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ORIGINAL ARTICLE

Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype

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RNA interference (RNAi) can be used to inhibit viral replication in mammalian cells and therefore could be a powerful new antiviral therapy. Small interfering RNA (siRNA) may be effective for RNAi, but there are some technical problems that must be solved in each case, for example, predicting the effective siRNA target site and targeting heterogeneous sequences in a virus population. We show here that diced siRNA generated from long double-stranded RNA (dsRNA) is highly effective for inducing RNAi in HuH-7 cells harboring hepatitis C virus (HCV) replicons and can overcome variations in the HCV genotype. However, in mammalian cells, long dsRNA induced an interferon response and caused cell death. Here we describe an improvement of this method, U6 promoter-driven expression of long hairpin-RNA with multiple point mutations in the sense strand. This can efficiently silence HCV RNA replication and HCV protein expression without triggering the interferon response or cell death normally caused by dsRNA. In conclusion, intracellular-diced dsRNA efficiently induces RNAi, and, despite the high rate of mutation in HCV, it should be a feasible therapeutic strategy for silencing HCV RNA.

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Keywords: *RNAi; long dsRNA; dicer; heterogeneous; interferon; U6 vector; HCV*

Introduction

An estimated 170 million people worldwide are persistently infected with hepatitis C virus (HCV).[1] Although the initial infection is frequently asymptomatic, there are several subsequent clinical manifestations, including fibrosis of the liver, cirrhosis and hepatocellular carcinoma. Although combination therapy with interferon (IFN)-α and ribavirin has markedly improved the clinical outcome, less than half of the patients with chronic hepatitis C can be expected to respond favorably to currently available agents.[2] Therefore, developing a new therapy for chronic HCV is a major public health objective.

The genome of HCV, a member of Flaviviridae family, is encoded in an approximately 9.6-kb single-stranded RNA with positive polarity that includes a 5′-untranslated region (UTR)[3] and a 3′-UTR containing a 3′X terminal sequence.[4] Hepatitis C virus displays a high rate of mutation and is classified into distinct genotypes (1–6) and subtypes, whose distribution varies both geographically and between risk groups.[5] Furthermore, several distinct but closely related HCV sequences coexist within each infected individual. These are referred to as quasispecies and reflect the high replication rate of the virus and the lack of a proofreading activity of the RNA-dependent RNA polymerase.[6,7]

Gene targeting with functional nucleic acids is commonly used to determine gene function and has potential as a treatment for viral diseases. Although antisense RNA and ribozyme technologies are successful in some situations, they have been difficult to apply universally and are less effective *in vivo*.[8] A possible alternative, sequence-specific post-transcriptional gene silencing by double-stranded RNA (dsRNA), also known as RNA interference (RNAi), has been found in plants, *Caenorhabditis elegans* and mammalian cells.[9,10] As RNAi with small interfering RNA (siRNA) can inhibit the replication of several viruses, including human immunodeficiency virus type 1 (HIV-1)[11] and poliovirus,[12] it may be a powerful new antiviral therapy. Recently, it has been demonstrated that replication of HCV RNA is also receptive to RNAi machinery,[13–18] but it has been difficult to design highly effective siRNAs against HCV because of the exquisite sequence specificity of the siRNAs coupled with the variation in HCV genotypes and the enormous diversity of HCV sequences between and within infected individuals.

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In this report, we show that dicer-generated siRNAs from long dsRNA can silence the replication of HCV RNA of different genotypes. Furthermore, we employed the modified long hairpin-RNA (hRNA) expression system to inhibit HCV replication and to avoid triggering the IFN response, which is normally caused by dsRNA. We demonstrated that intracellular-diced dsRNA can be used in mammalian cells to silence HCV RNA and, therefore, that long dsRNA-mediated RNAi could be useful as a therapeutic agent for natural viral infection by HCV.

Results

Synthetic small interfering RNA inhibits hepatitis C virus replication in HuH-7 cells containing replicating hepatitis C virus RNA

As siRNA-mediated RNAi is strictly sequence specific, an siRNA targeting site was selected in regions conserved among the various HCV genotypes. Of the HCV genome sequences, the 5′-UTR and the 3′X region are the most highly conserved.[19] Therefore, we selected six sites in the 5′-UTR or core coding regions (A–F) and three sites in the 3′X regions (G–I) (see Materials and methods). HuH-7 cells carrying the HCV replicon were established as described.[20] We also modified the replicon RNA derived from the HCV genotype 1b clone (GenBank accession number AY045702) by substituting the *neo*^{r} gene with the firefly luciferase gene fused to foot-and-mouth disease virus (FMDV) 2A and the *neo*^{r} gene (named the R6FLR-N replicon). This modification enables the sensitive and precise quantification of HCV replication levels using a luciferase assay.

To examine the ability of siRNAs to inhibit HCV replication, the nine synthetic siRNAs were transfected into R6FLR-N replicon cells (Figure 1a, left). Of the siRNAs, siE (nucleotides (nt) 325–344) was the most effective and it dose-dependently inhibited HCV replication (Figure 1a, right). Moreover, continuous transfection with siE but not the negative control p53m siRNA caused a gradual decrease in the HCV replicon titer up to the 23rd day (Figure 1b). Using Northern blot analysis, we confirmed that the effects of siRNAs on the luciferase activity are associated with siRNA-directed degradation of the HCV replicon RNA (data not shown). These results indicated that siE was the most potent siRNA for inhibiting HCV replication of the selected siRNA sites.

Effect of ex-vivo *dicer-generated small interfering RNAs from long double-stranded RNA*

We found that shifting the siRNAs 5′ or 3′ from the siE target position reduced the efficacy of siRNA-mediated RNAi (Figure 2a). Therefore, to overcome site specificity of the selected siRNAs, we prepared *ex-vivo* recombinant human dicer (rhDicer)-generated siRNAs (d-siRNAs) from long dsRNAs (Figure 2b).[21] R6FLR-N replicon cells were transfected with d-siRNAs targeting the HCV genome or p53 mRNA (negative control). Luciferase reporter assays indicated that d-siRNAs generated from the 5′-UTR of HCR6 sequences (D5-357, D5-197, and D5-50) silenced the HCV RNA more efficiently than siE. In contrast, the d-siRNAs generated from the 3′-UTR of the HCR6 sequences were less effective than siE. These

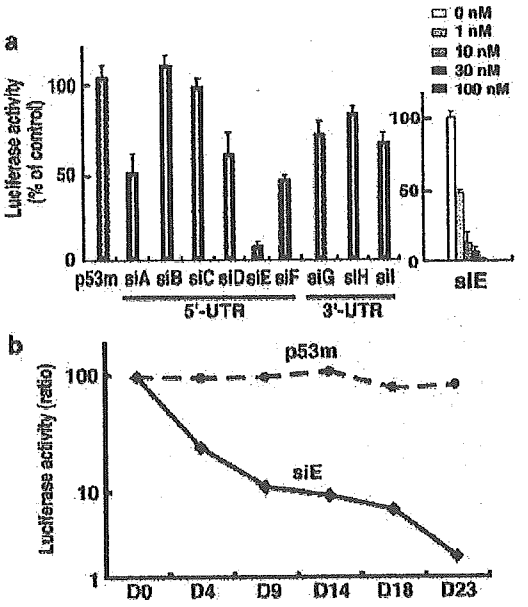

Figure 1 Effect of siRNA on HCV replication. (a) Inhibition of the HCV replicon by siRNAs in R6FLR-N replicon cells. Luciferase activity was measured 24 h after transfection using the Trans IT TKO reagent. Data represent means ± s.d. ($n = 3$) compared with mock-transfected cells. siA to siF, siRNA in the 5′-UTR or core cording regions; siG to siI, siRNA in the 3′-UTR; p53 m, negative control siRNA. (b) Long-term effect of siE. The R6FLR-N replicon cells were transfected with siRNAs every 4 days. Luciferase activity was measured on the indicated days.

results indicated that d-siRNAs generated from 5′-UTR containing the siE sequences, especially those generated from 197-bp dsRNAs, were more effective than the synthetic siE.

Dicer-generated siRNAs generated from 197-bp double-stranded RNA overcome hepatitis C virus genotype variation

Genotype 1b-derived d-siRNAs generated from the conserved sequence motifs within the NS5B sequence do not block the replication of HCV genotypes 1a and 2a.[14] To examine whether our selected d-siRNAs can overcome HCV genotype variation, we transfected genotype 2a-specific d-siRNAs into R6FLR-N replicon cells, which harbor the genotype 1b replicon. As shown in Figures 3a and b, the genotype 2a-derived d-siRNAs generated from 197-bp dsRNA efficiently inhibited genotype 1b replication, even though genotypes 2a and 1b differ by 15 bases within the 197-bp dsRNA sequences (sequence homology = 92%). In contrast, genotype 2a-derived siE, which harbors a single mutation at position 18 of the sense strand (sequence homology = 95%), showed a weak silencing activity against genotype 1b. These results demonstrated that d-siRNAs generated from the 197-bp dsRNA were highly effective for RNAi and could overcome HCV genotype variation.

Long double-stranded RNA transfection into HuH-7 replicon cells induces target-specific silencing

Dicer is a large multi-domain protein present in all eukaryotes.[22] Recently, Kim *et al*.[23] reported that syn-

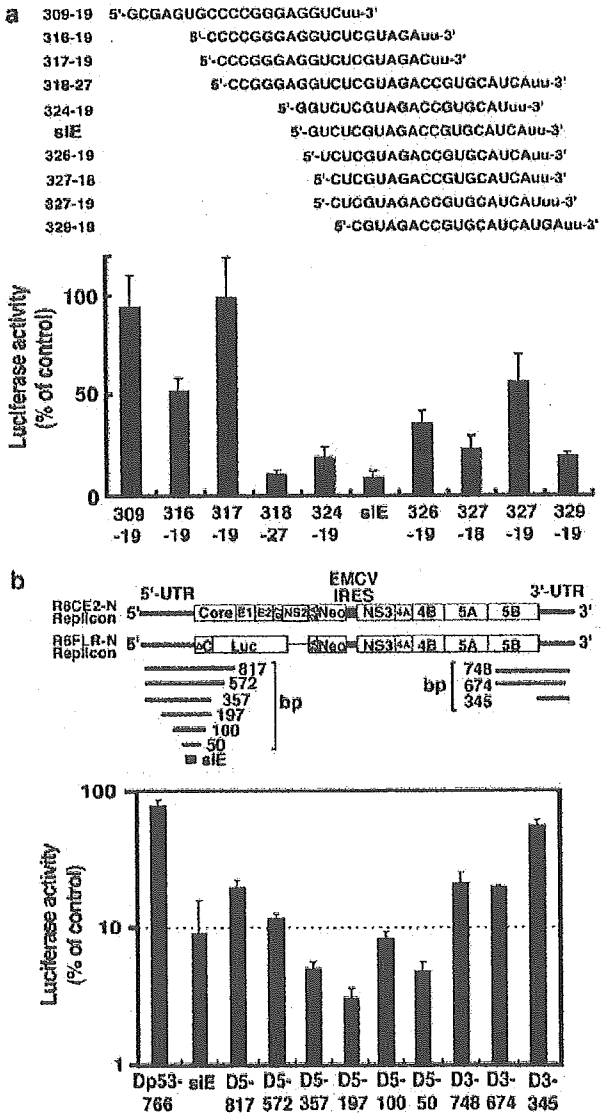

Figure 2 Small interfering RNAs cleaved by rhDicer from long dsRNA. (**a**) Effect of the positional variations in the siE region. R6FLR-N replicon cells were transfected using Lipofectamine 2000 with siRNAs in which the target position was shifted towards either the 5′- or 3′-end of the siE region. Luciferase activity assay measured 48 h after transfection with 1 nM siRNAs. Data represent means±s.d. compared with mock-transfected cells ($n=5$). (**b**) Upper panel, schematic representation of the long dsRNAs used for targeting different sites in the HCV genome RNA; lower panel, effect of d-siRNAs. The d-siRNAs were generated from the long dsRNAs by cleavage with rhDicer. R6FLR-N cells were transfected with d-siRNAs. Luciferase activity was measured after 48 h. Data represent means±s.d. compared with mock-transfected cells ($n=5$). Dp53-766, which targeted p53 mRNA (766 bp), was used as a negative control.

