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Multitarget ribozyme against the S1 genome segment of reovirus possesses novel cleavage activities and is more efficacious than its constituent mono-ribozymes

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Abstract

Two hammerhead motif containing ribozymes (Rzs) were constructed through recombinant techniques that were directed to cleave at the conserved sites of the reovirus S1 gene segment which encodes the cell attachment protein σ1. The two mono-ribozymes 553 and 984 cleaved the target RNA in a sequence specific manner, and Rz-553 being the more efficient. When the mono-Rzs were combined in direct tandem to make it a multitarget-Rz, very efficient cleavage of the S1 RNA was achieved that retained the specificity of the two mono-ribozymes. This cleavage was, as expected, Mg^{++}-dependent but protein-independent. Almost complete cleavage of the S1 RNA was observed with multitarget ribozyme alone. Although S1-Rz-984 cleaved the short S1 synthetic RNA, it failed to cleave the full length S1 RNA (1.4 kb). On the contrary, Rz-553 cleaved the short synthetic RNA as well as the full length S1 RNA with equal efficiency. Full length S1 RNA was, however, cleaved efficiently by the multitarget-ribozyme-S1-Rz-984–553 that cleaved at both the target sites. Thus, hybridization of one ribozyme (Rz-553) to a full length S1 RNA potentially opened up the 984-Rz target site that was otherwise inaccessible to the mono-Rz-984. Multitarget ribozyme expressing mammalian cells showed reduced amounts of S1 RNA that correlated well with the levels of reovirus σ1 protein. Potential uses of such multitarget-Rzs are discussed.

Keywords: Transcription; Gene cleavage; Pathogenesis

1. Introduction

Reovirus genome consists of ten double stranded RNA fragments and they encode ten or more proteins. Functions of most of the proteins, including protein σ1, are known. Genetic studies revealed that the major determinant for serotype specificity is determined by the S1 genome segment (Sharpe and Fields, 1985; Weiner and Fields, 1977). It is the cell attachment protein (Lee et al., 1981) that is present in the virus particles in extremely small ($<2\%$) amounts on the 12 vertices of the icosahedral. It is also made in extremely small amounts in the reovirus-infected

Abbreviations: RT-PCR, Reverse transcriptase-dependent PCR; Rz, Ribozyme.

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cells. The N-terminal of the S1 genome segment consists of several heptapeptide repeat units where position A and D are occupied by hydrophobic amino acids (Bassel-Duby et al., 1985). They form coiled coil structures while the globular C-terminal end protrudes from the surface of virus particles (Furlong et al., 1988). Electron microscopic studies of the virus particles or purified reovirus protein σ1, revealed structures that possess a fibrous tail on one end and a globular head at the other end (Furlong et al., 1988; Banerjea et al., 1988). This protein is oligomeric in nature and the domains responsible for oligomerization have been identified (Banerjea and Joklik, 1990; Leone et al., 1992). One of the reasons for the small amounts of protein σ1 that are synthesized by reovirus-infected cells is because the initiation codon AUG (methionine) of the S1 genome is placed in a weak Kozak context (Roner et al., 1989). Besides being the cell attachment protein, σ1 elicits the formation of neutralizing antibodies and generates cell-mediated immune responses (Fontana and Weiner, 1980; Finberg et al., 1979). Protein σ1 has recently been recognized as the major determinant for causing apopotosis (Tyler et al., 1995). Recent evidence suggests that other structural proteins could also be involved in causing apoptosis (Tyler et al., 1996), albeit to a smaller extent. Direct proof that reovirus devoid of protein σ1 is non-infectious was shown by characterizing the 13 particle species that differed in their electrophoretic mobilities (Larson et al., 1994). Therefore, any antiviral strategy aimed to specifically cleave the S1 RNA may reduce the infectivity of the virus and very likely protect cells from the cytopathic effects caused by reovirus infection. In order to test this hypothesis, we engineered ribozymes (Rzs) against this target gene.

Rzs are short catalytic RNA molecules that are capable of cleaving any target RNA in *trans* in a sequence specific manner (Cech, 1987). The hammerhead motif-containing Rzs have been tested against many genes (Beck and Nassal, 1995; Ferbeyre et al., 1995). We have also designed Rzs against the most important HIV-1 coreceptor, the chemokine receptor CCR5, and, by cell membrane fusion assays showed functional interference (Goila and Banerjea, 1998). Multitarget Rzs have earlier been used against HIV-1 envelope gene (Chen et al., 1992; Paik et al., 1997) and against multiple regions of HIV-1 using two different approaches, namely a shot-gun-type approach that released individual Rzs or by multiple Rzs expressed as a single RNA molecule (Ohkawa et al., 1993).

In the present study we compared the sequence-specific cleavage activities using all the three Rzs and their ability to inhibit the reovirus S1 gene expression. We showed that multitarget Rzs possess novel cleavage properties and that they are more efficacious than mono-ribozymes.

2. Materials and methods

2.1. Viruses and cells

Reovirus serotype 3 (strain Dearing) was obtained from ATCC, Maryland, USA, and grown in HeLa cells that were maintained in suspension using Joklik's MEM (GIBCO/BRL, MD) supplemented with 5% fetal calf serum. Cos-1 cells were grown in 75 cm^2 tissue culture flask as a monolayer.

