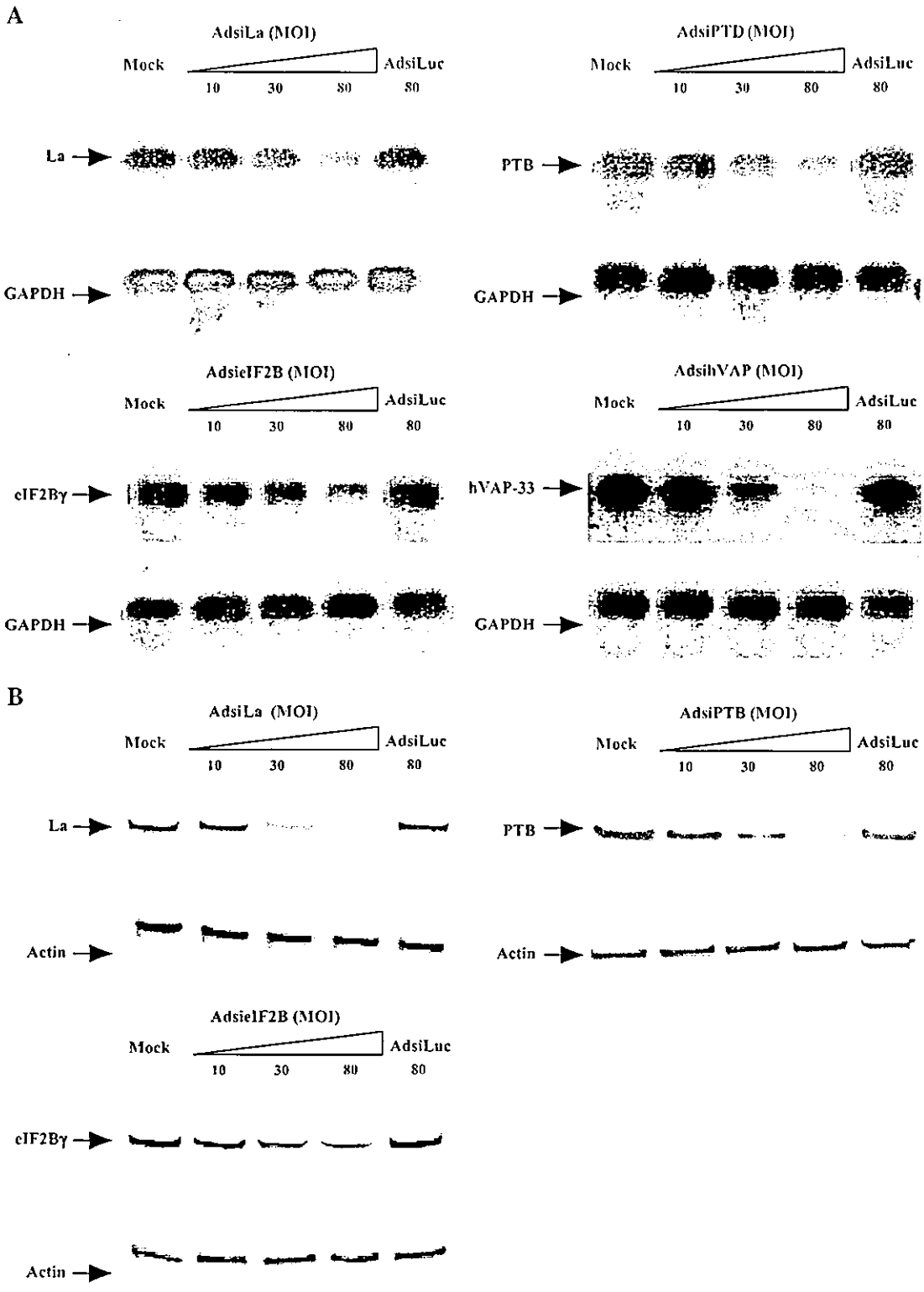

Fig. 3. Dose-dependent silencing of endogenous genes in Huh-NNRZ cells transduced with siRNA expression adenoviral vectors. Huh-NNRZ cells harboring HCV replicon were infected with each AdsiRNA at the MOI of 10, 30, and 80. The transduced cells were harvested at day 3 postinfection. The mRNA (A) and protein level (B) of the cellular factors were analyzed by Northern and Western blot. GAPDH and actin were served as controls for RNA and protein loading, respectively.

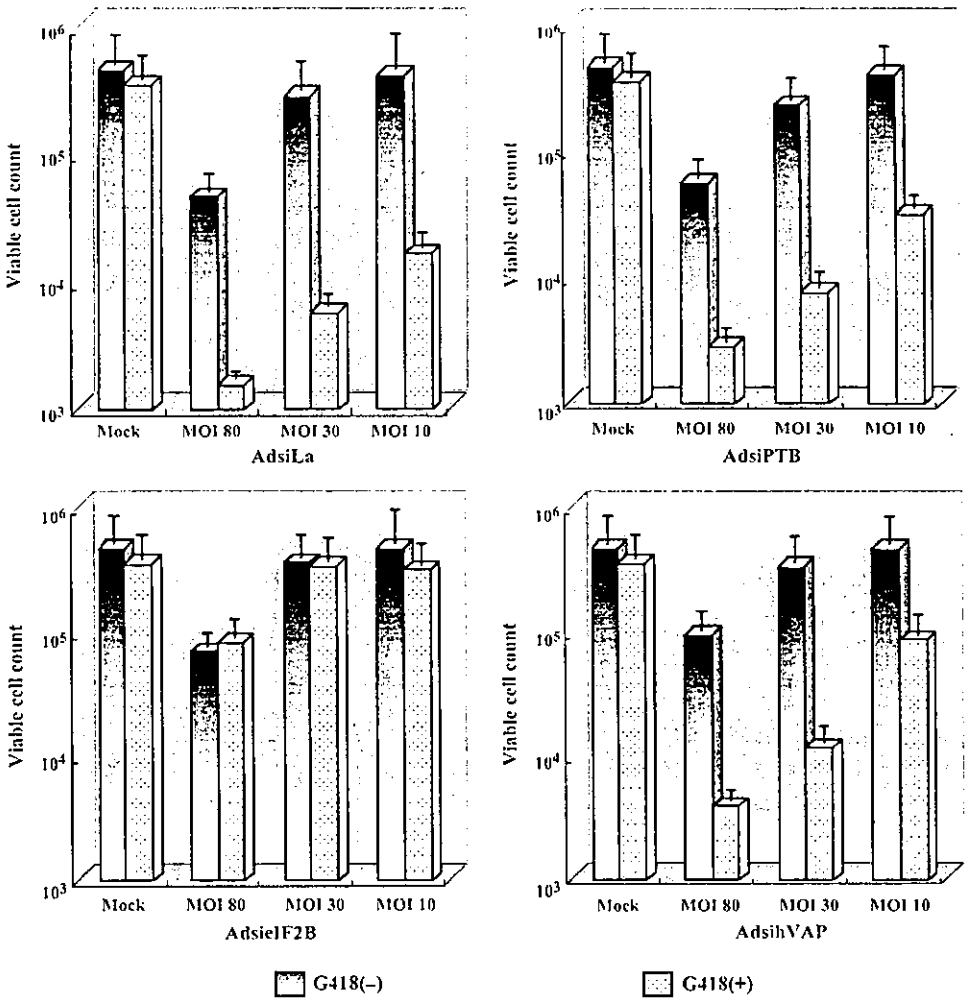

Fig. 4. Influence of the endogenous genes silencing on HCV replication. Huh-NNRZ cells coated on 12-well plate were infected with each AdsiRNA at the indicated MOI, and maintained either in the absence (shaded columns) or presence (dotted columns) of G418 selection. The viable cells were quantified after 10 days. Representative data are from three independent experiments.

