inhibitor was less effective at suppressing the replication of the JFH1 replicon than genotype 1b replicons.

Interactions between CyPB and JFH1 NS5B. Previously, we have shown that CyPB interacts with NS5B to promote HCV genome replication and that CsA inhibits this binding in a genotype 1b replicon (31). Here, we examined the association between CyPB and NS5B in a JFH1 replicon. Immunoprecipitation analysis revealed an interaction of CyPB with NS5B in JFH1#4-1 (JFH1/2a/SG) cells (Fig. 5A). This interaction was dissociated following the treatment of CsA, as observed with the genotype 1b replicon (Fig. 5B).

Role of CyPB in replication of the JFH1 replicon. Although we observed some differences of expression levels of endogenous CyPB among the replicon cells in the immunoblot analysis (Fig. 6A), there was no particular correlation between endogenous CyPB expression levels and replication sensitivity to CsA among cells. CyPB reportedly regulates HCV genome replication of the genotype 1b replicon (31). We then explored the requirement of CyPB for the replication of JFH1 replicon with RNA interference. Transfecting siRNAs designed to recognize several CyP subtypes [si-CyP(broad)] (Fig. 6B) reduced HCV RNA to <1/5 in MH14#W31 (NN/1b/SG) cells (Fig. 6C). Specific knockdown of CyPB but not CyPA (Fig. 6B) decreased HCV RNA in MH14#W31 (NN/1b/SG) cells, consistent with a previous report (Fig. 6C) (31). In contrast, HCV RNA in JFH1#4-1 (JFH1/2a/SG) cells was not altered following the suppression of either endogenous CyPA or CyPB (Fig. 6B and C). We observed a weak decrease of HCV RNA levels (around one-half) with si-CyP(broad) (Fig. 6C). These data suggests the possibility that the replication of the JFH1 replicon is independent of CyPB, in contrast to the genotype 1b replicon. In the previous study, it was reported that the doubling time, saturation density, and response to cell confluence of the replicon cells carrying JFH1 were different from those in cells carrying a genotype 1b replicon, suggesting the possibility that the coupling relationship between the replication and cell growth was different between genotype 1b and the JFH1 replicon (21). The introduction of either si-CyPB or si-CyP(broad), however, had little effect on cell growth in MH14#W31 (NN/1b/SG) or JFH1#4-1 (JFH1/2a/SG) cells (Fig. 6D). And we did not observe cells being confluent in the experiment period. The above results suggest that the different response to si-CyPB in the two lines is independent of the conditions of cell growth.

The role of CyPB in the RNA binding activity of JFH1 NS5B. CyPB regulates HCV genome replication of a genotype 1b replicon by promoting the RNA binding activity of NS5B (31). We examined the effect of CyPB on the RNA binding activity of NS5B in JFH1. NS5B in the replication complex was isolated from cells by treatment with digitonin-proteinase K, as described previously (31). This fraction was incubated with poly(U) RNA-Sepharose or protein G-Sepharose as a negative control for the detection of RNA binding NS5B in the replication complex. RNA-bound NS5B in this fraction from MH14#W31 (NN/1b/SG) cells was decreased drastically following treatment with CsA (Fig. 7A, lanes 5 and 6). However, the reduction of RNA binding of NS5B in the replication complex of JFH1#4-1 (JFH1/2a/SG) cells was not as prominent (Fig. 7A, lanes 11 and 12). We confirmed this result by an in vitro RNA binding assay, in which in vitro-synthesized NS5B was incubated with poly(U) RNA-Sepharose, together with

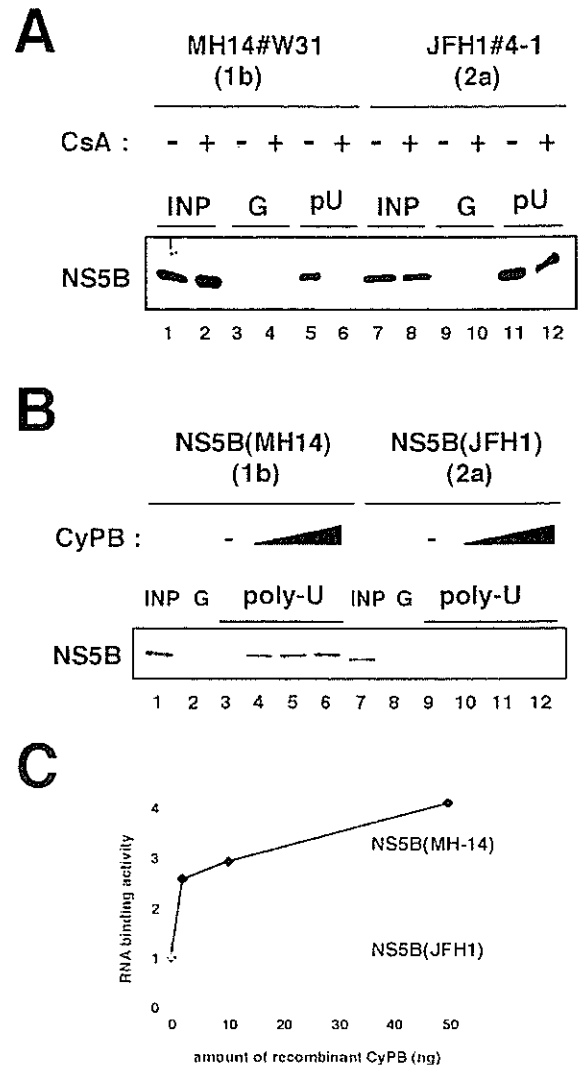

FIG. 7. RNA binding capacity of JFH1 NS5B was independent of CyPB. (A) An RNA-protein binding precipitation assay was performed using MH14#W31 (NN/1b/SG) cells (lanes 1 to 6) and JFH1#4-1 (JFH1/2a/SG) cells (lanes 7 to 12) as described in Materials and Methods. MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells preincubated without (lanes 1, 3, 5, 7, 9, and 11) or with (lanes 2, 4, 6, 8, 10, and 12) CsA were treated with digitonin, followed by digestion with proteinase K to isolate the replication complex. This fraction was then incubated with poly(U) RNA-Sepharose (lanes 5, 6, 11, and 12) or protein G-Sepharose as a negative control (lanes 3, 4, 9, and 10). Precipitates were detected by immunoblot analysis with anti-NS5B antibody. INP, one-sixth of the amount of cell lysate used in the precipitation assay; G and pU, samples with protein G-Sepharose and poly(U)-Sepharose, respectively. (B) An in vitro RNA binding assay was performed as described in Materials and Methods. In vitro-synthesized NS5B of MH-14 (lanes 1 to 6) or JFH1 (lanes 7 to 12) with the rabbit reticulocyte lysate in the presence of [^{35}S]methionine was incubated with protein G-Sepharose (lanes 2 and 8) or poly(U)-Sepharose in the absence (lanes 3 and 9) or presence of various amounts of purified recombinant GST-CyPB (2 ng in panels 4 and 10, 10 ng in panels 5 and 11, and 50 ng in panels 6 and 12). The resultant precipitates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by the detection of radiolabeled protein. (C) The density of the bands of NS5B in the RNA binding fraction was quantified and plotted against the amount of the recombinant GST-CyPB (in nanograms). Solid line, NS5B of MH-14; faint line, NS5B of JFH1.

