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Inhibition of Hepatitis E virus replication using short hairpin RNA (shRNA)

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ABSTRACT

Hepatitis E virus (HEV) is a non-enveloped, single-stranded, positive sense RNA virus, which is a major cause of water-borne hepatitis. RNA interference (RNAi) is a sequence-specific cellular antiviral defence mechanism, induced by double-stranded RNA, which we used to investigate knockdown of several genes and the 3′ cis-acting element (CAE) of HEV. In the present report, shRNAs were developed against the putative helicase and replicase domains and the 3′CAE region of HEV. Production of siRNA was confirmed by northern hybridization. The possible innate response induction due to shRNA expressions was verified by transcript analysis for interferon-β and 2′,5′-oligoadenylate synthetase genes and was found to be absent. Initially, the selected shRNAs were tested for their efficiency against the respective genes/3′CAE using inhibition of fused viral subgenomic target domain–renilla luciferase reporter constructs. The effective shRNAs were studied for their inhibitory effects on HEV replication in HepG2 cells using HEV replicon and reporter replicon. RNAi mediated silencing was demonstrated by reduction of luciferase activity in subgenomic target–reporter constructs and reporter replicon. The real time PCR was used to demonstrate inhibition of native replicon replication in transfected cells. Designed shRNAs were found to be effective in inhibiting virus replication to a variable extent (45–93%).

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1. Introduction

Hepatitis E virus (HEV), a sole member of genus Hepevirus in the family Hepeviridae (Emerson et al., 2004), is associated with feco-orally transmitted (Balayan et al., 1983), sporadic and epidemic, self-limiting acute hepatitis occurring in many parts of the world (Panda et al., 2007; Vishwanathan, 1957). Several animal species like swine, wild boar and deer have been proved to be zoonotic reservoirs for HEV (de Deus et al., 2006; Schielke et al., 2009; Takahashi et al., 2004; Tei et al., 2003). Its ~7.2 kb genome, comprised of a single-stranded, capped, polyadenylated positive sense RNA is encapsidated so as to form a 27–34 nm virion (Aye et al., 1993; Kabrane-Lazizi et al., 1999; Reyes et al., 1990; Tam et al., 1991). To date four genotypes of HEV have been identified belonging to one serotype only (Schlauder and Mushahwar, 2001).

HEV RNA is organized into three open reading frames (ORFs) (Fig. 1). The 27–35 nt long 5′ non-coding region (NCR) is followed by the largest ORF (*ORF1*), which encodes the non-structural polyprotein (Ansari et al., 2000; Aye et al., 1993; Panda et al., 2000; Reyes et al., 1993). Computer-assisted analyses of HEV *ORF1* suggested the presence of methyltransferase, putative cysteine protease, putative RNA helicase and RNA-dependent RNA polymerase (RdRp) or replicase domains (Koonin et al., 1992). Replicase and methyltransferase activities have been experimentally demonstrated (Agrawal et al., 2001; Magden et al., 2001; Rehman et al., 2008). *ORF2* encodes the major viral capsid protein, which contains a signal peptide and potential glycosylation sites (Jameel et al., 1996; Zafrullah et al., 1999). *ORF3* encodes a 114 amino acid cytoskeleton-associated phosphoprotein that may be involved in host interactions (Huang et al., 2007; Zafrullah et al., 1997). The 3′ NCR is 65–74 nt long containing cis-acting element for replicase binding, and ends in a poly (A) tail (Agrawal et al., 2001).

Information available on HEV biology is mainly derived from transient transfection system and animal models (Nanda et al., 1994; Panda et al., 2000; Purcell and Emerson, 2001). The replication of HEV occurs exclusively in the cytoplasm (Rehman et al., 2008). An initial step in the virus replication involves early polyprotein translation to form functional proteins. The interaction of replicase with the 3′ cis-acting element of the virus and synthesis of complementary negative sense RNA is critical for the viral replica-

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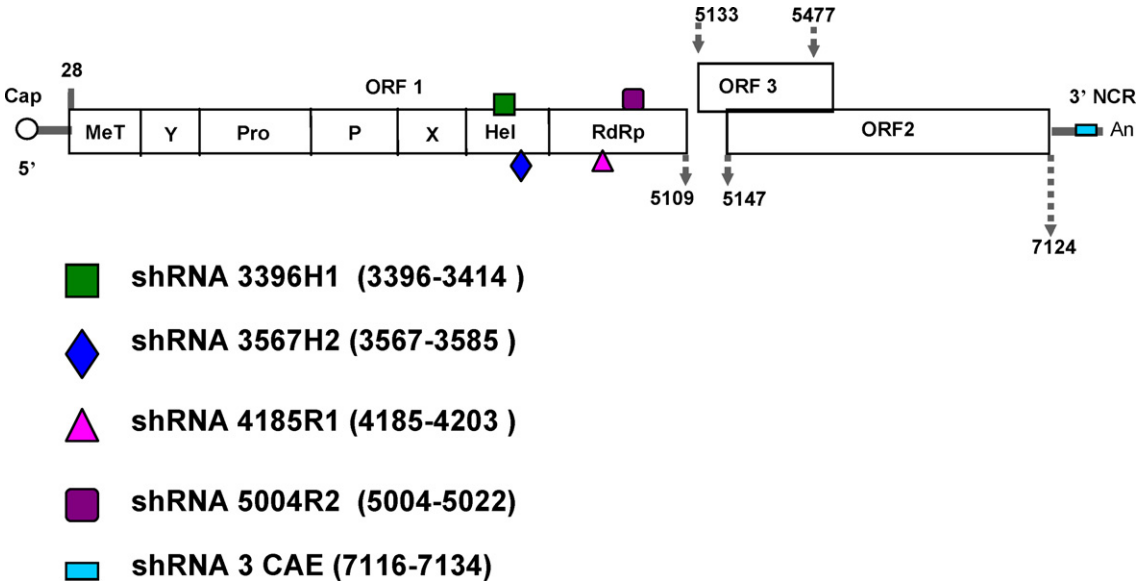

Fig. 1. RNAi targets and shRNA position on the HEV genome (genotype 1) (AF076239). Helicase, replicase (RdRp) and 3′ cis-acting element (CAE) were chosen as targets for RNAi. Position of shRNA is depicted by different geometric figures. Helicase and replicase are targeted with two shRNA constructs each.

tion (Agrawal et al., 2001). The replicase possibly binds at a putative subgenomic promoter of negative sense RNA to form bicistronic 3′ co-terminal subgenomic RNA that encodes both pORF2 and pORF3 (Graff et al., 2006).

RNA interference (RNAi) is a cellular endogenous system triggered by double-stranded RNA (dsRNA) leading to degradation of messenger RNA whose sequence is complementary to the small interfering RNA (siRNA) (Andino, 2003; Fire et al., 1998). Its unique features like ease to use, specificity and efficiency have made it a popular and efficient tool for gene silencing including inhibition of virus replication and growth (Hu et al., 2004; Stram and Kuzntzova, 2006).

HEV mostly causes a self-limiting illness. However, 2–3% mortality in general population (Krawczynski, 1993) and up to 20% in pregnant women has been observed (Khuroo et al., 1981). The virus has been found to be non-cytopathic and the disease is possibly immune mediated. It needs to be seen if inhibiting the virus replication thereby reducing the viral load can be of any help in preventing liver failure and death. Short hairpin RNA (shRNA) can be used to generate RNAi that can play such a role.