translation, RNA replication may also be the target event for siRNA action, especially in the case of siPTB, because a specific interaction with 3′-UTR and a potential role in HCV minus-strand synthesis were reported for PTB. On the other hand, because both HCV and EMCV IRES-directed translation were inhibited by siRNA-mediated depletion of hVAP-33 (Fig. 2), the degree of antiviral effect of sihVAP may be overestimated here. Nonetheless, the results presented here demonstrate that siRNA-mediated silencing of the endogenous genes such as La, PTB, and hVAP-33 can efficiently inhibit HCV replication in cultured cells.

Discussion

Recently, a couple of laboratories have demonstrated RNAi-based inhibition of HIV, poliovirus, and HCV, suggesting a potentially promising application of siRNA in antiviral therapy. However, it was shown that absolute conservation of the base-pairing in the siRNA target region is required for efficient cleavage, and if even a single nucleotide differs between an siRNA and its target, the effect is greatly diminished, or even eliminated completely. Therefore, in attacking HCV RNA directly, it is necessary to target viral RNA sequences that are conserved and normally invariant among different strains. Although the 5′-UTR and the 5′-end of core region are relatively conserved, considerable genetic diversity exists among various HCV isolates. The 5′-UTR region constitutes the IRES capable of initiating cap-independent translation of HCV. Highly ordered RNA structures and multiple sites participating in RNA–protein interaction within 5′-UTR have been documented to be critical for promoting internal initiation and/or its control, both of which might prevent recognition by siRNA. Thus, it may

be relatively difficult to choose optimal targets in HCV genome for therapeutic siRNA. Furthermore, because of the inaccuracy of RNA-dependent RNA polymerase, mutated progeny virus may rapidly emerge and escape from recognition by siRNA.

Here, we investigated whether siRNAs directed to cellular genes presumably essential for viral infection can inhibit HCV replication in cultured cells. Our results presented here showed that adenoviral-delivered siRNAs elicited potent RNAi effect on endogenous genes, and silencing of La, PTB, and hVAP-33 by AdsiRNA consequently blocked HCV replication in Huh-7 cells. Significantly, even a modest reduction of La and PTB expression by infection of AdsiRNA at the MOI of 10 also resulted in substantial inhibition of HCV replication, decreasing viable (infected) cells by 20- and 10-fold, respectively (Fig. 4). By demonstrating that the replication of the preexisting HCV replicon can be efficiently suppressed by cofactor-targeting siRNAs, it is conceivable that pretreatment of naive liver cells with these siRNAs could protect them from HCV infection. This would be particularly useful in preventing newly transplanted livers from reinfection with HCV.

Besides the attenuation of HCV IRES activity by siLa and siPTB, the effective anti-HCV effect may partially be attributed to the interference with HCV RNA replication. This is especially true in the case of siPTB, because PTB has been shown to bind to the 3′-end of the HCV RNA at two conserved stem–loop structures, which is critical for minus-strand synthesis (Tsuchihara et al., 1997; Ito and Lai, 1997). The data presented here showed a function relevance of hVAP-33 in HCV IRES-dependent translation, together with the previous study suggesting that hVAP-33 may participate in HCV replication by serving as a membrane receptor for replication complex (Tu et al., 1999), implicating that hVAP-33 may be a cellular factor playing a role in regulating the switch between translation and replication of HCV RNA. If this is the case, depletion of hVAP-33 by sihVAP may inhibit both translation and replication of HCV RNA, the processes which may be coupled, as that reported in poliovirus (Novak and Kirkegaard, 1994).

Different from the genomic structure of native HCV in which the viral polyprotein is translated under the control of its IRES, synthesis of the nonstructural protein in the replicon is initiated by EMCV IRES, whereas translation of the neomycin phosphotransferase is directed by HCV IRES. Theoretically, silencing of cellular factors, which are specific for HCV IRES, does not affect the expression level of nonstructural proteins, and consequently, replication of HCV replicon may not be altered. Accordingly, quantification of HCV RNA transcripts or protein expression would underestimate the authentic anti-HCV effect of the siRNAs against cofactors for HCV IRES. In view of this respect, we assessed anti-HCV efficacy of the siRNAs by measuring the loss of G418 resistance, which can be taken as a comprehensive measure of HCV infection including both translation and RNA replication. Further experiments with other HCV-permissive cell culture or small animal models of chronic hepatitis C are required to validate the anti-HCV function of these siRNAs. Additionally, it is important to confirm that silencing of these endogenous genes by siRNA has no serious deleterious effects. La protein is an autoimmune antigen that is transiently associated with the 3′ oligo (U) terminus of the RNA polymerase III transcripts and facilitates transcription termination and recycling of transcription complexes for reinitiation process (Maraia, 1996). One concern is whether La silencing may affect the transcription of tRNAs, 5SrRNA, and other transcripts synthesized by pol III. PTB is an RNA-binding protein recognizing pyrimidine-rich sequence, playing a role in regulating alternative splicing by selectively repressing 3′ splice sites (Singh et al., 1995). Also unknown is whether knockdown of PTB may lead to perturbation of cellular RNA processing. hVAP-33 has a typical SNARE structure, potentially involved in diverse vesicle trafficking between membrane compartments, although its precise functions are unknown currently. One possibility is that partial depletion of hVAP-33 may affect the vesicle transport functions of cells. Further studies are required to determine whether this endogenous gene silencing is truly limited by its roles in cells. Nonetheless, the results presented here provide proof of principle for the idea that siRNA directed to cellular cofactor can serve as a potential therapeutic agent for HCV infection, which can be used either alone or in combination with siRNA directed to HCV RNA genome. It is theoretically possible that siRNA simultaneously targeting cellular and viral gene could help to prevent the escape of mutant variants and provide additive or synergistic effects.

