

FIG. 8. Establishment of a *trans*-packaging system involving two-plasmid transfection. (A) Schematic representation of the plasmids used for the production of HCV-LP. HCV polyproteins are indicated by the open boxes. Bold lines indicate the HCV UTR. EMCV IRES is denoted by gray bars. The firefly luciferase gene (F Luc) is depicted as a gray box. CAG, CAG promoter; Pol I P, Pol I promoter; dC, 5' region of Core gene; Pol I T, Pol I terminator. (B) Luciferase activity in Huh7.5.1 cells inoculated with culture supernatant from cells transfected with the indicated plasmids. Luciferase activity is expressed in terms of relative luciferase units (RLU). The data for each experiment are averages of triplicate values with error bars showing standard deviations. (C) Culture supernatant from cells cotransfected with pHH/SGR-Luc and the indicated plasmids were collected 4 days p.t. The luciferase activity in Huh7.5.1 cells inoculated with culture supernatant was determined 3 days postinoculation and expressed as relative luciferase units (RLU). The RLU was normalized according to the luciferase activity observed in the pCAG/C-NS2-transfected sample (C-NS2), which was set at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations. (D) Huh7.5.1 cells were inoculated with HCVLP in the absence (-) or presence of 5 µg of anti-CD81 or anti-FLAG antibody/ml. The luciferase activity was determined 72 h postinoculation and is expressed as relative luciferase units (RLU). The RLU was normalized to the level of luciferase activity observed in the antibody-untreated sample (-), which was set at 100%. The data for each experiment are averages of triplicate values with error bars showing standard deviations.

clones exhibited little to no surface expression of CD81, one of the key features of HCV glycoprotein-mediated infection (Fig. 5). Defective expression of receptor molecules might be advantageous in generating stable cell lines for robust production of HCV. HCV-induced cytotoxicity has been reported (7, 45, 54). Persistent HCV infection was established after electroporation of JFH-1 genomic RNA, and a variable cytopathic effect was observed at the peak of acute HCV infection, as well as during the persistent phase of infection (54). A recent study has demonstrated that the cytopathic effect triggered by HCV RNA transfection and viral infection is characterized by massive apoptotic cell death with expression of several ER stress markers, such as GRP78 and phosphorylated eIF2-α (39). Therefore, in the present study, it is likely that selective forces to evade cell death during high levels of HCV replication produced cell populations resistant to virus infection. As a consequence, H751JFH1/Zeo cells maintained robust production of infectious HCV particles over a long period of time without gross cytopathic effects or changes in cell morphology.

Substantial evidence demonstrates that the mutation rate of the HCV genome produced in H751JFH1/Zeo was low (Fig. 6) presumably because of consistent expression of wild-type HCV RNA from the chromosomally integrated gene. Nevertheless, a considerable proportion of the genome was mutated, with

two nonsynonymous mutations in the E1 (V293A) and E2 (I414T) regions identified in the culture supernatant of H751JFH1/Zeo cells after 4 months of passages (Fig. 6). A I414T mutation has also been reported after long-term propagation of HCV in culture after JFH-1-RNA transfection (54). This mutation is located between the hypervariable regions 1 and 2 within the N terminus of E2 (51). Adaptive mutations in this region have been shown to enhance virus expansion, presumably by enabling more efficient virus entry (6, 36, 54). A possible CD81-independent mechanism for cell-to-cell transmission of HCV has been proposed (48, 50). However, the mechanisms governing cell-to-cell spread of HCV are not well understood. Further investigation into the importance of envelope protein mutations in HCV transmission independent of CD81 provide a better understanding of the complex interactions required for HCV infection.

In the present study we assessed the effects of N-linked glycosylation inhibitors on HCV production using H751JFH1/Zeo (Fig. 7) and found that an α-glucosidase inhibitor NN-DNJ inhibits the production of infectious HCV, which has also been observed in previous studies (43, 47). In contrast, HCV production is increased in the presence of an ER α-mannosidase inhibitor KIF, but not in the presence of the Golgi α-mannosidase inhibitors DMJ, DIM, and SWN. KIF inhibits α-man-

nosidase I, which primarily functions to remove the middle mannose branch from Man₉GlcNAc₂ to form Man₈GlcNAc₂ after the removal of glucose residues by glucosidases I and II (8, 24). Experiments to elucidate the role of mannose trimming of N-glycans in the HCV life cycle are currently under way.

It has recently been demonstrated that subgenomic replicons or defective genomes of HCV that have the potential of translation and self-replication can be encapsidated into infectious viruslike particles by *trans*-complementation of the viral structural proteins (1, 17, 32, 41, 44). In these studies, the viral RNAs were generally generated by *in vitro* transcription from linearized corresponding plasmids, followed by electroporation into the cells. Structural proteins or Core to NS2 proteins were then provided by DNA or RNA transfection, viral-vector-based transduction, or stable packaging cell lines established. Here, we achieved the replicon *trans*-encapsidation via transient cotransfection with two DNA plasmids. This system, which is apparently easier to manipulate and allows production of *trans*-encapsidated materials more rapidly compared to the systems published, can be applied to the study for understanding phenomenon and biological significance of a variety of naturally occurring HCV subgenomic deletion variants that possibly circulate in hepatitis C patients.

In summary, we have established a Pol I-based reverse-genetics system for the efficient production of infectious HCV. This methodology can be applied to develop (i) a stable HCV-producing cell line with a low mutation frequency of the viral genome and (ii) a simple *trans*-encapsidation system with the flexibility of genome packaging and improved biosafety. This may be useful for antiviral screening and may assist in the development of a live-attenuated HCV vaccine.

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Involvement of Creatine Kinase B in Hepatitis C Virus Genome Replication through Interaction with the Viral NS4A Protein[∇]

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Persistent infection with hepatitis C virus (HCV) is a major cause of chronic liver diseases. The aim of this study was to identify host cell factor(s) participating in the HCV replication complex (RC) and to clarify the regulatory mechanisms of viral genome replication dependent on the host-derived factor(s) identified. By comparative proteome analysis of RC-rich membrane fractions and subsequent gene silencing mediated by RNA interference, we identified several candidates for RC components involved in HCV replication. We found that one of these candidates, creatine kinase B (CKB), a key ATP-generating enzyme that regulates ATP in subcellular compartments of nonmuscle cells, is important for efficient replication of the HCV genome and propagation of infectious virus. CKB interacts with HCV NS4A protein and forms a complex with NS3-4A, which possesses multiple enzyme activities. CKB upregulates both NS3-4A-mediated unwinding of RNA and DNA *in vitro* and replicase activity in permeabilized HCV replicating cells. Our results support a model in which recruitment of CKB to the HCV RC compartment, which has high and fluctuating energy demands, through its interaction with NS4A is important for efficient replication of the viral genome. The CKB-NS4A association is a potential target for the development of a new type of antiviral therapeutic strategy.

Hepatitis C virus (HCV) infection represents a significant global healthcare burden, and current estimates suggest that a minimum of 3% of the world's population is chronically infected (4, 19). The virus is responsible for many cases of severe chronic liver diseases, including cirrhosis and hepatocellular carcinoma (4, 16, 19). HCV is a positive-stranded RNA virus belonging to the family *Flaviviridae*. Its ~9.6-kb genome is translated into a single polypeptide of about 3,000 amino acids (aa), in which the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B reside in the C-terminal half region (6, 34, 44). NS4A, a small 7-kDa protein, functions as a cofactor for NS3 to enhance NS3 enzyme activities such as serine protease and helicase activities. The hydrophobic N-terminal region of NS4A, which is predicted to form a transmembrane α -helix, is responsible for membrane anchorage of the NS3-4A complex (8, 44, 50), and the central region of NS4A is important for the interaction with NS3 (10, 44). A recent study demonstrated the involvement of the C terminus of NS4A in the regulation of NS5A hyperphosphorylation and viral replication (28).

The development of HCV replicon technology several years

ago accelerated research on viral RNA replication (7, 44). Furthermore, a robust cell culture system for propagation of infectious HCV particles was developed using a viral genome of HCV genotype 2a, JFH-1 strain, enabling us to study every process in the viral life cycle (27, 47, 54). RNA derived from genotype 1a, HCV H77, containing cell-culture adaptive mutations, also produces infectious viruses (52). Using these systems, it has been reported that the HCV genome replicates in a distinct, subcellular replication complex (RC) compartment, which includes NS3-5B and the viral RNA (2, 14, 33). The RC forms in a distinct compartment with high concentrations of viral and cellular components located on detergent-resistant membrane (DRM) structures, possibly a lipid-raft structure (2, 41), which may protect the RC from external proteases and nucleases. Almost all processes in viral replication are dependent on the host cell's machinery and involve intimate interaction between viral and host proteins. However, the functional roles of host factors interacting with the HCV RC in viral genome replication remain ambiguous.

To gain a better understanding of cellular factors that are components of the HCV RC and that function as regulators of viral replication, a comparative proteomic analysis of DRM fractions from HCV replicon and parental cells and subsequent RNA interference (RNAi) silencing of selected genes were performed. We identified creatine kinase B (CKB) as a key factor for the HCV genome replication. CKB catalyzes the reversible transfer of the phosphate group of phosphocreatine

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(pCr) to ADP to yield ATP and creatine and is known to play important roles in local delivery and cellular compartmentalization of ATP (48, 51). The findings obtained here suggest that recruitment of CKB to the HCV RC, through CKB interaction with NS4A, is essential for maintenance or enhancement of viral replicase activity.

MATERIALS AND METHODS

Cell lines, antibodies, and reagents. Human hepatoma cell line Huh-7.5.1 (54) was kindly provided by Francis V. Chisari. Cell lines carrying subgenomic replicon RNAs, namely, SGR-N (41) and SGR-JFH1 (23), were derived from the HCV-N (17) and JFH-1 strains (24), respectively. Mouse monoclonal antibodies (MAbs) against HCV NS3 (Chemicon, Temecula, CA), NS4A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), NS5A (Biodesign, Saco, ME), NS5B (2), FLAG (M2; Sigma-Aldrich, St. Louis, MO), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon), and Flotillin-1 (BD Biosciences, San Jose, CA) and polyclonal antibodies (PABs) against CKB (mouse [Abnova, Taipei, Taiwan], goat [Santa Cruz]), hemagglutinin (HA; Sigma-Aldrich), and FLAG (Sigma-Aldrich) were used. Cyclocreatine (Ccr; also known as 2-imino-1-imidazolidineacetic acid), pCr, and phosphopyruvic acid (pPy) were purchased from Sigma-Aldrich. Recombinant CKB and pyruvate kinase (PK) were obtained from Acris (Herford, Germany) and Calbiochem (San Diego, CA), respectively.

Proteome analysis. RC-rich membrane fractions of cells were isolated as described previously (2, 41). Briefly, cells were lysed in hypotonic buffer. After removing the nuclei, supernatants were treated with 1% NP-40 for 60 min, mixed with 70% sucrose, overlaid with 55 and 10% sucrose, and centrifuged at 38,000 rpm for 14 h. Proteins from membrane fractions were purified by using a 2D Clean-Up kit (GE Healthcare, Tokyo, Japan), followed by labeling with fluorescent dyes: Cy5 for replicon cells, Cy3 for parental cells, and Cy2 for the protein standard containing equal amounts of both cell samples. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was performed using Immobiline DryStrip as the first-dimension gel and 12.5% polyacrylamide gel as the second-dimension gel. The 2D-DIGE images were analyzed quantitatively using the DeCyder software (GE Healthcare). Student *t* test was performed on differences between the tested samples using DeCyder biological variation analysis module. Samples were analyzed in triplicate. The protein spots of interest were excised from the gel, subjected to in-gel digestion using trypsin or lysyl endopeptidase and analyzed by liquid chromatography (MAGIC 2002 System; Michrom Bioresources, Auburn, CA) directly connected to electrospray ionization-trap mass spectrometry (LCQ-decaXP; Thermo Electron Corp., Iwakura, Japan). The results were subjected to database (NCBIInr) search by Mascot server software (Matrix Science, Boston, MA) for peptide assignment.

