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Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs

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Small interfering RNAs (siRNAs) efficiently inhibit gene expression by RNA interference. Here, we report efficient inhibition, by both synthetic and vector-derived siRNAs, of hepatitis C virus (HCV) replication, as well as viral protein synthesis, using an HCV replicon system. The siRNAs were designed to target the 5′ untranslated region (5′ UTR) of the HCV genome, which has an internal ribosomal entry site for the translation of the entire viral polyprotein. Moreover, the 5′ UTR is the most conserved region in the HCV genome, making it an ideal target for siRNAs. Importantly, we have identified an effective site in the 5′ UTR at which ~80% suppression of HCV replication was achieved with concentrations of siRNA as low as 2.5 nM. Furthermore, DNA-based vectors expressing siRNA against HCV were also effective, which might allow the efficient delivery of RNAi into hepatocytes *in vivo* using viral vectors. Our results support the feasibility of using siRNA-based gene therapy to inhibit HCV replication, which may prove to be valuable in the treatment of hepatitis C.

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INTRODUCTION

Hepatitis C virus (HCV) is one of the main causes of liver-related morbidity and mortality. The virus establishes a persistent infection in the liver, leading to the development of chronic hepatitis, liver cirrhosis and hepatocellular carcinomas (Alter, 1997). A satisfactory treatment for HCV infection has yet to be developed because investigations of HCV have been hampered by the lack of a stable cell-culture system and of a small-animal model. An HCV replicon that has been reported recently is a selectable sub-genomic HCV RNA, which replicates efficiently and continuously in human hepatoma Huh7 cells (Lohmann *et al*., 1999). The development of the replicon system has allowed various molecular studies of HCV replication, host–cell interactions and antiviral strategies.

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing that is initiated by double-stranded RNA (dsRNA). RNAi is a multi-step process that involves the generation of small interfering RNA (siRNA), 21–23 nucleotides long, that results in degradation of RNA that is complementary to the siRNA (Sharp, 2001). In mammalian cells, however, this provokes a strong cytotoxic response, leading to the non-specific degradation of RNA transcripts and a general shutdown of host-cell protein translation (Baglioni & Nilsen, 1983; Williams, 1997). This problem has been overcome recently by using a synthetic siRNA that is long enough to mediate gene-specific suppression, but is short enough to evade the adverse effects of long dsRNAs (Elbashir *et al*., 2001a). RNAi has become a powerful tool for the analysis of gene function and has potential therapeutic applications. Recently, suppression of human immunodeficiency virus (HIV) and poliovirus replication by siRNA has been reported (Gitlin *et al*., 2002; Jacque *et al*., 2002). The successful use of siRNA in mammalian cells encouraged us to develop an siRNA expression vector (Miyagishi & Taira, 2002) and to apply RNAi to the exploration of anti-HCV strategies using the HCV replicon system as the target.

The HCV genome is a positive-stranded RNA that contains a single, long open reading frame that encodes structural and non-structural proteins. Translation of the viral genome is mediated by an internal ribosomal entry site (IRES), which is located in the untranslated region at the 5′ terminus (the 5′ UTR; Tsukiyama-Kohara *et al*., 1992). The HCV genome varies considerably between HCV strains. However, the 5′ UTR and the upstream portion of the core region are the most conserved parts of the genome, with a nucleotide identity of 99.6% (Choo *et al*., 1991; Okamoto *et al*., 1991). Because sequence mismatches between the siRNA and the target affect the efficiency of RNAi, the 5′ UTR would seem to be an ideal target for siRNA.

Here, we engineered siRNAs and DNA-based siRNA-expressing vectors to target HCV RNA, and evaluated the effects on viral replication using an HCV replicon system. We report that viral replication was inhibited successfully both by vector-derived siRNA and by an extremely low concentration of a synthetic siRNA that targets the conserved 5′ UTR of the HCV genome.

