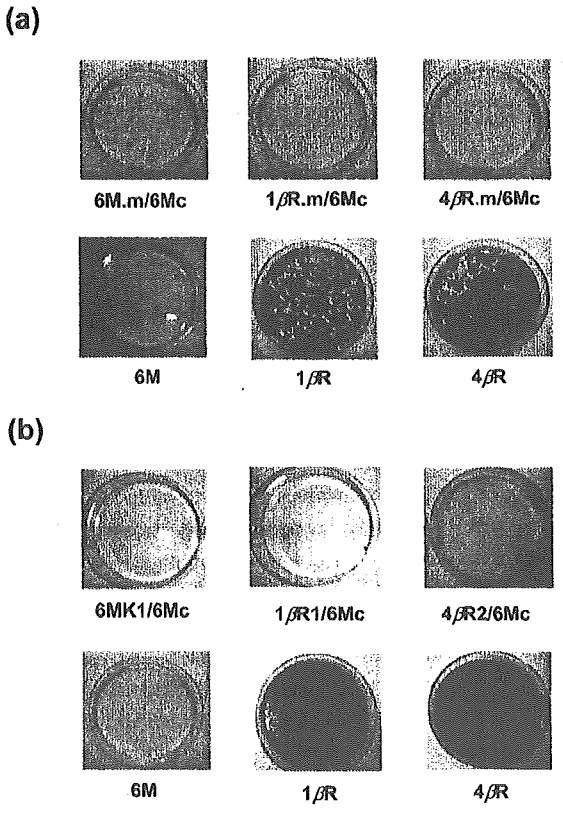

Fig. 1. IFN sensitivities of various HCV replicon-harbouring cells. (a) 6M.m/6Mc, 1βR.m/6Mc and 4βR.m/6Mc cells obtained as G418-resistant mixed colonies were treated with IFN-α (400 IU ml^{-1}) for 3 weeks in the presence of G418 (300 μg ml^{-1}). 6M, 1βR and 4βR cells were also used for control experiments. G418-resistant colonies were stained with Coomassie brilliant blue as described previously (Naganuma *et al.*, 2004). (b) IFN sensitivities of 6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc cells were examined as described in (a) except that they were treated with IFN-β (200 IU ml^{-1}).

3 weeks). Although many colonies were found to have survived in 1βR and 4βR cells after IFN-α treatment, only a few colonies survived in 1βR.m/6Mc and 4βR.m/6Mc cells or in 6M and 6M.m/6Mc cells (Fig. 1a). Similar results were obtained when these replicon-harbouring cells were treated with IFN-β (400 IU ml^{-1}) (data not shown), although two colonies (named 6βR and 7βR, described below) derived from 6M.m/6Mc and 4βR.m/6Mc cells, respectively, proliferated as highly IFN-resistant clones.

We further examined the IFN sensitivity of replicon-harbouring cells (6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc) established by the transfection of *in vitro*-synthesized replicon RNAs (6MK1, 1βR1 and 4βR2 obtained from 6M, 1βR and 4βR cells, respectively) (Namba *et al.*, 2004; Kato *et al.*, 2005) into 6Mc cells. The results revealed that few or no colonies survived in 1βR1/6Mc and 4βR2/6Mc cells, as in 6M and 6MK1/6Mc cells, whereas many colonies survived in 1βR and 4βR cells (Fig. 1b). These results suggested that cellular factors rather than viral factors contributed to the highly IFN-resistant phenotype of HCV replicon-harbouring cells. However, the present results obtained under a high concentration of IFN do not necessarily rule out a possible role for HCV mutations in conferring low degrees of IFN resistance, because effects of HCV mutations on IFN activity are presumably weaker than those of the cellular factors.

To obtain evidence in favour of the idea that alterations in cellular factor(s) are involved in the emergence of an IFN-resistant phenotype, we attempted to prepare cured cells from the replicon-harbouring cells possessing a highly IFN-resistant phenotype. Since phosphorylation of signal transducer and activator of transcription 1 (STAT1) occurred in the 1βR and 4βR cells treated with IFN-γ (Fig. 2a), these replicon-harbouring cells were treated with IFN-γ (500 IU ml^{-1}) for 3 weeks, and cured 1βRc and 4βRc cells were obtained. Western blot (Fig. 2b) and RT-PCR (data not shown) analyses showed that no replicons were detected in either type of cured cells. Analysis of a luciferase reporter assay indicated that the complete defect of the IFN-α/β signalling was not restored in the cured 1βRc and 4βRc cells (Fig. 2c).

To clarify whether or not the signalling defect in these replicon-harbouring cells was restricted to type I IFN, we examined the phosphorylation status of STAT3 in 6M, 1βR and 4βR cells treated with interleukin-6 (IL6). Since it has been reported that STAT3 is also activated by IFN-α treatment (Pfeffer *et al.*, 1997), the phosphorylation status of STAT3 in these replicon-harbouring cells after IFN-α treatment was also examined. Our results revealed that STAT3 was not phosphorylated in 1βR and 4βR cells treated with IFN-α, while phosphorylation of STAT3 was observed in 6M, 1βR and 4βR cells treated with IL6 and in 6M cells treated with IFN-α (Fig. 2d), indicating that only type I IFN signalling was defective in 1βR and 4βR cells. These results suggested that the initial reaction following the addition of IFN-α/β was defective in replicon-harbouring cells possessing a highly IFN-resistant phenotype.

Following up this suggestion, we examined the genetic status of tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1). Sequence analysis of TYK2 and JAK1 cDNAs obtained from 1βR and 4βR cells was performed after cloning into the pCXbsr vector (Akagi *et al.*, 2000), as described previously (Nozaki *et al.*, 2003). However, the results showed no mutations in these cDNAs (data not shown). We next focused on type I IFN receptors (IFNAR1 and IFNAR2c). Our results showed that the mRNA levels of the two receptors were almost equal among all examined replicon-harbouring cells including 6Mc cells (see Supplementary Fig. S1a, available in JGV Online). More than three independent clones of each cDNA (1708 bp for IFNAR1 and 1582 bp for IFNAR2c) were sequenced as described above. Table 1 shows a summary of sequence analysis of IFNAR1

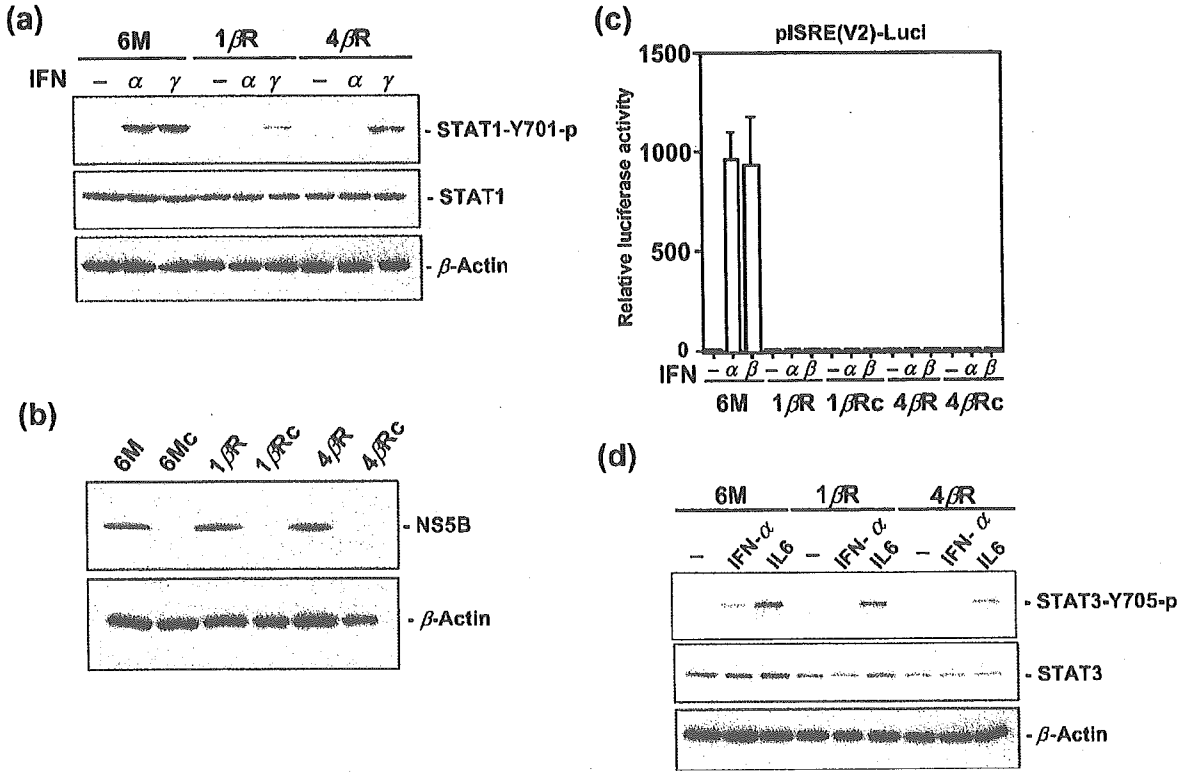

Fig. 2. Cellular factors rather than HCV replicons contribute to defects in type I IFN signalling. (a) Western blot analysis of STAT1 in 6M, 1βR and 4βR cells treated with IFN-α or -γ. The replicon-harbouring cells were treated with or without IFN-α or -γ (500 IU ml^{-1} each) for 30 min, and Western blot analysis for STAT1 and its phosphorylation status (Tyr-701) was then performed as previously described (Hijikata *et al.*, 1993). β-Actin was used as a control for the amount of protein loaded per lane. (b) Western blot analysis of NS5B. Anti-NS5B antibody was used for immunoblotting. β-Actin was used as described in (a). (c) Analysis of IFN signal transduction. Dual luciferase assays using pISRE(V2)-Luci (Dansako *et al.*, 2003) were performed as previously described (Naganuma *et al.*, 2000). Cells were treated with IFN-α or -β (500 IU ml^{-1} each) for 6 h. (d) The defect in the signalling pathway in 1βR and 4βR cells is restricted to type I IFN. Replicon-harbouring cells were left untreated or treated with IFN-α (500 IU ml^{-1}) or IL6 (100 ng ml^{-1}) for 30 min. Western blot analysis for STAT3 and its phosphorylation status (Tyr-705) was performed as described in (a).

and IFNAR2c mRNAs. Surprisingly, we found that nt 475 of IFNAR2c mRNA from 1βR cells had a U substituted for G in 13/13 clones, resulting in a nonsense mutation at codon 159 from glutamic acid (GAG) to the termination codon UAG (see Supplementary Fig. S1b). Furthermore, nt 319 of IFNAR1 mRNA from 4βR cells was also found to have a U substituted for G in 12/12 clones, resulting in a nonsense mutation at codon 107 from glutamic acid (GAA) to the termination codon UAA (see Supplementary Fig. S1c). However, interestingly, IFNAR1 and IFNAR2c mRNAs obtained from 1αR and 4αR cells, which were derived from clone 1 and clone 4 and were counterparts of 1βR and 4βR cells, respectively (see Supplementary Fig. S2, available in JGV Online), did not possess the nonsense mutations found in the mRNAs from 1βR and 4βR cells. In addition, several kinds of deletion and another nonsense mutation (lysine to a termination codon at codon 458) were found in approximately half of IFNAR1 cDNA clones obtained from 3βR and 5βR cells. In contrast to the finding of frequent mutations and deletions in IFNAR mRNAs from the βR series, such genetic abnormalities in IFN receptors were quite rare in the αR series (Table 1).