In the present study, we have investigated the inhibitory potential of shRNAs designed against non-structural genes and 3′ cis-acting element of HEV genome. This can help in understanding virus biology and could be useful in future therapeutics.

2. Materials and methods

2.1. DNA constructs

2.1.1. shRNA constructs

siRNA against Hepatitis E virus were designed using the online software siRNA converter at www.ambion.com (Fig. 1). Designed siRNA were checked for their annealing site in the target RNA using software RNA structure version 4.2 (Mathews, Zuker and Turner). siRNAs which optimally annealed to the target RNA by *in silico* analysis were selected. siRNA were converted into shRNA cassette by adding a loop (9 nt) and appropriate sites for cloning as per standard guidelines. Out of 179 designed siRNA, 5 siRNA were chosen and synthesized as DNA oligonucleotides on an automated oligonucleotide synthesizer to produce respective shRNA (Applied Biosystem Model 392B) using phosphoramidite chemistry (Table 1). Apa I and EcoR I sites were added at the 5′ and 3′ ends in such a way that they anneal to form terminal adapter for Apa I and EcoR I sites for cloning into the vector pSilencer 1.0-U6 (Ambion). Each upper and lower oligos were phosphorylated using T4 DNA kinase (Promega) as per the manufacturer's instructions, annealed and ligated to Apa I–EcoR I digested pSilencer 1.0-U6 vector to generate the required clones.

2.1.2. Target constructs for shRNA

The infectious cDNA clone pSG1–HEV (GenBank accession no. AF076239) (Panda et al., 2000) was used for the generation of HEV constructs. All clones generated were sequence- and expression-confirmed.

2.1.2.1. HEV helicase–replicase–renilla luciferase in-frame fusion construct (pSG1–(Hel–Rep)–Rluc). Renilla luciferase (*Rluc*) gene from pRnull vector (Promega) and 2827–5106 nt of HEV (helicase–replicase region) were PCR amplified with Pfu Turbo DNA polymerase (Stratagene) using their respective primer pairs with engineered restriction sites separately (Table 2). The *Rluc* gene was cloned into EcoR V site of pcDNA3 (Invitrogen) and subsequently released using Nhe I and Xho I enzymes. A three-way ligation was setup between EcoR I/Nhe I digested helicase–replicase amplicon, *Rluc* fragment generated by digestion with Nhe I/Xho I of the pcDNA3–Rluc described above, and linearized pSG1 vector purified after digestion with EcoR I and Xho I, creating an in-frame fusion construct pSG1–(Hel–Rep)–Rluc.

2.1.2.2. HEV 3′ end cis-acting element (CAE)–renilla luciferase in-frame fusion construct (pSG1–3′CAE–Rluc). HEV 3′ end (nt 7086–7194A_5) was released from pSG1–HEV (AF076239) using Sac I and Xho I enzymes and was subsequently cloned into equivalent sites of pSG1 to produce the pSG1–3′CAE construct. The *Rluc* gene was amplified from pRnull vector using a primer pair in which the forward primer has engineered Xho I site (Table 2). pSG1–3′CAE–Rluc construct was subsequently produced using standard cloning methods, such that the RNA transcribed shall express Rluc.

2.1.2.3. Full length HEV reporter replicon with in-frame fused Rluc in ORF2 region (pSG1–HEVRluc). The *Rluc* gene from pRnull vector was released by digestion with Nhe I and Xba I enzymes and subsequently end polished with Klenow enzyme (Invitrogen) as

Table 1
Sequences of shRNA used in study: constructs, nucleotide positions and their representative names. Only sense strand of each shRNA is shown.

shRNA constructs	Name	Position	Sequence
pSilencer1.0-U6 Helicase1	3396H1	3396–3414	5′ C**ACTAGTGTTCACCCAGGCG***TTCAAGAGA***CGCCTGGGTGAACACTAGT**TTTTTTG 3′
pSilencer1.0-U6 Helicase2	3567H2	3567–3585	5′ C**GTGGGTCATCATTGACGCA***TTCAAGAGA***TGCGTCAATGATGACCCAC**TTTTTTG 3′
pSilencer1.0-U6 Replicase1	4185 R1	4185–4203	5′ C**CAAGTTCACCACAGGTGAG***TTCAAGAGA***CTCACCTGTGGTGAACTTG**TTTTTTG 3′
pSilencer1.0-U6 Replicase2	5004R2	5004–5022	5′ C**CCTGATTGGCATGCTACAG***TTCAAGAGA***CTGTAGCATGCCAATCAGG**TTTTTTG 3′
pSilencer1.0-U6 3′CAE region	3CAE	7116–7134	5′C**CTCGGGAGTTATAGTTTAT***TTCAAGAGA***ATAAACTATAACTCCCGAG**TTTTTTG3′
pSilencer1.0-U6 5′ UTR Enterovirus 70	EV70 123-U	123–141	5′ C**ACTAATGTTCAACAGGAGG***TTCAAGAGA***CCTCCTGTTGAACATTAG**TTTTTTTG 3′

shRNA's name given in the Table are used throughout the paper. Bold letter indicates the sequence of passenger and guide siRNA strand. Italized letter indicates the loop sequence of short hairpin RNA.

per the Manufacturer's guidelines. The end polished *Rluc* fragment was ligated using T4 DNA ligase (Promega) to Hind III digested (nt 5679) and end polished pSG1–HEV (AF076239) creating full-length fusion construct pSG1–HEVRluc construct, with *Rluc* gene in-frame with ORF2 at 5679 nt position. Clones were sequence-confirmed and expression of *Rluc* and pORF2 was checked in HepG2 cells by renilla luciferase assay (Promega) and indirect Immunofluorescence (Thakral et al., 2005) before use.

2.1.2.4. In vitro production of full length capped transcripts. pSG1–HEV and pSG1–HEVΔ (4010–5141) (RdRp deficient mutant) (Thakral et al., 2005) were digested with Xho I to produce linear DNA templates for generating full length HEV run-off transcripts (Thakral et al., 2005). Linearized plasmids were *in vitro* transcribed using mMESSAGE mMACHINE T7 kits (Ambion) as per the manufacturer's instructions, to produce, capped and polyadenylated full length HEV RNA. The input DNA was removed by DNase (Ambion) treatment thrice and the product was cleaned using phenol–chloroform extraction. The removal of remnant plasmid template was checked both on a formaldehyde–agarose gel and by PCR using primers 4554 F: 5′ GCTAGGGTTTTGGAAGAAACACTC 3′ and 4705 R: 5′ ACTCACTGCAAAGCACTATCGAAT 3′ (152 bp) (data not shown).

2.2. Cell culture and transient transfections

Human hepatoma cell line (HepG2), was maintained in Dulbeco's Modified Eagle's Medium (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (Invitrogen), 100 U penicillin ml^{-1}, 10 μg streptomycin ml^{-1} and 25 μg amphotericin B ml^{-1} (Sigma–Aldrich). Cells were maintained in an atmosphere of 5% CO_2 in 37 °C incubator (Leec, UK). Cells plated in 35 mm or 60 mm petri dishes (Corning) at a confluence of 60–80%, were used for transfections. Transfections were performed by either using Lipofectamine 2000 reagent (Invitrogen) or FuGENE 6 (Roche, Germany) according to the Manufacturer's instructions. FuGENE 6 was used for native full length and its derivative reporter replicon, whereas all other transfections were performed using Lipofectamine 2000 reagent.

