Classification of mutations occurring in 50-1 and 1B-2R1 replicon RNAs during the long-term cell culture

To understand the mutation mode of the replicons in long-term cell culture, we examined the numbers of synonymous and non-synonymous mutations with transition or transversion. The results are summarized in Table 1. The ratio of synonymous to non-synonymous mutations in 50-1 replicon RNA was 0·81 to 1·50 (1·38±0·14 after 6 months in culture and 1·03±0·20 after 12 months in culture), and the ratio in 1B-2R1 replicon RNA was 0·63 after 6 months in culture and 0·59 after 12 months in culture. These values indicate that amino acid substitutions in the replicons occur frequently during the cell culture. The rate of mutations with transition in the 50-1 replicon was 1·82–4·06-fold (2·00±0·18 after 6 months in culture and 2·85±1·07 after 12 months in culture) greater than the rate of mutations with transversion. Similarly, the 1B-2R1 replicon showed a transition-to-transversion ratio of 2·69 (after 6 months in culture) or 2·86 (after 12 months in culture).

Regarding the mutation patterns over more than 12 months of culture, we observed that A→G and U→C mutations were the most and second-most common mutations, and these mutations were approximately two to three times more common than G→A and C→U mutations (Supplementary Table A, which is available as Supplementary material in JGV Online). The rarest mutation was G→U (Supplementary Table A).

Genetic diversity of the 50-1 and 1B-2R1 replicons arising during long-term cell culture

To clarify whether or not the replicons acquire a quasispecies nature during long-term cell culture, we estimated the genetic diversities of the 50-1 and 1B-2R1 replicon populations. First, based on the sequence data of all clones obtained in this study, we constructed phylogenetic trees for the first 2 kb region and the NS region. The results revealed that the genetic diversity of 50-1 replicon populations was expanded in a time-dependent manner (Fig. 4). Similar phylogenetic trees were obtained for the 1B-2R1 replicon populations as well (data not shown). Next, as another index of genetic diversity, we calculated the mean number of nucleotide differences among three independent clones at each time point. The schematic presentation of such analysis on the NS regions of 50-1 and 1B-2R1 replicon RNAs was shown in Supplementary Fig. A, which is available as Supplementary material in JGV Online. The results also showed a time-dependent expansion of genetic diversity. After 12 months in culture, 0·32 % (mean of three cell culture lines) and 0·55 % diversities in nucleotide sequences were observed in the NS region of 50-1 and 1B-2R1 replicon RNAs. A similar time-dependent expansion of genetic diversity was also observed in the first 2 kb regions of both replicon RNAs (data not shown). These results indicate that the quasispecies nature of replicon RNA was easily acquired during the replication of the replicons.

Table 1. Base substitutions occurring in 50-1 and 1B-2R1 replicon RNAs during long-term cell culture

The counting of base substitutions was performed by comparison with the consensus sequence obtained from the 0M series of 50-1 or 1B-2R1 replicon.

Replicon	series	No. base substitutions										Synonymous/ non-synonymous	Transition/ transversion
		Transition					Transversion						
		Synonymous		Non-synonymous		Non-coding region	Synonymous		Non-synonymous		Non-coding region		
		Neo^R	NS	Neo^R	NS		Neo^R	NS	Neo^R	NS			
50-1	4MK	1	13	0	8	4	0	5	0	6	2	1·36	2·00
	6MK	0	20	2	10	8	3	8	1	9	1	1·41	1·82
	12MK	3	29	6	19	13	4	9	4	9	2	1·18	2·50
	18MK	5	43	8	26	16	3	10	4	14	5	1·17	2·72
	6MD	3	20	3	9	2	0	5	4	7	1	1·22	2·18
	12MD	5	29	2	26	3	2	5	1	8	0	1·11	4·06
	6MN	2	19	2	8	3	2	4	0	8	3	1·50	2·00
	12MN	3	25	2	21	9	1	6	5	15	3	0·81	2·00
1B-2R1	6M	1	14	5	14	1	1	1	3	5	3	0·63	2·69
	12M	2	22	4	29	6	1	2	3	10	6	0·59	2·86

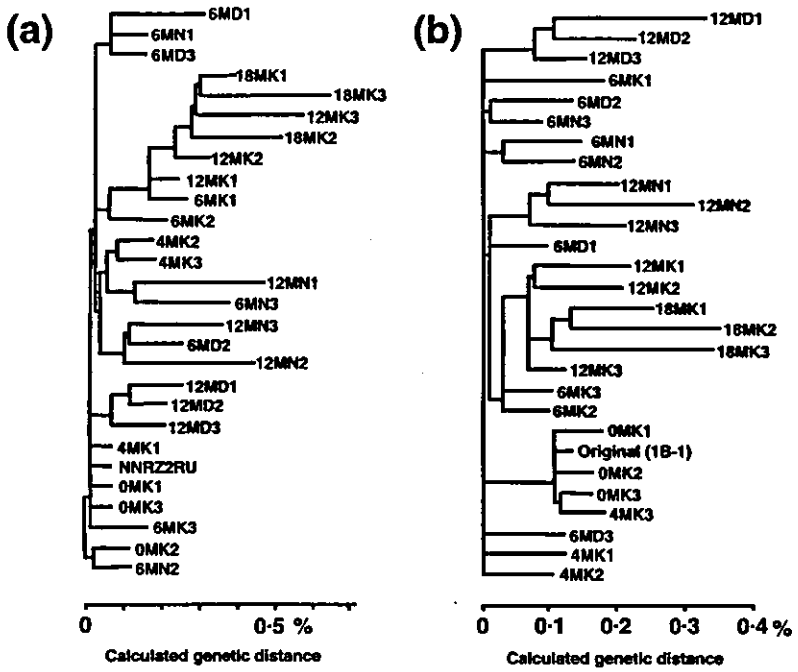

Fig. 4. Phylogenetic trees of 50-1 replicon populations obtained in long-term cell culture. The phylogenetic tree is depicted on the basis of nucleotide sequences of all replicon clones obtained by long-term culture of 50-1 cells. (a) The first 2·0 kb region of replicon RNA. NNRZ2RU indicates the original sequences of 50-1 replicon RNA, and the others indicate the names of clones. (b) The NS region of replicon RNA. Original (1B-1) indicates the original sequences of 50-1 replicon RNA, and the others indicate the names of clones.

Enhancement of HCV replication is associated with the expansion of the replicons' genetic diversity

To assess whether or not the mutations accumulating in the replicons increase the replication efficiencies of the replicons, the efficiency of colony formation (ECF) of the replicon was examined at each time point of the culture. An ECF assay was performed by transfection of total RNAs isolated from 50-1 and 1B-2R1 replicon cells at different time points into naïve HuH-7 cells. After 3 weeks of G418 selection, only a few colonies were obtained when RNAs from 50-1 replicon cells cultured less than 4 month were used (Fig. 5). However, ECF was apparently increased when RNAs from cells cultured 6 months, in particular the D and N cell culture lines, were used, and much higher numbers of colonies were obtained when RNAs from cells cultured 12 months were used (Fig. 5). Interestingly, ECFs of RNAs from D and N cell lines cultured more than 6 months were higher than those in the K cell culture line. These results indicated that ECF of the replicon was increased with the cultured periods of the replicon cells and suggested that ECF enhancement is associated with the expansion of the 50-1 replicon's genetic diversity.

In contrast to the case with 50-1 replicon cells, a number of colonies were obtained even when RNA from the initial culture of 1B-2R1 replicon cells was used (Fig. 5). In this replicon also, the ECF of RNA from cells cultured 12 months was apparently higher than those of RNA from the initial culture or 6 months of culture (Fig. 5). These results suggest that S2200R substitution, which was detected when the 1B-2R1 replicon was established (Kato *et al.*, 2003b), function as an adaptive mutation, and that the expansion of genetic diversity in the 1B-2R1 replicon also contributes to the enhancement of ECF, as was the case with the 50-1 replicon.

Effect of ribavirin and mizoribine on the genetic evolution and dynamics of the 50-1 replicon

Combined treatment of interferon plus ribavirin for patients with chronic hepatitis C has been shown to be more effective than treatment with interferon alone (McHutchison *et al.*, 1998), although it has been shown that ribavirin alone does not cause a decrease of HCV level in patients with chronic hepatitis C. Recently, several groups have reported that ribavirin might cause 'error catastrophe' of HCV genome (Contreras *et al.*, 2002; Tanabe *et al.*, 2004; Zhou *et al.*, 2003), however, controversial results have also been reported (Schinkel *et al.*, 2003). Therefore, to clarify whether or not ribavirin affects the genetic alterations of HCV, we cultured parent 50-1 cells (corresponding to 0M in Fig. 1) for 6 months in the presence of ribavirin (5 or 25 μM) or its derivative molecule, mizoribine (25 μM). As a control, the parent 50-1 cells were also cultured for 6 months in the absence of ribavirin or mizoribine. After 6 months in culture, the levels of replicon RNAs and HCV proteins were examined by Northern and Western blot analyses, respectively. As shown in Fig. 6(a), the level of replicon RNA in the cells treated with ribavirin or mizoribine was almost the same as that in the cells without ribavirin or mizoribine treatment. The NS3 and NS5B were also expressed at similar levels in the cells irrespective of ribavirin or mizoribine treatment (Fig. 6b). These results indicate that even 6 months of treatment with ribavirin or mizoribine did not prevent the replication of replicon RNA under the G418 selection pressure. Using the 50-1 cells cultured for 6 months with or without ribavirin or mizoribine, we performed sequence analysis of replicon

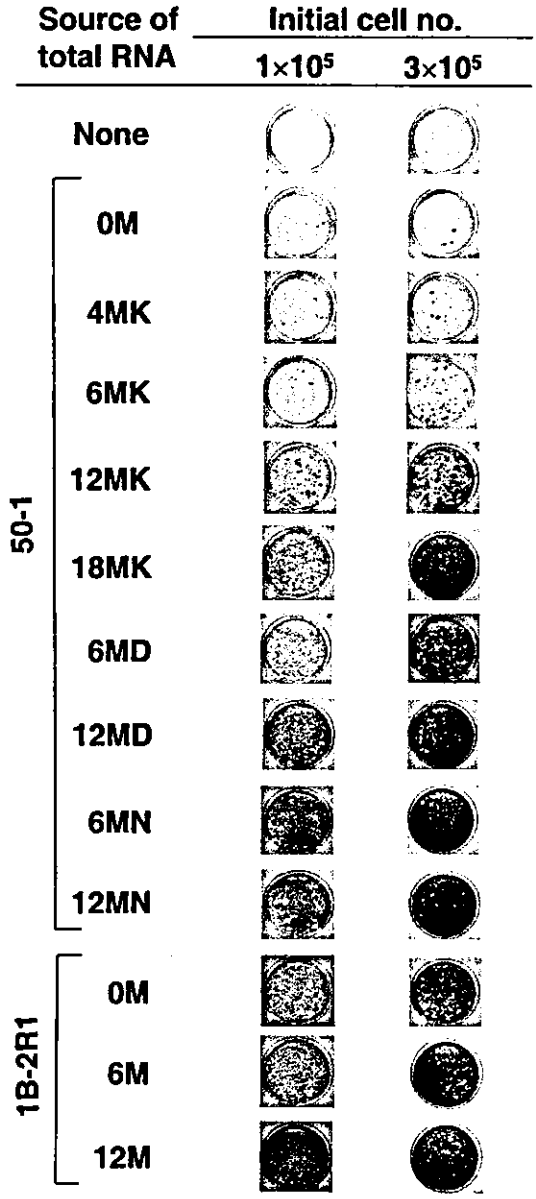

Fig. 5. ECF of the RNAs isolated from 50-1 and 1B-2R1 replicon cells at different time points in the culture. Total RNAs obtained from the replicon cells were transfected into HuH-7 cells as described in Methods. The panels show the cell colonies that were recovered after 3 weeks of G418 selection.