To evaluate the possibility that genetic mutants might pre-exist in the cloned replicon-harbouring cells (clones 1, 3, 4 and 5 shown in supplementary Fig. S2) or that genetic mutants had appeared during the IFN-β treatment of the cloned replicon-harbouring cells, we repeated IFN-β treatment of cloned 1, 3, 4 and 5 cells following the method described previously (Namba *et al.*, 2004). The result was almost identical to that obtained previously (Namba *et al.*, 2004), indicating the good reproducibility of the experiment with IFN-β treatment (data not shown). In the present study, each of the three colonies showing resistance to IFN-β was isolated and proliferated (see Supplementary Fig. S2) and we then performed sequence analysis of IFNAR mRNAs

Table 1. Genetic alterations of type I IFN receptors in HCV replicon-harbouring cell lines possessing IFN-resistant phenotypes

The determined nucleotide sequences were compared with those of 6M and 6Mc cells, which confirmed that the deduced amino acid sequences were identical to the human IFNAR1 (GenBank accession no. NM_000629) and IFNAR2c (GenBank accession no. L41942) sequences. NM, Not mutated.

Cell line	IFNAR1 mRNA			IFNAR2c mRNA		
	Nucleotide change and position	Effect on protein	Mutation frequency*	Nucleotide change and position	Effect on protein	Mutation frequency*
6M	NM	None	0/3	NM	None	0/3
1βR	NM	None	0/3	G to U at nt 475	E to stop at codon 159	13/13
3βR	Deletion of 5 nt at nt 376	Truncated (135 aa)	2/11	NM	None	0/3
	A to U at nt 1372	K to stop at codon 458	3/11			
4βR	G to U at nt 319	E to stop at codon 107	12/12	NM	None	0/3
5βR	Deletion of 176 nt at nt 201	Truncated (78 aa)	4/10	NM	None	0/3
	Deletion of 79 nt at nt 201	Truncated (67 aa)	2/10			
1αR	NM	None	0/3	NM	None	0/3
3αR	NM	None	0/3	NM	None	0/3
4αR	Deletion of 5 nt at nt 376	Truncated (135 aa)	1/3	NM	None	0/3
5αR	Deletion of 79 nt at nt 201	Truncated (67 aa)	1/3	NM	None	0/3
αRmix	NM	None	0/3	NM	None	0/3
6Mc	NM	None	0/3	NM	None	0/3

*Number of mutated or truncated clones/number of examined clones.

as described above. The results revealed that the nonsense mutations or deletions identified at this time (see Supplementary Table S1, available in JGV Online) were quite different from those obtained from the βR series (Table 1). Therefore, it is unlikely that the identified IFNAR mutants pre-existed in cloned 1, 3, 4 and 5 cells when these cells were obtained as colonies surviving IFN-α treatment.

To examine whether or not additional HCV replicon cell lines possessing the IFN-resistant phenotype could be obtained from HCV replicon-harbouring cells other than the parental replicon-harbouring cells used for the isolation of the αR and βR series, 6M.m/6Mc, 1βR.m/6Mc, 4βR.m/6Mc and 50-1 replicon-harbouring cells were treated with IFN-β (see Supplementary Fig. S2). Finally, we obtained four replicon-harbouring cell lines (6βR obtained from the 4βR.m/6Mc cells, 7βR obtained from the 6M.m/6Mc cells, and 8βR and 9βR obtained from the 50-1 cells) showing resistance to IFN-β. These results indicated that HCV replicon-harbouring cells showing the IFN-resistant phenotype were obtained from HCV replicon-harbouring cells established immediately. By sequence analysis of IFNAR1 and IFNAR2c cDNAs as described above, the E107stop nonsense mutation in IFNAR1 cDNA, which was the same mutation found in the 4βR cells, was found again in the 8βR and 9βR cells, while no IFNAR mutations were detected in the 6βR and 7βR cells (see Supplementary Table S1). The observation that IFNAR mutations occurred preferentially after IFN-β treatment is interesting. Since a variety of mutations and deletions in the IFN receptors were obtained from the cloned replicon-harbouring cells surviving after IFN-β treatment, such genetic alterations might occur accidentally in order to impair the antiviral states caused after IFN-β treatment. Thereafter, only replicon-harbouring cells possessing the IFNAR mutants might be able to proliferate in the presence of G418, resulting in the βR series.

To clarify whether or not the IFNAR mutations found in the βR series were determinants for the IFN sensitivity of HCV replicons, we prepared 4βR cells (possessing the IFNAR1 mutant) stably expressing wild-type IFNAR1 and examined its IFN sensitivity. Analysis of a luciferase reporter assay (see Supplementary Fig. S3a, available in JGV Online) clearly showed that IFN signalling in 4βR cells was restored by the expression of wild-type IFNAR1 in comparison with those of 4βR cells expressing the IFNAR1 mutant (see Supplementary Fig. S3b). The quantitative RT-PCR analysis of replicon RNA in the cells treated with IFN-β clearly showed that the level of 4βR replicon in cells expressing wild-type IFNAR1 was drastically decreased after IFN-β treatment, as was the level of 6M replicon in cells expressing wild-type IFNAR1 (see Supplementary Fig. S3c). In summary, we demonstrated that the IFNAR mutation found in 4βR cells was a major determinant for a strongly IFN-resistant phenotype of 4βR cells, suggesting that IFNAR mutations, which lead to the impairment of IFN signalling, convert HCV replicon-harbouring cells from an IFN-sensitive phenotype to a highly IFN-resistant phenotype.

IFNAR1 and IFNAR2c belonging to the class II cytokine receptor superfamily are structurally conserved transmembrane receptors located on the cell surface (see Supplementary Fig. S4a, available in JGV Online). However, since both the IFNAR1 E107stop mutant and the IFNAR2c E159stop mutant found in 4βR and 1βR cells, respectively, were N-terminally truncated and probably soluble forms, these truncated proteins may not be functional as IFN receptors or may act as dominant-negative inhibitors, and will lead to the interception of IFN signalling (see Supplementary Fig. S4b). Thus, the cause of the IFN-resistant phenotype of 1βR or 4βR cells appeared to be the functional disruption of IFNAR. The present results suggest that the downstream JAK/STAT pathway is intact, at least in 4βR cells.

Although for the most part we could clarify the mechanism underlying a highly IFN-resistant phenotype of HCV replicon-harbouring cells, at least in the case of 4βR cells, the mechanism underlying a partially IFN-resistant phenotype remains unclear, because IFNAR mutations were rare in the αR series. Since the expression levels of IFNAR, TYK2 and JAK1 were not decreased in the αR series, a functional deficiency of other cellular factor(s) involved in the IFN signalling may contribute to the acquisition of IFN resistance. Alternatively, certain HCV mutation(s) may account for the partially IFN-resistant phenotype of the αR series.

Since Machida *et al.* (2004a, b) recently reported that the frequency of genetic mutation was enhanced by HCV replication in *in vitro*-infected B cells and that the HCV core and NS3 were involved in the induction of a mutator phenotype mediated through the activation of inducible nitric oxide synthase, we cannot exclude the possibility that persistent HCV replication induces some irreversible genetic mutations. To clarify whether or not HCV acts as a mutagen for cellular factors, further study using an HCV RNA replication system (Ikeda *et al.*, 2002, 2005; Naka *et al.*, 2005) will also be necessary.

The HCV replicon-harbouring cells including 1βR and 4βR, in which IFN signalling is impaired, used or obtained in the present study may be useful for future studies, not only of the mechanism(s) underlying the IFN resistance of the replicons but also of the functional characterization of IFN receptors. Furthermore, these replicon cells may also be useful for screening novel anti-HCV reagents that act by mechanisms unrelated to IFN signalling.

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Inhibition of hepatitis C virus replication by pol III-directed overexpression of RNA decoys corresponding to stem-loop structures in the NS5B coding region

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Abstract

Increasing evidence has shown that the stem-loop (SL) structures in the NS5B coding region of hepatitis C virus (HCV) function as *cis*-replicating elements that are indispensable for viral replication. We have investigated whether small RNA molecules analogous to the SL structures could inhibit HCV replication. Reporter assays showed that both in vitro transcribed and pol III-directed transcripts corresponding to 5BSL3.1 and 5BSL3.2 efficiently inhibited HCV replicon-encoded luciferase expression. Mutagenesis studies revealed that mutation in 5BSL3.2 which debilitated its binding to NS5B also abolished the ability of 5BSL3.2 RNA to inhibit HCV replication, suggesting that SL RNA inhibits HCV by sequestering the replication complex. Further, adenoviral-mediated expression of the SL RNAs potently blocked the replication of HCV replicon in Huh-7 cells. Importantly, SL RNAs derived from HCV 2a, an evolutionarily distant genotype, were also shown to suppress the replication of HCV 1b replicon in spite of the genetic heterogeneity between the SL elements of the two viruses, implying the potential of SL RNA-based approach to inhibit a wide range of HCV isolates. These results suggest that SL RNA decoys may prove to be useful in the treatment of hepatitis C, which may be advantageous over other sequence-specific gene therapy modalities (such as antisense RNA and siRNA) in preventing the escape of genetic variants.

Keywords: RNA decoy; Hepatitis C virus; Stem-loop; Gene therapy

Introduction

Infection with hepatitis C virus (HCV), the main causative agent for non-A, non-B hepatitis, is a serious worldwide health problem. It is estimated that more than 200 million individuals are infected with HCV, and many of them are at a high risk for developing liver cirrhosis and hepatocellular carcinoma (Alter, 1997). Current therapeutic regimens using interferon-α, whether alone or in combination with ribavirin, have limited efficacy (McHutchison, 2002). Although small inhibitory molecules targeting serine protease, helicase, or RNA-dependent RNA polymerase (RdRp) are in development, one unavoidable problem is selection of resistant mutants conferred by single or multiple mutations due to the error-prone nature of RdRp (Lindenbach and Rice, 2003; Lu et al., 2004; Nguyen et al., 2003).

The potential of using small RNA molecules as therapeutic agents has been extensively explored, antisense RNA, ribozyme, and more recently siRNA have been demonstrated to be highly efficient in inhibiting a number of pathogenic viruses, including HCV (Gitlin et al., 2002; Jacque et al., 2002; Randall et al., 2003). However, because of the highly sequence-specific property intrinsic in these approaches, the antiviral efficacy is largely limited by emergence of escape variants due to high mutation rates of viruses (Gitlin et al., 2002; Wilson and Richardson, 2005).

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An alternative RNA-based approach to block the replication of RNA viruses is to overexpress small and structured viral RNA elements, which function as decoys to compete with the corresponding sequences in viral RNA for viral regulatory proteins binding, thereby inhibiting essential viral gene expression. It has been reported that overexpression of *trans*-acting response element (TAR) (Sullenger et al., 1990) and Rev response element (RRE) RNAs (Lee et al., 1992) rendered cells resistant to HIV replication by preventing the interaction of Tat and Rev proteins with the viral RNA.

