

Fig. 1. Schematic diagrams of T7-based minigenomes derived from HCV 1b (A), HCV 2a (B), chimeric minigenomes p1b-2a consisting of 5'-end of HCV 1b and 3'-end of HCV 2a (E) and p2a-1b consisting of 5'-end of HCV 2a and 3'-end of HCV 1b (F). HCV minigenome containing the antisense sequence of the *Renilla* luciferase gene and EMCV IRES flanked by the 5'-end and 3'-partial NS5B coding sequence-connected 3'-UTR was juxtaposed precisely at the T7 transcription start site and followed by the HDV ribozyme sequence. pnu-1b (C) and pnu-2a (D) were identical to p1b-1b and p2a-2a except for the 5'-end sequences deleted.

days posttransfection, protein expression was verified by Western blot analysis (Fig. 2) and the replication of minigenome was determined by luciferase assay and Northern blot analysis. The firefly luciferase activity from cotransfected pGL3-Control vector was simultaneously measured to normalize the transfection efficiency. As shown in Fig. 3A, only background level of *Renilla* luciferase (Rluc) activity was detected in cells transfected with the empty vector. Cotransfection of the plasmid encoding the polyprotein NS3 to NS5B (pNS3-51b) resulted in significant *Renilla* luciferase expression. Omission of pAM8-1 in the transfection mixture completely abrogated *Renilla* luciferase activity, largely ruling out the possibility that the minus-strand RNA used here as the mRNA for reporter gene expression was synthesized as a consequence of the transcription by a cryptic promoter. Consistent with the results reported previously [13], *Renilla* luciferase activity was also detected in Huh-NNRZ cells stably replicating the HCV subgenomic replicon, although it was lower than that in

cells *trans*-expressing the polyprotein. The fact that the replicase complex reconstituted by *trans*-expressed polyprotein could support more efficient replication of the minigenome may be attributable to the higher expression level of plasmid-encoded protein on a per-transfected-cell basis. Alternatively, the recruitment of the replication complex to the minigenome may be competed by the subgenomic replicon, because both of these share the replication machinery in replicon cells.

To further confirm the result of reporter assay, RNA was extracted from transfected cells and subjected to Northern blot analysis using digoxigenin-labeled antisense *Renilla* luciferase probes. Also, minus-strand RNA transcripts of the expected size were specifically detected in Huh-7 cells expressing NS 3–5 protein and Huh-NNRZ cells replicating HCV subgenomic replicon (Fig. 3B, lanes 2 and 4). These data demonstrate that *trans*-replication of HCV minigenome does not require replication of the helper viral RNA.

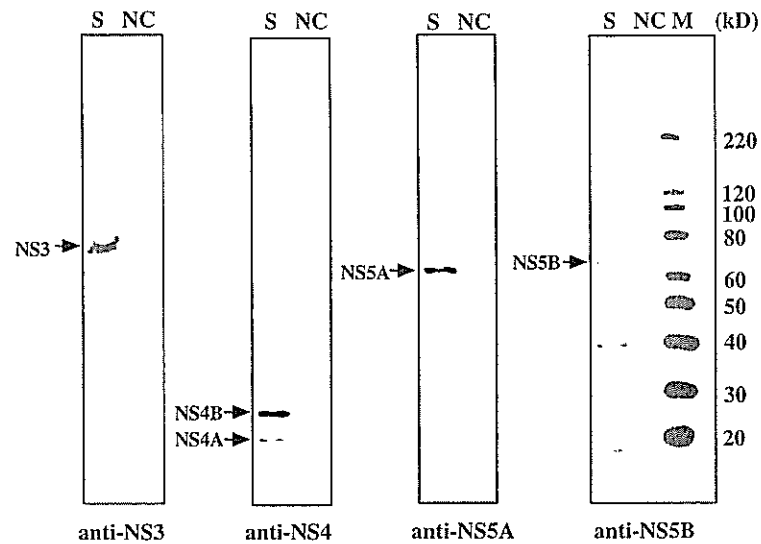


Fig. 2. Expression of NS proteins in Huh-7 cells transfected with plasmid encoding a polyprotein encompassing NS3 to NS5B. Huh-7 cells were transfected with pNS3-51b together with pAM8-1 plasmid expressing T7 RNA polymerase and harvested at day 3 posttransfection. Cell lysates of the transfected cells were analyzed by Western blot using antibodies against each NS protein. Protein standard is shown on the right, and the band corresponding to each NS protein is indicated by an arrowhead. Huh-7 cells transfected with the empty vector served as a negative control (NC).

To document that the reporter gene expression detected above was dependent on HCV replicase reconstituted by *trans*-expressed NS proteins, we employed inactive mutant pNS3-51b/dGDD (in which the GDD motif of NS5B was deleted) and AdsiNS5B expressing siRNA directed against NS5B [13] in the reporter assay. As shown in Fig. 3C, the deletion of GDD motif significantly attenuated the ability of NS proteins to support minigenome replication, and transduction with AdsiNS5B resulted in a substantial and dose-dependent reduction in luciferase expression. These results provide further evidence that the reporter gene was expressed as a result of replication of HCV minigenome by *trans*-supplied NS proteins.

Chimeric minigenomes as templates for HCV replication complex

Next, we were interested in investigating whether the replicase of HCV can recognize the heterologous signals for synthesis of minus-strand RNA. For this purpose, HCV minigenome from distantly related genotype 2a (Fig. 1B, p2a-2a), minigenomes with 5'-end deleted (Fig. 1C and D, pnu-1b and pnu-2a), and chimeric minigenomes whose 5'- or 3'-end was replaced by the respective region of the heterologous virus (Fig. 1E and F, p1b-2a and p2a-1b) were constructed. Huh-7 cells were transfected with these minigenomes together with the plasmid expressing HCV 1b- or 2a-derived NS proteins, pAM8-1, and *Renilla* luciferase activities were measured as fore-mentioned. Consistent with the results described above, replicase of HCV 1b and 2a accepted its respective minigenome as the template for synthesis of minus-strand RNA, and exchange of NS proteins between HCV1b- and 2a-derived minigenome systems

also led to reporter gene expression (Fig. 4), implying that the replication complex is not strictly specific for the homologous RNA template. Deletion of the 5'-end region in the minigenome fully abrogated its replication, both NS proteins from HCV 1b and 2a, however, could support the replication of chimeric minigenomes, suggesting that both RNA-protein interaction between replicase and viral genome and long range RNA-RNA interaction between 5'- and 3'-terminal sequence involved in HCV minus-strand RNA synthesis are functionally conserved between genotype 1b and 2a. Additionally, in all tested minigenomes, the NS proteins originated from HCV 1b constantly yielded higher levels of luciferase expression than that from 2a, suggesting that intrinsic differences in the replication capabilities of the replicase complex from different strains may exist. More likely, the superior capability of pNS3-51b in supporting the minigenomes replication may be attributable to the fact that the coding sequence in pNS3-51b was amplified from the replicon which harbors the adaptive mutations due to long-term culture, whereas the coding region in pNS3-52a was directly amplified from HCV 2a-infected serum.

Discussion

Successful establishment of the minigenome system has been described in a number of minus-stranded RNA viruses from different families and plus-stranded RNA viruses belonging to the *Coronaviridae* family, which has contributed greatly to the analysis of *cis*-acting sequences and *trans*-acting proteins essential for viral replication [11,12]. The rescue of synthetic minigenomes was achieved either through helper virus infection of minigenome-transfected

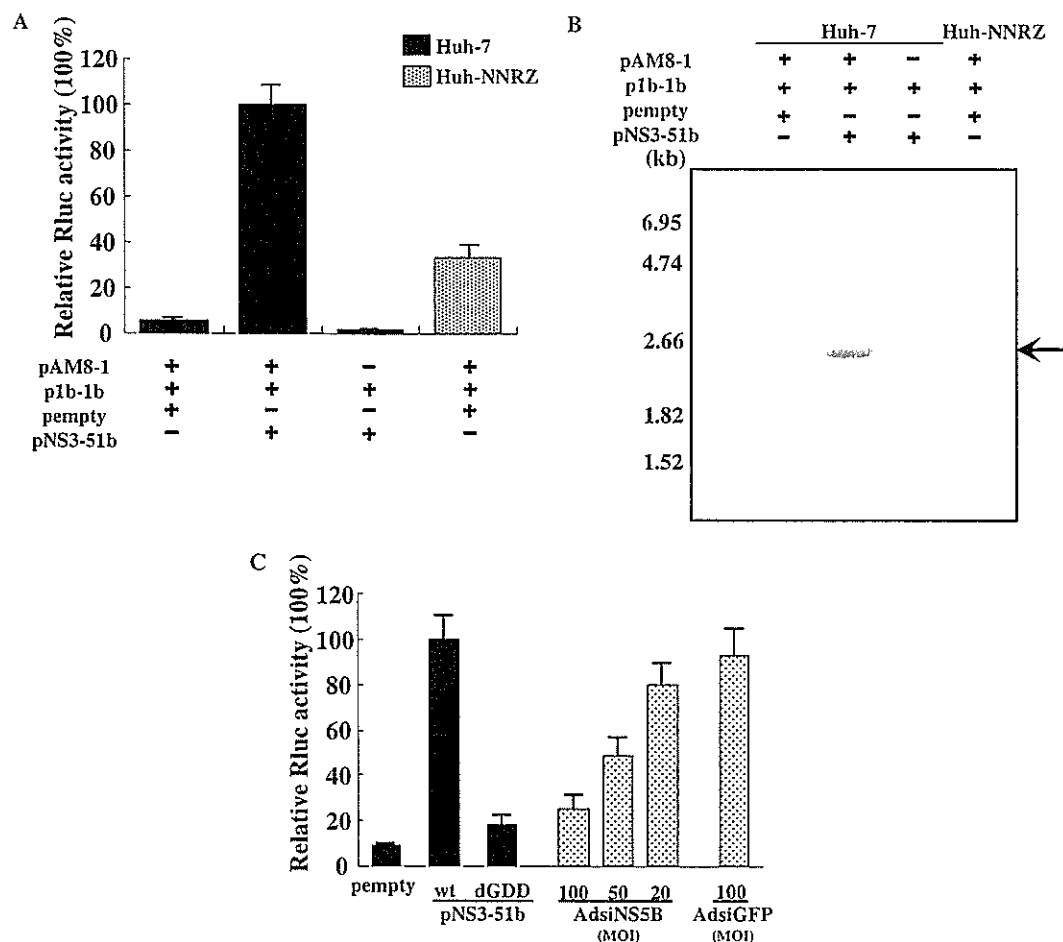


Fig. 3. Replication of HCV minigenome in Huh-7 cells expressing polyprotein NS3 to NS5B. (A) Huh-7 or Huh-NNRZ cells were transfected with p1b-1b, pNS3-51b expressing polyprotein NS3 to NS5B, together with or without pAM8-1. Relative *Renilla* luciferase activities in the lysates were determined at 72 h posttransfection. The columns and bars represent mean and standard deviation of four independent experiments. (B) Northern blot was performed on 8 μ g of extracted RNA using digoxigenin-labeled antisense *Renilla* luciferase RNA probe to detect minus-strand transcripts. RNA size markers are shown on the left, and the bands corresponding to minus-strand RNA are indicated on the right. (C) Huh-7 cells were transfected with p1b-1b, pAM8-1, and pempty, pNS3-51b or pNS3-51b/dGDD (column 1–3), or infected with AdsiNS5B at an MOI of 100, 50, and 20 (column 4–6) before transfection, and relative *Renilla* luciferase activities in the lysates were determined as described above.

cells with virus particles or through co-transfection of plasmids expressing viral proteins. For viruses of *Flaviviridae* family, however, a little has been reported in the development of similar approach except an *in vitro* replication system which utilizes cytoplasmic extracts from viral-infected cells and exogenous RNA template containing 5'- and 3'-terminal regions was described for dengue virus [17]. Together with those reported previously [13], the data shown here represent the first example of minigenome system for HCV, indicating that both the replicase complex supplied from replicating subgenomic replicon and that reconstituted by plasmid-encoded NS proteins are capable of supporting the replication of HCV minigenome.

The data shown here further confirm that the viral 5'- and 3'-end sequence together with the 3'-partial NS5B coding region represent sufficient *cis*-acting signals for minus-strand RNA synthesis. These results, however, do not rule out the possibility for the existence of *cis*-acting

elements in other coding region, which may act as regulatory elements (either enhancers or silencers) in RNA synthesis. The presence of noncontiguous *cis*-acting signals involved in viral RNA replication has been reported in the viral genome of the brome mosaic virus [18], tobacco mosaic virus [19], and the double-stranded RNA virus of yeast [20].