In addition to the therapeutic significance, the siRNA-based approach allowed us to provide further evidence for the functional requirement of La and PTB in the HCV IRES-mediated translation, not only in the reporter system, but also in the context of HCV replicon. In contradiction to those reported by Kruger et al. (2000), our data do not support a role of eIF2Bγ in regulating HCV IRES function. As shown in Fig. 4, the G418-resistant phenotype of replicon cells, which is conferred by HCV IRES-directed translation of neomycin phosphotransferase, was not affected by transduction with AdsieIF2B, although a significant silencing of endogenous eIF2Bγ was observed. These results substantially lessen, if could not rule out completely, the possibility that eIF2Bγ is a cofactor for HCV IRES. The basis for the discordance in the data presented by Kruger et al. and those presented here is currently unknown. Unexpectedly, our data showed a biological relevance of hVAP-33 in both HCV and EMCV IRES-mediated translation. To our knowledge, involvement of hVAP-33 in IRES-dependent translation has not been reported previ-

ously. Whether hVAP-33 is a universal internal initiation factor that is indispensable in every case of internal initiation is to be further investigated.

In conclusion, the results presented here demonstrate that adenoviral-delivered siRNA specific to cellular genes such as La, PTB, and hVAP-33 significantly inhibit viral replication in cultured Huh-7 cells. These data also suggest that siRNA-based approach is a powerful tool for identification of novel HCV cellular cofactors yet to be described, thereby facilitating the development of new antiviral therapy for HCV infection.

Materials and methods

Plasmids

The U6 pol III promoter was amplified by PCR from the genomic DNA of the HeLa cells with a sense primer containing a *Mfe*I site 5′-atgcaattgAAGGTCGGGCAG-GAAGA-3′ and an antisense primer containing an *Eco*RI and a *Bam*HI site 5′-agaattcggatccCGCGTCCTTTCCA-CAAGATA-3′. The PCR product was digested with *Mfe*I and *Eco*RI, and cloned into the *Mfe*I and *Eco*RI sites of pShuttle (Clontech) to create pShuttleU6. Target sequences for siRNAs were selected according to the Ambion web-based criteria and further analyzed by BLAST research to avoid a significant homology with other genes. For each endogenous gene (La, PTB, eIF2Bγ, or hVAP-33), sense and antisense oligonucleotides of self-complementary hairpin sequences (including the termination signal of five 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. The resultant vectors are termed pShuttlesiLa, pShuttlesiPTB, pShuttlesieIF2B, and pShuttlesihVAP, respectively. The sequences of these constructs were confirmed by nucleotide sequencing.

Adenovirus

Each siRNA-expressing cassette in pShuttlesiRNA, which is flanked by I-*Ceu*I and PI-*Sce*I sites, was digested with these two 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 all 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 units/ml penicillin and streptomycin in a 5% CO_2 humidified atmosphere. The cell line HepT, which stably expresses T7 RNA polymerase (Zhang et al., 1999), was grown in DMEM containing 5 μg/ml puromycin (Sigma). A Huh-7-derived cell line (Huh-NNRZ) stably replicating HCV subgenomic replicon (referred to as #50-1 cell clone in Kishine et al., 2002) was grown in DMEM containing 300 μg/ml G418 (Geneticin, Invitrogen).

Northern and Western blot analysis

Total RNAs were isolated from culture cells with Trizol reagent (Invitrogen), separated by denaturing agarose gel electrophoresis, and analyzed by Northern blot using Dig-labeled probes. The probes correspond to nucleotides 72–581 of the cDNA for La, 88–656 for PTB, 87–598 for eIF2Bγ, and 16–506 for hVAP-33. Western blot analysis was performed on total lysates from mock- or AdsiRNA-infected Huh-NNRZ cells. Protein was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel, transferred to Immobilon PVDF Transfer Membrane (Millipore). The blots were probed with monoclonal antibodies specific for La (clone La4B6, Progen Biotechnik GmbH), PTB (clone 1, Zymed Laboratories, Inc.), and eIF2Bγ (sc-9980, Santa Cruz Biotechnology, Inc.). Proteins were visualized by using Immun-Blot Assay Kit (Bio-Rad).

Transfection

HepT cells were seeded onto 35-mm-diameter tissue culture dishes 24 h before transfection. Four micrograms of each pShuttlesiRNA, 2 μg of the reporter vector, pNC371RL or pEMCVRL, and 0.2 μg of pGL3-Control vector were cotransfected into HepT cells with TransFast Transfection Reagent (Promega). The cells were harvested after 48 h, 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).

Quantification of cell viability

Cell viability was measured with Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer's instructions.

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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 S2321), 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 S2321 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-S2321. To generate the double mutants containing both the point mutation S2321 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-S2321. Because there were no optimal enzyme sites in the others, the point mutation S2321 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

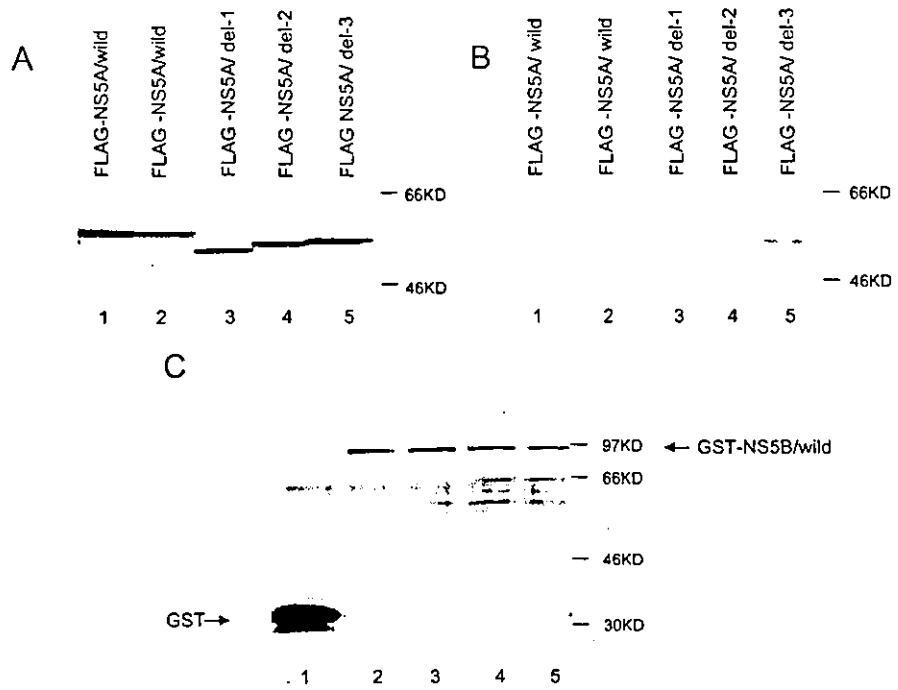

FIG. 1. Interaction between NS5A and NS5B of the isolate HCV M1LE and the regions essential for this interaction. COS1 cells were transiently cotransfected with mammalian expression vectors expressing FLAG-NS5A proteins (lanes: 1 and 2, wild type; 3, internal deletion mutant 1; 4, internal deletion mutant 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/S2321, harboring the point mutation S225P (35), a deletion of S229 (delS229) (24), and S2321 (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