2.2. Target sites for ribozymes

Two conserved sites, GUA and GUC, were chosen that are located at position 553 and 984 of the S1 genome segment corresponding to the B and C regions, respectively (Duncan et al., 1990). The cleavage is expected to take place after the A and C nucleotides (Fig. 1, panel A, shown by arrows).

2.3. Cloning & transcription of the reovirus S1 genome

We used two kinds of S1 substrate, a synthetic 72-nucleotide long fragment, and the full length S1 RNA (1.4 kb) that was derived from the plasmid S1/S1/a (Roner et al., 1989). The cloned genes were placed down stream of either SP6 or T7 polymerase promoter, as described in Fig. 2, panel A. When linearized at their 3′-ends and

subjected to in vitro transcription using either SP6 or T7 polymerase, correct size transcripts were synthesized.

2.4. Cloning and expression of ribozymes

The strategy to construct hammerhead Rzs was the same as described by us before (Goila and Banerjea, 1998). Briefly, a sequence of eight bases complementary to the target RNA was synthesized on either side of the target site (shown by arrow in Fig. 1) along with the conserved catalytic domain. Terminal primers were designed for amplifying the Rz-encoding DNA, the sequence of which is given in Fig. 1 of panel A. The amplified products were cloned into pTARGE™ (Promega Biotech., WI) in a manner that the expression was under control of the T7/CMV promoter (Fig. 2, panel B). The three oligonucleotides for constructing a multitarget-ribozyme were as follows:

1. Multitarget-Rz-oligonucleotide, 5′-GAAGCTTGAATAGGACTGATGAGTCCGTGAGGACGAAACAATTCCACGGTTATCTGATGAGTCCGTGAGGACGAAACGGATTGGGATCCG.
2. Forward terminal primer, GAAGCTTGAATAGGAC.
3. Reverse terminal primer, CGGATCCCAATCCGTTTCG.

When the cloned multitarget-Rz is linearized with *Sma*1 and subjected to in vitro transcription using T7 RNA polymerase, a 116-nucleotide long transcript is expected to be synthesized. The authenticity of the cloned ribozyme was checked by sequencing and the results are shown in Fig. 3,

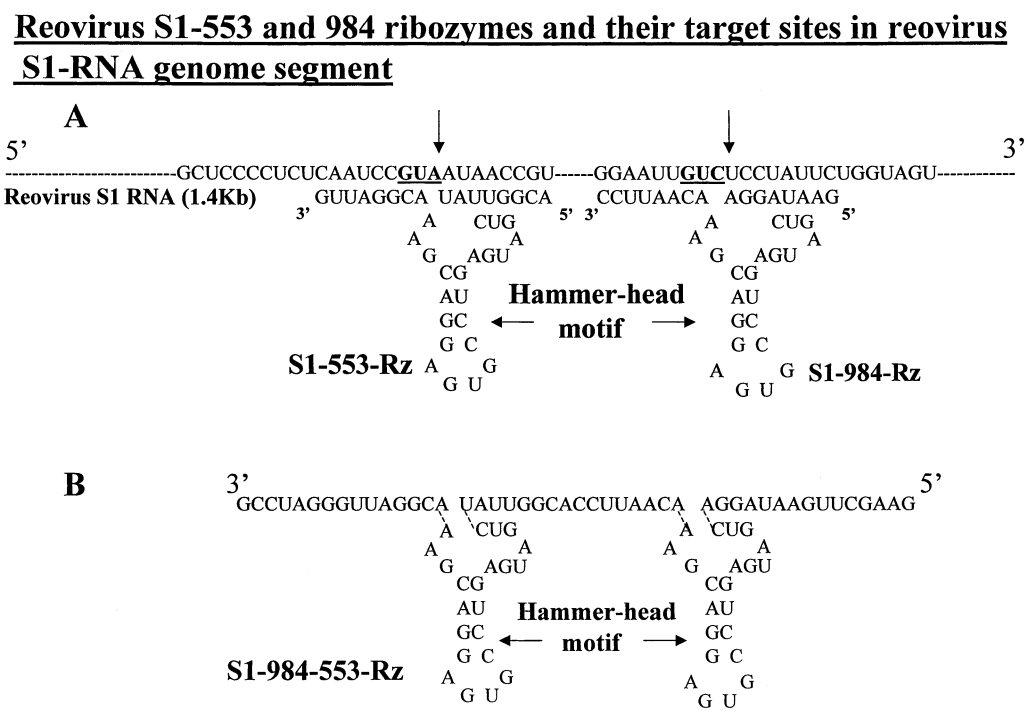


Fig. 1. Two mono-Rzs (Rz-553 and 984) possessing the earlier described hammerhead catalytic motif were synthesized using recombinant techniques. The two cleavage sites GUA and GUC located at positions 553 and 984 in the target RNA are shown in bold letters. Eight bases on either side of the target site is synthesized that are complementary to the target sequences and the cleavage is expected to take place after A and C nucleotides (shown by arrows) (Panel A). A di-Rz (984–553) was also constructed by placing the Rzs in direct tandem. They contained their own catalytic motif (Panel B). Recombinant clones harboring these Rzs were confirmed by sequencing.