Similar to that found in dengue virus, it was shown that deletion of the 5'-end region in the minigenome fully abrogated its replication, but substitution of the 5'-end with the respective sequence from heterologous virus (p1b-2a or p2a-1b) did not significantly affect its template ability, suggesting that the long range RNA-RNA interaction between 5'- and 3'-ends essential for RNA replication is functionally conserved between HCV 1b and 2a. In addition to homologous minigenome, both HCV 1b- and 2a-derived replicase were able to accept the heterologous and chimeric minigenomes as the templates for synthesis of minus-strand

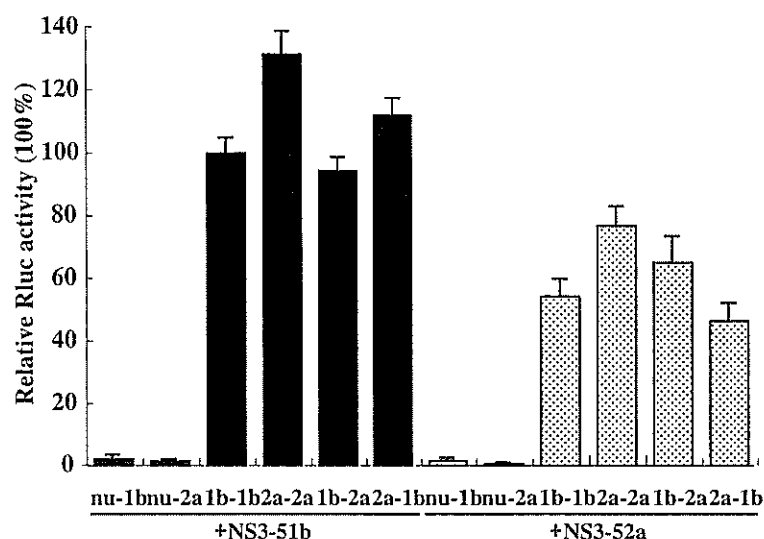


Fig. 4. Replication of chimeric HCV minigenomes. Huh-7 cells were transfected with each indicated minigenome, pAM8-1, and pNS3-51b or pNS3-52a expressing polyprotein NS3 to NS5B derived from genotype 1b or 2a. Relative *Renilla* luciferase activities in the lysates were determined as described for Fig. 3A. The columns and bars represent means and standard deviations of three independent transfections.

RNA, indicating that the replicase-catalyzed RNA synthesis is not strictly strain- or genotype-specific.

Using replicon system, Bartenschlager's group obtained the evidence showing that only mutations in NS5A, but not mutations in NS3, NS4B, and NS5B, could be rescued by *trans*-complementation [21]. Our data presented here indicate that replication of the minigenome can be supported by *trans*-expressed NS proteins. One scenario may make these two different findings compatible: the *cis*-expressed, lethally mutated NS proteins may exert dominant negative effect in reconstituting replication complex, and thus interfere with the incorporation of *trans*-supplied NS proteins into a functional replication complex, which may account for the failure of NS proteins (other than NS5A) to *trans*-complement HCV RNA replication; however, such a dominant negative effect does not exist in the minigenome system described here because there is no NS protein expressed in *cis*, and *trans*-expressed NS proteins might be able to reconstitute the functional replication complex to support minigenome replication. Further experiments are now in progress to substantiate this assumption.

It is generally believed that the HCV replication follows the pathway used by other plus-strand RNA viruses: the input RNA is first transcribed into a minus strand, which in turn serves as the template for production of progeny plus strand. The negative strand intermediates are postulated to exist as a dsRNA form. However, there is no direct evidence demonstrating this postulation in HCV, and whether there is free HCV-specific RNA of negative polarity in infected cells is still an issue to be elucidated. On the other hand, increasing evidence showed that the RNA in native replication intermediates of some positive strand RNA viruses is single-stranded. For example, in polio virus-infected cells, a careful electron microscope analysis

using a membrane-permeable cross-linking reagent demonstrated that the native replication intermediate *in vivo* has a predominantly single-stranded backbone attached to several nascent RNA chains with few or no regions of extensive base-pairing, although deproteinized (phenol-extracted) replication intermediate has a backbone mostly double-stranded [22]. More recently, Fujimura et al., reported that native replication intermediates of 20 S RNA virus have a single-stranded RNA backbone [23]. After completion of product-strand elongation, both the product and template strands are released from the replication complex as single-stranded RNA. The data presented here indicate that the minus strand RNA could serve as the mRNA for *trans*-gene expression, implying a similar scenario may also occur in HCV replication and minus strand RNA may be dissociated and present as a free single-strand form after RNA synthesis is completed.

One issue of concern in using minigenome to study the molecular mechanism of viral replication is whether the elements controlling viral replication in the context of minigenome could authentically reflect those that occurred in the context of full-length genome. Recently, differential effect of a point mutation in the replicase gene on genome and minigenome replication was reported in coronavirus, emphasizing the need to use full-length genome to validate the replication signals obtained from minigenome system [24]. Nonetheless, the HCV minigenome system described here represents a useful tool for identification of *cis*- and *trans*-acting factors involved in viral replication while eliminating biosafety constraints required for work with infectious systems. Additionally, it will be of interest to explore whether the HCV minigenome can be packaged by additional provision of the viral structural protein in *trans*, and its success will not only further broaden the

application of the HCV minigenome, but also facilitate the development of HCV-based gene delivery system.

We describe here a reverse genetic system for HCV that is based on T7-driven minigenome coupled with plasmid-encoded NS proteins. This system opens the possibility of manipulation of *cis*-acting signals and *trans*-acting factors involved in the control of HCV RNA synthesis, which may facilitate future studies aimed at investigation of the mechanisms involved in the replication of viral RNA.

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E6AP Ubiquitin Ligase Mediates Ubiquitylation and Degradation of Hepatitis C Virus Core Protein[∇]

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Hepatitis C virus (HCV) core protein is a major component of viral nucleocapsid and a multifunctional protein involved in viral pathogenesis and hepatocarcinogenesis. We previously showed that the HCV core protein is degraded through the ubiquitin-proteasome pathway. However, the molecular machinery for core ubiquitylation is unknown. Using tandem affinity purification, we identified the ubiquitin ligase E6AP as an HCV core-binding protein. E6AP was found to bind to the core protein *in vitro* and *in vivo* and promote its degradation in hepatic and nonhepatic cells. Knockdown of endogenous E6AP by RNA interference increased the HCV core protein level. *In vitro* and *in vivo* ubiquitylation assays showed that E6AP promotes ubiquitylation of the core protein. Exogenous expression of E6AP decreased intracellular core protein levels and supernatant HCV infectivity titers in the HCV JFH1-infected Huh-7 cells. Furthermore, knockdown of endogenous E6AP by RNA interference increased intracellular core protein levels and supernatant HCV infectivity titers in the HCV JFH1-infected cells. Taken together, our results provide evidence that E6AP mediates ubiquitylation and degradation of HCV core protein. We propose that the E6AP-mediated ubiquitin-proteasome pathway may affect the production of HCV particles through controlling the amounts of viral nucleocapsid protein.

Hepatitis C virus (HCV; a single-stranded, positive-sense RNA virus that is classified in the family *Flaviviridae*) is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (5, 26, 45). More than 170 million people worldwide are chronically infected with HCV (41). The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein (5, 54). The polyprotein is cleaved cotranslationally into at least 10 proteins by viral proteases and cellular signalases (6, 10).

The HCV core protein represents the first 1 to 191 amino acids (aa) of the polyprotein and is followed by two glycoproteins, E1 and E2 (6). The core protein plays a central role in the packaging of viral RNA (25, 40); modulates various cellular processes, including signal transduction pathways, transcriptional control, cell cycle progression, apoptosis, lipid metabolism, and the immune response (9, 40); and has transforming potential in certain cells (43). Mice transgenic for the HCV core gene develop steatosis (32) and later hepatocellular carcinoma (31). These findings suggest that HCV core protein plays a crucial role in hepatocarcinogenesis.

Two major forms of the HCV core protein, p21 (mature form) and p23 (immature form), can be generated in cultured cells (60). Cellular signal peptidase cleaves at the junction of the core/E1, releasing the immature form of the core protein from the polypeptide (12, 46). Signal peptide peptidase cleaves just before the signal sequence, liberating the mature form of the HCV core protein at the cytoplasmic face of the endoplasmic reticulum (29). Several different sites have been proposed as potential cleavage sites of signal peptide peptidase, such as Leu-179 (15, 29), Phe-177 (36, 37), Leu-182 (15), and Ser-173 (46). Further processing of the HCV core protein yields a 17-kDa product with a C terminus at around amino acid 152. A truncated form of the core protein, p17, was found in transfected cells (42, 52) and liver tissues from humans with hepatocellular carcinoma (59). The majority of this protein translocates to the nucleus. The C terminus of the core protein is important for regulating the stability of the protein (20, 52).

We previously showed that the C-terminally truncated forms of the core protein are degraded through the ubiquitin-proteasome pathway (52). We found that the mature form of the core protein, p21, also links to a few ubiquitin moieties, suggesting that the ubiquitin-proteasome pathway involves proteolysis of heterologous species of the core protein (52). Overexpression of PA28 γ (a REG family proteasome activator also known as REG γ or Ki antigen) enhances the proteasomal degradation of the HCV core protein (30). A recent study has shown that

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PA28 γ is involved in the degradation of the steroid receptor coactivator 3 (SRC-3) in an ATP- and ubiquitin-independent manner (27). It is still unclear what E3 ubiquitin ligase is responsible for ubiquitylation of the HCV core protein.

E6AP was initially identified as the cellular factor that stimulates ubiquitin-mediated degradation of the tumor suppressor p53 in conjunction with the E6 protein of cancer-associated human papillomavirus types 16 and 18 (14, 48). The E6-E6AP complex functions as a E3 ubiquitin ligase in the ubiquitylation of p53 (49). E6AP is the prototype of a family of ubiquitin ligases called HECT domain ubiquitin ligases, all of which contain a domain homologous to the E6AP carboxyl terminus (13). Interestingly, E6AP is not involved in the regulation of p53 ubiquitylation in the absence of E6 (55). Several potential E6-independent substrates for E6AP have been identified, such as hHR23A, Blk, and Mcm7 (23, 24, 35). E6AP is also a candidate gene for Angelman syndrome, which is a severe neurological disorder characterized by mental retardation (21).

This study aimed to identify endogenous ubiquitin-proteasome pathway proteins that are associated with HCV core protein. Tandem affinity purification and mass spectrometry analysis identified E6AP as an HCV core-binding protein. Here we present evidence that E6AP associates with HCV core protein *in vitro* and *in vivo* and is involved in ubiquitylation and degradation of HCV core protein. We propose that an E6AP-mediated ubiquitin-proteasome pathway may affect the production of HCV particles through controlling the amounts of HCV core protein.

MATERIALS AND METHODS

Cell culture and transfection. Human embryonic kidney 293T cells, human hepatoblastoma HepG2 cells, and human hepatoma Huh-7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 50 IU/ml penicillin, 50 μ g/ml streptomycin (Invitrogen), and 10% (vol/vol) fetal bovine serum (JRH Biosciences) at 37°C in a 5% CO₂ incubator. 293T cells and HepG2 cells were transfected with plasmid DNA using FuGene 6 transfection reagents (Roche). Huh-7 cells were transfected with plasmid DNA using TransIT LT1 transfection reagents (Mirus).

Plasmids and recombinant baculoviruses. MEF tag cassette (containing *myc* tag, the tobacco etch virus protease cleavage site, and FLAG tag) (16) was fused to the N terminus of the cDNA encoding core protein of HCV NIHJ1 (genotype 1b) (1). To express MEF-tagged core protein in mammalian cells, the genome coding for HCV core protein (amino acids 1 to 191) was amplified by PCR using pBR HCV NIHJ1 as a template. Sense oligonucleotide containing a Kozak consensus translation initiation codon and antisense oligonucleotide containing an in-frame translation stop codon were synthesized by PCR. The amplified PCR product was purified, digested with EcoRI and EcoRV, and then inserted into the EcoRI-EcoRV site of pcDNA3-MEF. FLAG-tagged HCV core expression plasmids based upon pCAGGS (34) were described previously (30). To express E6AP and the active-site cysteine-to-alanine mutant of E6AP in mammalian cells, pCMV4-HA-E6AP isoform II and pCMV4-HA-E6AP C-A were utilized (19). The C-A mutation was introduced at the site of E6AP C843. To express E6AP and E6AP C-A under the CAG promoter, the E6AP fragment and the E6AP C-A fragment were amplified by PCR, purified, digested with SmaI and NotI, and blunt ended using a DNA blunting kit (Takara). These PCR fragments were subcloned into pCAGGS.

