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Potent inhibition of influenza virus replication with novel siRNA-chimeric-ribozyme constructs

Prashant Kumar[a], Vikas Sood[b], Rajesh Vyas[a], Nidhi Gupta[b], Akhil C. Banerjea[b], Madhu Khanna[a,*]

[a] Department of Respiratory Virology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 110007, India
[b] Virology Lab I, National Institute of Immunology, Jawaharlal Nehru University Campus, Aruna Asaf Ali Marg, New Delhi 110067, India

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ABSTRACT

A multitarget approach is needed for effective gene silencing for RNA viruses that combines, more than one antiviral approach. Towards this end, we designed a wild-type (wt) chimeric construct, that consisted of small hairpin siRNA joined by a short intracellular cleavable linker to a known, hammerhead ribozyme (Rz), both targeted against M1 genome segment of influenza A virus. When this, wt chimeric RNA construct was introduced into a mammalian cell line, along with the M1 substrate, encoding DNA, very significant (67%) intracellular down regulation in the levels of target RNA was, observed. When the siRNA portion of this chimeric construct was mutated keeping the Rz region, unchanged, it caused only 33% intracellular reduction. On the contrary, when only the Rz was made, catalytically inactive, keeping the siRNA component unchanged, about 20% reduction in the target M1, specific RNA was observed. This wt chimeric construct showed impressive (>80%) protection against, virus challenge, on the other hand, the selectively disabled mutant constructs were less effective. Thus, in this proof of concept study we show that varying levels of protection against virus challenge was, observed with novel mutant versions of the chimeric constructs.

1. Introduction

Influenza A virus causes the most prevalent infection of the respiratory tract in humans (Lamb and Krug, 2001). The frequent changes in the antigenic structure (antigenic drift) and emergence of new recombinant strains by reassortment or mixing of two or more RNA genome segments between the two circulatory viruses creates a major public health problem globally.

Currently available vaccines are of limited value because of the rapidly changing antigenic structure of the virus. Similarly, although four antiviral drugs are currently available for the symptomatic treatment of infected individuals, their use is limited because of the undesirable side effects and possible emergence of mutants resistant to these drugs (Luscher-Mattli, 2000). Clearly, there is a need to develop novel strategies that can potently and specifically interfere with the replication of influenza virus.

Several nucleic acid based approaches are available to interfere with the gene expression in a sequence-specific manner. They include antisense (DNA or RNA), ribozymes (Rzs), DNA-enzymes (Dzs), aptamers and small interfering RNAs (Akkina et al., 2003; Kurreck, 2003; Joyce, 2004; Banerjea et al., 2004a,b; Kurreck et al., 2002). Of all the ribozymes, the hammerhead motif containing Rzs have been exploited by several investigators. Its smaller size and impressive catalytic properties make it a promising therapeutic molecule. Any target RNA with NUX (where N is any nucleotide and X is any nucleotide except guanosine) sequence can be targeted by the hammerhead Rz. This consists of 22 nt long conserved hammerhead Rz catalytic motif flanked by 8 nt long hybridizing arms that are made complementary to the target RNA sequences. A single nucleotide change in the hammerhead catalytic motif abolishes the catalytic activity of the Rz. Earlier some attempts were made to interfere with the replication of influenza virus using hammerhead Rzs (Lazarev et al., 1999; Tang et al., 1994).

RNAi is an evolutionary conserved process by which a Ds-RNA of a defined length (21–23 nt), called siRNA, directs sequence-specific degradation of any RNA (Scherer and Rossi, 2004; Elbashir et al., 2001; Pickford and Cogoni, 2003; Nishikura, 2001) via complicated steps involving the RNA induced silencing complex (RISC) (Hammond et al., 2000). Naturally occurring siRNAs are produced in the mammalian cells by the Ds-RNA specific endonuclease called DICER, which cleaves the long Ds-RNA into short Ds-RNA fragments (siRNAs). It is well-known now that RNAi phenomenon can be triggered in mammalian cells by introducing a preformed siRNA or shRNAs or via DNA expression vectors and achieves impressive inhibition of the expression of the desired genes (Ge et al., 2004; Tompkins et al., 2004). Few attempts have been made to interfere

* Corresponding author. Tel.: +91 11 27667441 132; fax: +91 11 27666549.
E-mail address: madhukhanna@hotmail.com (M. Khanna).

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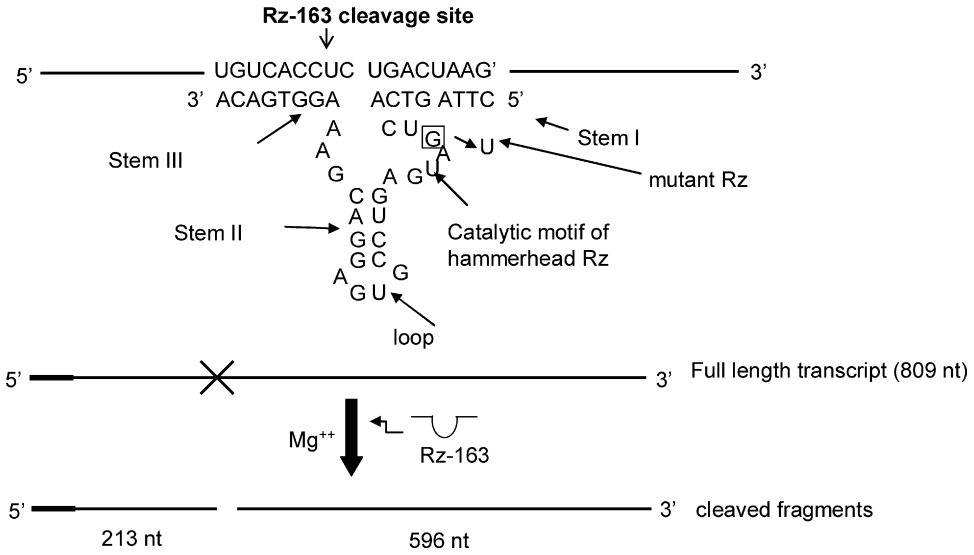

Fig. 1. Construction of a hammerhead against M1 RNA. The hammerhead catalytic motif (stem II and a loop) along with two hybridizing arms that constitute stems I and III is shown. The relevant target sequence involved in hybridization with ribozyme is shown. *In vitro* generated M1 RNA (809 nt long) when subjected to *in vitro* cleavage by ribozyme, two specific RNA fragments are expected in the presence of Mg^{2+}.

with the influenza A virus replication by siRNAs targeted against single influenza genes (Ge et al., 2003, 2004; Tompkins et al., 2004; Hui et al., 2004; Bennink and Palmore, 2004).