Table 2
Primer pairs used for amplification of Rluc gene and HEV helicase–replicase region.

Primer name	Restriction sites	Sequence
294-FP-RlucX	Xho I	5′ TATAGGCTACTCGAGATGACTTCGA 3′
294-FP-RLucN	Nhe I	5′ CTAGCTAGCATGACTTCGAAAGTTTA 3′
1263-RP-Rluc		5′ TCAATGTATCTTATCATGTCTGCTC 3′
HEV-2827 F	EcoR I, Xho I	5′ ATACTCGAGGAATTCGCAATGACAACAGC-GAATCT 3′
HEV-5106 R	Nhe I	5′ CTAGCTAGCCCATTCCACCCGACACA 3′

2.3. Optimization of expression constructs for shRNA mediated inhibition

Briefly, different amounts of column purified (Qiagen plasmid mini kit, Germany) plasmid DNA constructs; pSG1–(Hel–Rep)–Rluc, pSG1–3′CAE–Rluc and pSG1–HEVRluc (1–4 μg: 1 μg, 2 μg, 3 μg and 4 μg) were transfected individually along with 50 ng of pcDNA3–Fluc into HepG2 cells. The constructs containing domain specific regions were tested for luciferase expression at 48 h (pSG1–(Hel–Rep)–Rluc and pSG1–3′CAE–Rluc). The full length HEV reporter replicon (pSG1–HEVRluc) was tested at 72 h. Both firefly and renilla luciferase activities were measured using dual luciferase assay kit (Promega) as suggested by the manufacturer. The Fluc reading was used to normalize transfection efficiency.

Similarly, the shRNA expression were studied by inhibiting subgenomic and genomic reporter constructs expression using their respective optimized concentrations and 1–4 μg of shRNA constructs (3396H1, 3567H2, 4185R1, 5004R2 and 3CAE). The luciferase activities were measured at 48 h for (pSG1–(Hel–Rep)–Rluc, pSG1–3′CAE–Rluc) and 72 h for (pSG1–HEVRluc) constructs. The best concentration of shRNAs was found at 3 μg level per 35 mm petri dish (Corning).

2.4. Processing of shRNA into siRNA: northern hybridization

HepG2 cells were transfected with 3 μg of each shRNA constructs individually (3396H1, 3567H2, 4185R1, 5004R2 and 3CAE). A shRNA against Enterovirus 70 5′UTR (EV70 123-U) (a kind gift from Prof. Gita Sathpathy) was used as an unrelated control. Mock-transfected HepG2 cells were used as negative control. Transfections were carried out in 60 mm petri dishes. Total RNA was isolated 72 h post-transfection using Trizol reagent (Invitrogen).

For northern blot analysis, 30 μg of total cellular RNA from the above cells were resolved on 15% urea (8 M)-PAGE. Before loading onto the gel, RNA was denatured at 95 °C for 5 min and immediately placed on ice. After electrophoresis gel was stained with ethidium bromide (2 μg ml^{-1}). Gel picture was taken to ensure that the quality and quantity of RNA has been maintained. Subsequently the bands were transferred to positively charged nylon membrane (Hybond-XL, Amersham Pharmacia Biotech) by electroblotting, carried out at 60 mA for 1 h (0.65 mA/cm^2). Further, the RNA was cross-linked to the membrane under UV light (1200 μJ × 100) using Stratalinker (Germany). Pre-hybridization was carried out for 6 h

Table 3
Sequence and position of probe used in northern blotting.

Probe	Position	Sequence
3396 H1	3396–3414	5′ ACTAGTGTTCACCCAGGCG 3′
3567 H2	3567–3585	5′ GTGGGTCATCATTGACGCA 3′
4185 R1	4185–4203	5′ CAAGTTCACCACAGGTGAG 3′
5004 R2	5004–5022	5′ CCTGATTGGCATGCTACAG 3′
3CAE	7116–7134	5′ CTCGGGAGTTATAGTTTAT 3′

in 6× SSC, 5× Denhardt solution and 0.5% SDS at 68 °C followed by hybridization at 38 °C for 24 h in 20 ml of 6× SSC, 0.5% SDS in the presence of γ^{32}P-ATP (Board of Radiation and Isotope Technology, BARC, India) end-labelled oligonucleotides complementary to antisense strand of siRNA (Table 3). All five probes were prepared separately and hybrized to the blot individually. Finally, pictures were compiled together. After hybridization, the blot was given three washes of 6× SSC, 0.5% SDS at 38 °C for 15 min followed by one wash at 42 °C. Post washes, the blot was exposed to X-ray film (Kodak XO-MAT AR) and kept at −70 °C for 5–7 days and signals were detected using autoradiography (Fig. 2).

2.5. Reverse transcription-PCR (RT-PCR) assay for interferon-β (INF-β) and 2′,5′-oligoadenylate synthetase (OAS) genes

HepG2 cells were individually transfected with shRNA constructs (3 μg each: 3396H1, 3567H2, 4185R1, 5004R2 and 3CAE) using Lipofectamine 2000 (Invitrogen). The 320 mer ssRNAs were produced by *in vitro* transcription of linearized forward and reverse clones of pGEMTeasy-HEV (6875–7194 nt) using mMESSAGE mMACHINE T7 kit (Ambion). These complementary RNAs (320 mer ssRNA) were annealed to form dsRNA (320 bp) which was transfected into HepG2 cells to serve as a positive inductor of innate responses and native HepG2 cells served as a negative control. Cells treated with Lipofectamine 2000 but without shRNA construct (Mock transfection) was used as control to determine the effect of lipofectamine 2000 on innate response machinery. The pSilencer 1.0-U6 was transfected as plasmid control. The endogenous, β-actin mRNA was amplified as internal control using primer pairs mentioned below.

Briefly, total RNA, isolated at 24 h post-shRNA transfection, from the cells using Trizol reagent (Invitrogen), was used for RT-PCR assay. RNA (2 μg) was reverse-transcribed into cDNA with Superscript II RT (Invitrogen) using reverse primers specific for interferon-β (INF-β), 2′,5′-oligoadenylate synthetase (OAS) and β-actin (endogenous control). IFN-β-F: 5′ GATTCATCTAGCACTGGCTGG 3′; IFN-β-R: 5′ CTTCAGGTAATGCAGAATCC 3′ (186 bp) (Liu et al., 2007), OAS-F: 5′ AGTGCATCTTGGGGGAAAG 3′; OAS-R: 5′ CATTACCCTCCCATCAGGTGC 3′ (302 bp) and β-actin-F: 5′ GACTACCTCATGAAGATCCTCAC 3′; β-actin-R: 5′ ATTGCCAATGGTGATGACCTG 3′ (197 bp) (Liu et al., 2007). The RT product (2 μl) was used to perform DNA PCR for the above gene using their respective primer pairs mentioned above. The PCR products were analyzed on a 1.5% agarose gel and visualized after staining with ethidium bromide (Fig. 3).