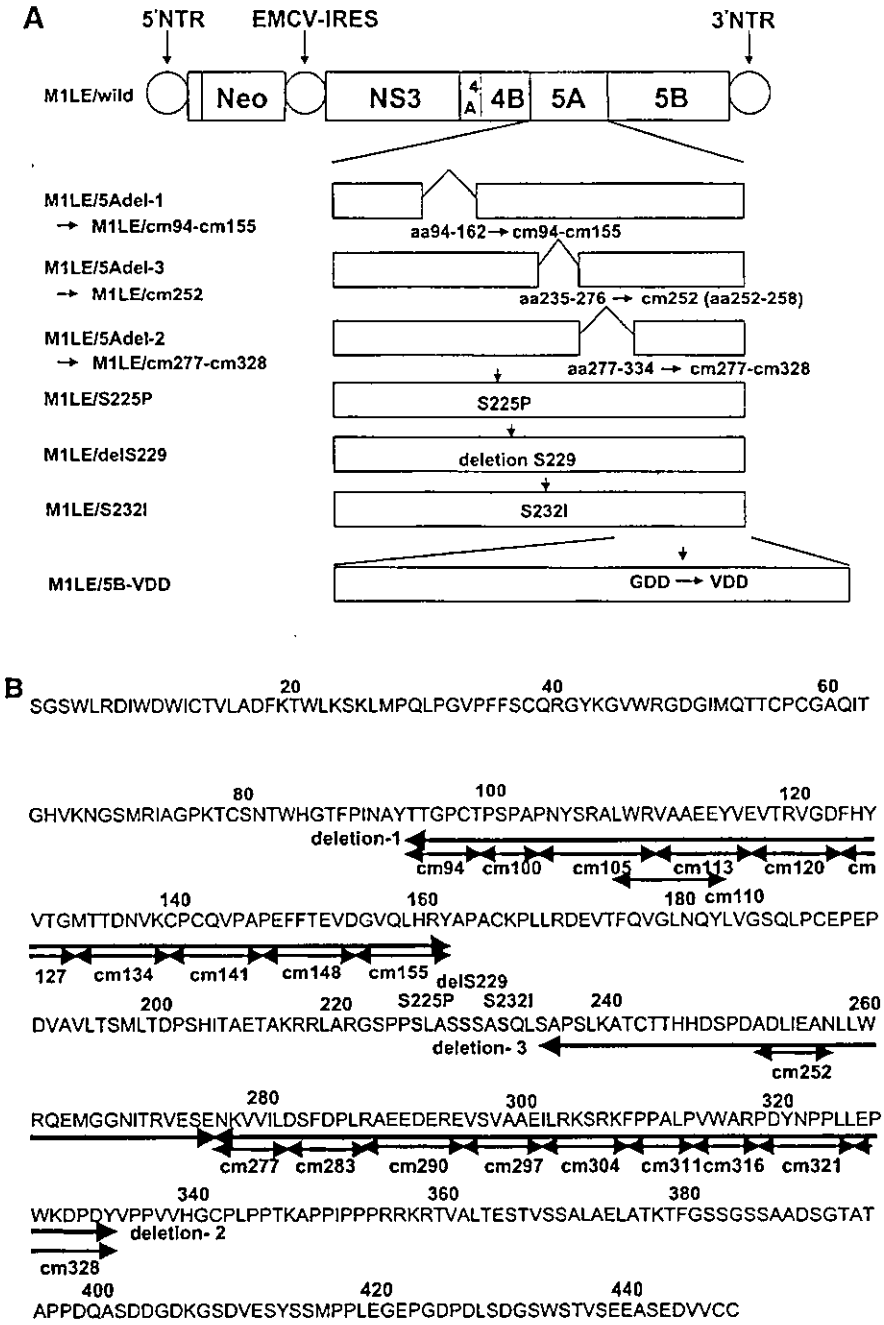

FIG. 2. (A) Schematic presentation of HCV mutant replicons used in this study. Wild-type M1LE contains the HCV M1LE wild-type sequence. M1LE/5Adel-1, M1LE/5Adel-2, and M1LE/5Adel-3 encode internal deletions of NS5A missing aa 94 to 162 (region 1), aa 277 to 334 (region 2), and aa 235 to 276 (region 3), 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.

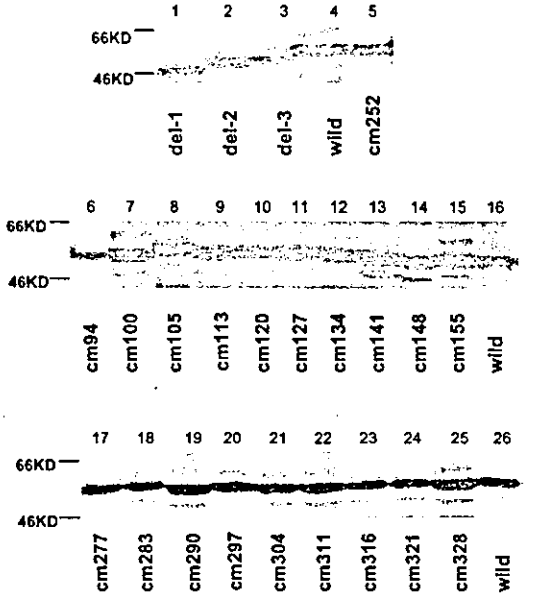

FIG. 3. Efficient translation of mutant FLAG-NS5As. COS1 cells were transiently transfected with mammalian expression vectors expressing FLAG-NS5A proteins that were prepared as described in Materials and Methods. Lanes: 4, 16, and 26, wild type; 1, internal deletion 1; 2, internal deletion 2; 3, internal 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