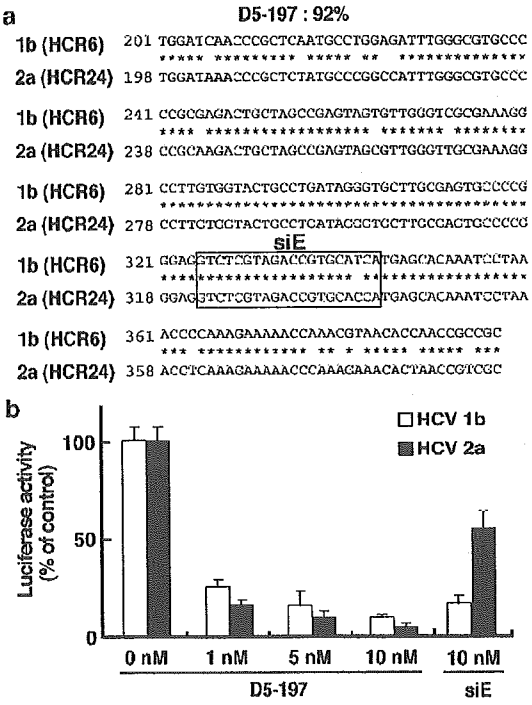

Figure 3 Dicer-generated siRNAs directed at the HCV genotype 2a can cause silencing of genotype 1b RNA. (**a**) The sequence homology between genotypes 1b and 2a was 92% within the 197-bp region (182/197 nt) and 95% within the 20-bp siE region (19/20 nt). (**b**) R6FLR-N cells harboring the genotype 1b HCV replicon RNA were transfected with the d-siRNAs generated from a 197-bp dsRNA directed at HCV genotype 2a (HCR24; accession number AY746460). Data represent means±s.d. compared with mock-transfected cells ($n=5$).

thetic RNA duplexes 25–30 nt in length are substrates of the dicer endonuclease, directly linking the production of siRNAs to incorporation in the RNA-induced silencing complex. We also expected that intracellular dsRNA duplexes longer than 50 nt would be recognized by dicer and thus induce RNAi. Therefore, we directly transfected long dsRNA into R6CE2-N replicon cells, which harbor the core to NS2 portion of the HCV genome (Figure 2b). The same amount of dsRNA was transfected into replicon cells, and the replicon copy number was determined by quantitative real-time detection (RTD)-polymerase chain reaction (PCR).[24] We found that, except for the 817-bp dsRNA, the long dsRNAs targeting sites in the HCV genome reduced the HCV RNA copy number. In contrast, an unrelated dsRNA targeting a site in endogenous p53 mRNA had no effect (Figure 4a). A luciferase assay in R6FLR-N replicon cells showed similar results for HCV-specific silencing (data not shown). On the other hand, immunoblot analysis with antibodies against p53 showed that p53-specific long dsRNA suppressed the level of p53 protein, whereas HCV-specific dsRNA had no effect on p53 expression (Figure 4b). These results indicated that in HuH-7 replicon cells, direct transfection of long dsRNA can specifically produce RNAi against HCV and reduce endogenous p53 expression.

Effect of long double-stranded RNA on the intracellular interferon response and cell death in HepG2 cells

In mammalian cells, Toll-like receptor (TLR) 3[25,26] recognizes dsRNA duplexes longer than 30 nt. This binding induces a type I IFN response, resulting in cell

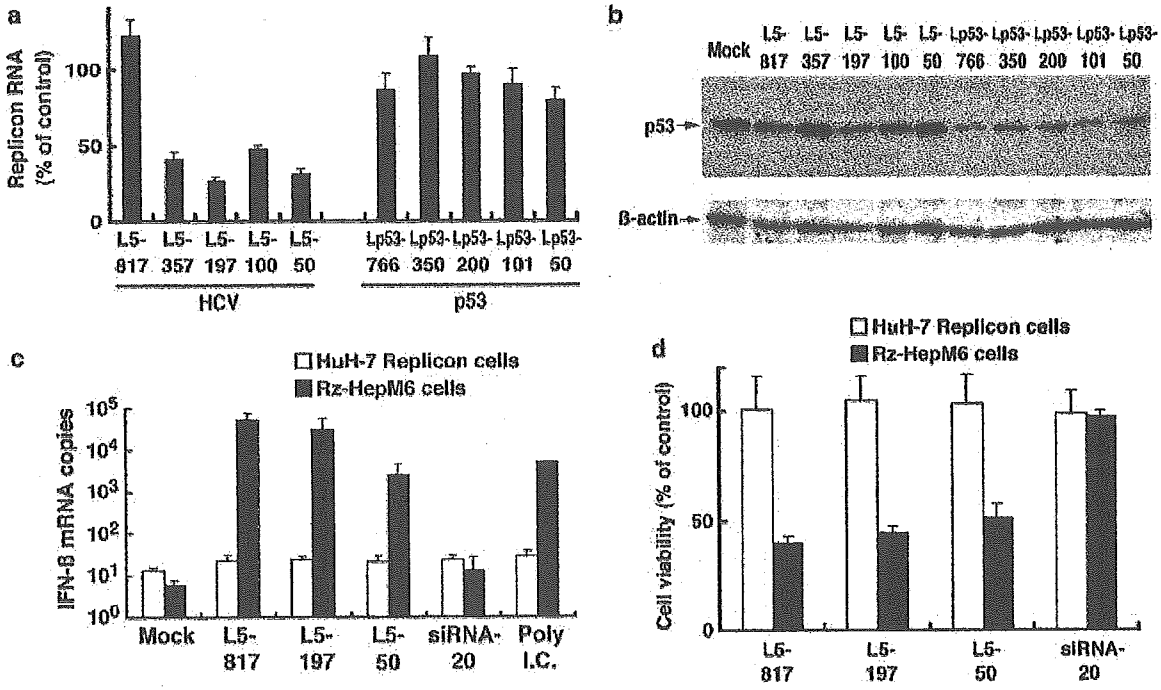

Figure 4 Transfection of long dsRNAs into HuH-7 replicon cells, which lack dsRNA-triggering IFN response, induces target-specific silencing. (a) R6CE2-N cells were transfected with long dsRNAs. Replicon RNA levels in cells transfected with 262 ng of dsRNA per 48-well dish were measured after 48 h by RTD–PCR. Data represent means ± s.d. ($n = 3$) of replicon levels compared with mock-transfected cells. (b) Immunoblot analysis of p53 and β-actin from replicon cells transfected with dsRNA targeting sites in the HCV genome or p53 mRNA. (c) Levels of human IFN-β mRNA were quantified by RTD-PCR 7 h after transfection with 50 ng of dsRNAs per 48-well dish. Values represent the mean copy number for each RNA per μg total RNA ± s.d. ($n = 5$). (d) Cell viability was determined after 48 h by WST-8 assay. Data represent means ± s.d. ($n = 3$) of WST conversion compared with mock-transfected cells.

death by apoptosis.[27] To examine the type I IFN response caused by direct transfection of dsRNA, we measured the intracellular IFN-β mRNA copy number and assessed cell viability. The IFN-β mRNA levels of R6FLR-N replicon cells (HuH-7 replicon cells) and the numbers of viable cells did not change following transfection with long dsRNAs or with the RNA duplex poly(rI):poly(rC) (Figures 4c and d). These results show that the dsRNA did not induce intracellular IFN-β mRNA or enhance apoptosis in HuH-7 replicon cells.

HuH-7 replicon cell lines are used as models for HCV replication and do not respond to the IFN signals.[28] We therefore investigated the effect of dsRNAs on the IFN-β response in another cell type. As an alternative model, we used HepG2 cells stably expressing the full genome HCV RNA (Rz-HepM6 cells).[29] Transfection with poly (rI):poly(rC) or long dsRNAs induced an IFN-β mRNA level of 10^3–10^5 copies per μg total RNA, whereas siRNA-20, a 20-nt duplex, induced only 10 copies per μg total RNA (Figure 4c). Furthermore, the number of viable Rz-HepM6 cells was reduced by transfection with long dsRNAs, but not with siRNA-20 (Figure 4d). These results indicated that direct transfection with dsRNA duplex longer than 50 nt induces IFN-β mRNA and causes cytotoxicity in Rz-HepM6 cells, but not in HuH-7 replicon cells. Therefore, to observe the knockdown efficiency of long-dsRNA against the HCV replicating model and the IFN response induced by long dsRNA, we tested the effects of RNAi in HuH-7 replicon, Rz-HepM6 and HepG2 cells.