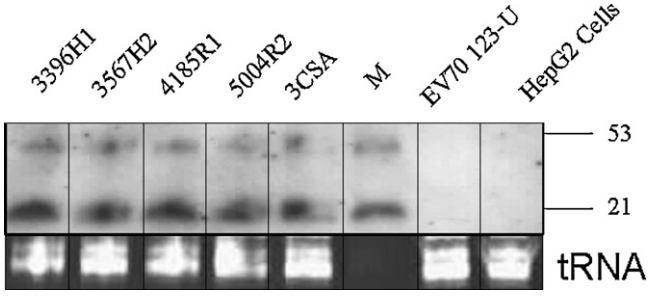

Fig. 2. Detection of siRNA production from shRNA by northern blotting. Northern analysis of transiently transfected shRNA constructs (3396H1, 3567H2, 4185R1, 5004R2 and 3CAE) in HepG2 cells, probed for antisense siRNA strand. Mock-transfected HepG2 cells and cells transfected with shRNA EV70 123-U were treated as negative and unrelated control. As size reference radiolabeled 21- and 53-mer oligos were used. Ethidium bromide staining of tRNA and other small ribosomal RNA band served as sample loading control.

2.6. Testing for anti-HEV potential of the shRNAs

All experiments were performed in triplicate and data were expressed as mean ± SD.

2.6.1. Effect of shRNA against helicase and replicase region by inhibition of Rluc expression using pSG1–(Hel–Rep)–Rluc construct

To analyse inhibitory activity of the RNAi induced from shRNAs (3396H1, 3567H2, 4185R1, 5004R2 and 3CAE), the decrease in luciferase activity was studied using co-transfection with in-frame reporter fusion constructs described in Section 2.1.2.1.

Briefly, HepG2 cells were transfected with a mixture of 2 μg of pSG1–(Hel–Rep)–Rluc plasmid and 3 μg of each short hairpin RNA expression constructs individually (3396H1, 3567H2, 4185R1 and 5004R2) and 50 ng of firefly luciferase expression plasmid (pcDNA3–Fluc). shRNA 3CAE as well as EV70 123-U were also included as unrelated controls. EV70 123-U is a pre-tested shRNA designed against 5′UTR of Enterovirus 70 (D00820). Its potency has been tested against pcDNA3–EV70-(1–150 nt)–Fluc construct using dual luciferase assay. It has been found to be 80% effective in silencing target transcript in Hep2 cells (unpublished data). Mock-transfected HepG2 cells were used as negative control. HepG2 cells, transfected with 2 μg of pSG1–(Hel–Rep)–Rluc construct, 3 μg of vector back bone (pSilencer 1.0-U6) and pcDNA3–Fluc (50 ng) were used as positive control.

After 48 h, cells were lysed, renilla and firefly luciferase activities were measured by dual luciferase kit (Promega) as per the Manufacturer's instructions. The firefly luciferase activity was used for normalization. Relative luciferase activity produced by the construct (pSG1–(Hel–Rep)–Rluc) in the presence of vector backbone only (pSilencer 1.0-U6) was set as 100% (Fig. 4A).

2.6.2. Testing of shRNA 3CAE on pSG1–3′CAE–Rluc construct

HepG2 cells were transfected with 3 μg of shRNA 3CAE, 2 μg of pSG1–3′CAE–Rluc construct and 50 ng of pcDNA3–Fluc. shRNA 5004R2 and EV70 123-U were transfected individually as unrelated controls. pSG1–3′CAE–Rluc transfected with the empty vector (pSilencer 1.0-U6) was taken as positive control. Dual luciferase assay was performed after 48 h of transfection as mentioned in Section 2.6.1 (Fig. 4B).

2.6.3. Testing of shRNAs on HEV full length reporter replicon (pSG1–HEVRluc)

To determine the functional efficiencies of characterized shRNA against HEV full length reporter replicon (pSG1–HEVRluc), HepG2 cells were co-transfected with 4 μg of pSG1–HEVRluc, 50 ng of pcDNA3–Fluc and 3 μg of each shRNA expression constructs (3396H1, 3567H2, 4185R1, 5004R2 and 3CAE) individually using FuGENE 6 (Roche), where DNA:FuGENE 6 ratio was 1:3. As unrelated control shRNA EV70 123-U (3 μg) was included in the study. pSG1–HEVRluc was co-transfected with the vector back bone (pSilencer 1.0-U6) (3 μg) as a reference control for positive reporter activity. In order to determine the cumulative/additive inhibitory effect of shRNAs, 3396H1 (1.5 μg) and 3567H2 (1.5 μg) were transfected with 4 μg of pSG1–HEVRluc construct along with 50 ng of pcDNA3–Fluc. Percentage silencing was determined 72 h post-transfection using dual luciferase assay kit (Promega) (Fig. 5A).

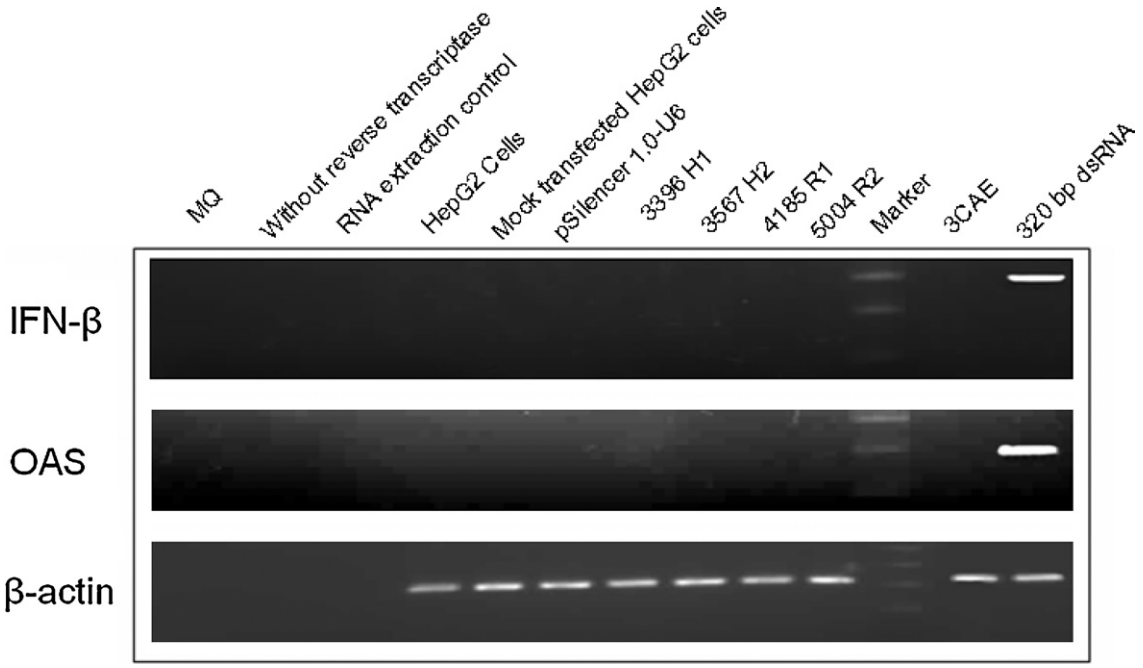

Fig. 3. Effect of shRNA on innate system. Total RNA was purified from HepG2 cells transfected with shRNA constructs (3396H1, 3567H2, 4185R1, 5004R2 and 3CAE). Interferon β (IFN-β), 2′,5′-oligoadenylate synthetase (OAS) expression levels were determined by RT-PCR, and products analyzed on 1.5% agarose gel. β-Actin mRNA expression served as an internal control. Appropriate PCR controls including a MilliQ water control, without reverse transcriptase and RNA extraction control were kept. Mock-transfected (Lipofectamine only) and empty vector (pSilencer 1.0-U6) were transfected in HepG2 cells to determine the effect of lipofectamine and plasmid on innate system. A 320 bp dsRNA was used as a positive control to induce innate responses. The 100 bp lane of the DNA ladder (Bangalore Genei) is shown as a size reference.