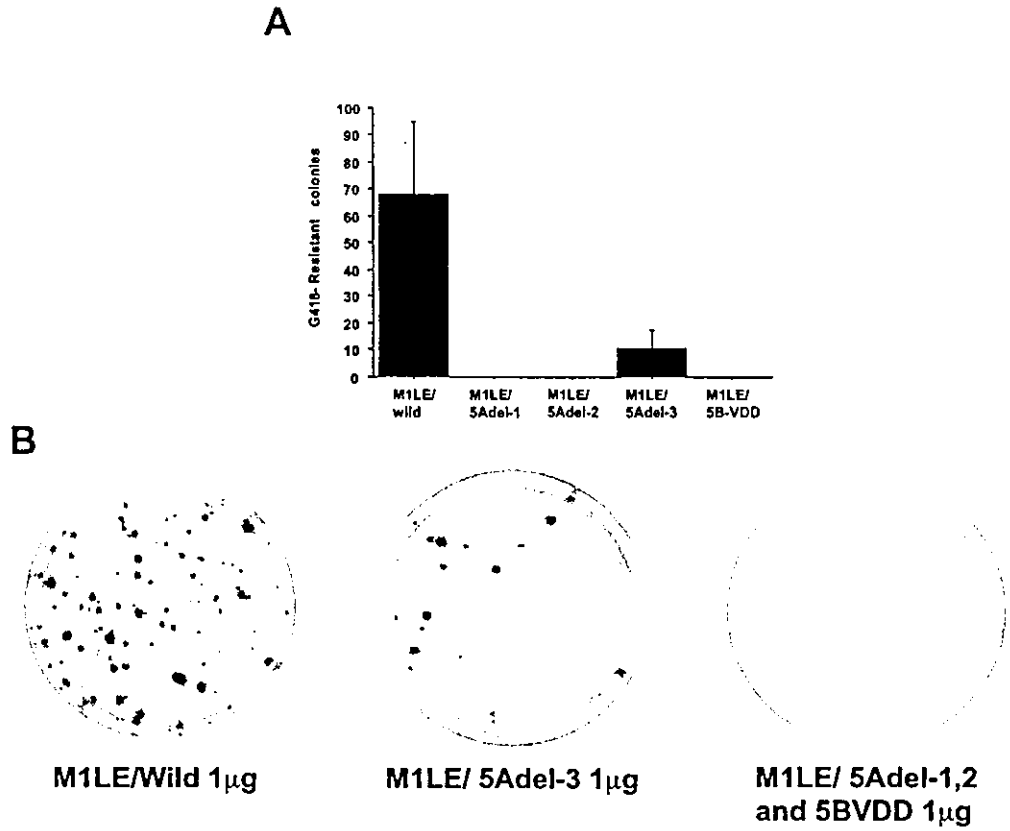

FIG. 4. Effect of internal deletion mutation on HCV RNA replication. Huh-7-DMB cells were transfected with 1 μg of in vitro-transcribed wild-type M1LE, 5Adel-1, 5Adel-2, 5Adel-3, and 5B-VDD RNA by electroporation, and G418-resistant cells were selected with a G418 concentration of 1 mg/ml. G418-resistant 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

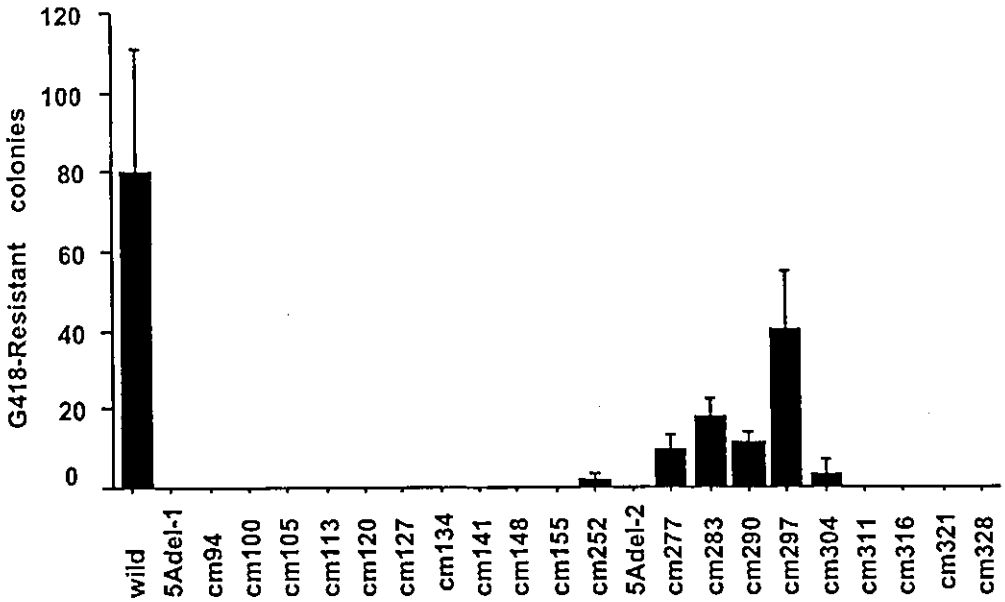

FIG. 5. Effect of clustered alanine-substitution mutations on HCV RNA replication. Huh-7-DMB cells were transfected with 1 μg of in vitro-transcribed wild-type M1LE, 5Adel-1, 5Adel-2, and cm 94, 100, 105, 113, 120, 127, 134, 141, 148, 155, 252, 277, 283, 290, 297, 304, 311, 316, 321, and 328 RNA, and 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.

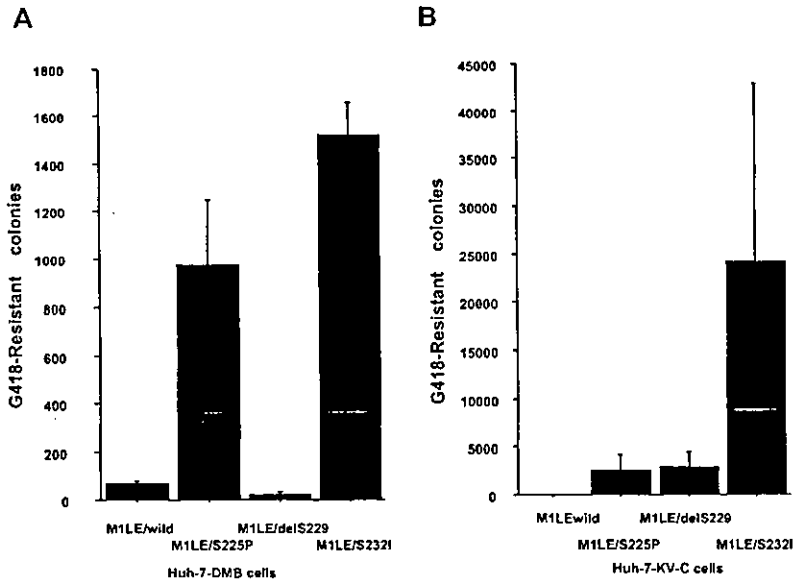

FIG. 6. Effects of three kinds of point mutation, S225P, deletion S229, and S232I, on HCV RNA replication in the Huh-7-DMB and KV-C sublines. 50-1 cells were cured of self-replicating subgenomic RNAs by IFN-α treatment, and then cured 50-1 cells, Huh-7-KV-C, were prepared. Huh-7-DMB and KV-C cells were 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.

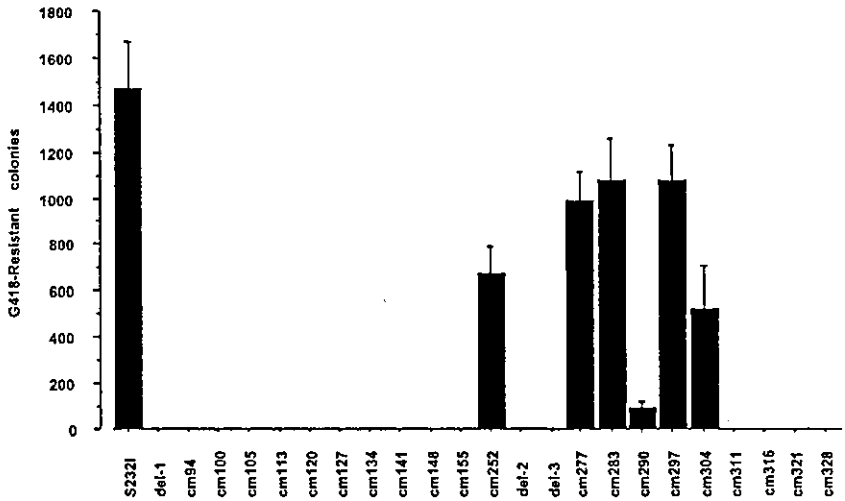

FIG. 7. Effect of M1LE/S232I plus internal deletion mutations or alanine-substitution mutations. Huh-7-DMB cells were transfected with 1 μg of in vitro-transcribed M1LE/S232I plus 5Adel-1, 5Adel-2, 5Adel-3, and cm 94, 100, 105, 113, 120, 127, 134, 141, 148, 155, 252, 277, 283, 290, 297, 304, 311, 316, 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