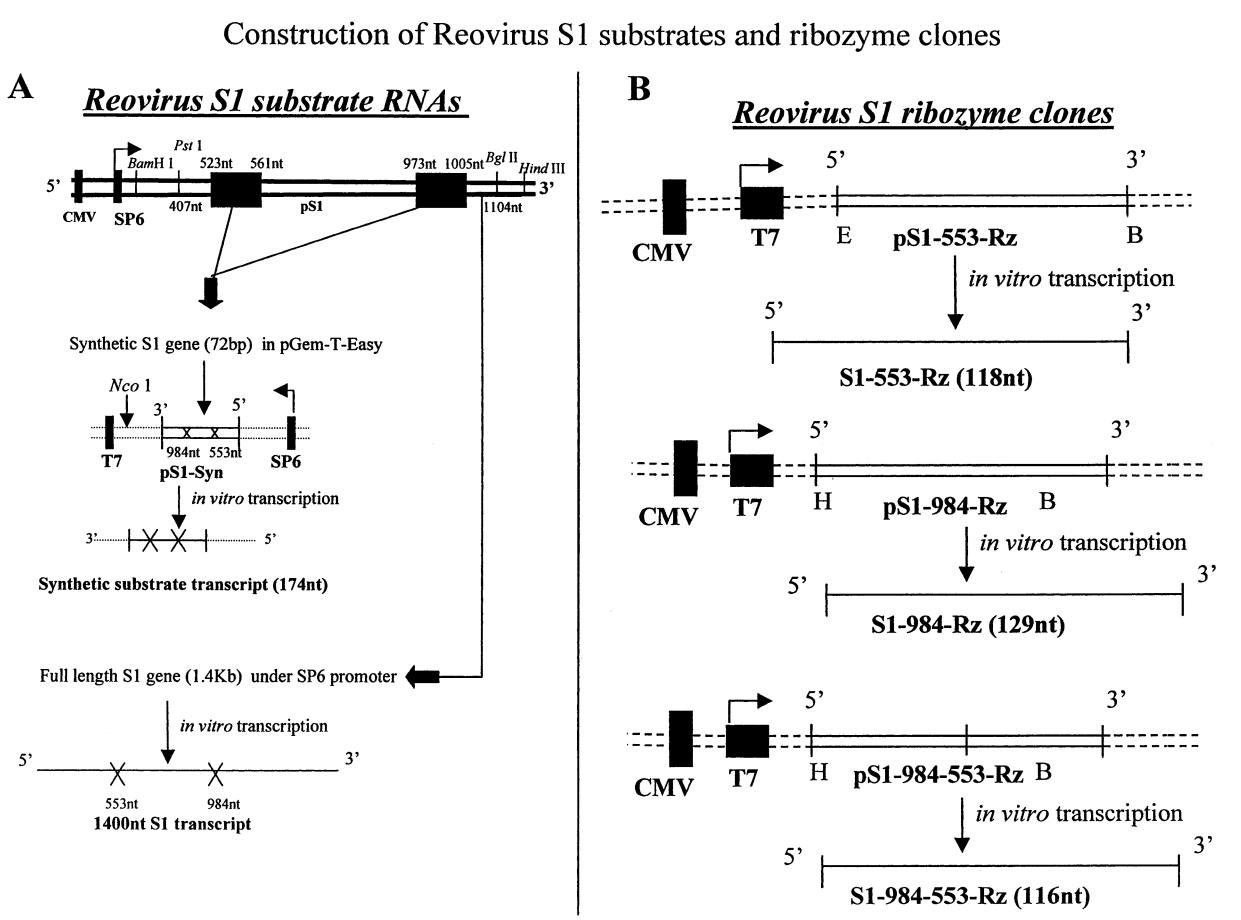


Fig. 2. Two kinds of reovirus substrates were used. A synthetic 72-bp region that possessed both the target sites, were generated by fusing two S1 RNA fragments (523–561 and 973–1005). This synthetic gene was cloned into pGEM-T-Ez (Promega Biotech. WI, USA). Upon linearization with *Nco*1 restriction enzyme, a 174-nt long transcript is expected that includes transcribed regions from the polylinker region as well. Full length S1 RNA (1.4 kb) could be synthesized after linearizing the pSG1-S1 plasmid with *Hind* III restriction site present at the end of the S1 genome segment (Panel A). Two mono- & a di-Rz clone were synthesized through recombinant techniques into pCDNA3 expression vector (Promega) that places all the Rzs downstream of a CMV promoter. After linearizing with an appropriate restriction enzyme, ribozyme transcripts with defined length were obtained (Panel B).

where the sequence of the catalytic motifs are indicated in bold letters at the bottom. In a similar manner, the mono-ribozymes (Rz-553 & 984) were also confirmed by sequencing (data not shown). The expression vector pTARGE™ (Promega Biotech.) was especially selected because it not only facilitates cloning of PCR product because of the T-tails but also places the Rzs under T7 as well as CMV promoter. While the former promoter is used for obtaining RNA by in vitro transcription, the latter is used to obtain intracellular expression of Rzs.