2.6.4. Inhibition of HEV replication using the validated shRNAs by real time PCR

In vitro transcribed capped, polyadenylated HEV RNA (AF076239) (3 μg) was transfected separately with each one of the shRNA constructs (3 μg each) i.e. 3396H1, 3567H2, 4185R1, 5004R2, 3CAE and EV70 123-U shRNA (as an unrelated control). The shRNA 3396H1(1.5 μg) and 3567H2 (1.5 μg) were used in combination against full length HEV RNA as well. pSG1–HEVΔ (4010–5141 nt) (RdRp deficient mutant) was used as a negative control for the replication. HEV RNA was co-transfected with the vector back bone (pSilencer 1.0-U6) (3 μg) as a reference control for replication. All the transfections were normalized using pcDNA3–Fluc construct (50 ng).

In the replication kinetics study, maximum positive sense:negative sense ratio was observed at 14 h (unpublished data). Therefore, at the end of 14 h post-transfection, the cells were washed thrice with PBS (pH 7.2) and harvested. Cell pellets were resuspended in 200 μl PBS. Forty microlitre of it was used for luciferase assay (Promega) as per the manufacturer's instructions, towards normalization of transfection efficiencies. Remaining 160 μl cell suspension was used for RNA isolation using Trizol reagent. The total RNA obtained was quantified spectrophotometrically at 260 nm. This RNA was subjected to strand-specific reverse transcription using 2714 RP 5′CCG ATCGGCACCTGGTATAT3′. Reverse transcription was carried out by using 2 μg of total cellular RNA with 50 U Superscript RT-III enzyme (Invitrogen) in a 20 μl reaction at 55 °C for 60 min. Standards for absolute quantification in real time PCR were made using serially diluted HEV transcripts (10^2–10^9) from pSG1–HEV clone and reverse-transcribed using primer 2714 RP (mentioned earlier). Following cDNA synthesis, the RNA was digested with 2.5 U RNase H (Ambion) and 0.75 μg RNase A (Ambion) at 37 °C for 30 min. Five microlitre of the RT-product was used in a 50 μl cDNA amplification reaction with 300 nM of 2589 FP 5′CGCCCCTGATTATAGGTTGGA3′ and 2704 RP (mentioned earlier) using SYBR Green PCR Core Reagents (1.25 U AmpliTaq Gold, 0.5 U AmpErase) (PE Biosystems, UK). The reactions were set up in MicroAmp Optical 96-well reaction plate (PerkinElmer Applied Biosystems) sealed and cycled on PerkinElmer ABI Prism 7700 Sequence Detection system (Applied Biosystems) with 40 cycles of annealing, extension (60 °C for 1 min) and denaturation (95 °C for 1 min) (Fig. 5B).

3. Results

3.1. Design and testing of anti-HEV shRNAs

Initially, siRNAs were designed *in silico* to cover the entire genome of HEV using the online software described in Section 2.1.1. Five of these were chosen, as they did not share significant homology with any other gene in the Genbank (Fig. 1). These were converted into shRNA by adding a nine nucleotide loop. These shRNAs were designed, synthesized, cloned and sequence-confirmed before use. We confirmed their processing into siRNA by northern hybridization (Fig. 2). To ensure equal loading, gel was stained with ethidium bromide, tRNA and other small RNA were visualized (Fig. 2). For detection of siRNA, 19-mer oligo complementary to the antisense strand of designed siRNAs were used. Radiolabelled 21 mer and 53 mer oligos were used as size markers. Conversion of shRNA to the ~21 mer siRNAs were detected in cells transfected with the corresponding HEV–shRNA constructs (Fig. 2). The bands of the precursor shRNAs were much fainter in the autoradiograph, indicating rapid and efficient processing of shRNA into siRNA (Fig. 2). To ensure the specificity of the shRNA, EV70 123-U was used as unrelated control. No signals were observed in the unrelated and mock-transfected cells (negative control) (Fig. 2).

3.2. Interferon response is not triggered by the shRNAs used in this study

It is well known that innate response can be activated by long dsRNAs (Daly and Reich, 1993). Therefore, before quantifying antiviral potential of shRNAs, we carried out RT-

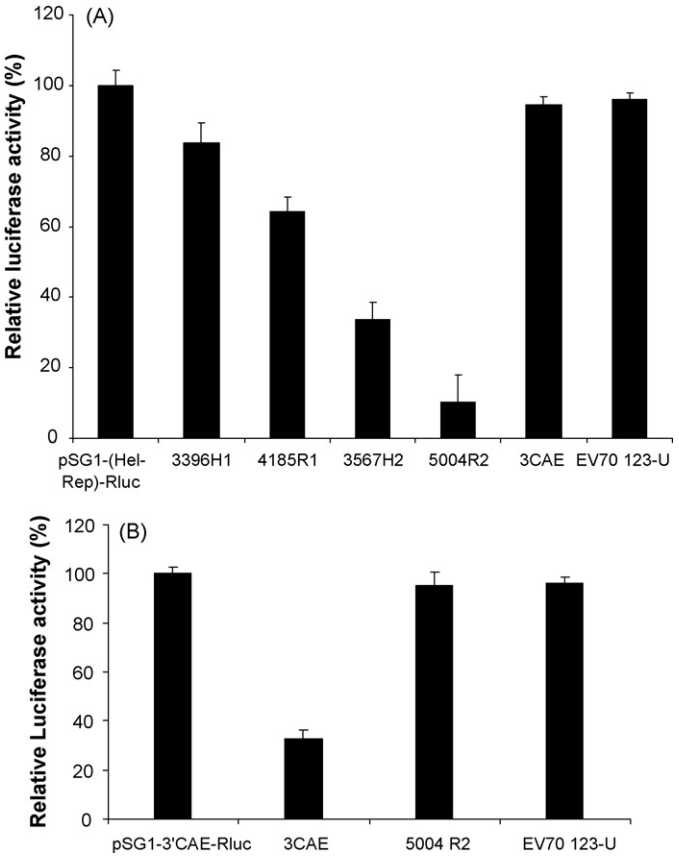

Fig. 4. Testing of shRNA against various genes and 3′ end cis-acting element of Hepatitis E virus. (A) Graph showing the effectiveness and specificity of designed shRNAs (3396H1, 3567H2, 4185R1 and 5004R2) against helicase and replicase regions of HEV. HepG2 cells were co-transfected with pSG1–(Hel–Rep)–Rluc construct, pcDNA3–Fluc (for normalization) and separately with each shRNA constructs (3396H1, 3567H2, 4185R1 and 5004R2). Transfections of pSG1–(Hel–Rep)–Rluc construct with vector backbone (pSilencer 1.0-U6) and shRNA 3CAE and EV70 123-U served as reference and unrelated controls respectively. At 48 h post-transfection luciferase activities were determined by dual luciferase assay. Average and standard deviations represent three independent transfections. (B) Graph showing the effect of shRNA 3CAE against target pSG1–3′CAE–Rluc. HepG2 cells were co-transfected with pSG1–3′CAE–Rluc construct, pcDNA3–Fluc (for normalization) and shRNA 3CAE. Transfections of pSG1–3′CAE–Rluc construct with vector backbone (pSilencer 1.0-U6) and shRNA 5004R2 and EV70 123-U construct served as reference and unrelated controls, respectively. Average and standard deviations represent three independent transfections.