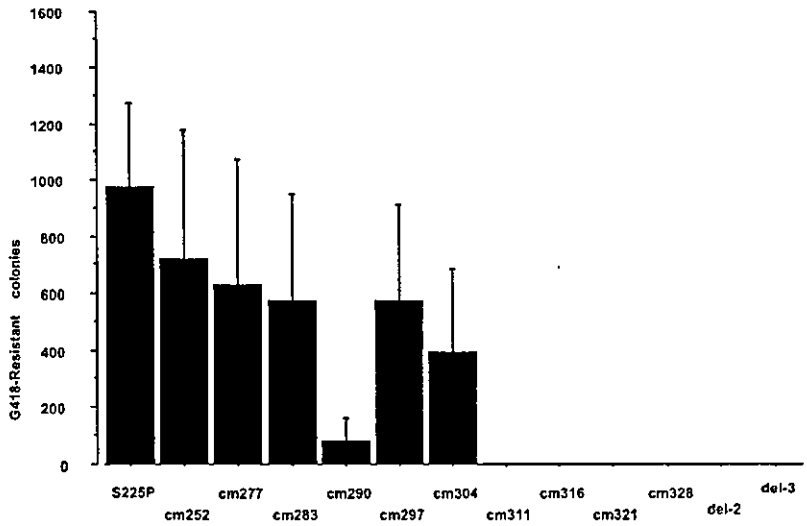

FIG. 8. Effect of M1LE/S225P plus internal deletion mutations or alanine-substitution mutations. Huh-7-DMB cells were transfected with 1 μg of in vitro-transcribed M1LE/S225P plus 5Adel-2, 5Adel-3, and cm 252, 277, 283, 290, 297, 304, 311, 316, 321, and 328 RNA. G418-resistant cells were selected with 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, S2321 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/S2321 construct. All of these mutants were more efficient in HCV RNA replication than M1LE/S2321 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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Association of Ubc9, an E2 ligase for SUMO conjugation, with p53 is regulated by phosphorylation of p53

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Abstract **Small ubiquitin-like modifier-1 (SUMO-1) conjugation to the tumor suppressor protein p53 seems to be regulated by murine double minute 2 homologue (Mdm2). It is thought that the physical association of Mdm2 with p53 is important for the enhancement of SUMO-1 conjugation to p53. However, mutant p53 that does not associate with Mdm2 is still sumoylated, albeit at a reduced level, suggesting that sumoylation of p53 is independent of the presence of Mdm2 and there is a direct association of ubiquitin-conjugating enzyme 9 (Ubc9), an E2 ligase for sumoylation, with p53. Here, we report evidence of the direct interaction of Ubc9 with p53. Furthermore, we observed that the interaction of Ubc9 with p53 was regulated by phosphorylation of p53. In particular, in cells treated with adriamycin that is a DNA damaging agent and that enhances phosphorylation of p53 at Ser-20, SUMO conjugation of p53 was severely impaired possibly by reduced affinity of Ubc9 to p53.**

Keywords: Small ubiquitin-like modifier conjugation; p53; Murine double minute 2 homolog; Ubiquitin-conjugating enzyme 9; Phosphorylation

1. Introduction

The tumor suppressor p53 plays a significant role in the cellular response to genome damage. The importance of p53 in maintaining genome integrity is emphasized by the high frequency of p53 mutations observed in many human tumors [1] and the high rate of tumor development seen in p53 knockout mice [2].

Although the level of p53 in cells is low under normal conditions, exposure to stress signals such as DNA damage and heat shock results in an increase in p53 levels because of its prolonged half-life [3]. The accumulation of p53 is responsible for increased transcription of p53 responsive genes, including proteins involved in cell-cycle regulation (e.g., $p21^{waf1}$) and apoptosis (e.g., Bax) [4–8]. Murine double minute 2 homologue (Mdm2), also a p53 responsive gene, is a critical negative regulator of p53 [9,10].

The ubiquitin-proteasome system is responsible for the degradation and rapid turnover of p53 [11], and this is mediated through the association of p53 with Mdm2 [12–14]. The interaction of Mdm2 and p53 plays a critical role in suppressing the transcriptional activity of p53 [15–17], as well as the ubiquitination and degradation of p53 [18].

The role of ubiquitination in protein degradation is well documented and it has recently become clear that a small ubiquitin-like protein, variously known as small ubiquitin-like modifier 1 (SUMO-1)/sentrin/GMP1/UBL1/PIC1, can be covalently linked to a variety of cellular proteins [19–22]. p53 has been found to be covalently modified by SUMO-1 in vitro and in vivo at lysine 386 [23,24]. This sumoylation is enhanced by the association of E3 ligases, PIAS family proteins [25,26]. However, SUMO-1 and ubiquitin modification do not occur on the same lysine residue in p53 although they do in the case of IkBα [27]. Also, SUMO-1 conjugation to p53 does not seem to alter its transcriptional activity. Some reports suggest increased p53 activity upon sumoylation, but this remains controversial.

Enhanced p53 sumoylation occurs through direct interaction with Mdm2 in cells [28] and a p53 mutant that does not interact with Mdm2 is poorly sumoylated. An Mdm2 mutant with a constitutively activated cryptic nucleolus localization signal targets p53 to the nucleolus and promotes p53 sumoylation. These data suggest that enhanced sumoylation of p53 by Mdm2 is mediated by targeting p53 to the nucleolus through the formation of the Mdm2/p53 complex. Additionally, the phosphorylation state of p53 affects its ability to interact with Mdm2. It is known that DNA-dependent protein kinase, which targets serine residues 15 and 37, reduces the affinity between p53 and Mdm2, and as a consequence, phosphorylated p53 is thought to be a poor substrate for ubiquitination and sumoylation under these conditions.

To clarify the phosphorylation sites on p53 that affects sumoylation, we focused on the Ser-20, a target of the checkpoint kinase 2 (Chk2) activated by DNA damage [29]. In addition, we analyzed the sumoylation of p53 mutants with serine to glutamic acid substitutions at residues 46 or 392, a change thought to mimic the phosphorylated state of p53.