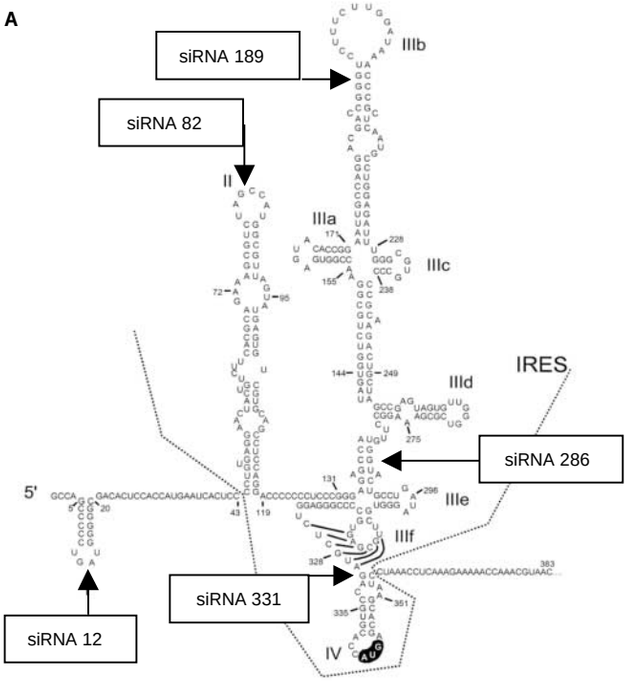

Fig. 1 | Design of small interfering RNAs and target constructs. (**A**) Predicted secondary structure of the 5′ internal ribosomal entry site (IRES) in the 5′ untranslated region (UTR) of the hepatitis C virus (HCV) genome (nucleotides 1–341) and target sites of small interfering RNAs (siRNAs; Brown *et al*., 1992). (**B**) Structures of the HCV IRES reporter and replicon plasmids. BGH pA, bovine growth hormone polyadenylation site; CMV P/E, cytomegalovirus early promoter/enhancer; ΔC, truncated HCV core region (nucleotides 342–377); EMCV, encephalomyocarditis virus; *FLuc*, firefly luciferase gene; *NPT*, neomycin phosphotransferase gene; *NS3*, *NS4*, *NS5A* and *NS5B*, genes that encode HCV non-structural proteins; pT7, T7 promoter; Rep-Feo, replicon that expresses a chimeric protein consisting of NPT and Fluc.

RESULTS

siRNAs directed against the HCV 5′ untranslated region

siRNAs were designed to target the 5′ UTR of HCV RNA (Table 1). The target sequences were directed to a single-stranded region, according to the secondary structure of the 5′ UTR predicted by Brown *et al*. (1992; Fig. 1A). Sequences of the form (AA/CA/GA)N_{19} (where N indicates any nucleotide) and with a GC content of less than 70% were selected from this region (Elbashir *et al*., 2001b). A guanine located after the 5′-AA/CA/GA is required for efficient RNA polymerase initiation when the siRNA is expressed in a DNA-based vector. The selected 19-nucleotide RNAs, followed by TT, were synthesized chemically.

Effect of siRNA oligonucleotides on pIRES–Fluc

First, we tested the HCV-directed siRNAs for their ability to suppress HCV IRES-mediated translation. An HCV IRES-reporter-gene vector, pIRES–Fluc, which expresses messenger RNA that consists of the HCV 5′ UTR and the upstream part of the core region (nucleotides 1–377), connected in-frame with the firefly luciferase (FLuc) gene (Fig. 1B), was used as the target. The pIRES–FLuc construct and the siRNA oligonucleotides were cotransfected into 293T cells. siRNAs 189 and 331 suppressed luciferase activity significantly in a dose-dependent manner in the range 2.5–125 nM

Table 1 | Sequences of small interfering RNAs used to target the 5′ untranslated region of hepatitis C virus RNA

Name	Sequence
siRNA 12	5′-gcccccgauuggggggcgacTT-3′
	3′-TTcgggggcuaaccccccgcua-5′
siRNA 82	5′-gcgucuagccauggcguuaTT-3′
	3′-TTcgcagaucgguaccgcaau-5′
siRNA 189	5′-ggacgaccgggguccuuucuTT-3′
	3′-TTccugcuggcccaggaaaga-5′
siRNA 286	5′-ggccuuugguacugccugTT-3′
	3′-TTccggaacaccaugacggac-5′
siRNA 331	5′-ggucucguagaccgugcacTT-3′
	3′-TTccagagcaucuggcacgug-5′
Control	5′-gcagcagcagcagcgggacTT-3′
siRNA	3′-TTcgucgucgucgucgcccug-5′
331-shuffle	5′-ucggggcacugcuagauccTT-3′
	3′-TTagccccgugacgaucuagg-5′
331-mutant	5′-ggucucguaugccgugcacTT-3′
	3′-TTccagagcauacggcacgug-5′

siRNA, small interfering RNA.
Uppercase letters indicate deoxyribonucleotides.