Plasmids. A human CKB cDNA (43; kindly provided by Oriental Yeast Corp., Tokyo, Japan) was inserted into the EcoRI site of pCAGGS, yielding pCAGCKB. To generate expression plasmids for HA-tagged versions of wild-type and deletion mutated CKB, the corresponding DNA fragments were amplified by PCR, followed by introduction into the BglII site of pCAGGS. A fragment representing the inactive mutant CKB-C283S was synthesized by PCR mutagenesis. To generate FLAG-tagged NS protein expression plasmids, DNA fragments encoding either NS3, NS4A, NS4B, NS5A, or NS5B protein were amplified from HCV strains NIHJ1 (1) and JFH-1 (23) by PCR, followed by cloning into the EcoRI-EcoRV sites of pcDNA3-MEF (20). To generate an HA-tagged NS3 expression plasmid, a fragment encoding NS3 with the HA tag sequence at its N terminus was inserted into pCAGGS.

siRNA transfection. The small interfering RNAs (siRNAs) targeted to CKB (CKB-1 [5'-UAAGACCUCCUGGUGUGGTT-3'] and CKB-2 [5'-CGUCACCCUUGGUAGAGUUTT-3']) and the scramble negative control siRNA to CKB-2 (5'-GGCGUACUAGCUUAUUCGCTT-3') were purchased from Sigma. Cells in a 24-well plate were transfected with siRNA using HiPerFect transfection reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The siRNA sequences for the other genes used in the siRNA screening are available upon request.

HCV infection. Culture media from Huh-7 cells transfected with in vitro-transcribed RNA corresponding to the full-length JFH-1 (47) was collected, concentrated, and used for the infection assay (3).

Quantification of HCV core protein and RNA. To estimate the levels of HCV core protein, aliquots of culture supernatants or of cell lysates were assayed by using HCV Core enzyme-linked immunosorbent assay kits (5). Total RNA was isolated from harvested cells using TRIzol (Invitrogen, Carlsbad, CA). Copy numbers of the viral RNA were determined by reverse transcription-PCR (RT-PCR) (2, 36, 46).

Immunoprecipitation, immunoblot analysis, and immunofluorescence microscopy. The analyses, as well as DNA transfection, were performed essentially as previously described (42). Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, 1 mM calcium acetate). For immunoprecipitation, supernatants of cell lysates were precipitated with anti-FLAG antibody and protein A-Sepharose Fast Flow beads (GE healthcare). For immunofluorescence microscopy, anti-CKB goat PAB and anti-NS4A MAb as primary antibodies and Alexa Fluor 555-conjugated donkey anti-goat immunoglobulin G (Invitrogen) and Alexa Fluor 488-conjugated rabbit anti-mouse immunoglobulin G (Invitrogen) as secondary antibodies were used and observed under an LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Immunoelectron microscopy. Postembedding immunostaining using the colloidal gold-labeling method was performed as described previously (38). Cells were fixed in 4% paraformaldehyde–1% glutaraldehyde at 4°C for 1 h. After dehydration through a graded series of ethanol, cells were embedded in LR White (London Resin Company, London, United Kingdom) and sectioned. After blocking, section grids were incubated with a mixture of anti-NS4A and anti-CKB antibodies at 4°C overnight, followed by treatment with a mixture of 18-nm colloidal gold-conjugated donkey anti-mouse immunoglobulin G and 12-nm colloidal gold-conjugated donkey anti-goat immunoglobulin G antibodies (Jackson ImmunoResearch, West Grove, PA) at 4°C overnight. The sections were stained with uranyl acetate and observed under a transmission electron microscope.

Measurement of CK activity and cellular ATP level. Cells were lysed with passive lysis buffer (Promega, Madison, WI), and CK activities were measured based on Oliver methods (40), in which the activity of converting creatine phosphate and ADP to creatine and ATP was measured. ATP levels in cell lysates were measured by using a CellTiter-Glo luminescent cell viability assay (Promega).

RNA replication assays in permeabilized replicon cells and in vitro. The RNA synthesis assay using permeabilized replicon cells was based on a previously described method (33). Briefly, SGR-JFH1 cells were treated with 5 μ g of actinomycin D/ml for 2 h, followed by permeabilization with 50 μ g of digitonin/ml for 5 min. The resulting mix was incubated with 500 μ M concentrations of ATP, GTP, and CTP; 10 μ Ci of UTP [α - 32 P]UTP]; 50 μ g of actinomycin D/ml; and 5 mM pCr with or without 20 U of CKB/ml for 4 h at 27°C. RNA was extracted by using TRIzol and analyzed by 1% formaldehyde agarose gel electrophoresis. The cell-free RNA replication assay was performed as described previously (2).

In vitro helicase assays. Helicase activity on double-stranded RNA (dsRNA) was investigated as described previously (11) with some modifications. The 5' end of the release strand was labeled with [γ - 32 P]ATP using T4 polynucleotide kinase (Ambion). The dsRNA substrate was obtained by annealing the labeled RNA with a template strand RNA at a molar ratio of 1:1. The helicase assay mixture contained 5 nM dsRNA, helicase enzyme (80 nM NS3 or NS3-4A [kindly provided by R. De Francesco]), 6 mM ATP, in the presence or absence of 20 U of CKB/ml in an assay buffer (25 mM MOPS-NaOH [pH 7.0], 2.5 mM dithiothreitol, 100 μ g of bovine serum albumin/ml, 3 mM MgCl₂, 5 mM pCr, 2.5 U of RNase inhibitor/ml). After the helicase reaction, samples were electrophoresed in a native 8% polyacrylamide gel and autoradiographed.

To determine the effect of PK/pPy system on the helicase activity, PK and pPy were used instead of CKB and pCr. Helicase activity on dsDNA was measured based on homogeneous time-resolved fluorescence quenching using a Trupoint helicase assay kit (Perkin-Elmer, Waltham, MA) according to the manufacturer's instructions.

In vitro protease assay. In vitro HCV protease activity of NS3-4A or NS3 was analyzed by using a SensoLyteHCV protease assay kit (AnaSpec, San Jose, CA) according to the manufacturer's instructions.

RESULTS

Identification of host factors involved in HCV RNA replication by comparative proteomic analysis of DRM fractions and RNAi silencing. To identify host proteins involved in the HCV RC, proteome profiles of the RC-rich membrane fraction in Huh-7 cells harboring subgenomic replicon RNA derived from genotype 1b, N isolate (SGR-N) were compared to those of parental cells by 2D-DIGE. We confirmed that the DRM fraction obtained from SGR-N cells is functionally active in a

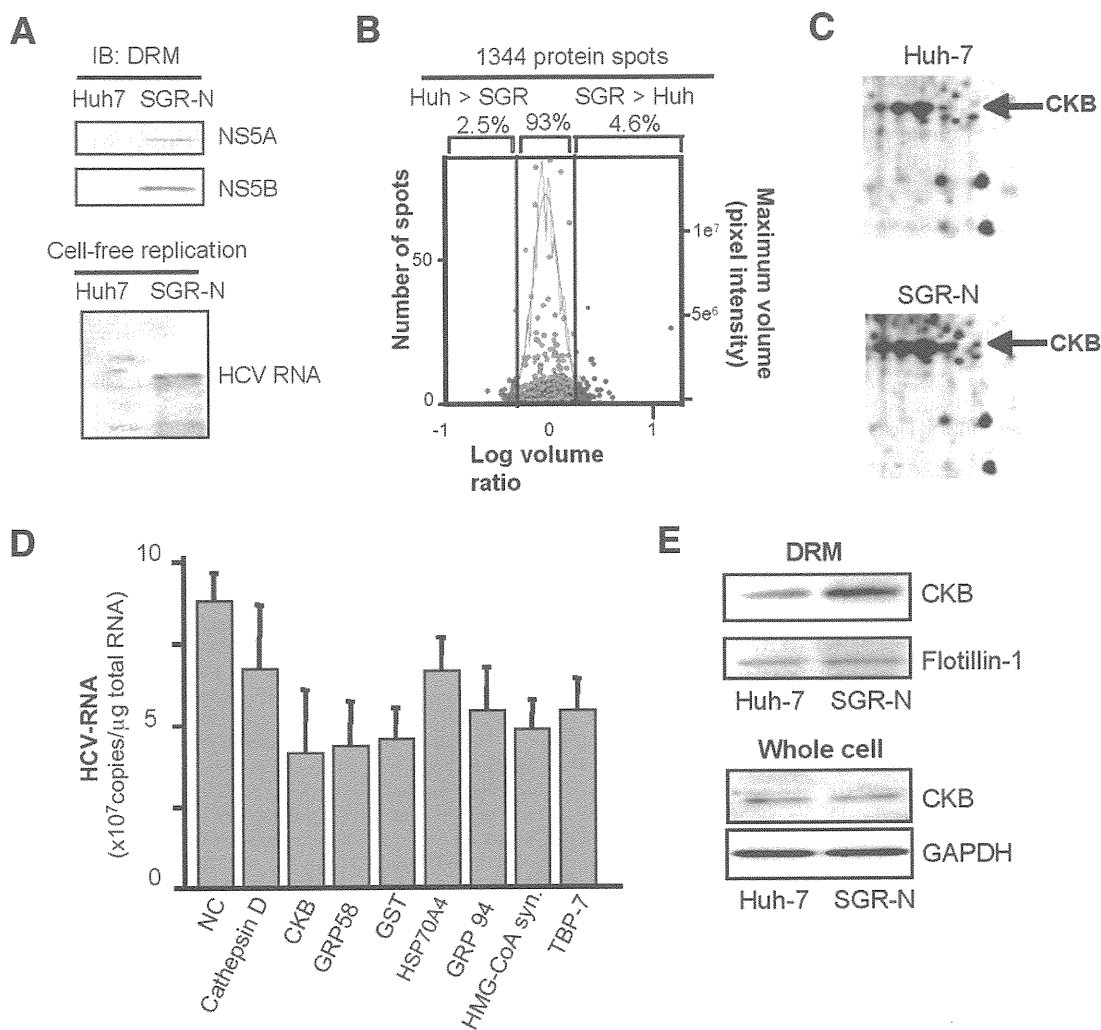


FIG. 1. Comparative proteomic analysis of DRM fractions and RNAi silencing. (A) Preparation of functionally active RC fraction for proteome analysis. DRM fractions obtained from SGR-N cells and parental Huh-7 cells were analyzed by immunoblotting with anti-NS5A and anti-NS5B antibodies (upper panel) and by the cell-free RNA replication assay (lower panel). (B) Histogram representation of proteins detected in 2D-DIGE. Images were analyzed quantitatively by the DeCyder software. The left and right y axis, respectively, indicate the spot frequency and the maximum volume of each spot, given against the log volume ratio (x axis). (C) Comparison of 2D-DIGE maps of proteins from DRM fractions of SGR-N cells and Huh-7 cells. Enlarged 2D-DIGE gel images of regions containing protein spots of CKB (arrows) are shown. (D) Effects of siRNAs of genes selected from comparative proteome analysis on HCV RNA replication. SGR-N cells were transfected with siRNA specific to cathepsin D, CKB (siCKB-1), GRP58, GST, Hsp70 protein 4, GRP94, HMG-coenzyme A synthase, or Tat binding protein 7 or with nontargeting (NC) siRNA. At 48 h posttransfection, total RNA was isolated and HCV RNA levels were assessed by real-time RT-PCR. (E) Enrichment of CKB in the DRM of HCV replicon cells. Equal amounts of DRM fractions from SGR-N and parental Huh-7 cells, or whole-cell lysates from both cells were analyzed by immunoblotting with antibodies against CKB, flotillin-1 or GAPDH.

cell-free replication assay (Fig. 1A). Three independent proteome experiments were performed for a reliable analysis of protein expression. Approximately 1,300 spots were resolved in each gel, and 4 to 5% of the protein spots represented a >2-fold increase in the membrane fraction of replicon cells in each experiment (Fig. 1B). The protein spots that exhibited high reproducibility (an example shown in Fig. 1C) were excised, digested by trypsin or lysyl endopeptidase, and analyzed by mass spectrometry, which identified the corresponding proteins in 27 cases (Table 1). Among the proteins implicated in a variety of functional categories, 10 were involved in protein folding, mainly as chaperones, 7 were metabolic and biosynthesis enzymes including proteins for redox regulation or en-

ergy pathways, 3 were involved in cytoskeleton organization, and 3 proteins were related to cellular processes, mainly proteolysis pathways. The viral NS proteins identified as differentially expressed proteins in the analysis were not listed.