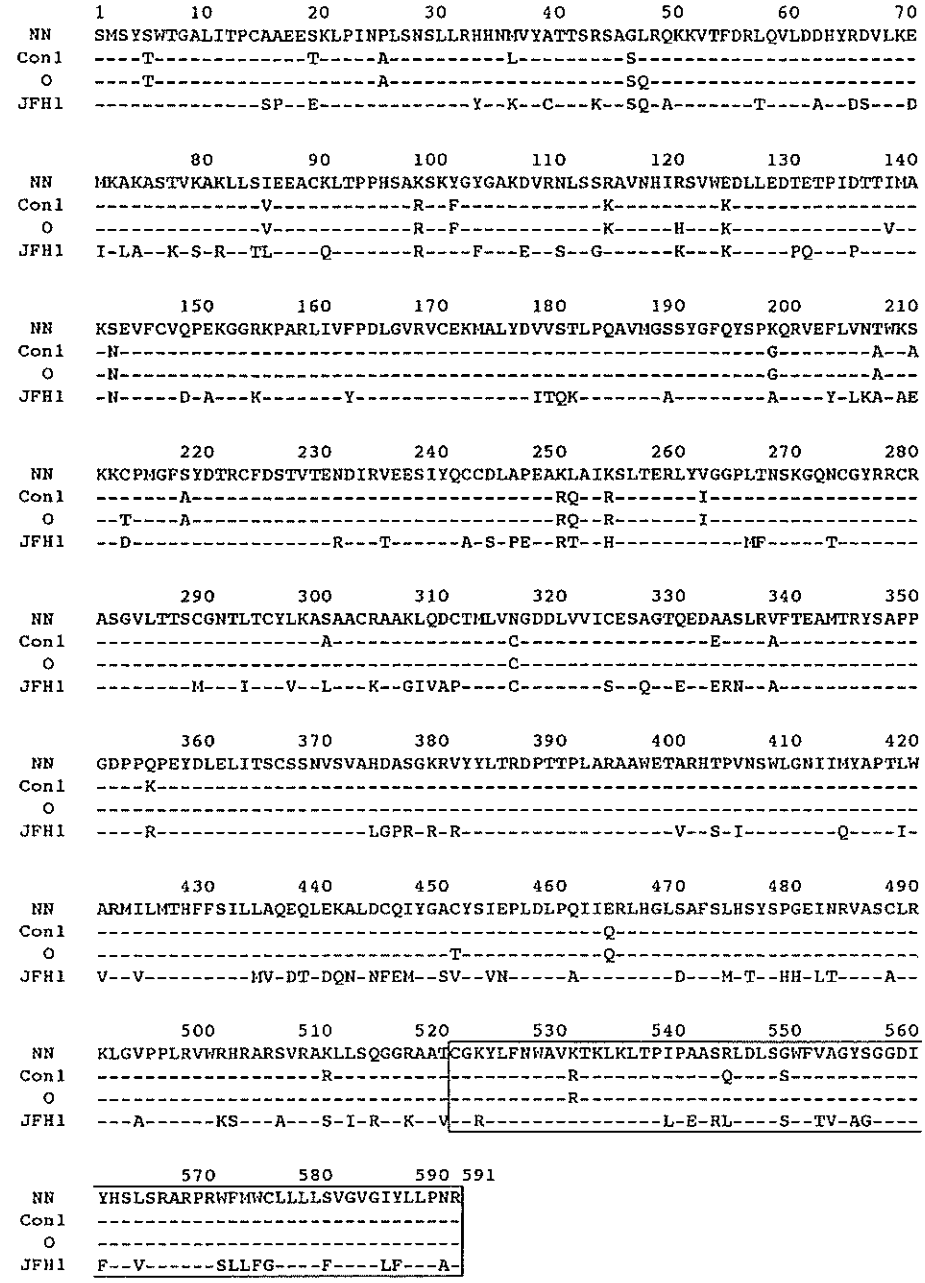

FIG. 8. Amino acid sequence alignment of NS5B encoded by HCV strains NN, Con1, O, and JFH1. The numbers above the sequence indicate the amino acid numbers. Conserved residues are shown by dashes. The region spanning 521 to 591 aa, which is involved in the interaction with CyPB, is boxed.

recombinant GST-CyPB. The addition of recombinant GST-CyPB increased the binding of genotype 1b NS5B to poly(U) RNA (Fig. 7B and C). However, this augmentation of RNA binding was not observed with NS5B from the JFH1 strain (Fig. 7B and C). From the above results, it is suggested that the RNA binding of JFH1 NS5B is free from regulation by CyPB.

DISCUSSION

Until now, we and another group have utilized subgenomic replicons carrying genotype 1b NN and HCV-N strains to demonstrate that CsA suppresses HCV genome replication (22, 29). This study reveals that CsA is effective on full-genome replicons to almost the same extent. In addition, other available genotype 1b replicons carrying the Con1 and O strains also have a high sensitivity to CsA, consistent with our proposal that HCV genotype 1b is highly sensitive to CsA. However, a fulminant-type genotype 2a replicon, JFH1, was less responsive to CsA, although a high dose of CsA suppressed the replication of this strain.

CyPB interacts with genotype 1b NS5B to stimulate its RNA

binding activity. In contrast, CyPB binds JFH1 NS5B but does not regulate the function of JFH1 NS5B. This is consistent with a previous speculation that genotype 1b and JFH1 replicons utilize the same cellular factors in distinct manners (21). The NS5B sequence of NN strain has 95.0, 95.9, and 70.4% homology to that of Con1, O, and JFH1, respectively (Fig. 8). The region spanning amino acids (aa) 521 to 591 of NS5B, which is involved in the interaction with CyPB (31), is highly conserved among genotype 1b strains NN, Con1, and O while that of JFH1 has 21 substituted residues in this region. The proline at 540 aa, which is important for CyPB binding (31), is conserved but the adjacent residues such as isoleucine at 539 aa and alanine at 541 aa are replaced by leucine and glutamic acid, respectively, in JFH1. Through molecular interactions, CyPB seems to make the conformation of NS5B of genotype 1b strains but not JFH1 suitable for RNA binding (31). The diverse regulation system of NS5B by CyPB among strains may be due to differences in either the sequence or the entire conformation of NS5B. Further study is important for elucidating the regulation mechanism of RNA binding activity of NS5B by CyPB.

Thus, replication in JFH1 replicon is independent of CyPB. Interestingly, human immunodeficiency virus type 1 (HIV-1) strains also have a diversity of CyP dependence on viral proliferation (3, 33). CyPA plays an important role in the life cycle of HIV-1. The interaction of the HIV-1 capsid protein with CyPA that resides within the target cells of infection is critical for HIV-1 replication (7, 24). In peripheral blood mononuclear cells or Jurkat T cells, CsA suppresses the proliferation of HIV-1 group main (M) strain (3). However, certain strains of group outlier (O), such as MVP5180 and MVP9435, are resistant to CsA (3, 33), suggesting the different dependency of the replication on CyPA. Authors have suggested that MVP5180 and MVP9435 clones adapt to replicate independently of CyPA and that this adaptation provides a significant replication advantage for the virus in vivo (3). In vesicular stomatitis virus (VSV) strains, a role for CyPA in virus replication also has been reported (2). CyPA is required for the infection of the VSV-NJ strain but not the VSV-IND strain. These authors proposed that during evolutionary divergence from the ancestral lineages that initially were dependent on CyPA for replication, VSV-IND may have adapted to reduce its dependency on CyPA (2). In the case of HCV, a fulminant type genotype 2a replicon (JFH1) replicates independently of CyPB. It has previously been reported that JFH1 has a much higher competency of replication in the cells than other strains (13). The adaptation to independence from CyPB may contribute to the high capacity of replication of JFH1.

Although the JFH1 replicon is less sensitive to CsA, high concentrations of CsA still suppress replication of the JFH1 replicon. Moreover, the introduction of the siRNA designed to recognize several CyP subtypes [si-CyP(broad)] moderately diminishes HCV RNA in the JFH1 replicon. We suspect that a CyP family member other than CyPB is involved in HCV genome replication. Further analysis is needed on the role of other CyP subtypes.

As there a replicon system for a fulminant-type genotype 1b replicon or chronic-type genotype 2a replicon does not yet exist, we cannot conclude whether chronic-type genotype 2a replicons or fulminant-type replicons are less sensitive to CsA or not. However, there is a clinical report describing cotreatment of patients with chronic hepatitis C with IFN and CsA that resulted in a higher sustained virological rate than with treatment of IFN alone (11). In this report, increase in the sustained virological rate was prominent with patients carrying genotype 1 HCV (51.7% versus 21.9%), while it was relatively weak in patients carrying genotype 2 HCV (66.7% versus 58.3%) (11). Thus, genotype may affect the sensitivity of HCV replication to CsA. However, we cannot exclude the possibility that the diminished sensitivity to CsA is a characteristic only of the fulminant-type genotype 2a strain.

Our results suggest that sensitivity to CsA and replication dependency to CyPB is different among HCV strains. This finding is an important insight into the diversity of the mechanism of HCV genome replication and its sensitivity to antiviral agents.

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Epigenetic silencing of interferon-inducible genes is implicated in interferon resistance of hepatitis C virus replicon-harboring cells

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***Background/Aims*: We previously established hepatitis C virus (HCV) replicon-harboring cell lines possessing two interferon (IFN)-resistant phenotypes: a partially resistant phenotype (αR series) and a severely resistant phenotype (βR series). We recently found that the severe IFN resistance of the βR-series cells is caused by the functional disruption of type I IFN receptors. Here, we aimed to clarify the mechanism(s) underlying the partial IFN resistance of the αR-series cells.**

***Methods*: αR-series cells were pre-treated with 5-azacytidine to evaluate the effects of DNA demethylation on IFN resistance. cDNA microarray analysis was carried out in order to compare 1αR cells, which belong to the αR series, treated with both 5-azacytidine and IFN-α with cells treated with 5-azacytidine or IFN-α alone.**

***Results*: We found that the IFN-resistant phenotype of αR-series cells was impaired by treatment with 5-azacytidine. cDNA microarray analysis identified seven IFN-stimulated genes, which were up-regulated by 5-azacytidine treatment. We demonstrated here that the ectopic expression of each of these seven genes in 1αR cells frequently weakened the IFN resistance of these cells.**

***Conclusions*: The present results suggest that the epigenetic silencing of IFN-stimulated genes is implicated in the acquisition of a partially IFN-resistant phenotype of HCV replicon-harboring cells.**

Keywords: **IFN resistance; HCV replicon; DNA methylation; cDNA microarray; Epigenetic silencing**

1. Introduction

Persistent infection by the hepatitis C virus (HCV) is a major cause of chronic hepatitis (CH) [1,2], which can progress to liver cirrhosis and hepatocellular carcinoma [3]. HCV is an enveloped RNA virus belonging to the family Flaviviridae, the genome of which consists of a positive-stranded 9.6-kb RNA encoding at least 10 structural and non-structural proteins [4]. Since, at least 170 million people are currently infected with HCV worldwide, this type of infection constitutes a global health problem [5]. Interferon (IFN)-α/ribavirin combination therapy is currently the standard clinical therapy for patients with CH C; however, the effectiveness of IFN is limited to approximately half of these patients [6]. This clinical finding suggests that HCV is resistant to the antiviral effects of IFN, and that HCV proteins directly or indirectly attenuate those effects [7].