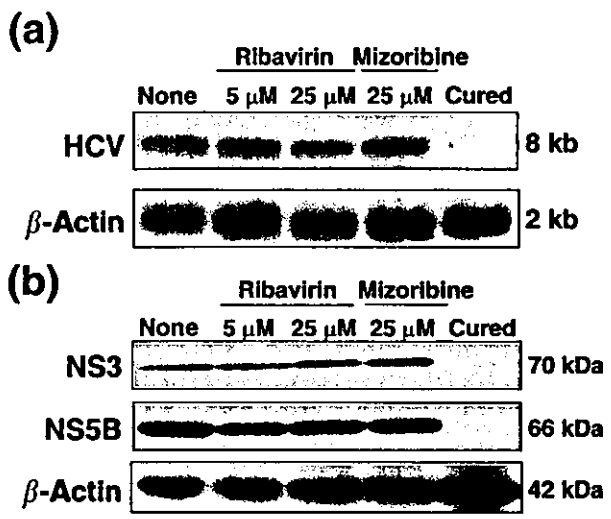

Fig. 6. Characterization of 50-1 cells cultured for 6 months in the presence of ribavirin or mizoribine. (a) Northern blot analysis. Total RNAs from 50-1 cells cultured for 6 months in the presence of ribavirin (5 and 25 μM) or mizoribine (25 μM), as well as total RNA from 50-1 cells cultured for 6 months in the absence of ribavirin and mizoribine were used for the analysis. Northern blot analysis was performed as indicated in Fig. 1(a). (b) Western blot analysis. The orders of specimens were the same as in (a). Western blot analysis was performed as indicated in Fig. 1(b).

RNAs as described above. As shown in Table 2, the results revealed that the numbers of mutations in the first 2·0 kb and NS regions of the replicon RNAs sequenced were not significantly different among the specimens, although the number in the NS region derived from the cells treated with 25 μM of ribavirin was a little lower than those of the other specimens. These results suggest that the treatment of replicon cells with either ribavirin or mizoribine does not increase the mutation rate of replicon RNA. The ratio of synonymous and non-synonymous mutations, and the ratio of transition and transversion mutations were also not altered by ribavirin or mizoribine treatment (data not shown). In addition, we did not observe any ribavirin- or mizoribine-specific common amino acid substitutions in either the first 2 kb or NS regions of the replicon RNA, although P1115L and E1966A were detected after 6 months in culture in all cell culture lines. The above-described analysis of genetic diversity among the replicon RNAs did

Table 2. Base substitutions occurred in 50-1 replicon RNA during 6 months culture in the presence of ribavirin or mizoribine

6MR5 and 6MR25 indicate the series treated with 5 and 25 μM of ribavirin, respectively. 6MM25 indicates the series treated with 25 μM of mizoribine. The counting of base substitutions was performed by the comparison with the original sequence of 50-1 replicon (Kishine *et al.*, 2002).

Series	First 2-kb region	NS region
6M (Fig. 2)	5·4±1·9*	16·4±1·8
6M	5·7±2·5	16·0±0·0
6MR5	5·7±1·5	16·3±1·5
6MR25	5·7±1·5	10·7±1·2
6MM25	3·7±0·6	18·7±4·0

*Numbers of base substitutions ± SD.

not reveal any significant differences between the specimens derived from the replicon cells with and those without ribavirin or mizoribine treatment (data not shown). Taken together, these results suggest that neither ribavirin nor mizoribine accelerated the mutation rate of HCV replicons or the development of their quasispecies nature.

DISCUSSION

In this study, we analysed the genetic evolution and dynamics of HCV replicons, and time-dependent genetic mutations of HCV replicons were observed. Time-dependent expansions of their genetic diversities were also revealed. Our results should provide useful fundamental information for understanding the remarkable genetic diversity and variation among the HCV genomes observed in patients with chronic hepatitis C.

Although RT-PCR techniques were used to amplify the replicon RNAs in this study, it is unlikely that the detected mutations were due to errors related to the use of the KOD-plus DNA polymerase in the PCR reaction, because we previously showed that KOD-plus DNA polymerase possessed a high proofreading activity (Alam *et al.*, 2002; Naganuma *et al.*, 2004). Furthermore, in the present study, we sequenced several clones (containing a 2·0 or 6·1 kb fragment) obtained by PCR using KOD-plus DNA polymerase and a single sequenced clone as a template, but no mutations were detected in these sequenced clones, indicating that KOD-plus DNA polymerase possesses extremely high fidelity. However, we are not able to completely exclude the possibility that some substitutions resulted from the erroneous use of KOD-plus DNA polymerase during the PCR. Even if such errors occurred, the error frequency is estimated to be less than one nucleotide per sequenced clone. This is explained as follows. Fig. 2 shows that the numbers of substitutions time-dependently increased with linearity in both HCV replicons. Interestingly, when these linear lines are extrapolated to zero base substitutions, the crossing points show approximately −2–−3 months in the time axis. These range of months is in accord with the time of initial electroporation of HCV replicon RNA to HuH-7 cells. Therefore, PCR-induced mutations are considered to be very rare and such mutations would have very little effect on the results shown in Fig. 2. In addition, to avoid a sampling effect, we sequenced three independent clones derived from each time point.

We showed that the mutation rates for the 50-1 and 1B-2R1 replicon RNAs were almost the same – about 3×10^{-3} base substitutions/site/year. However, the actual mutation frequency of the replicon RNAs would be higher than this value, because the mutations that occurred in positions that were critical for the replication of replicon RNA should not have been passed on to the progeny. Our observed mutation rates of the replicon RNAs were approximately two times higher than those previously obtained in chimpanzees and clinical patients with chronic hepatitis C (Major *et al.*, 1999; Ogata *et al.*, 1991; Okamoto *et al.*, 1992). Since the selective pressure of the immune system also functions *in vivo* (Kato *et al.*, 1993), the mutation rate in cell culture obtained in this study may be reasonable value as a potential mutation rate of HCV. However, direct comparison of these mutation rates would be difficult, because both the experimental model and analytical method were different in this study compared with the previous studies. It would be interesting to examine whether this mutation rate (3×10^{-3} base substitutions/site/year) would be maintained during longer-term culture of the replicon cells. If so, approximately 3 % of nucleotide sequences of the replicon RNAs might be mutated after 10 years in cell culture. Alternatively, the mutations might become saturated during further long-term culture of the replicon cells. To clarify this point, further long-term culture of replicon cells is in progress.

Although the mutations detected in this study were dispersed throughout the entire length of the replicon RNAs (Fig. 3), the mutation frequencies in the 5′ UTR and NS5B region were lower than those in other regions, and the NS5A region showed the highest mutation frequency. These observations are consistent with the genetic diversities of HCVs in patients with chronic hepatitis C reported to date (Kato, 2001). In addition, the positions in which amino acid substitutions were observed during the cell culture did not appear to be critical for replication of the HCV genome.

Time-dependent expansions of genetic diversities of HCV replicons were also found in this study. However, this finding seems to be different from the previous findings that HCV populations in the cells infected *in vitro* gradually altered with time and converged to the limited populations (Kato *et al.*, 1998; Kato, 2001). This gap may have been due to the differences in the HCV sources used: a patient's inoculum containing a quasispecies of HCV was used for the *in vitro* infection experiment, and a single HCV species was used for the replicon system. Alternatively, the gap may have been due to the overwhelming difference between the replication level of the HCV genome in the cells infected *in vitro* and that in the replicon cells.

To date, a number of amino acid substitutions belonging to adaptive mutations that enhance the frequency with which the replicon is established *in vitro* have been found in established HCV replicons (Bartenschlager, 2002; Blight *et al.*, 2000, 2003; Ikeda *et al.*, 2002; Krieger *et al.*, 2001; Lanford *et al.*, 2003; Lohmann *et al.*, 2001, 2003; Pflugheber *et al.*, 2002). Although none of the amino acid substitutions detected in the long-term cultures of the 50-1 and 1B-2R1 replicons were the same as those reported as adaptive mutations, ECF analysis of the replicons using naïve HuH-7 cells suggested that adaptive mutations accumulated in the replicon populations in a time-dependent manner. In particular, drastic enhancement of ECF was observed in the 50-1 replicon after 6 months of culture. However, this result suggests that the four common amino acid substitutions (P1115L, K1609E, V1896F and E1966A) do not contribute much to the drastic enhancement of ECF,

because the ECFs of 4MK and 6MK samples possessing these substitutions did not increase much. Therefore, we estimate that some uncommon amino acid substitutions accumulated as so-called adaptive mutations. The candidates for such adaptive mutations are culture-line-specific amino acid substitutions (Fig. 3b, *1–12), and many amino acid substitutions sporadically appeared in the replicons in the long-term cell cultures. To identify which amino acid substitution is the main contributor to the drastic enhancement of ECF, further transfection experiments using replicon RNAs possessing mutations will be needed. Based on the results of this study, S2200R substitution in the 1B-2R1 replicon is considered an adaptive mutation. This description is supported by the previous result that we were unable to obtain any G418-resistant colonies when the original 1B-2 replicon RNA library, used in the isolation of the 1B-2R1 replicon, was transfected into naïve HuH-7 cells (Kato *et al.*, 2003b). Since the ECF of 1B-2R1 replicon RNA from 12 months of culture was further enhanced, it may be that the I1097V substitution, detected commonly at 12 months of culture, functions as an additional adaptive mutation.

Interestingly, once a new mutation was observed in all three clones at a particular time point, the clones which went back to the original sequences were never obtained in the subsequent cell culture, except for one clone (a mutation in the HCV IRES region) derived from 1B-2R1 replicon cells after 12 months in culture (Fig. 3a). This finding suggests that the genetic evolution of HCV replicons is irreversibly progressing.

Although the mechanism of action of ribavirin for patients with chronic hepatitis C is ambiguous, an 'error catastrophe' theory of ribavirin has been proposed by several groups (Contreras *et al.*, 2002; Tanabe *et al.*, 2004; Zhou *et al.*, 2003). However, our results obtained in this study were not able to support this 'error catastrophe' theory, because ribavirin had no effect on the genetic variation and diversity of the 50-1 replicon. The concentration (5 and 25 μM) of ribavirin used in this study was considered to be reasonable, because the growth rate of 50-1 cells decreased at a ribavirin concentration of more than 50 μM, and approximately 10 μM of ribavirin is the maximum plasma concentration in current clinical usage (Tanabe *et al.*, 2004). Higher concentration (more than 50 μM) of ribavirin used in previous studies may be required for causation of the error catastrophe. Recently, a single amino acid substitution (F2834Y) was identified as a ribavirin-resistant NS5B mutation in genotype 1a (Young *et al.*, 2003); however, it is difficult to evaluate that finding in this study, because most of the HCV strains belonging to genotype 1b, including 1B-1 (50-1) and 1B-2 (1B-2R1), already possess a Tyr residue at position 2834. No amino acid substitution at position 2834 in NS5B was observed in the replicon cells treated with ribavirin.

