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Functional Interaction of Hepatitis C Virus NS5B with Nucleolin GAR Domain

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Hepatitis C Virus (HCV) non-structural proteins are major components of replication complex that is modulated by several host factors. We previously reported that nucleolin, a representative nucleolar marker, interacts with the NS5B through two separated sequences, amino acids (aa) 208–214 and 500–506, and that W208 in the former stretch is essential for both nucleolin-binding and HCV replication. Here we evaluated the role of the latter stretch aa 500–506 of WRHRARS in nucleolin-binding and HCV replication scanned by alanine-substituted clustered mutant (cm) or point mutant (pm). One tryptophan and three arginine residues in the sequence were found to be essential both for nucleolin-binding *in vivo* and HCV replication detected with a HCV subgenomic replicon transfected into Huh7 cells. NS5B-binding of nucleolin was further delineated by truncation and clustered mutants of nucleolin. Arginine-glycine-glycine (RGG) repeat in the Glycine arginine rich (GAR) domain were defined to be indispensable for NS5B-binding immunologically detected in *in vivo* and *in vitro* although short internal-truncations of RGG repeat are tolerable for NS5B-binding. These results indicate that nucleolin is a critical host factor for HCV replication through the direct interaction between W208 and several residues at the sequence, aa 500–505, of NS5B, and the long-turn motif including RGG repeat at nucleolin C-terminal.

Key words: HCV, NS5B, nucleoli, nucleolin, RGG.

Abbreviations: GAR, glycine arginine rich; HCV, hepatitis C virus; NS, non-structural; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; RBD, RNA-binding domain; RdRp, RNA-dependent RNA polymerase; RGG, arginine-glycine-glycine.

The Hepatitis C virus (HCV) is a major cause of chronic hepatitis around the world (*1*, *2*) Chronic infection with HCV can progress to cirrhosis, end-stage liver disease and hepatocellular carcinoma (*3–5*) HCV is classified in the genus *Hepacivirus* of the family *Flaviviridae*. It contains a single-stranded, and positive-sense RNA genome, ~9.6 kb in length, which composed of a 5′ non-translated region (5′NTR), a single open reading frame (ORF), and a 3′NTR. The 5′NTR contains an internal ribosome entry site (IRES), which mediates the translation of a single large polyprotein of ~3000 amino acid residues (*6*, *7*). During and after translation, the polyprotein is cleaved in the structural region by host cell enzymes, and the viral non-structural (NS) proteins are cleaved by viral proteases, giving rise to at least 10 different products, including the structural proteins, core protein, E1, E2, and followed by p7, and the NS proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (*8–11*). The 3′NTR has a tripartite structure, consisting of a variable region following the stop codon of the O′RF, a poly (U)-poly (UC) tract of variable length, and a highly conserved 98-nucleotide-long sequence designated the X tail, which is critical for HCV RNA replication and HCV infection (*12–15*).

HCV is unique among positive-strand RNA viruses in that it causes persistent and chronic infections. In addition, the high mutation rate of the gene encoding the E2 envelope protein allows it to escape host immune surveillance, which is strongly associated with chronic inflammation of the liver (*16–19*). As a result, HCV replication has become a target for treatment of chronically infected individuals. The RNA-dependent RNA polymerase (RdRp) NS5B is the core catalytic enzyme for HCV RNA replication. Several recombinant and catalytically active forms of NS5B have been expressed in and purified from insect cells and *Escherichia coli*, and these proteins have provided insights into the biochemical and catalytic properties of NS5B (*20–23*). Although studies of HCV replication *in vitro* have provided the critical information on the catalytic properties of NS5B, but they are limited in elucidating the molecular mechanism of *in vivo* HCV replication, since replication requires all or most NS proteins and/or host proteins and occurs at the specialized loci on the host cell membrane. Studies on the biology of HCV replication have been facilitated by the development of subgenomic and full-length HCV

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replicons, which express HCV proteins and replicate their RNA when transfected into a human hepatoma cell line, Huh7 cells, and other cell lines (*24–26*).

Nucleolin is an abundant protein of nucleolus and major nucleolar phosphoprotein that has been implicated in rDNA transcription, rRNA maturation, ribosome assembly and nucleolin specific antibodies have been used immunohistochemically to identify nucleoli (*27–29*). Nucleolin has been shown to be an RNA chaperone and/or shuttling protein for various host and viral components in the nucleoli, nucleoplasm, cytoplasm and the plasma membrane (*30–32*). Nucleolin seems to be composed of several modules. The amino terminal third of this protein contains alternating acidic and basic domains defined as nuclear localization signals (NLS), its middle part includes four consensus RNA-binding domains (RBD), and its carboxy-terminus is a distinctive glycine/arginine-rich (GAR) domain with several arginine-glycine-glycine (RGG) motifs. The biological role of the GAR domain remains uncertain although it was reported to interact with several factors including ribosomal proteins (*33*). We previously reported that the transient expression of NS5B caused the redistribution of endogenous nucleolin from the nucleolus to the cytoplasm, and that nucleolin and NS5B interact with each other *in vivo* and *in vitro* through two independent regions of NS5B, amino acids (aa) 208–214 and 500–506. Truncated nucleolin constructs having the C-terminal part could inhibit NS5B RdRp activity *in vitro* (*34*) in the situation that recombinant full-length nucleolin is not available. The result supported the relevance of the interaction, but it may not reflect the exact role of the interaction *in vivo* that remains elucidated (*35*).

To further evaluate the interaction of nucleolin and NS5B in HCV replication, we have focused on NS5B aa 500–506, and prepared a series of mutant NS5B proteins and replicons, in which each amino acid within the sequence was substituted to alanine(s). Here we report that the W500, R501, R503 and R505 residues are critical for, binding to nucleolin *in vivo* as well as for transient HCV replication.

To gain further insight into the NS5B-binding region of nucleolin, we prepared the various truncated and clustered mutated nucleolin constructs of the GAR domain harbouring the RGG repeats. We show that the long-turn motif harbouring the RGG repeat domain is indispensable for NS5B binding *in vivo* and *in vitro*.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The plasmid pNNRZ2RU (*36*) harbours a subgenomic replicon derived from the cell line MT-2C infected with HCV (genotype 1b, GenBank™ Accession Number AB080299, M1LE isolate) and contains M1LE/S232I, MA cDNA. MA is a replicon containing a neomycin resistance gene and the point mutation S232I in NS5A and was used as a wild-type replicon in this study (*37*). The plasmid pNNRZ2RU/S232I was digested with *Bgl*II and *Xba*I, and the obtained fragment was inserted into the *Bgl*II and *Xba*I sites of the vector pGL3 Basic (Promega) to create the plasmid pGL3-*Bgl*II-*Xba*I, which was used as an intermediate vector. All mutations of NS5B were introduced into the plasmid pGL3-*Bgl*II-*Xba*I. The plasmid containing each mutation was digested with *Bgl*II and *Xba*I, and the fragment was reinserted into the *Bgl*II and *Xba*I sites of pNNRZ2RU/S232I to create each mutant replicon cDNA. The plasmids pNNRZ2RU/S232I and each mutant pNNRZ2RU/S232I were used as templates for replicon RNA, MA and mutant MA, respectively.