Although HCV replicon system harboring autonomously replicating HCV subgenomic RNA containing the non-structural region [8] provides a powerful tool for various HCV studies, all of the HCV replicons established to date

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Abbreviations: aa, amino acid; 5-azaC, 5-azacytidine; CH, chronic hepatitis; HCV, hepatitis C virus; IFN, interferon; IFNAR, IFN receptor; IRF-1, IFN regulatory factor 1; ISG, IFN-stimulated gene; RT-PCR, reverse transcription-polymerase chain reaction.

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have been highly sensitive to IFN [9–12]. Based on our assumption that prolonged IFN treatment might change HCV replicons from the IFN-sensitive phenotype into an IFN-resistant phenotype, we established nine HCV replicon-harboring cell lines possessing two IFN-resistant phenotypes: a partially resistant phenotype (αR series; 1αR, 3αR, 4αR, 5αR, and αRmix) and a severely resistant phenotype (βR series; 1βR, 3βR, 4βR, and 5βR) obtained by IFN-α and IFN-β treatment, respectively [13]. αR- and βR-series cells were derived from clones 1, 3, 4, and 5, and 1αR, 3αR, 4αR, and 5αR cells were counterparts of 1βR, 3βR, 4βR, and 5βR cells, respectively [13]. Although genetic analysis of these replicons identified one common amino acid (aa) substitution and several cell line-specific aa substitutions, we failed to obtain the evidence of the direct involvement of these aa substitutions to IFN resistance [14]. However, we found frequent non-sense mutations and deletions in type I IFN receptor (IFNAR) genes (IFNAR1 and IFNAR2c) in the βR-series cells, but such mutations were rarely observed in the αR-series cells [14]. Since we demonstrated that the ectopic expression of wild-type IFNAR in the βR-series cells restored IFN signaling, we determined that the functional disruption of type I IFNAR was responsible for this type of resistance [14]. However, the mechanism underlying the partial IFN-resistance of αR-series cells remains unclear. Since, the expression levels of IFNARs, Tyk2, and Jak1 were not lower in the αR-series cells [13,14], the functional degeneration of other cellular factor(s) involved in IFN signaling or IFN-stimulated gene(s) (ISG) was thought to contribute to the acquisition of IFN resistance.

As one potential mechanism for the partial resistance, we assumed that the epigenetic silencing of some ISGs, which are known to be involved in the anti-HCV activity of IFN, by DNA methylation around the promoter region might convert HCV replicon-harboring cells from the IFN-sensitive phenotype to the IFN-resistant phenotype. To evaluate our hypothesis, we examined whether or not pre-treatment of αR-series cells with 5-azacytidine (5-azaC), an inhibitor of DNA methyltransferase and an inducer of gene suppressed by DNA methylation, could alter the IFN sensitivity of the cells.

Here, we report that the epigenetic silencing of ISGs is implicated in the IFN-resistance of αR-series cells. We have also identified several ISGs that are up-regulated by 5-azaC treatment and weaken the IFN resistance of αR-series cells.

2. Materials and methods

2.1. Cell cultures

HCV replicon-harboring cells and cured 6Mc cells [14], from which the HCV replicons had been eliminated by IFN-γ treatment, were maintained as described previously [13].

2.2. Analysis of IFN sensitivity

HCV replicon-harboring cells were treated with 5-azaC (2–10 μM) (A-2385, Sigma, St Louis, MO) for 2 weeks. Then, human IFN-α (I-2396; Sigma) was added to the cells (with or without pre-treatment with 5-azaC) as described previously [12,13]. After 3 weeks in culture, the colonies obtained on the culture dishes were stained with Coomassie brilliant blue (CBB) as described previously [15].

2.3. Quantitative analysis of HCV replicon RNA

The quantification of HCV RNA was carried out to monitor the antiviral effects of IFN-α, and was performed by real-time LightCycler polymerase chain reaction (PCR) as described previously [16,17].

2.4. Construction of replicon plasmid and RNA synthesis

The non-structural region (6.1 kb) fragment of a 1βR1 clone obtained from 1βR cells [13] was digested with SpeI and BsiWI, and the digested

Table 1
The primers used for RT-PCR analysis of mRNA expression

Gene	Direction	Nucleotide sequence	Products (bp)	Cycles
IFI27	Forward	gttttgcccctggccaggattgct	252	27
	Reverse	aatggagcccaggatgaacttggt		
9-27	Forward	tcttcttgaactggtgctgtctgggg	191	28
	Reverse	agagccgaataccagtgacaggat		
LMP2	Forward	atggaaccctgggaggaatgctg	145	27
	Reverse	gcaatagcgtctgtggtgaagcg		
LMP7	Forward	ctgggataagaagggtcctggac	293	27
	Reverse	tactggtgcagcaggtcactggac		
Viperin	Forward	tggagcgccacaaagaagtgtcct	240	27
	Reverse	ccagcttcagatcagccttactcc		
IFI44	Forward	tgtggctttgctcactcatgtgga	227	31
	Reverse	cagcccatagcattcgtctcagag		
IFIT2	Forward	aggccatccaccactttatagagg	272	28
	Reverse	tgggcaccacatctctattctcca		
ISG56	Forward	tagccaacatgtcctcacagac	396	32
	Reverse	tcttctaccactggtttcatgc		
GAPDH	Forward	gactcatgaccacagtccatgc	334	26
	Reverse	gaggagaccacctggtgctcag		

fragment (5.7 kb) was ligated into the plasmid pNSS1RZ2RU [12], which was predigested with SpeI and BsiWI. The obtained plasmid was linearized by XbaI and was used for RNA synthesis with T7 MEGAscript (Ambion) as previously described [12].

2.5. RNA transfection and selection of G418-resistant cells

The transfection of HCV replicon RNA synthesized in vitro into 6Mc cells was performed by electroporation, and the cells were selected in the presence of G418 (300 μg/ml; Invitrogen) for 3 weeks as described previously [14].

2.6. cDNA microarray analysis

The 1αR cells (5×10^5 cells), which were cultured for 2 weeks in the absence or presence of 5-azaC (10 μM), were plated onto a 10-cm diameter dish, and were cultured for 2 days in the absence of G418. Then the cells were treated with or without IFN-α (500 IU/ml) for 8 h. Total RNAs prepared from 80% confluent cells were subjected to cDNA microarray analysis (CodeLink™, Uniset human I containing 54840 spots of 30-mer oligonucleotides; Amersham Biosciences) as described previously [18].

2.7. Reverse transcription (RT)-PCR

RT-PCR and real-time LightCycler PCR were performed as described previously [18,19] using the primer sets in Table 1.

2.8. Expression vectors and retroviral infection

Retroviral vectors pCXbsr [20] and pC4bsr(IRES) (kindly provided by Dr T. Akagi), which contain the resistance gene for blasticidin, were used in this study. Retroviral infection and selection by blasticidin were performed as described previously [15].