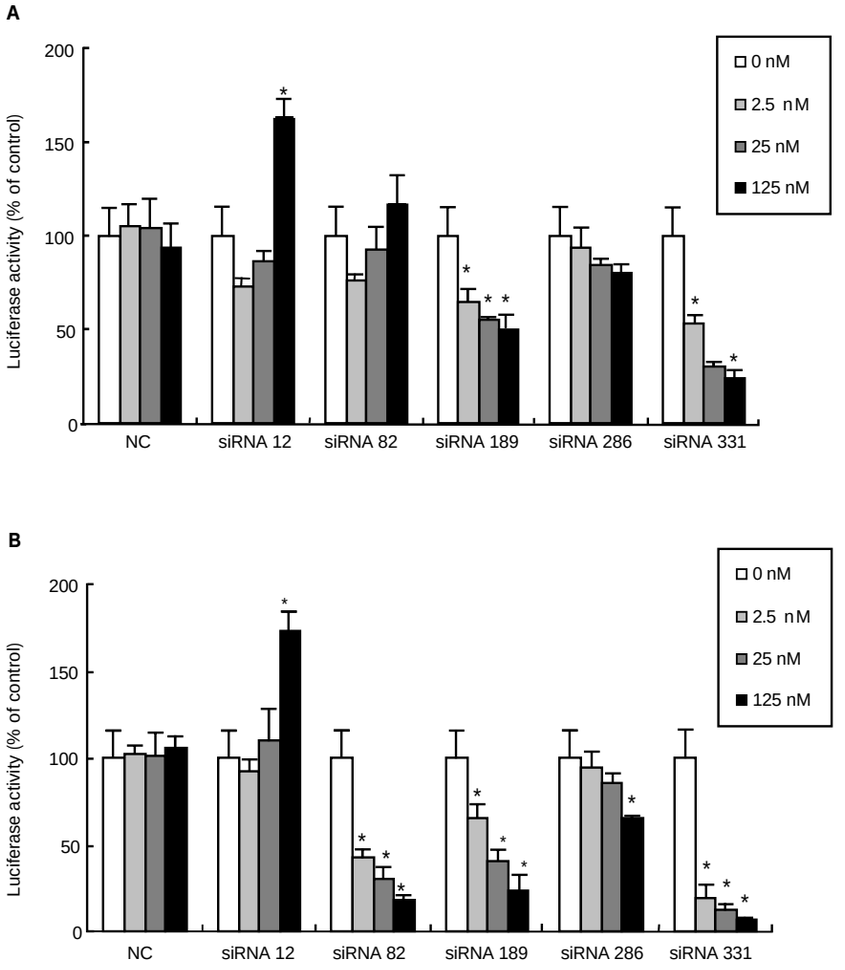

Fig. 2 | Effects of small interfering RNA oligonucleotides on the internal ribosomal entry site reporter and the hepatitis C virus replicon. (A) 293T cells were transfected with pIRES–FLuc, pRL-RSV and small interfering RNAs (siRNAs) at the concentrations indicated, or with a control siRNA (NC). The cells were harvested 48 h after transfection, and luciferase activities were measured. (B) Huh7 Rep-Feo cells were transfected with siRNA oligonucleotides or control siRNAs (NC). The internal luciferase activities were measured 48 h after transfection. Values are shown as percentages of the siRNA negative control, as the mean ± s.d. Asterisks indicate $p < 0.05$. FLuc, firefly luciferase; IRES, internal ribosomal entry site; pRL–RSV, *Renilla* luciferase expression plasmid; Rep-Feo, replicon that expresses a chimeric protein, consisting of NPT and Fluc.

