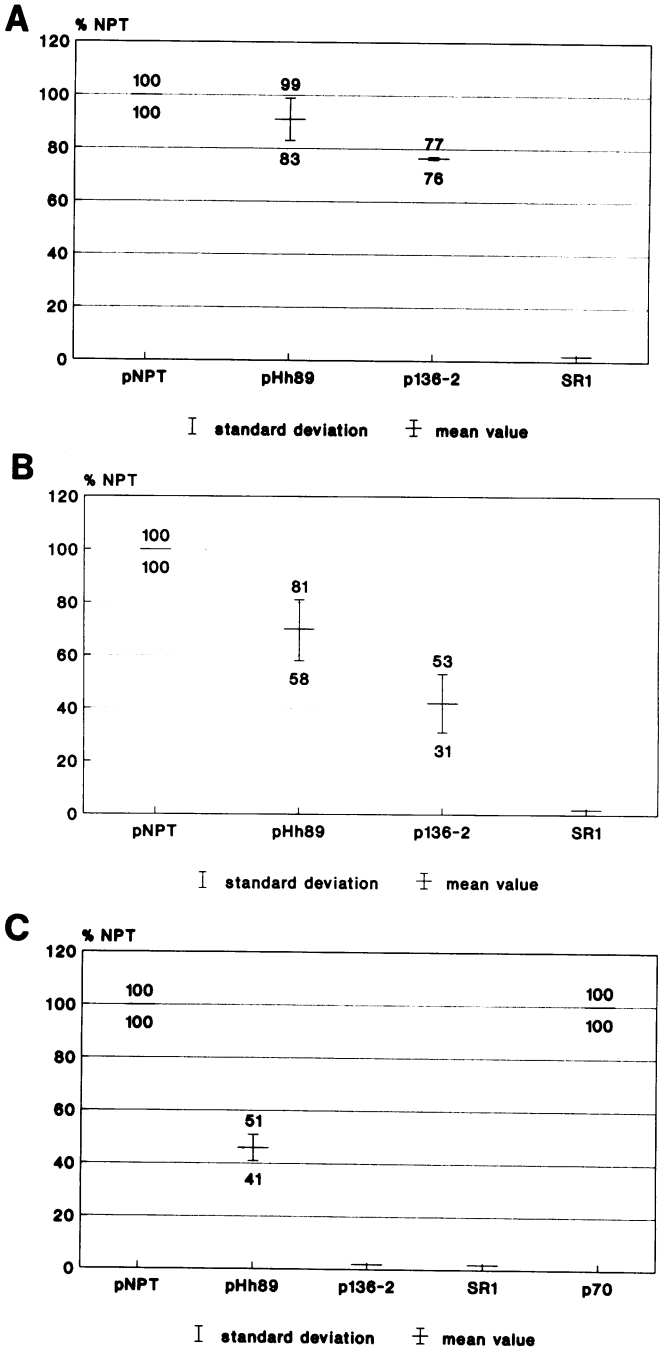

Fig. 4. Percent NPT enzyme activity of the co-transfected tobacco protoplasts. Protoplasts were isolated and transfected with 50 μg plasmid DNA per transfection with either a 10-fold molar or a 100-fold molar excess respectively, or an equal amount, of the plasmid encoding the anti-*npt* ribozyme over *npt* target mRNA-expressing vector. After incubation for 7 h, protoplasts were harvested and the NPT assay was performed. NPT activity was determined by direct measurement of the radioactivity bound to the phosphocellulose paper from the NPT test. The activity obtained by the transfection with pNPT alone was set at 100%. NPT activity in cells transfected with other plasmids was measured relative to that obtained by pNPT. The cotransfected plasmids containing the ribozyme genes are indicated, SR1 designates untransfected protoplasts. Average values with the standard deviation of the mean value of four (1:1) (**A**), (100:1) (**C**) and five (10:1) (**B**) experiments are given respectively.