In order to identify host factors involved in HCV replication, we examined the effects on viral RNA replication of transfection of SGR-N cells with siRNAs against genes encoding nine proteins belonging to diverse classes of biological functions (Table 1). Each siRNA reduced the HCV RNA level to 47 to 76% of the level of the siRNA control (Fig. 1D). None of the siRNAs tested exhibited considerable cytotoxicity against the replicon cells, ruling out overt toxicity as a mechanism for inhibition of viral RNA replication. Among the candidate

TABLE 1. Selected proteins that reproducibly increased in the DRM fraction of SGR-N cells^a

Avg ratio	P (Student <i>t</i> test)	Coverage (%)	Protein name	Molecular function	GI no.
5.56	0.04	27	GRP94	Protein folding	15010550
4.99	0.07	47	Hsp60	Protein folding	6996447
3.73	0.07	6	tRNA guanine transglycosylase	Metabolism	30583205
3.56	0.06	23	KIAA0088	Unknown	577295
3.32	0.07	4	Thioredoxin-related protein	Unknown	20067392
3.32	0.13	12	Tat binding protein 1 (TBP-1)	Cellular processes	20532406
3.06	0.14	22	Aldehyde dehydrogenase 1	Metabolism	2183299
3.06	0.14	14	Chaperonin TRiC/CCT, subunit 2	Protein folding	54696794
2.96	0.04	14	Heat shock 70-kDa protein 4 (HSPA4)	Protein folding	6226869
2.96	0.04	29	GRP58	Metabolism/protein folding	2245365
2.94	0.01	37	Mutant β -actin	Cytoskeleton organization	28336
2.65	0.17	33	Glutathione S-transferase (GST)	Catalytic activity	2204207
2.53	0.04	37	Keratin 19	Cytoskeleton organization	6226861
2.46	0.08	6	Heterogeneous nuclear ribonucleoprotein K	Nucleic acid modification	460789
2.45	0.001	13	HMG-coenzyme A synthase	Metabolism	30009
2.4	0.02	31	CKB	Energy pathway/metabolism	180570
2.4	0.02	11	Cathepsin D	Cellular processes	30582659
2.4	0.02	11	C8orf2	Unknown	37181322
2.36	0.1	38	Tropomyosin 4-anaplastic lymphoma kinase fusion protein	Cytoskeleton organization	14010354
2.36	0.1	6	Calreticulin	Protein folding	30583735
2.33	0.01	29	Quinolate phosphoribosyltransferase	Metabolism	30583301
2.29	0.04	25	Protein disulfide isomerase-related protein 5	Protein folding	1710248
2.29	0.04	16	Tat binding protein 7 (TBP-7)	Cellular processes	263099
2.05	0.11	24	Calumenin	Metabolism	2809324
2.05	0.12	10	TRiC/CCT, subunit 5	Protein folding	24307939
2.03	0.07	20	Hsp90 beta	Protein folding	34304590
2.01	0.07	10	TRiC/CCT, subunit 1	Protein folding	36796

^a The spectra obtained by tandem mass spectrometry were collected using data-dependent mode, and the results were subjected to database (NCBI) search by Mascot server software (Matrix Science, London, United Kingdom) for peptide assignment. Coverage, the ratio of the portion of protein sequence covered by matched peptides to the whole protein sequence. GI no., GenInfo identifier number.

genes examined, we observed a reproducible inhibition of HCV RNA replication by two independent siRNAs targeting CKB (see below).

CKB participates in HCV RNA replication and the propagation of infectious virus. CKB is a brain-type creatine kinase isoenzyme and is also detected in a variety of other tissues, including human liver (32). Steady-state levels of CKB in the DRM fraction, as well as in whole-cell lysate of SGR-N cells were compared to those from parental cells by Western blotting. The CKB level in the DRM fraction of replicon cells was higher than that in parental cells (Fig. 1E), confirming the results of the proteome analysis described above. In contrast, the CKB level in whole cells was similar in both cells (Fig. 1E). These results suggest participation of posttranslational modification, such as translocation to the DRM fraction, of CKB in replicon cells.

Figure 2A shows the inhibitory effect on HCV RNA replication of CKB siRNA; siCKB-2, the sequence of which does not overlap with the sequence of siCKB-1 used in the above siRNA screening (Fig. 1D). Transfection with siCKB-2 effectively decreased the cellular level of CKB enzymatic activity (data not shown), as well as the abundance of CKB protein (Fig. 2A), and resulted in 60% reduction in the viral RNA level in SGR-N cells compared to the cells treated with control siRNA. This inhibitory effect of siRNA on HCV RNA abundance was also observed in JFH-1-derived subgenomic replicon (SGR-JFH1) cells. The viral RNA level in the cells transfected with siCKB-2 decreased by 50% compared to the control (Fig. 2A). We also tested the CKB mutant, CKB-

C283S, in which Cys at aa 283, near the catalytic site, has been replaced with Ser (Fig. 3A) and which is known to be enzymatically inactive and to work in a dominant-negative manner (22, 29). As expected, overexpression of CKB-C283S resulted in a reduction in HCV RNA replication in SGR-N cells (Fig. 2B). We obtained a similar result in SGR-JFH1 cells, as described below (Fig. 3E).

To further examine the involvement of CKB in HCV RNA replication, we tested the effect of Ccr, a substrate analogue and possible inhibitor for CK in either SGR-N, SGR-JFH1 (Fig. 2C), or Huh7 cells transiently replicating luciferase-subgenomic replicon (data not shown). We found dose-dependent inhibition of HCV RNA replication but no observed effect on total cellular levels of protein and ATP (Fig. 2D) in the replicon setting used.

We next examined whether the knockdown of CKB or treatment with Ccr would abrogate the production of HCVcc. At 72 h posttransfection with siCKB-2, the HCV core level in cells infected with HCVcc was significantly reduced (Fig. 2E). Treatment of the infected cells with Ccr at various concentrations also reduced the intracellular and supernatant core level and subsequently decreased HCVcc production (Fig. 2F). These results demonstrate that suppression of the HCV RNA replication by the siRNA-mediated knockdown of CKB or treatment with CKB inhibitor leads to reduction of the production of infectious virus.

CKB interacts with HCV NS4A. Having established a role for CKB in HCV RNA replication, we then tried to determine to how CKB influences the HCV life cycle. It has been re-

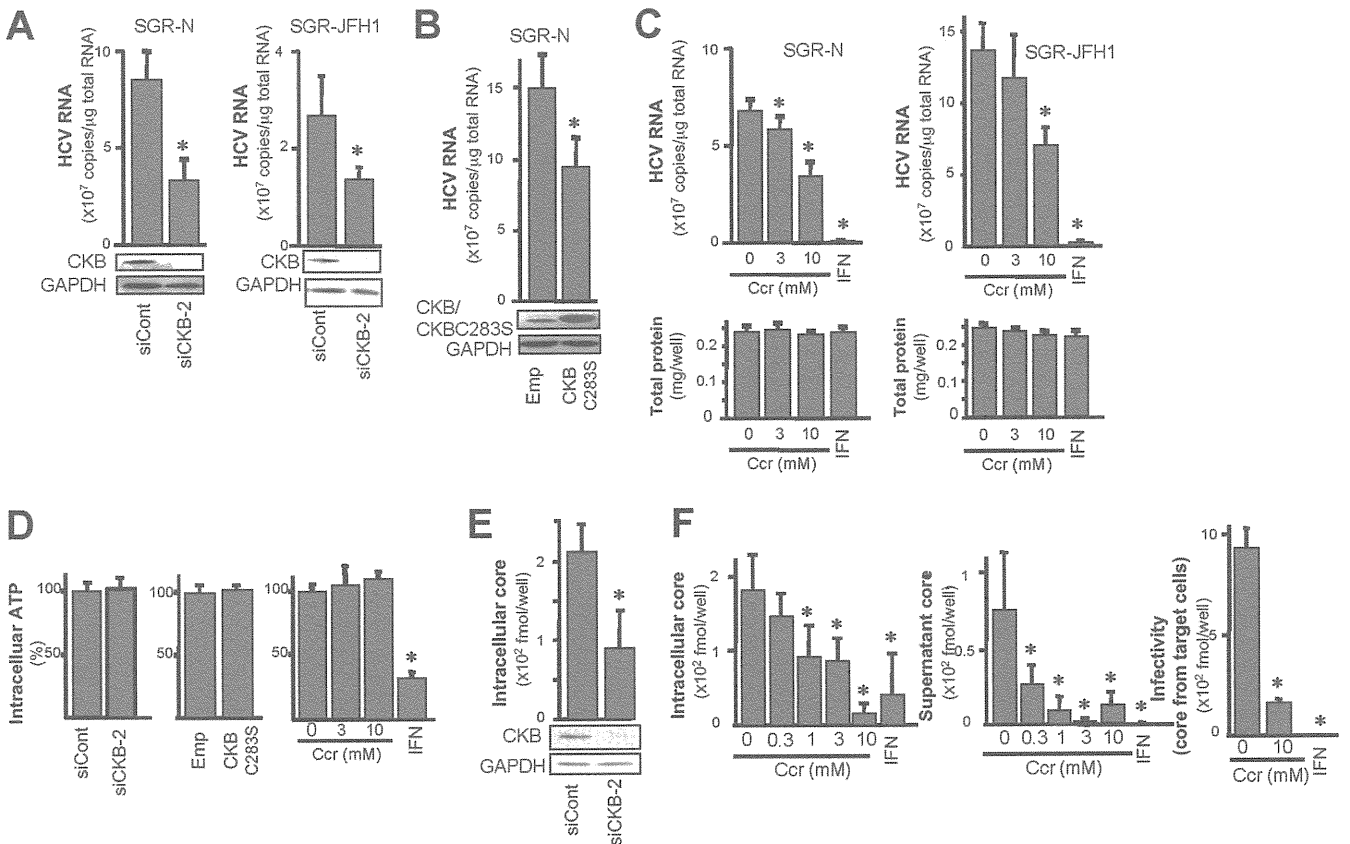


FIG. 2. Involvement of CKB in HCV replication. (A and E) Knockdown of endogenous CKB in SGR-N and SGR-JFH1 cells (A) or HCVcc-infected cells (E). Cells were transfected with siRNA against CKB (siCKB-2) or control siRNA (siCont) and were harvested at 72 h posttransfection. Real-time RT-PCR for HCV RNA levels and immunoblotting for CKB and GAPDH were performed. (B) SGR-N cells were transfected with pCAGCKB-C283S or empty vector, and HCV RNA levels and expression of CKB and CKB-C283S were determined 72 h posttransfection. SGR-N and SGR-JFH1 cells (C) or HCVcc-infected cells (F) were treated with Ccr at various concentrations for 72 h, followed by quantification of HCV RNAs and total cellular proteins. ATP levels (D) were determined after transfection with siCKB-2, pCAGCKB-C283S, or treatment with Ccr for 72 h in SGR-N cells. The ATP levels in the cells transfected with negative control siRNA (left), empty vector (middle), and no treatment (right) were set at 100%, respectively. (F) HCVcc-infected cells were treated with Ccr, and the viral core protein levels in cells (left) and supernatants (middle) were determined at 72 h postinfection. Collected culture supernatants were inoculated into naive Huh-7.5.1 cells after the removal of Ccr. After 72 h, the core proteins in cells were determined (right panel). All data are presented as averages and standard deviation values for at least triplicate samples. *, $P < 0.05$ against control such as transfection with siCont (A and E) or empty vector (B) or nontreatment (C, D, and F).