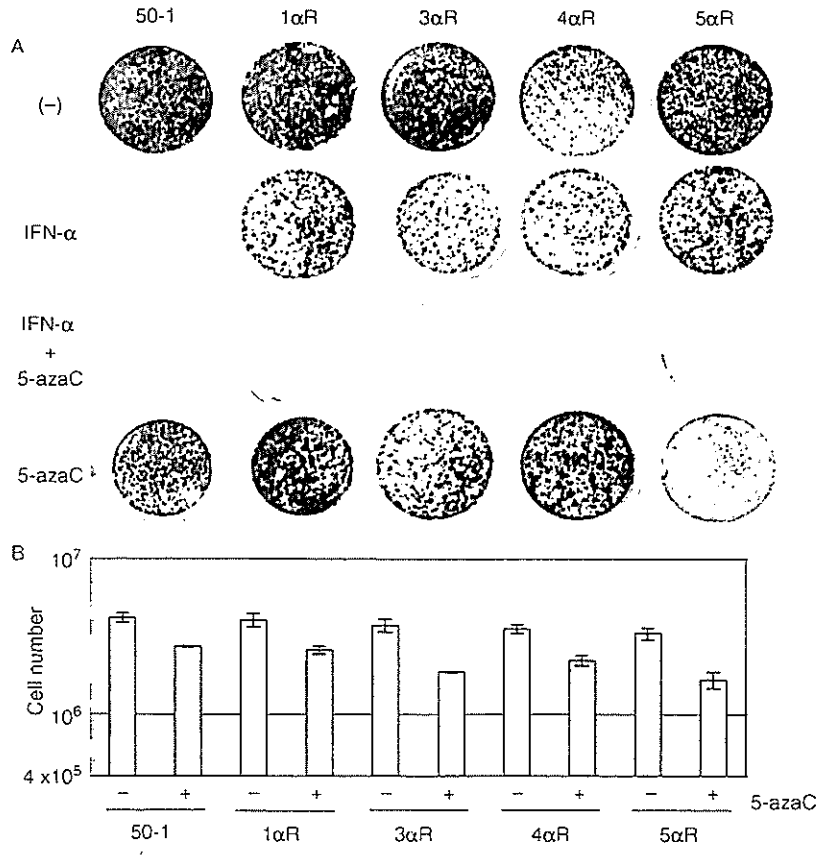

Fig. 1. 5-AzaC treatment converts αR-series cells from an IFN-resistant phenotype into an IFN-sensitive phenotype. (A) IFN sensitivities of various HCV replicon-harboring cells. αR-series cells (1αR, 3αR, 4αR, and 5αR) showing a partially IFN-resistant phenotype and the parent 50-1 cells with an IFN-sensitive phenotype were used for the analysis. These cells were plated onto 10-cm diameter dishes (2×10^4 cells/dish) and were cultured for 1 day before treatment with 5-azaC. 5-AzaC was added to the cultures at a final concentration of 10 μM (4 μM for 4αR cells, and 2 μM for 5αR cells) and the cells were then cultured for 2 weeks, and were subsequently treated with or without IFN-α (400 IU/ml) for 3 weeks in the presence of G418 (300 μg/ml). The HCV replicon-harboring cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase was produced by the efficient replication of the HCV replicon in the cells. Therefore, when an HCV replicon was excluded from the cells or levels of the replicon were low, the cells did not survive in the presence of G418. The panels show G418-resistant colonies stained with CBB as described previously [15]. (B) Effect of 5-azaC on the growth of HCV replicon-harboring cells. 50-1 and αR-series cells were plated onto 10-cm diameter dishes (4×10^5 cells/dish) and were cultured for 1 day before treatment with 5-azaC. These cells were cultured in the absence or presence of 5-azaC (10 μM for 50-1, 1αR, and 3αR cells, 4 μM for 4αR cells, and 2 μM for 5αR cells) for 3 days, and then the cell number was determined by a method described previously [27]. The data indicate means ± SD of triplicates from two independent experiments. [This figure appears in colour on the web.]

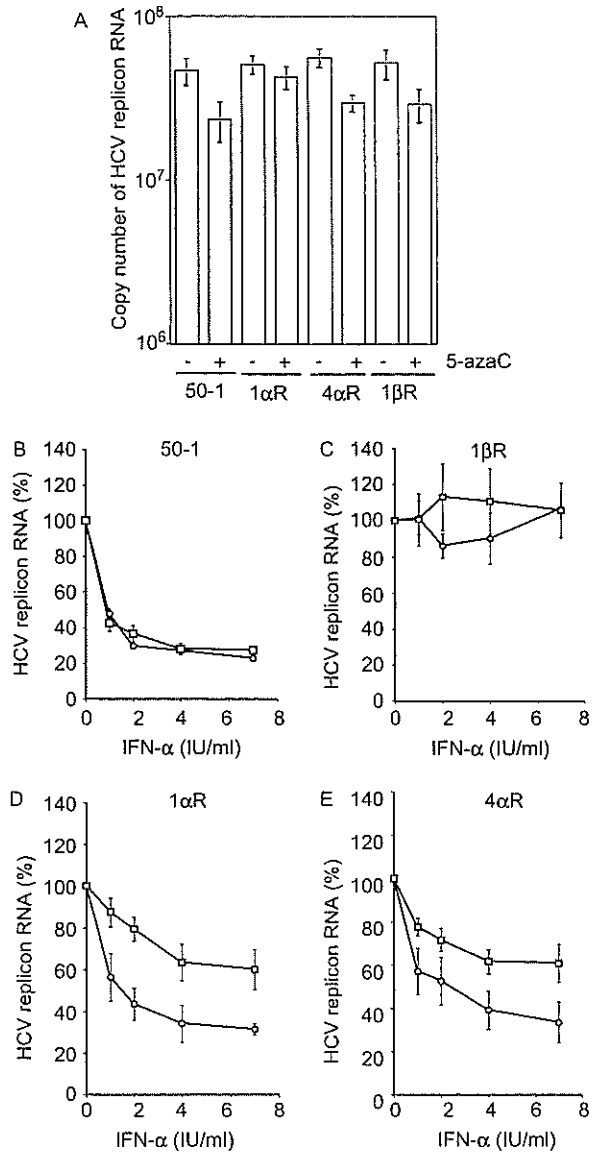

Fig. 2. IFN sensitivities of HCV replicons. (A) HCV replicon RNA levels of 50-1, 1βR, 1αR, and 4αR cells cultured in the presence or absence of 5-azaC (10 μM for 50-1, 1βR, and 1αR cells, 4 μM for 4αR cells) for 2 weeks. Real-time LightCycler PCR was carried out in order to quantitatively monitor the levels of replicon RNAs in the cells, as described previously [16,17]. The copy numbers of HCV replicon RNA per μg of total RNA determined by quantitative RT-PCR are shown. (B) 50-1, (C) 1βR, (D) 1αR, and (E) 4αR cells were cultured in the presence (open circles) or absence (open squares) of 5-azaC as described in (A), and then the cells were treated with IFN-α (0, 1, 2, 4, and 7 IU/ml each) for 3 days. Quantitative RT-PCR was carried out as described in (A). The relative level of HCV replicon RNA (%) calculated at each point, when the HCV replicon RNA level of IFN non-treated cells was assigned to be 100%, is shown here. The data indicate means±SD of triplicates from two independent experiments.

2.9. Western blot analysis and immunoprecipitation

The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis were performed as previously described [21].

Table 2
Genes whose elevated levels in the C vs. IFN+5-azaC experiment were more than 2.5-fold those in the C vs. IFN or C vs. 5-azaC experiment

Gene	C vs. IFN+ 5-azaC	C vs. IFN	C vs. 5azaC	Accession no.
IFI27	25.21[a]	1.56	1.72	NM_005532
9-27	2703.30	600.65	−0.04	NM_003641
LMP2	23.60	6.20	1.57	NM_002800
LMP7	22.27	8.79	3.72	NM_004159
Viperin	25.26	9.07	−2.26	NM_080657
IFI44	29.60	7.05	0.03	NM_006417
IFIT2	10.67	2.96	−3.02	NM_001547

The manufacturer's protocol (Amersham Biosciences) recommended the use of a ratio of at least 2:1 as a standard for the selection of genes showing significant differences in expression.
[a] Fold.

3. Results

3.1. IFN-resistant phenotype of HCV replicon-harboring cells is impaired by co-treatment with 5-azaC and IFN-α

Based on our assumption, we examined whether or not pre-treatment of αR-series cells with 5-azaC could convert these cells from the IFN-resistant phenotype to the IFN-sensitive phenotype. Parent HCV replicon-harboring cells (50-1) [22], and αR-series cells were treated with IFN-α and/or 5-azaC, and their IFN sensitivities were compared. After IFN-α treatment, many 1αR, 3αR, 4αR, and 5αR cell colonies survived, although only a few small colonies of 50-1 cells survived. However, when 5-azaC pre-treatment following IFN-α treatment was applied, almost none of the 1αR, 3αR, 4αR, or 5αR cell colonies survived, nor did the colonies of 50-1 cells (Fig. 1(A)). Although, we observed that 5-azaC treatment slightly lowered the growth rates of these cells (Fig. 1(B)), the cell viabilities exceeded 99%, and a number of colonies were finally obtained after treatment with 5-azaC alone (Fig. 1(A)). These results suggest that 5-azaC treatment converts αR-series cells from an IFN-resistant phenotype into an IFN-sensitive phenotype.