U6 promoter-driven expression of long hairpin-RNA with mutations in the sense strand causes gene silencing without triggering an interferon response or cell death

We examined the ability of a stable hairpin-type siRNA-expression vector[30–32] to silence the HCV genome. Recently, U6 promoter-driven transcription of hRNA with mutations in the sense strand has been reported to be more effective for RNAi than hRNA containing nonmutated sense strands.[32] Therefore, we constructed vectors for U6 promoter-driven expression of hRNAs containing multiple mutations (mhRNA) and examined their ability to cause gene silencing. To confirm the RNAi effect, we transfected the long mhRNA-expression vectors into R6FLR-N replicon cells. The 50- and 197-bp mhRNA vectors against the HCV sequence reduced luciferase activity as effectively as the siE-20-bp mhRNA vector (Figure 5a). Furthermore, in Rz-HepM6 cells, the 50- and 197-bp mhRNA vectors targeted to the HCV sequence specifically suppressed HCV core protein expression (Figure 5b). To avoid the inhibition of IFN-β activation by HCV itself,[28] we next examined the IFN response in original HepG2 cells. In contrast to the direct transfection of dsRNAs targeted to the same sequences, the 50- and 197-bp mhRNA vectors did not induce the expression of IFN-β mRNA (Figure 5c). Owing to palindrome structure-specific recombination in the mammalian gene,[33] it was not possible to construct stably transformed cells expressing hRNA vectors against the

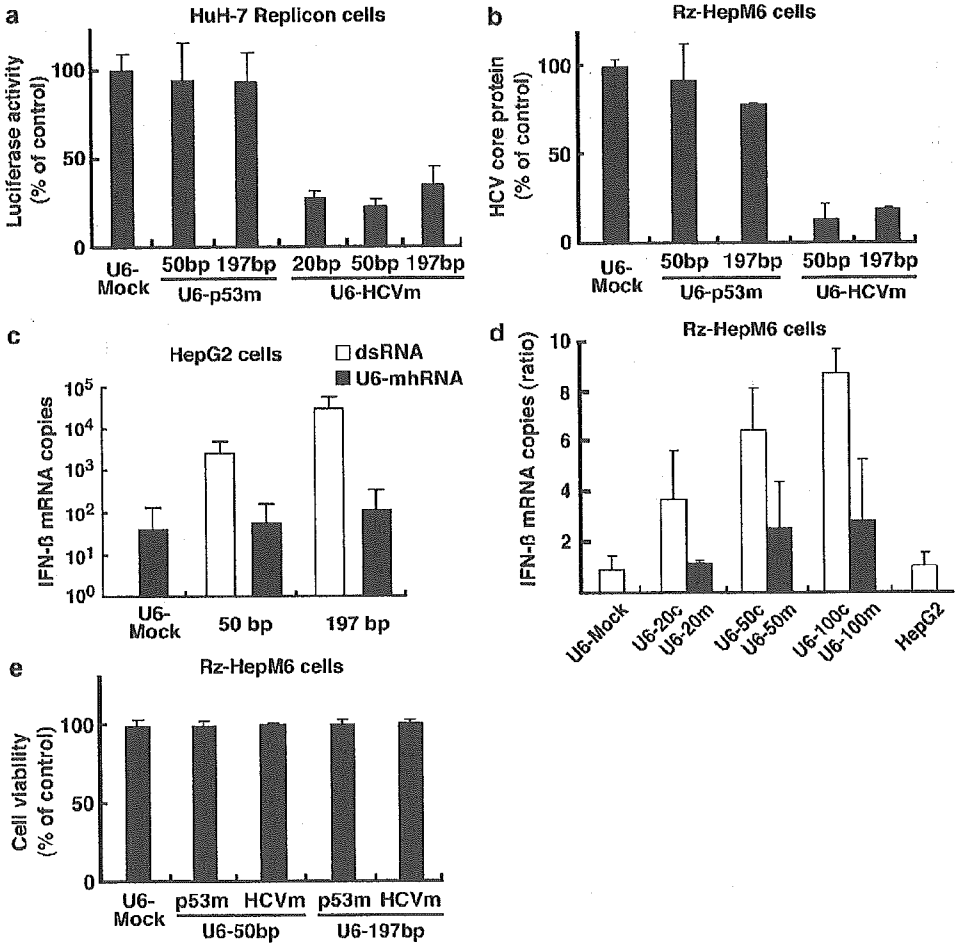

Figure 5 U6 promoter-driven expression of long mhRNA causes gene silencing without triggering an IFN response or cell death. (**a**) U6 promoter-driven transcription of long hRNAs containing multiple point mutations within the sense strand induced RNAi in R6FLR-N replicon cells. Luciferase activity was measured 96 h after transfection with 200 ng vector per 96-well dish. Data represent means±s.d. compared with control U6 vector-transfected cells ($n=3$). (**b**) Gene silencing for the long mhRNA-expression vector in Rz-HepM6 cells. All assays were performed 120 h after transfection with 600 ng vector per 48-well dish. Data represent means±s.d. compared with control U6 vector-transfected cells ($n=3$). (**c**) Interferon responses in original HepG2 cells following targeting of the same HCV sequences by direct transfection with dsRNA and U6 promoter-driven expression of mhRNA. The level of human IFN-β mRNA was measured by RTD-PCR 7 and 16 h after transfection. Values represent the mean copy numbers for each RNA per μg total RNA±s.d. ($n=3$). (**d**) U6 promoter-driven expression of mhRNA caused a lower IFN response in Rz-HepM6 cells than expression of hRNA containing non-mutated sense strands. The level of human IFN-β mRNA was measured by RTD-PCR 16 h after transfection. Values represent the mean ratios compared to control U6 vector-transfected cells±s.d. ($n=3$). U6-20c, HCVc-20bp; U6-20m, HCVm-20bp; U6-50c, U6-50m, U6-100c, and U6-100m are U6 vectors against the luciferase gene. (**e**) Cell viability of Rz-HepM6 cells was determined after 120 h by WST-8 assay. Data represent means±s.d. ($n=3$) of WST-8 conversion compared with control U6 vector-transfected cells.

HCV sequence containing non-mutated sense strands longer than 50 bp. Using control vectors against the luciferase gene (U6-50c, U6-50m, U6-100c and U6-100m), we confirmed an intracellular IFN response. In Rz-HepM6 cells, all of the mhRNA vectors (HCVm-20 bp, U6-50m and U6-100m) had a reduced IFN response compared to the hRNA vectors containing non-mutated sense strands (HCVc-20 bp, U6-50c and U6-100c; Figure 5d). Moreover, U6 promoter-driven expression of long mhRNAs against the HCV sequence was not cytotoxic (Figure 5e).

These results indicated that in IFN-responsive cells, U6 promoter-driven expression of modified long dsRNA, which be made by inserting multiple mutations in the sense strand of hRNA, can effectively and specifically silence HCV without triggering the IFN response or cell death.

Discussion

Previous studies have shown that HCV RNA can be suppressed by the RNAi machinery in replicon cells.[13–18] We demonstrated that there are two significant limitations for the use of siRNA-mediated RNAi as a therapy for HCV: first, it is difficult to predict which target site will be most effective for siRNA; and, second, it is difficult to target the other HCV genotypes with multiple sequences. We further examined the ability of d-siRNAs and intracellular-diced long dsRNAs to overcome these problems and inhibit HCV replication in HCV replicon

cells. We found that *ex-vivo* dicer-generated siRNAs generated from the 5′-UTR sequences are more effective for silencing than the most potent synthetic siRNA, siE. Our results further demonstrated that 50- and 197-bp dsRNA regions of the HCV genome are potential target areas for RNAi. Although dsRNA duplexes targeting the 50- to 357-bp sites in the HCV genome efficiently cause target silencing, dsRNA duplexes targeting the 817-bp HCV genome are less effective for HCV replication. This suggests that the area of the HCV genome that can be targeted by the RNAi machinery is restricted because of the formation of a complex internal ribosome entry site structure. Recently, Kim *et al.*[23] showed that 27-mer duplexes that are substrates of cellular dicer have enhanced RNAi potency and efficacy in mammalian cells. Our results also suggest that siRNAs generated by dicer from dsRNA duplexes longer than 50 nt are available in their natural form and, therefore, can have enhanced efficacy for RNAi.

In HuH-7 HCV replicon cells, which lack a long dsRNA-induced IFN response, the long dsRNAs were effective at causing RNAi of the HCV genome or endogenous p53. Therefore, we further examined the effect of dsRNA on HepG2 cells, in which dsRNA causes production of IFN-β and activates downstream signaling, including 2′–5′-oligoadenylate synthetase and protein kinase R.[29] Although transfection with dsRNA duplexes longer than 50 nt induced IFN-β and caused cell death, U6 promoter-driven expression of long hRNAs containing multiple point mutations in the sense strand (i.e., near-complementary inverted repeats) efficiently inhibited HCV replication, but was not cytotoxic. Moreover, the intracellular IFN-β mRNA titer was equivalent to that induced by the control U6 vector. The precise mechanism is now under investigation, but it is clear that this system allows intracellular-diced long dsRNA to induce RNAi without activating the IFN response in mammalian cells.

The genotype 2a-derived d-siRNAs generated from the 197-bp dsRNA were able to efficiently inhibit HCV genotype 1b replication. Thus, siRNAs generated from long dsRNA can cause silencing of heterogeneous viruses and should be able to overcome siRNA escape mutations. Long-term HIV-1 replication assays[34] revealed that, after 3–6 weeks of culture, siRNA-mediated RNAi-resistant viruses containing nucleotide substitutions or deletions in the target sequence arise. Wilson *et al.*[35] reported that HCV replicons escaped RNAi induced by subsequent treatment with the same siRNA directed against the NS5B cording region. In contrast, we also examined the long-term efficiency of long dsRNA-mediated RNAi using HCV replicon cells. When examined over 5 weeks with continuous transfection of 197-bp dsRNA, the HCV replicon RNA titer gradually decreased to a 100-fold reduction and never rebounded (data not shown). The degree of sequence conservation reflects the fact that the structural elements in the 5′- and 3′-terminal regions of the RNA are essential for viral replication.[36,37] Therefore, long dsRNA-mediated RNAi targeting a site in the 5′-UTR can avoid the problem of escape virus generation because extensive alterations in a conserved region of the viral genome would be required.

In summary, our results show that dicer-generated siRNAs from long dsRNA are highly effective for RNAi of the HCV genome and overcome genotype variations. We also showed that U6 promoter-driven expression of modified long dsRNA avoids activation of the IFN response and the induction of cell death normally caused by dsRNA. This strategy should be useful for therapy against natural viral infection by HCV and other RNA viruses, such as HIV-1, that display a high rate of mutation.

Materials and methods

Small interfering RNAs

We synthesized T7 siRNAs using the Silencer siRNA Construction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The sense sequences of siRNAs were as follows:

siA (nt 26–45), 5′-ACUCCACCAUAGAUCACUCCUU-3′;
siB (nt 53–73), 5′-GGAACUACUGUCUUCACGCAGUU-3′;
siC (nt 139–159), 5′-GCCAUAGUGGUCUGCGGAACC UU-3′;
siD (nt 278–299), 5′-AGGCCUUGUGGUACUGCCUGAU UU-3′;
siE (nt 325–344), 5′-GUCUCGUAGACCGUGCAUCAUU-3′;
siF (nt 368–387), 5′-AGAAAAACCAAACGUAACACUU-3′;
siG (nt 9517–9537), 5′-GGCUCCAUCUUAGCCCUAGU CUU-3′;
siH (nt 9540–9560), 5′-GGCUAGCUGUGAAAGGUCCG UUU-3′; and
siI (nt 9553–9572) and 5′-AGGUCCGUGAGCCGCAUGA CUU-3′.

The sense sequence of the p53 m siRNA, which contains two nucleotide mismatches in the target sequence,[38] was 5′-GACUCCAGUGAUAAUCUGCUU-3′ (nucleotide mismatches underlined).

Long double-stranded RNAs

Long dsRNAs were prepared by *in vitro* transcription of PCR-amplified DNA templates. A modified T7 promoter sequence was added to the 5′-end of each PCR primer for amplification (Table 1). The dsRNAs were produced from the purified DNA templates using an Ampliscribe T7 transcription kit (Epicenter Technologies, Madison, WI, USA). Single-stranded RNA was converted to dsRNA by allowing annealing the two strands. Purification of dsRNA was performed as described for dicer-generated siRNAs.