This study provided the fact that the genetic diversity of HCV replicons was enlarged in a time-dependent manner during long-term cell culture. Since all the HCV replicons established to date have been shown to be highly sensitive to interferon-α, -β and -γ (Kato *et al.*, 2003b), and most of the HCV replicons established to date are able to replicate in only HuH-7 cells, the extensive genetic polymorphism of HCV replicon populations obtained by long-term cell culture may change the sensitivity against interferon or the ability of replication in the cells except for HuH-7. In the future, it will be necessary to clarify these points. Thus, HCV replicon populations obtained by long-term cell culture may be useful not only for analysis of the genetic variations and dynamics of HCV but also for analysis of the variable properties of HCV.

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Effect of Interaction between Hepatitis C Virus NS5A and NS5B on Hepatitis C Virus RNA Replication with the Hepatitis C Virus Replicon

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Hepatitis C virus (HCV) NS5A has been reported to be important for the establishment of replication by adaptive mutations or localization, although its role in viral replication remains unclear. It was previously reported that NS5A interacts with NS5B via two regions of NS5A in the isolate JK-1 and modulates the activity of NS5B RdRp (Y. Shirota et al., J. Biol. Chem., 277:11149–11155, 2002), but the biological significance of this interaction has not been determined. In this study, we addressed the effect of this interaction on HCV RNA replication with an HCV replicon system derived from the isolate M1LE (H. Kishine et al., Biochem. Biophys. Res. Commun., 293:993–999, 2002). We constructed three internal deletion mutants, M1LE/5Adel-1 and M1LE/5Adel-2, each encoding NS5A which cannot bind NS5B, and M1LE/5Adel-3, encoding NS5A that can bind NS5B. After transfection into Huh-7 cells, M1LE/5Adel-3 was replication competent, but both M1LE/5Adel-1 and M1LE/5Adel-2 were not. Next we prepared 20 alanine-substituted clustered mutants within both NS5B-binding regions and examined the effect of these mutants on HCV RNA replication. Only 5 of the 20 mutants were replication competent. Subsequently, we introduced a point mutation, S225P, a deletion of S229, or S232I into NS5A and prepared cured Huh-7 cells that were cured of RNA replication by alpha interferon. Finally, with these point mutations and cured cells, we established a highly improved replicon system. In this system, only the same five mutants were replication competent. These results strongly suggest that the interaction between NS5A and NS5B is critical for HCV RNA replication in the HCV replicon system.

The hepatitis C virus (HCV) is a major cause of chronic hepatitis around the world (1, 9). Chronic infection with HCV results in liver cirrhosis and often hepatocellular carcinoma (50, 53). HCV is an enveloped positive-strand RNA virus belonging to the genus *Hepacivirus* in the family *Flaviviridae* (44). The HCV RNA genome is ~9.6 kb in length and consists of a 5′ nontranslated region (5′ NTR), a large open reading frame, and a 3′ NTR. The 5′ NTR contains an internal ribosome entry site (IRES) mediating translation of a single polyprotein of ~3,000 amino acid (aa) residues (57, 59). The polyprotein is cleaved by host and viral protease into at least 10 different products (2, 21, 22, 25, 26). The structural proteins core, E1, and E2 are located in the amino terminus of the polyprotein, followed by p7, a hydrophobic peptide with unknown function, and the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (41). The 3′ NTR consists of a short variable sequence, a poly(U)-poly(UC) tract, and a highly conserved X region, and it is critical for HCV RNA replication and HCV infection (17, 34, 65, 66).

HCV is unique among positive-strand RNA viruses in causing persistent infections, and a high mutation rate in E2 allows it to escape host immune surveillance. These phenomena are tightly associated with chronic inflammation of the liver (27, 32, 61, 62). Therefore, HCV RNA replication has been a target for treatment of HCV. NS5B is an RNA-dependent RNA polymerase (RdRp), the central catalytic enzyme in HCV RNA replication. Several recombinant forms of NS5B expressed and purified from insect cells and *Escherichia coli* are available and catalytically active, and studies with purified NS5B proteins provide insight into the biochemical and catalytic properties of NS5B (3, 16, 39, 64). However, the result that NS5B can initiate de novo RNA replication in vitro with both a non-HCV RNA template and an HCV RNA template may reflect the catalytic property of NS5B but not the tight regulation of HCV RNA replication initiation. Studies of HCV RNA replication in vitro have to overcome several difficulties, since replication requires all or most NS proteins and occurs at the membrane where all of the HCV NS proteins are recruited.

A second system used to study HCV RNA replication is the study of HCV RNA replicons in vivo, which utilizes autonomously replicating HCV-derived RNAs. These replicon RNAs have the authentic HCV 5′ and 3′ NTRs. The HCV IRES drives the translation of a selectable marker such as neomycin resistance, and an internal encephalomyocarditis virus IRES directs translation of NS3 to NS5B (38). In vitro-transcribed replicon RNAs are transfected into the human hepatoma cell line Huh-7 by electroporation and placed under selection. The emergence of neomycin-resistant cell colonies is indicative of

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RNA replication. Recently, adaptive mutations that dramatically enhance the ability of HCV RNA to replicate have been identified in NS3, NS4B, NS5A, and NS5B (4, 24, 35, 37, 40). Initially, only the replicon derived from a genotype 1b isolate, HCV-Con1, was replication competent in Huh-7 cells, and adaptive mutations were required for efficient replication (4, 35, 37, 40). Then, several replicon systems derived from a genotype 1b isolate, HCV-N, and a genotype 1a isolate, HCV-H77, were reported to replicate in Huh-7 cells (6, 23, 24, 29). Recently, a replicon system was established which uses an HCV sequence derived from the human T-cell line MT-2C infected with HCV (a genotype 1b isolate, M1LE) in vitro and isolated 50-1 cells replicating subgenomic RNAs with some amino acid mutations (31, 33, 43, 55).

It was previously reported that NS5A and NS5B interact in vitro and in vivo through two independent regions of NS5A and that NS5A modulates the activity of NS5B RdRp through this interaction in vitro in the isolate JK-1 (54). This ability of NS5A to modulate the RdRp activity in vitro may be consistent with the high frequency of adaptive mutations in NS5A which result in a much more efficient RNA replication in Huh-7 cells; however, the biological meaning of the interaction between NS5A and NS5B remains to be addressed in vivo. Here we report a modified HCV RNA replicon system derived from the isolate M1LE by introducing point mutations (S225P, a deletion of S229, and S232I), and curing 50-1 cells of HCV subgenomic RNA replication by interferon (IFN) treatment. By introducing internal deletion and substitution mutations into NS5A, we demonstrate that the regions essential for the interaction between NS5A and NS5B are also critical for HCV RNA replication in using the HCV replicon system.

MATERIALS AND METHODS

Construction of plasmids. pNNRZ2RU (33) harbors a subgenomic replicon derived from MT-2C cells infected with HCV (a genotype 1b isolate, M1LE; GenBank accession no. AB080299), and this plasmid contains cDNA of wild-type M1LE. For convenience, pNNRZ2RU was digested with *Mlu*I and *Bgl*II, and the obtained fragment was inserted into the *Mlu*I and *Bgl*II sites of the vector pGL3Basic (Promega) to create pGL3-*Mlu*I-*Bgl*II. pGL3-*Mlu*I-*Bgl*II was used as an intermediate vector. All mutations were introduced into pGL3-*Mlu*I-*Bgl*II, and then the fragments of pGL3-*Mlu*I-*Bgl*II digested by *Mlu*I and *Bgl*II containing each mutation were reintroduced into the *Mlu*I and *Bgl*II sites of pNNRZ2RU to create each mutant.

M1LE/5Adel-1 was generated by PCR with the primers 5Adel-1 For (containing a *Mlu*I site) and 5A Rev-3 and then inserted into the *Mlu*I and *Nhe*I sites of pGL3-*Mlu*I-*Bgl*II to create pGL3-*Mlu*I-*Bgl*II-5Adel-1. M1LE/5Adel-2 was generated by PCR with overlap extension with the primers 5Adel-2 For, 5A Rev-2, 5Adel-2 Rev, and 5A For-3 and then inserted into the *Nhe*I and *Sac*I sites of pGL3-*Mlu*I-*Bgl*II to create pGL3-*Mlu*I-*Bgl*II-5Adel-2. M1LE/5Adel-3 was generated by PCR with the primers 5A del-3 For (containing a *Nhe*I site) and 5A Rev-2 and then inserted into the *Nhe*I and *Sac*I sites of pGL3-*Mlu*I-*Bgl*II to create pGL3-*Mlu*I-*Bgl*II-5Adel-3.

An alanine scanning method was used to construct NS5A alanine-substituted mutants to minimize the effects of substituted amino acid residues (7). The positions of alanine-substituted clustered mutations (cm) of NS5A are shown in Fig. 2B. To generate M1LE/cm 94, 100, 105, 110, 113, 120, 127, 134, 141, 148, and 155, each mutation was introduced into the *Mlu*I and *Nhe*I sites of pGL3-*Mlu*I-*Bgl*II by site-directed mutagenesis with primers carrying the necessary nucleotide changes to create pGL3-*Mlu*I-*Bgl*II-cm94, -100, -105, -110, -113, -120, -127, -134, -141, -148, and -155, respectively. To generate M1LE/cm 252, 277, 283, 290, 297, 304, 311, 316, 321, and 328, each mutation was introduced into the *Nhe*I and *Sac*I sites of pGL3-*Mlu*I-*Bgl*II by site-directed mutagenesis with primers carrying the necessary nucleotide changes to create pGL3-*Mlu*I-*Bgl*II-cm252, -277, -283, -290, -297, -304, -311, -316, -321, and -328, respectively.

To generate M1LE/S225P and a deletion of S229 (delS229), the point mutations S225P and delS229 were introduced into the *Mlu*I and *Nhe*I sites of pGL3-*Mlu*I-*Bgl*II by site-directed mutagenesis with primers carrying the necessary nucleotide changes to create pGL3-*Mlu*I-*Bgl*II-S225P and -delS229. The point mutation S232I was introduced into the *Mlu*I and *Sac*I sites of pGL3-*Mlu*I-*Bgl*II by site-directed mutagenesis with primers carrying the necessary nucleotide changes to create pGL3-*Mlu*I-*Bgl*II-S232I. To generate the double mutants containing both the point mutation S232I plus an internal deletion mutation or cm, the *Eco*RI fragments of pGL3-*Mlu*I-*Bgl*II-cm94, -100, -105, -110, -113, -120, -127, -134, and -141 were inserted into the *Eco*RI sites of pGL3-*Mlu*I-*Bgl*II-S232I. Because there were no optimal enzyme sites in the others, the point mutation S232I was introduced into the *Mlu*I and *Sac*I sites of pGL3-*Mlu*I-*Bgl*II-5Adel-1, -del-2, -del-3, and -cm148, -155, -252, -277, -283, -290, -297, -304, -311, -316, -321, and -328 by site-directed mutagenesis with primers carrying the necessary nucleotide changes.

To create double mutants containing both the point mutation S225P plus an internal deletion mutation or cm (cm 252, 277, 283, 290, 297, 304, 311, 316, 321, and 328), the *Nhe*I and *Sac*I fragments of pGL3-*Mlu*I-*Bgl*II-5Adel-2, -5Adel-3, -cm252, -277, -283, -290, -297, -304, -311, -316, -321, and -328 were introduced into the *Nhe*I and *Sac*I sites of pGL3-*Mlu*I-*Bgl*II-S225P.