The mammalian expression vectors pNKFLAG and pNKGST (*38*) were used to express amino-terminally FLAG- and GST-tagged proteins, respectively.

The plasmid pNNRZ2RU/S232I was subcloned by PCR using the set of the primers, 5′-TATCGAGCTCGATGTC AATGTCCTACTCATGGACAGGT-3′ (NS5B For), which contains an artificial initiation codon downstream of the *Sac*I site, and 5′-ATGGATGGATCCGCGGGGTCGGGCG CGAGACAGGCT-3′ (NS5Bt Rev), which contains a *Bam*HI site. The amplified fragment, NS5Bt, containing full-length NS5B truncated by 21 aa at the C-terminus, was subcloned into the *Sac*I and *Bam*HI sites of pNKFLAG and pNKGST to create pNKFLAG/NS5Bt and pNKGST/NS5Bt, respectively.

To express FLAG-tagged mutant NS5Bt proteins, single substitution mutants of NS5B (W500A, R501A, H502A, R503A, R505A, S506A, W500S, W500F and W500Y), and a clustered alanine substitution mutant within aa 500–506, cm503, were constructed by introducing each mutation into the *Cfr*9I and *Hind*III sites of pNKFLAG/NS5Bt by site-directed mutagenesis using primers carrying the necessary nucleotide changes.

To create individual intermediate vectors pGL3-*Bgl*II-*Xba*I, harbouring each of these point substitution mutants of NS5B and cm503, the fragment of pNKFLAG/NS5Bt containing each NS5B mutation was introduced into the *Eco*81I and *Hind*III sites of pGL3-*Bgl*II-*Xba*I. The *Bgl*II-*Xba*I fragment of pGL3-*Bgl*II-*Xba*I containing each mutation was subsequently ligated into the *Bgl*II and *Xba*I sites of pNNRZ2RU/S232I to create each mutant replicon cDNA.

The plasmid pLMH14 (*39*) harbours a replicon cDNA in which the neomycin resistance gene of pNNRZ2RU/S232R was replaced by a luciferase gene. The expression plasmids of these point mutants (pm) and clustered mutants (cm) 503 in pLMH14 were constructed by introducing the *Bgl*II-*Xba*I fragments of pNNRZ2RU/S232I harbouring individual NS5B mutations into the *Bgl*II-*Xba*I sites of pLMH14. The plasmids pLMH14, pLMH14/GHD (*39*) and pLMH14 containing each NS5B mutation were used as templates for replicon RNA, LMH14, LMH14/GHD and mutant LMH14, respectively.

To express a series of truncated nucleolin mutants, we used mammalian expression vectors which have been described previously (*34*). To create the plasmids encoding each FLAG-tagged mutant nucleolin, internal-truncated and alanine-substitution mutations within the nucleolin RGG motif were inserted into the *Hind*III and *Bam*HI sites of pNKFLAG-nucleolin1234R (*34*). The main primers used to generate the nucleolin mutants are shown in Table 1.

The sequences of all of the constructs were confirmed by the dideoxy sequence method.

Table 1. **Sequence of the primers used in this study.**

Primer	Sequence
RGG Del 1 (aa 653–668)	For:5′-GGTGAAGGTGGCTTCGGGGGTGGTAGAGGAGGCCGAGGAGGA-3′
RGG Del 1 (aa 653–668)	Rev:5′-TCCTCCTCGGCCTCCTCTACCACCCCCGAAGCCACCTTCACC-3′
RGG Del 2 (aa 666–678)	For:5′-GCCTCCTCGCCCTCCAAAGCCTCCACCACCTCGTCCTCCAAAGCCGCC-3′
RGG Del 2 (aa 666–678)	Rev:5′-GGCGGCTTTGGAGGACGAGGTGGTGGAGGCTTTGGAGGGCGAGGAGGC-3′
RGG Del 3 (aa 679–691)	For:5′-CGAGGAGGATTTGGTGGCAGAGGCCGGGGAGGAGGAGGTGACCACAAG-3′
RGG Del 3 (aa 679–691)	Rev:5′-CTTGTGGTCACCTCCTCCTCCCCGGCCTCTGCCACCAAATCCTCCTCG-3′
RGG Del 12 (aa 653–678)	For:5′-GGTGAAGGTGGCTTCGGGGGTGGAGGCTTTGGAGGGCGAGGA-3′
RGG Del 12 (aa 653–678)	Rev:5′-TCCTCGCCCTCCAAAGCCTCCACCCCCGAAGCCACCTTCACC-3′
RGG Del 123 (aa 653–691)	For:5′-GGTGAAGGTGGCTTCGGGGGTGGAGGAGGAGGTGACCACAAG-3′
RGG Del 123 (aa 655–691)	Rev:5′-CTTGTGGTCACCTCCTCCTCCACCCCCGAAGCCACCTTCACC-3′
RGG Del 4 (aa 643–702)	For:5′-GACTGGGCCAAGACGAAGTTTGAA-3′
RGG Del 4 (aa 643–702)	Rev:5′-CCCCGGATCCTTCAAACTTCGTCTTGGCCCAGTCCAAGGTAAC-3′

Cell Culture—The COS cells were used for immunoprecipitation assays. To monitor HCV RNA replicons, we used two kinds of Huh7 cells, one derived from our laboratory's original Huh7 cell line, designated Huh7-DMB (*40*), and the other cured cell line by IFN of MH14, designated cured MH14 (*39*). Both types of Huh7 cell were grown as described (*40*). Huh7-DMB was used for colony formation assay, and cured MH14 for luciferase assay.

In vitro Transcription and Purification of RNA—All plasmids harbouring replicon cDNA were linearized with *Xba*I and purified by passage through a column (DNA purification kit; Promega). Replicon RNA was synthesized and purified as described (*40*).

RNA Transfection and Selection of G418 Resistant Cells—RNA was transfected by electroporation as described (*40*, *41*). To select G418-resistant cells, the medium was replaced with fresh medium containing 1 mg/ml of G418 (Geneticin; Gibco-BRL, Invitrogen Life Technologies) 24 h after transfection and the medium was changed twice a week. Finally 3 weeks after transfection, the colonies were stained with Coomassie brilliant blue (0.6 g/l in 50% methanol-10% acetic acid).