Since, we previously observed that none of the non-sense mutations in IFNAR genes found in all clones derived from 1βR and 4βR cells were detected in 1αR and 4αR cells, which were the counterparts of 1βR and 4βR cells, respectively [14], we focused on 1αR and 4αR cells to assess the effect of 5-azaC treatment on IFN sensitivity. Quantitative RT-PCR analysis of replicon RNA in cells treated with IFN-α was performed using 1αR, 4αR, 1βR (a counterpart of 1αR), and 50-1 cells pre-treated with or without 5-azaC. We confirmed that HCV replicon RNA levels in the cells pre-treated with 5-azaC were sufficient for analysis, although HCV replicon RNA levels were a little lower by 5-azaC treatment (Fig. 2(A)). The level of replicon RNA in 50-1 cells drastically decreased after IFN-α treatment, regardless of 5-azaC pre-treatment (Fig. 2(B)). Contrary to the level observed in 50-1 cells, that of replicon

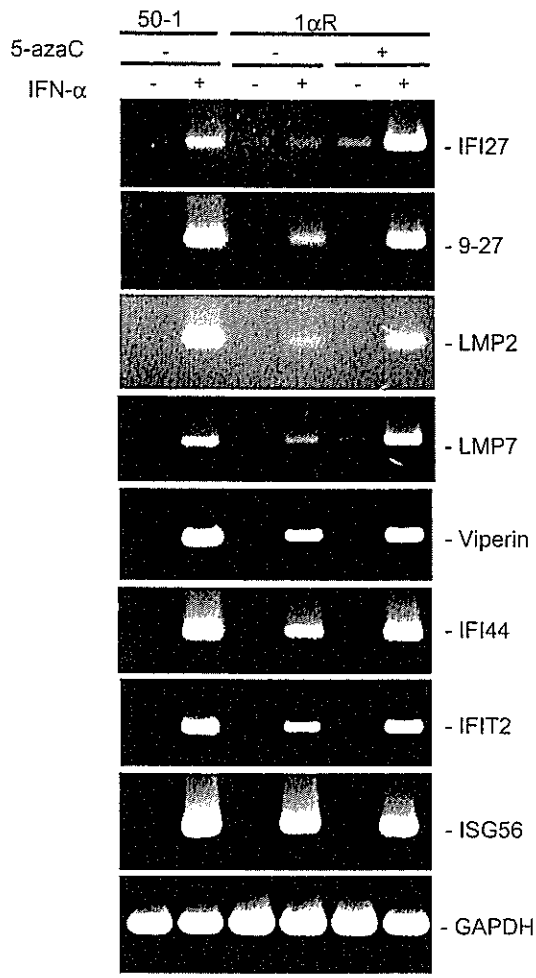

Fig. 3. RT-PCR analysis of mRNA expression of selected ISGs based on the microarray results. 1αR cells were cultured in the presence or absence of 5-azaC (10 μM) for 2 weeks. The 1αR cells treated with 5-azaC and the 50-1 cells were treated with or without IFN-α (500 IU/ml) for 8 h. The total RNAs extracted from these cells were subjected to RT-PCR using the primer sets listed in Table 1. ISG56 was used as the representative ISG, which was not selected by the microarray analysis. GAPDH was used as an internal control. RT-PCR products were detected by staining the samples with ethidium bromide after 3% agarose gel electrophoresis.

RNA in 1βR cells did not decrease after IFN-α treatment, regardless of 5-azaC pre-treatment (Fig. 2(C)). However, we observed that the level of replicon RNA in 1αR cells (Fig. 2(D)) and that in 4αR cells (Fig. 2(E)) pre-treated with 5-azaC had decreased more substantially after IFN-α treatment than that in the cells lacking 5-azaC pre-treatment. In general, these results indicate that 5-azaC treatment of αR-series cells (at least 1αR and 4αR cells) may convert the cells from a partially IFN-resistant phenotype to an IFN-sensitive phenotype; moreover, the present findings suggest that some ISGs known to contribute to the anti-HCV activity of IFN are suppressed by DNA methylation in αR-series cells.

3.2. cDNA microarray analysis using 1αR cells

Based on the results presented above, we attempted to identify those ISGs that are determinative of IFN sensitivity; to this end, cDNA microarray analyses were performed by comparing the following: 1αR cells with 1αR cells treated with 5-azaC (C vs. 5-azaC), 1αR cells with 1αR cells treated with IFN-α (C vs. IFN), and 1αR cells with 1αR cells treated with IFN-α after pre-treatment with 5-azaC (C vs. IFN+5-azaC). As the first step in this analysis, we selected the only already-known genes whose expression levels were up-regulated at a ratio of more than 10 in C vs. IFN+5-azaC. Then, from among those that were selected in the first step, we performed an additional selection of genes whose elevated levels in the C vs. IFN+5-azaC experiment were more than 2.5-fold, as compared with those in the C vs. IFN or C vs. 5-azaC experiment. Finally, seven genes (IFI27, IFI44, LMP2, LMP7, 9-27, Viperin, and IFIT2) were identified as genes that were highly and selectively induced by co-treatment with IFN-α and 5-azaC (Table 2).

In order to confirm the results of our microarray selection, we conducted RT-PCR analysis and real-time LightCycler PCR to examine the mRNA levels of the identified seven genes in the 1αR cells treated with or without IFN-α after pre-treatment with or without 5-azaC. As a control, parent 50-1 cells treated with or without IFN-α were used. The results (Fig. 3 and Table 3) confirmed the results of the microarray analysis (Table 2). The findings revealed that the levels of induction of these seven genes in 1αR cells treated with IFN-α were clearly lower than those in 50-1 cells treated with IFN-α. It is noteworthy that the expression of these seven genes in 1αR cells treated with IFN-α after 5-azaC pre-treatment was remarkably elevated, whereas the IFN-α-induced level of expression of the ISG56 gene, an immediate early antiviral ISG, was slightly enhanced after 5-azaC pre-treatment (Fig. 3 and Table 3). These results suggest that the epigenetic silencing of these ISGs is involved in the acquisition of the IFN-resistant phenotype, at least in 1αR cells, and this is also likely to be the case in other αR-series cells.

3.3. Characterization of additional HCV replicon-harboring cells possessing an IFN-resistant phenotype

In order to evaluate the reproducibility of the phenomenon observed in 1αR cells, additional HCV replicon-harboring 1βR1/6Mc cells, which were recently established independently [14], were used for IFN-α treatment (Fig. 4(A)). IFN-α-treated 1βR1/6Mc cells yielded several distinct IFN-resistant colonies, which were designated as 1βR1/6McαR mixed colonies.

To assess the effects of 5-azaC treatment on IFN-sensitivity, quantitative RT-PCR analysis of replicon RNA in the cells treated with IFN-α was performed using 1βR1/6Mc cells, 1βR1/6McαR cells, and 1βR1/6McαR cells pre-treated with 5-azaC (designated as 1βR1/6McαR+AZ cells). The level of replicon RNA in 1βR1/6Mc cells

Table 3
Real-time RT-PCR analysis of mRNA expression of selected ISGs based on the microarray results

Gene	50-1		1αR				
	−		−		+		5-azaC
	−	+	−	+	−	+	IFN-α
IFI27	4.8±1.9	100	6.4±3.2	11.9±1.9	22.9±5.4	380.4±18.9	
9-27	<1.0	100	<1.0	6.8±4.6	<1.0	46.0±6.7	
LMP2	<1.0	100	<1.0	11.7±0.9	2.7±0.3	60.5±4.5	
LMP7	2.3±0.5	100	2.1±0.3	19.8±2.0	8.8±1.3	92.6±18.7	
Viperin	n.d.	100	n.d.	7.4±2.0	<1.0	35.2±5.2	
IFI44	n.d.	100	n.d.	5.9±0.5	n.d.	56.0±7.1	
IFIT2	<1.0	100	<1.0	16.8±9.1	<1.0	48.4±13.3	
ISG56	<1.0	100	<1.0	76.2±4.1	<1.0	117.1±8.7	

The experiments were performed in at least triplicate. n.d., not detected. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of each mRNA concentration to that of GAPDH. The relative level (mean±SD) of each mRNA calculated, when the level of each mRNA of 50-1 cells treated with IFN-α was assigned to be 100, is shown here.

decreased markedly after IFN-α treatment (Fig. 4(B)), as had also been observed in the 50-1 cells (Fig. 2(B)). In contrast to the 1βR1/6Mc cells, 1βR1/6McαR cells exhibited a partially IFN-resistant phenotype (Fig. 4(B)), as had been observed in the 1αR cells (Fig. 2(D)) and 4αR cells (Fig. 2(E)). However, after IFN-α treatment, the level of replicon RNA in the 1βR1/6McαR+AZ cells was lower than that in 1βR1/6McαR cells (Fig. 4(B)). This result suggests that the treatment of 1βR1/6McαR cells with 5-azaC also contributed to a weakening of the IFN-resistant phenotype in these cells.