To make a fusion protein consisting of glutathione S-transferase (GST) fused to the N terminus of E6AP in *Escherichia coli*, the E6AP fragment was amplified by PCR and the resultant product was cloned into the SmaI-NotI site of pGEX4T-1 vector (Amersham Biosciences). To express a series of E6AP truncation mutants as GST fusion proteins, each fragment was amplified by PCR and cloned into the SmaI-NotI site of pGEX4T-1. To purify GST core protein efficiently by two-step affinity purification, we fused hexahistidine (His) tag to the C terminus of GST fusion proteins. To bacterially express HCV core (aa 1 to 173) protein as a fusion protein containing N-terminal GST tag and C-terminal

His tag, core fragment was amplified by PCR and the resultant product was cloned into the EcoRI-NotI site of pGEX4T-1 vector. The resultant plasmid was designated pGEX GST-C173HT. To express GST core (1-152)-His and GST-His in *E. coli*, pGEX core (1-152)-His and pGEX-His were constructed similarly. The resultant plasmids were designated pGEX GST-C152HT and pGEX GST-HT, respectively.

To generate recombinant baculoviruses expressing GST-E6AP, GST-E6AP fragment was excised from pGEX E6AP by digestion with SmaI and Tth1111 and ligated into the SmaI-Tth1111 site of pVL1392 (Invitrogen). To express GST-E6AP C-A, pVLGST-E6AP C-A was constructed similarly. To generate recombinant baculovirus expressing HCV core (aa 1 to 173) protein as a fusion protein containing N-terminal GST tag and C-terminal His tag, GST-C173HT fragment was amplified by PCR using pGEX GST-C173HT as a template, digested with BglII-XbaI, and subcloned into the BglII-XbaI site of pVL1392. To generate recombinant baculoviruses expressing GST-C152HT and GST-HT, cDNA fragments corresponding to GST-C152HT and GST-HT were amplified by PCR and subcloned into pVL1392, respectively. The resultant plasmids were designated pVLGST-C173HT, pVLGST-C152HT, and pVLGST-HT. To generate recombinant baculovirus expressing MEF-tagged E6AP, cDNA fragment encoding MEF-E6AP was subcloned into pVL1392. To express HCV core protein in the TNT-coupled wheat germ lysate system (Promega), HCV core cDNA was inserted in the EcoRI site of pCMVTNT (Promega). The primer sequences used in this study are available from the authors upon request. The sequences of the inserts were extensively verified using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Recombinant baculoviruses were recovered using a BaculoGold transfection kit (PharMingen) according to the manufacturer's instructions.

Antibodies. The mouse monoclonal antibodies (MAbs) used in this study were anti-hemagglutinin (anti-HA) MAb (12CA5; Roche), anti-FLAG (M2) MAb (Sigma), anti-c-myc MAb (9E10; Santa Cruz), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) MAb (Chemicon), anti-GST MAb (Santa Cruz), anti-ubiquitin MAb (Chemicon), anti-E6AP MAb (E6AP-330) (Sigma), anticore MAb (B2; Anogen), and another anti-core MAb (2H9) (56). Polyclonal antibodies (PABs) used in this study were anti-HA rabbit PAB (Y-11; Santa Cruz), anti-FLAG rabbit PAB (F7425; Sigma), anti-E6AP rabbit PAB (H-182; Santa Cruz), anti-DDX3 rabbit PAB (47), anti-PA28 γ rabbit PAB (Affinity), and anti-GST goat PAB (Amersham). Anticore rabbit PAB (TS1) was raised against the recombinant GST core protein.

MEF purification procedure. 293T cells were transfected with the plasmid expressing MEF core by the calcium phosphate precipitation method (4). After the cells were lysed, the expressed MEF core and its binding proteins were recovered following the procedure described previously (16). 293T cells transfected with pcDNA3-MEF core in four 10-cm dishes were lysed in 2 ml of lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (wt/vol) glycerol, 100 mM NaF, 1 mM Na₂VO₄, 1% (wt/vol) Triton X-100, 5 μ M ZnCl₂, 2 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 1 μ g/ml leupeptin. The lysate was centrifuged at 100,000 \times g for 20 min at 4°C. The supernatant was passed through a 5- μ m filter, incubated with 100 μ l of Sepharose beads for 60 min at 4°C, and then passed through a 0.65- μ m filter. The filtered supernatant was mixed with 100 μ l of anti-myc-conjugated Sepharose beads for the first immunoprecipitation. After incubation for 90 min at 4°C, the beads were washed five times with 1 ml of TNTG buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% [wt/vol] glycerol, and 1% [wt/vol] Triton X-100), twice with 1 ml of buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% [wt/vol] Triton X-100), and finally once with 1 ml of TNT buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% [wt/vol] Triton X-100). The washed beads were incubated with 10 U of tobacco etch virus protease (Invitrogen) in TNT buffer (100 μ l) to release bound protein complexes from the beads. After incubation for 60 min at room temperature, the supernatant was pooled and the beads were washed twice with 70 μ l of buffer A. The resulting supernatants were combined and incubated with 12 μ l of FLAG-Sepharose beads for the second immunoprecipitation. After incubation for 60 min at room temperature, the beads were washed three times with 240 μ l of buffer A, and proteins bound to the immobilized HCV core protein on the FLAG heads were dissociated by incubation with 80 μ g/ml FLAG peptide (NH₂-Asp-Tyr-Lys-Asp-Asp-Asp-Lys-COOH) (Sigma).

MS/MS. Proteins were separated by 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining. The stained bands were excised and digested in the gel with lysylendoprotease-C (Lys-C), and the resulting peptide mixtures were analyzed using a direct nano-flow liquid chromatography-tandem mass spectrometry (MS/MS) system (33), equipped with an electrospray interface reversed-phase column, a nanoflow gradient device, a high-resolution Q-time of flight hybrid mass spectrometer (Q-TOF2; Micromass), and an automated data analysis system. All the MS/MS

spectra were searched against the nonredundant protein sequence database maintained at the National Center for Biotechnology Information using the Mascot program (Matrixscience) to identify proteins. The MS/MS signal assignments were also confirmed manually.

Expression and purification of recombinant proteins. *E. coli* BL21(DE3) cells were transformed with plasmids expressing GST fusion protein or His-tagged protein and grown at 37°C. Expression of the fusion protein was induced by 1 mM isopropyl- β -D-thiogalactopyranoside at 37°C for 4 h. Bacteria were harvested, suspended in lysis buffer (phosphate-buffered saline [PBS] containing 1% Triton X-100), and sonicated on ice.

Hi5 cells were infected with recombinant baculoviruses to produce GST-C173HT, GST-C152HT, GST-HT, MEF-E6AP, and His-tagged mouse E1 (17). GST and GST fusion proteins were purified on glutathione-Sepharose beads (Amersham Bioscience) according to the manufacturer's protocols. His-tagged proteins were purified on nickel-nitrilotriacetic acid beads (QIAGEN) according to the manufacturer's protocols. MEF-E6AP and MEF-E6AP C-A were purified on anti-FLAG M2 agarose beads (Sigma) according to the manufacturer's protocols.

Immunoblot analysis. Immunoblot analysis was performed essentially as described previously (11). The membrane was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce).

HCV core protein and E6AP binding assays. To map the E6AP binding site on HCV core protein, 2.5 μ g of purified recombinant GST-E6AP expressed in Hi5 cells was mixed with 1,000 μ g of 293T cell lysates transfected with a series of FLAG-tagged HCV core deletion mutants as indicated. The protein concentration of the cells was determined using the bicinchoninic acid protein assay kit (Pierce). The mixtures were immunoprecipitated with anti-FLAG M2 agarose beads (Sigma), and proteins bound to the immobilized HCV core protein on anti-FLAG beads were dissociated with FLAG peptide (Sigma). The eluates were analyzed by immunoblotting with anti-GST PAb. To map the HCV core-binding site on E6AP, GST pull-down assays were performed as described previously (51).

In vivo ubiquitylation assay. In vivo ubiquitylation assays were performed essentially as described previously (57). FLAG-core was immunoprecipitated with anti-FLAG beads. Immunoprecipitates were analyzed by immunoblotting, using either anti-HA PAb or anticore PAb (TS1) to detect ubiquitylated core proteins.

In vitro ubiquitylation assay. For in vitro ubiquitylation of HCV core protein, purified GST-C173HT and GST-C152HT were used as substrates. Purified GST-HT was used as a negative control. Assays were done in 40- μ l volumes containing 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM ATP, 10 mM MgCl₂, 8 μ g of bovine ubiquitin (Sigma), 0.1 mM dithiothreitol, 200 ng mouse E1, 200 ng E2 (UbcH7), and 0.5 μ g each of MEF-E6AP or MEF-E6AP C-A. The reaction mixtures were incubated at 37°C for 120 min followed by purification with glutathione-Sepharose beads and immunoblotting with the indicated antibodies.

siRNA transfection. 293T cells or Huh-7 cells at 3×10^5 cells in a six-well plate were transfected with 40 pmol of either E6AP-specific short interfering RNA (siRNA; Sigma) or scramble negative-control siRNA duplexes (Sigma) using HiPerFect transfection reagent (QIAGEN) following the manufacturer's instructions. The siRNA target sequences were as follows: E6AP (sense), 5'-GGGUCUACACCAGAUUGCUTT-3'; scramble negative control (sense), 5'-UUGCGGGUCUAAUCACCGATT-3'.

CHX half-life experiments. To examine the half-life of HCV core protein, transfected 293T cells were treated with 50 μ g/ml cycloheximide (CHX) at 44 h posttransfection. The cells at zero time points were harvested immediately after treatment with CHX. Cells from subsequent time points were incubated in medium containing CHX at 37°C for 3, 6, and 9 h as indicated.

Infection of Huh-7 cells with secreted HCV. Infectious HCV JFH1 was produced in Huh-7.5.1 cells (61) as described previously (56). Culture supernatant containing infectious HCV JFH1 was collected and passed through a 0.22- μ m filter. Naive Huh-7 cells were seeded 24 h before infection at a density of 1×10^6 in a 10-cm dish. The cells were incubated with 2.5 ml of the inoculum (6.5×10^3 50% tissue culture infectious dose [TCID₅₀]/ml) for 3 h, washed three times with PBS, and supplemented with fresh complete Dulbecco's modified Eagle's medium. Then the cells were transfected with 6 μ g each of pCAGGS, pCAGG-HA-E6AP, or pCAGG-HA-E6AP C-A by using TransIT LTI (Mirus). The cells were trypsinized and replated in six-well plates at 1 day postinfection. The culture medium was changed every 2 days. The culture supernatants and the cells were collected at days 3 and 7 postinfection.

Quantitation of HCV RNA and core protein. We quantitated HCV core protein in cell lysate using the HCV core antigen enzyme-linked immunosorbent assay (ELISA) (Ortho-Clinical Diagnostics). Total RNA was extracted from cells

using TRIzol reagent (Invitrogen). To quantitate HCV RNAs, real-time reverse transcription-PCR was performed as described previously (53).

Infectivity assay. The TCID₅₀ was calculated essentially based on the method described previously (28). Virus titration was performed by seeding Huh-7 cells in 96-well plates at 1×10^4 cells/well. Samples were serially diluted fivefold in complete growth medium and used to infect the seeded cells (six wells per dilution). Following 3 days of incubation, the cells were immunostained for core with anticore MAb (2H9). Wells that expressed at least one core-expressing cell were counted as positive, and the TCID₅₀ was calculated.

Immunocytochemistry and fluorescence microscopy. Cells on collagen-coated coverslips were washed with PBS, fixed with 4% paraformaldehyde for 30 min at 4°C, and permeabilized with PBS containing 0.2% Triton X-100. Cells were preincubated with BlockAce (Dainippon Pharmaceuticals), incubated with specific antibodies as primary antibodies, washed, and incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin G (ICN Pharmaceuticals, Inc.) and Qdot 565-conjugated goat anti-mouse immunoglobulin G (Quantumdot) as secondary antibody. Then the cells were washed with PBS, counterstained with DAPI (4',6'-diamidino-2-phenylindole) solution (Sigma) for 3 min, mounted on glass slides, and examined with a BZ-8000 microscope (Keyence).

Knockdown of endogenous E6AP in HCV JFH1-infected Huh-7 cells. Naive Huh-7 cells at 10^6 cells/10-cm dish were inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5×10^3 TCID₅₀/ml) and cultured. The cells were replated in a six-well plate at 3×10^5 cells/well at day 11 postinfection and transfected with 40 pmol of E6AP siRNA or control siRNA. The culture medium was changed at 24 h after transfection. The cells were harvested at day 2 after transfection, and the intracellular core protein levels were quantitated using the HCV core antigen ELISA. The culture supernatants were collected at day 2 after transfection and assayed for TCID₅₀ determinations.