RNA Transfection and Luciferase Assay—RNA transfection for luciferase-replicon RNA was performed in using DMRIE-C reagent (Invitrogen Life Technologies), as described (*41*). Cell proteins were extracted in a lysis buffer supplied in the Dual-Luciferase Reporter Assay System (Promega), and their luciferase activity was measured. Each assay was performed at least in triplicate, and means and standard deviations were determined.

DNA Transfection—DNA transfection was performed using the calcium phosphate precipitation method as reported previously (*23*).

Co-transfection of Replicon RNA and Plasmid DNA—24 h after transfection of luciferase-replicon RNA, plasmid DNAs with 100 ng per 1 well of 48-well plate were transfected into cured MH14 cells using FuGENE6 transfection reagent (Roche Applide Science), according to the manufacturer's recommendations. 24 h and 72 h after RNA transfection, luciferase assay was done as described earlier.

Preparation for Cell Extracts, Co-precipitation with GST Resin, and Western Blot Analysis—Transient transfection of COS1 cells, an immortalized cell line of African green monkey kidney cells, was performed as described (*42*, *43*). The transfected cells were harvested, washed with phosphate-buffered saline (PBS) (−) and sonicated in lysis buffer (50 mM Tris–HCl, pH7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 10 mM leupeptin and aprotinin, and 1 mM DTT). Total cell lysate was diluted 15-fold with lysis buffer, mixed with 10 μl of glutathione-Sepharose 4B beads, 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 sulphate (SDS)-12% polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, and subjected to Western blot analysis with anti-FLAG M2 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 M2 antibody were reprobed with anti-GST monoclonal antibody (Zymed Laboratories), according to the manufacturer's instructions (Amersham Biosciences).

RESULTS

Interaction between Nucleolin and Substituted Mutants of NS5B—We previously reported that NS5B from JK-1 isolate binds to nucleolin through its two regions, aa 208–214 and 500–506, and that NS5B from M1LE isolate binds to nucleolin as same as JK-1 isolate, and aa 208–214 is essential for this interaction in M1LE isolate (*34*). Although it remained to be uncertain whether NS5B from M1LE isolate could bind nucleolin through its aa 500–506. To determine the essential regions/residues of NS5B required for its binding to nucleolin, we prepared FLAG-NS5Bt/cm503, in which all seven aa 500–506 from M1LE isolate were replaced by alanine residues and FLAG-NS5Bt/pmW500A, R501A, H502A, R503A, R505A and S506A, in which each individual amino acid residue was changed to alanine (except for aa 504, which is alanine in the wild-type

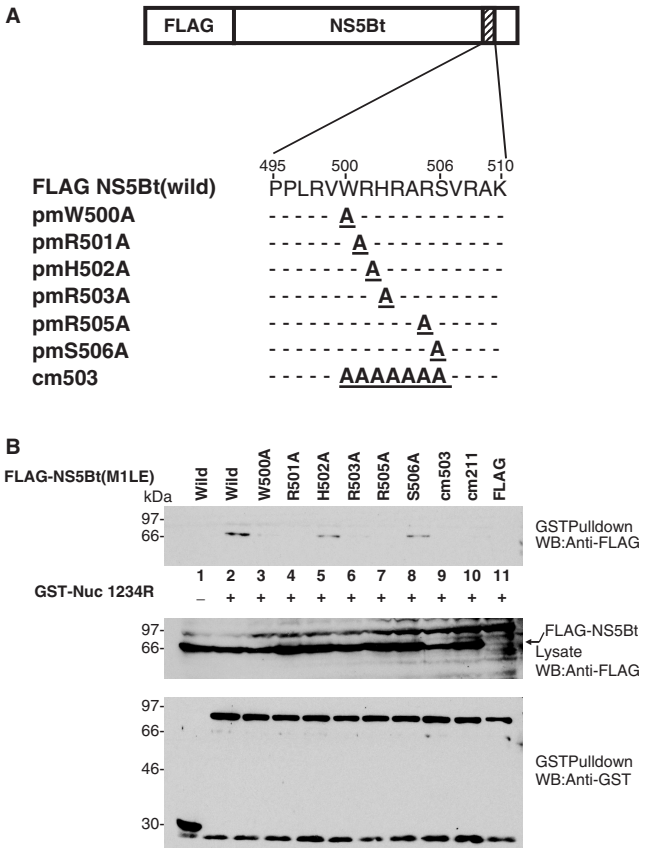


Fig. 1. **Interaction between nucleolin and NS5B and essential residue of NS5B for this interaction.** COS cells were transiently co-transfected with plasmids expressing FLAG-NS5Bt proteins (lanes 1 and 2, wild-type; lane 3, W500A; lane 4, R501A; lane 5, H502A; lane 6, R503A; lane 7, R505A; lane 8, S506A; lane 9, cm503; lane 10, cm211; lane 11, FLAG protein alone, respectively), and GST protein alone (lane 1) or GST-nucleolin1234R protein (lanes 2–11). (A), Schematic representation of FLAG-tagged mutant NS5Bts. To express each mutant NS5B protein, we introduced each mutation into pNKFLAG/NS5Bt (wild). (B), GST pull-down assay and Western blot analysis were carried out with anti FLAG antibody and anti GST antibody. Upper panel is output of FLAG-NS5Bt proteins. Co-precipitants on glutathione resin were washed with PBS (–) containing 1.0% Triton X-100, fractionated by SDS-12% PAGE, and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. Middle panel is input of FLAG-NS5Bt proteins. Total lysates were fractionated by SDS-12% PAGE and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. Lower panel is recovery of GST or GST-nucleolin 1234R proteins. The nitorocellulose used for Western blot analysis of co-precipitants with anti-FLAG M2 antibody was re-probed with anti-GST antibody. Molecular masses (kDa) are indicated on the left of each panel.

sequence) (Fig. 1A). GST-nucleolin 1234R and the recombinant FLAG-tagged NS5Bt, a soluble form of NS5B missing the C-terminal 21 aa (*23*) that is not essential for this interaction (*34*), were coexpressed in COS cells. In this study we used nucleolin 1234R missing N-terminal (NLS region) since the expression level of the construct is better than that of full-length and we have shown that the NLS is not essential for binding to NS5B (*34*). The cell lysates were subjected to GST pull-down assays, and the bound proteins were fractionated by SDS–PAGE and immunologically detected with anti-FLAG M2 and anti-GST antibodies (Fig. 1B upper, middle and lower panels). FLAG-NS5Bt/cm503 did not bind GST-nucleolin (Fig. 1B upper panel), indicating that aa 500–506 in both isolates is critical for binding to nucleolin. The NS5B protein harbouring alanine substitution at W500, R501, R503 or R505 failed to bind GST-nucleolin 1234R, although that with H502A and S506A could bind it as like the wild one (Fig. 1B upper panel).