(Fig. 2A). siRNA 331, which is directed against a region just upstream of the translation start codon, was the most effective, and decreased luciferase activity by 74% compared with the control. By contrast, siRNA 12, which was directed against helix 1 of the 5′ UTR, increased luciferase expression when used at 125 nM.

Effect of siRNA on HCV Feo-replicon cells

To assess the effects of the siRNAs on the intracellular replication of HCV, an HCV replicon was used as the target. We constructed a replicon that expresses a chimeric protein consisting of neomycin phosphotransferase (NPT) and Fluc (Rep-Feo; Fig. 1B). The fusion protein, which we called Feo, enables the selection of cells that continously carry the replicon, and also enables the quantification of replication levels by measuring luciferase activity. Transfection of the siRNA into Huh7 Rep-Feo cells, which stably express the HCV Feo replicon, showed that siRNAs 82, 189, 286 and 331 inhibited replication of the HCV RNA significantly in a dose-dependent manner (Fig. 2B). siRNA 82, which was ineffective against pIRES–FLuc, suppressed the replicon. Suppression profiles for luciferase expression obtained from assays using Huh7–Rep-Feo cells closely matched those obtained from the cotransfection of siRNA and pIRES–FLuc. The most effective siRNA, siRNA 331, suppressed luciferase activity by 81% at a concentration as low as 2.5 nM, and the suppression rate increased to 94% at 125 nM. The siRNAs suppressed the expression of the HCV replicon more potently than they did that of pIRES–FLuc. The levels of suppression of the replicon as compared with those of pIRES–Fluc, respectively, using 125 nM of the siRNAs were: 82% compared with 17% for siRNA 82; 77% compared with 50% for siRNA 189; and 94% compared with 75% for siRNA 331.

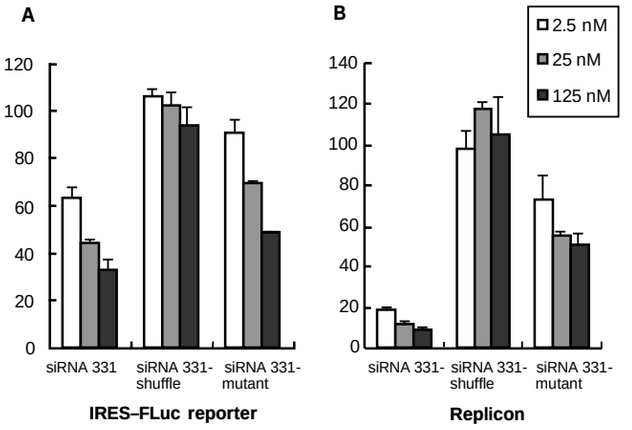

Fig. 3 | Reversal of suppression by negative-control small interfering RNAs. Two small interfering RNA (siRNA) controls were used to analyse further the effects of siRNA 331. The controls used were an siRNA with a shuffled sequence of siRNA 331 (331-shuffle) and an siRNA with mutations at the tenth and eleventh nucleotides from the 5′ end of the siRNA 331 sequence (331-mutant). siRNAs 331, 331-shuffle and 331-mutant were cotransfected with pIRES-Fluc into 293 cells (A) or were transfected into Huh7 Rep-Feo cells (B). Luciferase assays were performed 48 h after transfection. pIRES–FLuc, a reporter-gene vector that expresses messenger RNA consisting of the hepatitis C virus 5′ untranslated region and the upstream part of the core region, connected in-frame with firefly luciferase.