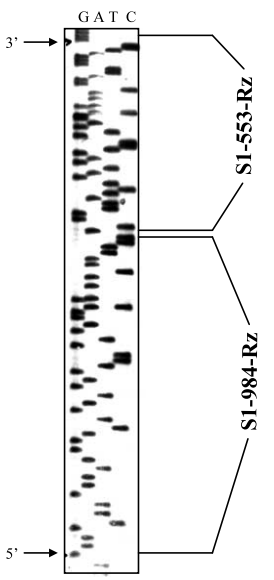
2.5. In vitro cleavage of the target RNA

Recombinant plasmids encoding Rzs or substrate RNAs were linearized at their 3′-ends and subjected to in vitro transcription as described earlier (Banerjea and Joklik, 1990). The cleavage reaction was carried out using equimolar amounts (0.3 pmols) of labeled S1 RNA and unlabeled Rz under standard conditions of cleavage (50 mM Tris–HCl, pH 7.5 per 10 mM $MgCl_2$) in a final volume of 10 μl as described (Goila and Banerjea, 1998). Varying concentrations of $MgCl_2$ were

used to find out how $MgCl_2$ affected the cleavage efficiency. The cleaved RNA fragments were analyzed by 7 M urea-6% PAGE using the MiniPROTEAN II system from Bio-Rad.

2.6. Antiviral effects of Rzs

Cos-1 cells were grown to 60% confluency in a 6-well plate and transfected with Rz encoding DNA using lipofectin (GIBCO/BRL) in the absence of serum, as described by the Manufacturer. Twelve hours later, the cells were challenged with 5 plaque forming units per cell of reovirus ST3 and incubated at 37 °C for an additional 12 h. Thereafter, the cells were harvested and total RNA was recovered using Trizol reagent (Promega). Using reverse transcriptase-dependent PCR (RT-PCR) techniques, the levels of target RNA in comparison with the control RNA (hGAPDH) were estimated as described (Goila and Banerjea, 2001) using specific primers. Equivalent amounts of lysates were also subjected to western blot analyses using polyclonal antiserum to reovirus ST3 (Strain Dearing), as described earlier (Banerjea et al., 1988).



5'⟶3' sequence :

GAAGCTTGAATAGGA**CTGATGAGTCCGTGAGGACGAA**ACAATTCCA
CGGTTAT**CTGATGAGTCCGTGAGGACGAA**ACGGATTGGGATCCG

Fig. 3. Sequence analysis of a cloned di-Rz. Briefly a di-Rz oligonucleotide was synthesized and with the help of two terminal primers, it was amplified by PCR and cloned into pCDNA3. Recombinant clones with correct size insert was purified on a Qiagen column (Qiagen) and subjected to sequencing using T7 primer. It is clear from the Figure that the two Rzs were cloned in tandem. The catalytic motifs of both Rzs are shown in bold letter at the bottom of the Figure.

3. Results

3.1. In vitro cleavage of the S1 synthetic RNA by mono- and multitarget ribozymes

When a 72-nt long S1 synthetic gene harboring the ribozyme cleavage sites is linearized with *Nco*1 and subjected to in vitro transcription using SP6 polymerase (Promega Biotech., WI), a 174-nt long transcript is expected that includes transcribed regions from the polylinker region of the vector (Fig. 2, panel A). When allowed to interact with equimolar amounts of Rzs, RNA fragments of varying sizes could be expected as depticted in Fig. 4, panel A. Note that cleavage with multitarget-Rz would generate mono-Rz specific fragments as well as a 20-nt long fragment when both the target sites are cleaved either simultaneously or sequentially.

A comparison of the cleavage efficiencies of the S1 Rzs is shown in Fig. 4, panel B. Lane 1 shows the synthesis of 174-nt long labeled S1 synthetic substrate RNA, lanes 2, 3 and 4 represent the synthesis of labeled Rz-984-553 (116-nt), Rz-553 (118-nt) and Rz-984 (129-nt), respectively. Labeled substrate was mixed with unlabeled Rz-S1-553, 984 and 553-984, respectively, and subjected to in vitro cleavage reaction for either 15 min (lanes 5, 6 and 7) or 2 h (lanes 8, 9 and 10). Sequence specific cleavage products could be seen at both time points by all the three Rzs and, as expected, more cleaved products could be observed after 2 h of incubation. The most interesting results were observed with the multitarget-Rz (lane 10) that yielded RNA