RESULTS

Identification of E6AP as an HCV core-binding protein. To identify the molecular machinery for HCV core ubiquitylation, we searched for endogenous ubiquitin-proteasome pathway proteins that associated with HCV core protein. HCV core-binding proteins (i.e., MEF core and its binding proteins, recovered from lysed cells) were purified by a tandem affinity purification procedure using a tandem tag (known as MEF tag) (16). Ten proteins were reproducibly detected (Fig. 1A, lane 2), but none were recovered from lysed control cells transfected with empty vector alone (Fig. 1A, lane 1).

To identify the proteins, silver-stained bands were excised from the gel, digested by Lys-C, and analyzed using a direct nanoflow liquid chromatography-MS/MS system. Nine proteins were identified: two known HCV core-binding proteins, human DEAD box protein DDX3 (38) and proteasome activator PA28 γ (30), and seven potential HCV core-binding proteins. E6AP was identified (Fig. 1A, lane 2) on the basis of five independent MS/MS spectra (Table 1). Immunoblot analyses confirmed the proteomic identification of E6AP, DDX3, PA28 γ , and MEF-core (Fig. 1B to E).

E6AP binding domain for HCV core protein. The E6AP binding domain for HCV core protein was investigated. Figure 2A is a schematic representation of E6AP and known motifs in E6AP. A series of deletion mutants of E6AP as GST fusion proteins were expressed in *E. coli*. GST pull-down assays found that the carboxyl-terminal deletion mutant E6AP (1–517), but not E6AP (1–418) (Fig. 2C, lanes C and D), and the amino-terminal deletion mutant E6AP (418–875), but not E6AP (517–875) (Fig. 2C, lanes J and K), were able to bind to the core protein. The signal was absent when unprogrammed wheat germ extracts (the negative control) were used as a source of proteins (data not shown). GST pull-down assays (Fig. 2B) found that the region from aa 418 to aa 517 is important for binding to the HCV core protein. An assay of the

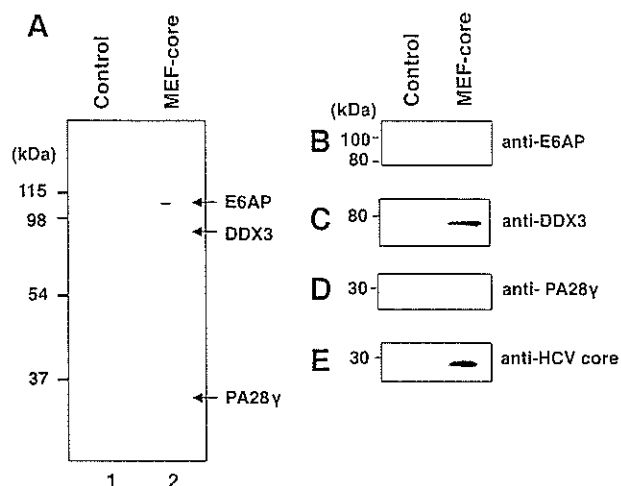


FIG. 1. HCV core protein associates with E6AP in vivo. (A) 293T cells were transfected with pcDNA3-MEF-core or empty plasmid, incubated for 48 h, and then harvested. The expressed MEF-core and binding proteins were recovered using the MEF purification procedure. Proteins bound to the MEF-core immobilized on anti-FLAG beads were dissociated with FLAG peptides, resolved by 9% SDS-PAGE, and visualized by silver staining. Control experiments were performed using 293T cells transfected with vector alone. The positions of E6AP, DDX3, and PA28 γ are indicated by arrows. (B to E) The proteins detected in panel A were confirmed by immunoblotting with appropriate antibodies: E6AP (B), DDX3 (C), PA28 γ (D), and MEF-core (E).

ability of GST-E6AP (418–517) to bind to the HCV core protein was confirmatory (Fig. 2C, lane N) and led to the conclusion that the HCV core-binding domain of E6AP was aa 418 to aa 517.

The HCV core-binding domain for E6AP. By use of a panel of HCV core deletion mutants (Fig. 3A), GST-E6AP was found to coimmunoprecipitate with all of the FLAG-core proteins (Fig. 3A, lanes A to H) except FLAG-core (72–191) or FLAG-core (92–191) (Fig. 3A, lanes I and J). No association of control GST protein with any FLAG-core proteins was observed (data not shown). These data suggest that the aa-58-to-aa-71 segment of the HCV core binds to E6AP. The ability of GST-core (58–71) to associate with purified MEF-E6AP confirmed that the core (aa 58–71) was the site for E6AP binding on the HCV core protein (Fig. 3B).

E6AP decreases steady-state levels of HCV core protein in 293T cells and HepG2 cells. One of the features of HECT domain ubiquitin ligases is direct association with their substrates (50). Thus, we hypothesized that E6AP would function as an E3 ubiquitin ligase for the HCV core protein. We as-

TABLE 1 Identification of E6AP by tandem mass spectrometry^a

Peptide <i>m/z</i>	Sequence determined	Residues
720.9	VFSSAEALVQSFRR	156–168
922.4	AACSAAAMEEDSEASSSR	196–213
774.9	MMEFQQLITYK	339–350
1,053.1	ITVLYSLVQGGQLNPYLR	507–524
809.4	EFVISYSYDYLNK	712–724

^a The protein was ubiquitin protein ligase E3A (E6AP) isoform 2 (GenBank accession no. NP_000453).

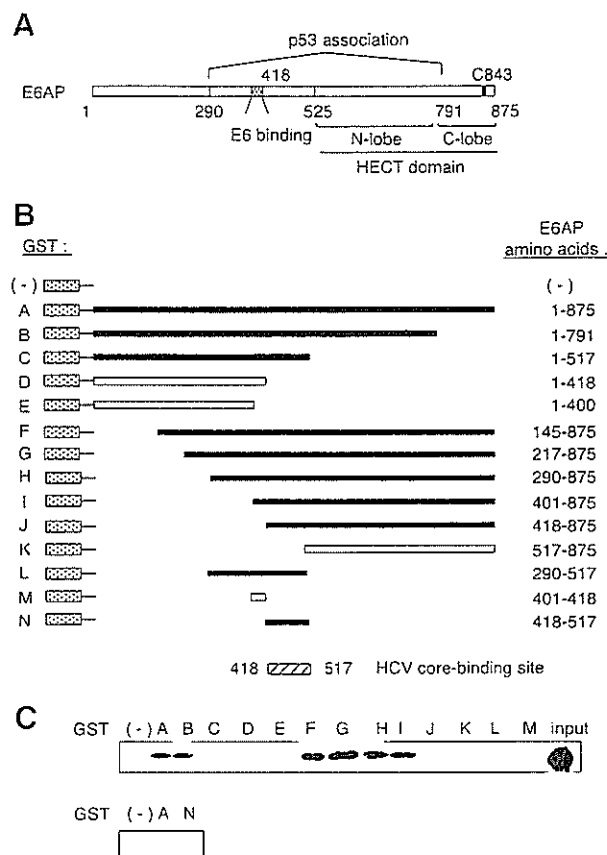


FIG. 2. Mapping of the HCV core-binding domain for E6AP. (A) Structure of E6AP. Shown is a schematic representation of the regions of E6AP isoform II that mediate E6 binding (aa 401 to 418), E6-dependent association with p53 (aa 290 to 791), and the HECT catalytic domain (aa 525 to 875). The catalytic cysteine residue is located at aa 843. (B) Schematic representation of GST-E6AP proteins. GST proteins A through N contain the E6AP amino acids indicated to the right. The shaded region of each represents the GST sequence. Closed boxes represent proteins that are bound specifically to HCV core protein, and open boxes represent those that are not bound. (C) Binding of HCV core protein to GST-E6AP proteins A through N. In vitro-translated core protein (aa 1 to 173) was assayed for association with GST (-) or the GST-E6AP fusion proteins A through N. Association of core protein was detected by immunoblotting with anti-core MAb.

sessed the effects of E6AP on the HCV core protein in 293T cells. FLAG-core (1–191) together with HA-tagged wild-type E6AP, catalytically inactive mutant E6AP, E6AP C-A (19), or WWP1 (another HECT domain ubiquitin ligase) (22) was introduced into 293T cells, and the levels of the core protein were examined by immunoblotting. The steady-state levels of the core protein decreased with an increase in the amount of E6AP plasmids (Fig. 4A and B). However, neither E6AP C-A mutant nor WWP1 decreased the steady-state levels of the core protein, suggesting that E6AP enhances degradation of the core protein.

To verify the critical need for endogenous E6AP in the core degradation, expression of E6AP was knocked down by siRNA and the expression of the core protein and E6AP was assayed by immunoblotting. Transfection of the E6AP-specific siRNA

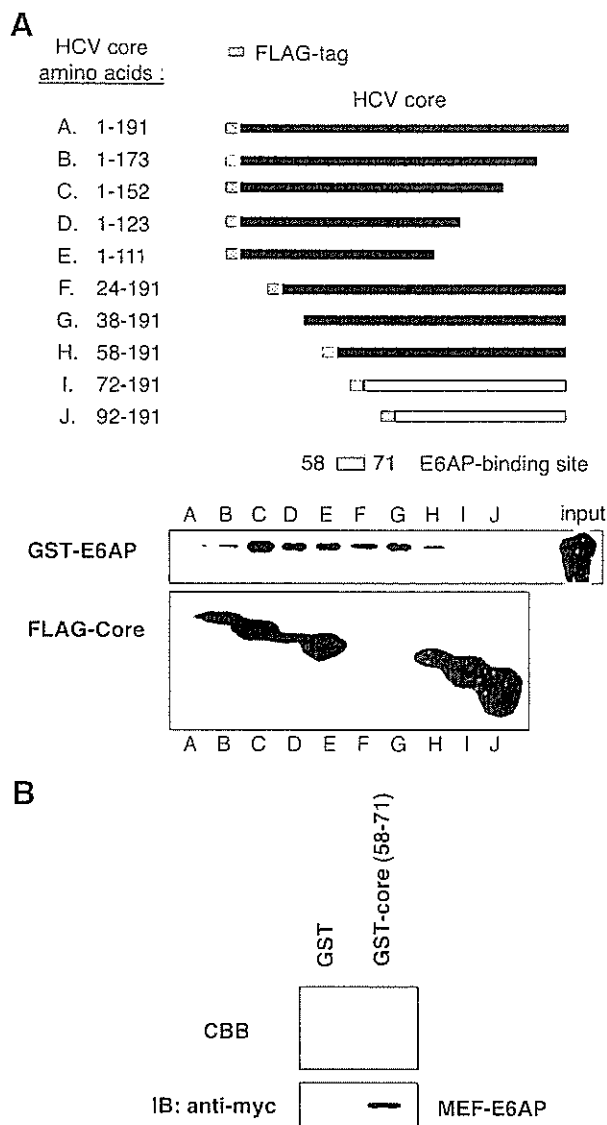


FIG. 3. Mapping of the E6AP binding domain for HCV core protein. (A) In vitro binding of E6AP to HCV core protein. 293T cells were transfected with each plasmid indicated in the upper panel. At 48 h posttransfection, cell lysates were mixed with purified GST-E6AP, immunoprecipitated with anti-FLAG beads, and then immunoblotted with anti-GST PAb (middle panel) or anti-FLAG MAb (bottom panel). The last lane (input) represents GST-E6AP used in this assay (middle panel). (B) Binding of GST-core (aa 58 to aa 71) to purified MEF-E6AP. GST served as a negative control for binding. Upper panel, Coomassie blue-stained SDS-PAGE of GST and GST-core (58-71). Lower panel, results of the GST pull-down assay. MEF-E6AP was detected by anti-myc MAb. CBB, Coomassie brilliant blue; IB, immunoblot.

duplex reduced the protein level of E6AP by 90% at 48 h posttransfection (Fig. 4C, middle panel). Immunoblotting revealed a 4.1-fold increase in the level of the core protein in the cells transfected with E6AP siRNA (Fig. 4C, top panel), suggesting that endogenous E6AP plays a role in the proteolysis of the HCV core protein.