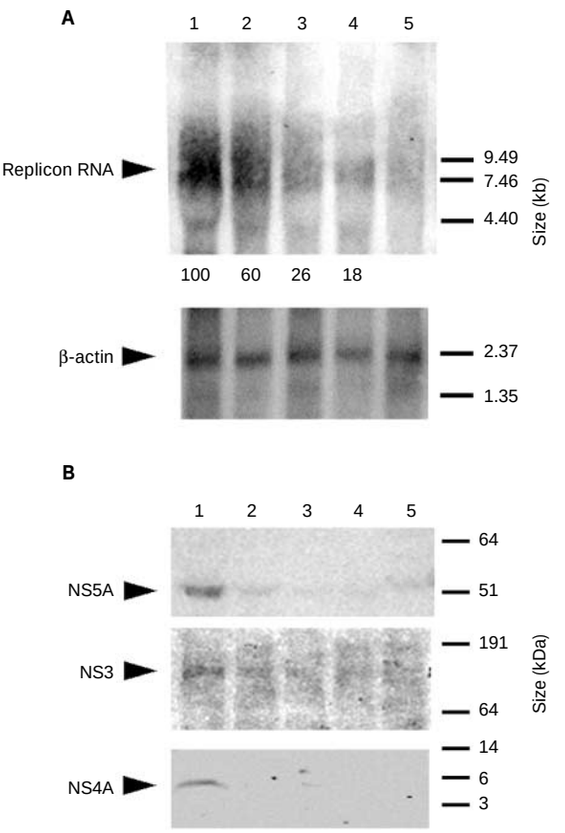

Fig. 4 | Suppression of replicon RNA and replicon-derived hepatitis C virus non-structural-protein synthesis by small interfering RNA 331. (A,B) Human hepatoma Huh7 Rep-Feo cells (which stably express the hepatitis C virus (HCV) Feo replicon) were mock-transfected (lane 1) or were transfected with 2.5 nM (lane 2), 25 nM (lane 3) or 125 nM (lane 4) of small interfering RNA (siRNA) 331. Lane 5, untransfected Huh7 cells. The cells were harvested 48 h after transfection. (A) Northern blotting of the HCV replicon and β-actin RNA. Numbers below the replicon fluorogram show the results of densitometric analysis shown as a percentage of the mock-transfected control. (B) Western blotting using the monoclonal anti-NS5A, anti-NS3 and anti-NS4A antibodies. Densitometry readings (as percentages of the mock-transfected control) for lanes 1–4 were 100, 30, 10 and 10, respectively, for NS5A, 100, 25, 56 and 43 for NS3, and 100, 4, 10, 0 for NS4A.

The control siRNA, which was unrelated to the HCV RNA sequence, did not have any inhibitory effects on the activity of the IRES–FLuc reporter or on the replication of the HCV replicon. For further analysis of the suppression at the most effective site (that targeted by siRNA 331), two additional control siRNAs were tested; these were an siRNA in which the sequence of siRNA 331 was shuffled (331-shuffle), and a mutant version of siRNA 331, which had mismatches at the tenth and eleventh nucleotides from the 5′ end (331-mutant; Table 1). The siRNAs were cotransfected with pIRES–Fluc into 293T cells or were transfected into Huh7 Rep-Feo cells (Fig. 3). The 331-shuffle siRNA had no effect on the activity of IRES–FLuc or on the replication of the HCV replicon, but unexpectedly, the 331-mutant siRNA had a partial inhibitory effect. Because the reduction of the replicon RNA level by 331-mutant siRNA was not obvious by northern blotting (see supplementary information online), the 331-mutant snRNA might function, at least in part, as a translational repressor by acting as a small temporal RNA (Doench *et al*., 2003). The MTS (see Methods section) assays of the cells transfected with the siRNAs showed no significant effects on cell growth and viability (data not shown). These data showed that the decrease in luciferase activity was due to specific suppressive effects of the siRNAs on HCV replication, and not due to cell death induced by the siRNAs.

Northern and western blotting analyses

In northern blotting analyses (Fig. 4A), levels of the Feo-replicon RNA, which was detectable in mock-transfected control cells, were reduced substantially after transfection of siRNA 331 at 2.5 nM, 25 nM and 125 nM. Densitometric analysis of the replicon RNA showed its intracellular levels in Huh7 Rep-Feo cells correlated well with the luciferase activities. Similarly, in western blotting analyses (Fig. 4B), levels of the HCV non-structural proteins NS3, NS4A and NS5A, which are translated from the HCV replicon, were decreased by corresponding amounts in response to treatment with the siRNA 331.