The replication of HCV requires a membrane-associated replication complex of viral and cellular proteins as well as a viral RNA template containing *cis*-acting replication elements (CRE). In addition to RNA signals reside in the 5′ and 3′ untranslated region (UTR) of HCV RNA, the highly conserved and stable stem-loop (SL) structures in the C-terminal NS5B coding region have also been demonstrated to contain CREs that are indispensable for HCV replication (Friebe et al., 2005; Lee et al., 2004; You et al., 2004). The function of *cis*-acting RNA elements is frequently mediated by binding of viral or cellular proteins. In fact, recent studies have provided evidence showing a correlation between the RdRp-binding ability and replication competency of the 5BSL mutants (Lee et al., 2004), suggesting that the replication complex containing RdRp is likely to exert its function by interacting with the 5BSL domain(s). If this is the case, overexpression of the RNA species encoding such SL structure(s) could act as decoys to sequester viral and/or host cell factors and prevent their binding to corresponding sequences in the viral RNA, thereby inhibiting HCV infection. Unlike the fore-mentioned sequence-specific antiviral molecules, such a strategy targeting protein–viral RNA interaction should be minimally affected by the genetic heterogeneity of HCV isolates.

In this study, we investigated the therapeutic feasibility of small RNA molecules encoding the defined SL structures in NS5B region. Our results demonstrate that adenoviral-delivered small RNA molecules corresponding to 5BSL3.1 and 5BSL3.2 efficiently inhibit HCV RNA replication in Huh-7 cells that stably replicate the subgenomic replicon.

Results

Down-regulation of HCV replication by in vitro transcribed RNA corresponding to 5BSL3.1 and 5BSL3.2

By using various computational analysis and phylogenetic comparisons, several different groups have analyzed the HCV genome for the presence of stable and highly conserved RNA secondary structures in the NS5B coding region (Hofacker et al., 1998; Tuplin et al., 2002), and moreover the predicted SL structures were recently demonstrated to contain CRE that is crucial for replication of subgenomic replicon in cultured cells. To explore whether the small RNA species encoding the SL sequences can act as therapeutic decoys, RNAs corresponding to the defined SL structures (5BSL3.1, 5BSL3.2, and 5BSL3.3), VR-U/UC containing variable region plus polyU/UC tract, and X-tail were transcribed in vitro, and transfected into Huh-7 cells together with the replicon RNA in vitro transcribed from pLMH14 (Murata et al., 2005), which contains the 5′ UTR, the first 36 nucleotides of the core region fused directly with the firefly luciferase (Fluc) reporter gene, the IRES element from encephalomyocarditis virus (EMCV) that directs translation of the HCV proteins from NS3 to NS5B and the 3′ UTR of HCV RNA. Additionally, the small RNA encoding the sequence from nt 9129 to 9189 (S9129), which encompasses a structure predicted by Tuplin et al. (referred to as SL8926 in Tuplin et al., 2002) but not identified by You et al. (2004), was also included. RNA transcribed from a replication-deficient construct pLMH14GHD carrying an inactive GHD motif in RdRp served as a negative control. The replicon-encoded luciferase expression, which was used as a read out of HCV RNA levels, was assayed at 3 and 72 h posttransfection. The luciferase levels at 3 h posttransfection were used to correct transfection efficiency. Transfection with RNA encoding 5BSL3.1 or 5BSL3.2 significantly inhibited the luciferase expression from replicon, causing a reduction in relative Fluc activity to 25.5% and 20.7%, respectively, compared with the control in which SL RNA was absent (Fig. 1B). There was no significant inhibition of Fluc activity in cells transfected with the corresponding amount RNA of S9129, 5BSL3.3, or VR-U/UC, whereas X-tail RNA caused a marginal inhibition (75.3%) of Fluc activity. This result provides first evidence supporting our hypothesis that the replication complex can be functionally inactivated by sequestering them from the HCV RNA by virtue of small RNAs encoding SL structures such as 5BSL3.1 and 5BSL3.2.

Significantly, the small RNAs corresponding to the 5BSL3.1 and 5BSL3.2 domains of HCV 2a, which differ from those in HCV 1b in 13 out of 44 nucleotides and 6 out of 48 nucleotides (Fig. 1A), also inhibited the replication of replicon RNA from pLMH14 (HCV 1b) although the inhibition extent was less (48.3% and 37.1%, respectively). These data indicate that HCV 2a-derived SL RNAs could cross-interact with the RdRp of HCV 1b despite the genetic diversity between the SL elements of the two viruses, suggesting the potential of SL RNA-based approach to inhibit a wide range of HCV isolates.

Inhibition of HCV replication by pol III-directed SL RNAs

In gene inhibition protocol with small RNA structures, RNA polymerase III (pol III)-based transcription is desirable because pol III promoter is highly productive and capable of generating complex RNA structures with high integrity. For this reason, we next cloned the coding sequence for the SL structure into an expression vector demonstrated to express

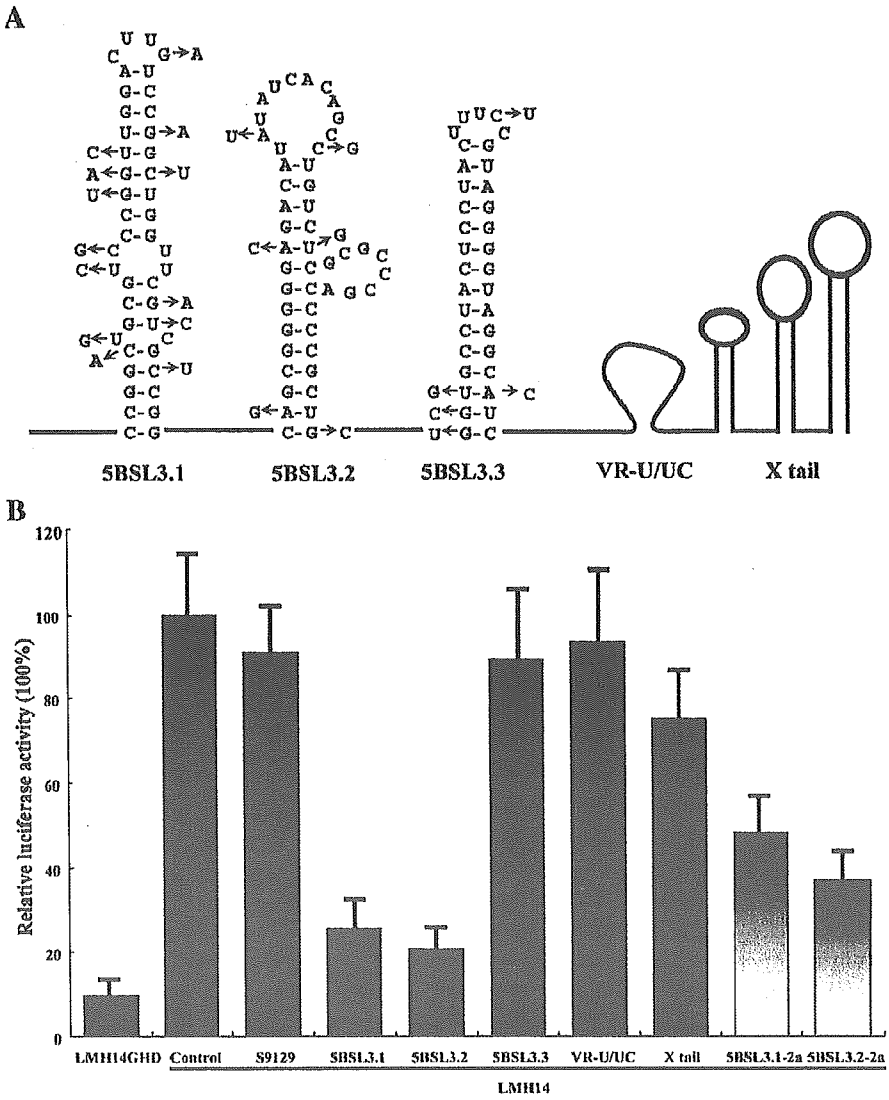


Fig. 1. Effects of in vitro transcribed RNA on HCV replicon-encoded luciferase expression. (A) The sequence and proposed secondary structure in the C-terminal NS5B coding region of HCV 1b replicon. The nucleotides in HCV 2a that differ from those in HCV 1b are indicated. The SL structure prediction and designation are from You et al. (2004) and Friebe et al. (2005). VR-U/UC, variable region plus U/UC tract. (B) Huh-7 cells were transfected with non-replicating replicon RNA (LMH14GHD), or replicating replicon RNA (LMH14) together with indicated in vitro transcribed RNA. Luciferase activities were measured at 3 h and 72 h posttransfection. Luciferase activities at 3 h were used to normalize the transfection efficiency. Relative luciferase activity at 72 h in cells transfected with control (scrambled) RNA was defined as 100%, and those from other transfectants are expressed as relative percentage. The columns and bars represent the means and standard deviations of three independent triplicate transfections. S9129, RNA encoding the sequence from nt 9129 to 9189.

high level of structured RNA molecule under the control of human U6 promoter (Zhang et al., 2004), creating pShuttleSL vectors. Huh-7 cells were transfected with each SL expressing vector, *Xba*I-linearized pLMH14, pAM8-1 plasmid expressing T7 RNA polymerase (Zhang et al., 1999), and pRL-TK (Promega) vector. Instead of in vitro transcribed replicon RNA, we used here a linear DNA replicon template combined with T7 polymerase expressing plasmid because it elicited more efficient replication of HCV replicon in our preliminary study. Transfected cells were harvested and Fluc activities were measured after 24, 48, 72, 96, and 120 h. The Renilla luciferase (Rluc) activity from cotransfected pRL-TK vector was simultaneously measured at 24 h posttransfection to normalize the transfection efficiency. Fig. 2 shows the time course of the replication level of HCV RNA, as indicated by luciferase activity. Huh-7 cells transfected with pShuttle empty vector support the replication of replicon RNA from pLMH14, as evidenced by a constant or even elevated level of Fluc activity after 72 h. Because the RdRp encoded in replication-deficient replicon (pLMH14GHD) is dysfunctional, luciferase expression from pLMH14GHD would depend on the transiently expressed T7 RNA polymerase and decrease with the diminution of T7 polymerase.

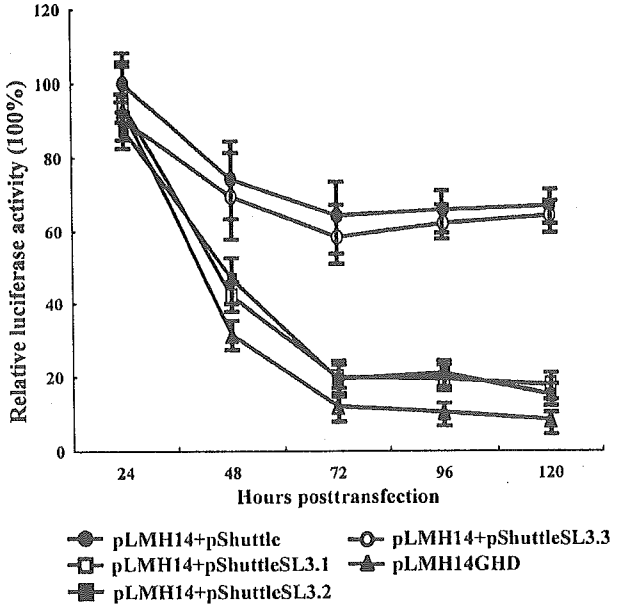


Fig. 2. Inhibition of HCV replication by pol III-directed 5BSL3.1- and 5BSL3.2-containing transcripts. Huh-7 cells were transfected with linearized pLMH14GHD (replication-deficient), or pLMH14 (replication-competent), pAM8-1 plasmid expressing T7 RNA polymerase, and pShuttle empty or pShuttleSL vector. Relative luciferase activities in the lysates were determined at the indicated time points. The data represent the means of two independent experiments.