2. Materials and methods

2.1. Cell line

HEK-293T cells (adenovirus-transformed human embryo kidney cell line containing endogenous p53) were maintained in DMEM (Nissui)

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Abbreviations: Mdm2, murine double minute 2 homologue; SUMO, small ubiquitin-like modifier; Ubc9, ubiquitin-conjugating enzyme 9; GST, glutathione *S*-transferase; HA-tag, hemagglutinin epitope tag

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supplemented with 10% fetal calf serum (MBL) and 200 μg/ml of kanamycin (Meiji) at 37 °C in 5% CO_2 atmosphere.

2.2. Plasmid construction

A pcDNA3 (Invitrogen) based plasmid expressing FLAG-tagged human p53 (wild type) was provided by Dr. Y. Ariumi. The p53 mutants were produced individually using the site-directed mutagenesis system, Mutan Super Express Km (Takara), together with the generated pkF18K-p53 as a template. Mutagenetic oligonucleotides (Invitrogen) used in LA-PCR were: p53S20A, 5′-pGGAAACATTTGCAGACCTATG-3′; p53S20E, 5′-pCAGGAAACATTTGAAGACCTATGG-3′; p53S46E, 5′-pGATTTGATGCTG-GAGCCGGACG-3′ and p53S392E, 5′-pCAGAAGGGCCTGACGAAGACTGACATTCTCCAC-3′. All plasmids were sequenced to confirm successful mutagenesis (ABI prism). FLAG-tagged p53 mutants were subcloned into pcDNA3. Plasmids encoding Myc-tagged ubiquitin-conjugating enzyme 9 (Ubc9) and hemagglutinin epitope tag (HA)-tagged SUMO-1 were generated as described previously [30].

2.3. Immunoprecipitations

HEK-293T cells (1×10^5 per 6 cm-diameter dish) were transfected using FuGENE6 (Roche) according to the manufacturer's instructions. To detect the sumoylated forms of p53, cells were lysed in 1 ml of RIPA buffer [25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM dithiothreitol (DTT), 5 mM ethylenediamine tetra-acetic acid (EDTA), 10 mM *N*-ethylmaleimide, 200 mM indole-3-acetic acid, and a complete protease inhibitor cocktail tablet (Roche)] for 30 min on ice. Cell debris was removed by centrifugation for 15 min. Lysates were pre-cleared with protein G beads for 30 min, followed by incubation with antibodies for 1 h at 4 °C. Finally, the antibody complexes were captured with protein G beads for 1 h. Beads-bound proteins were washed four times with RIPA buffer, and immunoprecipitates were eluted and analyzed by immunoblot (IB). For co-immunoprecipitations, cells were transfected with 2 μg pcDNA3-FLAG-p53 expression plasmids with or without 2 μg pcDNA3-Myc-Ubc9 expression plasmid. After 36 h of culture, cells were lysed in 1 ml of immunoprecipitated (IP) buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM EDTA, and a complete protease inhibitor cocktail tablet]. Lysates were analyzed by immunoprecipitation followed by IB assay.

2.4. Adriamycin treatment

HEK-293T cells were transfected with the expression plasmid encoding FLAG-tagged p53WT. After 24 h of culture, cells were treated with 0.64 μM of adriamycin for another 24 h, and cell lysates were prepared for immunoprecipitation and IB assay.

2.5. Materials

Rat anti-HA (3F10, Roche), mouse anti-Myc (9E10, Santa Cruz), mouse and rabbit anti-FLAG (Sigma) antibodies were purchased. Phospho-Ser20-p53 antibody was generously provided by Dr. Y. Taya. Horseradish peroxidase (HRP)-linked goat antibodies to rat IgG were acquired from Jackson ImmunoResearch Lab. HRP-linked goat antibodies to mouse or rabbit IgG were purchased from Amersham Biosciences. Adriamycin was purchased from Sigma.

3. Results and discussion

To examine the sumoylation of FLAG-tagged p53, plasmids encoding wild-type or mutant p53 and HA-tagged SUMO-1 were transiently co-expressed in cells. Expression of FLAG-tagged p53 was confirmed by immunoprecipitation followed by immunoblotting. Two bands, one with the expected size of wild-type FLAG-p53 and another more slowly migrating band, were detected (Fig. 1). The apparent molecular weight of the upper band was higher in cells exogenously expressing SUMO-1. Because the upper band was detected by anti-HA, this band was the sumoylated form of p53 (Fig. 1, middle panel). Cells expressing p53S46E and p53S392E were similar to wild-type p53, but in the cells producing p53S20E, the upper band was very weak. This was further confirmed by the observation that the upper band in cells expressing p53S20E, but not p53S20A, was detected as a weak signal (Fig. 1, lower panel). These data suggested that phosphorylation of p53 at Ser-20 severely impaired SUMO-1 conjugation. Previously, it was shown that sumoylation of p53 was affected by its interaction with Mdm2 in cells [28]. A p53 mutant that poorly binds Mdm2 undergoes deficient sumoylation. These data suggest that Mdm2 plays an important role in the enhancement of p53 sumoylation. Since a p53 mutant that does not interact with Mdm2 is still sumoylated in vitro, Mdm2 does not seem to be an essential component for the sumoylation of p53 but rather enhances sumoylation. Since Ubc9, the E2 ligase for the SUMO-conjugation reaction, was shown to associate with a target molecule for sumoylation, the association of Ubc9 with

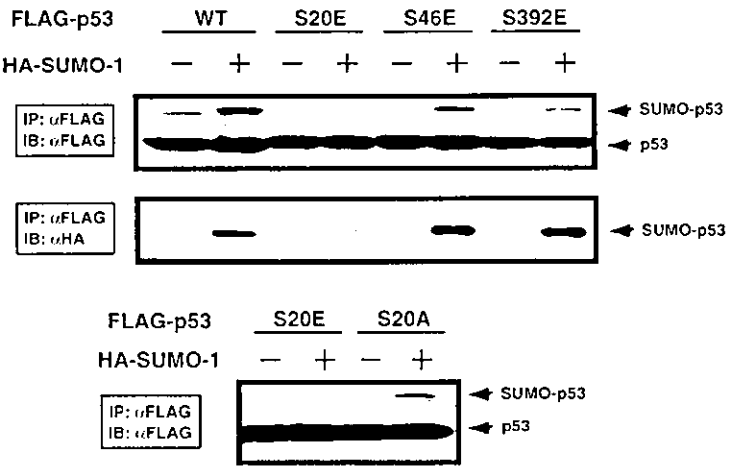

Fig. 1. Phosphorylation mimicked form of p53 at Ser-20 suppresses SUMO-1 conjugation. HEK-293T cells were transfected with 2 μg of plasmid expressing FLAG-tagged wild-type p53 (WT), or the p53 mutants S20E, S20A, S46E, or S392E with (+) or without (−) plasmids expressing HA-SUMO-1. Thirty six hours after transfection, cell lysates were prepared and IP with anti-FLAG mouse monoclonal antibody. The immunoprecipitates were subjected to 7.5% SDS–PAGE followed by analysis by IB using the anti-FLAG rabbit polyclonal antiserum (top panel). After stripping the filter shown in the top panel, the same filter was re-probed with the anti-HA antibody (middle panel).