Although several investigators have individually used both Rzs and siRNAs, we reasoned that by combining these two most powerful antiviral approaches against the influenza virus matrix protein M1, we may be able to inhibit the viral gene expression in a more powerful manner. We targeted M1 genome for our antiviral approaches as M1 protein plays an important role in several stages of virus replication (Hui et al., 2004). We engineered a well-known intracellular cleavable linker in our novel siRNA-chimeric-Rz construct. We show that our siRNA-chimeric-Rz construct inhibited the influenza virus replication very potently and using selectively disabled versions of this construct, it was possible to modulate the extent of virus inhibition.

2. Methods

2.1. Cells and viruses

MDCK cells (purchased from NCCS Pune, India) were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO, USA) and 100 μg/ml of gentamycin. Influenza virus strain A/PR/8/34 (H1N1) was obtained from CDC, Atlanta, GA, USA. The virus was propagated in 10-day-old embryonated chicken eggs and virus containing allantoic fluid was collected and stored at −70 °C.

2.2. Cloning and in vitro transcription of M1 gene

Viral RNA was isolated using QIAamp viral RNA isolation mini kit (Qiagen, Hamburg, Germany). cDNA was prepared using IM-PROM reverse transcription kit (Promega, Madison, WI, USA). cDNA specific for M1 genome segment was amplified by PCR using specific primer (1) forward 5′-GGCgaattcATGAGTCTTCTAACCGAGGTCGAA-3′ (EcoR1 site is written in lowercase) and (2) reverse 5′-GGCgagctcTCACTTGAACCGTTGCATCTGC-3′ (XhoI site is written in lowercase). PCR amplified M1 gene was subjected to EcoR1 and XhoI restriction digestion and cloned into pcDNA3 vector (Promega, Madison, WI, USA) that was also digested with same two enzymes. This placed the M1 gene under the expression of CMV and T7 promoter. This plasmid was called M1-pcDNA3 which was linearized with appropriate enzymes and *in vitro* transcribed using the transcription kit from Promega Biotech, as described earlier (Banerjea and Joklik, 1990).

2.3. Selection of target site, construction and cloning of Rz-163

Several predicted secondary structures of M1 RNA were obtained using M-fold programme and site 161-CUC-163 was chosen as a target of ribozyme because it was located in single-stranded loop region of predicted secondary M1 RNA structure. For construction of ribozyme, a sequence of eight bases complementary to the M1 genome segment was synthesized on either side of target site along with the central conserved catalytic domain (Goila and Banerjea, 2004) (Fig. 1). The ribozyme sequence was amplified and cloned in pcDNA3.1 (+) vector which was digested with HindIII and BamH1. This plasmid was called pcDNA3.1-Rz-163.

2.4. In vitro cleavage of M1 RNA by Rz-163

The cleavage reaction was carried out by mixing equimolar amounts (100 pmol each) of [^{32}P] UTP labeled M1 substrate RNA and unlabeled Rz under standard conditions of cleavage (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$) in a volume of 10 μl at 37 °C for an hour as mentioned earlier (Shahi et al., 2001). The cleaved products were resolved by 7 M urea–6% PAGE using sequencing apparatus from Life Technologies (Hayward, CA, USA). The gel was dried and exposed to X-ray film. The M1 transcripts were allowed to undergo cleavage reaction in the presence of various concentrations of $MgCl_2$ ranging from 0 to 50 mM.

2.5. Synthesis of novel siRNA-chimeric-Rz constructs and its mutants

Strategy for synthesizing the various constructs were same as described earlier (Anderson et al., 2003). Briefly, an oligonucleotide template consisting of T7 promoter at 3′-end followed by siRNA sequence linked to ribozyme with the help of a cleavable linker sequence was synthesized (Leirdal and Sioud, 2002) (Fig. 2a). This oligonucleotide was annealed with T7 primer and subject to *in vitro* transcription using T7 RNA polymerase. To determine the efficacy of individual Rz or siRNA component of the molecule the catalytic