Dicer-generated small interfering RNAs

Digestion with rhDicer (Gene Therapy Systems, San Diego, CA, USA) was carried out according to the manufacturer's protocol. The rhDicer-cleaved siRNAs and dsRNAs were separated by electrophoresis on a nondenaturing 12% polyacrylamide gel and detected by ultraviolet shadowing on a Fluor-coated thin-layer chromatography plate (Ambion). The rhDicer-cleaved siRNAs migrating as 20- to 21-bp bands were excised from the gel and extracted at 37°C for 4 h in extraction buffer (0.5 M ammonium acetate, 1 mM EDTA and 0.2% SDS). Following buffer exchange and desalting by gel filtration with Sephadex G-25 (Amersham Biosciences, Piscataway, NJ, USA), the rhDicer-cleaved siRNAs were dissolved in TE buffer. The cleaved siRNAs were then quantified by adsorption at 260 nm and stored at −70°C.

Table 1 Templates, PCR primers and amplicons used for the generation of dsRNAs

Template (source[a])	*Primer set*	*Primer sequence (5′ to 3′)*	*Amplicon name*	*Start position*	*Stop position*	*dsRNA size (nt)*
HCR6 genotype 1b (AY045702[a])	F-1	GCG TAA TAC GAC TCA CTA TAG GGA GAG AGT GCC CCG GGA GGT CTC GTA GAC	L5-50	311	360	50
	R-1	GCG TAA TAC GAC TCA CTA TAG GGA GAT TAG GAT TTG TGC TCA TGA TGC ACG				
	F-2	GCG TAA TAC GAC TCA CTA TAG GGA GAT AGT GTT GGG TCG CGA AAG GCC TTG	L5-100	261	360	100
	R-1	GCG TAA TAC GAC TCA CTA TAG GGA GAT TAG GAT TTG TGC TCA TGA TGC ACG				
	F-3	GCG TAA TAC GAC TCA CTA TAG GGA GAT GGA TCA ACC CGC TCA ATG CCT GGA	L5-197	201	397	197
	R-2	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGG CGG TTG GTG TTA CGT TTG G				
	F-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA CTC CCC TGT GAG GAA CTA CTG TCT	L5-357	41	397	357
	R-2	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGG CGG TTG GTG TTA CGT TTG G				
	F-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA CTC CCC TGT GAG GAA CTA CTG TCT	L5-572	41	612	572
	R-3	GCG TAA TAC GAC TCA CTA TAG GGA GAC CCT CGT TGC CAT AGA GGG GCC A				
	F-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA CTC CCC TGT GAG GAA CTA CTG TCT	L5-817	41	857	817
	R-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA ACC GGG CAA ATT CCC TGT TGC ATA				
	F-5	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGG GGG AGA GAT ATA TCA CAG C	L3-345	9267	9611	345
	R-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA CAT GAT CTG CAG AGA GGC CAG T				
	F-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA GGA TGA TTC TGA TGA CCC ATT TCT	L3-674	8864	9537	674
	R-7	GCG TAA TAC GAC TCA CTA TAG GGA GAG ACT AGG GCT AAG ATG GAG CCA CCA				
	F-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA GGA TGA TTC TGA TGA CCC ATT TCT	L3-748	8864	9611	748
	R-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA CAT GAT CTG CAG AGA GGC CAG T				
HCR24 genotype 2a (AY746460[a])	F-2a	GCG TAA TAC GAC TCA CTA TAG GGA GAT GGA TAA ACC CGC TCT ATG CCC GGC	2a-197	198	394	197
	R-2a	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGA CGG TTA GTG TTT CTT TGG G				
p53 (NM_000546[a])	F-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC ATC ACA CTG GAA GAC TCC AG	Lp53-50	1013	1062	50
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p2	GCG TAA TAC GAC TCA CTA TAG GGA GAG TGT AAC AGT TCC TGC ATG GG	Lp53-101	962	1062	101
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p3	GCG TAA TAC GAC TCA CTA TAG GGA GAG TAT TTG GAT GAC AGA AAC ACT TTT CGA C	Lp53-200	863	1062	200
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p4	GCG TAA TAC GAC TCA CTA TAG GGA GAC ACC CGC GTC CGC GCC ATG G	Lp53-350	713	1062	350
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p5	GCG TAA TAC GAC TCA CTA TAG GGA GAG CAA TGG ATG ATT TGA TGC TG	Lp53-766	366	1131	766
	R-p2	GCG TAA TAC GAC TCA CTA TAG GGA GAC CCC TTT CTT GCG GAG ATT C				

dsRNA = double-stranded RNA; PCR = polymerase chain reaction.
[a]GenBank accession number.

Construction of U6 vectors

Plasmids containing a human U6 promoter were prepared as described previously.[30] A series of long-hairpin–RNA expression vectors was constructed by inserting a sense sequence between the U6 promoter and the corresponding antisense sequence. Sequences downstream of the U6 promoter were as follows (nucleotide substitutions underlined and loop sequence indicated in lowercase letters):

HCVc-20 bp, 5′-GTCTCGTAGACCGTGCATCAtagaatt acatcaagggagatTGATGCACGGTCTACGAGACTTTTT-3′;

HCVm-20 bp, 5′-GTCTTGTAGATTGTGTATTAtagaatt acatcaagggagatTGATGCACGGTCTACGAGACTTTTT-3′;

p53m-50 bp, 5′-CATTACATTGGAGGATTCCAGTGGT GATCTATTGGGGCGGAGTAGCTTTGgtgtgctgtccCA AAGCTGTTCCGTCCCAGTAGATTACCACTGGAGT CTTCCAGTGTGATGTTTTT-3′;

HCVm-50 bp, 5′-GAGTGTTCTGGGAGGTTTCGTAG ATCGTGTATCGTGAGTACAAGTTCTAAgtgtgctgtccT TAGGATTTGTGCTCATGATGCACGGTCTACGAGA CCTCCCGGGGCACTCTTTTT-3′;

p53m-197 bp, 5′-GTGTTTGGGTGATAGAGACACCTC TCGGCATGGTGTGGTGGTGTCTTATGAGTCGCT TGGGGTTGGTTCTGATTGTATCACTATCTATTACA GCTACGTGTGTGATAGTTCTTGTATGGGTGGCATG GACCGGGGGTCCATTCTCATCATTATCGCACTGG GAGATTCTAGTGGTGATCTATTGGGGCGGGACGG CTTTGgtgtgctgtccCAAAGCTGTTCCGTCCCAGTAG ATTACCACTGGAGTCTTCCAGTGTGATGATGGTG AGGATGGGCCTCCGGTTCATGCCGCCCATGCAG GAACTGTTACACATGTAGTTGTAGTGGATGGTGG TACAGTCAGAGCCAACCTCAGGCGGCTCATAGG GCACCACCACACTATGTCGAGAAGTGTTTCTGTC ATCCAAATACTTTTT-3′;

HCVm-197 bp, 5′-ATGGGTCAGCTCGTTCAATGCTT GGAGGTTTGGGTGTGTCCTCGTGAGATTGCTAGT CGAGTGGTGTTGGGTTGCGGAAGGTCTTGTGGTG CTGTCTGATGGGGTGTTTGTGAGTGTCCTGGGAG GTTTCGTTGACTGTGCATTATGAGTACAGATCCTA GACCTCAGAGAAGGACCAGACGTGACATCAACT GCCGCgtgtgctgtccGCGGCGGTTGGTGTTACGTTTG GTTCTTCTTTGGGGTTTAGGATTTGTGCTCATGAT GCACGGTCTACGAGACCTCCCGGGGCACTCGCA AGCACCCTATCAGGCAGTACCACAAGGCCTTTC GCGACCCAACACTACTCGGCTAGCAGTCTCGCG GGGGCACGCCCAAATCTCCAGGCATTGAGCGGG TTGATCCATTTTT-3′;

U6-50c, 5′-GCCTTCAGGATTACAAGATTCAAAGTG CGCTGCTGGTGCCAACCCTATCTttcaagagaGAATA GGGTTGGCACCAGCAGCGCACTTTGAATCTTGTA ATCCTGAAGGCTTTTT-3′;

U6-50m, 5′- GCCTTTAGGATTATAAGGTTCAAAGTG TGCTGTTGGTGTCAACTCTATCTttcaagagaGAATAG GGTTGGCACCAGCAGCGCACTTTGAATCTTGTAA TCCTGAAGGCTTTTT -3′;

U6-100c, 5′-GATTTCGAGTCGTCTTAATGTATAGATT TGAAGAAGAGCTGTTTCTGAGGAGCCTTCAGGA TTACAAGATTCAAAGTGCGCTGCTGGTGCCAACC CTATTCttcaagagaGAATAGGGTTGGCACCAGCAGC GCACTTTGAATCTTGTAATCCTGAAGGCTCCTCA GAAACAGCTCTTCTTCAAATCTATACATTAAGAC GACTCGAAATCTTTTT-3′ and

U6-100m, 5′-GATTTCGGGTTGTCTTGATGTATGGGT TTGGAGAGGAGTTGTTTCTGGGGAGTCTTTAGGA TTATAAGGTTCAAAGTGTGCTGTTGGTGTCAACT CTATTCttcaagagaGAATAGGGTTGGCACCAGCAGC GCACTTTGAATCTTGTAATCCTGAAGGCTCCTCA GAAACAGCTCTTCTTCAAATCTATACATTAAGAC GACTCGAAATCTTTTT-3′.

Construction of recombinant plasmids for expressing the hepatitis C virus replicon

The HCV genotype 1b replicon pRep-R6FLR-NRz was assembled and cloned from pRep-R6Rz and the 1bneo/delS plasmid.[39] Replicon pRep-R6Rz was engineered from pHCR6-Rz[29] as described previously,[20] and replicon pRep-R6-NRz was engineered by replacing a NS3-NS5B fragment (nt 3420–7996; *Mfe*I site) in pRep-R6Rz with a NS3-NS5B fragment (nt 3420–7996; *Mfe*I site) from the 1bneo/delS plasmid. The final replicon, pRep-R6FLR-NRz, was constructed by replacing the neomycin phosphotransferase (*neo*^{r}) gene of pRep-R6-NRz with a chimeric gene encoding firefly luciferase protein fused in-frame with the 2A genes of FMDV and *neo*^{r}.

The HCV genotype 1b replicon pRep-R6CE2-NRz was assembled and cloned from pRep-R6-NRz and pHCR6-Rz. Plasmid pRep-R6CE2-NRz was engineered by replacing the HCV internal ribosome entry site gene (nt 1–389) in pRep-R6-NRz with a Core-NS2 gene (nt 1–3030; *Rsr*II site) from the pHCR6 plasmid. The pRep-R6CE2-NRz replicon was constructed by fusing the HCV NS2 protein gene in-frame with the genes for FMDV 2A protein and *neo*^{r}.