ported that interaction of CKB with some cellular proteins is required for local availability of CKB activity and local generation of ATP (22, 29). To examine the possible interaction of CKB with HCV NS proteins, HA-tagged CKB (HA-CKB) was coexpressed with FLAG-tagged NS proteins (NIHJ1 strain), followed by immunoprecipitation with an anti-FLAG antibody. CKB was shown to specifically interact with NS4A. No or little interaction was observed between CKB and either NS3, NS4B, NS5A, or NS5B (Fig. 3B). CKB-NS4A interaction was also found with the JFH-1 strain (Fig. 3C).

To identify the CKB region required for the interaction with NS4A, various deletion mutants of CKB were generated (Fig. 3A). An immunoprecipitation assay indicated that NS4A was coimmunoprecipitated with either a full-length CKB, a C-terminal deletion (aa 1 to 357), an N-terminal deletion (aa 297 to 381), or CKB-C283S, but not with aa 1 to 296, aa 1 to 247, or aa 1 to 184 (Fig. 3D, upper middle panel). Further, internal deletions of CKB (CKBdel297-357 and CKB-C283Sdel297-357) failed to interact with NS4A (Fig. 3D, lower panel), sug-

gesting that aa 297 to 357 of CKB are important for its interaction with NS4A. It is noted that the expression of CKB aa 297 to 357 in cells was undetected, presumably due to its misfolding and/or instability. To verify a role for CKB-NS4A interaction in HCV RNA replication, we further determined the effect of expression of either CKB-C283S or its internal deletion lacking aa 297 to 357 (CKB-C283Sdel297-357) on viral replication in SGR-JFH1 cells. As expected, the HCV RNA level was significantly decreased by CKB-C283S, whereas this effect was not observed by CKB-C283Sdel297-357 (Fig. 3E).

NS4A is a 54-residue small protein composed of three domains: the N-terminal membrane anchor, the central domain responsible for interacting with NS3, and the C-terminal acidic domain. To define the portion in NS4A responsible for its interaction with CKB, we constructed three NS4A deletion mutants, each separately expressing one of the NS4A domains, with a FLAG tag (Fig. 3F). CKB proved to interact with the central domain, aa 21 to 39, of NS4A, which is involved in

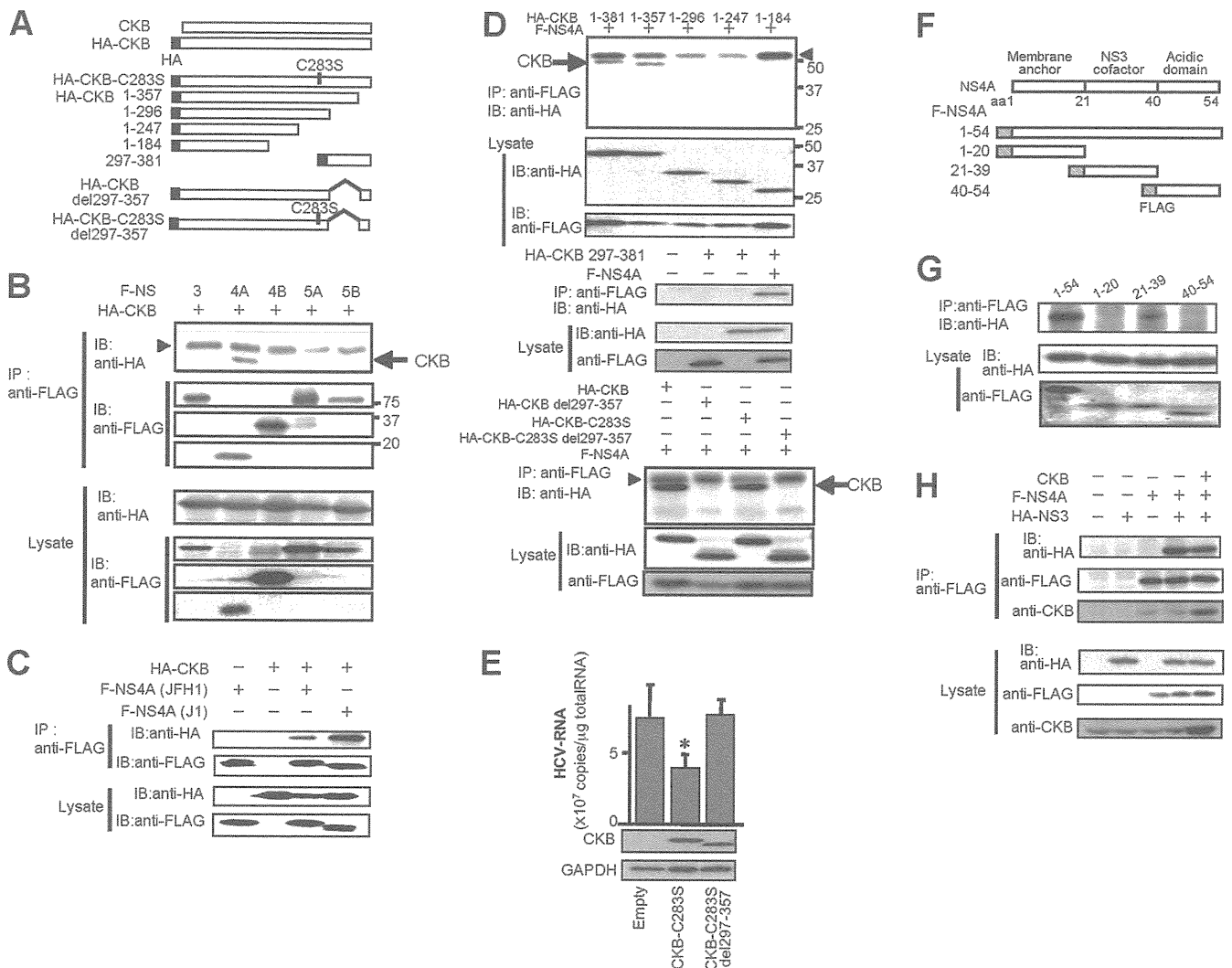


FIG. 3. CKB interacts with HCV NS4A. (A) Structures of CKB constructs used in the present study. A full-length wild-type CKB without an epitope tag (CKB) or with an N-terminal HA tag (HA-CKB), HA-CKB with deletions (aa 1 to 357, aa 1 to 296, aa 1 to 247, aa 1 to 184, and aa 297 to 381 and del297-357), CKB mutant at the catalytic site, Cys-283 (CKB-C283S) or CKB-C283S lacking aa 297 to 357 (CKB-C283Sdel297-357) are shown. HA-CKB was coexpressed with FLAG-tagged versions of each NS protein of strain NIHJ1 (B) or with NS4A of strain JFH-1 (C) in 293T cells and immunoprecipitated (IP) with an anti-FLAG antibody. Immunoprecipitates were subjected to immunoblotting (IB) with anti-HA or anti-FLAG antibody. (D) Each CKB deletion mutant was coexpressed with FLAG-NS4A in 293T cells. Immunoprecipitates were analyzed by immunoblotting. Arrow, CKB; arrowhead, immunoglobulin heavy chain. (E) SGR-JFH1 cells were transfected with the expression plasmid for CKB-C283S, CKB-C283Sdel297-357 or empty vector. At 72 h posttransfection, HCV RNA levels and the expression of CKB and CKB-C283S were determined by real-time RT-PCR and immunoblotting with anti-HA antibody, respectively. For HCV RNA quantitation, data are indicated as averages and standard deviations ($n = 3$). *, $P < 0.05$ against the empty vector control. (F) Structure of NS4A and NS4A constructs. FLAG-tagged NS4A (aa 1 to 54) or its truncated mutants (aa 1 to 20, aa 21 to 39, or aa 40 to 54) are shown. (G) Each NS4A deletion mutant was coexpressed with HA-CKB and analyzed as described above. (H) FLAG-NS4A was coexpressed with HA-NS3 or HA-NS3 and CKB, followed by immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were analyzed by immunoblotting with anti-HA, anti-FLAG or anti-CKB antibody.

formation of the NS3-NS4A complex (Fig. 3G). We therefore investigate whether NS3-NS4A interaction is affected in the presence of CKB and found that exogenous expression of CKB has no influence on NS3-NS4A interaction, and a putative NS3-NS4A-CKB complex was detected in the coimmunoprecipitation analysis (Fig. 3H). Collectively, these results strongly suggest that CKB plays a key role in HCV RNA replication via interaction with NS4A.