PCR analysis of IFN-β and OAS to rule out any innate response.

The ethidium bromide (EtBr) stained agarose gels showed absence of amplification products from these genes in total cellular RNA isolated from shRNA transfected cells (Fig. 3), whereas cells transfected with 320 bp dsRNA, used as positive control showed amplification. β-Actin, used as internal control, showed similar amplification in all experiments and controls (Fig. 3). HepG2 cells transfected with Lipofectamine and plasmid (pSilencer 1.0-U6) in two different experiments were used as negative control and showed no amplification (Fig. 3).

3.3. Efficiency and specificity of shRNAs

After demonstrating that the designed shRNA against HEV was processed into siRNA without triggering innate immune response (Figs. 2 and 3), we proceeded to determine their silencing effect on reporter tagged subgenomic domain-specific constructs.

To test the silencing capacity of shRNAs, pSG1–(Hel–Rep)–Rluc (2.1.2.1) and pSG1–3′CAE–Rluc (2.1.2.2) constructs were used. shRNAs 3396H1, 3567H2, 4185R1 and 5004R2 were individually tested with pSG1–(Hel–Rep)–Rluc construct (Fig. 4A) and shRNA 3CAE was targeted to pSG1–3′CAE–Rluc construct (Fig. 4B). All the shRNA except for 3396H1 (~16%) showed significant inhibitory effect varying between 66 and 90% as determined by decrease in normalized renilla luciferase activity when compared with target–vector backbone (Fig. 4A and B). Unrelated and negative controls did not show any significant decrease in renilla luciferase activity (Fig. 4A and B).

3.4. Inhibition of HEV replication

Silencing efficiency is known to be associated with the local structure of RNA (Schubert et al., 2005; Shao et al., 2007). So, the characterized shRNAs either individually or in combinations were tested against the full length HEV reporter construct (pSG1–HEVRluc), expressing Rluc in-frame with *ORF2*. The full length HEV-reporter construct along with individual shRNA and pcDNA3–Fluc (normalization standard) were co-transfected and reporter signals were monitored 72 h post-transfection. Significant decrease in relative luciferase activity was observed with 5004R2 (~75%), 3567H2 (~70%) and 4185R1 (~58%) shRNA. Maximum (~82%) and minimum (~35%) inhibitions were observed with shRNA 3CAE and 3396 H1, respectively (Fig. 5A). To check the combinatorial effect of shRNAs in silencing the target (pSG1–HEVRluc), shRNA 3396H1 and 3567H2 were used together, and the maximum inhibition (~90%) was observed (Fig. 5A). shRNA EV70 123-U was included in the study as unrelated control and showed no significant inhibition (Fig. 5A).

3.5. Inhibition of HEV replication by shRNA using real time quantitative analysis

The shRNA constructs were finally tested for their silencing efficacy against the full length transcribed, capped, polyadenylated HEV RNA replicon (AF076239). The shRNA and replicon were co-transfected and viral RNA copy numbers were detected by real time PCR at 14 h post-transfection. A maximum decrease in copy number in cells transfected with shRNA 3CAE (~93%) was observed followed by 5004R2 (~88%), and 3567H2 (~88%). Significant silencing was observed with 4185R1 (~60%) (Fig. 5B). The least decrease in viral load was associated with shRNA 3396H1 (~45%) (Fig. 5B). Additive effect was observed when HEV replicon was targeted with both shRNAs i.e. 3396H1 and 3567H2 (~94%) (Fig. 5B). There was no significant decrease in HEV RNA copy number with the unrelated control (EV70 123-U) (Fig. 5B).

4. Discussion

The present study investigated the possibility of effective and significant inhibition of HEV replication using shRNA based siRNA strategy, targeting the helicase and replicase domains as well as the 3′ cis-acting element (3′CAE) of the HEV genome. We have previously demonstrated that replicase initiates negative sense replicative intermediate synthesis by recognizing specific secondary structures at the 3′ end of HEV (nt 7084–7194A_n) genome (Agrawal et al., 2001). Putative helicase domain was hypothesized to function in the unwinding of the positive and negative strand duplex formed during replication (Koonin et al., 1992). Therefore, the indispensable nature of the replicase and helicase proteins and 3′ cis-acting element in HEV replication cycle make their gene domains the most plausible target for siRNA mediated virus inhibition (Fig. 1).

RNAi has become a standard tool for sequence-specific inhibition of gene expression (Gomase and Tagore, 2008; Shrey et al., 2009). It can be induced by transfection of dsRNA in the form of