DNA-vector-based siRNAs suppressed HCV replication

On the basis of the results obtained with the siRNA oligonucleotides, we constructed DNA-based vectors that expressed the siRNA 331 sequence. This was done by modifying previously reported methods, and using two different vectors, the tandem type (Miyagishi & Taira, 2002) and the stem–loop type (Brummelkamp *et al*., 2002). The tandem-type vector contained 19-nucleotide

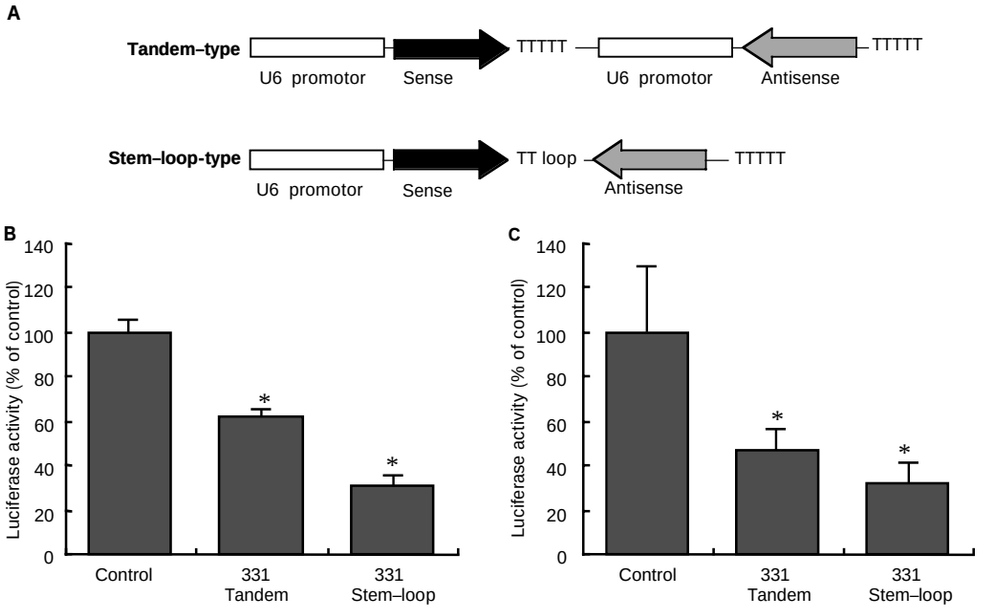

Fig. 5 | Effects of expressing small interfering RNA 331 from a DNA-based vector on hepatitis C virus internal-ribosomal-entry-site-mediated translation and replication. The structures of small interfering RNA (siRNA) expression vectors are shown in (A). Tandem- or stem–loop-type siRNA 331 expression vectors were co-transfected with pIRES-Fluc and pRL–RSV (a *Renilla*-luciferase expression plasmid) into 293T cells (panel (B), or were transfected into Huh7 Rep-Feo cells (C). Luciferase activities were measured 48 h after transfection. Values are shown as percentages of the siRNA-negative control as the mean ± s.d. Asterisks indicate $p<0.05$. pIRES–FLuc, a reporter-gene vector that expresses messenger RNA consisting of the hepatitis C virus 5′ untranslated region and the upstream part of the core region, connected in-frame with firefly luciferase.

sense and antisense siRNA sequences with a 3′ overhang of 4 uridines. Each of the sequences was placed under the control of the U6 promoter. The stem–loop-type vector expressed siRNA hairpins under control of the U6 promoter, which contained the 3′ end of the sense strand and the 5′ end of the antisense strand, connected by a nine-nucleotide loop sequence (Fig. 5A). The siRNA-331-expressing vectors were cotransfected with pIRES–FLuc into 293T cells. Both the tandem and stem–loop siRNA-expressing vectors suppressed luciferase expression, but the stem–loop type was more efficient than the tandem type (Fig. 5B). In addition, both the tandem-type and stem–loop-type vectors significantly suppressed HCV RNA replication in the replicon cells (Fig. 5C).