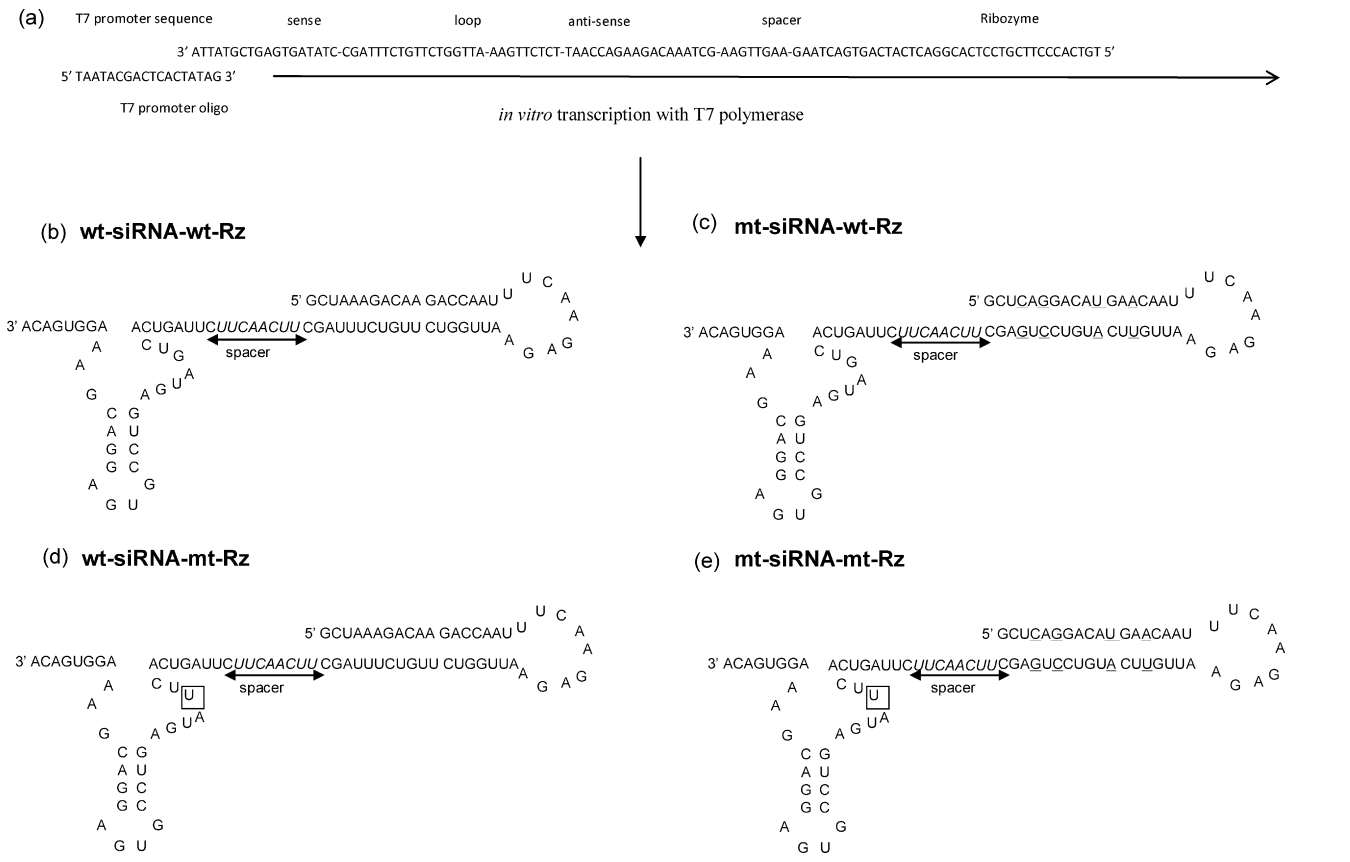

Fig. 2. Strategy for designing novel chimeric constructs. (a) An oligonucleotide having Rz sequence linked to siRNA with a cleavable spacer sequence and T7 promoter sequence (complement) at the 3′-end was assembled. Transcription was initiated after annealing T7 primer and four different chimeric constructs (b–e) were designed. A four point mutation in the stem region (underlined) of siRNA was introduced and a single nucleotide substitution in the catalytic motif was introduced to inactivate Rz (boxed, d and e). The siRNA-specific 21 nt were targeted immediately downstream of AUG (initiation codon) of M1 RNA.

motif of the two mutant constructs were sequentially mutated. The first construct possessed a single G to U mutation in ribozyme keeping siRNA motif active (wt-siRNA-mt-Rz) (Fig. 2c) but the second construct possessed a four point mutation in siRNA portion with active Rz motif (mt-siRNA-wt-Rz) (Fig. 2d). The *in vitro* derived chimeric constructs were treated with RNase free DNase, dissolved in nuclease free water and kept at −70 °C for further use. The size of the siRNA-chimeric-Rz construct was confirmed by agarose gel electrophoresis using standard RNA marker (data not shown).

2.6. Cleavage of chimeric construct at spacer sequence in the presence of cell extract

50 ng of radio-labeled chimeric construct was incubated with or without cytoplasmic protein extract (20 μg) for the period of 1, 2 and 5 min at room temperature. The reaction mixture was quenched with phenol–chloroform and subjected to polyacrylamide gel electrophoresis (Leirdal and Sioud, 2002).

2.7. Inhibition of target gene expression by RT-PCR and real time RT-PCR

MDCK cells were plated in a 6 well plate at density of 0.5×10^6 cells/well and grown to 70% confluency. Thereafter, they were co-transfected with 500 ng of M1-pcDNA3 plasmid DNA (along with equivalent amounts of unrelated RNA of same length) and various concentrations ranging from 1.5 to 5 μg of siRNA-chimeric-Rz constructs with lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Total cellular RNA was isolated 24 h post-transfection using Trizol (Invitrogen, Grand Island, NY, USA) reagent and treated with appropriate concentration of DNase. The concentrations and the quality of the RNA from each well were determined by measuring their absorbance at 260 and 280 nm, respectively. One μg of RNA from each experimental well was taken and reverse transcribed using IM-PROM reverse transcription kit (Promega). The cDNAs prepared from each experimental well were used for conventional PCR of M1 gene. As a control, human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) was amplified simultaneously using gene specific primers (forward 5′-ACCACCATGGAGAAGGCTGG-3′ and reverse 5′-CTCAGTGTAGCCCAGGATGC-3′). The PCR products obtained were analyzed on 1.2% agarose gel. The control reporter plasmid DNA, pSVβ-gal (Promega, Madison, WI, USA) was used for ensuring uniform transfection efficiency. The transfection efficiency achieved was usually in the range of 70–80% in MDCK cells. RT-PCR experiment was also performed after transfection of 2.5 μg each of wild-type siRNA-chimeric-Rz construct or its selectively disabled versions in different wells along with 500 ng of M1-pcDNA3.

A SYBR green-based real time RT-PCR protocol was also employed using an iScript One-Step RT-PCR kit with SYBR green (Bio-Rad Laboratories, Hercules, CA, USA) to confirm the down regulation of M1 gene. Reaction mixtures containing 20 ng total RNA and appropriate amounts of reagents and primers were first incubated at 50 °C for 30 min to synthesize cDNA, heated at 95 °C for 8 min to inactivate the reverse transcriptase, and then subjected to 40 thermal cycles (95 °C for 20 s, 50 °C for 30 s and 72 °C for 30 s) of PCR amplification followed by generation of melting curve with an iCycler iQ real time PCR detection system (Bio-Rad Corp., Hercules, CA, USA). At least two replicate reactions were performed for each sample.

2.8. Ribonuclease protection assay

Ribonuclease protection assay (RPA) was performed using a kit from Ambion Inc. (Austin, TX, USA), according to manufacturer instructions. Briefly, MDCK cells were transfected with M1-pcDNA3 plasmid DNA (along with equivalent amounts of unrelated siRNA-Rz RNA), with or without various chimeric constructs. Total cellular RNA from each well was isolated and 5 μg of RNA was allowed to hybridize with full-length ^{32}P labeled M1-specific probe, digested with RNase H and analyzed on 7 M urea, 12% polyacrylamide gel. Equal amount of yeast t-RNA was used as nonspecific control RNA.

2.9. Virus challenge, cell-staining, FACS and assessment of virus yield by PAGE and western blot

MDCK cells were seeded in six well plates (0.5×10^6 cells per plate) (Hui et al., 2004). After attaining 70–80% confluency, cells were transfected with 5 μg of wild-type or sequentially mutated siRNA-chimeric-Rz RNA constructs that were complexed with lipofectamine-2000 (Invitrogen) according to manufacturer's instruction. 24 h post-transfection, cells were infected with 20 μl of A/PR/8/34 strain of influenza A virus (MOI of 0.1) in a total volume of 150 μl of 1× PBS containing 2 μg/ml TPCK treated trypsin, for 1 h at 37 °C and 5% CO_2. Cells were harvested 10 h post-infection and subjected to FACS analysis using primary (1:100 dilutions) mouse monoclonal antibody influenza type A (cat. #VS2208), obtained from CDC, Atlanta, GA, USA and (1:100 dilution) of rabbit-antimouse IgG-FITC labeled (Serotec, Oxford, UK) as secondary antibody. Unstained cells infected with virus were taken as negative control while stained cells infected with virus but without any transfected chimeric construct were taken as a positive control. FACS results were analyzed by WinMDI software.