p53 was examined. p53 was synthesized by in vitro translation in the presence of ^{35}S-methionine. The interaction of Ubc9 with ^{35}S-labeled wild-type p53, p53S20E and p53S20A was analyzed by GST pull-down analysis (Fig. 2A). Ubc9 bound all the p53 products tested with slightly varying affinity. The affinity of Ubc9 towards p53S20E was reduced by half (Fig. 2B). Because there is no Mdm2 in this assay system, there appears to be a direct interaction between Ubc9. The interaction of Ubc9 and p53 was also observed in vivo (Fig. 2C). Cells were co-transfected with Myc-tagged Ubc9 together with FLAG-tagged p53, p53S20A or p53S20E. Cells lysates prepared from these cells were then analyzed by immunoprecipitation followed by IB. Comparing to the amount of wild-type p53 and p53S20A in the complexes co-precipitated with Ubc9, that of p53S20E was significantly reduced. However, this result may not support the possible direct interaction of p53 with Ubc9, since Ubc9 is known to interact with Mdm2.

The Ser-20 of p53 can be directly phosphorylated by Chk2 in response to DNA damage [29]. To address whether sumoylation of p53 is suppressed by phosphorylation of p53 at Ser-20, we analyzed the sumoylation of p53 after adriamycin treatment. In order to determine whether adriamycin treatment led to phosphorylation of Ser-20 of p53, HEK-293T cells were transfected with a plasmid expressing FLAG-tagged wild-type p53 and treated with 0.64 μM of adriamycin at 24 h post-transfection. Adriamycin treatment was performed for 24 h and the whole cell lysates were prepared for analysis by IB assay with phospho-Ser20 p53 antibody (Fig. 3, upper panel). Ser-20 phosphorylation following adriamycin treatment was observed. We next analyzed sumoylation of p53 in cells treated with adriamycin. Sumoylation of p53 was significantly decreased after adriamycin treatment (Fig. 3, lower panel),

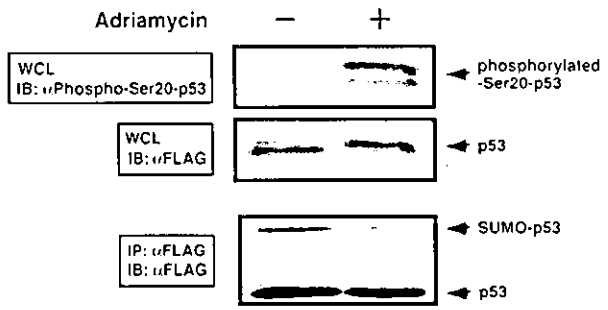

Fig. 3. Sumoylation of p53 is repressed by adriamycin treatment. HEK-293T cells were transfected with 5 μg of plasmid expressing FLAG-tagged wild-type p53. Twenty-four hours after transfection, cells were treated with (+) or without (–) 0.64 μM adriamycin for 24 h, and cell lysates were prepared and IP with anti-FLAG mouse monoclonal antibody. The immunoprecipitates as well as the whole cell lysates were subjected to 7.5% SDS–PAGE and then analyzed by IB using anti-FLAG rabbit polyclonal antiserum.

suggesting that it was repressed by Chk2-mediated phosphorylation of p53 at Ser-20.

It seems that Mdm2 binding to p53 is important for the enhancement of SUMO conjugation to p53 in cells. Since Mdm2 associates with Ubc9, it is possible that Mdm2 enhances the recruitment of Ubc9 to p53. However, this is less likely because sumoylation of p53 in vitro in the presence of Ubc9 was not enhanced by Mdm2. We observed a direct interaction of Ubc9 with p53 in vitro and this interaction was affected by the phosphorylation state of p53. Considering these results and previous reports, it is likely that Ubc9 directly associates and functions to sumoylate p53. Mdm2 may regulate

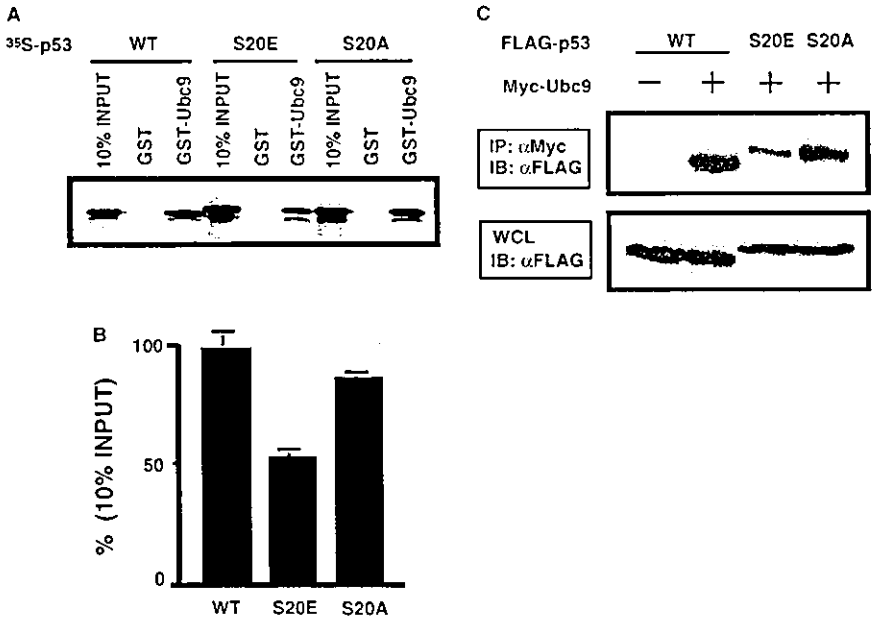

Fig. 2. Association of Ubc9 with wild-type and mutant p53 in vitro and in vivo. (A) ^{35}S-labeled wild-type p53 and p53 mutants, S20E and S20A, were incubated with GST or GST-Ubc9. (B) The GST pull-down complexes were quantitated by imaging analyzer. The experiment was conducted three times independently and data were shown with error bars. (C) HEK-293T cells were transfected with 2 μg of plasmid expressing FLAG-tagged wild-type p53 (WT), S20E, or S20A mutants together with (+) or without (–) Myc-tagged Ubc9 expression plasmid. Thirty six hours after transfection, cell lysates were prepared and subjected to IP with anti-Myc antibody. The immunoprecipitates and the whole cell lysates were subjected to 7.5% SDS–PAGE followed by IB using anti-FLAG rabbit polyclonal antiserum.

this process through at least two mechanisms, enhanced p53 nuclear localization and a mechanism yet to be clarified.

In the present study, we suggest that phosphorylation of p53 at Ser-20 reduces sumoylation. This may result from either the lack of an interaction of p53 with Mdm2 or reduced affinity of Ubc9 to p53 in vivo. Although the physiological roles of sumoylation of p53 are yet to be fully determined, the reduced sumoylation of p53S20E suggests an intrinsic role of sumoylation upon stress induced conditions including DNA damage.

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