Then we examined whether E6AP reduces the steady-state

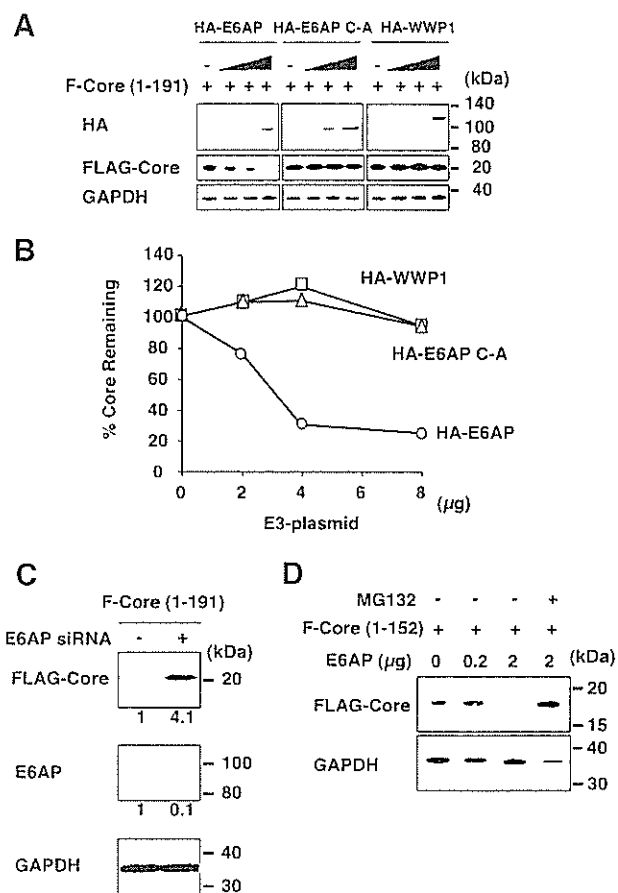


FIG. 4. E6AP decreases steady-state levels of HCV core protein in 293T cells and in HepG2 cells. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μ g of pCAG FLAG-core (1-191) along with either pCAG-HA-E6AP, pCAG-HA-E6AP C-A, or pCAG-HA-WWP1 as indicated. At 48 h posttransfection, protein extracts were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA PAb (top panel), anti-FLAG MAb (middle panel), and anti-GAPDH MAb (bottom panel). (B) Quantitation of data shown in panel A. Intensities of the gel bands were quantitated using the NIH Image 1.62 program. The level of GAPDH served as a loading control. Circles, E6AP; triangles, E6AP C-A; squares, WWP1. (C) Knockdown of endogenous E6AP by siRNA inhibits degradation of HCV core protein in 293T cells. 293T cells (3×10^5 cells/six-well plate) were transfected with 40 pmol of E6AP-specific duplex siRNA (or control siRNA) as described in Materials and Methods. The cells were transfected with 2 μ g of FLAG-core (1-191) expression plasmid and cultured for 24 h, harvested, and analyzed by immunoblotting. Shown is immunoblot detection of FLAG-tagged core protein (top panel), E6AP protein (middle panel), and GAPDH (bottom panel) in control siRNA-treated 293T cells or E6AP-siRNA-treated 293T cells. The relative levels of protein expression were quantitated by densitometry and indicated below in the respective lanes. GAPDH served as a loading control. (D) HepG2 cells (2×10^5 cells/six-well plate) were transfected with pCAG FLAG-core (1-152) along with either empty vector or pCMV E6AP as indicated. The cells were harvested at 44 h posttransfection. Where indicated, cells were treated with 25 μ M MG132 or with dimethyl sulfoxide control 14 h prior to collection. Equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-FLAG MAb (upper panel) or anti-GAPDH MAb (lower panel).

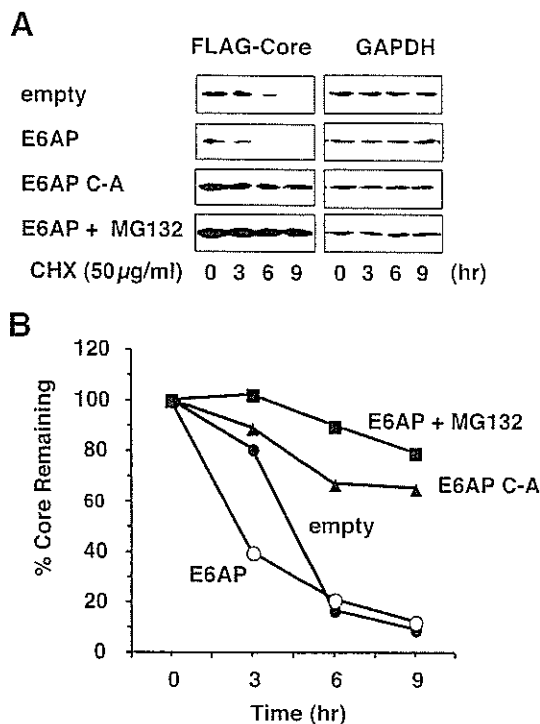


FIG. 5. Kinetic analysis of E6AP-dependent degradation of HCV core protein. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 µg of pCAG-FLAG core (1–152) plus 4 µg of empty vector, pCMV-HA-E6AP, or pCMV-HA-E6AP C-A. The cells were treated with 50 µg/ml CHX at 44 h after transfection. Cell extracts were collected at 0, 3, 6, and 9 h after treatment with CHX, followed by immunoblotting. (B) Specific signals were quantitated by densitometry, and the percent remaining core at each time was compared with that at the starting point. The level of GAPDH served as a loading control. Open circles, E6AP; closed circles, empty plasmid; closed triangles, E6AP C-A; closed squares, E6AP with MG132 treatment. Data are representative of three independent experimental determinations.

levels of the core protein in hepatic cells as well as in 293T cells. Exogenous expression of E6AP resulted in reduction of the core protein in human hepatoblastoma HepG2 cells (Fig. 4D). Treatment of the cells with the proteasome inhibitor MG132 increased the core protein level, suggesting that the core protein was degraded through the ubiquitin-proteasome pathway. These results indicate that E6AP enhances proteasomal degradation of the HCV core protein in both hepatic cells and nonhepatic cells.

Kinetic analysis of E6AP-dependent degradation of HCV core protein. To determine whether the E6AP-induced reduction of the core protein is due to an increase in the rate of core degradation, we performed kinetic analysis using the protein synthesis inhibitor CHX. HCV core protein together with wild-type E6AP or inactive mutant E6AP C-A was expressed in 293T cells. At 44 h after transfection, cells were treated with either 50 µg/ml CHX alone or 50 µg/ml CHX plus 25 µM MG132 to inhibit proteasome function. Cells were collected at 0, 3, 6, and 9 h following treatment and analyzed by immunoblotting (Fig. 5A). Overexpression of E6AP resulted in rapid degradation of the core protein, whereas inactive mutant

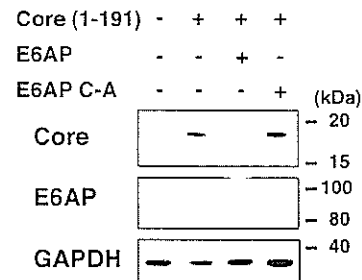


FIG. 6. E6AP promotes degradation of full-length HCV core protein in Huh-7 cells. Huh-7 cells (2×10^5 cells/six-well plate) were transfected with 0.5 µg of pCAG-core (1–191) together with 2 µg of pCMV-HA-E6AP or pCMV-HA-E6AP C-A. At 48 h posttransfection, cells were harvested and analyzed by immunoblotting with anticore MAb (top panel), anti-E6AP PAb (middle panel), or anti-GAPDH MAb (bottom panel).

E6AP C-A increased the half-life of the core protein (Fig. 5B), suggesting that the inactive E6AP inhibited degradation of the core protein in a dominant-negative manner, which is in agreement with previous studies (19, 55). Treatment of the cells with MG132 inhibited the degradation of the core protein (Fig. 5B). Reverse transcription-PCR to determine mRNA levels of the HCV core gene and GAPDH gene found that neither wild-type E6AP nor inactive E6AP changed mRNA levels of the HCV core gene and GAPDH gene (data not shown). These results indicate that E6AP enhances proteasomal degradation of the core protein.

E6AP promotes degradation of the full-length core protein in Huh-7 cells. To determine whether the full-length HCV core protein expressed in hepatic cells is degraded through an E6AP-dependent pathway, human hepatoma Huh-7 cells were transfected with pCAG HCV core (1–191) along with either E6AP or E6AP C-A. To rule out the effects of N-terminal FLAG tag on the core degradation, HCV core protein was expressed as untagged protein. Expression of wild-type E6AP resulted in reduction of the core protein (Fig. 6). On the other hand, HCV core protein was not decreased after transfection of inactive E6AP, indicating that the full-length core protein expressed in Huh-7 cells is also degraded through an E6AP-dependent pathway.

E6AP mediates ubiquitylation of HCV core protein in vivo. To determine whether E6AP can induce ubiquitylation of HCV core protein in cells, we performed in vivo ubiquitylation assays. 293T cells were cotransfected with FLAG-core (1–191) and either E6AP or empty plasmid, together with a plasmid encoding HA-tagged ubiquitin to facilitate detection of ubiquitylated core protein. Cell lysates were immunoprecipitated with anti-FLAG MAb and immunoblotted with anti-HA PAb to detect ubiquitylated core protein (Fig. 7A). Only a little ubiquitin signal was observed on the core protein in the absence of cotransfected E6AP (Fig. 7A, lane 3). In contrast, coexpression of E6AP led to readily detectable ubiquitylated forms of the core protein as a ladder and a smear of higher-molecular-weight bands (Fig. 7A, compare lane 3 with lane 4). Immunoblot analysis with anticore PAb confirmed that FLAG-core proteins were immunoprecipitated (Fig. 7B, lanes 2 to 4, short exposure) and that higher-molecular-weight bands con-

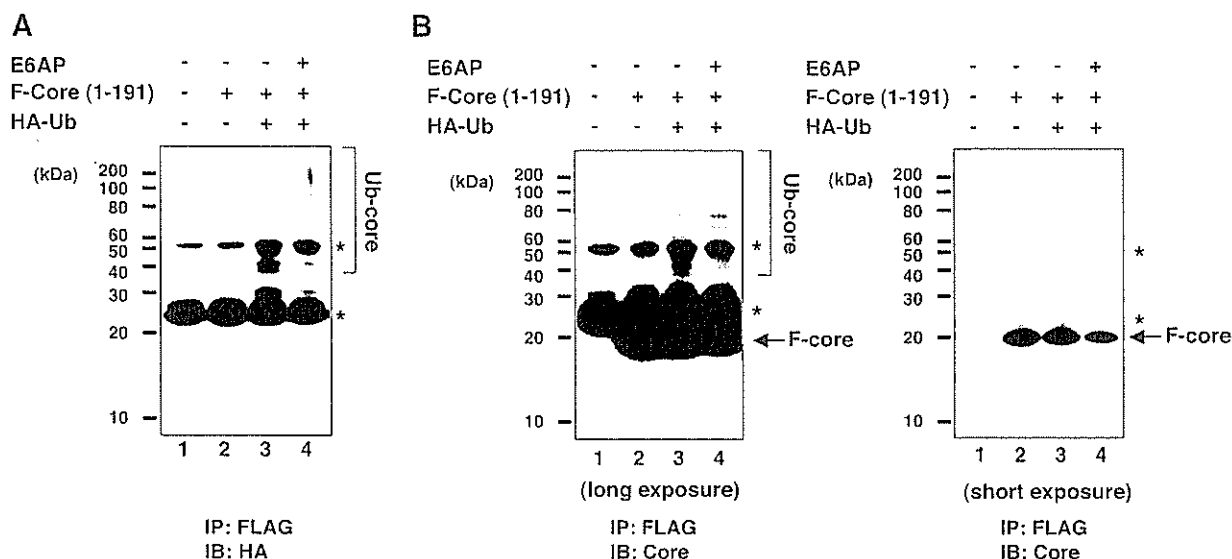


FIG. 7. E6AP-dependent ubiquitylation of HCV core protein in vivo. 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μ g of pCAG FLAG-core (1–191) together with 2 μ g of plasmid encoding E6AP as indicated. Each transfection also included 2 μ g of plasmid encoding HA-ubiquitin. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb (A) or anticore PAb (B). A shorter exposure of the core blot shows immunoprecipitated FLAG-core protein (B, right panel). A longer exposure of the core blot shows the presence of a ubiquitin smear (B, left panel). Asterisks indicate cross-reacting immunoglobulin light chain or heavy chain. Arrows indicate FLAG-core. IB, immunoblot; IP, immunoprecipitation.

jugated with HA-ubiquitin were indeed ubiquitylated forms of the core protein (Fig. 7B, lanes 3 and 4, long exposure).