At 24 h post-infection, the cultural fluid were harvested for virus yield assessment and clarified by centrifugation at 3000 rpm for 10 min. 2 ml of the supernatant was layered on the top of 1.5 ml of 20% sucrose in 0.1 M NaCl, 0.01 M Tris–HCl pH 7.4 buffer and centrifuged at 21,000 rpm for 90 min at 4 °C. The pellet was dissolved in dissociation buffer and analyzed on 12% polyacrylamide gel. The gel was stained with coomassie blue and bands corresponding to the viral M1 protein were compared to assess the virus yield (Rudneva et al., 1996).

The cells were also harvested at 24 and 72 h post-infection and whole cell lysate was prepared using mammalian cell lysis buffer [0.1 M NaCl, 0.01 M Tris Cl (pH 7.6), 0.001 M EDTA (pH 8.0), 1 mM protease inhibitor cocktail, 100 μg/ml PMSF]. The protein concentration was determined by BCA protein assay (AMRESCO, Solon, OH, USA) and whole cell lysate (40 μg/lane) was fractionated on 12% polyacrylamide for western blotting. The blot was developed using primary (1:100 dilutions) goat monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) against M1 protein of influenza A virus and horseradish peroxide conjugated bovine-anti-goat IgG (1:1000 dilutions) as secondary antibody.

The cytopathic effect (CPE) of the virus on the cells transfected with different chimeric constructs was observed at 72 h post-infection and effect of the constructs on virus multiplication was analyzed.

3. Results

3.1. Selection of target site, construction and cloning of Rz-163

Several secondary structures of M1 RNA were obtained using M-fold program and the target site CUC (161–163 nt) was chosen to construct a hammerhead ribozyme because it was located in the single-stranded loop region of predicted secondary structures (data not shown). For construction of a hammerhead ribozyme, a sequence of eight bases complementary to the M1 genome segment was synthesized on either side of target site (CUC nt position 163; Fig. 1) along with the central conserved catalytic domain as described earlier (Goila and Banerjea, 2004). The ribozyme sequence was amplified and cloned in pcDNA3.1 (+) vector at HindIII and BamH1 restriction sites to yield plasmid pcDNA3.1-Rz-163.

3.2. Synthesis of novel siRNA-chimeric-Rz constructs and its mutants

Strategy for synthesizing the various constructs were same as described earlier (Anderson et al., 2003). Briefly, an oligonucleotide template consisting of T7 promoter at 3′ end followed by siRNA, cleavable linker sequence and ribozyme was synthesized as described before (Leirdal and Sioud, 2002). This oligonucleotide was annealed with T7 primer and subject to *in vitro* transcription using T7 RNA polymerase (Fig. 2a). To determine the efficacy of either Rz or siRNA component of this chimeric construct, we synthesized several mutant versions of this construct. The first construct possessed wild-type (wt) siRNA-chimeric-Rz (wt-siRNA-wt-Rz) (panel b); the second construct possessed a four point mutation in siRNA portion but the ribozyme motif was unaltered (mt-siRNA-wt-Rz) (panel c); the third chimeric construct possessed a single G to U mutation in the catalytic motif of ribozyme (boxed) but keeping siRNA motif unaltered (wt-siRNA-mt-Rz) (panel d); and the last construct consisted of mutant Rz and mutant siRNA (mt-siRNA-mt-Rz) (panel e). The *in vitro* derived chimeric constructs were treated with RNase free DNase, dissolved in nuclease free water and kept at −70 °C for further use. Gel electrophoretic analysis confirmed that all of the chimeric constructs were synthesized correctly and with equal efficiency (data not shown).

3.3. Sequence-specific cleavage of M1 RNA with Rz-163

M1-pcDNA3 (substrate encoding DNA) when subjected to *in vitro* transcription after linearization in the presence of labeled UTP a 809 nt long transcript is expected which also includes 50 nt at the 5′-end derived from the multiple cloning region of the vector (Fig. 3a, b, and c, lane 1). The 809 nt long ^{32}P labeled M1 RNA was specifically cleaved in the presence of cold Rz-163 (100 pmol each) under standard conditions (Santoro and Joyce, 1997). Two cleaved products corresponding to the expected size (596 and 213 nt long RNA fragments) were obtained (Fig. 3a, lane 2). The disabled ribozyme carrying a single point mutation in its catalytic motif failed to cleave the target RNA (Fig. 3b, lane 2). We then subjected the substrate RNA to cleavage with Rz-163 in the absence and presence of varying amounts of $MgCl_2$ (0–50 mM) as indicated at the top of the lanes (Fig. 3c). Enhanced cleavage was observed with increasing amounts of $MgCl_2$ (Fig. 3c, lanes 2–5). No cleavage was observed in the absence of $MgCl_2$. We conclude that Rz-163 cleaves the target RNA specifically in Mg^{2+} dependent manner.

3.4. In vitro cleavage of chimeric construct at spacer sequence in the presence of cell extract

In vitro synthesized chimeric constructs (50 ng) were incubated with cytoplasmic protein extract (20 μg) for the period of 5 min at room temperature. Briefly, the cells were washed twice with PBS and lysed at 4 °C with lysing buffer (0.2% NP40 in PBS + protease inhibitors) as described before (Leirdal and Sioud, 2002). All the four constructs were processed with equal efficiency with cellular nucleases as judged by the appearance of a ∼45 nt long RNA fragment due to cleavage in the middle of the chimeric RNA construct. Cold RNA molecular weight marker (Low range ssRNA ladder, New

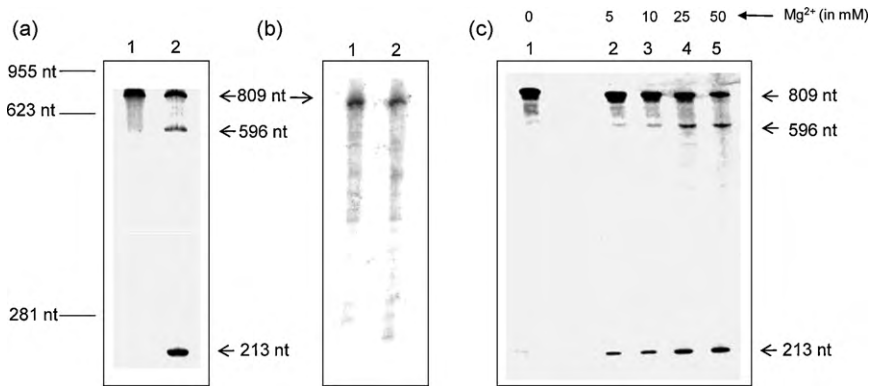

Fig. 3. Cleavage of target M1 RNA with Rz-163. (a) Autoradiograph showing full-length M1 RNA (lane 1) and cleaved fragments of target RNA with Rz (lane 2). Numbers on the left indicate size of RNA marker (Promega, Madison, WI, USA). (b) Lane 1 shows full-length M1 RNA as before. Mutant Rz-163 failed to cleave the target RNA (lane 2). (c) The same transcript of M1 RNA was subjected to cleavage by Rz-163 in the presence of varying amounts of Mg^{2+} concentrations as shown on top of each lane.