Scanning of aa 500–506 of NS5B in HCV Subgenomic Replicon—We previously showed that the sequence aa 500–506 is one of two amino acid stretches critical for nucleolin-binding (*34*). To determine the importance of this sequence in HCV replication, we used the HCV RNA subgenomic replicon, MA, in which the serine residue at aa 232 of NS5A had been replaced by isoleucine (S232I), because MA could replicate more efficiently than the original construct in Huh7-DMB cells (*37*, *40*). The replicon MA/NS5Bcm503, in which all 7 aa of NS5B were changed to alanine, and MA/W500A, R501A, H502A, R503A, R505A and S506A in which each individual amino acid residue was changed to alanine (except for aa 504, which is alanine in the wild-type sequence), were individually transfected into Huh7-DMB cells, and the cells were cultured in the presence of G418 (Fig. 2A). We observed G418 resistant colonies in the cells transfected with MA/S506A, but not in the cells transfected with a RNA replicon having one of the other five point mutations or with MA/cm503, or in the negative control, the mutant replicon M1LE/VDD defective in RdRp (*40*, *41*). These findings indicate that all the residues except S506 of the NS5B sequence, aa 500–506, are all critical for replication (Fig. 2B and C). The non-critical role of S506 of NS5B in HCV replication seems to be consistent to the fact that not S506 but A506 is more often used among the HCV isolates (Fig. 3).

Transient Replication of HCV Replicons Carrying Luciferase Reporter Gene—To further confirm these results, we evaluated the effect of these mutations in HCV replication using a transient replication assay with a replicon in which the neomycin-resistant gene was replaced by a luciferase gene. Using this system, the luciferase activity in a lysate of transfected cells could be used to directly monitor HCV RNA replication, as the copy number of the reporter gene should be determined by the level of replicaion of the replicon. We introduced the several point mutations into NS5B aa 500–506 in LMH14 (Fig. 4A) and measured luciferase activity of the lysates 24 h and 72 h after transfection. The activity at 24 h was used to monitor the efficiency of each transfection, and the ratio of the activity at 72 h to that at 24 h was used to monitor the extent of HCV replication. As shown in Fig. 4B, the ratio was high when a subline of Huh7 cells, cured MH14, was transfected with a wild–type replicon, LMH14 RNA, whereas it was low with LMH14/GHD, a mutant RNA replicon of LMH14 defective in RdRp activity. The relative ratio of the luciferase activities was low with all the tested mutants except LMH14/S506A, with which the ratio is the same as in the cells transfected with the wild replicon (Fig. 4B).

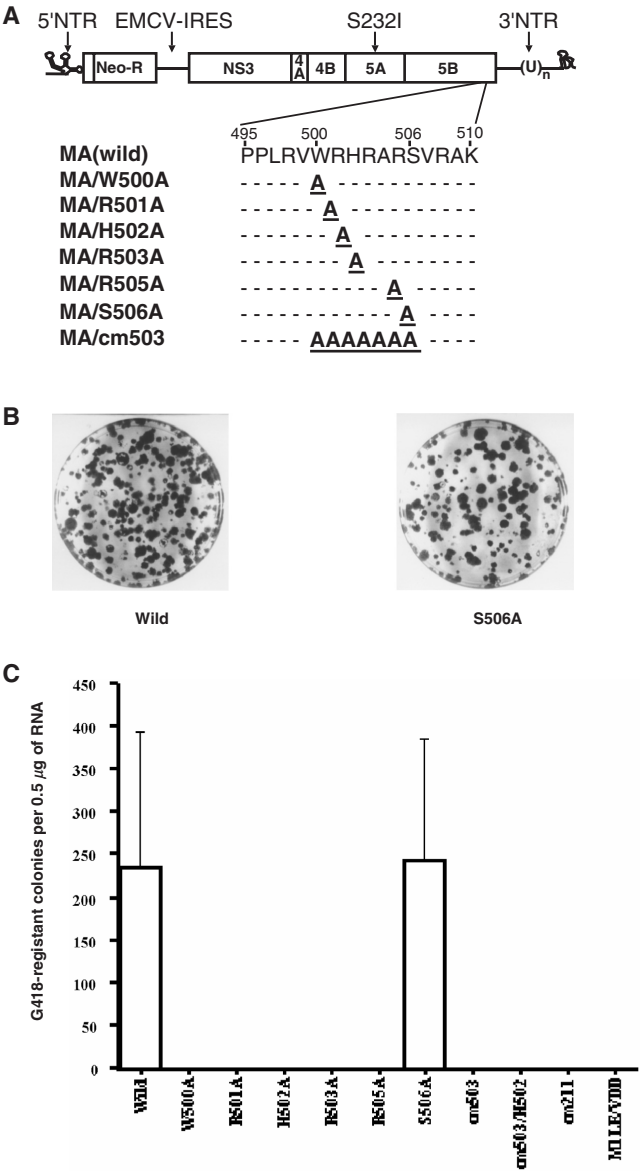


Fig. 2. **Effect of NS5B mutations within nucleolin-binding region on the HCV RNA replication.** The 0.5 μg of each *in vitro* transcribed RNA were transfected into 4.0×10^6 Huh7-DMB cells by electroporation. G418-resistant cells were selected with 1 mg/ml G418 and stained 3 week later. The numbers of G418 resistant cell colony were used as the indications of HCV RNA replication. (A) Schematic representation of the HCV mutant replicons used in this study. MA is a replicon carrying the point mutation S323I in NS5A and was used as wild-type replicon. The mutant replicons, MA/W500A, MA/R501A, MA/H502A, MA/R503A, MA/R505A MA/S506A and MA/cm503 carry each NS5B mutations as shown. Numbering starts from the beginning of NS5B. (B) Visualization of G418-resistant colonies. The protocol is described in EXPERIMENTAL PROCEDURES. (C) This figure shows the mean number of G418-resistant cell colonies per 10-cm-diameter cell culture dish per 0.5 μg each replicon RNA. Error bars indicate the standard deviations of the results from at least three independent experiments. M1LE/VDD was used as a negative control (*37*).