Cell culture and transfection

We maintained the human hepatoma cell line HuH-7 in complete Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA). G418 was added to a final concentration of 500 μg/ml to cell lines carrying HCV replicons.[20] Replicon cells were transfected with synthetic siRNA using Trans IT TKO reagent (Mirus, Madison, WI, USA) or with modified siE, dicer-generated siRNAs, long dsRNA and DNA vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Also, Rz-HepM6 cells[29] were transfected with various amounts of dsRNAs or DNA vector using Lipofectamine 2000.

Luciferase assays

The luciferase assay was performed using the Steady-Glo or Bright-Glo luciferase assay systems (Promega). Luciferase activities were quantified using a luminometer (Mithras LB940; Berthold Technologies, Wildbad, Germany).

Cell viability assay

To evaluate the cytotoxic effects of dsRNAs, cell viability was measured by metabolic conversion of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) using a Cell Counting Kit-8 (Wako, Tokyo, Japan) according to the manufacturer's protocol.

Immunoblot analysis

Immunoblot analysis was performed as described previously.[29] Anti-p53 (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the primary antibodies.

Real-time detection–polymerase chain reaction analysis

The HCV genome RNA and IFN-β mRNA were quantified using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) as described previously.[24,29]

Quantification of hepatitis C virus core protein

Hepatitis C virus core protein was assessed in cell lysates using a fluorescent enzyme-linked immunosorbent assay.[40]

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Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system ☆

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Abstract

Recently we reported a subgenomic hepatitis C virus (HCV) replicon derived from HCV (HCV-O strain) infected in non-neoplastic human hepatocyte PH5CH8. In this study, we developed a genome-length dicistronic HCV RNA from HCV-O. A cured HuH-7 cell line (sOc) was obtained from a cloned subgenomic replicon cell line (sO) by interferon (IFN) treatment and used for transfection with genome-length HCV RNA. One cloned cell line, O, was successfully selected by G418 treatment following the introduction of genome-length HCV RNA into sOc cells, and the robust expression of HCV RNA and proteins was confirmed. Oc, a cured cell line, was also obtained from the cloned cell line (O) by IFN treatment. The number of colonies increased drastically when genome-length HCV RNA was introduced into Oc cells. However, the cloned cured cell lines, sOc and Oc, differed in their colony formation efficiency despite their common origin. This result suggests that even a cloned cell line can change its characteristics during cell culture. Sequence analysis of HCV RNA from the O cells revealed an amino acid substitution in the NS3 helicase region (K1609E). This substitution worked as an adaptive mutation in transient reporter and colony formation assays. Using the advantages of this adaptive mutation and of Oc cells in colony formation, we established the first cell line in which genome-length dicistronic HCV RNA encoding a luciferase gene replicated efficiently. This culture system is useful tool for the study of HCV replication and mass screening for anti-HCV reagents.

Keywords: Hepatitis C virus; Genome-length HCV RNA; Replication; Cured cells; Reporter assay

Persistent infection with hepatitis C virus (HCV) causes liver cirrhosis and progresses to hepatocellular carcinoma. The low level of response to interferon therapy by chronic hepatitis C patients remains a worldwide threat to public health. One obstacle to the development of new therapy has been the lack of an efficient HCV replication system. HCV is a positive-stranded RNA virus of the family *Flaviviridae*. The HCV genome encodes a long polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1]. Translation of the HCV open reading frame (ORF) is mediated via the 5′ untranslated region (UTR) and a part of the core coding region carrying the internal ribosomal entry site (IRES). The studies on the mechanism of HCV replication became active after the subgenomic HCV replicon was developed in 1999 [2]. Genetic analysis using this replicon revealed that about 100 nucleotides from the 5′ and 3′ ends are essential RNA elements for replication [3–5]. More recently it was found that conserved nucleotides within NS5B worked as *cis*-acting replication elements (Cre) [6].

☆ The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. AB191333.

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After the first Con-1 replicon, subgenomic HCV replicons derived from N, H77, 1B-1, and JFH-1 strains were developed and tissue, genotype, and host ranges were expanded [7–14]. We also previously reported a subgenomic HCV replicon (1B-2R1) derived from the 1B-2 strain (newly designated as HCV-O in this paper) [15]. The sequence of 1B-2R1 is derived from HCV RNA in non-neoplastic human hepatocyte PH5CH8 inoculated with HCV-O [16]. To facilitate the monitoring of a subgenomic HCV replicon's replication level, several attempts have been made using replicons possessing a reporter gene, such as secreted alkaline phosphatase (SEAP), luciferase, or β-lactamase [17–19]. These subgenomic replicon systems are useful for understanding the mechanism underlying HCV replication and for evaluating the effectiveness of anti-HCV reagents. Subgenomic HCV replicons were used to accumulate information about viral and cellular factors in HCV replication [20–22]. But in attempts to see what happens in HCV-infected human liver, subgenomic HCV replicons were insufficient because they lacked the effects of HCV structural proteins. A genome-length HCV RNA replication system, on the other hand, may reflect the phenomena that HCV-infected human liver undergoes. So far, three genome-length HCV RNA replication systems, using N, Con-1, and H77 strains, have been reported [8,11,23]. We have also tried to develop a genome-length HCV RNA replication system derived from the HCV-O strain. The purpose of this study was to characterize our genome-length HCV RNA replication system and to develop a replication system of genome-length HCV RNA encoding a reporter gene for the simple monitoring of HCV replication levels and for the mass screening of anti-HCV reagents.

Materials and methods

Cell culture system. HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM). Cells supporting subgenomic and genome-length HCV RNAs were maintained in the presence of G418 (300 μg/ml; Geneticin, Invitrogen) and passaged twice a week at a 5:1 split ratio.

Plasmid constructions. The plasmid pON/C-5B contains neomycin phosphotransferase (Neo) downstream of HCV IRES and the full-length HCV-O polyprotein coding sequence downstream of encephalomyocarditis virus (EMCV) IRES. We first constructed an authentic genome-length HCV-O, pHCV-O, using two fragments: the *Eco*RI–*Mlu*I fragment (corresponding to positions 45–2528 of the HCV genome) from pBR322/16-6, which was previously described [24], and the *Mlu*I–*Spe*I fragment (corresponding to positions 2528–3420 of the HCV genome) from the PCR product of serum 1B-2. These two fragments were ligated into the *Eco*RI–*Spe*I fragment of pNSS1RZ2RU with 1B-2R1 sequence (a generous gift from Drs. K. Shimotohno, Kyoto University, and K. Sugiyama, Saitama Medical School), which was previously described [15]. To make a fragment for pON/C-5B, overlapping PCR was used to fuse EMCV IRES to the core protein-coding sequence. The resulting DNA was digested with *Rsr*II and *Cla*I, and then ligated with the *Xba*I–*Rsr*II fragment of pNSS1RZ2RU into the *Cla*I–*Xba*I fragment of pHCV-O.

The plasmids pORN/3-5B/KE and pORN/C-5B/KE were constructed from pON/3-5B/KE and pON/C-5B/KE, respectively, by introducing the PCR product of *Renilla* luciferase (Promega) into the *Asc*I site before the Neo gene.

The K1609E mutation was introduced, and 10 amino acids (MLVNGDDLVV), including the GDD motif, were deleted by QuickChange mutagenesis (Stratagene, La Jolla, CA) as previously described [11].

To construct pOF/3-5B, the Neo gene was replaced with the firefly luciferase gene at the *Asc*I and *Pme*I sites in pON/3-5B.

RNA synthesis. Plasmid DNAs were linearized by *Xba*I and used for RNA synthesis with the T7 MEGAscript Kit (Ambion, Austin, TX). After precipitation with lithium chloride, RNA was washed with 75% ethanol and dissolved in RNase-free water.

RNA transfection and selection of G418-resistant cells. For electroporation, HuH-7 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended at 10^7 cells/ml in PBS. RNA was mixed with 500 μl of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad, Hercules, CA). The mixture was immediately subjected to two pulses of current at 1.2 kV, 25 μF, and maximum resistance. Following 10 min of incubation at room temperature, cells were seeded into 10-cm dishes. Cells were selected in complete DMEM with 300 μg/ml G418.

Northern blot analysis. Total RNAs from the cultured cells were extracted with the RNeasy Mini Kit (Qiagen) and quantified by spectrophotometry at 260 nm. Four micrograms of RNA was used for the detection of HCV RNA and β-actin with reagents included in the Northern Max Kit (Ambion) according to the manufacturer's suggested protocol as described previously [11]. After samples were blotted onto positively charged Hybond-N+ nylon membranes (Amersham-Pharmacia Biotech, Piscataway, NJ), RNAs were immobilized on the membranes by UV cross-linking (Stratagene) and stained with ethidium bromide to locate 28S rRNA on the membrane. The membrane was cut approximately 1 cm below the 28S rRNA band. The upper part of the membrane, containing the HCV RNA, was hybridized with digoxigenin-labeled negative-sense RNA riboprobe complementary to the NS5B region. The lower part of the membrane, containing β-actin mRNA, was hybridized with a digoxigenin-labeled, β-actin-specific riboprobe. For the detection of riboprobe, membranes were incubated with anti-digoxigenin alkaline phosphatase-conjugate, reacted with CSPD (Roche Molecular Biochemical, Indianapolis, IN), and exposed to X-ray film. The synthetic RNAs transcribed from pON/3-5B and pON/C-5B (10^8 genome equivalent spiked into normal cellular RNA) were used to compare the levels of replicon RNA and genome-length HCV RNA.

Western blot analysis. Preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed as described previously [15]. The antibodies used in this study were those against core (Institute of Immunology, Tokyo), anti-E1 (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-E2 [25], anti-NS3 (Novocastra Laboratories, UK), anti-NS4A (a generous gift from Dr. A. Takamizawa, Research Foundation for Microbial Diseases, Osaka University), anti-NS5A [26], anti-NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), and β-actin (Sigma). Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

IFN treatment. The preparation of cured cells (1B-2R1C; designated as sOc in this paper) from subgenomic replicon cells (1B-2R1; designated as sO in this paper) was described previously [15]. To prepare cured cells from genome-length HCV RNA replicating cells (O), O cells were plated onto 6-well dishes for 24 h before IFN treatment. Human IFN-α (Sigma) was added to the cells at a final concentration of 500 IU/ml. The cells were cultured for 2 weeks without

G418 and with the addition of IFN-α (500 IU/ml) at 4-day intervals. The cured cells were named Oc cells.

To monitor the anti-HCV effect of IFN-α on ORN/3-5B/KE and ORN/C-5B/KE cells, 2×10^4 cells were plated onto 24-well plates and cultured for 24 h. Then the cells were treated with IFN-α at a final concentration of 0, 1, 10, and 100 IU/ml for 24 h, and subjected to luciferase and reverse transcription (RT)-PCR assay.