We next considered the expression levels of the seven genes that had been identified as epigenetically suppressed genes in 1αR cells; here, IFN-α-treated 1βR1/6Mc and 1βR1/6McαR cells were compared. The results obtained by RT-PCR analysis revealed that the induction of these seven genes by IFN-α in the 1βR1/6McαR cells was weaker than

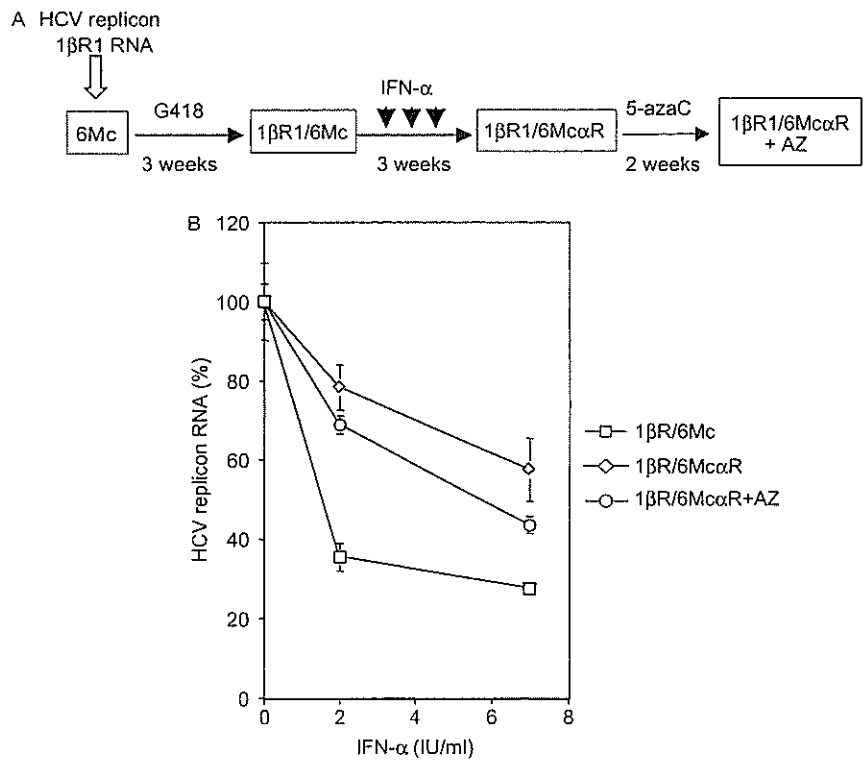

Fig. 4. Effect of 5-azaC treatment on IFN resistance in newly isolated HCV replicon-harboring cells showing a partially IFN-resistant phenotype. (A) Outline of the isolation of HCV replicon-harboring cells showing partial IFN resistance. HCV replicon-harboring cells (designated as 1βR1/6Mc cells) [14], established by the transfection of in vitro-synthesized replicon RNA (1βR1 obtained from 1βR cells) [13] into 6Mc cells, were treated with 400 IU/ml of IFN-α for 3 weeks in the presence of G418, and several colonies survived as cells with an IFN-resistant phenotype (1βR1/6McαR cells). The 1βR1/6McαR cells were treated with 5-azaC (4μM) for 2 weeks. (B) IFN sensitivities of HCV replicons. The HCV replicon-harboring cells treated with IFN-α (0, 2, and 7 IU/ml each) for 3 days were subjected to quantification of HCV replicon RNA, as described in Fig. 2.

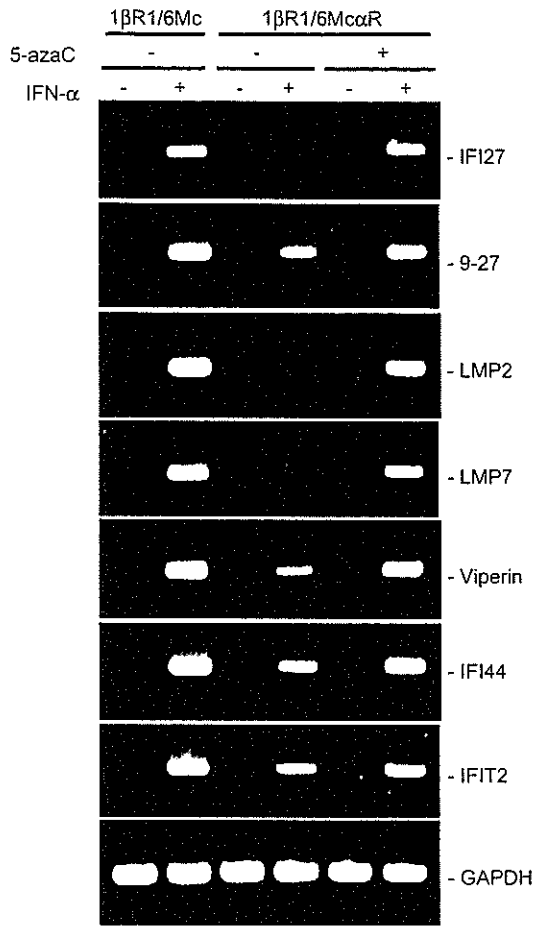

Fig. 5. RT-PCR analysis of mRNA expression of seven ISGs selected by microarray analysis. 1βR1/6McαR cells were cultured in the presence or absence of 5-azaC (4 μM) for 2 weeks. The 1βR1/6McαR cells were treated with 5-azaC and the 1βR/6Mc cells were treated with or without IFN-α (500 IU/ml) for 8 h. The total RNAs extracted from the cells were subjected to RT-PCR using the primer sets listed in Table 1. GAPDH was used as an internal control. RT-PCR products were detected by staining the samples with ethidium bromide after 3% agarose gel electrophoresis.

that in the parent 1βR1/6Mc cells (Fig. 5). However, these seven genes were induced by IFN-α in 1βR1/6McαR cells pre-treated with 5-azaC (Fig. 5). These results were confirmed by real-time LightCycler PCR (Table 4), and were similar to those obtained in the study of 1αR cells (Fig. 3 and Table 3), thus demonstrating that the appearance of the IFN-resistant phenotype is reproducible. Furthermore, these results suggest that some form of epigenetic silencing such as DNA methylation is frequently involved in the acquisition of the IFN-resistant phenotype.

3.4. Ectopic expression of epigenetically suppressed genes weakens the IFN resistance of 1αR cells

To evaluate the effects of epigenetically suppressed genes on the IFN sensitivity of HCV replicons, we prepared 1αR cells stably expressing IFI27, IFI44, LMP2, LMP7, 9-27, Viperin, or IFIT2 using retroviral gene transfer system [15]. The ectopic expression of each ISG protein was confirmed by Western blot analysis or immunoprecipitation following Western blot analysis (Fig. 6(A)). The results obtained by quantitative RT-PCR analysis revealed that the level of HCV replicon RNA decreased to about half that in the cells expressing Viperin. Also, in LMP2-, LMP7-, and IFIT2-expressing cells, significant reductions of HCV replicon RNA were detected (Fig. 6(B)). These results indicate that the simple expression of these genes into the cells interferes with the level of HCV replicon RNA, and that the combination of ISGs contributes to the IFN resistance of the HCV replicon. Therefore, 1αR cells expressing each ISG protein were initially treated with IFN-α, and then the level of HCV replicon RNA was monitored by quantitative RT-PCR analysis. Interestingly, the results revealed that the level of HCV replicon RNA in the 1αR cells expressing Viperin or LMP7 was largely decreased to the level in parent 50-1 cells (Fig. 6(C)). Furthermore, we observed that the 1αR cells expressing IFIT2, 9-27, LMP2, or IFI44 also partially changed to the IFN-sensitive phenotype (Fig. 6(C)). These results suggest that the transcriptional suppression of ISGs such as LMP-7

Table 4
Real-time RT-PCR analysis of mRNA expression of seven ISGs selected by microarray analysis

Gene	1βR1/6Mc		1βR1/6McαR				5-azaC
	−		−		+		
	−	+	−	+	−	+	IFN-α
IFI27	4.0±0.8	100	13.7±2.0	18.4±3.7	60.9±10.7	383±16.4	
9-27	<1.0	100	<1.0	14.8±0.2	1.0±0.2	101.7±26.3	
LMP2	1.1±0.1	100	<1.0	6.9±0.6	3.0±0.3	112.2±38.1	
LMP7	3.5±0.4	100	<1.0	9.9±0.7	8.7±0.9	96.5±11.2	
Viperin	n.d.	100	n.d.	23.2±9.4	<1.0	104.8±14.0	
IFI44	n.d.	100	n.d.	4.5±0.9	n.d.	57.3±1.9	
IFIT2	<1.0	100	<1.0	10.2±16	<1.0	67.9±9.2	

The experiments were performed in at least triplicate. n.d., not detected. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of each mRNA concentration to that of GAPDH. The relative level (mean±SD) of each mRNA calculated, when the level of each mRNA of 1βR1/6Mc cells treated with IFN-α was assigned to be 100, is shown here.