Discussion

We designed experiments to prove that hammerhead ribozymes can act endonucleolytically *in trans* on a target mRNA *in vivo* and as a consequence cause reduced gene expression of the target gene. In a recent publication, Eckner *et al.* (1991) first demonstrated RNA cleaving activity of a *cis*-acting ribozyme. Our results demonstrate the ability

of an engineered *trans*-acting RNA catalyst, embedded into a chimeric gene, to function in a complex cellular milieu in the desired fashion. In previous *in vivo* studies of ribozyme action, inhibition of gene expression was also reported. However, these studies failed to demonstrate a concomitant endonucleolytic activity and thus one had to assume that an antisense effect was responsible for the phenotypic results. We therefore included a mutant ribozyme in our study to investigate if the catalytic activity of our wildtype ribozyme is significantly enhanced by antisense activity in reducing gene expression. Both ribozyme genes were expressed equally well. The inactive ribozyme does not hydrolyse its target RNA but still reduces gene expression, presumably due to an antisense effect. This is not unexpected since the formation of helices I and III (Figure 1A) is a prerequisite for mediating hydrolytic cleavage and by itself potentially gives rise to an antisense effect. If the reduction of gene expression by the mutant ribozyme is used to estimate the contribution of antisense effect by the hydrolytically active ribozyme, the different thermodynamic values of the two complexes need to be considered. In this case, it diminishes the relevance of antisense action for the wildtype ribozyme in mediating inhibition of *npt* gene expression. When the endonucleolytic activity of a ribozyme gene has been demonstrated unequivocally *in vivo*, further investigations will concentrate on minimizing the unavoidable antisense effect and enhancing efficiency of the cleavage reaction. It is potentially worthwhile to study other specific mutations to elevate cleavage. Furthermore it is not clear whether proteins are involved in the cleavage reaction; it seems reasonable that helix II or the hammerhead structure resembles a protein recognition site as suggested for other similar hairpins (Uhlenbeck, 1990; Cheong *et al.*, 1990). The transient expression assay presented in this study provides a good basis for testing this hypothesis. Furthermore it will be tested if the cleavage activity can be employed in transgenic plants to downregulate expression of physiologically relevant genes. We also expect that ribozyme genes will repress gene activity in transgenic animal cells since we do not envisage that ribozymes exclusively work in plant cells. Hence, the present study is the first proof that ribozymes can be used as tools for the specific inhibition of gene expression and supports the potential of ribozymes as antiviral therapeutic agents, and their use to reduce or abolish gene expression of an individual gene within a highly related gene family.

Materials and methods

Materials

Cellulase R-10 and Macerozyme R-10 were purchased from Serva and radioisotopes from Amersham. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer.

Construction of the ribozyme genes and the npt gene

The deoxyoligonucleotide encoding ribozyme Rz_{89}, 5′-AGCTTGGATCCCCGTCGCTCTGACGAGTCCCTTAAAGGACGAGACAGCCGGAACGAATTCAA-3′, and the complementary oligonucleotide, 5′-AGCTTTGAATTCGTTCCGGCTGTCTCGTCCTTTAAGGGACTCGTCAGAGCGCAGGGGATCCA-3′, were phosphorylated, annealed and cloned into plasmid pMEX001 (B.Reiss, personal communication) containing a cassette contributed by the CaMV 35S promoter and its homologous transcription termination signal, resulting in plasmid pHh89. The ribozyme $Rz_{136\text{-}2}$ and the plasmid p136-2 are essentially identical to Rz_{89} and pHh89 but contain an A and a T instead of a G and a C respectively at the positions underlined in the oligonucleotides mentioned above.

The oligonucleotide encoding ribozyme Rz_{70}, 5′-GGATCCAGATTTGACTGATGAGTCCCGTGAGGACGAAACCAGTGAAAATCTAGA-3′, and the complementary oligonucleotide were phosphorylated and cloned in *Bam*HI and *Xba*I double digested pSPT19 vector DNA (Boehringer) containing a CaMV 35S promoter and the transcription termination and polyadenylation signals of the nopaline synthase gene from the *Agrobacterium tumefaciens* Ti plasmid extracted from plasmid pPCV702 (Koncz *et al.*, 1989), resulting in plasmid p70. The *npt* gene was obtained as a *Bam*HI–*Sma*I fragment from pkm4 (Reiss *et al.*, 1984a) and was subcloned into pMEX001 leading to plasmid pNPT. The size marker represents restriction fragments of ϕ174 bacteriophage DNA digested by *Taq*I and labelled by a fill-in reaction with [α-^{32}P]dCTP and Klenow. Cloning and DNA manipulation procedures were essentially as described by Sambrook *et al.* (1989).

Preparation of RNA probes

Antisense probes for RNase protection analysis were prepared from appropriate plasmids by *in vitro* transcription with SP6 polymerase (Melton *et al.*, 1984) using [α-^{32}P]CTP (800 Ci/mmol), and purified by gel electrophoresis.