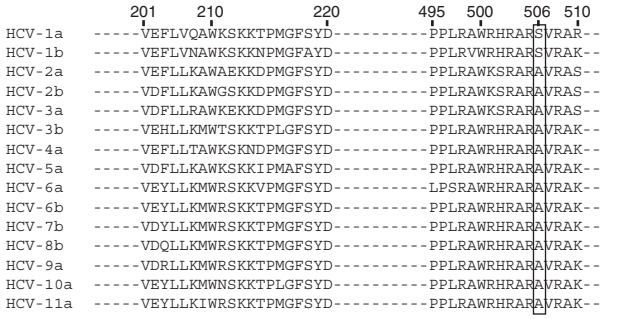


Fig. 3. **Partial sequence alignment among several genotypes of HCV NS5B.** Amino acid residues common to all genotypes are denoted by asterisks below the sequence. The sequence were obtained from HCV-1a (AF0096), HCV-1b (AB016785), HCV-2a (D00944), HCV-2b (AB030907), HCV-3a (AB031663), HCV-3b (D49374), HCV-4a (Y11604), HCV-5a (Y13184), HCV-6a (Y12083), HCV-7b (D84263), HCV-8b (D84264), HCV-9a (AB016785), HCV-10a (D63821) and HCV-11a (D63822).

This result is well consistent to that with the cm and pm mutations monitored by the colony formation assay (Fig. 2B and C).

Dissection of the C-terminal Region of Nucleolin Critical for HCV NS5B-binding in vivo—We previously mapped the region of nucleolin necessary for NS5B-binding within the RBD4-GAR region (4R) as the FLAG-4R protein exhibited the ability to bind GST-NS5B of JK1 isolate (*34*). To further map the NS5B-binding region within RBD4-GAR, we prepared the mutant nucleolin in which RBD4 was internally deleted. This mutant could bind NS5B, indicating that GAR, not RBD4 would be the essential region for this binding (data not shown) and then GAR domain was further delineated with several truncated and internal-deletion constructs within the domain (Fig. 5A). COS cells were transfected with wild-type or mutated FLAG-nucleolin 1234R, together with GST-NS5Bt or GST, the cell lysates were subjected to GST pull-down, and the bound proteins were immunologically detected with anti-FLAG M2 and anti-GST antibodies. We found that nucleolin 1234R and three nucleolin internal-truncation mutants, 1234R Del653-665, 1234R Del666-678 and 1234R Del679-691, were bound to GST-NS5Bt, whereas all mutants missing the GAR domain, namely the internal-truncation mutants 1234R Del653-678, 1234R Del653-691 and 1234R Del643-702, did not bind GST-NS5Bt (Fig. 5B upper, middle and lower panel). To confirm the critical role of the RGG repeats of nucleolin in NS5B binding, the bacterial recombinant FLAG-tagged nucleolin 1234R and mutant nucleolin-1234R proteins were prepared and subjected to GST pull-down assays using GST-tagged NS5Bt. The wild-type construct could bind NS5Bt, but the mutants missing the long-turn motif at the GAR domain could not (data not shown), indicating that the long-turn motif harbouring the RGG repeats is critical for binding to NS5B. As truncation analysis may affect the integrity of the secondary structure of the region, a version of alanine substitution mutants were constructed to further delineate the sequences critical for NS5Bt binding of the C-terminus of nucleolin (*44*).

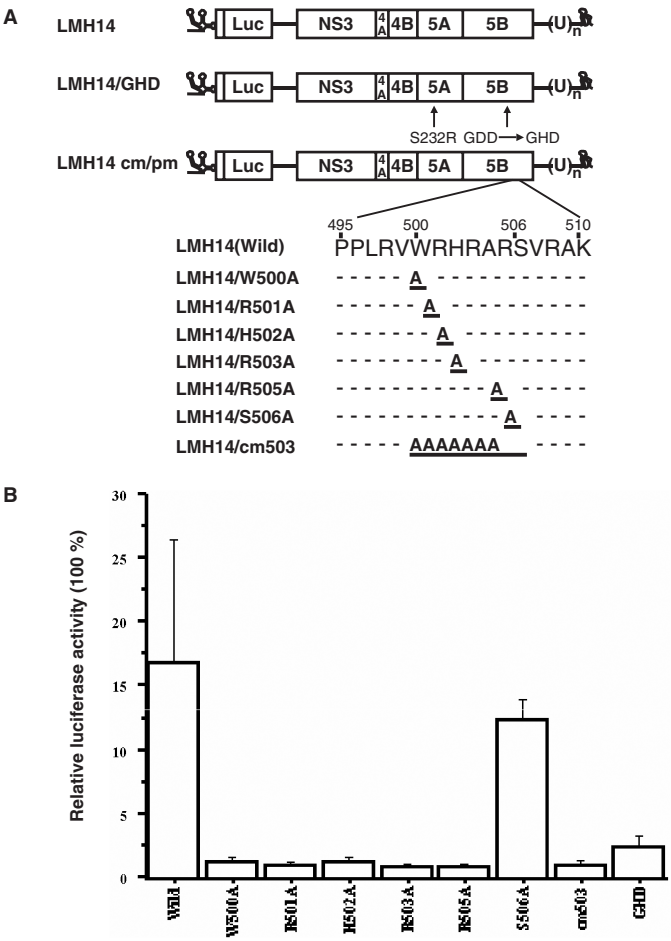


Fig. 4. **Effect of NS5B mutations within the nucleolin-binding region on transient HCV RNA replication.** (A) Schematic representation of the luciferase replicon. In the LMH14 replicon, the neomycin resistance gene of replicon MA was replaced by a luciferase gene and S232 of NS5A was replaced by R. In the LMH14/GHD replicon, the NS5B GDD motif in LMH14 was changed to GHD, and used as a negative control. The mutant LMH14 replicons W500A, R501A, H502A, R503A, R505A, S506A and cm503, carry the NS5B mutations shown. Numbering starts from the beginning of NS5B. (B) Cured MH14 cells were transfected with *in vitro* transcribed LMH 14 or LMH14 mutant replicons, using DMRIE-C reagent, and luciferase activity (relative light units; RLU) was measured 24 and 72 h after transfection. Shown are the rations of activity (%) at 72 h relative to 24 h. Error bars indicate the standard deviations of the results from at least three independent experiments.