As expected, the Fluc expression from pLMH14GHD decreased with time and reached a background level at 72 h after transfection, although a comparable Fluc activity was observed at 24 h posttransfection. Compared with the cells transfected with pShuttle empty vector, diminished luciferase expression was detected in cells transfected with pShuttleSL3.1 and pShuttleSL3.2, especially at the time points after 72 h posttransfection. In agreement with the reporter assay using in vitro transcribed RNA, luciferase expression was not significantly changed in the cells transfected with pShuttleSL3.3.

Because translation of Fluc in pLMH14 is under the control of HCV IRES, and synthesis of the NS proteins is directed by EMCV IRES, thus down-regulation of HCV IRES- or EMCV IRES-mediated translation may also lead to a reduction in replicon-encoded Fluc expression. To investigate whether the inhibitory effect of SL RNA on Fluc expression was due to its influence on HCV IRES- or EMCV IRES-dependent translation, we performed reporter assays with pHCVRLNS5B and pEMCVRLNS5B, in which the expression of Rluc gene is directed by HCV IRES (nucleotides 1–377) and EMCV IRES (Zhang et al., 2002) identical to that inserted in the replicon, respectively. As a positive control, we used SL e + f element of HCV IRES domain III, which was reported to inhibit HCV IRES-mediated translation by interacting with the ribosomal S5 protein and preventing the recruitment of the 40S ribosomal unit (Ray and Das, 2004). While *trans*-expression of the SL e + f RNA inhibited HCV IRES-dependent Rluc expression, neither HCV IRES- nor EMCV IRES-directed Rluc expression was significantly affected by overexpression of SL RNAs, indicating that the suppressive activity of 5BSL3.1 and 5BSL3.2 was specific to HCV replication rather than to HCV IRES- or EMCV IRES-mediated translation (Figs. 3A and B). Additionally, to rule out the possibility that the SL RNA-mediated HCV inhibition was a consequence of RNAi effect, reporter assay with pNS5BRL, which contains the NS5B coding sequence encompassing the SL structures studied here fused in frame directly to the N-terminal of Rluc gene, was also conducted as described above. Although a significant reduction in Rluc activity was observed in cells transfected with pShuttlesiRL expressing siRNA against Rluc gene, Rluc expression from pNS5BRL was not inhibited by cotransfection with each pShuttleSL, indicating that the inhibitory activity of small SL RNA was not due to RNAi effect (Fig. 3C). Taken together, these observations provide further evidence supporting our hypothesis that small RNAs encoding SL structures such as 5BSL3.1 and 5BSL3.2 can inhibit HCV replication by competing with HCV RNA for replication complex binding.

Correlation between NS5B-binding ability and anti-HCV activity of mutant 5BSL3.2

To address the issue whether, as hypothesized, SL RNAs inhibit HCV replication by sequestering replication complex, we analyzed the effects of sequence alterations on the function of corresponding SL RNA decoys. For this purpose, two mutant 5BSL3.2 RNAs were in vitro transcribed and subjected to reporter assay and gel shift assay in parallel. As shown in Fig. 4A, 5BSL3.2m-1 contains two A to U substitutions in the upper loop region, which would disrupt the presumed kissing-loop interaction with SL2 in the X-tail; 5BSL3.2m-2 was generated by deletion of 3 nucleotides (CGC) in the internal bulge. Fig. 4B shows that the ability of 5BSL3.2 to inhibit replicon-encoded luciferase expression was not significantly affected by a double point mutation in the upper loop (5BSL3.2m-1). However, a CGC trinucleotide deletion in the bulge (5BSL3.2m-2) abolished the ability of the corresponding RNA to inhibit the luciferase expression from HCV replicon. Gel shift assay was carried out with cytoplasmic extract from HepG2 cells expressing myc-tagged NS5B protein and labeled NS5B (nt 9067–9374) RNA. The result showed that RNA–protein complexes of slower electrophoretic mobility were formed in the binding mixture, which were supershifted by mAb (9E10) against the myc-epitope tag (Fig. 4C, lane 3). The formation of RNA–protein complex was significantly inhibited by an excess of unlabeled homologous NS5B RNA, indicating the specificity of the observed RNA–protein binding. The binding of labeled NS5B RNA to myc-tagged NS5B protein was also competed away by the presence of an excess of unlabeled wild-type 5BSL3.2 or 5BSL3.2m-1, albeit to less extent. In contrast, an increasing

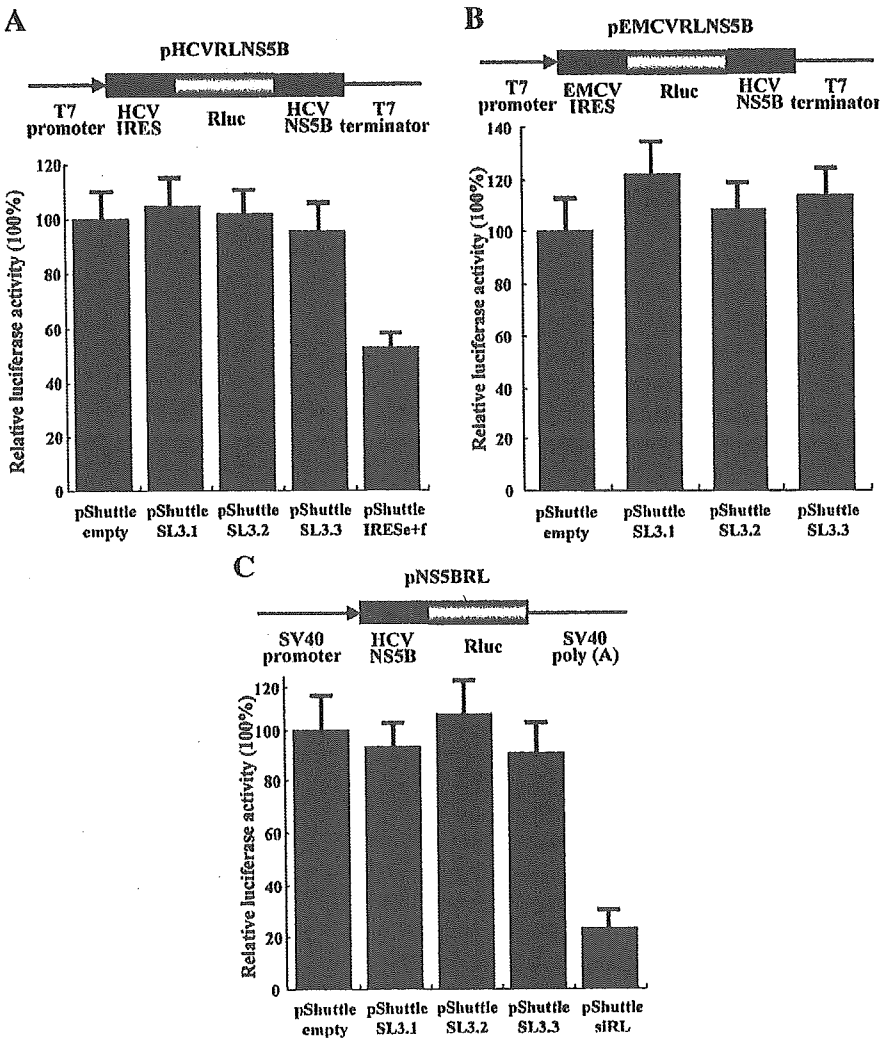


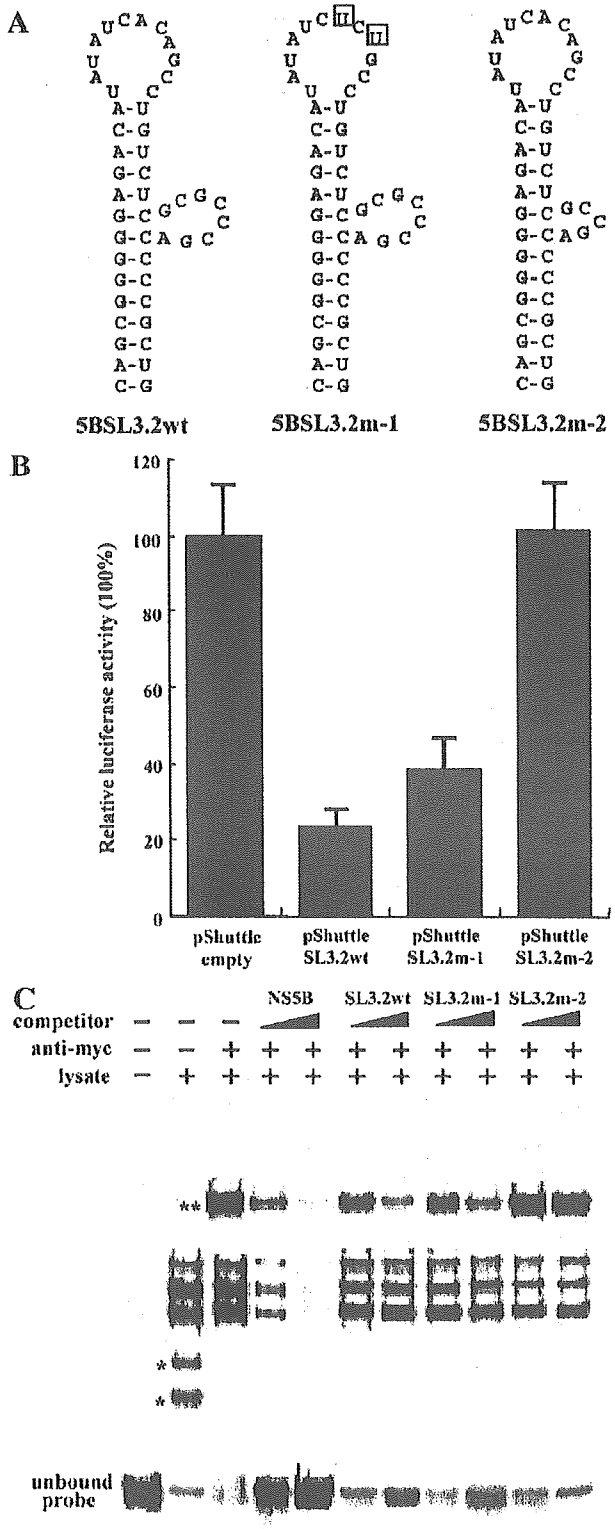
Fig. 3. Effects of SL RNAs on HCV IRES- (A), or EMCV IRES-dependent translation (B). Huh-7 cells were transfected with pHCVRLNS5B (A) or pEMCVRLNS5B (B), which contains T7 promoter, IRES element from HCV or EMCV, and Renilla luciferase (Rluc) gene followed by NS5B coding sequence, pAM8-1, and pShuttle empty or pShuttleSL vector. (C) The antiviral activity of SL RNAs was not due to RNAi effect. Huh-7 cells were transfected with pNS5BRL, which contains NS5B coding sequence fused in frame with the Rluc gene between SV 40 promoter and SV 40 poly (A), and pShuttle empty, pShuttleSL, or pShuttlesiRL vector expressing siRNA against Rluc gene. Relative Renilla luciferase activities in the lysates were determined as described for Fig. 1. The columns and bars represent the means and standard deviations of two separate triplicate transfections.

amount of 5BSL3.2m-2 with trinucleotide deletion in the bulge, which was shown to abolish the ability of 5BSL3.2 to inhibit HCV replication, did not attenuate the formation of the complexes between the labeled NS5B RNA and myc-tagged NS5B protein in the lysate. Taken together, these results support our hypothesis that inhibition of HCV in cells expressing SL RNA decoys occurs by interfering with the interaction between RdRp containing replicase complex and HCV RNA.