To generate M1LE/5B-VDD, a point mutation changing the GDD motif of NS5B to VDD was introduced at the *Nde*I and *Sma*I sites of pGL3-*Mlu*I-*Bgl*II by site-directed mutagenesis with primers carrying the necessary nucleotide changes to create pGL3-*Mlu*I-*Bgl*II-5B-VDD.

All of the mammalian expression vectors were derived from pSG5UTPL (36). The vector pNKFLAG (49) was used to express amino-terminally FLAG-tagged proteins. The vector pNKGST (49) was used to express glutathione *S*-transferase (GST)-fused proteins.

pNNRZ2RU was subcloned by PCR with the primers NS5A For, NS5A Rev, NS5B For, and NS5B Rev. NS5A For contains an artificial initiation codon downstream of the *Mun*I site, and NS5B For contains one artificial initiation codon downstream of the *Sac*I site. NS5A Rev contains a *Bgl*II site, and NS5B Rev contains a *Bam*HI site. Full-length NS5A was subcloned into the *Eco*RI and *Bam*HI sites of pNKFLAG to create pNKFLAG-5A/wild, and full-length NS5B was subcloned into the *Sac*I and *Bam*HI sites of pNKGST to create pNKGST-5B/wild.

To create NKFLAG-5A/del-1, and -cm94, -100, -105, -113, -120, -127, -134, -141, -148, and -155, the *Mlu*I and *Nhe*I fragments of pGL3-*Mlu*I-*Bgl*II mutants were introduced at the *Mlu*I and *Nhe*I sites of pNKFLAG-5A/wild. To create NKFLAG-5A/del-2, /del-3, -cm252, -277, -283, -290, -297, -304, -311, -316, -321, and -328, the *Nhe*I and *Sac*I fragments of pGL3-*Mlu*I-*Bgl*II mutants were inserted into the *Nhe*I and *Sac*I sites of pNKFLAG-5A/wild.

The sequences of all of the constructs were confirmed by the dideoxy sequence method. The main primers used for plasmid construction are shown in Table 1.

In vitro transcription and purification of RNA. Plasmids were linearized with *Xba*I and purified by passage through a column (PCR purification kit; Qiagen) prior to transcription. RNA was synthesized with T7 MEGAScript reagents (Ambion) by following the manufacturer's directions, and the reaction was stopped by digestion with RNase-free DNase. The synthesized RNA was passed

TABLE 1. Sequences of primers used in the present study

Primer	Sequence (5′–3′)
5Adel-1 For	ATATATCAACGCGTACCCGGCGTGTAAACCTCTCCTACGG
5Adel-2 For	GTGGAGTCAGAGAACGTTCCTCCGGTGGTACACGGGTGCCCA
5Adel-2 Rev	TACCACCGGAGGAACGTTCTCTGACTCCACGCGGGTGATGTT
5Adel-3 For	ATATATATGCTAGCCAGTTGAAGGTAGTAATTCTGGACTCTTTC
5A For-3	ATCCTTCCCACATTACAGCA
5A Rev-2	CTCAACGTCGGATCCCTTGT
5A Rev-3	GGTCAGCGTCCGGGGAGTCATG
NS5A For	ATATCAATTGCATGTCCGGCTCGTGGCTAAGGGATATT
NS5A Rev	ATATAGATCTGCAGCAGACGACGTCCTCACTAGCCTC
NS5B For	TATCGAGCTCGATGTCAATGTCCTACTCATGGACAGGT
NS5B Rev	ATATGGGATCCCCGGTTGGGGAGCAGGTAGATGCCTAC

through a column (RNeasy mini kit; Qiagen) and dissolved in RNase-free water. The concentration was determined by measuring the optical density at 260 nm, and RNA integrity was checked by nondenaturing agarose gel electrophoresis.

RNA transfection and selection of G418-resistant cells. Subconfluent Huh-7 cells were trypsinized, washed once with phosphate-buffered saline (PBS) (−), and resuspended at 10^7 cells/ml in OPTI-MEM (Gibco-BRL, Invitrogen Life Technologies). Then, 10 to 1,000 ng of transcript was adjusted with total RNA from naive Huh-7 cells to a final amount of 10 μg, which was mixed with 400 μl of the cell suspension in a cuvette with a gap width of 0.4 cm (Bio-Rad). The mixture was immediately transfected into Huh-7 cells by electroporation with GenePulser II system (Bio-Rad) set to 270 V and 975 μF. Following 10 min of incubation at room temperature, the cells were transferred into 10 ml of growth medium and then seeded into a 10- or 15-cm-diameter cell culture dish. For the selection of G418-resistant cells, the medium was replaced with fresh medium containing 0.5 to 1 mg of G418 (Geneticin; Gibco-BRL, Invitrogen Life Technologies)/ml after 24 to 48 h and the medium was changed twice a week. Four weeks after transfection, colonies were stained with Coomassie brilliant blue (0.6 g/liter in 50% methanol–10% acetic acid).

IFN treatment. To stop the replication of HCV subgenomic RNA, 50-1 cells were treated with 10,000 U of IFN-α2b (kindly provided by Schering-Plough)/ml in the absence of G418. After 2 weeks of IFN treatment, the absence of HCV RNA was determined from the results of Northern hybridization, reverse transcription-PCR, and sensitivity to G418.

Cell culture. We used two kinds of Huh-7 cells, one derived from our own laboratory's original Huh-7 cell line, designated Huh-7-DMB, and the cured clone of 50-1 cells, designated Huh-7-KV-C. Both types of Huh-7 cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, 100 U of penicillin, and 100 μg of streptomycin.

Preparation of cell extracts, coprecipitation with glutathione resin, and Western blot analysis. The transient transfection of COS1 cells was carried out by using the calcium-phosphate method. The cells were harvested, washed with PBS (−), and sonicated in PBS lysis buffer [PBS (−) containing 250 mM NaCl, 1.0% Triton X-100, 1 mM EDTA, and 1 mM dithiothreitol] with 10 mg (each) of aprotinin and leupeptin per ml. Total cell lysate was diluted 10-fold with PBS lysis buffer, mixed with 40 μl of glutathione-Sepharose 4B beads (glutathione resin) (Amersham Biosciences), and then incubated for 3 h on a rotator in a cold room. After an extensive wash with PBS (−) containing 1.0% Triton X-100, the bound proteins were eluted, fractionated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE), transferred onto nitrocellulose membranes, and subjected to Western blot analysis with anti-FLAG monoclonal antibody. The proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences). The nitrocellulose membranes used for Western blot analysis with anti-FLAG monoclonal antibody were reprobed with anti-GST monoclonal antibody (Zymed Laboratories) according to the manufacturer's instructions (Amersham Biosciences).

RESULTS

Interaction between NS5A and NS5B. It was previously reported that NS5A and NS5B associate through two discontinuous regions of NS5A (aa 105 to 162 and 277 to 334) and that NS5A weakly stimulates the activity of NS5B RdRp in vitro initially (at a molar ratio to NS5B of less than 0.1) and then inhibits the activity in a dose-dependent manner (54). To examine the effect of this interaction on HCV RNA replication, we used an HCV RNA replicon system derived from the isolate M1LE and 50-1 cells (33). We prepared a hybrid replicon of JK-1 and M1LE which harbors the JK-1 sequence from aa 92 (*Mlu*I site) of NS5A to the end of NS5B. However, the hybrid replicon did not produce any G418-resistant colony with the Huh-7 cell line (data not shown), so we constructed various mutated versions of NS5A of the RNA replicon derived from M1LE.

First, we confirmed whether the association between NS5A and NS5B through the two discontinuous regions of NS5A occurs with the sequence derived from M1LE. COS1 cells were transiently cotransfected with mammalian expression vectors, pNKFLAG-5A/wild, /del-1, /del-2, and /del-3; pNKGST or pNKGST-5B/wild; and the cell lysates were subjected to a GST pull-down assay. pNKFLAG-5A/wild encodes the full-length and wild-type NS5A proteins of M1LE. pNKFLAG-5A/del-1 encodes the internally deleted NS5A protein missing aa 94 to 162, a deletion 11 aa longer than that reported for JK-1 for the convenience of mutagenesis (aa 105 to 162). pNKFLAG-5A/del-2 encodes the internally deleted NS5A protein missing aa 277 to 334, the same region reported for JK-1. pNKFLAG-5A/del-3 encodes the internally deleted NS5A protein missing aa 235 to 276, a region nonessential for the interaction with NS5B in JK-1. pNKGST-5B/wild encodes the full-length and wild-type NS5B proteins of M1LE, and pNKGST encodes only a GST protein. Under conditions in which the expression levels of FLAG-NS5A proteins (input) were similar and the recovery of the GST-NS5B proteins was almost the same (Fig. 1A, lanes 1 to 5, and C, lanes 2 to 5), coprecipitated NS5A proteins (output) were examined (Fig. 1B, lanes 1 to 5). FLAG-NS5A/wild bound to GST-NS5B (Fig. 1B, lane 2) but not to GST alone (Fig. 1B, lane 1). This result demonstrates that NS5A and NS5B also interact not only in JK-1 but also in M1LE in vivo. Very little FLAG-NS5A/del-1 or /del-2 was recovered (Fig. 1B, lane 3 and 4); however, FLAG-NS5A/del-3 was efficiently pulled down (Fig. 1B, lane 5). When larger amounts of proteins were used for this assay, both FLAG-NS5A/del-1 and /del-2 were weakly detected in the fraction pulled down with GST-NS5B but much significantly weaker than FLAG-5A/wild and /del-3. These results demonstrate that aa 94 to 162 (defined as region 1) and aa 277 to 334 (region 2), but not aa 235 to 276 (region 3), of NS5A seem to be essential for binding NS5B in M1LE as observed in JK-1.

Effect of binding NS5B on HCV RNA replication. To examine the effect of the interaction between NS5A and NS5B on HCV RNA replication in the replicon system, we prepared three kinds of internal deletion mutants, M1LE/5Adel-1, M1LE/5Adel-2, and M1LE/5Adel-3, missing regions 1, 2, and 3 of NS5A, respectively (Fig. 2). M1LE/5Adel-1 and M1LE/5Adel-2 are impaired in their binding to NS5B, but M1LE/5Adel-3 is not. As a negative control, we prepared M1LE/5B-VDD, in which the GDD motif of NS5B was mutated to VDD.

FLAG-tagged wild-type and internally deleted NS5A proteins were efficiently expressed in transiently transfected COS1 cells (Fig. 3). When wild-type M1LE and M1LE/5Adel-3 were transfected by electroporation into our laboratory's Huh-7 cell line, Huh-7-DMB, G418-resistant colonies emerged after selection at a concentration of 1 mg/ml. In the case of M1LE/5Adel-3, the number of G418-resistant colonies was about seven times fewer than in wild-type M1LE. In contrast, no colonies emerged when M1LE/5Adel-1, M1LE/5Adel-2, and M1LE/5B-VDD were transfected into Huh-7-DMB cells, indicating that both of the NS5B-interacting regions of NS5A are critical for HCV RNA replication (Fig. 4). The fact that M1LE/5Adel-3 was replication competent but less efficient than the wild type in Huh-7-DMB cells may reflect some roles of region 3 in HCV RNA replication or a conformational change introduced by the internal deletion (see Discussion).