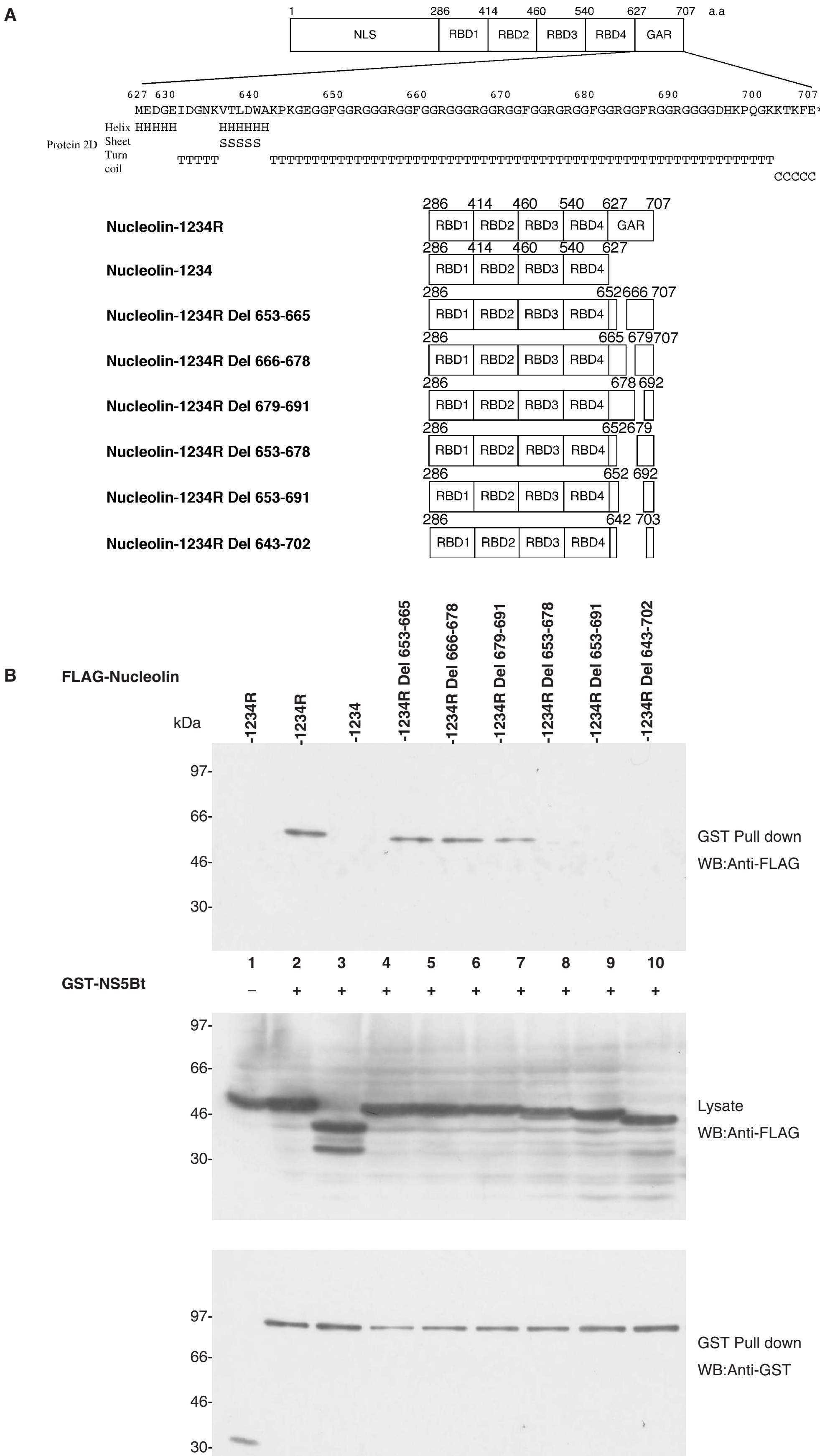
All of arginine (R) or phenyl alanine (F) residues of three subregions in the nucleolin GAR domain were scanned by introducing alanine substitutions (Fig. 6A). Nucleolin 1234R cm1 (all R and F residues within aa 653–665 were substituted to alanine), cm2 (all R and F residues were substituted within aa 666–678) and cm3 (all R and F residues were substituted within aa 679–691), exhibited binding ability to GST-NS5Bt, whereas cm12 (all R and F residues were substituted within aa 653–678) and cm123 (all R and F residues were substituted within aa 653–691) did not bind or weakly bound to GST-NS5Bt (Fig. 6B upper, middle and lower panel). All of these cm nucleolin mutants in the FLAG-fused form were expressed at the similar level in transiently transfected COS cells (Fig. 6B middle panel). The results confirmed that the long RGG repeat of nucleolin in the GAR domain is contributing to the binding ability of nucleolin to NS5B (Fig. 5B).

Effect of Wild and Truncated Mutant Nucleolin on HCV Replication—To evaluate whether nucleolin affects HCV replication by its binding to NS5B. The FLAG tagged plasmid DNA which encode nucleolin full-length, N terminal (NLS) alone, RBD1234GAR (Nuc 1234R), RBD1234 (Nuc 1234), RBD4GAR (Nuc 4R), and GAR (Nuc R) alone were transiently coexpressed with LMH14. However, neither wild-type nor the truncated nucleolin mutants, had much effect on luciferase activity (Fig. 7). Similarly the transiently coexpressed nucleolin construct missing 4R or R could not affect much HCV replication in the HCV subgenomic replicon system (data not shown). The result suggests that transient overexpression of truncated nucleolin mutants may not efficiently compete out endogenous nucleolin that is in excess to be required for HCV replication in the cells.

DISCUSSION

Nucleolin is involved in a variety of cellular functions, including RNA metabolism, regulation of chromatin structure, trafficking of telomerase and transcriptional regulation (*29*, *45–49*). We previously reported the direct interaction of NS5B and nucleolin through two sequences (aa 208–214 and aa 500–506) of NS5B and the C-terminus of nucleolin *in vivo* and *in vitro* (*34*), and the important role of W208 within aa 208–214 of NS5B for both HCV replication and nucleolin-binding (*41*). These results strongly support the notion that nucleolin is critical for HCV replication. To gain further insights into the role of nucleolin in HCV replication, here we

Fig. 5. **Interaction between NS5Bt and mutant nucleolin1234R and essential regions of nucleolin for this interaction.** COS cells were transiently co-transfected with plasmids expressing mutant FLAG-nucleolin1234R proteins and GST protein alone or GST-NS5Bt protein. (A) Schematic diagram of wild-type nucleolin protein and deletion mutants. The secondary structure within the nucleolin RGG domain predicted by DNASIS (HITACHI) software is shown in upper panel. A series of internal deletion mutants are shown. (B) GST pull-down assay and Western blot analysis were carried out with anti FLAG antibody and anti GST antibody. Upper panel is output of FLAG-nucleolin 1234R proteins. Co-precipitants on glutathione resin were washed with lysis buffer, fractionated by SDS-12% PAGE and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. Middle panel is input of FLAG-nucleolin 1234R proteins. Total lysates were fractionated by SDS-12% PAGE and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. Lower panel is recovery of GST or GST-NS5Bt proteins. The nitrocellulose used for Western blot analysis of co-precipitants with anti-FLAG M2 antibody was re-probed with anti-GST antibody. Molecular masses (kDa) are indicated on the left. NLS, nuclear localization signal; RBD, RNA binding domain RGG; arginine/glycine rich domain; H, helix; S, sheet; T, turn.