Potent inhibition of HCV replication by adenoviral-mediated expression of SL RNAs

To further investigate the therapeutic utility of small SL RNA in combating HCV infection, we cloned each SL RNA expression cassette into recombinant adenovirus vector, and the effect of adenoviral-delivered SL RNA expression on HCV RNA replication was investigated by quantification of HCV replicon RNA levels and viable cell count in G418 selection assay. Huh-NNRZ cells, which autonomously replicate HCV subgenomic replicon carrying neomycin resistant gene, were transduced with each SL RNA expressing adenovirus at the multiplicity of infection (MOI) of 50, 25, and 10 and maintained for a total of 5 days in the absence of G418 selection. Cells were then harvested and total RNAs were subjected to real-time RT-PCR. As shown in Fig. 5A, transduction with AdSL3.1 and AdSL3.2 resulted in a substantial and dose-dependent reduction in replicon RNA copies, reducing HCV transcripts by 35- and 38-fold, respectively, at the MOI of 50. HCV

RNA level was not significantly changed in cells transduced with AdSL3.3, even at the MOI of 50, fully consistent with the results from reporter assay using HCV replicon carrying the luciferase gene.



The antiviral effect of AdSL3.1 and AdSL3.2 was further confirmed in G418 selection assay. The G418 resistance of Huh-NNRZ cells is conferred by persistent expression of neomycin phosphotransferase from replicating HCV RNAs, thus the ability of Huh-NNRZ cells to grow in G418-containing medium can be taken as an indirect measure of HCV replication. In the absence of G418, viability of the cells transduced with each AdSLRNA was comparable to that of mock-infected cells, indicating that overexpression of SL RNA in Huh-NNRZ cells had no adverse effect on cell growth (see discussion). After a 10-day exposure to G418, cells transduced with AdSL3.1 and AdSL3.2 showed a marked and dose-dependent reduction in viable cell count, decreasing viable cells by 18- and 20-fold, respectively, at the MOI of 50 (Fig. 5B). Again, no significant effect on cell growth in the presence of G418 was observed by transduction with AdSL3.3, paralleling the result of the real-time RT-PCR. These results demonstrate that adenoviral-mediated overexpression of 5BSL3.1 and 5BSL3.2 could efficiently inhibit HCV replication in cultured cells.

Discussion

The SL structures in the NS5B coding region were recently demonstrated to function as CRE, and moreover the mutation in SL domain which abrogated its binding to RdRp has also been found to abolish the replication ability of replicon, suggesting that HCV RNA replication likely involves physical interaction between RdRp and SL domain containing CRE. Whether binding directly or indirectly via other viral and/or cellular component of replication complex, it is reasonable to assume that overexpression of RNA species mimicking the SL structures could act as decoy to sequester the replication complex containing RdRp, thereby inhibiting HCV replication. The data presented here support this hypothesis by showing that both in vitro transcribed RNAs and pol III-directed transcripts corresponding to 5BSL3.1 and 5BSL3.2 efficiently inhibited HCV replicon-encoded luciferase expression, and moreover, coupled with recombinant adenovirus delivery, overexpression of

Fig. 4. Correlation between NS5B-binding ability and anti-HCV activity of mutant 5BSL3.2. (A) The sequence and secondary structure of wild- and two mutant-type 5BSL3.2. Nucleotide alterations are indicated by boxes. (B) Huh-7 cells were transfected with linearized pLMH14, pAM8-1, and pShuttle empty or pShuttleSL vector. Relative luciferase activities in the lysates were determined at 72 h posttransfection. The columns and bars represent the means and standard deviations of three independent triplicate transfections. (C) Gel shift analysis of the NS5B-binding ability of 5BSL3.2 RNA. Labeled NS5B RNA was incubated with cytoplasmic lysate from HepG2 cells expressing myc-tagged NS5B protein alone or in the presence of 25- or 200-fold molar excess of unlabeled homologous NS5B RNA, wild-, or mutant-type 5BSL3.2 RNA. The reaction mixtures were left alone or further incubated with mAb against the myc-epitope. The final reaction mixtures were analyzed by electrophoresis on a non-denaturing polyacrylamide gel. Shifted and supershifted complexes are indicated by single and double asterisks, respectively.

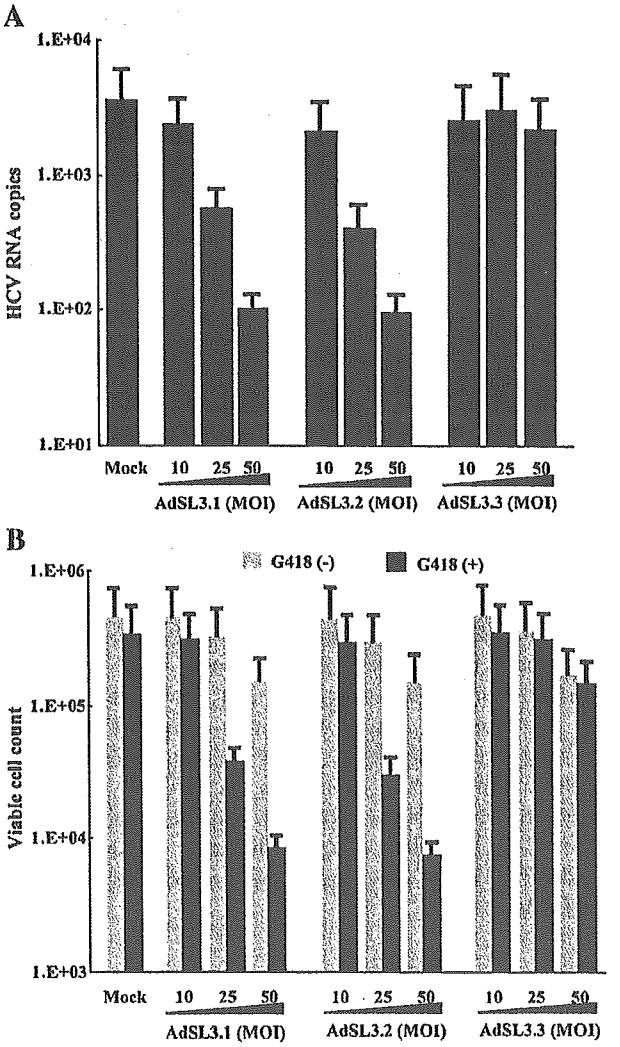


Fig. 5. Dose-dependent inhibition of HCV replication by adenoviral-mediated expression of 5BSL3.1 and 5BSL3.2 RNA. Huh-NNRZ cells were mock infected or infected with each AdSLRNA at the MOI of 10, 25, and 50. (A) After a 5-day culture in the absence of G418 selection, the transduced cells were harvested and HCV replicon RNAs were quantified with real-time RT-PCR. (B) Following a 10-day culture either in the absence (grey columns) or presence (black columns) of G418 selection, the viable cells were quantified. Representative data are from three separate experiments.

5BSL3.1 and 5BSL3.2 RNAs potently blocked HCV replication, as evidenced by significant reduction in HCV transcripts (by 35- and 38-fold, respectively) and marked decrease in viable cell count (by 18- and 20-fold, respectively) in the G418 selection assay.

Overexpression of SL RNAs to prevent the binding of RdRp to its physiological target represents a novel anti-HCV approach with obvious advantage. Distinct from sequence-specific anti-HCV molecules, such as antisense RNA and siRNA, the efficacy of SL RNA approach targeting RNA–protein interaction should be less affected by the extensive variability encountered among HCV isolates. Indeed, SL RNAs derived from HCV 2a, an evolutionarily distant genotype, were also found to inhibit the replication of HCV 1b replicon used here in spite of the genetic heterogeneity between them (Fig. 1). Moreover, since SL RNA–RdRp interaction is essential for RdRp-catalyzed HCV RNA replication, any mutation in the NS5B gene that blocks its binding to the SL RNA also blocks its binding to the SL sequence in viral RNA. To circumvent SL RNA-mediated inhibition, double mutation, one in NS5B and another compensatory one in the SL sequence, is simultaneously required, thus making the chance of generation of escape mutants much lower. Additionally, since the SL RNA molecule is a part of the viral genome, if administered to patients harboring HCV RNA, it would not give rise to immune response, which is problematic in other "intracellular immunization" strategies such as dominant-negative mutants and antisense RNA (Dove, 2002).

Among the SL structures studied here, both 5BSL3.1 and 5BSL3.2 RNA were found to strongly inhibit the replication of HCV replicon, which is compatible with that reported by Lee and coworkers, who determined two SL structures (designated SL-VI and SL-V, which correspond to 5BSL3.1 and 5BSL3.2, respectively) that are essential for HCV replication (Lee et al., 2004). The mutagenesis data reported by You et al. and later by Friebe et al., however, showed that mutational disruption of 5BSL3.2 blocked RNA replication but no significant phenotypic alteration was observed by mutation of 5BSL3.1, thus they concluded that only 5BSL3.2 out of all SL structures predicted in NS5B coding region is indispensable for HCV replication (Friebe et al., 2005; You et al., 2004). This discordance may be partially attributable to the fact that because the SL elements reside in NS5B coding sequence and mutations disrupting SL structures in most cases would change the amino acid sequence of NS5B, and the mutational analyses conducted in those studies were largely limited to preserve the amino acid sequence of the NS5B protein. Alternatively, the association of 5BSL3.1 with RdRp, and more likely with cellular cofactors (see below) may be relatively robust, with less sequence dependence. Based on the finding that HCV 2a-derived 5BSL3.1 also efficiently inhibited HCV 1b replicon despite the nucleotide dissimilarities, together with the fact that most of the nucleotide substitutions in 5BSL3.1 from HCV 2a are compensatory base pair changes, it is suggested that the SL structure of 5BSL3.1 rather than the primary sequence my be important for its function. Additionally, it was recently reported that kissing-loop interaction between the upper loop sequence of 5BSL3.2 and that of the SL2 in the X-tail is essential for HCV replication (Friebe et al., 2005). If so, 5BSL3.2 element is more likely to function as the part of the pseudoknot formed by interacting with the complementary sequence in SL2 of the X-tail. The data presented here show that 5BSL3.2 RNA can act as a potent decoy independently, and moreover the ability of 5BSL3.2 to inhibit the replication of HCV replicon was not significantly affected by introduction of two A to U

mutation in the upper loop (Fig. 4), which would disrupt the presumed kissing-loop interaction. These results largely lessen, if could not rule out completely, the possibility that SL RNA acts by interfering RNA–RNA kissing interaction.

In view of the observation that inhibition of HCV replication always correlated with the expression of the RNA corresponding to 5BSL3.1 or 5BSL3.2, and mutant SL RNAs with debilitated protein-binding activity were also unable to inhibit HCV replication (Fig. 4), it is suggested that HCV replication was inhibited in SL RNA expressing cells because RdRp-catalyzed RNA synthesis was competitively squelched by the presence of excess of SL RNA which sequestered the component(s) of replicase complex. In addition to RdRp, the SL RNAs could potentially function by competing with the viral nascent transcript for association with cellular factors involved in HCV RNA replication. In fact, multiple proteins have been suggested to be associated with the formation of the replication complex, such as polypyrimidine tract-binding protein (Tsuchihara et al., 1997), autoantigen La (Spangberg et al., 1999), and human VAMP-associated protein, a SNARE-like protein (Gao et al., 2004). The possibility that cellular factors associate with SL domain may bring the safety of SL RNA-based approach into question. As judged by morphologic observation and viability measurement (Fig. 5B), however, no discernible adverse effects was found in Huh-NNRZ cells expressing SL-containing transcripts, thereby reducing the concern that overexpression of SL RNA may sequester essential cellular factors and be consequently deleterious to cell viability and cell function. Nevertheless, further studies are required to determine whether the SL RNA-based inhibition strategy is limited by its sequestration of essential cellular factors. If this is the case, it is necessary to engineer altered SL RNA with diminished ability to bind cellular factors but retaining the capability to inhibit HCV replication.