Reverse transcription and PCR. RT-PCR was performed separately in two parts; one part covered from HCV 5′UTR to NS3, with a final product of approximately 5.1 kb. The other part covered from NS2 to most of HCV 3′UTR, with a final product of about 6.1 kb. These fragments overlapped at the NS2 and NS3 regions, and were used for sequence analysis for HCV ORF following subcloning into pBR322. For RT of both parts, the antisense primers 290ROK, 5′-ATTAT TCTAGATCGACCTGGTTCCTGTCCCG-3′ and 386R, 5′-AATG GCCTATTGGCCTGGAG-3′ were used, respectively. The primer pair of 21X, 5′-ATTATTCTAGAGCCAGCCCCCGATTGGGGG CG-3′ and NS3RXOK 5′-ATTATTCTAGAGGCCTGTGAGACT AGTGATGATGC-3′ was used for the PCR of the first part. The primer pair of NS2XOK 5′-ATTATTCTAGACGTGTGGGGACAT CATCTTGGGTC-3′ and 9388RX 5′-ATTATTCTAGAATGGCCT ATTGGCCTGGAGTG-3′ was used for PCR of the second part. KOD-plus DNA polymerase was used for PCR (45 cycles), and each PCR cycle consisted of annealing at 64 °C for 30 s, primer extension at 68 °C for 7 min, and denaturation at 94 °C for 15 s.

cDNA cloning and sequencing. Two PCR products (5.1 and 6.1 kb) were digested with *Xba*I and then subcloned into the *Xba*I site of pBR322MC as previously described [13]. Plasmid insertions were sequenced in both the sense and antisense directions using the Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer Life Sciences) on an ABI PRISM 310 genetic analyzer (Applied Biosystems).

Quantification of HCV RNA. The RNAs were prepared from HCV RNA replicating cell lines, and 2 μg of each total RNA was used for RT with SuperScript II using primer 319R as previously described [27]. One-twentieth of the synthesized cDNA was subjected to real-time LightCycler PCR using primer pairs 104 and 197R as described previously [27].

Luciferase reporter assay. ORN/3-5B/KE and ORN/C-5B/KE cells were prepared as shown above in IFN treatment. After 24 h of this treatment, the cells were harvested with *Renilla* lysis reagent (Promega) and subjected to luciferase assay according to the manufacturer's protocol.

Results

Replication of genome-length HCV-O RNA in G418-resistant cells

Recently we reported the subgenomic replicon derived from genotype 1b virus, HCV-O (previously described as 1B-2) [15]. The source of the replicon RNA was HCV-O infected in the human hepatocyte PH5CH8 cell line. The RNA was prepared from PH5CH8 cells at 8 days post-infection of HCV-O. The sO (previously described as 1B-2R1), one of the cloned replicon cells, was obtained after 3 weeks of G418 selection. Based on the subgenomic replicon (ON/3-5B in Fig. 1) in sO cells, we tried to develop a genome-length HCV-O RNA (ON/C-5B in Fig. 1). To construct it, a structural region was synthesized by RT-PCR using RNA from HCV-O-infected PH5CH8 cells as described previously [24]. EMCV IRES and the core-encoding region were fused by overlapping PCR (see Materials and methods). The gene organization of the subgenomic replicon (ON/3-5B) was the same as that of the dicistronic genome-length HCV-O (ON/C-5B), except that only ON/C-5B contained the structural protein-encoding region (Fig. 1).

In the initial experiment, we used cured subgenomic replicon cells (sOc), because cured cells enhanced the colony formation of the subgenomic replicon more than did parental HuH-7 cells (data not shown). Ten micrograms of ON/C-5B transcripts was electroporated into sOc cells. After 3 weeks of G418 selection, only one colony was obtained. In repeated experiments, the number of G418-resistant colonies reproducibly was one or zero, so in this condition the efficiency of colony formation (ECF) was estimated at less than 0.1 colonies/μg RNA. We designated this cell line as 'O.'

To examine the replication level of ON/C-5B in O cells, total RNA extracted from O cells was subjected to Northern blot analysis for the detection of HCV RNA. As shown in Fig. 2A, the presence of a substantial abundance of HCV-specific RNA with a length of approximately 11 kb was detected in the extracts of total cellular RNA prepared from O cells. The cells contained more ON/C-5B RNA than subgenomic replicon RNA. To determine the level of HCV proteins produced from O cells, Western blot analysis was performed. Abundant structural proteins, core, E1, and E2 were detected in O cells (Fig. 2B). The detection of the nonstructural proteins—NS3, NS4A, NS5A, and NS5B—was also demonstrated in O and sO cells at almost uniform levels (Fig. 2B). These results revealed that the expression levels of HCV RNAs and HCV proteins differed somewhat between O and sO cells, suggesting that the stabilities of

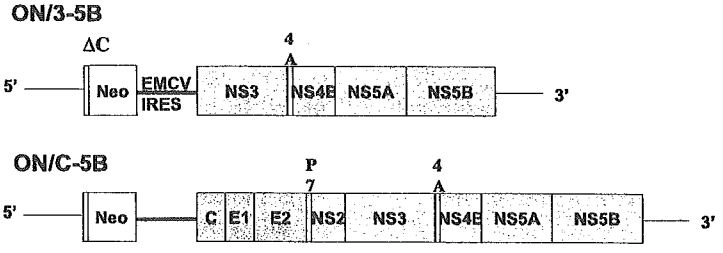

Fig. 1. Organization of subgenomic HCV replicon and genome-length HCV RNA derived from HCV-O. Open reading frames, untranslated regions, EMCV IRES, and Neo genes are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES.

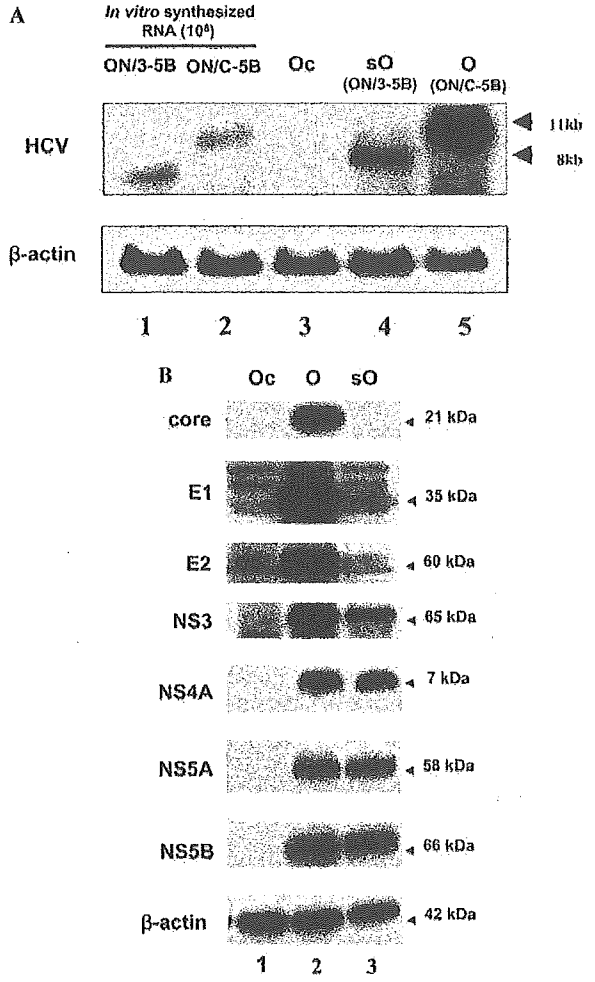

Fig. 2. Detection of HCV RNA and proteins in G418-resistant cell line. (A, top panel) Northern blot analysis of HCV-specific RNA in G418-resistant genome-length HCV RNA-replicating cell line. Lanes 1 and 2, synthetic RNA transcribed from ON/3-5B and ON/C-5B, respectively; lane 3, cured HuH-7 cells (Oc); and lanes 4 and 5, clonally isolated subgenomic HCV replicon and genome-length HCV RNA replicating cell lines by G418 selection, sO (ON/3-5B) and O (ON/C-5B), respectively. (Bottom panel) Northern blot analysis of β-actin mRNA using β-actin-specific RNA probe. (B) Western blot analysis of HCV proteins. Production of core, E1, E2, NS3, NS4A, NS5A, and NS5B in Oc cells (lane 1), O cells (lane 2), and sO cells (lane 3) was analyzed by immunoblotting using anti-core, anti-E1, anti-E2, anti-NS3, anti-NS4A, anti-NS5A, and anti-NS5B antibodies, respectively. β-actin was used as a control for the amount of protein loaded per lane.

genome-length HCV RNA and subgenomic replicon RNA or the efficiency of translation differs between O and sO cells. In summary, we showed the efficient replication of genome-length HCV-O RNA in O cells.

A cloned cell line changed characteristics during cell culture with G418 selection

The O cells were expected to possess the same cellular background as the sO and sOc cells. To examine this, we first obtained the cured cells (Oc) by treating O cells with IFN-α (500 IU/ml) for 2 weeks, and then confirmed that ON/C-5B RNA in Oc cells was not detected by RT-PCR (Figs. 3A and B). In addition, we confirmed that ON/3-5B RNA in sOc cells was also not detected by RT-PCR (Fig. 3B).

We tested the ECFs of sOc and Oc cells by reintroducing ON/C-5B RNA and ON/3-5B RNA into them. Unexpectedly, G418-resistant colonies were produced from ON/C-5B RNA-introduced Oc cells even with 0.02 μg RNA, and the number of colonies increased in an RNA-dose-dependent manner (Fig. 3C). The ECF of ON/C-5B in Oc cells is estimated to be about 50 colonies/μg RNA. In contrast, the ECF of ON/C-5B in sOc cells was less than 0.1 colonies/μg of RNA, although a number of G418-resistant colonies were produced from ON/3-5B RNA-introduced sOc and Oc cells (Fig. 3C). These results suggest that Oc cells possess overwhelming advantages in the replication of genome-length HCV RNA.