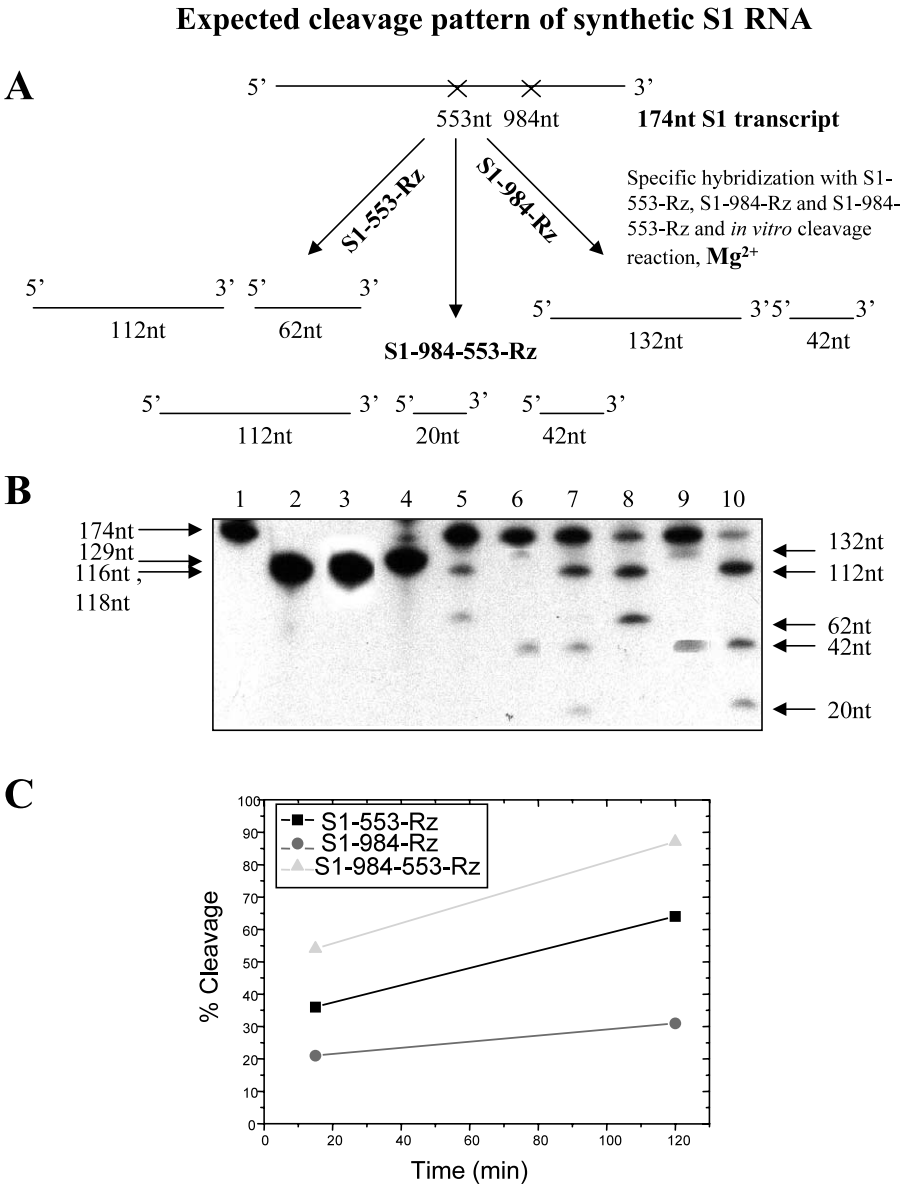


Fig. 4. Synthetic S1 RNA (174 bases long) was used as substrate to test the efficacy of mono- and di-Rz, and the expected cleavage pattern is shown in panel A. Note that the di-Rz should release two mono-Rz specific fragments (112 and 42 bases long), besides a 20 nucleotide long fragment, when the cleavage occurs simultaneously at two target sites. Lanes 1–4 from panel B are control lanes that show 174-nt long S1 RNA (174 bases long) alone (lane1); 116-nt long labeled di-Rz (lane 2); 118-nt long Rz-553 (lane 3) and 129-nt long Rz-984 (lane 4). Equimolar amounts (0.3 pmoles each) of Rz and substrate RNA (^{32}P labeled) were mixed and subjected to cleavage for 15 min (lanes 5, 6 and 7) or 2 h (lanes 8, 9 and 10). Specific cleavage products could be observed that match the pattern described in panel A. Approximately 30 to 40% more cleavage was observed after 2 h of cleavage reaction (compare lanes 5, 6, 7 with 8, 9, 10). The efficiency of the cleavage by each Rz is depicted in panel C. It is clear that di-Rz is most efficient, followed by Rz-553, Rz-984 being the least effective.

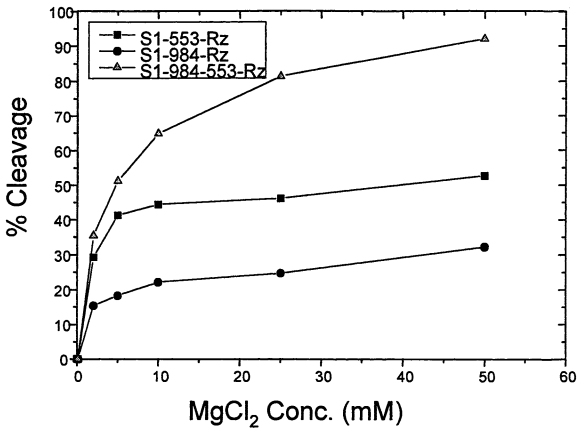


Fig. 5. Cleavage efficiency of each Rz was tested in presence of varying amounts of $MgCl_2$ as described in the text. Di-Rz showed almost 90% cleavage in presence of 50 mM $MgCl_2$, as compared with 50% and 30% with Rz-553 and Rz-984. Cleavage efficiencies were calculated on the basis of the input substrate left after the reaction as described in the text.

fragments specific for Rz-553 and 984. Also note the presence of a 20-nt long RNA fragment and almost a complete disappearance of the S1-synthetic substrate RNA (lanes 7 and 10). A comparison of the cleavage efficiencies clearly revealed that the multitarget-Rz was the most effective, followed by Rz-553, Rz-984 being the least effective (Fig. 4, panel C). This was evident from the extent of S1 RNA (substrate) left after the action of Rzs. We conclude that all the three Rzs possess sequence-specific cleavage activities and that the di-Rz is the more effective under identical experimental conditions.