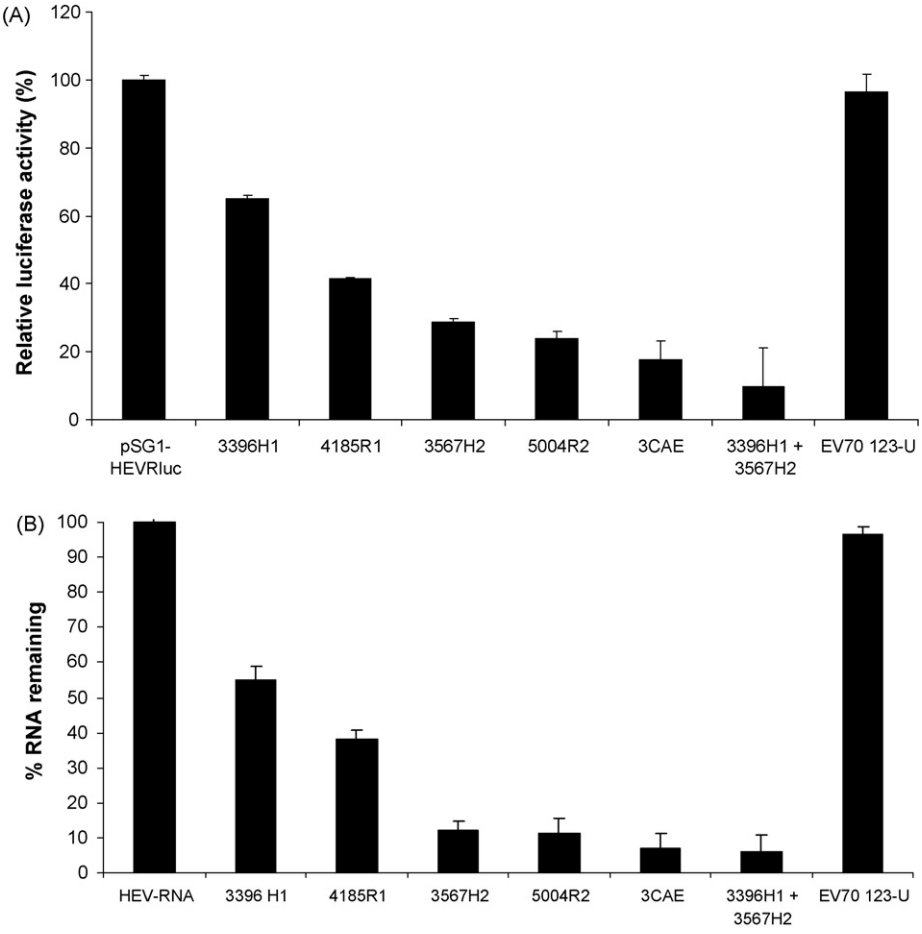

Fig. 5. Inhibition of HEV replication by shRNA constructs. (A) Effect of shRNA on HEV replication determined by monitoring the expression of ORF2 in-frame Rluc protein produced from pSG1–HEVRluc construct (indirect measure of HEV replication). HepG2 cells were co-transfected with pSG1–HEVRluc construct, pcDNA3–Fluc (for normalization) and individually with each shRNAs (3396H1, 3567H2, 4185R1, 5004R2 and 3CAE). shRNA 3396H1 and 3567H2 were also tested in combination against pSG1–HEVRluc. Transfections of pSG1–HEVRluc construct with vector backbone (pSilencer 1.0-U6) and shRNA EV70 123-U served as reference and unrelated controls, respectively. Average and standard deviations represent three independent transfections. (B) Graph showing the reduction of viral RNA by shRNA. HepG2 cells were transfected with HEV replicon, pcDNA3–Fluc (for normalization) and individually with each shRNA (3396H1, 3567H2, 4185R1, 5004R2 and 3CAE). shRNA 3396H1 and 3567H2 were also tested in combination against HEV RNA. Transfections of pSG1–HEV replicon with vector backbone (pSilencer 1.0-U6) and shRNA EV70 123-U served as reference and unrelated controls, respectively. Average and standard deviations represent three independent transfections.

synthetic siRNA, long dsRNA or shRNA expressed from eukaryotic expression vectors (Dykxhoorn et al., 2003). We opted for shRNA over other methods, for (i) being a promoter driven system, ensuring continuous production of siRNA processed from shRNA (Sui et al., 2002) (ii) not inducing IFN machinery at low doses (Zhang et al., 2004) (iv) its DNA nature makes it more stable as compared to synthetic siRNA (Rao et al., 2009b).

Initially, shRNA designed against HEV gene domains coding for helicase (3396H1 and 3567H2), replicase (4185R1 and 5004R2) along with one directed against the 3′ cis-acting element (3CAE), were targeted to the corresponding HEV genome fragments cloned with in-frame fused renilla luciferase gene. All shRNAs except for 3396 H1 were found to be effective in inhibiting the expression of corresponding viral gene/cis-acting element as observed by inhibition/reduction of reporter expression (Fig. 4A and B). We also demonstrated, processing of shRNA into siRNA and non-induction of cellular immunity (Figs. 2 and 3).

Replication of positive sense RNA viruses can be readily assessed with a viral replicon carrying the reporter gene in place of the viral structural gene or in-frame with the structural gene (Kanda et al., 2004; Khromykh, 2000). We developed the HEV renilla luciferase construct (pSG1–HEVRluc) and checked its expression in HepG2 cells. Such DNA-based constructs provide a constant source of full length HEV RNA which after exiting from the nucleus initiates the viral replication cycle via negative sense RNA synthesis followed by replication (Khromykh, 2000). shRNAs were targeted against this reporter–replicon construct (Fig. 5A).

However, questions still remains about the reliability of a DNA based replicon for a positive sense RNA virus. Such viruses including HEV mostly replicate in the cell cytoplasm (Rehman et al., 2008). Therefore, we validated our results of inhibition obtained from pSG1–HEVRluc using a native HEV RNA replicon (AF076239) by real time PCR (Fig. 5B). We produced capped and polyadenylated RNA replicon using *in vitro* transcription of linearized pSG1–HEV (Section 2.1.2.4). The question may be raised regarding residual plasmid DNA which may complicate our real time PCR results. Therefore in order to prove that only RNA replicon was transfected in those experiments, we eliminated residual plasmid DNA by three successive Dnase treatments (data not shown).

The inhibitory ability of individual shRNAs obtained using pSG1–HEVRluc (reporter replicon construct) were ranked similar to those obtained by using the native HEV replicon (Fig. 5). Therefore, the reporter replicon system developed by us can be used to study drugs that can have replication inhibitory activity on HEV. However, the reading of renilla luciferase gave an inhibition which was always less than that observed by real time PCR (viral RNA level). This may be due to differences in sensitivity of the assay, and the time points at which the results of these assays were recorded.

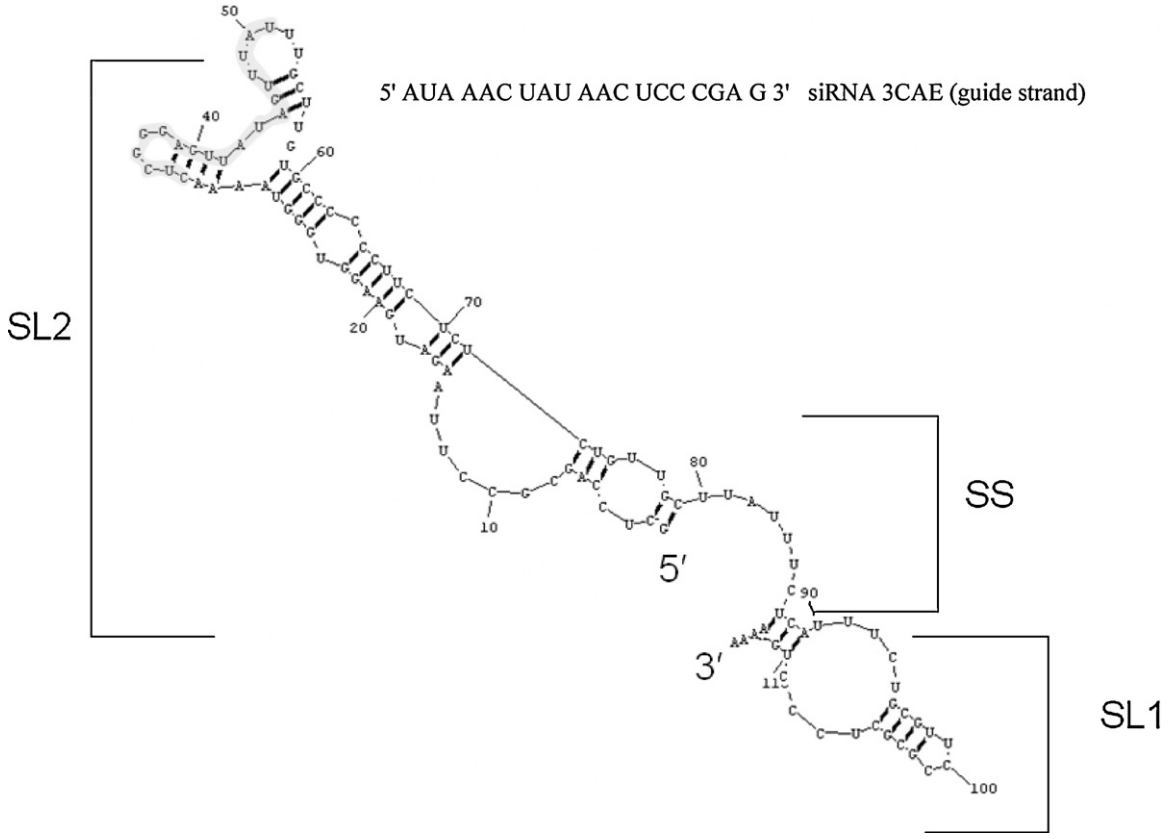

Fig. 6. Secondary structure prediction of the 3′ end of HEV (AF076239) using the software RNA structure version 4.2 (Mathews, Zuker and Turner). Annealing site of guide strand of siRNA 3CAE is highlighted in grey. Free energy of the predicted structure is 24.3 kcal/mol. Description of structure is given in the text. The SL1 = Stem Loop1, SL2 = Stem Loop2 and SS = single strand region.