To minimize the effect of the internal deletion and further delineate the sequence(s) critical for HCV RNA replication, we used the alanine-scanning method (7). All residues of the two regions were scanned by introducing alanine substitution

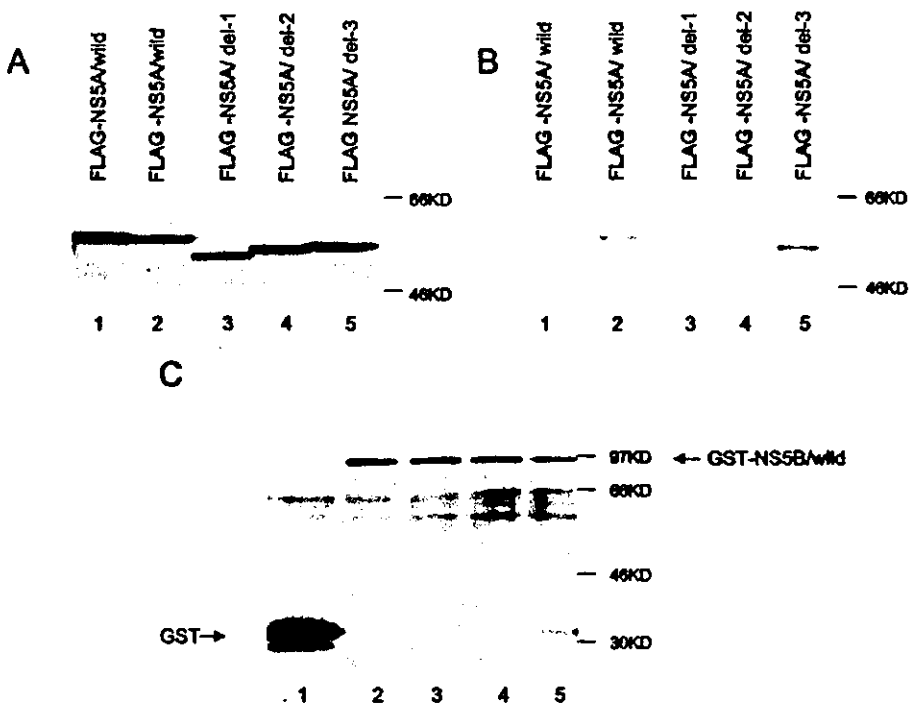
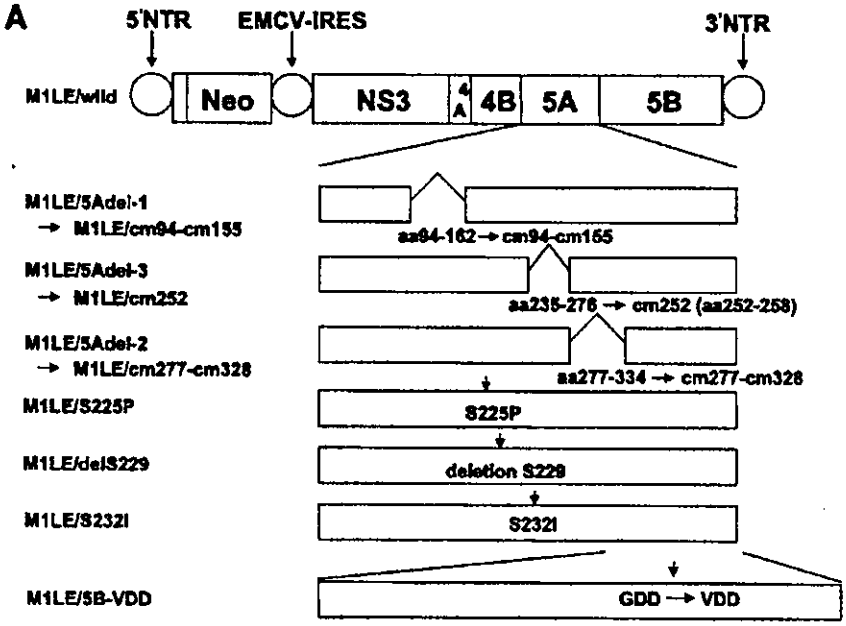


FIG. 1. Interaction between NS5A and NS5B of the isolate HCV M1LE and the regions essential for this interaction. COS1 cells were transiently cotransfected with mammalian expression vectors expressing FLAG-NS5A proteins (lanes: 1 and 2, wild type; 3, internal deletion mutant 1; 4, internal deletion mutant 2; 5, internal deletion mutant 3) and GST protein alone (lane 1) or GST-NS5B proteins (lanes 2, 3, 4, and 5). (A) Input of FLAG-NS5A proteins. Total lysates were fractionated by SDS–10% PAGE and subjected to Western blot analysis with anti-FLAG monoclonal antibody. (B) Output of FLAG-NS5A proteins. Coprecipitants by glutathione resin were washed with PBS (−) containing 1.0% Triton X-100, fractionated by SDS–10% PAGE, and subjected to Western blot analysis with anti-FLAG monoclonal antibody. (C) Recovery of GST or GST-NS5B proteins. The nitrocellulose membrane used for Western blot analysis of coprecipitants with anti-FLAG antibody was reprobed with anti-GST antibody. Molecular masses (in kilodaltons) are indicated to the right of each panel.

cm in addition to one cm mutant within region 3 as a control (Fig. 2). All of these cm mutants in FLAG-tagged forms, 10 in region 1, 9 in region 2, and 1 in region 3, were similarly expressed in transiently transfected COS1 cells (Fig. 3). When these 20 mutants were transfected by electroporation into Huh-7-DMB cells, only M1LE/cm 252, 277, 283, 290, 297, and 304 were found to be replication competent, although less so than wild-type M1LE. All other mutants were replication incompetent (Fig. 5). The regions of cm 252, 277, 283, 290, 297, and 304 are predicted to form a helical structure by DNASIS-Mac, version 3.2 (Hitachi Software Engineering Co.). The competence of replication may be due to this original structure (see Discussion). To rule out this possibility, we constructed another cm mutant, M1LE/cm 110. In this mutant, the region from aa 110 to 117 of NS5A is predicted to form a helical structure, were all changed to alanines, and after the transfection into Huh-7-DMB cells, no colonies emerged (data not shown). These results support the notion that the inability of the internal deletion mutants, M1LE/5Adel-1 and M1LE/5Adel-2, to replicate is due not to conformational change induced by the deletions but to the absence of interaction between NS5A and NS5B. It is also unlikely that these results are due to an increased cytotoxicity associated with the mutant NS5As, because we observed no decrease in transfection efficiency or ability to establish colonies by using the plasmid encoding a drug resistance marker along with the wild or the mutant NS5A protein (data not shown).

Improvement in the HCV replicon system. The results clearly showed that two discontinuous regions of NS5A are essential for HCV RNA replication by using the HCV replicon system with Huh-7-DMB cells; however, the number of G418-resistant colonies per microgram of transfected RNA was much smaller than previously reported (24, 29, 35, 37). It remains unclear whether some mutants were replication competent but too inefficient to be detected in the system we applied. Therefore, we tried to improve the assay system in two ways, by the introduction of point mutations to NS5A and by the selection of Huh-7 cells cured of HCV RNA replication by IFN treatment.

We constructed three mutants, M1LE/S225P, M1LE/delS229, and M1LE/S232I, harboring the point mutation S225P (35), a deletion of S229 (delS229) (24), and S232I (4), respectively, all defined as adaptive mutations in other HCV replicon systems (Fig. 2). Next, the 50-1 cells, an HCV subgenome-replicating subclone, were cured of HCV RNA by treatment with IFN for 2 weeks (as described in Materials and Methods), and then the absence of HCV RNA was determined from the results of Northern hybridization, reverse transcription-PCR, and sensitivity to G418 (data not shown). The 50-1 cells cured of HCV RNA by treatment with IFN, designated



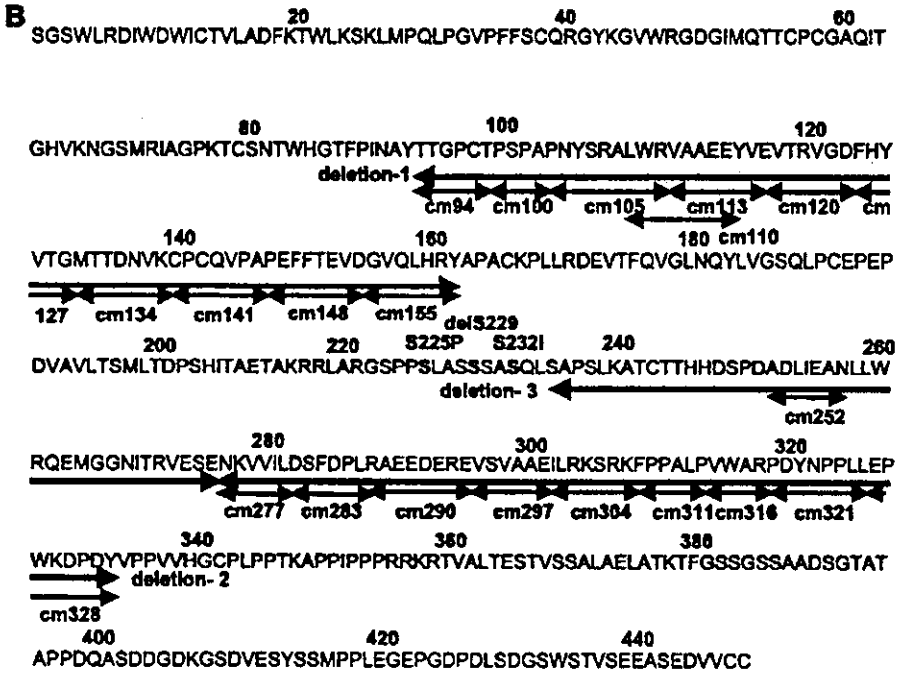


FIG. 2. (A) Schematic presentation of HCV mutant replicons used in this study. Wild-type M1LE contains the HCV M1LE wild-type sequence. M1LE/5Adel-1, M1LE/5Adel-2, and M1LE/5Adel-3 encode internal deletions of NS5A missing aa 94 to 162 (region 1), aa 277 to 334 (region 2), and aa 235 to 276 (region 3), respectively. All amino acids within region 1 were replaced with alanines, and 11 cm mutants were prepared. All amino acids within region 2 were replaced, and 9 cm mutants were prepared. The aa 252 to 258 within region 3 were replaced, and M1LE/cm 252 was prepared. The point mutation S225P, a deletion of S229, and S232I were introduced into wild-type M1LE, and then M1LE/S225P, M1LE/delS229, and M1LE/S232I were prepared. M1LE/5B-VDD encodes NS5B in which the GDD motif was mutated to VDD. (B) Summary of the NS5A mutations. This figure shows the entire amino acid sequence of NS5A of M1LE and the positions of mutations. Numbering starts from the beginning of NS5A. Internal deletions 1, 2, and 3, and point mutations S225P, delS229, and S232I, and cm 252 were introduced as described for panel A. All amino acids within region 1 were replaced with alanines, and then M1LE/cm 94, 100, 105, 110, 113, 120, 127, 134, 141, 148, and 155 were prepared. All amino acids within region 2 were replaced with alanines, and M1LE/cm 277, 283, 290, 297, 304, 311, 316, 321, and 328 were prepared. The positions of the substituted amino acids in each cm mutant are shown in panel B.