3.2. Effect of $MgCl_2$ on cleavage efficiency

Labeled substrate RNA and Rzs were mixed in equimolar amounts as described earlier in the presence of varying amounts of $MgCl_2$ (range 2–50 mM) at 37 °C for 2 h and subjected to gel analysis. Almost complete disappearance of the input substrate was obtained with the multitarget-Rz, $\sim$50% by Rz-553 and $\sim$30% by Rz-984 at the highest concentration of $MgCl_2$ tested (Fig. 5). No cleavage was obtained by any of the above Rzs if $MgCl_2$ was omitted from the cleavage reaction (data not shown).

3.3. In vitro cleavage of the full length S1 RNA

Full length S1 RNA was derived after linearizing the plasmid S1/S1/a (Roner et al., 1989) by in vitro transcription. The cleavage pattern obtained by all the Rzs are depicted in panel A of Fig. 6. Panel B shows the cleavage results obtained with the two mono-ribozymes. Lane 1 shows the synthesis of full length S1 RNA (1.4 kb). Specific cleavage products (847 and 553 nt) could be observed in the presence of Rz-553 (lane 2). Using similar cleavage conditions, Rz-984 failed to cleave the S1 RNA. We then wanted to know if the multitarget-Rz possessed the ability to cleave the same S1 RNA, and the results are shown in panel C. Lane 1 is the same as lane 1 of panel B. When the substrate RNA was reacted with multitarget-Rz, specific cleavage products (553, 431 and 416 nt) could be observed (lane 2). We conclude that the multitarget-Rz cleaved the S1 RNA at both the target sites.

3.4. Intracellular reduction of reovirus S1 RNA

Multitarget-Rz expressing and control cells were challenged with reovirus (Strain Dearing, ST3) at five plaque forming units per cell and the levels of S1 RNA were estimated using primers that span the *BamH*1 site and *Bgl* II site of the S1 RNA [7] (panel A of Fig. 7). This combination will amplify $\sim$1.00 Kb DNA fragment by RT-PCR. Equivalent amounts of cell lysates were also tested for the levels of house keeping gene (human GAPDH) using specific primers as described earlier (Goila and Banerjea, 2001). Lane 1 of panel B shows the sizes of the DNA standards (PCR marker, Promega). No S1 specific signal was observed from plain cell lysates (lane 2) or if the RT was omitted from the reaction (lane 3). As expected, an intense S1 specific band was observed in cells that were infected with reovirus (lane 4). A sharp reduction in the S1 RNA was observed when 1 μg of multitarget-Rz was used (lane 5) that was further reduced when 4 μg of the same multitarget-Rz were used (lane 6). The levels of the control

RNA in the corresponding lanes (4, 5 and 6) were, however, not changed. We conclude that the reduction in the intracellular levels of S1 RNA is specific due to the action of Rzs. Intracellular reduction with the mono-Rz-553 was also observed but only at 4 μg, whereas mono-Rz-984 failed to interfere with the expression of S1 RNA (data not shown).

3.5. Intracellular reduction in reovirus protein σ1

Equivalent amounts of cell lysates from ribozyme-expressing and untreated cells that were infected with reovirus, were subjected to Western-blot analysis. Lane 1 of Fig. 8 shows all the three classes (λ, μ and σ) of reovirus proteins including σ1. When 1 μg (lane 2) or 4 μg (lane 3) of