England Biolabs, Ipswich, MA, USA) was used to determine the size. We concluded that all the four constructs were processed correctly with equal efficiency (Fig. 4).

3.5. Novel chimeric construct and its mutants mediated modulation of intracellular M1 expression

MDCK cells were transfected with 500 ng of M1-pcDNA3 alone (along with 5 μg of unrelated RNA of similar length–91 nt) or co-transfected with wild-type chimeric construct (wt-siRNA-wt-Rz) at concentration of 1.5 or 5 μg and a significant decrease in the levels of target RNA was observed (Fig. 5a). The expression of M1 gene was reduced by 32 and 84% at concentration of 1.5 and 5 μg/ml, respectively (Fig. 5a, right panel). In order to define the relative roles of Rz or siRNA in the chimeric construct, a suboptimal dose (2.5 μg) of wild-type and sequentially mutated chimeric constructs were used. Selectively disabled mt-siRNA-wt-Rz construct diminished the target gene expression up to 33%, but wt-siRNA-mt-Rz construct was able to reduce only 20% of the target RNA expression. On the contrary, the wild-type construct (both siRNA and Rz component being wt) showed ~67% reduction in the levels of target RNA. This level of inhibition is clearly more than additive and exhibits synergistic effect. It is noteworthy that cells that received the M1 encoding DNA (M1-pcDNA3) also received 2.5 μg of unrelated RNA of similar length. No target-specific amplification was observed when RT was omitted from the PCR reaction (data not shown). The levels of control RNA (hGAPDH) remained unchanged in all the corresponding lanes. These are representative results from three independent experiments. The reduction in level of intracellular M1 RNA was further confirmed by ribonuclease protection assay. Control lane (Fig. 5c and left panel) shows a prominent M1-specific probe as expected. siRNA-chimeric-Rz construct showed up to 81% reduction (lane 5); mt-siRNA-wt-Rz construct showed 62% reduction (lane 3); 39% reduction was observed in case of wt-siRNA-mt-Rz (lane 4) and mt-siRNA-mt-Rz construct showed 17% reduction (lane 2). These are representative results from three independent experiments.

The same was also confirmed by Real Time RT-PCR with RNA isolated from MDCK cells transfected with different siRNA-ribozyme constructs. The PCR was performed in triplicate using iScript SYBR Green from Bio-Rad. The level of M1 RNA in various experiments was compared by calculating $\Delta\Delta$Ct value for each experiment. The RNA level of M1 in cells transfected with only M1 clone was taken as the reference whose value was "1" and RNA levels in all other transfected cells were compared with it. There was 54% decrease in the M1 RNA content in the presence of wt-siRNA-wt-Rz and ~30% decrease in the RNA content was observed in the presence of mt-siRNA-wt-Rz and wt-siRNA-mt-Rz (Fig. 6).

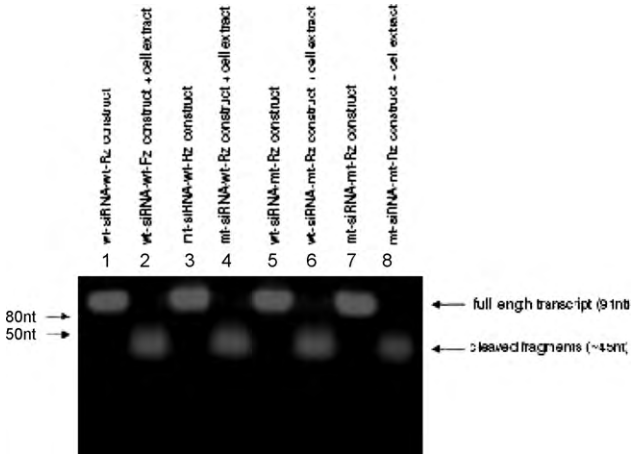

Fig. 4. *In vitro* synthesized chimeric constructs (50 ng) were incubated with cytoplasmic protein extract (20 μg) for the period of 5 min at room temperature and cleavage products were analyzed by gel electrophoresis. Lanes 1, 3, 5 and 7 represent uncleaved product and lanes 2, 4, 6 and 8 represent cleaved product of 50 and 45 nucleotides.

3.6. Variable protection against virus challenge with novel siRNA-chimeric-Rz constructs

MDCK cells were transfected with or without various chimeric constructs (indicated at the top of each panel) for 24 h and subjected to virus challenge (MOI of 0.1) as described earlier in materials and methods. Thereafter the cells were subjected to FACS analysis and results are shown in Fig. 7. The unstained control cells showed almost no fluorescent staining (panel a) and the cells that received virus (along with equivalent amounts of unrelated RNA) showed >83% virus-specific staining, double mutant showed no significant reduction (~11%, compare panels b and c); mt-siRNA-wt-Rz treatment reduced it to 31% (panel d); wt-siRNA-mt-Rz showed 47% staining (panel e) but our wild-type construct showed only 15% staining (panel f).

The cell supernatant was analyzed for virus yield at 24 h post-infection by PAGE. Intensity of the bands corresponding to viral proteins revealed that the amount of virus proteins in the collected fluid was maximum in the cells transfected with double mutant chimeric construct and the level of virus protein reduced to minimum in the cells transfected with the wt-siRNA-wt-Rz chimeric construct (Fig. 8a).