In this study, we have shown that expression of small RNAs corresponding to 5BSL3.1 and 5BSL3.2 in trans can efficiently inhibit HCV replication, which suggests that use of SL RNA decoys is a promising new stratagem for combating HCV infection. Further studies are required to determine the range and duration of SL RNA-mediated inhibition for natural HCV isolates and to explore the feasibility of combining the therapeutic SL RNAs with other modalities to obtain additive or synergistic anti-HCV effect. Additionally, the use of SL RNAs also represents a novel and complementary approach to investigate the molecular mechanism of HCV RNA replication.

Materials and methods

Plasmids

The pShuttleU6 was created as previously described (Zhang et al., 2004). pGEMEX-1 vector (Promega) was modified by deletion of all of the T7 gene 10 and introduction of *Bam*HI and *Eco*RI sites between T7 promoter and T7 terminator (Zhang et al., 1999). Sense and antisense oligonucleotides of SL-coding sequence plus 5 thymidines, which contain cohesive ends for *Bam*HI and *Eco*RI at the 5′- and 3′-ends, were synthesized and annealed by heating at 95 °C for 10 min and slowly cooled down to room temperature. After a gel electrophoresis purification, these annealed oligonucleotides were inserted into the *Bam*HI and *Eco*RI sites of pShuttleU6 and modified pGEMEX, creating pShuttleSL and pGEMEXSL vectors, respectively. The sequences of these constructs were confirmed by nucleotide sequencing.

Adenovirus

Each expression cassette in pShuttleSL, which is flanked by I-*Ceu*I and PI-*Sce*I sites, was digested with these two restriction enzymes, and ligated to the E1- and E3-deleted Adeno-X viral DNA (I-*Ceu*I and PI-*Sce*I digested) (Adeno-X Expression System, Clontech). The resultant adenoviral DNAs were digested with *Pac*I and then transfected into low-passage 293 cells. Seven days following transfection, crude virus was prepared from the transfected cells by three cycles of freeze–thawing, and further amplified in 293 cells by several rounds of infection. The purified virus was aliquoted and stored at −80 °C before use. The authenticity of recombinant adenoviral DNAs was verified before preparing high-titer viral stocks.

Cells

The cell lines Huh-7 and 293 were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum and 50 u/ml penicillin and streptomycin in a 5% CO_2 humidified atmosphere. A Huh-7-derived cell line (Huh-NNRZ) stably replicating HCV subgenomic replicon was grown in DMEM medium containing 300 μg/ml G418 (Geneticin, Invitrogen).

In vitro transcription

pGEMEXSL plasmids were linearized by digestion with *Eco*RI, and then transcribed in vitro using T7 RNA polymerase according to the protocol supplied by the manufacturer (Roche). For transcription of replicon RNA, pLMH14 was linearized at *Xba*I site located immediately downstream of the HDV ribozyme, and the fragment was used as the template for run-off RNA synthesis. After transcription, 10 U of RQ DNaseI (Promega) were added to the reaction mixture to digest DNA templates. The mixture was extracted with phenol–chloroform and RNA was precipitated with ethanol–7.5 M ammonium acetate.

Transfection

Huh-7 cells were seeded onto 6- or 12-well plates 24 h before transfection. Two microgram of each pShuttleSL, 0.5 μg of *Xba*I-linearized pLMH14, pLMH14GHD, pHCVRLNS5B, or pEMCVRLNS5B, 0.5 μg of pAM8-1, and 0.1 μg of pRL-TK vector were cotransfected into cells with TransFast Transfection Reagent (Promega). For RNA transfection, 2 μg of each in vitro transcribed SL RNA and 1 μg of replicon RNA in vitro transcribed from pLMH14 or pLMH14GHD were cotransfected into Huh-7 cells with Lipofectin Reagent (Invitrogen). The cells were harvested at the indicated time points, and cell lysates were assayed for luciferase activity as described below.

Luciferase assay

Cell lysates were prepared from transfected cells, centrifuged briefly, and 20 μl of the supernatants was used for luciferase assays with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured using a TD-20/20 Luminometer (Promega).

Gel mobility shift assay

NS5B RNA (nt 9067–9374) probe was synthesized by in vitro transcription as described above. Cytoplasmic lysate was prepared from HepG2 cells expressing myc-tagged NS5B protein and incubated with labeled RNA probe at room temperature for 30 min in a buffer containing 10 mM HEPES (pH 7.6), 40 mM KCl, 3 mM $MgCl_2$, 2 mM dithiothreitol, 10% glycerol, and 200 μg/ml yeast RNA. To perform gel mobility supershift analysis, following RNA–protein binding, 3 μl of a mAb against myc-tag (9E10, Santacruz) was added and further incubated at room temperature for 30 min. For competition assay, a 25- or 200-fold molar excess of unlabeled homologous NS5B RNA, wild-, or mutant-type 5BSL3.2 was added. The final reaction mixture was resolved by electrophoresis on a non-denaturing polyacrylamide gel.

Real-time RT-PCR

RNAs were isolated from cultured cells with Trizol reagent (Invitrogen) and treated with RNase-free DNase (Promega). One microgram of DNase-treated total RNA was reverse transcribed using a primer complementary to NS5B 5′-ACGGAGCGGATGTGGTTGAC-3′. After incubation at 95 °C for 5 min, the resulting cDNA was quantified with SYBR GREEN according to the protocol supplied by the manufacturer (Takara). PCR was performed with a primer 5′- TGGTCTACGCCACAACATCC-3′ and the primer used in RT reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level in each sample was simultaneously quantified to normalize the value of HCV replicon RNA.

G418 selection assay

Huh-NNRZ cells were mock infected or infected with each AdSLRNA. Twenty-four hours later, the medium was changed with fresh DMEM containing 0 or 300 μg/ml G418. After an additional 10-day culture, cell viability was measured with cell proliferation reagent WST-1 (Roche) according to the manufacturer's instructions.

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Cyclophilin B Is a Functional Regulator of Hepatitis C Virus RNA Polymerase

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Summary

Viruses depend on host-derived factors for their efficient genome replication. Here, we demonstrate that a cellular peptidyl-prolyl *cis-trans* isomerase (PPIase), cyclophilin B (CyPB), is critical for the efficient replication of the hepatitis C virus (HCV) genome. CyPB interacted with the HCV RNA polymerase NS5B to directly stimulate its RNA binding activity. Both the RNA interference (RNAi)-mediated reduction of endogenous CyPB expression and the induced loss of NS5B binding to CyPB decreased the levels of HCV replication. Thus, CyPB functions as a stimulatory regulator of NS5B in HCV replication machinery. This regulation mechanism for viral replication identifies CyPB as a target for antiviral therapeutic strategies.

Introduction

CyP was originally discovered as a cellular factor with high affinity for the immunosuppressant cyclosporin A (CsA) (Handschumacher et al., 1984; Schreiber, 1991). CyPs are a family of PPIases, which catalyze the *cis-trans* interconversion of peptide bonds amino-terminal to proline residues, facilitating changes in protein conformation (Fischer et al., 1998; Takahashi, 1999; Waldmeier et al., 2003). In mammals, CyPs include more than ten subtypes (Braaten and Luban, 2001; Takahashi, 1999; Waldmeier et al., 2003). CyP family members function in numerous cellular processes, including transcriptional regulation, immune response, protein secretion, and mitochondrial function (Braaten and Luban, 2001; Brazin et al., 2002; Colgan et al., 2004; Duina et al., 1996; Rycyzyn and Clevenger, 2002; Waldmeier et al., 2003). In this study, we report the involvement of CyPB in HCV genome replication and propose its molecular mechanism.

HCV is a major causative agent of liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Liang et al., 1993). HCV, a member of the Flaviviridae family, has a positive-strand RNA genome. The genome encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least ten functional viral proteins: core, envelope (E)1, E2, p7, nonstructural protein (NS)2, NS3, NS4A, NS4B, NS5A, and NS5B (Grakoui et al., 1993). NS5B is an RNA-dependent RNA polymerase (RdRp), which is crucial in viral genome replication (Bartenschlager and Lohmann, 2001; Tellinghuisen and Rice, 2002). Until recently, research into HCV genome replication has been hampered by the lack of a cell culture system that can efficiently reproduce HCV infection and proliferation. Even now, we do not have an in vitro system that efficiently produces infectious HCV viral particles. Lohmann et al. previously established the HCV subgenomic replicon system in which the HCV subgenomic RNA autonomously replicates in Huh-7 cells (Lohmann et al., 1999), which we refer to throughout this report as HCV replicon cells. This replicon system enables us to investigate HCV genome replication in a cell culture system. By using this system, several groups have recently reported that HCV genome replication occurs in a distinct, subcellular replication complex (RC), which includes viral genome RNA and HCV proteins (Aizaki et al., 2004; Egger et al., 2002; El-Hage and Luo, 2003; Gosert et al., 2003; Miyanari et al., 2003), in a manner similar to other RNA viruses (Noueiry and Ahlquist, 2003). The RC is formed on intracellular membranes, including the endoplasmic reticulum (ER) membrane. This membrane structure protects the RC from external nucleases and proteases, which seems advantageous for efficient viral genome replication (Aizaki et al., 2004; El-Hage and Luo, 2003; Miyanari et al., 2003). However, the role of cellular factors directly regulating the activity of the RC has remained unclear.

We and other groups have previously reported that CsA has the potential to suppress HCV genome replication (Nakagawa et al., 2004; Watashi et al., 2003a). In this study, we used CsA as a bioprobe to identify cellular factors involved in HCV genome replication. This strategy revealed that CyPB, one of the cellular targets of CsA, was required for the efficient replication of the HCV genome in the cells. Investigation into the molecular mechanism showed that CyPB interacted with NS5B and directly promoted its RNA binding activity. The CyPB-NS5B association is a potent target for the development of an antiviral therapeutics.