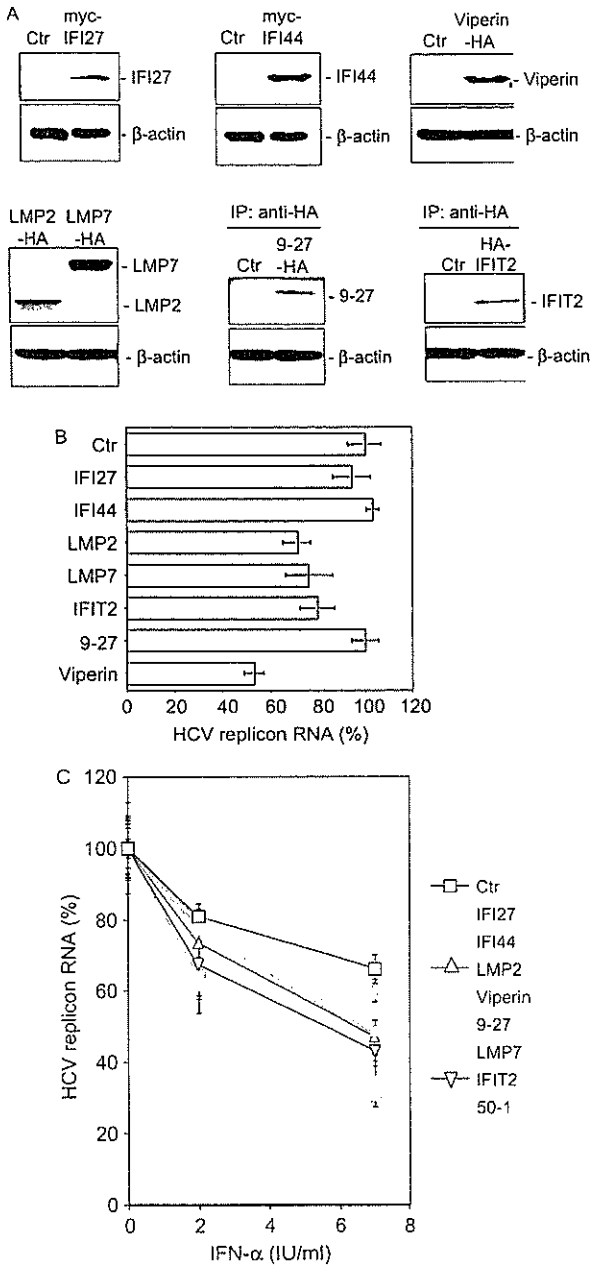

Fig. 6. IFN sensitivities of HCV replicons in 1αR cells expressing epigenetically silenced ISG. (A) Ectopic expression of epigenetically silenced ISG in 1αR cells introduced by retrovirus-mediated gene transfer. The DNA fragments encoding myc-tagged IFI27 and IFI44, and influenza hemagglutinin-tagged (HA)-LMP2, LMP7, 9-27, and IFIT2 were obtained by the PCR amplification of cDNAs derived from HCV replicon-harboring cells using primer sets designed from the nucleotide sequences (see the Accession no. in Table 2). The obtained DNA fragments were cloned into the EcoRI and NotI sites of pCXbsr, as described previously [28]. The DNA fragment encoding the HA-IFIT2 was cloned into the EcoRI and NotI sites of pC4bsr(IRES). The sequences of the plasmid inserts were confirmed by Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Western blot analysis or immunoprecipitation (IP) following Western blot analysis of 1αR cells infected with pCXbsr retroviruses encoding myc-IFI27, myc-IFI44, Viperin-HA, LMP2-HA, LMP7-HA, 9-27-HA, and HA-IFIT2 was performed by anti-HA (rat monoclonal 3F10; Roche, Mannheim, Germany) or anti-myc (mouse monoclonal PL14; Medical and Biological Laboratories, Nagoya, Japan) antibodies. In order to detect 9-27-HA and HA-IFIT2, IP was performed by using anti-HA affinity matrix (3F10, Roche). pCXbsr or pC4bsr (IRES) retrovirus-infected 1αR cells were used as a control (Ctr). β-actin was detected by anti-β-actin antibody (AC-15, Sigma) as a control for the amount of protein loaded per lane. (B) The quantification of HCV replicon RNA in 1αR cells expressing epigenetically silenced ISG was performed as described in Fig. 2. pCXbsr retrovirus-infected 1αR cells were used as a control (Ctr). (C) IFN sensitivities of HCV replicon RNA in 1αR cells expressing epigenetically silenced ISG. ISG-expressed 1αR cells treated with IFN-α (0, 2, and 7 IU/ml each) for 3 days were subjected to quantification of HCV replicon RNA, as described in Fig. 2. pCXbsr retrovirus-infected 1αR cells were used as a control (Ctr). The relative level of HCV replicon RNA (%) calculated at each point, when the HCV replicon RNA level of IFN non-treated cells expressing each ISG was assigned to be 100%, is shown here. [This figure appears in colour on the web.]

and Viperin, among other ISGs, is involved in the partial IFN resistance of HCV replicon-harboring cells.

4. Discussion

Although, we identified several ISGs, which are expected to contribute to the acquisition of the IFN-resistant phenotype, the relationships among these ISGs remain unclear, in particular as regards issues such as anti-HCV activity and the mechanisms of induction of these ISGs after 5-azaC treatment. In this context, several plausible explanations can be considered: (1) all seven ISGs are independently induced by 5-azaC treatment; (2) one of the seven ISGs is induced by 5-azaC treatment, and then this ISG activates the other ISGs; (3) a certain transcriptional factor (e.g. IFN regulatory factor 1 (IRF-1), IRF-7, etc.) is induced by 5-azaC treatment, and then this factor activates the seven ISGs; (4) a certain combination of a identified ISG and an unidentified ISG in the present study exhibits anti-HCV activity; and/or (5) a certain combination of a number of ISGs identified in the present study exhibits anti-HCV activity. As regards possibilities (1) and (2), we examined the expression levels of seven ISGs in 1αR cells ectopically expressing one of these seven ISGs. We did not obtain evidence demonstrating that one of the seven ISGs activates the other ISGs (data not shown); therefore, possibility (1) was supported and possibility (2) was excluded (data not shown). As regards option (3), it was noted that ectopic IRF-1 expression suppresses the replication of the HCV replicon via the activation of ISGs [23]. However, we did not observe any significant induction of the mRNA for the IRF-1 gene in 1αR cells co-treated with IFN-α and 5-azaC (data not shown). Moreover, no induction of the IRF-7 gene was observed in 1αR cells co-treated with IFN-α and 5-azaC (data not shown). Therefore, possibility (3) also appears unlikely, although the possibility of the induction of another transcriptional factor(s) cannot be excluded. As regards

option (4), a number of ISGs that were not selected by the present microarray analysis could be considered as candidates for contributing to anti-HCV activity. However, at least with respect to the representative 2′-5′-oligoadenylate synthetase (2′-5′-OAS) and double-stranded RNA-specific adenosine deaminase (ADAR1), no significant differences in mRNA induction of 2′-5′-OAS or ADAR1 genes by IFN-α treatment were observed between 50-1 and 1αR cells, regardless of 5-azaC pre-treatment (data not shown). As regards option (5), it is likely that a combination of several ISGs is involved in the conversion to the IFN-sensitive phenotype of these cells, because the expression of LMP-2, LMP-7, or Viperin alone was associated with reductions in the level of HCV replicon RNA (Fig. 6(B)). Therefore, in order to clarify this issue, future studies involving the co-expression of these ISGs will also be necessary.

Among the genes selected in this study, LMP2 and LMP7 are of interest; these genes are known as catalytic subunits of immunoproteasome, which is induced by IFN-γ [24]. These genes have recently been identified as being suppressed in HCV replicon-harboring cells [18]. The down-regulation of these genes may contribute to persistent viral infection due to the acquisition of the IFN-resistant phenotype.

Viperin is a third gene of interest identified in this study. Viperin is induced by IFN-α/β, IFN-γ, as well as by human cytomegalovirus (HCMV) infection [25]. Stable expression of Viperin in fibroblasts inhibited HCMV infection and downregulated several HCMV proteins, although its molecular mechanism remains unclear at present [25]. Since, HCV replication complexes have been associated with inner cellular lipid membrane structures [26], viperin may contribute to the inhibition of HCV replication.

The present study using HCV replicon-harboring cell lines to investigate the mechanisms of IFN resistance may contribute to further diagnostic study of IFN sensitivity among patients with CH C. However, additional comparisons of ISG expression levels in IFN responders and non-responders with CH C will be required to understand the mechanisms underlying IFN resistance associated with HCV.