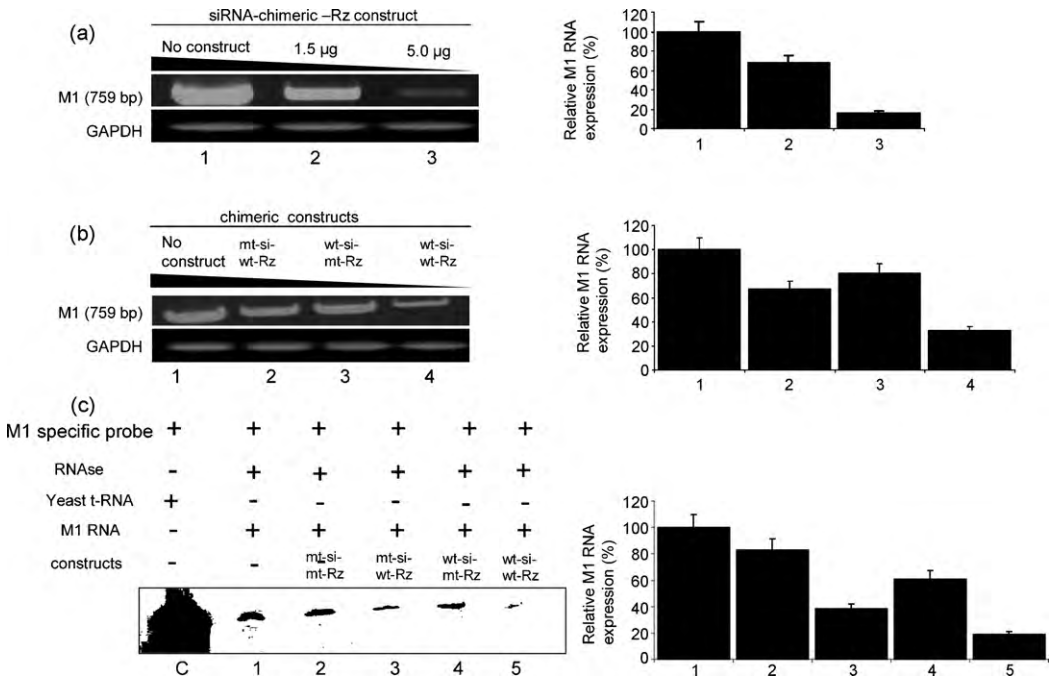

Fig. 5. Intracellular reduction in expression of M1 RNA with chimeric constructs in MDCK cells. RNA level in MDCK cells were shown by RT-PCR and RPA. (a) MDCK cells were transfected with 500 ng of M1-pcDNA3.1 along with 5 μg of unrelated RNA (lane 1); along with 1.5 and 5 μg of siRNA-chimeric-Rz construct (lanes 2 and 3, respectively). Total cell RNA was isolated 24 h post-transfection using Trizol reagent. They were divided into two equal parts. One set was used to estimate the levels of M1 RNA by RT-PCR and the other for estimating the housekeeping gene (huGAPDH). PCR was carried out using target specific primers. (b) The nature of the experiment is same as described in (a) except 2.5 μg of various chimeric constructs were used as indicated on top of the lanes. RT-PCR was carried out as described in (a). (c) MDCK cells were co-transfected with M1-pcDNA3 plasmid DNA with or without 5 μg of chimeric constructs as shown on top of each lane. 24 h post-transfection total cell RNA was isolated and subjected to RPA as described earlier (Goila and Banerjea, 2004). ^{32}P labeled antisense to M1 probe was generated by *in vitro* transcription of pGEM-T-Ez-M1 plasmid DNA using SP6 polymerase enzyme. Equal amount of yeast-tRNA was taken as control (lane 1). Quantification of the target RNAs is shown on the right side for each experiment. These are representative data from 3 separate experiments (mean ± SD).

The cells were harvested at 10, 24 and 72 h post-infection and analyzed by western blotting for the viral M1 protein. An efficient inhibition of M1 protein expression in the presence of wt-siRNA-wt-Rz construct till 72 h post-infection (Fig. 8b) was observed and reduced inhibition was shown in the presence of wt-siRNA-mt-Rz and mt-siRNA-wt-Rz constructs (Fig. 8c).

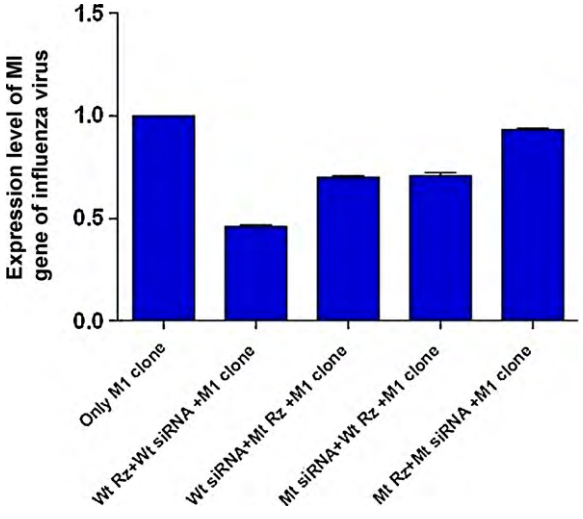

Fig. 6. Real time RT-PCR shows the modulation of expression level of M1 gene of influenza virus in the presence of various siRNA-Rz constructs. SYBR green-based real time RT-PCR was performed in triplicate and the level of M1 RNA in various experiments was compared by calculating ΔΔCt value for each experiment. The RNA level of M1 in cells transfected with only M1 clone was taken as the reference, the value of which was "1" and RNA levels in all other transfected cells were compared with it.

The cytopathic effect of the virus on the cell lines transfected with different chimeric constructs showed that the multiplication of the virus was efficiently inhibited in the presence of the constructs and maximum inhibition was observed in the presence of wt-siRNA-wt-Rz constructs (Fig. 9a–e).

4. Discussion

RNAi technology has been exploited against several RNA viruses including influenza A virus. Since influenza virus is prone to extensive mutation, targeting a single region in the genome of the virus by one antiviral approach may result in rapid generation of variants that may be resistant to the specific siRNA. Resistant viruses were generated in response to specific siRNA against HIV (Boden et al., 2003; Das et al., 2004). Earlier investigators have shown down regulation of influenza virus gene expression using mono-specific siRNA (Tompkins et al., 2004; Ge et al., 2003; McCown et al., 2003) or ribozyme (Lazarev et al., 1999). A bispecific siRNA construct against different genes of HIV was also reported previously (Anderson et al., 2003).