Combination of adaptive mutation in NS3 and cured cells enhances the efficiency of colony formation

Information on adaptive mutation has been accumulated so far by using subgenomic replicons [20,22,28], but there has been no systematic analysis of mutations in a genome-length HCV RNA replication system. We therefore performed a sequence analysis of HCV RNA replicating in O cells. RNAs extracted from O cells were subjected to RT-PCR, and then two fragments (5.1 and 6.1 kb) amplified for ORF were subcloned into plasmid for sequence analysis, as described in Materials and methods. The sequences of three independent clones were determined and compared with each other to avoid PCR error and to find conserved mutations. Only one common mutation with an amino acid substitution was detected, and it was in the NS3 helicase region at amino acid position 1609 (Fig. 4). This mutation, from lysine to glutamic acid (K1609E), was seen in previously reported Con1 and 1B-1 replicons, in which the mutation seemed to have little impact on ECF [22]. We examined the effect of this mutation in ON/C-5B on ECF. In the initial experiment, we introduced the ON/C-5B/KE transcript into sOc cells. As shown in Figs. 5C and D, ON/C-5B/KE RNA-introduced sOc cells produce G418-resistant colonies (the ECF is estimated to be about 75 colonies/μg RNA), although no G418-resistant colonies were obtained in ON/C-5B/wt RNA–introduced sOc cells (Figs. 5A and B). These results indicated that the K1609E mutation worked as an adaptive mutation. Furthermore, when ON/C-5B/KE RNA was introduced into Oc cells, the ECF was significantly enhanced (Figs. 5G and H). The estimated ECF of K1609E with Oc was about 1500 colo-

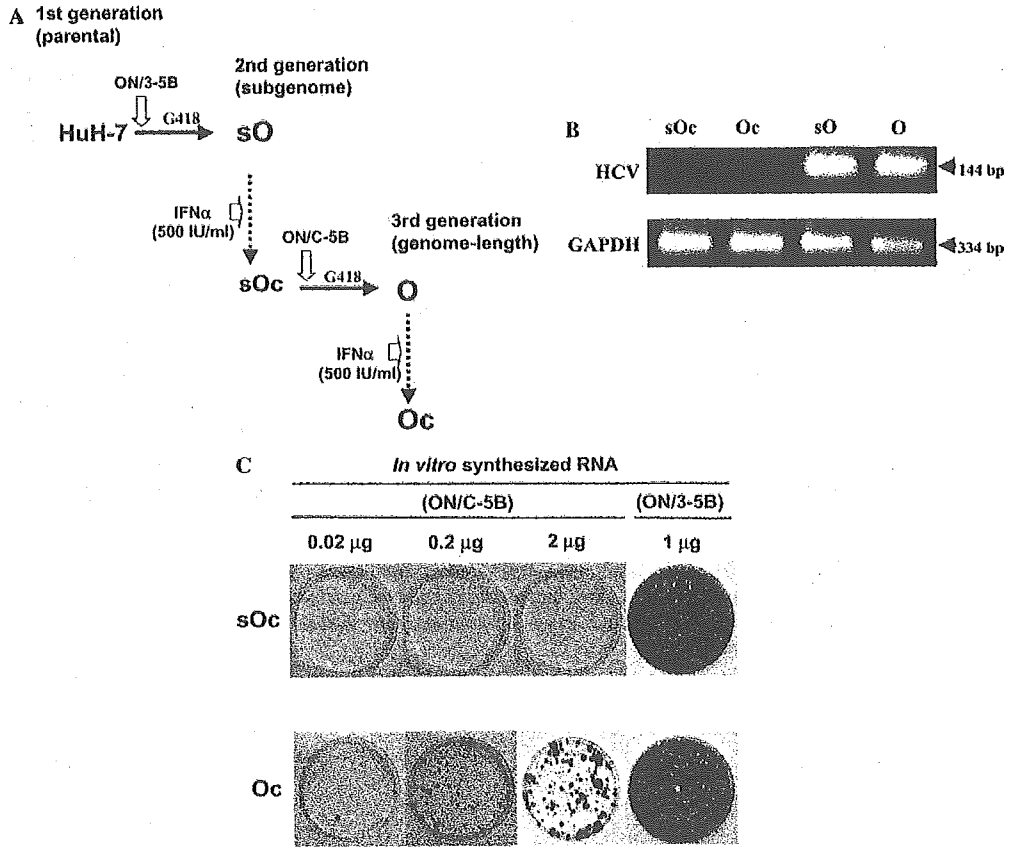

Fig. 3. Characterization of cured cells. (A) Lineage of cured cells. The procedures of G418 and IFN-α treatments are schematically shown. The solid line is the G418 (300 μg/ml) treatment for 4 weeks, and the dotted line is the IFN-α (500 IU/ml) treatment for 2 weeks. The sOc, Oc, sO, and O cells used in this study were obtained in order by the treatment of the indicated reagents, respectively. (B) RT-PCR analysis for the detection of subgenomic and genome-length HCV RNAs. Total RNAs were extracted from sO, sOc, O, and Oc cells, and then RT-PCR was performed as previously described [27]. RT-PCR products (144 bp for HCV and 344 bp for GAPDH) were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (C) Different ECF between sOc and Oc cells. ON/C-5B RNA (0.02, 0.2, and 2 μg per 10-cm dish) and ON/3-5B RNA (1 μg per 10-cm dish) were transfected into sOc cells (top panel) and Oc cells (bottom panel) as described in Materials and methods. The panels show G418-resistant colonies that were stained with Coomassie brilliant blue at 3 weeks after transfection of RNAs [22].

nies/μg RNA. Moreover, a number of G418-resistant colonies were also obtained in ON/C-5B/wt RNA—introduced Oc cells (Figs. 5E and F). These results suggest that Oc cells are superior to sOc cells regarding the intracellular replication of genome-length HCV RNA.

To examine how this effect of K1609E on ECF correlates with early events (i.e., those immediately after electroporation), we constructed subgenomic HCV replicons with the firefly luciferase gene for transient assay (Fig. 6A). The subgenomic replicon with K1609E showed better replicability than the wild-type replicon in Oc cells (Fig. 6B). This indicates that the ECF reflects the effect of adaptive mutation in early events, so the transient assay for adaptive mutations might be suitable for evaluating the establishment of a persistent HCV RNA replication system.

Genome-length dicistronic HCV-O RNA encoding luciferase gene facilitates the monitoring of HCV replication in HuH-7 cells

Thus far, HCV RNA replication systems possessing persistently expressing reporter genes have been limited to studies of subgenomic HCV replicons, and there is no report of a genome-length HCV RNA replication system possessing a reporter gene. The combination of a K1609E adaptive mutation and Oc cells demonstrated a great impact on ECF (Fig. 5). This result encouraged us to construct a convenient genome-length HCV RNA replication system in which a *Renilla* luciferase gene is introduced to facilitate the monitoring of HCV replication.

Ten micrograms of in vitro transcripts from pORN/3-5B/KE and from pORN/C-5B/KE (Fig. 7A) was elec-

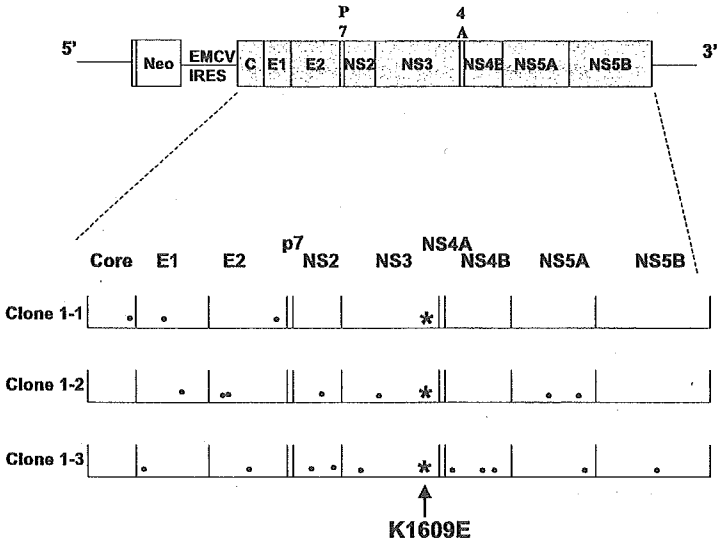

Fig. 4. Amino acid substitutions detected in the genome-length HCV RNA derived from O cells. HCV ORF derived from O cells was amplified by RT-PCR using HCV-specific primer sets. After subcloning, three independent clones were subjected to sequence analysis. A common amino acid substitution (indicated by the asterisk) from lysine to glutamic acid was found at amino acid position 1609 in the NS3 helicase region. The dots indicate clone-specific amino acid substitutions.

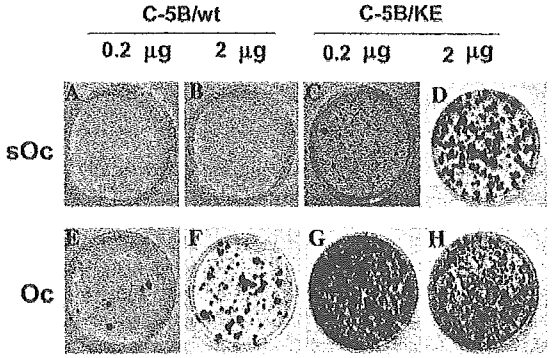

Fig. 5. Effect of K1609E on ECF. ECF was examined using cured cell lines, sOc and Oc. G418-resistant colonies were stained with Coomassie brilliant blue at 3 weeks after electroporation of RNA transcripts without (C-5B/wt; A, B, E, and F) or with (C-5B/KE; C, D, G, and H) the K1609E mutation (0.2 and 2 μg per 10-cm dish) into sOc cells (top panel) or into Oc cells (bottom panel) [22].

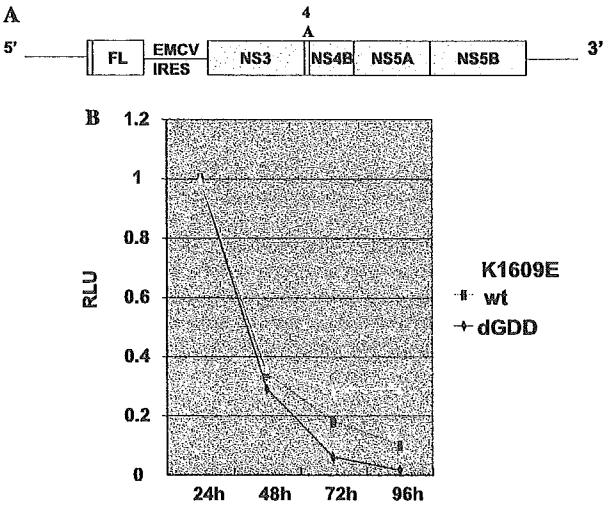

Fig. 6. Effect of K1609E in NS3 on transient replication. (A) The construct of reporter subgenomic HCV replicon carries firefly luciferase instead of Neo. (B) The reporter subgenomic HCV replicon with K1609E was compared with wild-type for transient replication in Oc cells. dGDD indicates the deletion of the GDD motif in the NS5B polymerase, and the subgenomic HCV replicon with the deletion of GDD was used as a negative control.

troporated into Oc cells, and ORN/3-5B/KE and ORN/C-5B/KE cells were selected as polyclonal cell lines by G418 (300 μg/ml) for 4 weeks. The ECF of ORN/C-5B/KE in Oc cells was about 7 colonies/μg RNA. To confirm the presence of HCV RNA in ORN/3-5B/KE and ORN/C-5B/KE cells, Northern blot analysis was performed with total RNA from these cells. As shown in Fig. 7B, 9 and 12 kb of HCV-specific RNA were detected for RNAs from ORN/3-5B/KE and ORN/C-5B/KE cells, respectively. The production of HCV proteins was also detected for the ORN/3-5B/KE cell line with anti-NS3 and anti-NS5B antibodies, and for ORN/C-5B with anti-core, anti-NS3, and anti-NS5B antibodies (Fig. 7C).