E6AP mediates ubiquitylation of HCV core protein in vitro. To rule out the possibility that E6AP contributes to core protein degradation by inducing degradation of inhibitors of core turnover, we determined whether E6AP functions directly as a ubiquitin ligase by testing the ability of purified MEF-E6AP to mediate in vitro ubiquitylation of the purified recombinant HCV core protein. HCV core protein was expressed as a fusion protein containing N-terminal GST tag and C-terminal His tag and purified as described in Materials and Methods. GST-C173HT (aa 1–173) and GST-C152HT (aa 1–152) (see Materials and Methods) were used to determine whether the mature core protein and the C-terminally truncated core protein are targeted for ubiquitylation in vitro. The validity of this assay was established by demonstrating that E6AP but not E6AP C-A induced ATP-dependent ubiquitylation of GST-core protein. When in vitro ubiquitylation reactions were carried out either in the absence of MEF-E6AP or in the presence of MEF-E6AP C-A, no ubiquitylation signal was detected (Fig. 8A, lanes 4 and 5). However, inclusion of purified MEF-E6AP in the reaction mixture resulted in marked ubiquitylation of GST-C173HT (Fig. 8A, lane 6), while no ubiquitylation was observed in the absence of ATP (Fig. 8A, lane 7). No signal was detected when GST-HT was used as a substrate (Fig. 8A, lane 8). The higher-molecular-weight species of GST-core proteins were reactive with both anti-ubiquitin MAb (Fig. 8B, right panel, lanes 2 and 4) and anti-GST MAb (Fig. 8B, left panel, lanes 2 and 4). Both GST-C152HT and GST-C173HT were polyubiquitylated by E6AP in vitro (Fig. 8B), indicating that both the C-terminally truncated core and the mature core are polyubiquitylated by E6AP in vitro. These results revealed

that E6AP directly mediated ubiquitylation of HCV core proteins in an ATP-dependent manner.

Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. We used a recently developed system for the production of infectious HCV particles using the HCV JFH1 strain (28, 56, 61) to examine whether E6AP can promote degradation of HCV core protein expressed from infectious HCV. E6AP-dependent core degradation was assessed in Huh-7 cells inoculated with the culture supernatant containing HCV JFH1. Levels of HCV core protein were detectable at day 3 postinfection and increased with time. Immunofluorescence staining for the core protein indicated that the percentage of HCV core-positive cells in the Huh-7 cells was almost 100 at day 7 postinfection. Transfection efficiency was 50 to 60% as measured with GFP-expressing plasmid. At day 7 postinfection, exogenous expression of E6AP reduced the intracellular core protein level by about 60% compared to the empty plasmid-transfected control cells (Fig. 9A). Inactive E6AP had little effect on the core protein levels. Total protein levels in the cells (Fig. 9B) and intracellular HCV RNA levels (Fig. 9C) did not change after transfection of wild-type E6AP or inactive E6AP. The immunofluorescence study revealed that HCV core protein was variably detected and the intensity of core staining was reduced in the cells staining positive for wild-type E6AP compared with neighboring cells staining negative for E6AP (Fig. 9E). Using inactive E6AP revealed colocalization of the core protein and E6AP in the perinuclear region (Fig. 9F) of HCV-infected cells. These results suggest that E6AP enhanced degradation of HCV core protein expressed from infectious HCV. Then we titrated HCV infectivity in the culture supernatant at day 7 postinfection by limiting

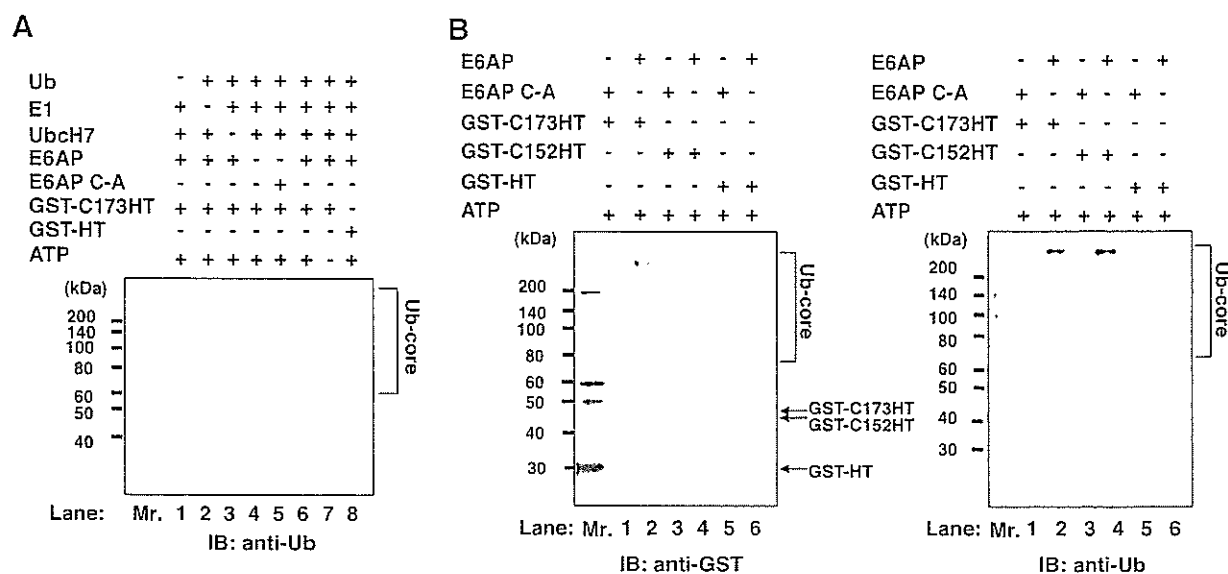


FIG. 8. In vitro ubiquitylation of HCV core protein by recombinant E6AP. For in vitro ubiquitylation of HCV core protein, purified GST-C173HT and GST-C152HT were used as substrates. Purified GST-HT was used as a negative control. Assays were done in 40- μ l volumes containing each component as indicated. The reaction mixture is described in Materials and Methods. The reaction was carried out at 37°C for 120 min followed by purification with glutathione-Sepharose beads and analysis by immunoblotting with the indicated antibodies. Arrows indicate GST-C173HT, GST-C152HT, and GST-HT, respectively. Ubiquitylated species of GST-core proteins are marked by brackets. IB, immunoblot.

dilution assays. Exogenous expression of E6AP reduced the supernatant infectivity titer, whereas inactive E6AP had no effect on its infectivity titer (Fig. 9D), suggesting that the E6AP-dependent ubiquitin proteasome pathway affects the production of HCV particles through downregulation of the core protein.

E6AP silencing increases the levels of intracellular HCV core protein and supernatant infectivity titers in HCV-infected Huh-7 cells. Finally, to further validate the role of E6AP in HCV production, expression of endogenous E6AP was knocked down by siRNA and the HCV infectivity titers released from HCV JFH1-infected cells were examined. Knock-down of E6AP by siRNA led to an increase in intracellular core protein levels (Fig. 10A) and supernatant HCV infectivity titers (Fig. 10B). Taken together, our results suggest that E6AP mediates ubiquitylation and degradation of HCV core protein in HCV-infected cells, thereby affecting the production of HCV particles.

DISCUSSION

HCV core protein is a major component of viral nucleocapsid, plays a central role in viral assembly (25, 40), and contributes to viral pathogenesis and hepatocarcinogenesis (9). Therefore, it is important to clarify the molecular mechanisms that govern the cellular stability of this viral protein. We have previously reported that processing at the C-terminal hydrophobic domain of the core protein leads to efficient polyubiquitylation of the core protein (52). In this study, we identified E6AP as an HCV core-binding protein and showed that HCV core protein interacts with E6AP in vivo and in vitro, that E6AP enhances ubiquitylation and degradation of the mature core protein as well as the C-terminally truncated core protein, and that HCV core protein expressed from infectious HCV is

degraded via E6AP-dependent proteolysis. HCV core protein and E6AP were found to colocalize in the cytoplasm, especially in the perinuclear region. Moreover, exogenous expression of E6AP reduces intracellular core protein levels and supernatant HCV infectivity titers in HCV-infected Huh-7 cells. Knock-down of endogenous E6AP by siRNA increases intracellular core protein levels and supernatant infectivity titers in HCV-infected cells. These findings suggest that E6AP mediates ubiquitylation and degradation of HCV core protein, thereby affecting the production of HCV particles.

HCV core protein interacts with E6AP through the region of the core protein between aa 58 and aa 71. These 14 amino acids are highly conserved, with the first nine amino acids (PRGRRQPIP) present in the core protein of all the HCV genotypes (3). This result suggests that E6AP-dependent degradation of HCV core protein is common to all HCV genotypes and plays an important role in the HCV life cycle or viral pathogenesis. Our data indicated that HCV core proteins of genotypes 1b and 2a are subjected to proteolysis through an E6AP-mediated degradation pathway. We are currently examining whether E6AP promotes degradation of HCV core proteins of other genotypes.

Studies in addition to ours have reported that other HCV proteins, such as NS5B (8), the unglycosylated cytosolic form of E2 (39), NS2 (7), and F protein (58), are degraded through the ubiquitin-proteasome pathway. These studies suggest that the ubiquitin-proteasome pathway plays a role in the HCV life cycle or viral pathogenesis. To our knowledge, the present study is the first to demonstrate that the ubiquitin-proteasome pathway affects the HCV life cycle.

PA28 γ was found to interact with HCV core protein in hepatocytes and promote proteasomal degradation of HCV core protein (30). PA28 γ , however, has been shown to function

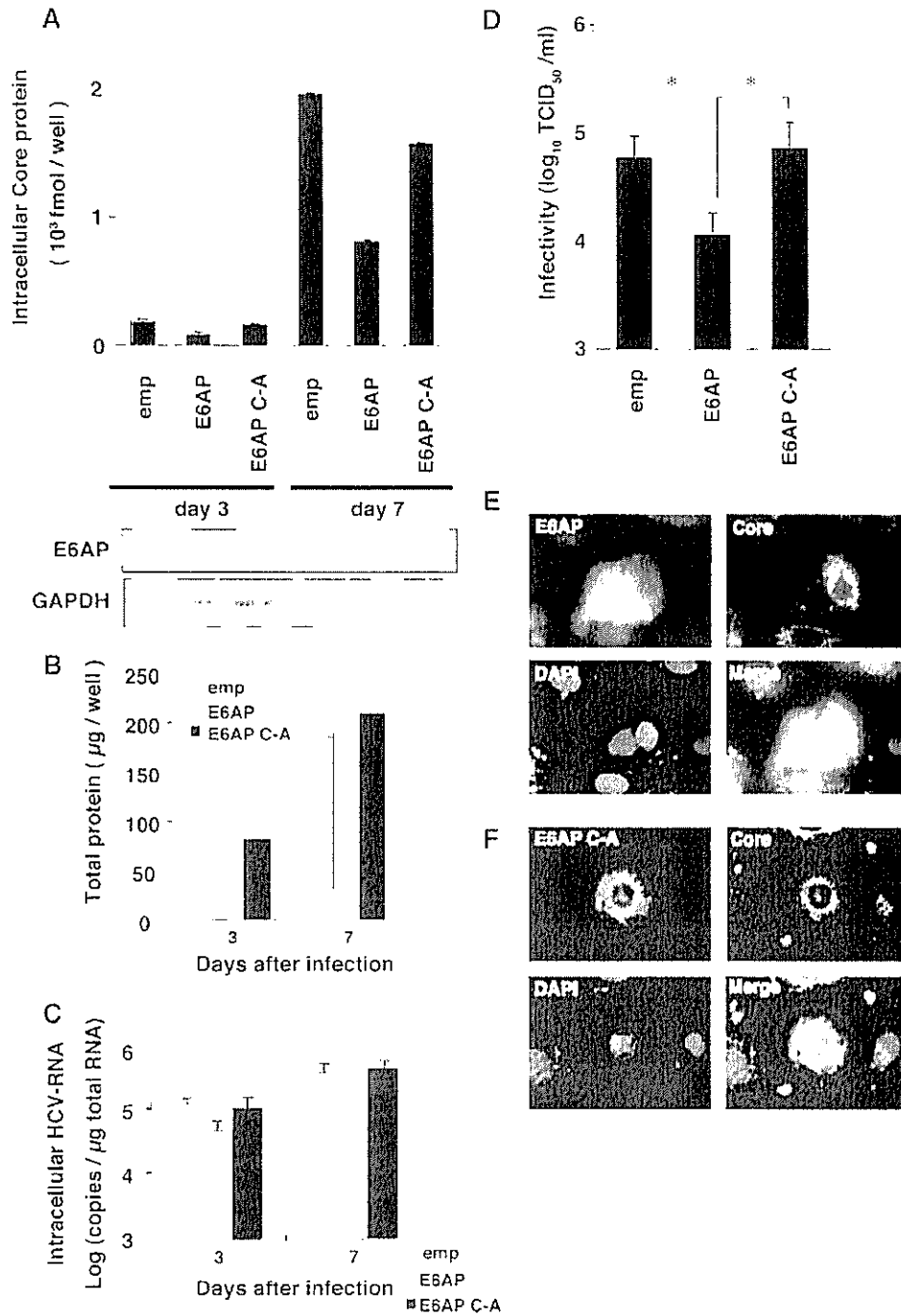


FIG. 9. Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. Naïve Huh-7 cells were seeded as described in Materials and Methods; inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5×10^3 TCID₅₀/ml); and transfected with 6 μg of empty plasmid, pCAG-HA-E6AP, or pCAG-HA-E6AP C-A. The culture supernatant and the cells were collected at days 3 and 7 postinfection. (A) Intracellular HCV core protein levels. (B) Levels of total protein. (C) Levels of intracellular HCV RNA in HCV-infected Huh-7 cells. Data represent the averages of three experiments with error bars. (D) Supernatant infectivity titers. At day 7 postinfection, culture supernatants were collected and assayed for TCID₅₀ determinations. The difference between empty vector and E6AP or between E6AP and E6AP C-A was significant ($P < 0.05$, Student's *t* test). (E and F) HCV JFH1-infected Huh-7 cells were transfected with either MEF-E6AP plasmid or MEF-E6AP C-A plasmid, grown on coverslips, fixed, and processed for double-label immunofluorescence for HCV core and MEF-E6AP (E) or MEF-E6AP C-A (F). Anticore MAb (2H9) and anti-FLAG PAb were used as primary antibodies. Nuclei were visualized by staining the cells with DAPI. All the samples were examined with a BZ-8000 microscope. Representative images of individual cells are shown with merge images. emp, empty vector