A
1 286 414 460 540 627 707 a.a
NLS RBD1 RBD2 RBD3 RBD4 GAR
627 630 640 650 660 670 680 690 700 707
MEDGEIDGNKVTLDWAKPKGEGGFGGRGGGRGGFGGRGGGRGGRGGFGGRGRGGFGGRGGFRGGRGGGGDHKPQGKKTKFE*
Protein 2D
Helix HHHHH HHHHHH
Sheet SSSSS
Turn
coil
Nucleolin-1234R
Nucleolin-1234
Nucleolin-1234R Del 653-665
Nucleolin-1234R Del 666-678
Nucleolin-1234R Del 679-691
Nucleolin-1234R Del 653-678
Nucleolin-1234R Del 653-691
Nucleolin-1234R Del 643-702
B
FLAG-Nucleolin
kDa
-1234R
-1234R
-1234
-1234R Del 653-665
-1234R Del 666-678
-1234R Del 679-691
-1234R Del 653-678
-1234R Del 653-691
-1234R Del 643-702
97- 66- 46- 30-
GST Pull down
WB:Anti-FLAG
1 2 3 4 5 6 7 8 9 10
GST-NS5Bt
– + + + + + + + + +
Lysate
WB:Anti-FLAG
GST Pull down
WB:Anti-GST