To demonstrate the correlation between levels of luciferase activity and HCV RNA, a luciferase reporter assay and real-time LightCycler PCR were performed. At 24 h after IFN-α treatment, the *Renilla* luciferase

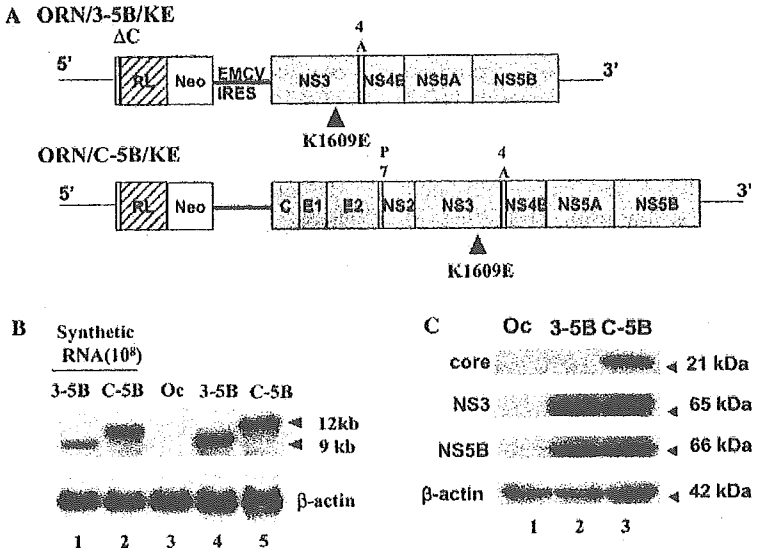

Fig. 7. Characterization of genome-length HCV RNA encoding the *Renilla* luciferase gene as a reporter. (A) Schematic gene organization of subgenomic and genome-length HCV RNA. The basic constructs are described in Fig. 1; the *Renilla* luciferase gene (RL) is depicted as a striped box and is expressed as a fusion protein with Neo. (B) Northern blot analysis was performed for Oc (lane 3), ORN/3-5B/KE cells (lane 4), and ORN/C-5B/KE cells (lane 5) using the HCV RNA-specific probe as shown in Fig. 2. In vitro transcripts of ORN/3-5B/KE (lane 1) and ORN/C-5B/KE (lane 2) were used as size markers. (C) Western blot analysis was performed for Oc cells (lane 1), ORN/3-5B/KE cells (lane 2), and ORN/C-5B/KE cells (lane 3) with anti-core, anti-NS3, and anti-NS5B antibodies as shown in Fig. 2.

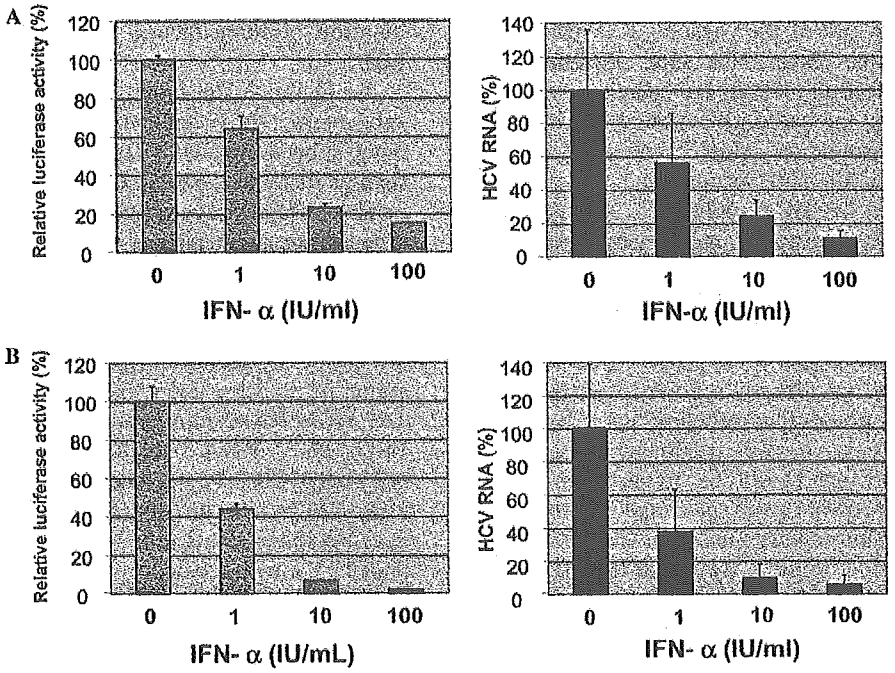

Fig. 8. The effect of IFN-α on the subgenomic HCV replicon and the genome-length HCV RNA replication system possessing the *Renilla* luciferase reporter. (A) The replication level of the subgenomic HCV replicon encoding the *Renilla* luciferase gene was monitored by luciferase reporter assay (left panel) and real-time LightCycler PCR (right panel) at 24 h after IFN-α treatment. (B) The replication level of genome-length HCV RNA encoding the *Renilla* luciferase gene was monitored by luciferase reporter assay (left panel) and real-time LightCycler PCR (right panel).

activity and HCV RNA concentration were examined. As shown in Fig. 8, luciferase activity correlated well with HCV RNA concentration, and IC_{50} of IFN-α was less than 10 IU/ml. The concentration of IC_{50} was compatible with the findings of a previous study [29].

Discussion

In this study, we established a dicistronic genome-length HCV RNA replication system using HCV RNA from HCV-O infected in non-neoplastic human hepatocyte PH5CH8 cells. The characterization of O cells supporting ON/C-5B RNA replication revealed the presence of an adaptive mutation (K1609E) in the NS3 helicase region and a high ECF in cured Oc cells. The combination of the adaptive mutation and cured cells led us to develop a genome-length HCV RNA replication system that stably expresses luciferase as a reporter to facilitate HCV replication monitoring.

For the cells into which genome-length HCV RNA was to be introduced, we chose the cloned cell line sOc, prepared by IFN treatment from subgenomic HCV replicon-supporting cells, since sOc had a higher ECF than its parental HuH-7 cells in a study of subgenomic HCV replicons [30]. In our initial study, only one colony (O cells) was produced after 3 weeks of G418 selection. Oc cells were obtained in 2 weeks of IFN treatment for O cells. We expected that sOc and Oc cells would have similar backgrounds. However, our colony formation experiments using these two cell lines showed unexpected results. The ECF of Oc cells was at least 500 times higher than that of the parental sOc cells. There are several possible reasons for such differences: (1) G418 used for selection affected the cellular factors, (2) IFN treatment for the cured cells altered something in the cellular background, (3) HuH-7 cells lacked a mismatch repair function and accumulated mutations in the genes required for HCV replication, and (4) HCV proteins inhibited the mismatch repair. Concerning possibilities (1) and (2), there are no reliable reports to date that these two reagents (G418 and IFN) worked as mutagens. Concerning (3), many cancer-derived cell lines were reported to lack a mismatch repair function [31,32]. As for (4), we recently reported that the HCV core protein promoted microsatellite instability [33]. However, further comparative experiments using subgenomic HCV replicons would be required to clarify whether the core protein causes the changes in the cellular background. Although we are able to clarify whether or not this alteration occurred by one of the factors described above or through more than one factor synergistically, the results in this study have indicated that even cloned HuH-7 cells have the potential to change their cellular background during culture. On the other hand, it might lead to the chance for us to select the cell line with the stronger ability to support virus replication from heterogeneous cell populations.

Information on adaptive mutations in subgenomic HCV replicons has been accumulated, but the present study is the first to examine precisely the adaptive mutation in genome-length HCV RNA. In this study, we analyzed mutations in genome-length HCV RNA in O cells and found that K1609E in the NS3 helicase region worked as an adaptive mutation. The adaptive mutation of K1609E was also reported by Lohmann et al. [22] but its impact on colony formation was only about 4 times that of the wild-type replicon [22]. We also reported the same mutation in our previous study of the 1B-1 replicon, although we did not assess ECF at that time [13]. In the present study, we found that the ECF of ON/C-5B/KE was significantly enhanced, to about 500 times that of the wild-type ON/C-5B. Also, in the transient reporter assay of the subgenomic HCV replicon, the introduction of K1609E enhanced the efficiency of replication. It was noteworthy that the introduction of S2204I in NS5A, which was reported as an adaptive mutation in HCV-N and Con1 replicons, had little impact on the transient reporter assay of the HCV-O replicon (data not shown). The differences in the effects of adaptive mutation might be due to the differences in HCV strains. To further improve the conditions of colony formation, the ECF in the combination of K1609E mutation and cured Oc cells was tested using ON/C-5B. This combination drastically enhanced the ECF of ON/C-5B. These results suggested that not only viral but also cellular factors were selected during culture with G418 for robust replication of HCV, and that the combination of these factors synergistically enhanced the ECF.

To facilitate the monitoring of the replication of a subgenomic HCV replicon, several groups have developed subgenomic HCV replicons with reporter genes such as luciferase, SEAP, or β-lactamase [17–19]. These persistent replication systems could save time and facilitate the mass screening of anti-HCV reagents. However, until now there has been no genome-length HCV RNA replication system with a reporter gene. One of the obstacles to the development of such a system may be low ECF, depending on the size of HCV RNA, or the ability of replicase complexes, including NS5B, to replicate HCV RNA. Our preliminary data showed that a 14-kb HCV RNA, which contained Core-E1-E2-p7-NS2-NS3-NS4A-NS4B fused to Neo at the first cistron instead of Neo in the subgenomic HCV-N replicon, produced colonies containing smaller HCV RNA, i.e., less than 12 kb, with a deletion at the first cistron (M. Ikeda and S.M. Lemon, unpublished data). In our trial to develop a genome-length HCV RNA replication system with a reporter gene, genome-length HCV RNA encoding the firefly luciferase gene (about 12.6 kb in total) failed to produce a G418-resistant colony, although we did obtain colonies containing subgenomic HCV replicon RNA encoding the firefly luciferase gene (about 9.6 kb in total) (data not shown). However, both subgenomic HCV replicon RNA and genome-length HCV RNA encoding the *Renilla* luciferase gene (about 9 and 12 kb in total, respectively) successfully produced

G418-resistant colonies. These results suggest that the NS5B polymerase of HCV-O possesses a limited elongation ability (probably up to a total length of 12 kb). We established a genome-length HCV RNA replication system with *Renilla* luciferase as a reporter using a newly discovered advantage in the combination of the K1609E adaptive mutation and cured Oc cells. The cell line supporting ORN/C-5B/KE derived from Oc cells demonstrated the usefulness of IFN-α's anti-HCV effect, since the values of *Renilla* luciferase correlated well with the level of HCV RNA at 24 h after IFN treatment. One of the most striking advantages of this system is that it allows us to investigate the effect of structural proteins on viral replication. In addition, anti-HCV activity in this system is reflected in the inhibitory level of HCV replication.

We developed a genome-length HCV RNA replication system from HCV-O infected in the non-neoplastic human hepatocyte line PH5CH8. Adaptive mutation was selected among a heterogeneous viral pool during replication, and a cell clone supporting robust HCV replication was selected from a heterogeneous pool of cells during culture. These viral and cellular factors contributed to the enhancement of colony formation and led to the establishment of a genome-length HCV RNA replication system with a reporter. This system has the longest RNA construct reported so far. In conclusion, this genome-length HCV replication system with a reporter gene, developed through the characterization of ON/C-5B, should be a useful tool for the study of HCV replication and for the mass screening of anti-HCV reagents.

Acknowledgments

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