DISCUSSION

RNAi is an ancient defence mechanism of plant and invertebrate cells and silences foreign gene expression, such as that from viruses or transposons. Since the recent success of highly specific RNAi in mammalian cells using an siRNA (Elbashir *et al*., 2001a), the technique has been applied to pathogenic human viruses such as HIV and poliovirus, which are suitable targets. Recently, a fragment of the HCV NS5B RNA polymerase gene, which was transiently cotransfected with siRNA into mouse liver by hydrodynamic injection, was reported to be cleaved after treatment with siRNA (McCaffrey *et al*., 2002). However, this experiment only showed suppression of protein expression from a plasmid that was transfected exogenously, and it has not been shown that siRNA can block HCV replication itself. Here, taking advantage of a newly developed HCV replicon system, we showed that siRNA targeted the HCV 5′ UTR efficiently and cleaved the target specifically. More importantly, it was shown that the cleavage of HCV RNA not only suppressed viral protein synthesis, but also blocked the replication of sub-genomic viral RNA. Although, during the review of this manuscript, other studies have demonstrated the effectiveness of siRNA against HCV replication (Randall *et al*., 2003; Seo *et al*., 2003), we present here the effectiveness of vector-derived siRNAs and synthetic siRNAs and describe the most effective site for the inhibition of HCV replication. As shown in Fig. 2B, more than 80% suppression was obtained using an siRNA concentration of only 2.5 nM.

Viruses, particularly RNA viruses such as HCV, are notoriously prone to errors during their replication, and continuously produce mutated viral proteins to escape immune-system defence mechanisms (Carmichael, 2002). These mutations may also escape attack by siRNAs. The protein-coding sequence of the HCV genome that was targeted in the study by McCaffrey *et al*. (2002) varies considerably among different HCV genotypes, and even among strains of the same genotype (Okamoto *et al*., 1991). In addition, given the high error rate of the non-proofreading HCV RNA-dependent RNA polymerase, so-called ‘siRNA escape mutants’, which have silent mutations in the protein-coding sequence, could emerge quickly. By contrast, the 5′ UTR, which was selected as the target in the present study, is almost identical among the known strains of HCV. Moreover, structural constraints on the 5′ UTR, in terms of its ability to direct internal ribosome entry and translation of viral proteins, would not permit escape mutations. Therefore, the 5′ UTR of the HCV genome appears to be an ideal target for siRNA in clinical applications.

Not all 5′-UTR-directed siRNAs were equally effective; among the siRNAs tested, siRNA 331, which is directed against a region upstream

of the start codon, was the most efficient, whereas siRNA 82, which is directed against helix II, had almost no effect on viral genome expression. These results may be due in part to the highly folded structure of the 5′ UTR, which may leave few single-stranded gaps that siRNAs can access. We reported previously that the target region of siRNA 331 is also an efficient target site for a catalytic RNA, a hammerhead ribozyme, for the suppression of HCV protein expression (Sakamoto *et al*., 1996). Our results suggest that the secondary structure of the HCV RNA genome influences the efficiency of siRNAs at least in part.

Our results showed that the siRNAs suppressed the expression of an HCV replicon more potently than they did the IRES reporter vector (Fig. 2). This stronger suppressive effect of siRNA on the HCV replicon might be due to several effects on its autonomous replication mechanism. The blockage of the IRES-mediated synthesis of the nonstructural proteins, which are essential for viral RNA synthesis, and the cleavage of elements in the 5′ UTR that are necessary to prime complementary RNA strand synthesis, may result in further suppression of viral replication. Thus, our siRNAs not only reduced viral protein synthesis, but also abolished intracellular replication of the viral genomic RNA, raising the possibility that RNAi could achieve the elimination of viruses from persistently infected host cells.

Cleavage of the HCV IRES by siRNAs may lead to complicated effects on protein translation. Treatment with a high concentration of siRNA 12, which was directed against helix 1 of the 5′ UTR, increased HCV protein expression and viral replication (see also supplementary information online). It has been reported that the most 5′ part of the 5′ UTR may negatively regulate the IRES function (Honda *et al*., 1996). Moreover, deletion of the nucleotides that make up helix 1 leads to an increase in IRES-mediated translation (Rijnbrand *et al*., 1995; Wang *et al*., 1993). We speculate that the cleavage of helix I by siRNA 12 led to an enhancement of IRES-mediated translation through the inactivation of *cis*- or *trans*-acting negative regulatory elements of the IRES. Our results demonstrate clearly, for the first time, that careful selection of target sequences for siRNAs is mandatory, not only to achieve maximum efficiency (as with siRNA 331), but also to avoid adverse effects (as with siRNA 12) in therapeutic applications.