A

```
GAR    :MEDGEIDGNKVTLDWAKPKGEGGFGGRGGGRGGFGGRGGGRGGRGGFGGRGRGGFGGRGGFRGGRGGGGDHKPQGKKTKFE*
cm1    :MEDGEIDGNKVTLDWAKPKGEGGFGGAGGGAGGAGGAGGGRGGRGGFGGRGRGGFGGRGGFRGGRGGGGDHKPQGKKTKFE*
cm2    :MEDGEIDGNKVTLDWAKPKGEGGFGGRGGGRGGFGGRGGGAGGAGGAGGAGAGGFGGRGGFRGGRGGGGDHKPQGKKTKFE*
cm3    :MEDGEIDGNKVTLDWAKPKGEGGFGGRGGGRGGFGGRGGGRGGRGGFGGRGRGGAGGAGGAAGGAGGGGDHKPQGKKTKFE*
cm12   :MEDGEIDGNKVTLDWAKPKGEGGFGGAGGGAGGAGGAGGGAGGAGGAGGAGAGGFGGRGGFRGGRGGGGDHKPQGKKTKFE*
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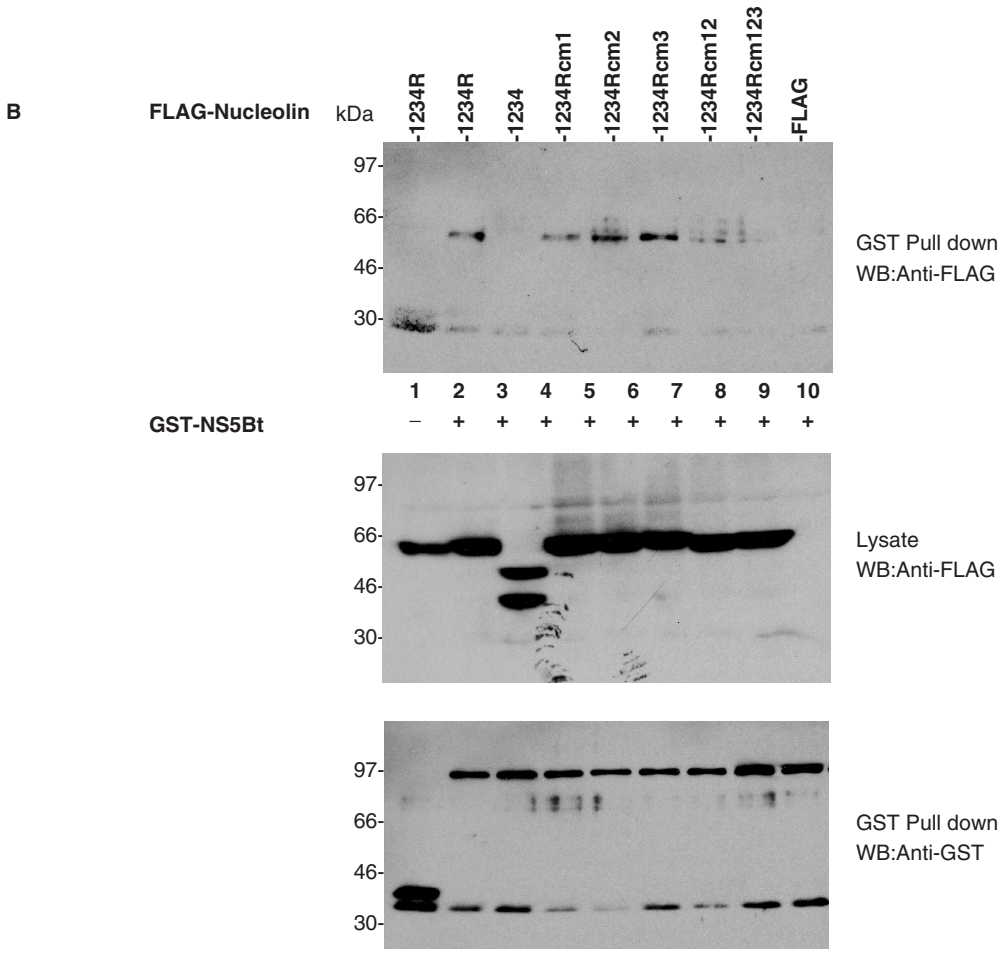

Fig. 6. **Interaction of NS5Bt with clustered mutants (cm) of nucleolin 1234R.** COS cells were transiently co-transfected with plasmids expressing FLAG-nucleolin 1234R proteins and GST protein alone or GST-NS5Bt protein. (A) Schematic diagrams of wild-type nucleolin and clustered mutants. This figure shows a series of clustered mutants. All of arginine and phenylalanine residues in three regions within nucleolin GAR domain were substituted to alanines in each mutants. (B) GST pull-down assay and Western blot analysis were carried out with anti FLAG antibody and anti GST antibody. Upper panel is output of FLAG-nucleolin 1234R proteins. Co-precipitants on glutathione resin were washed with lysis buffer, fractionated by SDS-12% PAGE and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. Middle panel is input of FLAG-nucleolin 1234R proteins. Total lysates were fractionated by SDS-12% PAGE and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. Lower panel is recovery of GST or GST-NS5Bt proteins. The nitrocellulose used for Western blot analysis of co-precipitants with anti-FLAG M2 antibody was re-probed with anti-GST antibody. Molecular masses (kDa) are indicated on the left.

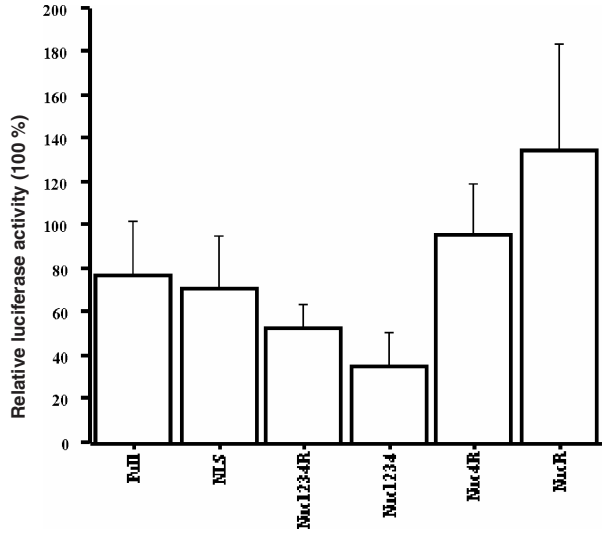

Fig. 7. **Effect of truncated wild and mutant nucleolin on HCV RNA replication.** Cured MH14 cells were transfected with *in vitro* transcribed LMH14 RNA using DMRIE-C reagent. 24 h after RNA transfection, plasmids encoding flag-tagged wild or mutant nucleolin proteins were transfected using FuGENE6 reagent. Luciferase activity (relative light units; RLU) was measured 24 and 72 h after RNA transfection. Shown are the rations of activity (%) at 72 h relative to 24 h. Error bars indicate the standard deviation of the results from at least three independent experiments.

report that another essential NS5B sequence in nucleolin-binding, aa 500–506, is indispensable for HCV replication, and that one tryptophan and three argnine residues, W500, R501, R503 and R505 in the sequence are critical both for its interaction with nucleolin and for HCV replication. We also confined that the long-turn motif harbouring the RGG repeat in the nucleolin GAR domain is essential for NS5B binding. These results indicate that nucleolin is a critical host factor for HCV replication through the direct interaction between W208 (*41*) and several residues in aa 500–505 of NS5B, and the long-turn motif, including the RGG repeat, at the C-terminus of nucleolin.

According to the crystal models, the sequence aa 500–506 is at the bottom part of the thick thumb of NS5B, which comprises the four helixes of the armadillo repeat (*50*). It is noteworthy that the sequence in the helix, apart from the active pocket of the catalytic centre, was found to be extremely sensitive to RdRp activity and/or HCV replication. The neck of the big thumb seems to be critical for the conformational change in NS5B, involving the second GTP-binding site and the dimeric interaction of NS5B (*38*, *50*). Interestingly, five residues (W500, R501, H502, R503 and R505) within the sequence were found to be critical for HCV replication by alanine scanning in the two HCV subgenomic replicon assays. All the residues except H502 are essential for the binding ability of NS5B to nucleolin, clearly demonstrating the indispensable role of the interaction between NS5B and nucleolin in HCV replication. The critical property of H502 and R503 in HCV replication has been demonstrated in the previous reports (*50*, *51*). H502 is essential for the homomeric interaction of NS5B that is prerequisite to RdRp activity (*37*, *38*), and R503 is one of the residues forming the GTP-specific binding pocket (*50*, *52*). By scanning the pm covering aa 500–506 of NS5B, only H502 was found to be critical for RdRp activity *in vitro* in the standard assay conditions (*38*), where concentrations of enzyme and substrates are much higher than those in HCV replication *in vivo*. The interaction of nucleolin and NS5B may be necessary for the active conformation of RdRp *in vivo* when local concentrations of RdRp are limited. It is uncertain at present whether NS5B interacts with NS5B and nucleolin at the same time, but the interaction between NS5B and nucleolin may happen before the conformational change of NS5B, or may be an intermediate complex for the active conformation of RdRp or the dimerization of NS5B. This notion seems to be consistent to the facts that R503 is required both for GTP-binding (*38*) and nucleolin-binding, and that the critical residues for nucleolin-binding are distributed in different surfaces of the helix which may interfere with the homomeric interaction of NS5B. We tried to evaluate the importance of tryptophan residue at aa 500 of NS5B by introducing substitution, the mutant substituted to phenylalanine or tyrosine but not to serine could bind GST-1234R in the transient coexpression system, suggesting the importance of the aromatic residue at aa 500 in nucleolin-binding, although NS5B harbouring W500F or W500Y could not support HCV replication (data not shown). The result may imply that the nucleolin-binding at aa 500 is not a sole function, or that only W500, not W500F or W500Y may support the nucleolin-binding in HCV replication *in vivo* when concentration of NS5B would be much lower than that in the transient overexpression system we applied.

We found that the sequence of nucleolin critical for NS5B-binding lies within the GAR domain. The GAR domain consists of an RGG repeats interspaced with different amino acids and forms a beta-turn structure (*53*). The GAR domain has been reported to interact with various host factors, including single- and double-stranded RNA and DNA (*53*), and several proteins such as a ribosomal protein L3, hnRNP A1 and some other hnRNP proteins (*28*). In some cases, modification of arginine residue(s) of the GAR domain has been shown to be involved in the interaction (*28*), although such modification is not necessary for the NS5B-binding as the interaction was observed among the bacterial recombinant forms *in vitro* (*34*). Our results here indicate that the NS5B-binding of nucleolin seems to require several stretches of the RGG repeat within GAR domain since at least two-thirds of RGG repeats of the domain are important for binding NS5B by scanning with versions of internal truncation and alanine substitution mutations of arginine and phenylalanine residues. The requirement of rather long sequence in the GAR domain for NS5B-binding may be consistent to the fact that the separated residues, W208 at the exposed surface at the neck of palm and four residues clustered at the bottom of thumb, are all required for the nucleolin-binding.

We tried to evaluate the critical role of the interaction between nucleolin and NS5B in HCV replication by the

truncated mutant of nucleolin missing the GAR domain or the GAR domain alone in a transient over expression system. However, both types of nucleolin mutants did not affect much HCV replication. The result may be due to the reason that HCV replication requires limited amount of nucleolin in the cells as suppression of endogenous nucleolin by siRNA only partially affected HCV replication (*41*). Alternatively, the truncated mutants may not efficiently compete out endogenous nucleolin in the system because the truncated mutants may not take the same traffic pathway as that of endogenous nucleolin. Biological functions of nucleolin have been reported to play many roles in trafficking of RNA and/or RNA-binding proteins, transcription activation, and chromatin remodelling, and nucleolin has been reported to be required for replication of some viruses (*54*, *55*), therefore, a possibility that some designs targeting to nucleolin could inhibit HCV replication still remains further explored.

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