Subcellular localization of CKB and NS4A in cells replicating HCV RNA. CKB is distributed throughout cells but is mainly localized in the perinuclear area (31), whereas NS4A is

predominantly localized at the endoplasmic reticulum and mitochondrial membranes (37). We examined the possible subcellular colocalization of CKB and NS4A in SGR-N cells by immunofluorescence staining (Fig. 4A). CKB tended to gather in the perinuclear area of HCV replicating cells and was partially colocalized with NS4A in the area, sharing a dotlike structure. To further analyze the subcellular compartments in which CKB and NS4A coexist, we used double-labeling immunoelectron microscopy on SGR-N cells using antibodies against CKB and NS4A, with secondary antibodies coupled to 12- and 18-nm gold particles, respectively. One fraction of

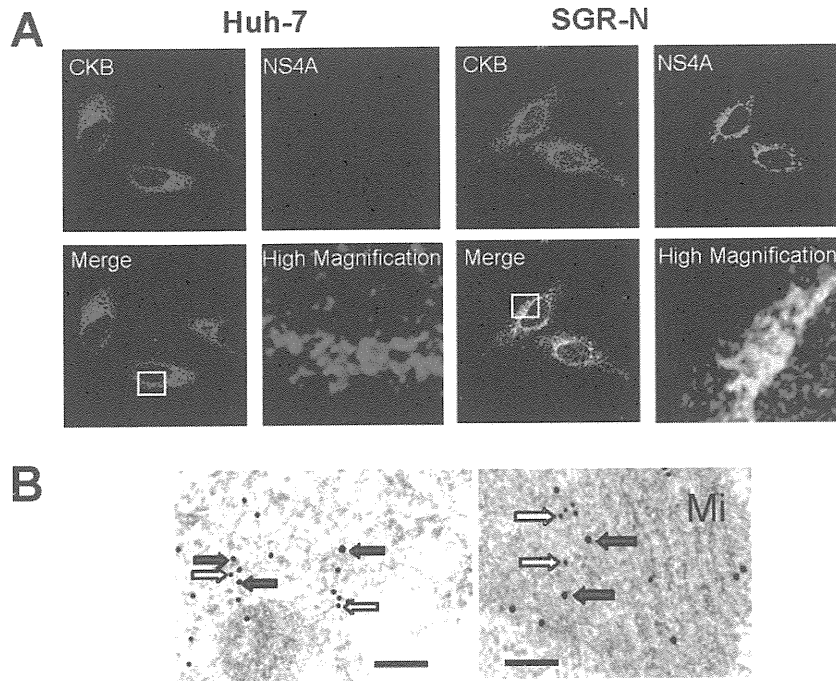


FIG. 4. Colocalization of CKB with HCV NS4A. (A) Indirect immunofluorescence analysis. The primary antibodies used were anti-CKB goat PAb (red) and anti-NS4A MAb (green). Merged images of red and green signals are shown. High-magnification panels are enlarged images of white squares in the merge panels. (B) Immunoelectron microscopic localization of CKB and NS4A. SGR-N cells were double-immunolabeled for CKB (12-nm gold particles; white arrows) and for NS4A (18-nm gold particle; gray arrows). Mi, mitochondria. Bars, 200 nm.

CKB colocalized with NS4A in the cytoplasmic electron-dense regions, presumably derived from altered or folded membrane structures (Fig. 4B, left panel) and mitochondria (Fig. 4B, right panel).

CKB enhances functional HCV replicase and NS3-4A helicase. NS4A is known to mediate membrane association of the NS3-4A complex and to function as a cofactor in NS3 enzyme activity. To understand the mechanism(s) underlying positive regulation of HCV RNA replication through CKB via its interaction with NS4A, we first investigated whether CKB modulates NS3-4A helicase activity. NS3-4A helicase is a member of the superfamily-2 DexH/D-box helicase, which unwinds RNA-RNA substrates in a 3'-to-5' direction. During RNA replication, the NS3-4A helicase is believed to translocate along the nucleic acid substrate by changing its protein conformation, utilizing the energy of ATP hydrolysis (9). We then tested the effect of CKB on RNA- or DNA-unwinding activity using purified recombinant full-length NS3 and NS3-4A complex (12). As shown in Fig. 5A (left middle panel), both NS3 and NS3-4A helicase activity unwound dsRNA substrate most efficiently when CKB, ATP, and pCr were added to the reaction mixture. The enhancing effect of CKB was observed in the presence of pCr but not in the absence of it, suggesting that catalytic activity of CKB is important for its effect on the HCV helicase activity. Similar results were obtained from the DNA helicase assay using dsDNA substrate (Fig. 5B). To address the specificity of the stimulation by the CKB/pCr system, effects of PK and pPy, which are also involved in the ATP generation, were determined (Fig. 5A, right panels). Exogenous PK and pPy at the same concentrations as those of CKB and pCr

used in the study exhibited no effect on the HCV helicase activity.

The effect of CKB on NS3-4A serine protease activity, which is considered to be ATP-independent, was also assessed in an *in vitro* protease assay using the purified viral proteins as mentioned above (Fig. 5C). As expected, NS3-4A complex exhibited significantly higher activity than NS3 alone; however, CKB did not affect the protease activities of NS3 or NS3-4A.

Finally, we investigated loss and gain of function of CKB in HCV replicase activity, which requires high-energy phosphate, in the context of semi-intact replicon cells. Miyanari et al. (33) reported that the function of the active HCV RC can be monitored in permeabilized replicon cells treated with digitonin. Thus, permeabilized replicon cells in the presence or absence of exogenous CKB were incubated with [α - 32 P]UTP to detect newly synthesized RNA. As indicated in Fig. 5D, an ~8-kb band corresponding to HCV subgenomic RNA was most abundant in cells in the presence of exogenous CKB, ATP and pCr. The enhancing effect of CKB was observed in the presence but not in the absence of pCr, suggesting that catalytic activity of CKB is important for its effect on the replicase activity. As for the RNA helicase assay, exogenous PK and pPy did not enhance the replicase activity (data not shown). HCV replicase activity in permeabilized cells to which we had introduced siCKB-2 was diminished compared to that in siRNA control-treated cells. Interestingly, the replicase activity in the CKB-depleted cells was recovered by the addition of CKB. Thus, our findings suggest that CKB functions as a key regulator of HCV genome replication by controlling energy-dependent viral enzyme activities.

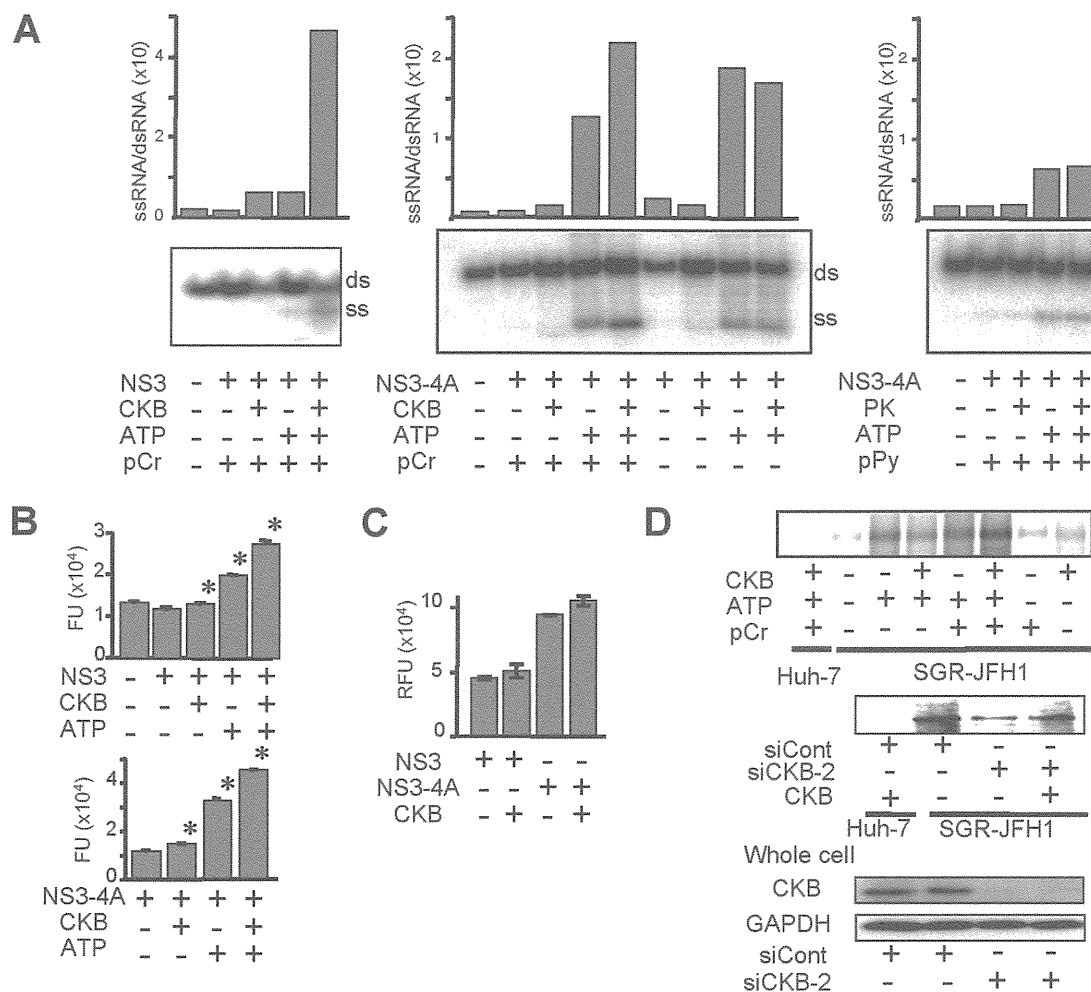


FIG. 5. CKB enhances NS3-4A helicase and HCV replicase activities. (A) In vitro RNA helicase activity of NS3-4A or NS3 was determined by detecting unwound single-strand RNA (ss) derived from the partially dsRNA substrate (ds). Band intensities corresponding to unwound products and those to dsRNA substrates were determined by ImageQuant 5.2 (Molecular Dynamics), and the ssRNA/dsRNA ratios were calculated. The results are representative of three similar experiments. (B) In vitro DNA helicase activity of NS3-4A or NS3 was analyzed by using a commercially available kit. The data represent averages and standard deviations ($n = 3$). *, $P < 0.05$ against the value without supplementation of CKB and ATP. (C) The in vitro HCV protease activity of NS3-4A or NS3 in the presence or absence of CKB was analyzed. Error bars represent standard deviations ($n = 3$). (D) Replicase activity in permeabilized replicon cells. The upper panel shows the activity for synthesis of HCV subgenomic RNA in the digitonin-permeabilized SGR-JFH1 cells with or without supplementation of CKB was measured. The middle panel shows results for SGR-JFH1 or Huh-7 cells that were transfected with siCKB-2 or siCont and permeabilized at 72 h posttransfection. The permeabilized cells with or without supplementation of CKB were subjected to the replicase assay. The lower panel shows the immunoblotting results for whole-cell lysates of siRNA-transfected cells.

DISCUSSION

Viral replication requires energy and macromolecule synthesis, and host cells provide the viruses with metabolic resources necessary for their efficient replication. Thus, it is highly likely that interaction of viruses with host cell metabolic pathways, including energy-generating systems, contributes to the virus growth cycle. In the regulation of HCV genome replication, the functions of the viral NS proteins that comprise the RC might be regulated by association in individual host cell factors. For example, hVAP-A and -B function as cofactors of modulating RC formation via interacting with NS5A and NS5B (13, 18). Cyclophilin B is involved in stimulating viral RNA binding activity via interacting with NS5B (49). FKBP8 (39) and hB-ind1 (45) play an important role in recruiting Hsp90 to

RC via interacting with NS5A. However, the association of viral protein(s) with the cellular energy-generating system to directly regulate the activity of the RC has not been well understood.

In the present study, the accumulation of CKB, an ATP-generating enzyme, in the HCV RC-rich membrane fraction of viral replicating cells and its importance in replication of the HCV genome and production of infectious virions have been demonstrated. Enzymatic analyses with semi-intact replicon cells and purified NS3-4A protein revealed that CKB enhances the functional replicase and helicase of HCV. Its enhancing effect was observed in the presence of pCr but not in its absence, suggesting that the catalytic activity of CKB is important for enhancing the replicase and

helicase activities. Moreover, we clearly detected a CKB-NS4A complex using anti-tag antibodies in cotransfection experiments, but the endogenous complex could not be immunoprecipitated from cells expressing only endogenous levels of CKB, probably because of the inefficiency of the available antibodies. Further, a deletion of the NS4A-interacting region within an inactive mutant of CKB (CKB-C283S) resulted in the loss of its dominant-negative effect on HCV replication.