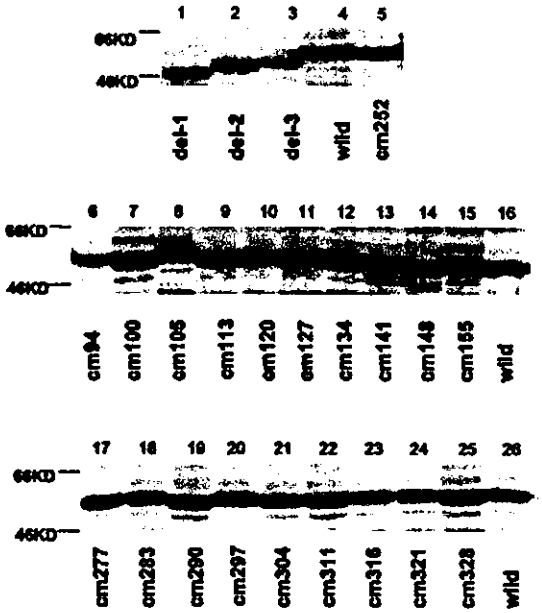


FIG. 3. Efficient translation of mutant FLAG-NS5As. COS1 cells were transiently transfected with mammalian expression vectors expressing FLAG-NS5A proteins that were prepared as described in Materials and Methods. Lanes: 4, 16, and 26, wild type; 1, internal deletion 1; 2, internal deletion 2; 3, internal deletion 3; 5, cm 252; 6, cm 94; 7, cm 100; 8, cm 105; 9, cm 113; 10, cm 120; 11, cm 127; 12, cm 134; 13, cm 141; 14, cm 148; 15, cm 155; 17, cm 277; 18, cm 283; 19, cm 290; 20, cm 297; 21, cm 304; 22, cm 311; 23, cm 315; 24, cm 321; 25, cm 328. Total lysates were fractionated by SDS–10% PAGE and subjected to Western blot analysis with anti-FLAG monoclonal antibody. Molecular masses (in kilodaltons) are indicated to the left of the panels.

Huh-7-KV-C, were evaluated. Wild-type and these mutant HCV replicons were transfected into Huh-7-DMB and Huh-7-KV-C cells. In Huh-7-DMB cells, M1LE/S225P and M1LE/S232I actually increased the efficiency of transduction to some extent, but M1LE/delS229 did not (Fig. 6A). M1LE/S232I was the most effective. In Huh-7-KV-C cells, interestingly, no colonies emerged after selection with G418 when wild-type M1LE was transfected, whereas in the case of M1LE/S225P, delS229, and S232I, 2,500, 3,000, and 25,000 colonies/μg of RNA emerged, respectively (Fig. 6B). These results indicate that two point mutations, S225P and S232I, can be categorized as the adaptive mutations in the isolate M1LE and that the cells cured of HCV RNA by treatment with IFN, Huh-7-KV-C, show higher permissiveness for M1LE/S225P, delS229, and S232I than Huh-7-DMB cells. In this way, we established highly improved replicon systems.

Delineation of important sequences of NS5A for HCV RNA replication. To examine the effect of internal deletions and alanine substitutions on HCV RNA replication with this improved replicon system, double mutants with S232I plus internal deletion mutations or alanine-substituted cm's in M1LE were constructed and transfected into Huh-7-DMB and Huh-7-KV-C cells by electroporation. After G418 selection, in Huh-7-DMB cells, some 400 to 1,000 colonies/μg of RNA emerged with the double mutants of M1LE/S232I plus cm 252, 277, 283, 297, and 304, but only about 100 colonies/μg of RNA emerged with the double mutant M1LE/S232I plus cm 290. No colonies emerged with the double mutant M1LE/S232I plus other cm's, del-1, del-2, and del-3 (Fig. 7; data not shown for Huh-7-KV-C cells). The double mutant M1LE/S232I plus cm 110 was also replication incompetent in Huh-7-DMB cells. To further examine the replication competence of these mutants, double mutants of M1LE/S225P plus an internal deletion mutation or cm were constructed and then transfected into Huh-7-DMB cells. The results were almost the same as those with the double mutants with S232I, although the efficiencies of these mutants were around one-half of those with S232I (Fig. 8). The similar results in the double mutants with S225P were obtained with Huh-7 KV-C cells (data not shown). The replication-defective property of the mutants with deletions of regions 1 and 2, the cm mutants within region 1, and the cm mutants in the C-terminal part of region 2 was also observed with both Huh-7-DMB and Huh-7-KV-C cells, indicating that the replication incompetence of these mutants is not due to the low efficiency of the original assay system. The cm mutants at the N-terminal part of region 2, cm 277, 283, 290, 297, and 304, were replication competent in the absence of the adaptive mutation in Huh-7-DMB cells and also in the presence of the adaptive mutation in Huh-7-DMB and Huh-7-KV-C cells to some extent. Taken together, these results suggest that the interaction with NS5B through regions 1 and 2, probably through its C-terminal part, is also essential for HCV RNA replication. We examined the interaction between cm mutants of FLAG-NS5A and GST-NS5B, but the difference among wild-type and cm mutants was weak in the pull-down assay. Differential binding would be possible if the two partner proteins were lower in concentration or together with other NS proteins, as those occur in vivo in HCV-RNA-replicating cells.

Interestingly, two quantitative differences were observed with M1LE/5Adel-3, cm 252, and 290 with and without the adaptive mutations. M1LE/5Adel-3 was weakly replication competent in Huh-7-DMB cells but incompetent in Huh-7-DMB and Huh-7-KV-C cells when the adaptive mutations were introduced. In contrast, M1LE/cm 252 was weakly replication competent in Huh-7-DMB cells but as high as that of the other replication-competent cm mutants in the presence of the adaptive mutations in Huh-7-DMB and Huh-7-KV-C cells (data not shown for Huh-7-KV-C cells; see Discussion).

DISCUSSION

HCV NS5A is a viral regulatory protein that modulates viral RNA replication and host processes by interacting directly and indirectly with a variety of host regulatory factors (10, 19, 42, 56, 58, 67). The important role of NS5A in HCV RNA replication has been clearly demonstrated by high or clustered incidence of adaptive mutations in NS5A detected in HCV RNA replicon systems, although the molecular mechanism involved remains unknown (4, 24, 35, 37, 40). Shirota et al. previously reported direct interaction between NS5A and NS5B through two binding regions of NS5A expressed in mammalian cells and in vitro with a purified recombinant and that NS5A could modulate the activity of NS5B RdRp in vitro through this direct interaction (54). Here we demonstrated the critical role of regions essential for the NS5A-NS5B interaction in HCV RNA replication with an HCV subgenomic replicon by introducing several internal deletion mutations into

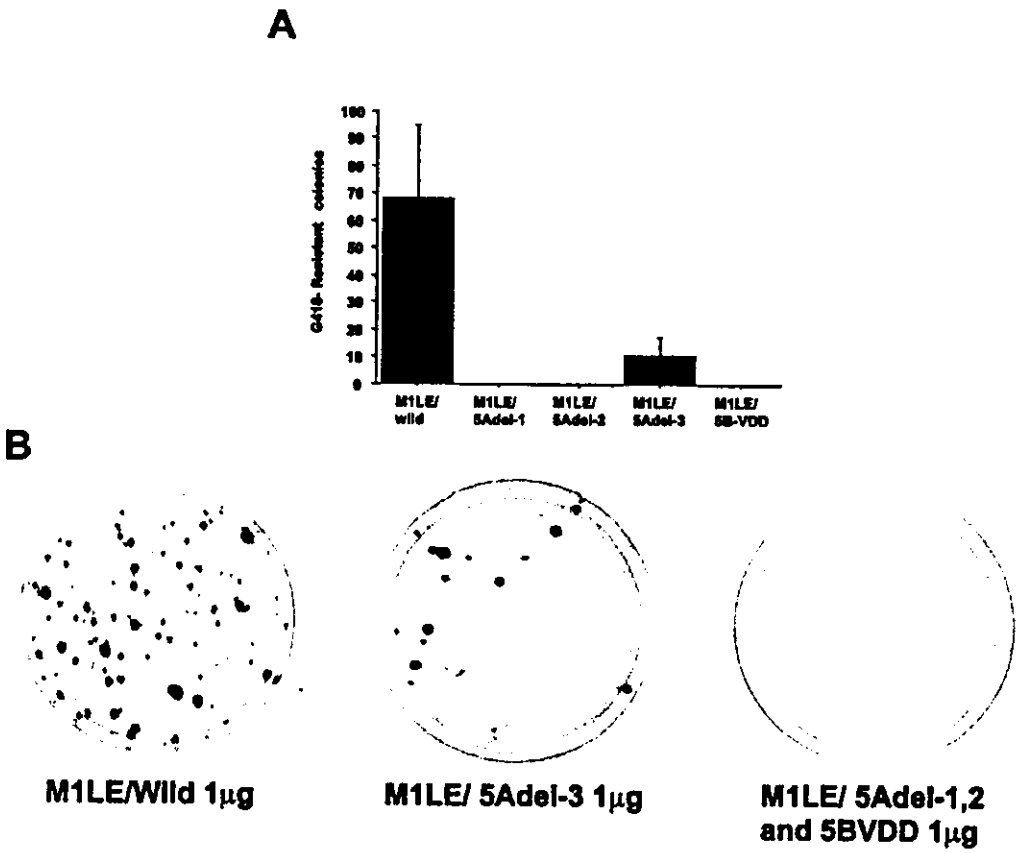


FIG. 4. Effect of internal deletion mutation on HCV RNA replication. Huh-7-DMB cells were transfected with 1 μg of in vitro-transcribed wild-type MILE, 5Adel-1, 5Adel-2, 5Adel-3, and 5B-VDD RNA by electroporation, and G418-resistant cells were selected with a G418 concentration of 1 mg/ml. G418-resistant cell colonies were stained 4 weeks after transfection. (A) This figure shows the mean number of G418-resistant cell colonies isolated per 10-cm-diameter cell culture dish per 1 μg of RNA. Error bars indicate the standard deviations of the results from at least three independent experiments. (B) G418-resistant colonies were visualized by staining cells as described in Materials and Methods.

NS5A. Our results provide clear evidence that NS5A is indispensable for HCV RNA replication probably through its interaction with NS5B, since the mutants missing the NS5B-binding regions, regions 1 and 2, could not produce G418-resistant colonies, but the mutants missing region 3, which is not essential for this interaction, could. The critical role of NS5A in HCV RNA replication in this report is consistent with the previous one that an amino-terminal amphipathic α-helix of NS5A is essential for HCV RNA replication in the replicon system, localizing NS5A to a membrane (13). The use of cm mutants strongly suggests that all of region 1 and the C-terminal part of region 2 are critical for HCV RNA replication. However, it is difficult to exclude the possibility that the substitution of eight amino acids in a row may induce structural change. Then the critical regions defined by cm mutants may simply reflect the structural integrity necessary for the function, although a structural evaluation is difficult at present, since no crystal model of NS5A is available. Further mutational analysis is necessary to test these possibilities.