Transfection of plant protoplasts

Nicotiana tabacum SR1 cv. Petit Havana SR1 (Maliga *et al.*, 1973) were grown in 0.5×MS (Murashige and Skoog, 1962) medium. Protoplasts were isolated from 4–8 week-old plants by incubating leaf pieces in B5 medium (Gamborg, 1970) containing 0.4 M sucrose, 1.5% cellulase and 0.5% Macerozyme. After 16 h at 27°C in the dark, the cell suspension was filtered through 250 μm and 100 μm sieves and was then transferred to 12 ml centrifuge tubes (Nunc) (10 ml/tube). After centrifugation at 60 *g* for 5 min the banded protoplasts were washed with 10 ml B5 medium, 0.4 M sucrose, and centrifuged as above. The lower phase was removed and the protoplasts were collected in 10 ml W5 medium (154 mM NaCl, 125 mM $CaCl_2$, 5 mM glucose, 5 mM KCl, adjusted to pH 5.6). After a second centrifugation the pelleted protoplasts were resuspended in MaMg solution (0.45 M mannitol, 15 mM $MgCl_2$, 0.1% MES, pH 5.6) to a final concentration of 3×10^6 protoplasts/ml. Transfection by the polyethylene glycol method was carried out as described by Negrutiu *et al.* (1987). After heat shock for 5 min at 45°C followed by 45 s on ice, 0.35 ml protoplasts were distributed in 10 ml centrifuge tubes (Nunc). Each sample was mixed with either 100 μg plasmid DNA for RNA extraction or 50 μg plasmid DNA for protein extraction. After 10 min 0.35 ml PEG solution [0.4 M mannitol, 0.1 M $Ca(NO_3)_2$, 40% PEG 4000] was added. After 20 min the mixture was transferred into Petri dishes and diluted with 4 ml B5 medium, 0.4 M sucrose. The protoplasts were incubated at 27°C for 7–8 h in the dark. Protein was then extracted (Reiss *et al.*, 1984) or total protoplast RNA was isolated by the acid guanidinium–phenol–chloroform extraction method according to Goodall and Filipowicz (1989) and treated with DNase I to eliminate any traces of remaining plasmid.

Analysis of ribozyme gene expression and cleavage of npt mRNA by RNase protection assay

Protoplast RNA was analysed with ^{32}P-labelled, complementary RNA probes. Hybridization reactions (20 μl) containing 1–5 μg RNA and the ^{32}P-labelled probe (20–100 000 c.p.m.) were carried out in 40 mM PIPES, pH 6.4, 80% formamide, 0.4 M NaOAc and 1 mM EDTA. Samples were heated for 5 min at 95°C and then incubated overnight at 45°C for ribozyme detection or at 48°C for *npt* mRNA detection respectively. RNase treatment was performed following the detailed instructions provided by the supplier of the RNase protection assay kit (Ambion Inc., Austin, Texas). Briefly, 200 μl of a 1:50 dilution of the RNase A/T1 digestion mixture including 30 U RNase T2 (Gibco BRL) were added to each sample and incubated for 40 min at 37°C. After RNase digestion, 10 μl proteinase K (10 mg/ml)/yeast RNA and 10 μl 20% SDS were added followed by further incubation for 15 min at 37°C. Then the RNA was extracted with phenol–chloroform–isoamylalcohol (50:48:2) and precipitated with ethanol. The pellet was resuspended in 8 μl formamide plus dye and analysed on a 6% polyacrylamide–7 M urea gel. Gels were exposed to X-ray films at −70°C with an intensifying screen.

NPT enzyme activity assay

N.tabacum protoplasts were harvested and the NPT assay was performed (Reiss *et al.*, 1984b; Schreier *et al.*, 1985). NPT activity was determined by direct measurement of the radioactivity bound to the phosphocellulose paper from the NPT test.

Analysis of RNA secondary structures

A VAX 8600 and a VAX 8800 computer with the Sequence Analysis Software Package of the Genetics Computer Group of Caballero and colleagues (University of Wisconsin) were used. Minimal free energy folds

of RNA were performed using the program LINALL (Steger *et al.*, 1984) with the free energy values as defined by Freier *et al.* (1986). Graphic representations were obtained with SQUIGGLES.

Acknowledgements

We wish to thank Jeff Schell for constant support, and Dorothee Wegener, Peter Loss, Jeff Dangl and Jeff Schell for critical discussion. We gratefully acknowledge the help of Gerhard Steger for advice with the computer simulations and making his programs available. The award of a fellowship from Bayer to Th.H. is gratefully acknowledged.