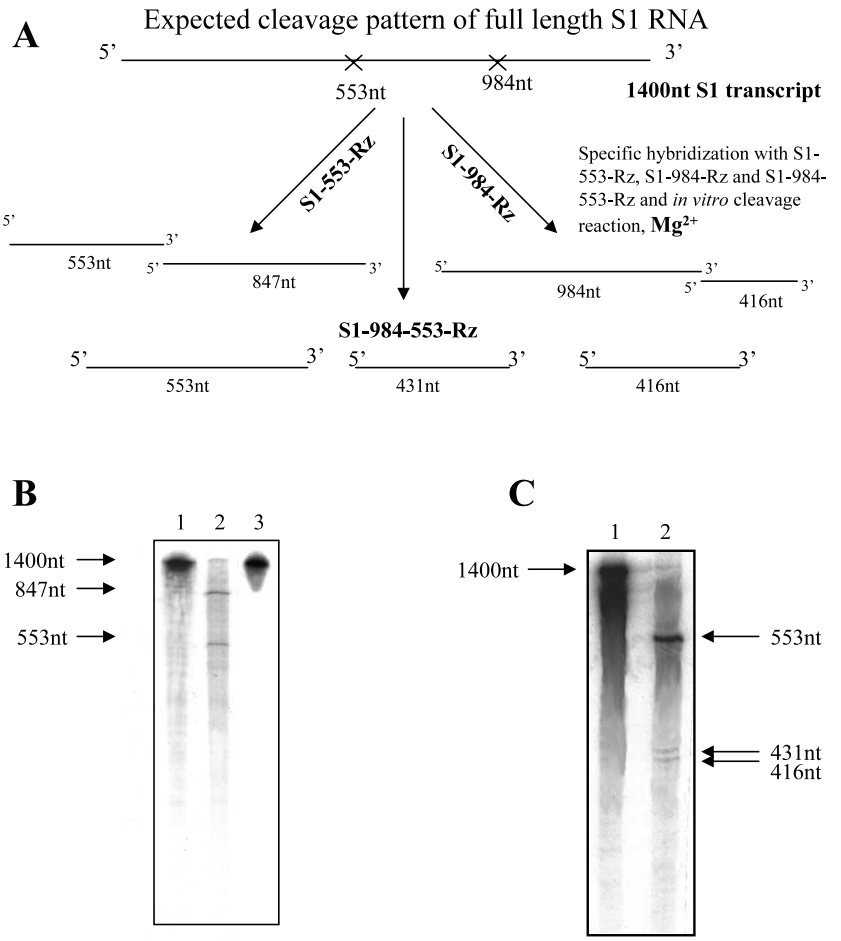


Fig. 6. The efficacy of all the Rzs were also tested on full length S1 RNA and the expected cleavage pattern is shown in panel A. Rz-553 should generate RNA fragments that are 553 and 847-nt long, whereas Rz-984 should yield 984 and 416-nt long RNA fragments. The di-Rz should yield mono-Rz-specific fragments along with a 431-nt long fragment when the two target sites are cleaved (panel A). Panel B depicts the cleavage pattern obtained with the two mono-Rzs when equimolar amounts of ribozyme and labeled RNA were used. Lane 1 shows the synthesis of full length S1 RNA. When this RNA was used for cleavage by Rz-553, specific products (847 and 553-nt long RNA fragments) could be observed (lane 2). Under exactly similar conditions, Rz-984 failed to cleave the 1.4 kb long S1 RNA (substrate). Panel C represents the cleavage obtained by di-Rz under identical experimental conditions (lane 2); lane 1 shows the synthesis of 1.4 kb long RNA fragment (lane1). Cleaved RNA fragments obtained after gel analysis resemble the predicted pattern of cleavage as described in panel A.

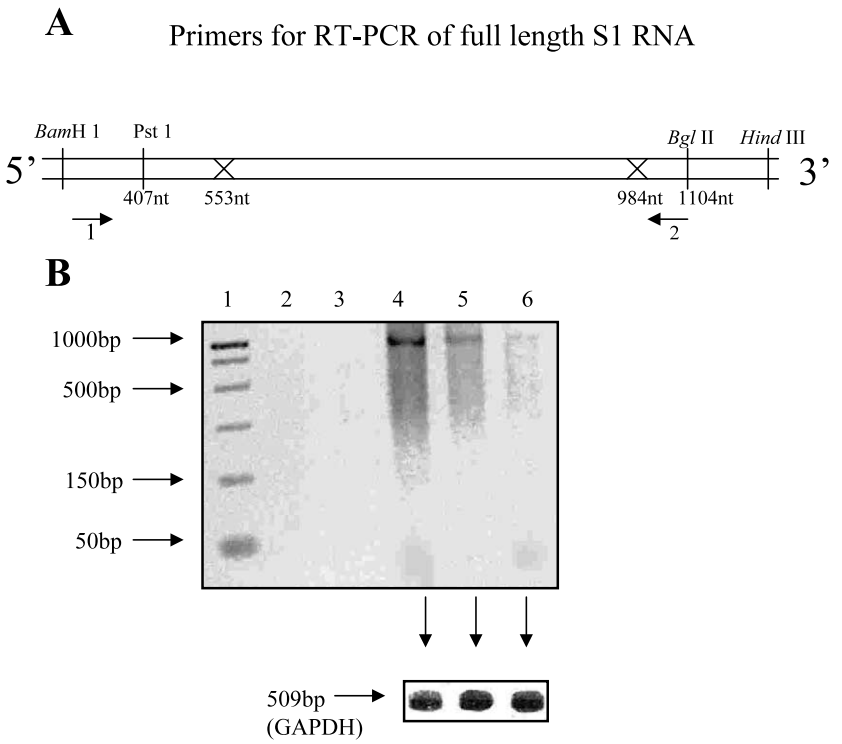


Fig. 7. Ribozyme expressing mammalian cells were challenged with 5 PFU per cell of reovirus ST3 (strain Dearing) for 12 h post transfection. Total RNA was isolated and the levels of S1 RNA were determined by RT-PCR techniques using S1 genome specific primers(Primer # 1 and 2, panel A). Equivalent amounts of cell lysates were quantitated for house keeping gene (human GAPDH) also using specific primers (see text). Plain Cos-1 cell lysate, as expected, failed to amplify any S1 RNA specific band (panel B, lane 2), and the same was true when the RT was omitted from PCR reaction (lane 3). Strong reovirus specific signal was obtained from cells that were infected with reovirus (lane 4). When cells were treated with either 1 μg (lane 5) or 4 μg (lane 6), a dose-dependent decrease in the signal could be observed. The levels of control RNA (GAPDH) in the three lanes (4, 5 and 6), however, remain unchanged.

multitarget-Rz was used, a complete absence of protein σ1 was observed, but the levels of the remaining structural proteins remained unaltered. Mono-Rz-553 could interfere with the expression of σ1 protein only at 4 μg, whereas the mono-Rz-984 failed to interfere at either 1 or 4 μg (data not shown).