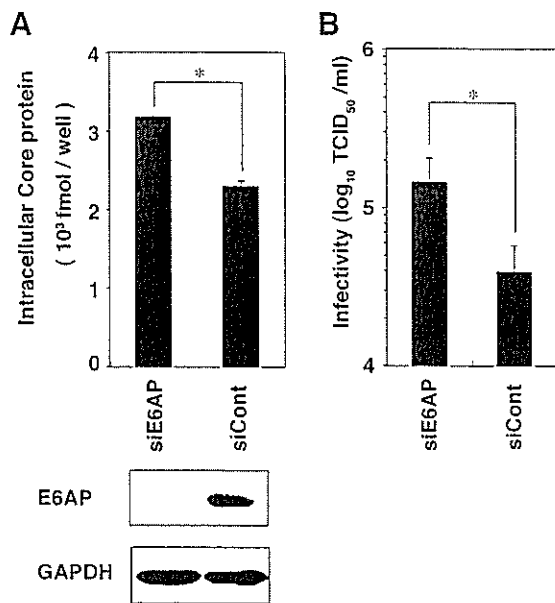


FIG. 10. E6AP silencing leads to an increase in the level of intracellular HCV core protein and supernatant infectivity titer in HCV-infected Huh-7 cells. (A) HCV JFH1-infected cells were replated in a six-well plate at 3×10^5 cells/well and transfected with 40 pmol of E6AP siRNA or control siRNA. The culture medium was changed at 24 h after transfection. The cells were harvested at day 2 after transfection, and the intracellular core protein levels were quantitated using the HCV core antigen ELISA. Equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-E6AP MAb or anti-GAPDH MAb. (B) Culture supernatants were collected at day 2 after transfection and assayed for TCID₅₀ determinations. For both panels, the difference between E6AP siRNA and control siRNA was significant (*, $P < 0.05$, Student's *t* test).

in a ubiquitin-independent, ATP-independent, and 20S proteasome-dependent pathway (27). There have been reports that several cellular factors, such as p53 (2), p73 (2), and RPN4 (18), are degraded through two alternative pathways, the ubiquitin-dependent 26S proteasome-dependent pathway and the ubiquitin-independent 20S proteasome-dependent pathway. Here we provide evidence that E6AP mediates ubiquitylation of HCV core protein. Still unclear is whether the PA28 γ -dependent pathway requires polyubiquitylation of HCV core protein. HCV core protein is predominantly localized in the cytoplasm, especially at the endoplasmic reticulum membrane, on the surface of lipid droplets, and on mitochondria and mitochondrion-associated membranes (51). In HCV JFH1-infected cells, HCV core was found to localize in the cytoplasm and frequently to accumulate in the perinuclear region and the lipid droplets (44). Our results indicated that E6AP colocalized with HCV core protein especially in the perinuclear region. PA28 γ was found to colocalize with HCV core protein in the nucleus. Functional differences may exist between the E6AP-dependent pathway and the PA28 γ -dependent pathway in the stability control of HCV core protein. The functional role of the E6AP-dependent pathway and the PA28 γ -dependent pathway remains to be elucidated.

The HCV core-binding region of E6AP was mapped to the region between aa 418 and aa 517. The multicopy maintenance protein 7, Mcm7, interacts with E6AP through a short motif,

termed the L2G box (aa 412 to 414), that lies within the E6 binding site of E6AP (23). Our data indicated that the E6 binding region containing the L2G motif is not required for interaction between HCV core protein and E6AP (Fig. 2C, lane M).

We propose here that E6AP may affect the production of HCV particles through controlling the amounts of HCV core protein. This mechanism may contribute to persistent infection. The E6AP binding domain of the core protein resides in the RNA-binding domain and binding domains for many host factors (40). These factors may affect the binding between E6AP and HCV core protein, resulting in control of E6AP-dependent core degradation. Another possibility is that HCV core protein may affect the normal function of E6AP, thereby contributing to pathogenesis. It will be intriguing to investigate whether HCV core protein has any effect on E6AP-dependent degradation of host factors. The other intriguing possibility is that HCV core-E6AP complex may function as an E3 ligase-like E6-E6AP complex to target host factors for proteasomal degradation and contribute to viral pathogenesis.

In conclusion, we have demonstrated that E6AP interacts with HCV core protein *in vitro* and *in vivo* and mediates ubiquitin-dependent degradation of the core protein, leading to downregulation of HCV particles. We propose that the E6AP-mediated ubiquitin-proteasome pathway may play a role in affecting the production of HCV particles through controlling the amounts of viral nucleocapsid protein. Identification of the specific E3 ubiquitin ligase may contribute to gaining a better understanding of the biology of the HCV life cycle as well as molecular details of the ubiquitin-dependent degradation of HCV core protein.

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Critical role of PA28 γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis

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Hepatitis C virus (HCV) is a major cause of chronic liver disease that frequently leads to steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC). HCV core protein is not only a component of viral particles but also a multifunctional protein because liver steatosis and HCC are developed in HCV core gene-transgenic (CoreTg) mice. Proteasome activator PA28 γ /REG γ regulates host and viral proteins such as nuclear hormone receptors and HCV core protein. Here we show that a knockout of the PA28 γ gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of CoreTg mice and disrupts development of both hepatic steatosis and HCC. Furthermore, the genes related to fatty acid biosynthesis and *srebp-1c* promoter activity were up-regulated by HCV core protein in the cell line and the mouse liver in a PA28 γ -dependent manner. Heterodimer composed of liver X receptor α (LXR α) and retinoid X receptor α (RXR α) is known to up-regulate *srebp-1c* promoter activity. Our data also show that HCV core protein enhances the binding of LXR α /RXR α to LXR-response element in the presence but not the absence of PA28 γ . These findings suggest that PA28 γ plays a crucial role in the development of liver pathology induced by HCV infection.

fatty acid | proteasome | sterol regulatory element-binding protein (SREBP) | RXR α | LXR α

Hepatitis C virus (HCV) belongs to the Flaviviridae family, and it possesses a positive, single-stranded RNA genome that encodes a single polyprotein composed of \approx 3,000 aa. The HCV polyprotein is processed by host and viral proteases, resulting in 10 viral proteins. Viral structural proteins, including the capsid (core) protein and two envelope proteins, are located in the N-terminal one-third of the polyprotein, followed by nonstructural proteins.

HCV infects >170 million individuals worldwide, and then it causes liver disease, including hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (1). The prevalence of fatty infiltration in the livers of chronic hepatitis C patients has been reported to average \approx 50% (2, 3), which is higher than the percentage in patients infected with hepatitis B virus and other liver diseases. However, the precise functions of HCV proteins in the development of fatty liver remain unknown because of the lack of a system sufficient to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and HCC in transgenic mice (4–6). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and HCC.

Recent reports suggest that lipid biosynthesis affects HCV replication (7–9). Involvement of a geranylgeranylated host protein, FBL2, in HCV replication through the interaction with NSSA suggests that the cholesterol biosynthesis pathway is also important for HCV replication (9). Increases in saturated and monounsaturated fatty acids enhance HCV RNA replication, whereas increases in polyunsaturated fatty acids suppress it (7). Lipid homeostasis is regulated by a family of steroid regulatory element-binding proteins (SREBPs), which activate the expression of >30 genes involved in

the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. Biosynthesis of cholesterol is regulated by SREBP-2, whereas that of fatty acids, triglycerides, and phospholipids is regulated by SREBP-1c (10–14). In chimpanzees, host genes involved in SREBP signaling are induced during the early stages of HCV infection (8). SREBP-1c regulates the transcription of acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, leading to the production of saturated and monounsaturated fatty acids and triglycerides (15). SREBP-1c is transcriptionally regulated by liver X receptor (LXR) α and retinoid X receptor (RXR) α , which belong to a family of nuclear hormone receptors (15, 16). Accumulation of cellular fatty acids by HCV core protein is expected to be modulated by the SREBP-1c pathway because RXR α is activated by HCV core protein (17). However, it remains unknown whether HCV core protein regulates the *srebp-1c* promoter.

We previously reported (18) that HCV core protein specifically binds to the proteasome activator PA28 γ /REG γ in the nucleus and is degraded through a PA28 γ -dependent pathway. PA28 γ is well conserved from invertebrates to vertebrates, and amino acid sequences of human and murine PA28 γ s are identical (19). The homologous proteins, PA28 α and PA28 β , form a heteroheptamer in the cytoplasm, and they activate chymotrypsin-like peptidase activity of the 20S proteasome, whereas PA28 γ forms a homoheptamer in the nucleus, and it enhances trypsin-like peptidase activity of 20S proteasome (20). Recently, Li and colleagues (21) reported that PA28 γ binds to steroid receptor coactivator-3 (SRC-3) and enhances the degradation of SRC-3 in a ubiquitin- and ATP-independent manner. However, the precise physiological functions of PA28 γ are largely unknown *in vivo*. In this work, we examine whether PA28 γ is required for liver pathology induced by HCV core protein *in vivo*.

Results

PA28 γ -Knockout HCV Core Gene Transgenic Mice. To determine the role of PA28 γ in HCV core-induced steatosis and the development of HCC *in vivo*, we prepared PA28 γ -knockout core gene transgenic mice. The PA28 γ -deficient, PA28 γ ^{-/-} mice were born without

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Abbreviations: CoreTg, HCV core gene-transgenic; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LXR, liver X receptor; LXRE, liver X receptor-response element; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; RXR, retinoid X receptor; SRC-3, steroid receptor coactivator-3; SREBP, steroid regulatory element-binding protein.

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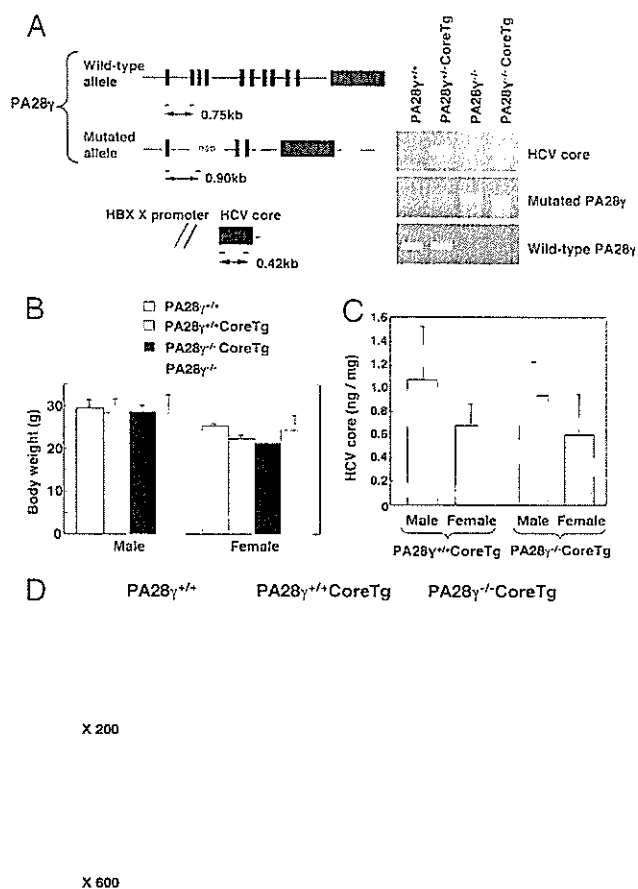


Fig. 1. Preparation and characterization of PA28 γ -knockout HCV core-transgenic mice. (A) The structures of the wild-type and mutated PA28 γ genes and the transgene encoding the HCV core protein under the control of the HBV X promoter were investigated. Positions corresponding to the screening primers and sizes of PCR products are shown. PCR products of the HCV core gene as well as wild-type and mutated PA28 γ alleles were amplified from the genomic DNAs of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice. (B) Body weights of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$ mice at the age of 6 months. (C) HCV core protein levels in the livers of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice were determined by ELISA (mean \pm SD, $n = 10$). (D) Localization of HCV core protein in the liver. Liver sections of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, and PA28 $\gamma^{-/-}$ CoreTg mice at the age of 2 months were stained with anti-HCV core antibody.

appreciable abnormalities in all tissues examined, with the exception of a slight retardation of growth (22). HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg) mice were bred with PA28 $\gamma^{-/-}$ mice to create PA28 $\gamma^{-/-}$ CoreTg mice. The PA28 $\gamma^{+/+}$ CoreTg offspring were bred with each other, and PA28 $\gamma^{-/-}$ CoreTg mice were selected by PCR using primers specific to the target sequences (Fig. 1A). No significant differences in body weight were observed among the 6-month-old mice, although PA28 $\gamma^{-/-}$ mice exhibited a slight retardation of growth (Fig. 1B). A similar level of PA28 γ expression was detected in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{+/+}$ mice (see Fig. 5B). The expression levels and molecular size of HCV core protein were similar in the livers of PA28 $\gamma^{-/-}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1C; see also Fig. 5B).