References

Branch,A.D. and Robertson,H.D. (1984) *Science*, **223**, 450–455.
Brawerman,G. (1990) *Trends Biotechnol.*, **8**, 171–174.
Buzayan,J.M., Gerlach,W.L. and Brüning,G. (1986) *Nature*, **323**, 349–353.
Cameron,F. and Jennings,P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9139–9143.
Cech,T.H. and Bass,B. (1986) *Annu. Rev. Biochem.*, **55**, 599.
Cheong,C., Varani,G. and Tinoco,Jr,I. (1990) *Nature*, **346**, 680–682.
Cotten,M. (1990) *Trends Biochem. Sci.*, **8**, 171–174.
Cotten,M. and Birnstiel,M.L. (1989) *EMBO J.*, **8**, 3861–3866.
Cotten,M., Schaffner,G. and Birnstiel,M.L. (1989) *Mol. Cell. Biol.*, **9**, 4479–4487.
Davies,C., Haseloff,J. and Symons,R.H. (1990) *Virology*, **177**, 216–224.
Ecker,J.R. and Davis,R.W. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5372–5376.
Eckner,R., Ellmeier,W. and Birnstiel,M. L. (1991) *EMBO J.*, **10**, 3513–3522.
Forster,A.C. and Symons,R.H. (1987) *Cell*, **49**, 211–220.
Freier,S.M., Kierzek,R., Jaeger,J.A., Sugimoto,N., Caruthers,M.H., Neilson,T. and Turner,D.H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9373–9377.
Fritz,C.C., Herget,T., Wolter,F.P., Schell,J. and Schreier,P.H. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4458–4462.
Gamborg,O.L (1970) *Plant Physiol.*, **45**, 372.
Goodall,G.J. and Filipowicz,W. (1989) *Cell*, **58**, 473–483.
Haseloff,J. and Gerlach,W. (1988) *Nature*, **334**, 585–591.
Koizumi,M., Iwai,S. and Ohtsuka,E. (1988) *FEBS Lett.*, **239**, 285–288.
Koncz,C., Martini,N., Mayerhofer,R., Koncz-Kalman,Z., Körber,H., Redei,G.P. and Schell,J. (1989) *Proc. Natl. Acad. Sci. USA*, **68**, 8467–8471.
Lamb,J.W. and Hay,R.T. (1990) *J. Gen. Virol.*, **71**, 2257–2264.
Maliga,P., Sz.-Breznovits,A. and Marton,L. (1973) *Nature*, **244**, 29–30.
Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R.(1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Murashige,T. and Skoog,F. (1962) *Physiol. Plant*, **15**, 473–497.
Negrutiu,I., Shillito,R., Potrykus,I., Biasini,G. and Sala,F. (1987) *Plant Mol. Biol.*, **8**, 363–373.
Prody,G.A., Bakos,J.T., Buzayan,J.M., Schneider,I.R. and Brüning,G. (1986) *Science*, **231**, 1577–1580.
Proudfoot,N.J. (1989) *Trends Biotechnol.*, **14**, 105–110.
Reiss,B., Sprengel,R., Schaller,H. (1984a) *EMBO J.*, **3**, 3317–3322.
Reiss,B., Sprengel,R., Will,H., Schaller,H. (1984b) *Gene*, **30**, 211–218.
Ruffner,D.E., Stormo,G.D. and Uhlenbeck,O.C. (1990) *Biochemistry*, **29**, 10695–10702.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sarver,N., Cantin,E.M., Chang,P.S., Zaia,J.A., Ladne,P.A., Stephens,D.A. and Rossi,J.J. (1990) *Science*, **247**, 1222–1225.
Saxena,S.K. and Ackerman,E.J. (1990) *J. Biol. Chem.*, **265**, 17106–17109.
Schreier,P.H., Seftor,E.A., Schell,J. and Bohnert,H.J. (1985) *EMBO J.*, **4**, 25–32.
Sioud,M. and Drlica,K. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 7303–7307.
Steger,G., Hofman.,H., Förtsch,F., Gross,H.J., Randles,J.W., Sänger,H.L. and Riesner,D. (1984) *J. Biomol. Struct. Dynamics*, **2**, 543–572.
Symons,R.H. (1989) *Trends Biochem. Sci.*, **14**, 445–450.
Uhlenbeck,O. (1987) *Nature*, **328**, 596–600.
Uhlenbeck,O. (1990) *Nature*, **346**, 613–614
Werntges,H., Steger,G., Riesner,D. and Fritz,H.-J. (1986) *Nucleic Acids Res.*, **14**, 3773–3790.
Wolter,F.P., Fritz,C.C., Willmitzer,L., Schell,J. and Schreier,P.H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 846–850.
Zaug,A., Been,M. and Cech,T.H. (1986) *Nature*, **324**, 4229–4233.

Received on October 28, 1991; revised on December 20, 1991