4. Discussion

Two mono-Rzs and a multitarget ribozyme against the reovirus S1 genome segment were constructed through recombinant techniques and were tested for their sequence-specific cleavage activities and their ability to interfere with the intracellular expression of S1 RNA following virus infection. Sequence-specific cleavage was obtained by all the three Rzs when tested on a synthetic S1 RNA. Multitarget-Rz was more effective as compared with the two mono-Rzs, as evident from almost complete disappearance of the input substrate RNA. Similar observations were made earlier, when a multitarget ribozyme was tested against the envelope gene of HIV-1 (Chen et al., 1992; Paik et al., 1997). Out of the two mono-Rzs, Rz-553 was ~15% more efficient than Rz-984. This could be due to the different secondary structures at the two target sites of the S1 RNA. The cleavage was dependent upon the presence of $MgCl_2$ and, as expected, all the Rzs showed increased cleavage efficiency in presence

of increasing amounts of $MgCl_2$. The important feature about all the Rzs was that all of them showed cleavage activity on synthetic S1 RNA in the presence of 1 and 3 mM $MgCl_2$ that is close to the physiological levels in humans (Santoro and Joyce, 1997).

When the same three Rzs were tested on a full length S1 RNA, only one of the two mono-Rzs, namely Rz-553, showed sequence-specific cleavage activity, which is quite different from the results obtained with the shorter synthetic S1 RNA, where all the three Rzs showed excellent cleavage activities. This could be because of a completely different secondary structure at the 984-Rz target site in the full length S1 RNA (1.4 kb) that prevented hybridization by the Rz-984, which was not the case when the shorter synthetic S1 RNA was used. This strongly suggests that the cleavage activities of the Rzs can not be predicted solely from the results obtained with the shorter transcripts, and, as far as possible, full-length authentic target RNAs should be used. Quite interesting results were obtained when the multi-target ribozyme was used to cleave the full length S1 RNA. This ribozyme cleaved at both the target sites in the S1 RNA as evident from the mono-Rz-specific cleavage products. When both the mono-Rzs were added simultaneously to the full length S1 RNA, evidence for cleavage at both the target sites were obtained, but the efficiency was lower than what was observed with the di-Rz (data not shown). Thus, a mono-Rz may be completely nonfunctional on a given target RNA but when placed in tandem with another efficient Rz, it may be able to cleave at the other target site with equal efficiency as the other Rz. Furthermore, an unrelated di-Rz with similar length and hammerhead catalytic motif failed to cleave the S1 RNA (both short synthetic S1 RNA and the full length S1 RNA). It also failed to interfere with the intracellular expression of S1 RNA (data not shown). Antisense constructs (32 nt long) devoid of the two hammerhead catalytic motifs showed modest reduction of intracellular RNA (data not shown). This multitarget approach is an important antiviral strategy especially when the target RNA is known to mutate frequently as was exploited earlier by designing novel heterodimeric maxizymes that simultaneously cleaved HIV-1 Tat RNA (Kuwabara et al., 1999).

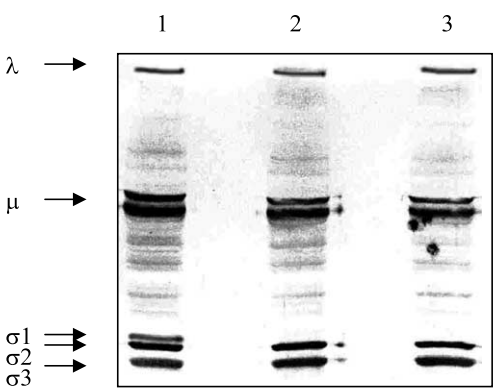


Fig. 8. Western blot analysis was carried out for determining the levels of reovirus protein σ1 using the polyclonal antiserum against reovirus ST3 (see text). Lane 1 shows all the three classes of reovirus proteins λ, μ and σ) including the minor component reovirus protein σ1 from cells that were infected with reovirus ST3 (5 PFU per cell). When either 1 μg (lane 2) or 4 μg (lane 3) of DNA encoding the di-Rz was used, only reovirus protein s1 was not synthesized.

The Rzs were able to interfere with the intracellular expression of the S1 RNA after virus challenge. This correlated very well with the specific reduction of reovirus protein σ1. Multitarget-Rzs were more effective in specific interference of the S1 gene and required four fold less amounts than Rz-553 under similar experimental conditions (data not shown). An additional reason for the significant reduction in S1 RNA and σ1 protein could be the generation of non-infectious reovirus particles containing reduced amounts of σ1 protein. Remarkably, multitarget ribozyme-treated cells showed protection against the cytopathic effects caused by the reovirus infection that is manifested by characteristic rounding of cells and a reduction of about 2.5–3 log in the infectious yields of the virus, as determined by plaque titration on LM-mouse fibroblast cell line (data not shown). Here again, the multitarget Rz was more efficacious than Rz-553. Rz-984, as expected, was not effective at all (data not shown). The levels of ribozyme RNA remained high (> 10 ng from 1×10^5 cells) during the entire experiment as detected by RT-PCR and nuclease protection assays, as described earlier (Goila and Banerjea,

2001). It may be possible to obtain more efficient Rzs by placing 3 Rzs in sequence, but further addition may interfere with the efficiency as reported earlier (Ohkawa et al., 1993).

This is the first multitarget Rz that was used against a virus that belongs to reoviridae family that are characterized by the presence of multiple double stranded RNA genome segments. This family includes reoviruses, rotaviruses that are the major cause for infantile diarrhea and blue tongue virus that is an extremely important pathogen for cattle. Not only these novel Rzs could be used as an antiviral approach but they can also be used as a tool to delineate specific functions of a target gene.

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