Creatine kinase, an evolutionarily conserved enzyme, is known to be critical for the maintenance and regulation of cellular energy stores in tissues with high and rapidly changing energy demands (48). In mammals, three cytosolic and two mitochondrial isoforms of CK, which share certain conserved regions, are expressed (35). The brain-type CK, CKB, plays a major role in cellular energy metabolism of nonmuscle cells, reversibly catalyzing the ATP-dependent phosphorylation of creatine and, hence, providing an ATP buffering system in subcellular compartments of high and fluctuating energy demand (21, 29). CKB is overexpressed in a wide range of tumor tissues and tumor cell lines, including hepatocellular carcinoma (32), and is used as a prognostic marker of cancer.

Although CK and creatine phosphate have been supplemented to *in vitro* replicase assays of some RNA viruses (15, 33), understanding of CKB function in the virus life cycle has been limited. One study indicated that the CK substrate analog, Ccr, exhibits antiviral activity against several herpesviruses but not influenza viruses or vesicular stomatitis virus (26). We have demonstrated here that HCV genome replication is downregulated by either treatment with Ccr, siRNA-mediated knockdown of CKB, or the exogenous expression of CKB-C283S. Coimmunoprecipitation experiments revealed that the essential domain within NS4A for the interaction with CKB is the NS4A central domain, aa 21 to 39, which is also responsible for NS3-4A complex formation. However, the NS3-4A interaction was not impaired by overexpression of CKB, and CKB was found to be able to form a complex with NS3-4A (Fig. 3H). Since CKB does not directly interact with NS3 (Fig. 3A), it is likely that NS3-4A-CKB association occurs through two interactions of NS3-4A and NS4A-CKB. We examined whether the formation of the ternary complex affects HCV enzymatic activities, possibly through conformational changes in the viral proteins, and found that CKB has no influence on NS3-4A protease activity (Fig. 5C). With regard to helicase activity, the effect of CKB on RNA unwinding activity by NS3-4A was similar to the effect of NS3 alone in the presence of ATP (Fig. 5A). It is conceivable that interaction with CKB causes no or little global change in the NS3-4A conformation and does not affect the viral helicase and protease activities.

In general, translation initiation in eukaryotes includes an ATP-dependent process such as unwinding the secondary structure in the 5'-untranslated region to permit assembly of 48S ribosomal complexes. It was reported, however, that 48S complex formation on the HCV internal ribosome entry site (IRES) has no requirement for ATP hydrolysis (25). In fact, we found that Huh-7 cells with or without gene silencing of CKB exhibited the same level of HCV IRES activity by transfection with IRES-reporter constructs (data not shown).

Collectively, we conclude that CKB is targeted to the HCV RC through its interaction with NS4A and functions as a pos-

itive regulator for the viral replicase by providing ATP. It is likely that the catalytic activity of CKB that associates with the viral RC is important for enhancing the RNA replication. The role of CKB-NS4A interaction in the enhancing effect seems to be limited. Although either knocking down CKB, expression of the dominant-negative mutant of CKB, or Ccr treatment resulted in the reduction of HCV replication (Fig. 2A to C), the total cellular ATP levels were not changed under these conditions (Fig. 2D). This suggests that CKB contributes to enhancing HCV replication through controlling the ATP level in the particular RC compartment. A tight coupling of a fast ATP regeneration and delivery system to the viral RC is advantageous for achieving efficient replication of the viral genome. To our knowledge, the findings presented here provide the first experimental evidence of the involvement of viral protein in recruiting an ATP generating/buffering system to the subcellular compartment for viral genome replication, a site with high-energy turnover. Given that the levels of HCV RNA were not dramatically diminished by the knocking down, dominant-negative mutant or Ccr, CKB may not be absolutely critical for the viral replication. One would argue that energy required for HCV genome replication can be partly complemented from the intracellular ATP pool.

Although there are several isoforms of CK as described above, the most abundant CK species expressed in Huh-7 cells in the present study was CKB, and no other isoenzymes, including mitochondrial CK, were detected by an isoform analysis based on the overlay gel technique (32; data not shown). Thus, the CKB isoenzyme appears to be a key molecule in the energy metabolism of HCV replicating cells. To identify potential HCV RC components, we used a comparative proteome analysis of the DRM fraction in cells harboring HCV subgenomic replicon and the DRM fractions in parental cells and then identified proteins that were more abundant in the fraction of HCV replicating cells. In agreement with similar previously reported approaches using the DRM or lipid raft fraction (30, 53), the functional categories of identified proteins included protein folding or assembly, cell metabolism and biosynthesis, cellular processes, and cytoskeleton organization (Table 1). Interestingly, Mannova et al. found that CKB was upregulated in the fraction of Huh-7 cells carrying the genotype 1b Con1 isolate-derived HCV replicon, as determined using stable isotope labeling by amino acids combined with one-dimensional electrophoresis (30). However, the effect of CKB on regulation of the HCV life cycle was not examined in that study.

In conclusion, CKB interacts with HCV NS4A and is important for efficient replication of the viral genome. Recruitment of CKB to the HCV replication machinery through its interaction with NS4A may have important implications for the maintenance or enhancement of the functional replicase activity in the RC compartment, where high-energy phosphoryl groups are required. A strategy for specific interception of energy supply at the subcellular site of HCV genome replication by disruption of the NS4A-CKB interface may lead to development of a new type of antiviral agent.

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Evaluation of Hepatitis C Virus Core Antigen Assays in Detecting Recombinant Viral Antigens of Various Genotypes[∇]

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A single substitution within the hepatitis C virus core antigen sequence, A48T, which is observed in ~30% of individuals infected with genotype 2a virus, reduces the sensitivity of a commonly used chemiluminescence enzyme immunoassay. Quantitation of the antigen is improved by using a distinct anticore antibody with a different epitope.

Hepatitis C virus (HCV) is a major cause of chronic liver disease throughout the world. Accurate diagnosis of HCV infection is important due to the morbidity associated with the virus, and determining the level of viral replication is important in predicting and monitoring the effect of antiviral treatment. Although quantifying viral RNA represents the standard method for identifying active infection (5, 8, 13), several sensitive immunoassays that detect the viral core antigen (Ag) have now been developed as an alternative to HCV RNA testing (3, 4, 6, 9, 10, 12, 16). The amino acid sequence of the core Ag is largely conserved among different viral isolates (14); however, genetic variability of the virus constitutes one of the major challenges to using core Ag assays for diagnosis. In this study, we examined the effects of sequence heterogeneity on the sensitivity of diagnostic kits for detection of the core Ag by using recombinant Ag derived from each of the major HCV genotypes. Expression plasmids for epitope-tagged core Ag were generated by inserting cDNA for the full-length core region of genotype 1a (17; GenBank accession no. AF011751), 1b (1; D89815), 2a (7; AB047639), 2b (AB030907), or 3a virus, with a FLAG tag sequence attached at its 5' end, into the EcoRI site of the pCAG mammalian expression vector (11). HEK293T cells transiently transfected with the expression plasmids were harvested 48 h after transfection using a passive lysis buffer (Promega, Madison, WI). Centrifugation was performed to remove the debris after ultrasonication. Total protein was quantified in aliquots of cell lysate by using the bicinchoninic acid method (Pierce, Rockford, IL) and then used for determining the concentrations of HCV core Ag.

Figure 1A shows comparable levels of core Ag in each sample of cell lysate, as determined by immunoblotting with anti-FLAG antibody (Ab). The ability of HCV core Ag assays to detect five different HCV genotypes were compared using a commercially available chemiluminescence enzyme immuno-

assay (CLEIA) (Lumipulse II HCV core assay [assay detection range, approximately 50 to 50,000 fmol/liter]; Fujirebio, Japan) (15) and enzyme-linked immunosorbent assay (ELISA) (Ortho HCV Ag ELISA test [assay detection range, approximately 44.4 to 3,600 fmol/liter]; Ortho-Clinical Diagnostics, Japan) (2) to detect HCV core Ag in cell lysate. As shown in Fig. 1B,

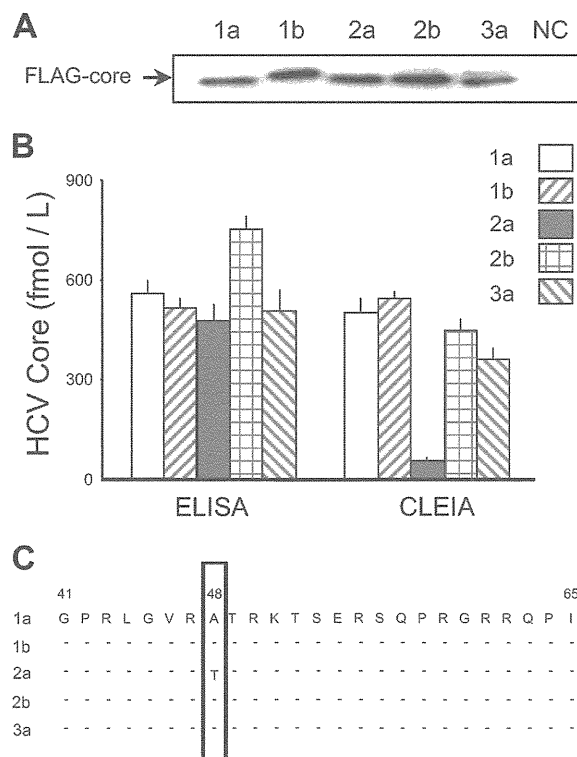


FIG. 1. Detection of recombinant HCV core Ag derived from genotype 1a, 1b, 2a, 2b, and 3a isolates by immunoblotting using an anti-FLAG Ab (A) as well as ELISA and CLEIA (B). The data shown in panel B represent the mean values and standard deviations ($n = 3$). NC, negative control. (C) The amino acid sequence from amino acids 41 to 65 of the core Ag used in this study. Key residues at the 48th position are boxed. Hyphens indicate conservation.

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TABLE 1. Comparison of the 48th residues of HCV core Ags of genotypes 1a, 1b, 2a, 2b, and 3a

Genotype	No. of isolates	No. (%) of isolates with residue at 48th position		
		T	A	Other
1a	263	9 (3.5)	254 (96.5)	0 (0)
1b	298	2 (0.7)	294 (98.6)	2 (0.7)
2a	17	5 (29.5)	12 (70.5)	0 (0)
2b	17	0 (0)	17 (100)	0 (0)
3a	23	0 (0)	23 (100)	0 (0)
Total	618	16 (2.6)	600 (97.1)	2 (0.3)

although the ELISA measured similar concentrations of core Ag in all samples, apparent low levels of the genotype 2a core Ag, originally from an isolate known as the JFH-1 isolate (7), were detected using the CLEIA method, suggesting that some differences in the amino acid sequences corresponding to particular HCV genotypes or isolates may influence the sensitivity of core Ag detection. A comparison of the core Ag sequences, including the monoclonal Ab epitopes used in the development of CLEIA, revealed conservation of alanine at the 48th position in four clones, of genotypes 1a, 1b, 2b, and 3a, but not genotype 2a, for which there is a threonine at this position (Fig. 1C). Based on our analysis of sequences available from the HCV database (<http://hcv.lanl.gov/content/sequence/NEWALIGN/align.html>), alanine is highly conserved at the 48th residue of the core Ag for HCV isolates of genotypes 1a, 1b, 2b, and 3a (Table 1). In contrast, alanine and threonine occur in this position in 70.5% and 29.5%, respectively, of genotype 2a isolates. To examine whether the low sensitivity of the CLEIA method might be due to this particular amino acid change, we next replaced threonine with alanine at the 48th position of the JFH-1 core Ag (for

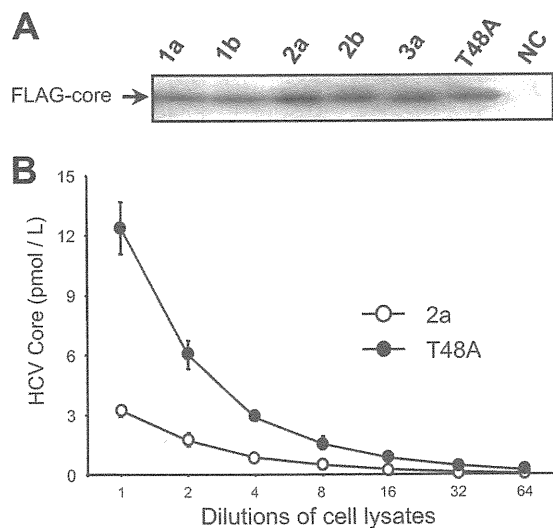


FIG. 2. Effect of T48A substitution in the core Ag of the JFH-1 isolate with regard to sensitivity of the CLEIA method. Samples of wild-type or mutated core Ag cell lysate were analyzed by immunoblotting (A) and CLEIA (B). The data shown in panel B represent the mean values and standard deviations ($n = 3$). NC, negative control.