RNAi efficiency is usually influenced by the local RNA structure of the target sequence (Schubert et al., 2005; Shao et al., 2007). The 3′ end of HEV forms a structural motif recognized by replicase (Agrawal et al., 2001). Our earlier structure probing experiments reveal that the 3′ end of HEV (nt 7084–7194A_n) forms characteristic stem loop structures SL1 and SL2 comprising nt 7173–7194 and 7089–7163, respectively, and the single strand region (SS) extends from 7164 to 7172 nt (Agrawal et al., 2001). It is known that 3′ end of the mRNA target sequence is initially recognized and bound by the RISC/siRNA complex. This is critical for siRNA efficiency (Haley and Zamore, 2004; Westerhout and Berkhout, 2007). Position of the annealing site of the guide strand of 3CAE siRNA on 3′ CAE of HEV shows a free 3′ end of the target which can be easily accessed by this siRNA (Fig. 6). Thus, the target site for 3CAE siRNA (indicated by grey area in Fig. 6) does not appear to be repressive. However, there have been successful RNAi targets even in the highly structured region of IRES of Hepatitis C virus (HCV) (Prabhu et al., 2006).

Not all the shRNAs investigated by us were found to be equally effective in inhibiting the virus replication (Figs. 4 and 5). All siRNA designs were based on the same algorithm provided by Ambion (http://www.ambion.com). This precludes any design-based explanation for the ineffectiveness of 3396H1 shRNA. The relative inefficiency may be contributed by binding of siRNA with RNA binding proteins (Dykxhoorn et al., 2003) as well as secondary and tertiary structure of the target RNA, which may restrict its accessibility to the RNAi complex (Kusov et al., 2006; Shao et al., 2007; Schubert et al., 2005).

We have observed that a combination of 3396H1 and 3567H2 shRNA is more effective in comparison to each of them individually (Fig. 5A and B). Error-prone replication of HEV by replicase may lead to viable escape mutants (Grandadam et al., 2004). Further selection of escape mutant under the influence of a single shRNA or siRNA is quite common (Boden et al., 2003; Wilson and Richardson, 2005). Therefore, the combination of shRNAs targeting various regions of virus can be useful in restricting evolution of escape mutants. However, instead of targeting a viral RNA by multiple individual shRNA constructs, one can use the combinatorial RNAi approach which will ensure the delivery of multiple shRNAs inside a single cell. There are several combinatorial means for producing multiple functional siRNAs derived from shRNA expressed in cell (Grimm and Kay, 2007a) e.g., multiple shRNA expression cassettes can be cloned in a single expression vector (ter Brake et al., 2006; Henry et al., 2006) or multiple siRNAs can be cloned like polycistronic microRNA transcript which will be processed to generate multiple siRNAs (Liu et al., 2008). Such combinatorial RNAi approaches have been successfully used against HIV-1 (Liu et al., 2009), HCV (Watanabe et al., 2006) and HBV (Weinberg et al., 2007).

During the preparation of this report, a study was published by Huang et al. (2009) showing effective inhibition of HEV replication in A549 lung carcinoma cells targeting the replicase region. The shRNA expressed from the DNA-based vector in the study of Huang et al. (2009) had a different target sequence in HEV replicase as compared to ours. However, our results are consistent with theirs. Further, our experiments have the significant advantage over Huang's study in that the helicase domain and 3′ cis-acting element of HEV are additionally targeted without eliciting an IFN response in HepG2 cells. The HEV sequence used in our study was genotype 1 which is more prevalent in India compared to the genotype 4 of HEV of swine used by Huang et al. (2009).

Present shRNA expression systems provide good target knockdown in experimental systems (Snøve and Rossi, 2006a). Only limited success has been documented in their *in vivo use*. The deliv-

ery of shRNA expression plasmid into the liver has been achieved by use of hydrodynamic tail-vein injection in mice (Wang et al., 2005) and by virosomes (Subramanian et al., 2009). But such approach is not possible or less efficient in case of other organs. Infective properties of viruses are used for the efficient tissue-specific delivery of shRNAs *in vivo* (Snøve and Rossi, 2006a). Both non-integrating viral vectors (adenovirus based vector, Huang et al., 2004) and integrating viral vectors (Oncoretrovirus derived vectors, Thomas et al., 2003) have been used to deliver the shRNA clone into desired target cells. Choosing these vectors depend upon the nature of the target cells/organ. Immunogenic nature of the viral proteins expressed from these vectors, competition of shRNA with the cellular micro-RNA machinery (Grimm et al., 2006) and off target effects they can initiate need to be addressed for shaping shRNA as a safe therapeutic modality. Immunogenic nature of these vectors, competition of shRNA with the cellular microRNA machinery (Grimm et al., 2006) and their off target effects need to be addressed for shaping shRNA into a safe therapy.

It is worth mentioning that overexpression of shRNAs can lead to the saturation of endogenous cellular RNAi machinery involved in microRNA transport across the nucleus and its processing (Grimm et al., 2006). Thus, there is need to optimize dose of shRNA to get maximum potency without saturating microRNA machinery (Snøve and Rossi, 2006b). Further, use of helper dependent adenoviral vector instead of adeno-associated viral vector for shRNA delivery, has reported to get significant silencing without altering levels of cellular microRNA (Grimm et al., 2006; Witting et al., 2008). microRNA saturation by shRNA expression can be avoided by the use of an RNA polymerase II driven shRNA expression constructs (Giering et al., 2008; Rao et al., 2009a). There are several advantages of using RNA polymerase II driven promoters. They are well studied, tightly regulated and can drive tissue-specific shRNA expression (Grimm and Kay, 2007b; Wiznerowicz et al., 2006). shRNA expression driven by RNA pol II leads to the production of polyadenylated shRNA which undergoes similar Drosha/DGCR8 processing in the nucleus, thus subjected to normal cellular regulatory process of nuclear transport and its loading to RNA-induced silencing complex in the cytoplasm (Rao et al., 2009a; Zeng et al., 2005).

Further, HEV is a self-limiting disease in most instances. Only recently persistent infection has been observed in renal and liver transplant recipients and other chronic immuno-suppressed individuals (Dalton et al., 2009; Haagsma et al., 2009; Kamar et al., 2008). Therefore, in case of HEV, transient suppression using shRNA should suffice. The main concern, however, remains toxicity and the effects on the host genes. All *in silico and in vitro* studies cannot really predict the *in vivo* effect in a human patient.

In conclusion, we have developed shRNA against helicase, replicase domains and 3′ cis-acting element of the HEV which strongly inhibit HEV replication in HepG2 cells without activating the IFN machinery. Thus, shRNA based RNAi can be a valuable tool for the study of viral pathogenesis and a safe antiviral therapy for combating HEV infection.

Acknowledgments

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