Results

Functional Inhibition of CyP Suppressed HCV Genome Replication

As HCV does not efficiently infect cultured cells, we studied viral replication by using HCV replicon cells. We have previously reported that CsA treatment suppressed HCV genome replication (Watashi et al., 2003a). A number of studies have shown that CsA has three major cellular targets: CyP, the calcineurin (CN)-NF-AT pathway, and P-glycoprotein (P-gp) (Loor et al., 2002; Takahashi, 1999) (Figure 1A). We initially determined which of these cellular targets of CsA were involved in HCV genome replication. We examined the effect of several CsA mutants (functional characteristics are shown in Figure 1A) on HCV genome replication by real-time RT-PCR and immunoblot analyses. CsA, (8′-OH-MeBmt[1])Cs,

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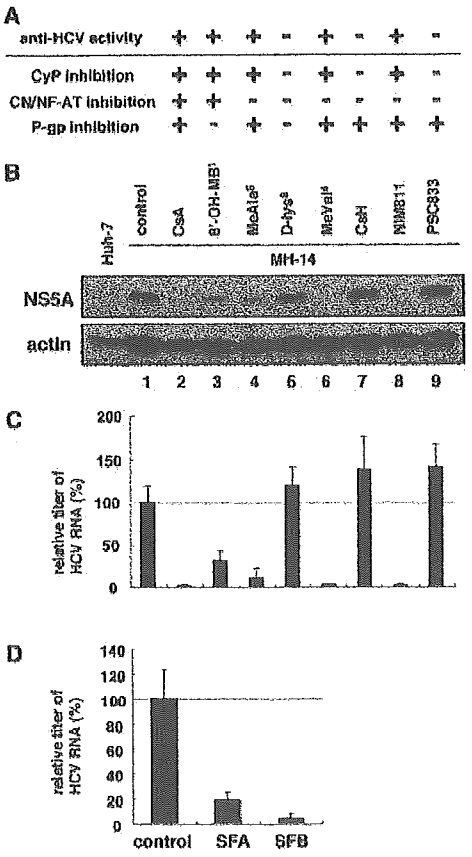

Figure 1. Functional Inhibition of Cyclophilin Suppressed HCV Genome Replication

(A) Characteristic features of cyclosporin A (CsA) and its derivatives, 8'-OH-MeBmt[1]-Cs (8'-OH-MB[1]), MeAla[6]-Cs (MeAla[6]), D-lys[8]-Cs (D-lys[8]), MeVal[4]-Cs (MeVal[4]), cyclosporin H (CsH), NIM811, and PSC833. "Anti-HCV activity" summarizes the potential for each derivative to decrease HCV replication, defined as + (effective), or – (not effective). The inhibition activity to cellular cyclophilin (CyP), calcineurin (CN)-NF-AT pathway, and P-glycoprotein (P-gp) is similarly defined by ±.

(B and C) MH-14 cells, carrying the HCV subgenomic replicon, were treated with 1 μg/ml CsA or its derivatives for seven days. Levels of NS5A (B, top) and actin as an internal control (B, bottom) in these cells were detected by immunoblot analysis. HCV RNA titers were quantified by real-time RT-PCR analysis, and relative titer is shown in (C). The data represent the means of three independent experiments. Error bars represent the SD.

(D) HCV RNA titers in MH-14 cells treated with 3 μg/ml sanglifehrin (SF)A and SFB for seven days were measured as described in (C). Error bars represent the SD.

(MeAla[6])Cs, (MeVal[4])Cs, and NIM811 had anti-HCV activity, decreasing both HCV protein production and HCV RNA titer in HCV replicon cells (Figures 1B and 1C, lanes 2, 3, 4, 6, and 8). In contrast, (D-lys[8])Cs, CsH, and PSC833 had little effect on HCV genome replication (Figures 1B and 1C, lanes 5, 7, and 9). A comparison of anti-HCV activity with the characteristics of these CsA derivatives (Figure 1A) indicates that the anti-HCV activity correlated with inhibition of CyP, but not the CN-NF-AT pathway or P-gp. Sanglifehrin (SF)A and SFB, additional CyP inhibitors (Sanglier et al., 1999), also decreased HCV RNA titers in replicon cells (Figure 1D), suggesting that CyP is linked to HCV genome replication.

CyPB Played a Significant Role in HCV Genome Replication

In mammalian cells, the CyP family contains more than ten subfamilies (Braaten and Luban, 2001; Takahashi, 1999; Waldmeier et al., 2003). CyPA and CyPB are the most abundant subtypes (Waldmeier et al., 2003). We applied RNAi methods to investigate the role of CyPs in HCV genome replication. By introducing small interfering RNA (siRNA) designed to recognize several CyP subtypes (si-CyP[broad]) (see Experimental Procedures), both endogenous CyPA and CyPB proteins, but not actin, were downregulated in replicon cells (Figure 2A, lane 2). The titer of HCV RNA in these cells was diminished to approximately one-fifth of the levels seen in the cells transfected with a randomized siRNA (si-control) (Figure 2B). Specific knockdown of endogenous CyPB also significantly reduced HCV RNA titer, whereas depletion of endogenous CyPA did not (Figure 2A, lanes 3 and 4, and Figure 2B). Depletion of CyPB did not affect cell proliferation (data not shown). A similar result was obtained in another HCV replicon cell clone, 50-1 (Watashi et al., 2003a) (data not shown). Introduction of siRNAs specific for other CyP family members, CyPC, CyPE, or CyPH (Figure 2C), had no effect on HCV RNA titer in replicon cells (Figure 2D). These data suggest that among the CyP family, CyPB is specifically linked to HCV genome replication. Also, in a system in which HCV genome replication can be monitored by luciferase activity (Murata et al., 2005) (Figure 2E), depletion of CyPB inhibited HCV genome replication (Figure 2F). This effect was reversed by the exogenous overproduction of FLAG-tagged CyPB (Figure 2G). These results further indicate that CyPB plays a significant role in HCV genome replication. A weak, nonspecific cross-silencing effect of si-CyPA on CyPB (Figure 2A, lane 3) did not affect HCV RNA titer, suggesting the possibility that the expression level of CyPB has a threshold to support HCV genome replication.

CyPB Interacted with HCV NS5B

To clarify the mechanisms underlying CyPB regulation of HCV genome replication, we analyzed the molecular interaction between CyPB and HCV proteins expressed in HCV replicon cells by GST pull-down. Human immunodeficiency virus (HIV)-1 Gag, which binds CyP family proteins (see Discussion) (Braaten and Luban, 2001; Luban et al., 1993), was used as a positive control (Figure 3A, panel e). As a result, the GST fusion of CyPB (GST-CyPB) coprecipitated only the NS5B viral protein (Figure 3A, panel d). The interaction between NS5B and CyPB was confirmed by immunoprecipitation of NS5B from replicon cells ectopically expressing CyPB (Figure 3B, lane 4). In addition, endogenous CyPB copurified with NS5B from replicon cells (Figure 3C, lane 2). Endogenous CyPB also associated with exogenously expressed NS5B in Huh-7 cells (Figure 3D, lane 4). In contrast, we could not detect the binding of CyPA to NS5B by using either a GST pull-down assay or an immuno-

precipitation assay (Figure 3A, panel d and Figure 3B, lane 8). These results suggest that CyPB specifically interacts with NS5B. CyPBΔPPI, which was previously shown to lose the catalytic activity (Rycyzyn and Clevenger, 2002), had a strong binding to NS5B (Figure 3B, lane 6).

GST pull-down assays demonstrated that the CyPB-NS5B interaction was reduced in a dose-dependent manner after treatment with CsA, which is an inhibitor of HCV genome replication (Figure 3E). Immunoprecipitations demonstrated that CyPB completely dissociated from NS5B in the presence of CsA (Figure 3F, lanes 2 and 4). We then determined the region of the NS5B molecule responsible for binding to CyPB by deletion analysis. The region comprising amino acids (aa) 71–591 of NS5B, which is deficient in RNA binding (Ishii et al., 1999), coprecipitated with GST-CyPB (Figure 3G, panel b). 401–591 and 521–591 aa, but not 201–400 aa, regions could be precipitated with GST-CyPB (Figure 3G, panels c–e). These results indicate that the carboxy (C)-terminal region of NS5B (521–591) interacts with CyPB. On the other hand, aa 1–570, which lack the C-terminal hydrophobic region, had little affinity for GST-CyPB (Figure 3G, panel f). Analysis of additional deletion mutants (Figure 3G, panels g–i) suggests that the whole 521–591 aa region rather than only the C-terminal 21 region (571–591 aa) of NS5B is likely to be important for the interaction with CyPB.

CyPB Associated with NS5B, which Is Functional for HCV Genome Replication

We next examined the subcellular localization of CyPB. In contrast to CyPA, which exhibited a diffuse distribution throughout the cell (Figure 4A, panel a), CyPB concentrated in the perinuclear region (Figure 4A, panel d). CyPB was colocalized with NS5B (Figure 4A, panels c–e), which reportedly resides on the cytoplasmic face of the ER membrane (Schmidt-Mende et al., 2001). To investigate whether CyPB is also located on the cytoplasmic face of the intracellular membrane, we next performed modified immunofluorescence analysis in which we pretreated the cells with digitonin to permeabilize the plasma membrane, but not intracellular membranes, followed by washing out the cytosol (see Experimental Procedures) (Watashi et al., 2001). PDI, a protein in the lumen of the ER, was not detected in this assay (Figure 4B, panel g), whereas CyPB as well as NS5B could be detected around the perinuclear region (Figure 4B, panels i and k). Also in Huh-7 cells, CyPB was observed in the perinuclear region (Figure 4B, panel o). This suggests that a fraction of CyPB is located on the cytoplasmic face of the intracellular membrane (see Discussion).

We labeled the site of newly synthesized HCV RNA by treating the cells with actinomycin D followed by addition of bromouridine (BrU) (El-Hage and Luo, 2003; Gosert et al., 2003; Restrepo-Hartwig and Ahlquist, 1996). In treated replicon cells, labeling was detected in the perinuclear region as previously reported (El-Hage and Luo, 2003; Gosert et al., 2003) (Figure 4A, panel q, shown by green), whereas no signal was observed in Huh-7 cells or replicon cells in the absence of BrU (Figure 4A, panels p and r). This signal merged with the localization of both NS5B and CyPB (Figure 4A, panels g–l).

In cells, a fraction of NS5B is involved in viral genome replication, but another fraction is not (Aizaki et al., 2004; El-Hage and Luo, 2003; Miyanari et al., 2003). These two fractions can be distinguished by digitonin/protease treatment; NS5B that does not function in HCV genome replication is sensitive to digitonin/protease treatment, whereas that participating in the viral RC and functioning for replication is protected from digitonin/protease digestion. After treatment with digitonin/proteinase K followed by washing and permeabilization with Triton X-100 (effective digitonin permeabilization and proteinase K digestion were confirmed by the complete loss of cytoplasmic protein IκBα detection [data not shown]), the signal intensities of NS5B and CyPB staining were reduced. Even under these conditions, a portion of NS5B, CyPB, and BrU was still detected merging in the perinuclear region (Figure 4A, panels m–o, and data not shown), although CyPB in the lumen of the ER could also be detected in this system (the localization of the NS5B-CyPB interaction is discussed further below). By using replicon cells treated with digitonin/proteinase K, NS5B and endogenous CyPB still coprecipitated (data not shown). Moreover, HCV RNA copurified with anti-CyPB as well as anti-NS5B antibody from the replicon cells crosslinked by formaldehyde (Figure 4C), suggesting the association between CyPB and NS5B-HCV RNA complex in the cells. These data cumulatively suggest that CyPB associates with a fraction of NS5B that is functional for viral genome replication.

CyPB Stimulated RNA Binding Activity of NS5B

To investigate which function of NS5B was modulated by CyPB, we first assumed the possibility that CyPB might alter formation of the RC like hVAP-33 does (see Discussion) (Gao et al., 2004). We estimated the amount of HCV proteins in the RC by examining the levels of digitonin/protease-resistant NS5A and NS5B as described previously (Miyanari et al., 2003). The amount of NS5A and NS5B resistant to digitonin/proteinase K digestion, however, was not affected by treatment with CsA, which inactivates CyPB function (Figure 5A). We did not observe any significant effect on the total protein levels of NS5A and NS5B after serial treatment with CsA for up to 24 hr (Figure 5A, lanes 1 and 6; Figure 5B, lower right panels; and Figure 5D, upper panel lanes 1 and 2). CsA treatment also had no effect on the subcellular localization of NS5B (data not shown). The RNA synthesis activity of the isolated RC, however, was significantly reduced after this CsA treatment condition (Figure 5B).