TABLE 2. Comparison of the modified CLEIA with the original version for detection of the core Ags of genotypes 1a, 1b, 2a, 2b, and 3a^a

Genotype	CLEIA	HCV core antigen concn (fmol/liter) in serially diluted cell lysates at indicated fold dilution							
		1	2	4	8	16	32	64	128
1a	Original	11,147	5,527	2,611	1,484	691	403	195	101
	Modified	10,511	5,700	2,676	1,420	716	444	200	111
1b	Original	11,612	5,618	3,081	1,551	779	409	223	113
	Modified	11,192	6,028	2,824	1,522	804	431	197	101
2a	Original	3,216	1,710	844	480	232	104	48	36
	Modified	12,101	6,255	3,153	1,676	805	422	212	106
2b	Original	10,559	5,635	2,811	1,286	762	387	194	94
	Modified	10,977	6,179	3,381	1,624	842	437	219	129
3a	Original	11,478	5,891	2,922	1,414	756	422	212	112
	Modified	11,208	6,225	3,126	1,555	791	445	215	100

^a Data represent the mean values in triplicate measurements.

the mutant JFH-1coreT48A) and measured the HCV core Ag concentration in cells expressing both mutated and wild-type JFH-1 core Ag. After confirming comparable levels of FLAG-tagged core Ag in the cell lysate samples by immunoblotting (Fig. 2A), HCV core Ag was quantified in the samples by serial dilution via the CLEIA method. As shown in Fig. 2B, the core Ag concentrations of JFH-1coreT48A were assessed to be 3.2- to 3.8-fold higher than those of the wild-type core Ag, suggesting that the sensitivity of HCV core Ag detection may have been affected by the 48th residue in the core Ag. Data for samples derived from genotypes 1a, 1b, 2b, and 3a were analogous to data for JFH-1coreT48A (data not shown). Although HCV isolates with threonine at the 48th position of the core Ag sequence comprise a relatively small proportion of the major genotype population, only 2.6% of the genotype 1a, 1b, 2a, 2b, and 3a isolates here (16 of 618 isolates; Table 1), attempts to overcome this problem would improve the overall sensitivity and usefulness of the assay. To achieve this aim, another monoclonal anticore Ab, whose epitope is comprised of amino acids 50 to 65, which are completely conserved among all the genotypes examined (Fig. 1C), was therefore used as a second Ab in a modified version of the CLEIA. We compared this modified assay with the original version by measurement of core Ag concentrations of the various genotypes (Fig. 2A) as illustrated in Table 2. The modified assay was able to quantify core Ag from genotypes 1a, 1b, 2a, 2b, and 3a with no significant differences observed between Ag levels in samples from different genotypes at each dilution.

It has been demonstrated that the HCV core Ag assay is a useful alternative to HCV RNA quantification for the diagnosis of hepatitis C and for monitoring the antiviral effects of treatment. Compared to various reverse transcription-PCR methods, HCV core assays are less expensive and easier to perform, without the requirement of sophisticated laboratory equipment and specially trained laboratory personnel. In addition, the core Ag assay can be used to measure a more diverse set of blood samples, such as sera stored for a long period of time, because the viral Ag is generally more stable than the RNA in sera or plasma. Despite the adequate performance of core Ag assays, we have shown that a single amino acid substitution at the 48th position of the core Ag changes the detection sensitivity. It is also noted that, although the original CLEIA should be improved, the ELISA used in this study may be substituted for it.

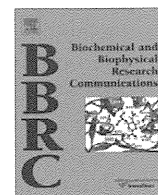
In conclusion, we have identified a distinct anticore Ab with a different epitope that might enable improved detection across all of the major HCV isolates. The findings of this study would provide useful information for the development of an improved assay with greater accuracy.

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Copy number of adenoviral vector genome transduced into target cells can be measured using quantitative PCR: Application to vector titration

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ABSTRACT

Both transfection and adenovirus vectors are commonly used in studies measuring gene expression. However, the real DNA copy number that is actually transduced into target cells cannot be measured using quantitative PCR because attached DNA present on the cell surface is difficult to distinguish from successfully transduced DNA. Here, we used Cre/loxP system to show that most of the transduced DNA was in fact attached to the cell surface; in contrast, most of the viral vector DNA used to infect the target cells was present inside the cells after the cells were washed according to the conventional infection protocol. We applied this characteristic to adenoviral vector titration. Current methods of vector titration using the growth of 293 cells are influenced by the effect of the expressed gene product as well as the cell conditions and culture techniques. The titration method proposed here indicates the copy numbers introduced to the target cells using a control vector that is infected in parallel (relative vector titer: rVT). Moreover, the new titration method is simple and reliable and may replace the current titration methods of viral vectors.

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1. Introduction

Transfection is the most commonly used method of choice for examining the nature and function of a gene *in vivo* because this technique is very simple to perform and easy to manipulate. However, the copy numbers of DNA that successfully reach the inside of the target cells cannot be measured using quantitative PCR (qPCR), since experiments using qPCR cannot effectively distinguish DNA present inside the cells from DNA attached to and present on the cell surface. The first-generation adenovirus vector (FG AdV) is now commonly used for gene expression experiments, mainly because the resulting expression level is much higher than that achieved using transfection. Another reason is that the data offered by this vector is quantitative for a linearity range that is about 20-fold wider [1]. However, the vector system is also thought to be unsuitable for qPCR for the same reason mentioned above.

There are several methods for using FG AdV. The most popular titration methods are bioassays of plaque-forming unit (PFU) [2,3] and end-point cytopathic effect (CPE) assay or 50% tissue-culture

infectious dose (TCID₅₀) assay [3,4]. These methods were actually developed for the titration of wild-type adenoviruses, and not for the titration of FG AdV. At least 4 days or up to 2 weeks are required to obtain the endpoint, and the results often vary depending on the conditions of the 293 cell lines, researchers and laboratories. The immunofluorescent focus assay using a fluorescent microscope [5,6] and the immunospot assay using 3,3'-diaminobenzidine staining [7] (TaKaRa Bio kit), count the foci of infected 293 cells expressing viral hexon protein. Although the titration can be completed in 2 days, these methods also rely on viral replication in 293 cells. The amount of AdV particles has been measured based on the optical density at 260 nm (OD₂₆₀) [8], although this method can only be used for purified virus stock. Because the AdVs replicate rapidly in growing 293 cells in all these methods, the titration results are sometimes influenced by the expressed product of an inserted gene if it disturbs viral replication or the growth of 293 cells. Consequently, sometimes the results do not reflect the actual copy number that was transferred to the target cells, which is undoubtedly the most important ability of a "vector".

qPCR has been used to calculate the copy numbers of AdV in viral stocks [9]. In the preparation of helper-dependent AdV (HD AdV), the contaminated helper virus (an FG AdV) in the viral stock has been measured using qPCR [10,11]. Another category of the qPCR method obtains the viral titer not by measuring AdV DNA in the viral stock, but by quantifying the copy numbers of *transduced viral genomes in the target cells* (genomic infectious titer,

Abbreviations: FG, first-generation; AdV, adenoviral vector; PFU, plaque-forming unit; TCID₅₀, 50% tissue-culture infectious dose; CPE, cytopathic effect; qPCR, quantitative real-time PCR; HD-AdV, helper-dependent AdV; GIT, genomic infectious titer; rVT, relative vector titer; MOI, multiplicity of infection; NLS, nuclear localization signal; OTC, Ornithine transcarbamylase.

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GIT); the total DNA of the infected cells were extracted, and the viral DNA were detected using slot-blot hybridization [12] or qPCR [10,13]. Although all these methods are intended to measure the copy number of the viral genome, there are two problems with using them for the titration of FG AdV. One reason is similar to that described above for transfection but is more crucial: the obtained copy numbers include not only the viral genome of the internally transduced viral particles, but also the DNA of *non-infectious particles* and unpackaged naked DNA that is present either freely in the viral stock or attached to the surface of the target cells. The other problem is that the GIT fluctuates markedly depending on the target cell concentration and conditions; hence, GITs obtained at different times and places are difficult to compare. Therefore, both of these problems must be solved to establish a reliable GIT method. In this paper, we propose a new titration method that solves these problems.

2. Materials and methods

2.1. Cell lines and recombinant adenovirus

The human embryo kidney cell line 293 [14] constitutively expresses adenoviral E1 genes. The cell line CV-1 is derived from African green monkey kidney. HeLa cells are derived from human cervical cancer. The cell line NIH-3T3 was established from an NIH Swiss mouse embryo. AxCANCre, a Cre-expressing AdV tagged with a nuclear localization signal [15], and AxEFDsR, a dsRed-expressing AdV [16], have been described previously. The GFP-expressing AdV AxCAGFP was generated using the COS-TPC method [17]. The AdV AxEFLNLdsRed is identical to AxCALNLZ [15] except that the CAG promoter and the LacZ gene were replaced by the EF1 α promoter, and the dsRed gene, respectively. The TCID₅₀ was measured according to the protocol described by Kanegae et al. [4]. The plasmid pA14cw contains the AdV genomic DNA of pAdex1w [17] from map units 0 to 14.

2.2. Southern blotting analysis

CV-1 cells in a 6-cm dish were infected with AxCANCre. After 24 h, the cells were infected with AxEFLNLdsRed or transfected with 1 μ g of the plasmid pxEFLNLdsRed per 6-cm dish using Transfast (Promega). The total DNA was prepared from the dish [18]. Before alkaline treatment, the agarose gel was exposed to 0.1-N HCl for partial depurination causing DNA fragmentation to several hundred base pairs (bp) to obtain the complete transfer to the membrane [19]; the DNA was then transferred to the nylon membrane Hybond-N (Amersham GE) using the capillary-transfer method [20]. Specific DNA was detected using a DIGDNA Labeling and Detection Kit (Roche Diagnostics). The 0.6-kb *XmnI* fragment derived from the EF1 α promoter region was labeled with digoxigenin-UTP, and specific DNA was detected using the chemiluminescence of CDP-Star (Roche Diagnostics). The bands were visualized using LAS-4000 (Fuji Film) and the densitometry was performed using an image analysis program (Multi Gauge version 3X, Fuji Film). The linear correlation between the DNA amounts and the intensity of the bands was confirmed (Fig. S1 of Supplementary Data), showing that the Southern analysis was quantitative.