In the present study, we show that our novel siRNA-Rz chimeric construct against the M1 RNA interfered with influenza A virus gene expression very efficiently. It is noteworthy Rz-163 cleaved the target RNA in the presence of 1–2 mM $MgCl_2$ which is the cellular concentration (Santoro and Joyce, 1997). This property may have contributed to its high efficacy against the virus challenge. Further, we asked if it was possible to modulate the target gene expression or inhibit virus replication using selectively disabled versions of the chimeric construct. In order to achieve this objective it was important to establish that this novel wt chimeric RNA construct was processed correctly with nucleases present in the cytoplasmic extracts of a mammalian cell. This was impor-

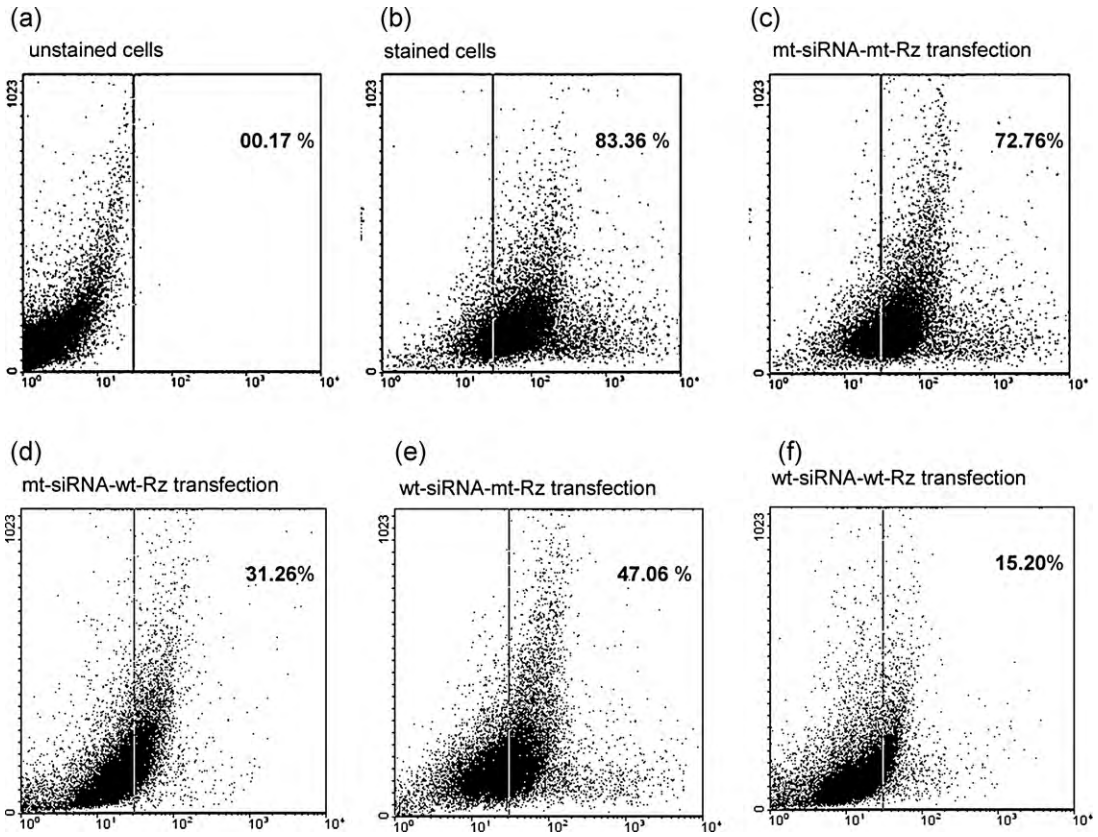

Fig. 7. Wild-type mutant constructs show inhibition of virus-specific immuno-fluorescence. Protection against influenza A/PR8/34 (H1N1) virus challenge using chimeric constructs was studied using MDCK cells. The various siRNA-chimeric-Rz constructs (5 μg/ml) were transfected as indicated at the top of each panel and then challenged with a fixed dose of virus (MOI of 0.1). The cells were harvested 10 h post-infection and viral protein levels were monitored by FACS analysis. (a–f) Represent the type of constructs used for transfection and the levels were monitored by FACS analysis.

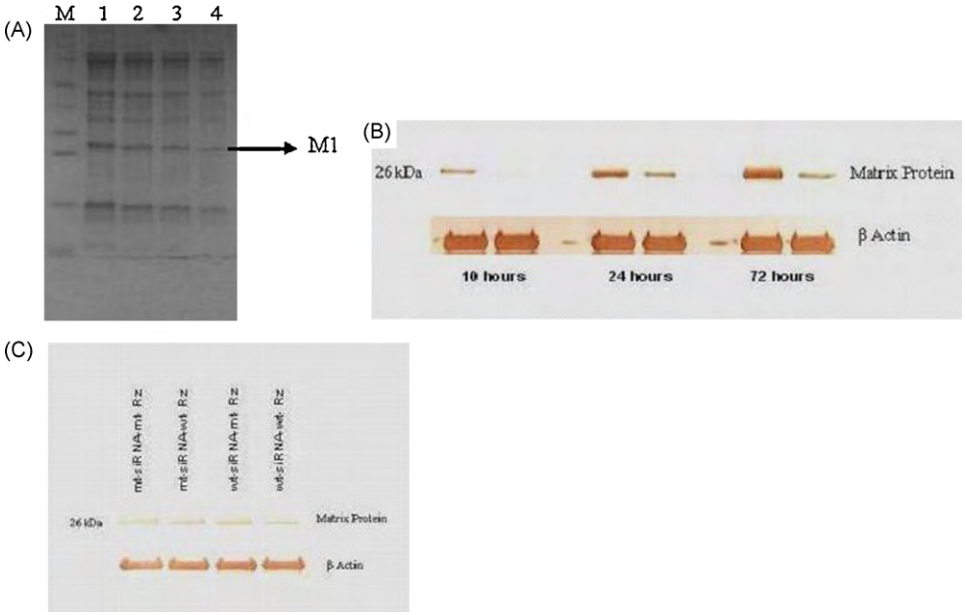

Fig. 8. The chimeric constructs show inhibition of viral protein synthesis in the infected cells: The various siRNA-chimeric-Rz constructs (5 μg/ml) were transfected in the MDCK cells seeded in six well plates followed by infection with influenza virus at a MOI of 0.1. (a) The culture supernatant were harvested at 24 h post-infection and analyzed by polyacrylamide gel electrophoresis (PAGE). M: Protein marker. Lanes 1–4: Virus proteins harvested from cells transfected with mt-siRNA-mt-Rz, mt siRNA-wt-Rz, mt-Rz-wt-siRNA and wt-Rz-wt-siRNA-chimeric construct, respectively. At 10, 24 and 72 h post-infection, the cells were harvested and the whole cell lysates were subjected to western blotting using primary goat monoclonal antibody against M1 protein of influenza A virus and horseradish peroxide conjugated bovine-anti-goat IgG as secondary antibody. (b) The expression level of viral M1 protein was analyzed in the presence of wt-siRNA-wt-Rz construct at 10 h (lanes 1 and 2), 24 h (lanes 3 and 4) and 72 h (lanes 5 and 6) post-infection. (c) The M1 protein expression was compared between the cells transfected with mt-siRNA-mt-Rz (lane 1), mt-siRNA-wt-Rz (lane 2), wt-siRNA-mt-Rz (lane 3) and wt-siRNA-wt-Rz (lane 4).