At present, several potential HCV therapies are under development. These include inhibitors of the NS3 protease (Sulkowski, 2003) and the NS5B RNA polymerase (Dhanak *et al*., 2002), and recombinant vaccines (Choo *et al*., 1994). Delivery methods for siRNAs to cells *in vivo* that are efficient and safe enough to suppress HCV replication in all infected cells have not been established. Chemically modified synthetic siRNA might easily be made and delivered into cells on their own; it was reported recently that serum (ribonuclease)-resistant modified siRNA can be delivered into cells without a cationic lipid carrier (Capodici *et al*., 2002). Importantly, we demonstrated for the first time that DNA-based siRNA expression vectors are effective against HCV, and might allow the efficient delivery of RNAi to hepatocytes *in vivo* using viral vectors. As an alternative approach, we are at present analysing siRNA-331-encoding DNA constructs within adenovirus and adeno-associated virus vectors, as reported in Xia *et al*. (2002). Our preliminary data indicate that an adenovirus vector expressing siRNA 331 efficiently suppresses HCV replication *in vitro*.

In conclusion, the efficiency of our siRNAs and siRNA-expressing vectors in inhibiting HCV replication in cells suggests that this RNA-targeting approach might provide an effective therapeutic option for HCV infection, especially at the optimal site (the siRNA 331 target-site) within the conserved 5′ UTR.

METHODS

Preparation of siRNAs and DNA-based vectors expressing siRNA. Sense and antisense strands of siRNA oligonucleotides were synthesized, and were then annealed at 95 °C for 1 min, followed by slow cooling in PBS, pH 6.8, containing 2 mM $MgCl_2$. To construct siRNA-expressing vectors, inserts were made by PCR. These contained the human U6 promoter in the case of the tandem-type vector, and the loop sequence (5′-TTCAAGAGA-3′) flanked by sense and antisense siRNA sequences in the case of the stem–loop-type vector. These were inserted immediately downstream of the U6 promoter in pUC19. siRNA and siRNA-expressing vectors for an unrelated target, the Machado–Joseph Disease gene, were used as negative controls (Table 1).

HCV replicon expressing chimeric reporter genes. An HCV replicon plasmid, pHCVIbneo-delS, was derived from an infectious HCV clone, HC-N, genotype Ib (Guo *et al*., 2001). The pHCVIbneo-delS plasmid was reconstructed by substituting the *NPT* gene with a fusion of *FLuc* and *NPT* (pRep–Feo; Fig 1B).

Cell culture and transfection. The human hepatoma cell line Huh7 and the human embryonic kidney cell line 293T were maintained in DMEM (Sigma), supplemented with 10% FCS, at 37 °C with 5% CO_2. Transfections of the siRNA oligonucleotides and the plasmids were performed in 24-well plates using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. Thirty nanogrammes of the pIRES–FLuc construct and 2.5–125 nM of siRNA or 0.5 μg of siRNA-expressing vectors were transiently transfected with 10 ng of the *Renilla* luciferase expression plasmid (pRL–RSV; Promega). For transfection into Huh7 Rep-Feo cells, 2.5–125 nM of siRNA or 0.5 μg of siRNA-expressing vectors were transfected with 10 ng of pRL–RSV. In each transfection, FLuc activity was adjusted using the *Renilla* luciferase activity to normalize the transfection efficiency.

Luciferase assays. Luciferase activities were quantified using a luminometer (Lumat LB9501; Promega) using the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate, and the results were expressed as means ±s.d. as percentages of the controls.

MTS (dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium) assays. To evaluate the cytotoxic effects of the siRNAs, MTT assays were performed 48 h after siRNA transfections using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega).

Northern hybridization. Total cellular RNA was extracted from cells using ISOGEN (Wako). The RNA was separated by denaturing agarose–formaldehyde-gel electrophoresis and transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech.). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labelled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for β-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche Molecular Biochemicals) and were visualized using a Fluoro-Imager (Roche).

Western blotting. 10 μg of total cell lysate was separated using NuPAGE 4.12% Bis–Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with monoclonal anti-NS5A (BioDesign), anti-NS3 and anti-NS4A antibodies (Virogen), and detection was carried out in a chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche).

Statistical analyses. Statistical analyses were performed using the student's *t*-test; *p* values of less than 0.05 were considered as statistically significant.

Supplementary information is available at *EMBO reports* online (www.emboreports.org).

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