HCV RNA replication would take place in a distinctly altered membrane structure of the endoplasmic reticulum, a membranous web (12), as recently reported by Gosert et al. (20). All NS proteins might be recruited to the membrane structure via their own membrane association domains or by the help of NS4A in the case of NS3 (8, 13, 20, 28, 30, 51, 52, 63). Recently, Dimitrova et al. (11) reported that all six NS proteins interact with each other through their multiple interacting surfaces. NS5B is HCV RdRp and has been reported to interact with NS proteins and some host proteins. Such interaction(s) may modulate the activity of NS5B RdRp in various ways. The critical role of the homomeric interaction of NS5B in RdRp activity was demonstrated by us and another group (48, 60). Piccininni et al. (47) reported that NS5B interacts with NS3 and NS4B as positive and negative regulators in the replication complex. Previously, it was reported that the direct binding of NS5A and NS5B in the isolate JK-1 weakly stimulated the activity of NS5B RdRp in vitro at first (at a molar ratio to NS5B of less than 0.1) and then inhibited the activity in a dose-dependent manner (54). In the present study, we showed that the two regions of NS5A are important for binding NS5B and are essential for HCV RNA replication in the isolate M1LE by HCV replicon assays. The weak stimulation by NS5A of RdRp activity through the binding of NS5B observed in vitro may reflect the essential role of NS5A in HCV RNA replication, or the interaction between NS5A and NS5B is important for the dynamic assembly of NS proteins in the HCV

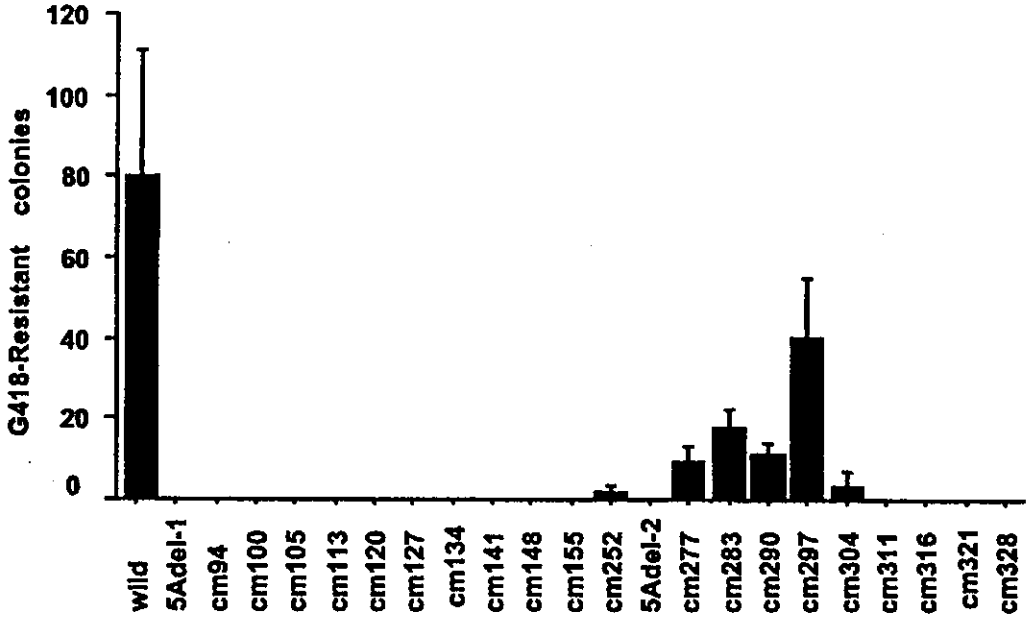


FIG. 5. Effect of clustered alanine-substitution mutations on HCV RNA replication. Huh-7-DMB cells were transfected with 1 μg of in vitro-transcribed wild-type M1LE, 5Adel-1, 5Adel-2, and cm 94, 100, 105, 113, 120, 127, 134, 141, 148, 155, 252, 277, 283, 290, 297, 304, 311, 316, 321, and 328 RNA, and G418-resistant cells were selected with a G418 concentration of 0.5 mg/ml. G418-resistant cell colonies were stained 4 weeks after transfection. This figure shows the mean number of G418-resistant cell colonies isolated per 15-cm-diameter cell culture dish per 1 μg of RNA. Error bars indicate the standard deviations of the results from at least three independent experiments.

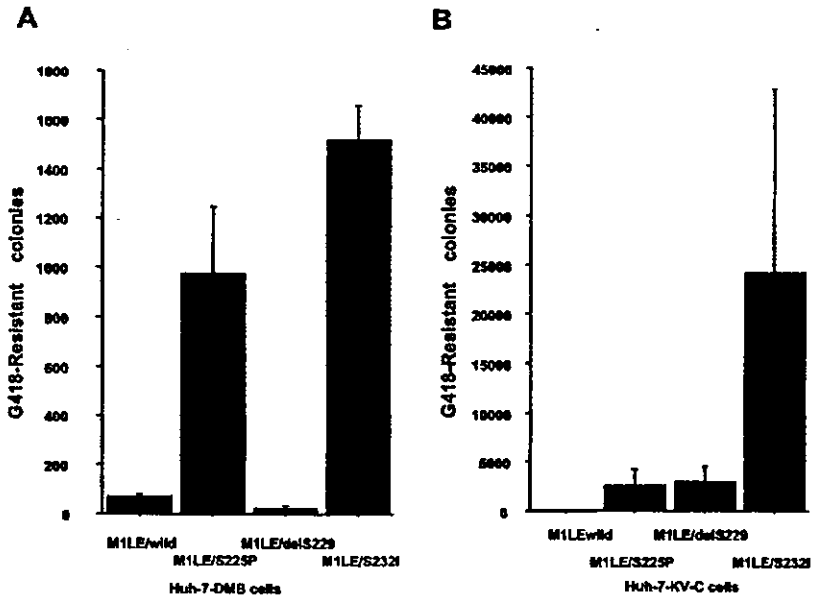


FIG. 6. Effects of three kinds of point mutation, S225P, deletion S229, and S232I, on HCV RNA replication in the Huh-7-DMB and KV-C sublines. 50-1 cells were cured of self-replicating subgenomic RNAs by IFN-α treatment, and then cured 50-1 cells, Huh-7-KV-C, were prepared. Huh-7-DMB and KV-C cells were transfected with 10 ng to 1 μg of in vitro-transcribed wild-type M1LE, M1LE/S225P, M1LE/delS229, and M1LE/S232I RNA. G418-resistant cells were selected with a G418 concentration of 1 mg/ml. G418-resistant cell colonies were stained 4 weeks after transfection. (A) This figure shows the mean number of G418-resistant cell colonies isolated per 10-cm-diameter cell culture dish per 1 μg of RNA when each in vitro-transcribed mutant RNA was transfected into Huh-7-DMB cells. Error bars indicate the standard deviations of the results from at least three independent experiments. (B) This figure shows the mean number of G418-resistant cell colonies isolated per 10-cm-diameter cell culture dish per 1 μg of RNA when each in vitro-transcribed mutant RNA was transfected into Huh-7-KV-C cells. Error bars indicate the standard deviations of the results from at least three independent experiments.

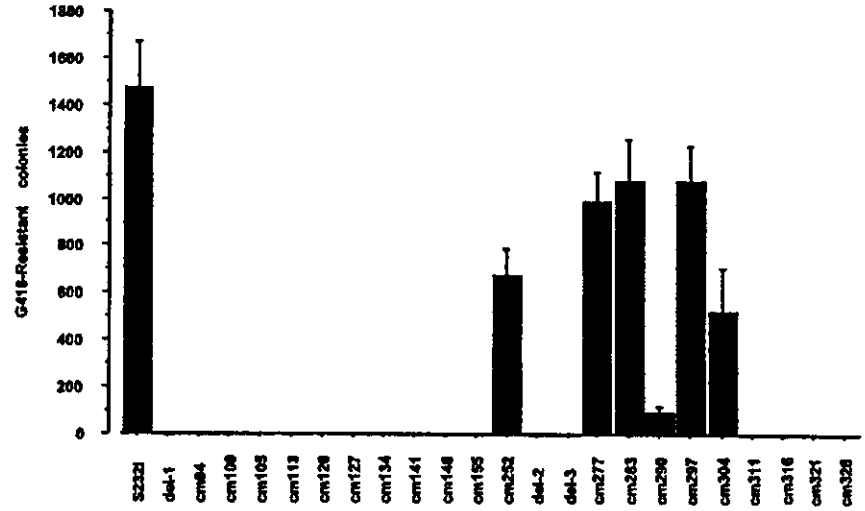


FIG. 7. Effect of M1LE/S232I plus internal deletion mutations or alanine-substitution mutations. Huh-7-DMB cells were transfected with 1 μg of in vitro-transcribed M1LE/S232I plus 5Adel-1, 5Adel-2, 5Adel-3, and cm 94, 100, 105, 113, 120, 127, 134, 141, 148, 155, 252, 277, 283, 290, 297, 304, 311, 316, 321, and 328 RNA. G418-resistant cells were selected with a G418 concentration of 1 mg/ml and stained 4 weeks after transfection. This figure shows the mean number of G418-resistant cell colonies isolated per 10-cm-diameter cell culture dish per 1 μg of RNA. Error bars indicate the standard deviations of the results from at least three independent experiments. The number of G418-resistant cell colonies in M1LE/S232I is derived from the results shown in Fig. 6A.

replication complex. Alternatively, it cannot be excluded that the internal deletion and cm mutants may be defective in other unknown function(s) essential for HCV RNA replication.

Adaptive mutations that increase the efficiency of HCV RNA replication have been accumulated in different HCV replicon systems (4, 24, 35, 37, 40). We introduced several point mutations into NS5A of M1LE and found that two, S232I and S225P, positively affected colony formation as adaptive mutations in two different Huh-7 sublines. Interestingly, a deletion of S229 had an effect distinct from those of the other two mutations, since it was only effective in the cured cells, the KV-C subline. Also, it is noteworthy that wild-type M1LE could not replicate in the Huh-7-KV-C subline. The effect of the deletion of S229 and the replication incompetence of wild-type M1LE may be of interest for the elucidation of the phenotypic or genetic change(s) in the cured cells. In combination with the adaptive mutation, the deletion and cm mutants exhibited distinct phenotypes in HCV RNA replication.

First, the mutant missing region 3 was replication incompetent in the presence of S232I or S225P, which was in contrast

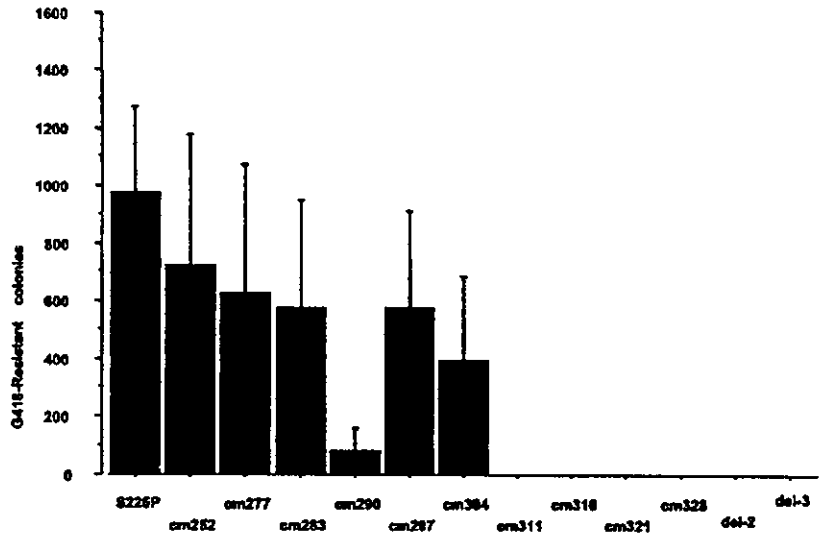


FIG. 8. Effect of M1LE/S225P plus internal deletion mutations or alanine-substitution mutations. Huh-7-DMB cells were transfected with 1 μg of in vitro-transcribed M1LE/S225P plus 5Adel-2, 5Adel-3, and cm 252, 277, 283, 290, 297, 304, 311, 316, 321, and 328 RNA. G418-resistant cells were selected with a G418 concentration of 1 mg/ml and stained 4 weeks after transfection. This figure shows the mean number of G418-resistant cell colonies isolated per 10-cm-diameter cell culture dish per 1 μg of RNA. Error bars indicate the standard deviations of the results from at least three independent experiments. The number of G418-resistant cell colonies in M1LE/S225P is derived from the results shown in Fig. 6A.

to the dramatic increase in HCV RNA replication efficiency obtained by introducing the adaptive mutation alone. A similar but more moderate phenotype was observed with cm 252 when the adaptive mutation was present. Our result is different from the reported enhancement of G418-resistant colony formation caused by a large 47-aa deletion in HCV-Con1 (4) and by a natural 4-aa insertion in HCV-N (29). Region 3 contains the IFN sensitivity-determining region, within which mutations have been reported to be associated with sensitivity to IFN therapy in patients with chronic HCV infection in Japan (14, 15). Region 3 is almost the same as a protein kinase R (PKR)-binding domain (18). It has been reported that NS5A can disrupt the dimerization of PKR through binding, resulting in the repression of PKR function, and efficient HCV RNA replication may involve a block in PKR-dependent signaling (18, 46). In this context, the adaptive mutations can greatly augment HCV RNA replication and thus may induce PKR, which could be inactivated via interaction with the PKR-binding region of NS5A, region 3, but not with those mutants defective in PKR-binding, such as del-3 and cm 252.