2.3. qPCR

The infected total cell DNA was prepared from cells, as described previously [18,21]. Alternatively, we confirmed the total cell DNA prepared using a DNA preparation kit (Macherey–Nagel through TaKaRa Bio). qPCR was performed to detect the AdV genome using a probe for the pIX gene [16] (Fig. 2A). The amount of

chromosomal DNA was simultaneously measured to correct the Ct values of the viral genome per cell, and the corrected Ct was shown throughout. The probes were derived from the sequence of the human β -actin gene for HeLa, the human OTC gene for CV-1 [16], and the mouse GAPDH gene for NIH-3T3 (Applied Biosystems, catalog number 7000-1). The qPCR reaction was performed according to the manufacturer's protocol: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min (Applied BioSystems).

2.4. Generation of a standard curve using qPCR

The copy numbers of the plasmid pA14cw containing the pIX sequences and the cosmid pAxcwit2 [22] containing the full-genome of the FG AdV [22] were calculated according to Puntel et al. [11]. The plasmid and cosmid were serially diluted (Fig. 2B and C). The equivalency between the molecular weight of the plasmid pA14cw and the number of copies was calculated by considering the pA14cw molecular weight and the equivalency between base pairs (pA14cw [4, 247 bp]) and Daltons (Da) ($1\text{bp}_{\text{Ad5}} = 678\text{ Da}$). $\text{Mass}_{\text{pA14cw}}(\text{Da}) = 4, 247\text{ bp/molecule} \times 678\text{ Da/bp} = 2.88 \times 10^6\text{ Da/molecule}$. We obtained the equivalency of mass $2.88 \times 10^6\text{ Da/molecule} \times 1.66 \times 10^{-18}\text{ }\mu\text{g/Da} = 4.78 \times 10^{-12}\text{ }\mu\text{g/molecule}$. The copy numbers of the cosmid pAxcwit2 (42, 698 bp) were similarly calculated.

3. Results and discussion

3.1. Quantification of internally transduced viral copies in target cells

To establish a reliable GIT method, determining the ratio of successfully internalized viral DNA to DNA that has physically attached to the cell surface (that is, naked viral DNA or DNA in inactivated viral particles in AdV-infected target cells) is essential. To estimate the amounts of the former and the latter, we utilized the Cre/loxP system. CV-1 cells were infected with the AdV AxCANCre expressing Cre at an MOI of 5. Then, 24 h later, the cells were infected with the target AdV AxEFLNLdsRed at an MOI of 7.5 or were transfected with 1 μ g of the target plasmid pxEFLNLdsRed as a control. The target unit in the AdV and the plasmid contains the same sequences of the EF1 α promoter and the dsRed gene flanked by two loxPs (Fig. 1A). The total cell DNA was extracted after the indicated number of days and digested with *BglIII*; the DNA of the target unit was detected using a Southern technique (Fig. 1B and C). The 2.8-kb band (S2.8) indicates the substrate originally present before Cre-mediated recombination, i.e., the unprocessed substrate, while the 1.5-kb band (R1.5) shows the presence of the recombined product. We considered that the viral DNA and the transfected DNA that are physically attached to the cell surface cannot be processed by Cre and remain as unprocessed substrate.

When the cells were transfected with the target plasmid, the majority of the target DNA remained unprocessed even after 72 h (Fig. 1C, column 6). A densitometry analysis showed that only $9 \pm 2\%$ ($n = 7$) of the DNA was processed substrate. Also, a preliminary experiment showed that when using 3 μ g of plasmid DNA, the percentage was 12% (data not shown). These results suggested that most of the transfected DNA was possibly present on the cell surface and that the DNA copy number after transfection did not reflect that of the internalized DNA molecules. In contrast, most of the target viral DNA was processed using Cre-mediated recombination by 2 or 3 days after infection (Fig. 1B, columns 5 and 6); the recombination efficiency was $92 \pm 3\%$ ($n = 4$). Considering that the recombination efficiency must not be 100%, the result suggested that at least 92% of the target viral DNA was present inside the infected cells. Similar results were obtained when using

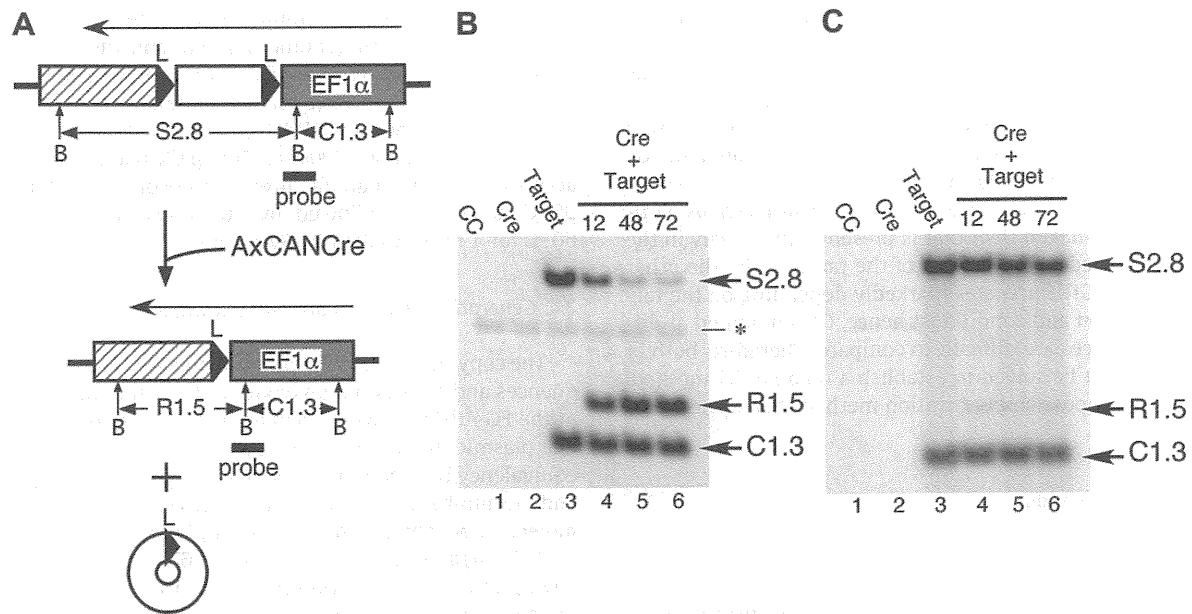


Fig. 1. (A) Structure of the target unit of recipient virus and plasmid. White box, stuffer DNA consisting of neo gene plus polyadenylation (poly(A)) sequence; shadowed box, cDNA plus poly(A) sequence; solid triangle, loxP sequence. Arrows show the direction of transcription. (B) Cre-mediated recombination using AdV. CC, uninfected; Cre, AxCANCre; Target, AxEFLNLDsRed. The numbers show the hours after infection. The asterisk shows the sequence of the EF1 α gene in the human chromosome. (C) Cre-mediated recombination using plasmid transfection. The representations are the same in (B). For the densitometry four and seven independent experiments in (B) and (C) were performed, respectively.

different MOIs from 1 to 10 (Fig. S2). Therefore, the result showed that nearly all the detected viral DNA was derived from internalized, actively infectious viral particles; hence, the quantification of viral genomes using qPCR can be reliably used for titration.

3.2. Measurement of AdV-genome copies in infected cells using qPCR

A set of TaqMan PCR primers and probes were designed for detecting the AdV genome in the viral pIX coding region, since this gene is present closest to the target insert and encodes a viral structural protein that is essential for stable viral particles (Fig. 2A). The method for quantifying the copy number of FG AdV DNA in infected cells was essentially that described by Ma et al. [9]. The sensitivity of detection and the linear range of the quantification of this qPCR were determined by serial dilution of the 4.2-kb plasmid pA14cw containing the pIX gene and the 42.7-kb cosmid pAxcwit2 containing the full-length genome of the AdV backbone (Fig. 2B and C). The linear range for quantification was found to be between at least 10^3 and 10^9 copies for the plasmid template pA14cw per 5 μ L; a plot of the plasmid copy number versus the Ct value between 7 and 27 was linear on a logarithmic scale with a coefficient correlation (r^2) of 0.99 (Fig. 2B). The cosmid template Axcwit2 produced an identical result (Fig. 2C), confirming that the Ct value accurately depended on the copy numbers irrespective of the DNA size. Based on these results, the Ct values obtained using our PCR system can be converted to the copy numbers for a given DNA sample.

As the next step in the titration using qPCR, CV-1 cells in the well of a 24-well plate (2.0×10^5 cells) were infected using the suspension method (see below) with 10 μ L of serially diluted stocks of AxCAGFP virus ("control virus stock" for the conversion of the viral Ct value to the copy number when using our particular qPCR machine), and the total infected cell DNA was extracted and the Ct values of the viral genome corrected using the Ct value of the cell DNA (see Section 2) were measured (Fig. 2D). A linear correlation of the Ct values to the dilutions was observed from 18 to at least 28. Therefore, together with the results shown in Fig. 2C, the

GIT, i.e., the copy number of the transduced viral genome per cell for this cell concentration of 2.0×10^5 cells, can be calculated. For example, a 10^{-2} dilution of 10 μ L of viral stock produced a Ct value of 24.34 according to the equation shown in Fig. 2D ($y = 2.97x + 18.4$), and the same Ct value using that shown in Fig. 2C ($y = 24.34 = -3.4x + 38.2$) corresponded to $10^{4.08} \approx 1.2 \times 10^4$ copies of viral genome in 5 μ L, i.e., the GIT titer of the control virus stock was 2.4×10^8 copies/mL ($1.2 \times 10^4 \times 10^3 / 5 \times 1 / 10^{-2}$). As described above, because the equations for the plasmid (Fig. 2B) and the cosmid (Fig. 2C) were practically identical, not the cosmid but a plasmid containing the pIX gene can be used for the conversion of the vital Ct value to the copy number. This method of obtaining the GIT of a control virus is essential for rVT because the Ct value differs depending on the qPCR machine. If the TCID₅₀ titer of this control virus is known, the TCID₅₀ titer can be converted to the copy number/mL (see Section 3.5).

3.3. Establishment of the method for relative vector titer (rVT)

To examine the effect of the cell concentration on the GIT, HeLa cells at densities of 0.6, 2.0 and 6.0×10^5 (full sheet condition) per 6-well plate were infected in parallel with a virus of unknown titer (testing virus) and the virus AxCAGFP (control virus, the same virus used in Fig. 2D). Three days later, the total cell DNA was extracted. For each DNA sample, the Ct values of not only the viral DNA but also the cell chromosome DNA were simultaneously measured to correct for fluctuations in the cell numbers (see Section 2). The transduced copy numbers/mL, i.e. the GIT, vary markedly from 1 to 3.2 and 4.9 in their ratio as reported by Sandig et al. [13] (columns "GIT ratio", lines "HeLa"). This is the second reason why the GIT method cannot be directly used as a titration method. Similar result was obtained when using CV-1 cells (data not shown). In addition to human HeLa cells, monkey CV-1 cells and mouse NIH-3T3 cells were infected with the testing and control viruses and GITs, i.e., the transduced number of copies, were measured (lines "HeLa 6.0", "CV-1 6.0" and "NIH-3T3 6.0"). While the GITs of CV-1 and NIH-3T3 cells were 3-fold and 17-fold lower than that