These results raised the possibility that CyPB directly regulated the function of NS5B within the RC. NS5B binds HCV RNA as a template to function as an RdRp. We therefore investigated the effect of CyPB on the RNA binding activity of NS5B in replicon cells by using poly-U Sepharose beads as a model RNA substrate, as described previously (Ishii et al., 1999; Lohmann et al., 1997; Qin et al., 2001). The association of NS5B with poly-U RNA was confirmed by using replicon cells (Fig-

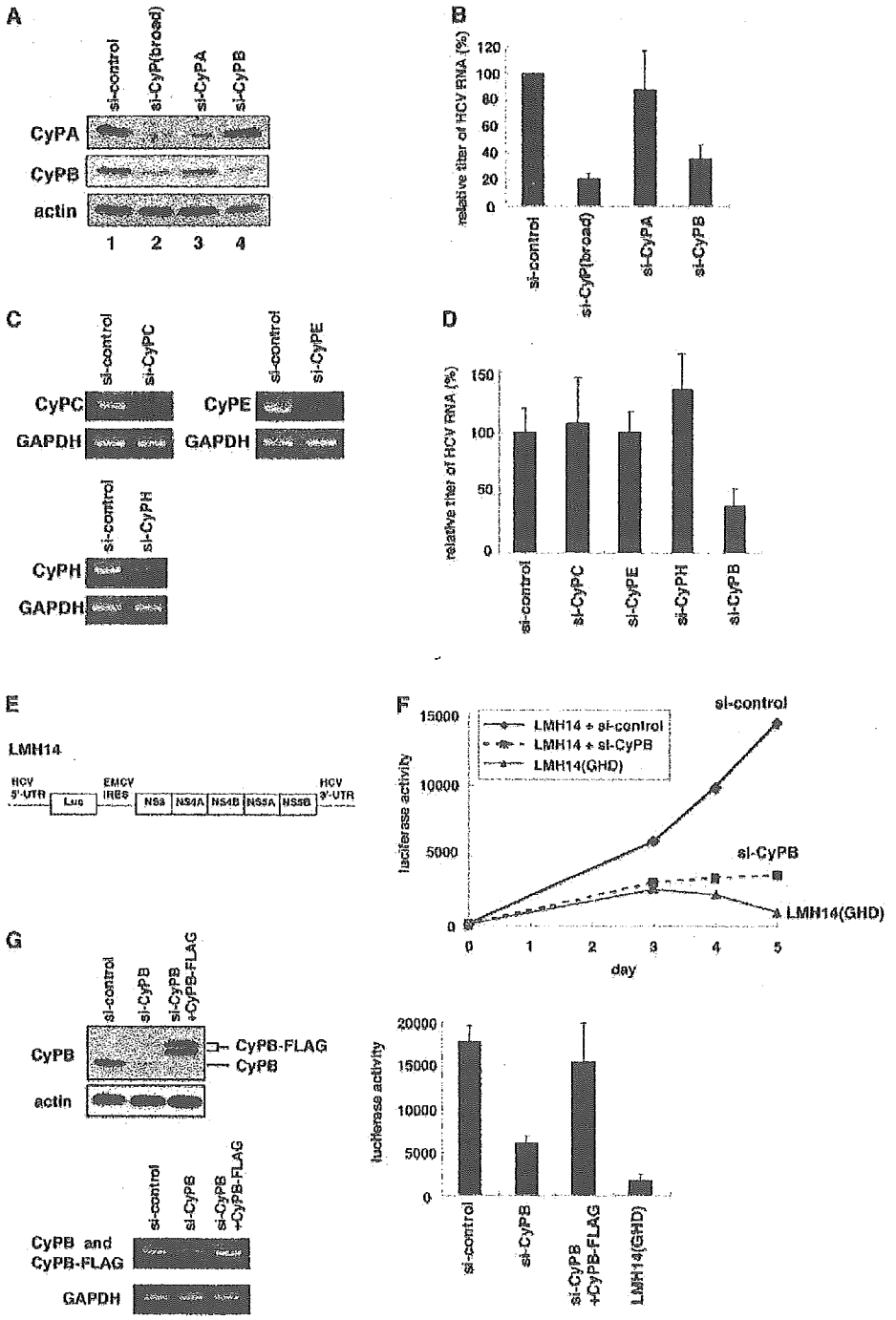

Figure 2. CyPB Regulated HCV Genome Replication

(A) Knockdown of endogenous CyPA and CyPB proteins. MH-14 cells were transfected with siRNA specific for CyPA (si-CyPA), CyPB (si-CyPB), or a broad range of CyP subtypes (si-CyP[broad]) or with a randomized siRNA (si-control). At 72 hr posttransfection, CyPA (top), CyPB (middle), and actin as an internal control (bottom) were detected in total cell lysates by immunoblot analysis.

(B) Depletion of CyPB decreased HCV RNA titers. At 5 days posttransfection of siRNA, HCV RNA titers were quantified by real-time RT-PCR analysis. The data represent the means of three independent experiments. Error bars represent the SD.

(C) siRNA constructs specific for CyPC (si-CyPC), CyPE (si-CyPE), CyPH (si-CyPH), or si-control were introduced into MH-14 cells. At 72 hr posttransfection, we analyzed the mRNA levels of CyPC, CyPE, CyPH, and GAPDH as an internal control by RT-PCR analysis.

(D) The experimental procedure is the same as described in (B). Error bars represent the SD.

(E) Schematic representation of the LMH14 RNA construct, which carries the luciferase gene driven by the HCV 5′-untranslated region (UTR)

ure 5C, upper panel lane 6). Endogenous CyPB, but not CyPA, also coprecipitated with poly-U RNA (Figure 5C, middle and lower panels lane 6). In Huh-7 cells, however, such CyPB-RNA binding was not observed (Figure 5C, lower panel lane 4), suggesting that CyPB binding to RNA is dependent on NS5B. The RNA binding affinity of NS5B isolated from the digitonin/protease-resistant fraction was largely abolished by CsA treatment (Figure 5D, upper panel lane 6), which abrogated the interaction of CyPB with NS5B (Figure 3F, lane 4). A similar result was obtained in another replicon cell clone, 50-1 (data not shown). siRNA-mediated knockdown of the endogenous CyPB also reduced the NS5B-poly-U RNA binding within the digitonin/protease-resistant fraction (Figure 5E, lane 6). These data suggest that CyPB promotes the binding of NS5B to RNA. This effect of CyPB was further documented by using an in vitro RNA binding assay. The binding of NS5B to either poly-U (Figure 5F) or poly-A (data not shown) RNA was increased by the addition of recombinant GST-CyPB. RNA binding, however, was unaffected by the addition of either recombinant GST-CyPA or GST-CyPBΔPPI, a PPIase-inactive point mutant of CyPB (Rycyzyn and Clevenger, 2002) that retained strong binding to NS5B (Figure 3B, lane 6) (Figure 5F). In contrast, CyPB did not augment the RNA binding activity of NS5B(1–570), which had little affinity for CyPB (Figure 3G, panel f) (Figure 5G). The stimulatory effect of CyPB on the RNA binding activity of NS5B was negated by treatment with CsA (Figure 5H). Although the conditions in this in vitro system differ from the cellular environment, these results clearly demonstrate that CyPB can augment RNA binding activity of NS5B even in vitro.

Association of CyPB with NS5B Was Critical for Efficient Replication of HCV Genome

To examine the physiological relevance of the association of CyPB with NS5B in HCV genome replication, we identified a point mutant of NS5B that was unable to bind to CyPB by alanine scanning mutation analysis on the 521–591 aa region of NS5B. In a GST pull-down assay, NS5B(P540A), which bore the replacement of the proline at 540 aa of NS5B by alanine, had little affinity for GST-CyPB (Figure 6A). We confirmed that NS5B(P540A) did not bind CyPB by immunoprecipitation (Figure 6B, lane 6). This P540A mutation decreased the RNA binding activity of NS5B in replicon cells (Figure 6C, lane 6), supporting our conclusion that CyPB plays a stimulatory role in RNA binding of NS5B. Because the P540A mutation may induce the overall conformational change of NS5B protein and destroy its function, we examined the basal function of NS5B(P540A). The levels of protein production and subcellular localization of exogenously produced NS5B(P540A) in Huh-7 cells were, however, similar to those seen in cells with wild-type (wt) NS5B (data not shown). This mutation did not cripple the molecular function of NS5B itself, including RNA polymerase activity and RNA binding activity in vitro (data not shown). The introduction of this point mutation into the NS5B sequence should not destroy the stem-loop RNA structure of the *cis*-acting replication element in the coding region for NS5B, which is present downstream of the P540 codon (Lee et al., 2004; You et al., 2004). The association of NS5B with the ER membrane via its 571–591 aa region is reportedly essential for HCV replication in cells (Moradpour et al., 2004). NS5B(P540A) still associated with intracellular membrane fractions (data not shown). Thus, NS5B(P540A) possessed basal functions of NS5B as far as we examined. However, the replication competency of HCV replicon RNA carrying this one point mutation was significantly decreased from levels observed for the wt in the luciferase assay system (Figure 6D). In this assay, we did not find any reversions at the second site or back to the wt within the NS5B-coding region in HCV replicon RNA of the transfected cells (data not shown). The colony formation assay also demonstrated a reduction in HCV genome replication with the NS5B P540A mutant (Figure 6E). These data suggest that the functional regulation of NS5B by CyPB is critical for the efficient replication of the HCV genome in the cells.

Discussion

Viruses exploit cellular factors for their efficient proliferation. Although it was recently reported that a SNARE-like protein, hVAP-33, augmented HCV replication by modulating RC formation (Gao et al., 2004), cellular factors directly regulating the activity of the RC have remained unknown. By using CsA, a compound exerting a strong anti-HCV potential, we determined that CyPB is required for efficient replication of the HCV genome. This cellular factor regulates HCV genome replication through modulation of the RNA binding activity of NS5B. CsA impaired this regulation of NS5B-RNA binding by CyPB to inhibit HCV genome replication. Previously, the CyP family was known to regulate the function of specific substrates such as calcineurin (Schreiber, 1992), prolactin (Rycyzyn and Clevenger, 2002), Itk (Brazin et al., 2002; Colgan et al., 2004), adenine nucleotide translocase (Waldmeier et al., 2003), and steroid receptors (Duina et al., 1996) by altering

and the coding region for HCV NS3 to NS5B whose expression is regulated by the EMCV IRES. By using this RNA construct, HCV replication can be monitored by measuring resultant luciferase activity as described previously (Murata et al., 2005).

(F) Suppression of HCV replication after CyPB knockdown. Cured MH-14 cells were transfected with LMH14 or LMH14(GHD) RNA together with si-control or si-CyPB. After 3, 4, or 5 days, luciferase activities were measured to plot against the time course. Solid line, transfected with LMH14 RNA and si-control; broken line, LMH14 RNA and si-CyPB; and faint line, LMH14(GHD) RNA, a replication-deficient mutant of LMH14 used as a negative control.

(G) On the right, cured MH-14 cells were transfected with LMH14 RNA plus si-control (si-control), LMH14 RNA plus si-CyPB (si-CyPB), LMH14 RNA plus si-CyPB and the expression plasmid for FLAG-tagged CyPB (si-CyPB + CyPB-FLAG), or LMH14(GHD) RNA (LMH14[GHD]). Luciferase activities were then quantified at 5 days posttransfection. Error bars represent the SD. Left panels show the immunoblot analysis (upper panels) by using anti-CyPB and anti-actin antibody and RT-PCR analysis (lower panels) by using the primer detecting CyPB and GAPDH upon the transfection of si-control, si-CyPB, and si-CyPB with CyPB-FLAG.