Second, cm 290 was less replication competent than the other cm mutants in region 2 when the adaptive mutation, S232I or S225P, was present. The highly charged sequence mutated in cm 290 may be critical for HCV RNA replication in the presence of the adaptive mutation. To address this point, four different combinations of three to four alanine substitutions in 7 aa residues were introduced into the M1LE/S232I construct. All of these mutants were more efficient in HCV RNA replication than M1LE/S232I plus cm 290 but still less so than the other replication-competent mutants in region 2 (data not shown), suggesting that all or most amino acids in the sequence contribute to its critical role in HCV RNA replication in the presence of the adaptive mutations. This result may suggest a functional linkage of the sequence mutated in cm 290 to the adaptive mutations.

In HCV subgenomic replicons, some groups have found that cured cell clones showed a high permissiveness for HCV RNA replication (5, 45) while another has not (40). Between the recipient sublines we used, the cured cells (Huh-7-KV-C) shared the nonpermissive property for wild-type M1LE; however, the DMB subline was permissive for wild-type M1LE and/or adaptive mutations with lower efficiency than the KV-C subline. These results suggest that several different genetic or phenotypic alterations in recipient cells emerge under IFN treatment or multiple ways for cells to be permissive to HCV RNA replication.

In summary, we established a highly efficient HCV replicon system derived from the isolate M1LE and demonstrated that the two regions critical for the interaction between NS5A and NS5B are also indispensable for HCV RNA replication in an HCV replicon system. Our results strongly suggest that NS5A is involved in the HCV replication complex and acts as a positive modulator of HCV RNA replication through its interaction with NS5B. The molecular mechanism of this positive effect by NS5A remains to be elucidated and may lead to the design of new drugs that inhibit HCV RNA replication.

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Down-regulation of viral replication by adenoviral-mediated expression of siRNA against cellular cofactors for hepatitis C virus

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Abstract

Small interfering RNA (siRNA) is currently being evaluated not only as a powerful tool for functional genomics, but also as a potentially promising therapeutic agent for cancer and infectious diseases. Inhibitory effect of siRNA on viral replication has been demonstrated in multiple pathogenic viruses. However, because of the high sequence specificity of siRNA-mediated RNA degradation, antiviral efficacy of siRNA directed to viral genome will be largely limited by emergence of escape variants resistant to siRNA due to high mutation rates of virus, especially RNA viruses such as poliovirus and hepatitis C virus (HCV). To investigate the therapeutic feasibility of siRNAs specific for the putative cellular cofactors for HCV, we constructed adenovirus vectors expressing siRNAs against La, polypyrimidine tract-binding protein (PTB), subunit gamma of human eukaryotic initiation factors 2B (eIF2Bγ), and human VAMP-associated protein of 33 kDa (hVAP-33). Adenoviral-mediated expression of siRNAs markedly diminished expression of the endogenous genes, and silencing of La, PTB, and hVAP-33 by siRNAs substantially blocked HCV replication in Huh-7 cells. Thus, our studies demonstrate the feasibility and potential of adenoviral-delivered siRNAs specific for cellular cofactors in combating HCV infection, which can be used either alone or in combination with siRNA against viral genome to prevent the escape of mutant variants and provide additive or synergistic anti-HCV effects.

Keywords: Small interfering RNA; Hepatitis C virus; Cellular factor; Antiviral agents

Introduction

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B viral hepatitis (Choo et al., 1989), which frequently leads to liver cirrhosis and hepatocellular carcinoma (Saito et al., 1990). It has been estimated that more than 3% of the world population is infected with HCV. The only current medical therapy for HCV infection is IFN either alone or in combination with ribavirin. Unfortunately, only a small fraction of treated patients develop a sustained remission. Thus, development of new therapies for HCV infection is of great clinical and economic significance.

RNA interference (RNAi) is an evolutionarily conserved phenomenon of posttranscriptional gene silencing (PTGS) that has been described in plants, invertebrates, and vertebrates. In this process, double-stranded RNA (dsRNA) is cleaved into small interfering RNAs (siRNAs) of 21–23 nt by an RNaseIII-like enzyme known as Dicer, followed by incorporation of siRNA into a RNA-induced silencing complex (RISC) that recognizes and cleaves the target. In mammals, however, it has been reported that long dsRNAs (larger than 30 nt in length) induce an antiviral interferon response and, in turn, lead to the nonspecific translational shutdown. Thus, its application to mammalian cells is largely limited. A crucial insight came from Elbashir et al. (2001), who found that specific gene silencing in mammalian cells can be mediated by siRNAs of 21 nt, which can bypass dsRNA-induced nonspecific interferon response. This significantly facilitated the use of siRNA technology in mammalian cells. As a powerful reverse genetic approach, siRNA contributes greatly to linking of gene sequence with biological function. Additionally, the potential of using siRNA for prevention and treatment of viral infection has also proved to be promising. A couple of

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laboratories have demonstrated inhibitory effect of siRNAs on respiratory syncytial virus (Bitko and Barik, 2001), HIV (Jacque et al., 2002), poliovirus (Gitlin et al., 2002), and more recently on HCV (Randall et al., 2003). Although these studies have taken us to a fascinating new field in antiviral therapy, one outstanding drawback of the approach using siRNA direct against viral genome is that genetic heterogeneity due to high mutation rates of viruses may lead to emergence of escape variants resistant to siRNA. Indeed, it was shown that a single point mutation in the siRNA target region conferred escape in poliovirus (Gitlin et al., 2002). Alternatively, cellular factors involved in the viral life cycle provide more attractive candidates for siRNA targeting to ensure a sustained antiviral effect. For example, it has been reported that siRNAs targeted to the HIV-1 main receptor, CD4 (Novina et al., 2002), or coreceptor, CCR5 (Qin et al., 2003), can suppress the entry and replication of HIV-1.

In this study, we investigated the therapeutic feasibility of siRNAs specific for the putative cellular cofactors for HCV, including La, polypyrimidine tract-binding protein (PTB), subunit gamma of human eukaryotic initiation factors 2B (eIF2Bγ), and human VAMP-associated protein of 33 kDa (hVAP-33). Our results demonstrate that knockdown of the endogenous La, PTB, or hVAP-33 with adenoviral-delivered siRNA efficiently inhibits HCV RNA replication in cultured cells.

Results

Silencing of the endogenous genes in Huh-7 cells

Although the exact significance is not fully understood, there is increasing experimental evidence that showed specific interaction between various cellular factors and HCV RNA or viral protein, implicating potential roles of these proteins in HCV RNA replication and/or translation. Among these cellular factors, La antigen was shown to bind to the 5′-untranslated region (UTR) of HCV (Ali and Siddiqui, 1997), PTB was shown to interact with both 5′- and 3′-UTR (Ali and Siddiqui, 1995; Tsuchihara et al., 1997), and hVAP-33, a SNARE-like protein, was found to specifically bind to NS5A and NS5B proteins, suggesting that it may be responsible for the association of the HCV replication complexes with the membrane (Tu et al., 1999). Additionally, eIF2Bγ was identified as a cofactor of HCV cap-independent translation (Kruger et al., 2000). To explore whether these presumed HCV cofactors can be used as functional targets for therapeutic siRNA, we first screened the RNAi activity of several synthetic siRNA molecules for each target gene (data not shown) and the most potent one was further used to construct siRNA expression plasmids (Fig. 1A). The small-hairpin RNAs are transcribed from each of these plasmids under the control of human U6 promoter. Huh-7 cells were transfected with each siRNA-expressing plasmid, and the expression level of each endogenous gene in the transfected cells was assessed by Northern blot analysis. When compared with the empty vector pShuttle, siRNA-expressing plasmid reduced the mRNA level of their respective target genes, albeit with different degree (Fig. 1B). The mRNA level of GAPDH, which served as an internal control for RNA loading, remained constant in each lane. Expectedly, each siRNA had no effect on the expression level of the irrelevant genes, confirming a specific RNAi effect induced by expressed siRNA.

Effects of the endogenous gene silencing on HCV internal ribosome entry site-dependent translation

Both La and PTB were shown to bind to HCV RNA in 5′-UTR, which constitutes internal ribosome entry site (IRES), indicating their potential roles in regulating HCV IRES-mediated translation. However, while Siddiqui and co-workers reported that La antigen (Ali et al., 2000) and PTB (Anwar et al., 2000) greatly enhance HCV IRES-mediated translation, Kaminski et al. (1995) contradicted the role of PTB in translation of HCV RNA, and Isoyama et al. (1999) found low requirement of La protein for HCV IRES activity. Translation is one of the processes in viral replication, and authentic cofactors for HCV IRES function are promising targets for therapeutic siRNA. To further clarify the conflicting issue on the functional role of these proteins in IRES-mediated translation, HepT cells stably expressing T7 RNA polymerase (Zhang et al., 1999) were transfected with each pShuttlesiRNA and the reporter vector pNC371RL (Zhang et al., 2002), which contains a T7 promoter, nt 1–371 of HCV sequence fused in frame with the Renilla luciferase (Rluc) gene. The cell lysates were assayed for the HCV IRES-dependent Rluc expression 48 h following transfection. To analyze the influence of the siRNA-mediated gene silencing on cap-dependent translation, pGL3-Control vector was cotransfected and the firefly luciferase (Fluc) activity was measured simultaneously. Compared with the Rluc activity in cells transfected with the empty vector pShuttle (100%), the Rluc activities in cells transfected with pShuttlesiLa, pShuttlesiPTB, pShuttlesieIF2B, and pShuttlesihVAP were 57.8%, 62.6%, 98.2%, and 66.0%, respectively (Fig. 2A). While the cap-dependent Fluc expression was comparable in these transfectants, except a marginal reduction in cells transfected with pShuttlesieIF2B. To test the specificity of inhibition by siRNAs, reporter assay with pEMCVRL, in which the translation of Rluc gene is directed by encephalomyocarditis virus (EMCV) IRES identical to that inserted in the HCV replicon, was also conducted as described above. As shown in Fig. 2B, the EMCV IRES-directed Rluc expression was not significantly affected by expression of siLa, siPTB, and sieIF2B, whereas a moderate inhibition of EMCV IRES-dependent translation was observed in cells expressing sihVAP. These results provide further evidence of La and