PA28 γ Is Required for Degradation of HCV Core Protein in the Nucleus and Induction of Liver Steatosis. HCV core protein has been detected at various sites, such as the endoplasmic reticulum, mitochondria, lipid droplets, and nucleus of cultured cell lines, as well as in hepatocytes of PA28 $\gamma^{+/+}$ CoreTg mice and hepatitis C patients

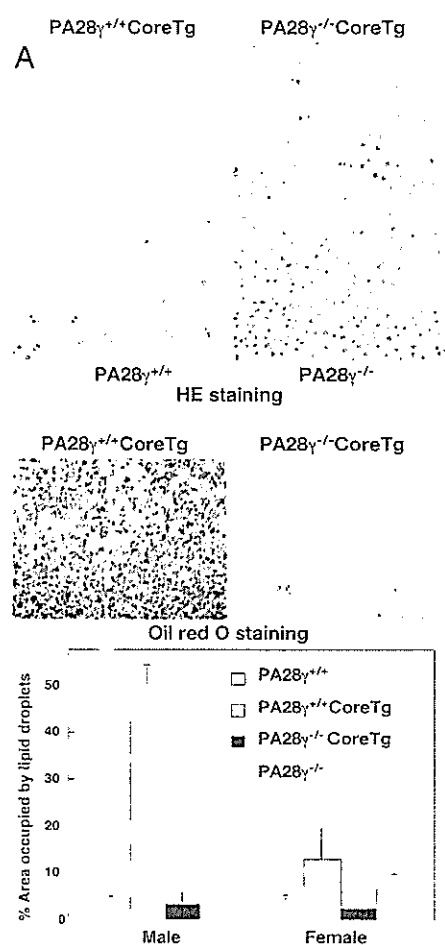


Fig. 2. Accumulation of lipid droplets by expression of HCV core protein. (A) Liver sections of the mice at the age of 6 months were stained with hematoxylin/eosin (HE). (B) (Upper) Liver sections of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice at the age of 6 months were stained with oil red O. (Lower) The area occupied by lipid droplets of PA28 $\gamma^{+/+}$ (white), PA28 $\gamma^{-/-}$ CoreTg (gray), PA28 $\gamma^{-/-}$ CoreTg (black), and PA28 $\gamma^{-/-}$ (dark gray) mice was calculated by image-Pro software (MediaCybernetics, Silver Spring, MD) (mean \pm SD, $n = 10$).

(6, 23, 24). Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of PA28 $\gamma^{+/+}$ CoreTg mice, as reported in ref. 6, in the present study a clear accumulation of HCV core protein was observed in the liver cell nuclei of PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1D). These findings clearly indicate that at least some fraction of the HCV core protein is translocated into the nucleus and is degraded through a PA28 γ -dependent pathway. Mild vacuolation was observed in the cytoplasm of the liver cells of 4-month-old PA28 $\gamma^{+/+}$ CoreTg mice, and it became more severe at 6 months, as reported in ref. 25. Hematoxylin/eosin-stained liver sections of 6-month-old PA28 $\gamma^{+/+}$ CoreTg mice exhibited severe vacuolating lesions (Fig. 2A), which were clearly stained with oil red O (Fig. 2B Upper), whereas no such lesions were detected in the livers of PA28 $\gamma^{-/-}$ CoreTg, PA28 $\gamma^{+/+}$, or PA28 $\gamma^{-/-}$ mice at the same age. The areas occupied by the lipid droplets in the PA28 $\gamma^{+/+}$ CoreTg mouse livers were ≈ 10 and 2–4 times larger than those of male and female of PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$ and PA28 $\gamma^{-/-}$ CoreTg mice, respectively (Fig. 2B Lower). These results suggest that PA28 γ is required for the induction of liver steatosis by HCV core protein in mice.

PA28 γ Is Required for the Up-Regulation of SREBP-1c Transcription by HCV Core Protein in the Mouse Liver. To clarify the effects of a knockout of the PA28 γ gene in PA28 $\gamma^{+/+}$ CoreTg mice on lipid

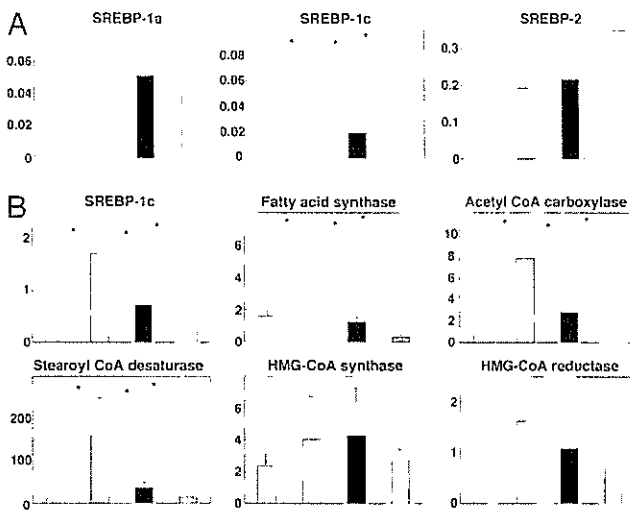


Fig. 3. Transcription of genes regulating lipid biosynthesis in the mouse liver. (A) Total RNA was prepared from the livers of 2-month-old mice; and the transcription of genes encoding SREBP-1a, SREBP-1c, and SREBP-2 was determined by real-time PCR. (B) The transcription of genes encoding SREBP-1c, fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CoA desaturase, HMG-CoA synthase, and HMG-CoA reductase of 6-month-old mice was measured by real-time PCR. The transcription of the genes was normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activity ($n = 5$; *, $P < 0.05$; **, $P < 0.01$). The transcription of each gene in PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$ mice is indicated by white, gray, black, and dark gray bars, respectively.

metabolism, genes related to the lipid biosyntheses were examined by real-time quantitative PCR. Transcription of SREBP-1c was higher in the livers of PA28 $\gamma^{+/+}$ CoreTg mice than in those of PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice at 2 months of age, but no such increases in SREBP-2 and SREBP-1a were observed (Fig. 3A). Although transcription of SREBP-1c and its regulating enzymes, such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, was also enhanced in the livers of 6-month-old PA28 $\gamma^{+/+}$ CoreTg mice compared with the levels in the livers of PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice, no statistically significant differences were observed with respect to the transcription levels of cholesterol biosynthesis-related genes that are regulated by SREBP-2 (e.g., HMG-CoA synthase and HMG-CoA reductase) (Fig. 3B). These results suggest the

following: (i) the up-regulation of SREBP-1c transcription in the livers of mice requires both HCV core protein and PA28 γ ; and (ii) the nuclear accumulation of HCV core protein alone, which occurs because of the lack of degradation along a PA28 γ -dependent proteasome pathway, does not activate the *srebp-1c* promoter.

HCV Core Protein Indirectly Potentiates *srebp-1c* Promoter Activity in an LXR α /RXR α -Dependent Manner. LXR α , which is primarily expressed in the liver, forms a complex with RXR α and synergistically potentiates *srebp-1c* promoter activity (16). Activation of RXR α by HCV core protein suggests that cellular fatty acid synthesis is modulated by the SREBP-1c pathway, although HCV core protein was not included in the transcription factor complex in the electrophoresis mobility shift assay (EMSA) (17). To analyze the effect of HCV core protein and PA28 γ on the activation of the *srebp-1c* promoter, we first examined the effect of HCV core protein on the binding of the LXR α /RXR α complex to the LXR-response element (LXRE) located upstream of the SREBP-1c gene (Fig. 4A). Although a weak shift of the labeled LXRE probe was observed by incubation with nuclear extracts prepared from 293T cells expressing FLAG-tagged LXR α and HA-tagged RXR α , a clear shift was obtained by the treatment of cells with 9-*cis*-retinoic acid and 22(*R*)-hydroxycholesterol, ligands for LXR α and RXR α , respectively. In contrast, coexpression of HCV core protein with LXR α and RXR α potentiated the shift of the probe irrespective of the treatment with the ligands. Addition of 500 times the amount of nonlabeled LXRE probe (competitor) diminished the shift of the labeled probe induced by the ligands and/or HCV core protein. Furthermore, coinubcation of the nuclear fraction with antibody to FLAG or HA tag but not with antibody to either HCV core or PA28 γ caused a supershift of the labeled probe. These results indicate that HCV core protein does not participate in the LXR α /RXR α -LXRE complex but indirectly enhances the binding of LXR α /RXR α to the LXRE.

The activity of the *srebp-1c* promoter was enhanced by the expression of HCV core protein in 293T cells, and it was further enhanced by coexpression of LXR α /RXR α (Fig. 4B). Enhancement of the *srebp-1c* promoter by coexpression of HCV core protein and LXR α /RXR α was further potentiated by treatment with the ligands for LXR α and RXR α . The cells treated with 9-*cis*-retinoic acid exhibited more potent enhancement of the *srebp-1c* promoter than those treated with 22(*R*)-hydroxycholesterol. HCV core protein exhibited more potent enhancement of the *srebp-1c* promoter in cells treated with both ligands than in those treated with either ligand alone. These results suggest that HCV core protein poten-

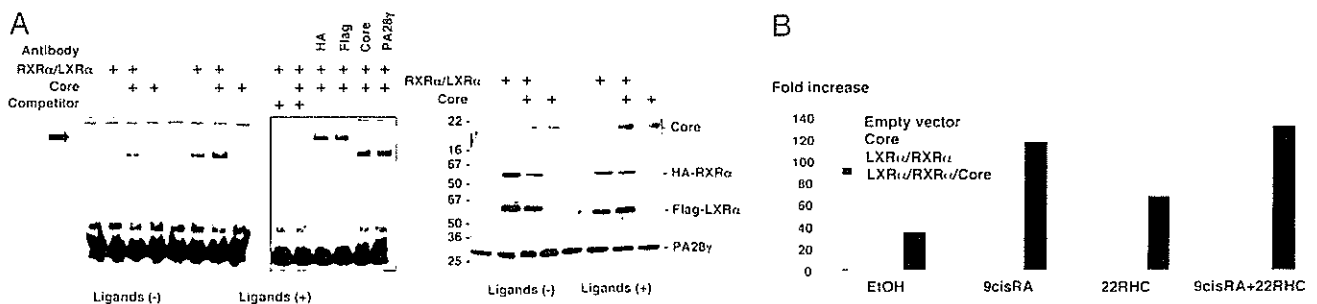


Fig. 4. Activation of the *srebp-1c* promoter by HCV core protein. (A) FLAG-LXR α and HA-RXR α were expressed in 293T cells together with or without HCV core protein. Ligands for LXR α and RXR α dissolved in ethanol [Ligands (+)] or ethanol alone [Ligands (-)] were added to the culture supernatant at 24 h posttransfection. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA in the presence or absence of antibody (100 ng) against HA, FLAG, HCV core or PA28 γ , or nonlabeled LXRE probe (Competitor). (Left) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase/streptavidin. The mobility shift of the LXRE probe and its supershift are indicated by a gray and black arrow, respectively. (Right) Expression of HCV core, HA-RXR α , FLAG-LXR α , and PA28 γ in cells was detected by immunoblotting. (B) Effects of ligands for RXR α , 9-*cis*-retinoic acid (9cisRA), and for LXR α , 22(*R*)-hydroxycholesterol (22RHC), on the activation of the *srebp-1c* promoter in 293T cells expressing RXR α , LXR α , and/or HCV core protein. Ligands were added into the medium at 24 h posttransfection at a concentration of 5 μ M, and the cells were harvested after 24 h of incubation.