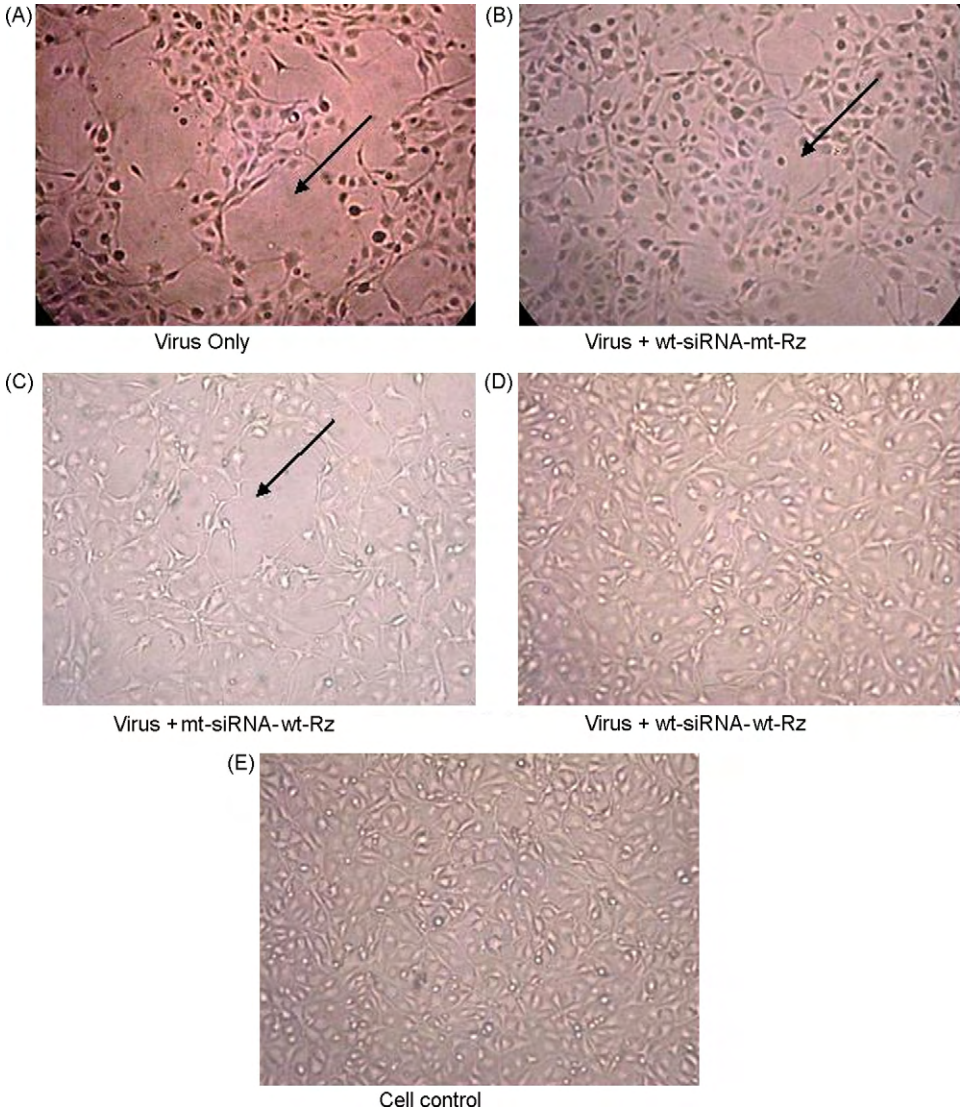

Fig. 9. Treatment of cell line with chimeric constructs inhibits the multiplication of virus (infected at a MOI of 0.1) and reduces its cytopathic effect (CPE). Arrow in the figures shows the CPE at 72 h post-infection. (a) Maximum CPE was observed in the infected cells that were not transfected with any construct. (b and c) CPE was inhibited in the infected cells transfected with partially mutated constructs viz. wt-siRNA-mt-Rz and mt-siRNA-wt-Rz. (d) The CPE in the infected cells transfected with wt-siRNA-wt-Rz construct was comparable to the control cells (e) that were not infected with the virus.

tant because the two antiviral components (the siRNA and the Rz components present in the chimeric construct) need to be separated from each other before they exert their ability to inhibit gene expression. The fact that most of the *in vitro* synthesized wt-chimeric RNA is cleaved specifically in the linker region in less than 5 min in the presence of cytoplasmic extracts (data not shown), strongly suggests that the same may be happening inside the mammalian cells also in a rapid manner. As expected, our wt chimeric construct interfered with the target gene expression or viral progeny production very significantly based on our virus challenge data.

The transfection studies with selectively disabled chimeric constructs allowed us to conclude that both siRNA and Rz components played an important role towards the inhibition of gene expression, the major role being played by the ribozyme. Our chimeric constructs which possessed wt-Rz catalytic motif retained the ability to cleave the target RNA specifically which was about 20% less than that was achieved with Rz-163 alone under *in vitro* conditions (data not shown). This is expected because of the large size of the catalytic RNA. It is very likely that after cleavage of the target RNA by either Rz or siRNA, the cleaved fragments become more susceptible to cleavage and get rapidly degraded intracellularly. Highly potent and synergistic activity of our wild-type chimeric construct may be attributed due to this activity.

It is important to note that siRNA against M1 gene was earlier shown to afford impressive protection against virus challenge only when high titers of lentiviral vectors were used (Hui et al., 2004). We could achieve similar levels of protection with our transient transfection approach using wild-type chimeric construct. It is noteworthy that we selected the siRNA target site immediately downstream of AUG of the target RNA which is usually more accessible towards siRNA-mediated down regulation. These chimeric constructs can be delivered to the desired cell via lentiviral vectors for achieving specific gene suppression as shown previously by one of us (Banerjea et al., 2004a,b).

Selectively disabled mutant versions may allow us to modulate the extent of target gene suppression and be exploited for variety of studies, especially for those genes that are temporally regulated. Although impressive inhibition of target gene expression was achieved with our chimeric construct, this still needs to be

tested against different virus strains with varying challenge doses and also against the previously described siRNAs.

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