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Oligomerization of Hepatitis C Virus Core Protein Is Crucial for Interaction with the Cytoplasmic Domain of E1 Envelope Protein[▽]

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Hepatitis C virus (HCV) contains two membrane-associated envelope glycoproteins, E1 and E2, which assemble as a heterodimer in the endoplasmic reticulum (ER). In this study, predictive algorithms and genetic analyses of deletion mutants and glycosylation site variants of the E1 glycoprotein were used to suggest that the glycoprotein can adopt two topologies in the ER membrane: the conventional type I membrane topology and a polytopic topology in which the protein spans the ER membrane twice with an intervening cytoplasmic loop (amino acid residues 288 to 360). We also demonstrate that the E1 glycoprotein is able to associate with the HCV core protein, but only upon oligomerization of the core protein in the presence of tRNA to form capsid-like structures. Yeast two-hybrid and immunoprecipitation analyses reveal that oligomerization of the core protein is promoted by amino acid residues 72 to 91 in the core. Furthermore, the association between the E1 glycoprotein and the assembled core can be recapitulated using a fusion protein containing the putative cytoplasmic loop of the E1 glycoprotein. This fusion protein is also able to compete with the intact E1 glycoprotein for binding to the core. Mutagenesis of the cytoplasmic loop of E1 was used to define a region of four amino acids (residues 312 to 315) that is important for interaction with the assembled HCV core. Taken together, our studies suggest that interaction between the self-oligomerized HCV core and the E1 glycoprotein is mediated through the cytoplasmic loop present in a polytopic form of the E1 glycoprotein.

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis C, leading to steatosis, cirrhosis, and hepatocellular carcinoma. It is estimated that over 170 million people are infected with HCV worldwide (5, 18, 37). HCV is an enveloped single-stranded plus-sense RNA virus in the *Hepacivirus* genus of the family *Flaviviridae*, which also includes the flaviviruses and pestiviruses (36). The genome of HCV encodes a polyprotein of approximately 3,000 amino acids which is cotranslationally and posttranslationally processed to generate at least 10 viral proteins (12). The structural proteins, the core and E1 and E2 envelope glycoproteins, are encoded in the N-terminal portion of the polyprotein, and the nonstructural proteins, thought to be required for replication of the viral genome, are encoded in the C-terminal region (11). The core protein, which interacts with viral RNA (47) to form the nucleocapsid, is liberated from the N terminus of the polyprotein by signal peptidase cleavage in the downstream E1 protein (at position 191), and the C-terminal transmembrane region of the core protein (residue 164 to 191) is further cleaved at residues 177 or 179 by the signal peptide peptidase (16, 43). The remaining hydrophobic region of the core protein (domain II; residues 119 to 174) has been shown to affect the efficiency of signal peptide peptidase cleavage and the intracellular localization of core protein (14, 44). Although the C-terminal transmembrane region of core protein and E1 were reported to interact with each other within the intramembrane space (25), the central hydrophobic region from residues 119 to 152 within domain II was also suggested to be responsible for the interaction between core and E1 (27).

Recently, in vitro replication of a JFH1 clone of HCV genotype 2a derived from a patient with fulminant hepatitis C was reported in a cell line that had been cured of its HCV replicon by treatment with interferon (23, 50, 51). However, this reverse genetics system is limited to the JFH-1 clone of genotype 2a and specific cell lines. Robust and reliable in vitro replication of other major genotypes of HCV such as genotypes 1a and 1b has yet to be developed. So far, biological functions of HCV envelope proteins have been characterized by using recombinant envelope proteins expressed in vitro, HCV-like particles produced in insect cells, and the pseudotyped virions based on vesicular stomatitis virus and retroviruses (8). The HCV polyprotein precursor must be specifically threaded through the membrane of the endoplasmic reticulum (ER) to undergo maturation to form the mature envelope glycoproteins (7). In the polyprotein, the C-terminal regions of E1 and E2 each contain a membrane-spanning domain as well as the hydrophobic signal peptide of the downstream viral protein (E2 and p7, respectively). These domains form hairpin structures that pass through the membrane twice, to allow processing by signal peptidase in the ER lumen. Upon signal peptidase cleavage, the C termini are thought to translocate into the cytoplasm to generate the type I membrane topology of the mature glycoproteins. The mature E1 and E2 glycoproteins

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remain noncovalently associated, interacting in part through their C-terminal transmembrane domains, which also mediate retention of the E1-E2 complex in the ER. Based on this model of membrane topology, the HCV envelope glycoproteins possess little or no cytoplasmic region. However, a physical association between E1 and the cytosolic core protein has been reported (25, 27), suggesting that the E1 glycoprotein is able to expose a cytoplasmic domain of sufficient length to interact with the core. In addition, the presence of the core protein has been shown to affect the folding of E1 (32).

We have previously suggested that the E1 glycoprotein may adopt a polytopic (double membrane-spanning) topology that coexists with the dominant type I form (35). In this study, we provide genetic evidence for a polytopic form of the E1 glycoprotein and for exposure of a centrally located cytoplasmic domain. Furthermore, we show that the cytoplasmic region of the polytopic form of E1 is required for interaction with amino acid residues 72 to 91 of the core protein.

MATERIALS AND METHODS

Cell culture. 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 2 mM L-glutamine, penicillin, and streptomycin and supplemented with 10% fetal bovine serum.

Plasmids. A cDNA of E1 glycoprotein was amplified from HCV type 1b strain J1 (1) by PCR using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and inserted between NheI and BamHI sites of pJW4303, which contains the signal sequence of tissue plasminogen activator and the bovine growth hormone polyadenylation signal (a kind gift from J. M. Mullins), to generate pJW383. For the deletion analysis, the plasmids pJW360 and pJW288 encoding residues 192 to 360 and 192 to 288, respectively, were amplified by PCR and cloned into pJW4303. The plasmids pJW383d1 and pJW383d2, containing deletions in residues 261 to 286 and 289 to 340, respectively, were generated from pJW383 by splicing of overlapping extensions (13, 15) as described previously (44). A cDNA fragment encoding core to E2 proteins of HCV strain J1 was amplified by PCR and cloned into pCAGGs-PUR (28), and glycosylation site mutations in the E1 protein were generated by the method of splicing by overlapping extension. For the yeast two-hybrid assay, pGBKT7HCVCore173 was used as bait, as described previously (38). The gene encoding amino acids 288 to 346 of HCV E1 protein was amplified from cDNA of strain J1 and introduced into NdeI and EcoRI sites of a pGADT7 vector (Clontech, Palo Alto, CA). In the same way, deletion mutants of core protein encoding residues 1 to 151, 1 to 25, 24 to 173, 38 to 173, 58 to 173, 72 to 173, and 92 to 173 were amplified by PCR and cloned into a pGBKT7 vector. The FLAG sequence was introduced between amino acids 195 and 196 of the cDNA encoding residues 1 to 383 of the HCV polyprotein and replaced Ala^{383} with Arg to avoid processing by signal peptidase and spacer amino acids (Gly-Gly-Gly-Ser), and influenza virus hemagglutinin (HA) sequence was added at the C terminus. The resulting cDNA fragment encoding core protein, FLAG tag, E1, and HA tag was cloned into a pcDNA3.1(+) vector and designated Flag-core-E1-HA (see Fig. 2D, below) and used for in vitro transcription and translation. Similarly, the FLAG sequence was introduced into the cDNA encoding residues 151 to 383 of the HCV polyprotein, and the HA sequence was added at the C terminus. The resulting cDNA fragment encoding the C-terminal hydrophobic/transmembrane region of the core protein, FLAG tag, E1, and HA tag was designated Flag-E1-HA (see Fig. 3A, below). The DNA fragments encoding residues 1 to 191 with amino acids 72 to 91 deleted were generated by splicing via overlapping extension and cloned into pCAGGS (CoreΔ72-91) (see Fig. 4A, below) (42). The DNA fragment encoding the cytoplasmic domain of the E1 protein with a C-terminal HA tag was amplified by PCR and introduced at HindIII and SacII sites of pEGFP-C3. pCAGGS plasmids encoding core to p7 replacing residues 304 to 307, 308 to 311, 312 to 315, 316 to 319, 320 to 323, 324 to 327, or 328 to 331 with Ala were generated by using splicing with overlapping extension (see Fig. 6A, below).

Antibodies. Mouse monoclonal antibody to HA tag (HA11) and anti-FLAG antibody (M2) were purchased from Covance (Richmond, CA) and Sigma, respectively. Mouse monoclonal antibodies to core protein (clones 11-7, 11-10, and 11-14) were gifts from S. Yagi (2). Anti-E1 mouse monoclonal antibody (clone 0726) was prepared by immunization using the membrane fraction of the CHO L10 cell line, which constitutively expresses HCV envelope proteins (30). Anti-E2 monoclonal antibody (clone 187) was a generous gift from M. Kohara.

Yeast two-hybrid assay. A yeast two-hybrid assay was carried out by using Matchmaker system 3 (Clontech) according to the manufacturer's protocol. The bait vector pGBKT7HCVcore 173 (38) or empty plasmid was transfected into *Saccharomyces cerevisiae* strain AH109 together with the prey vectors, pGADT7-based constructs (see Table 1, below). The yeast cells possessing pGBKT7/p-53 and pGADT7/large T antigen were used as positive controls, while yeast cells possessing pGBKT7 and pGADT7 were the negative controls. These transfected yeast colonies were cultivated on dropout plates lacking Trp, Leu, His, and Ade (test plates) or plates lacking Trp and Leu (control plates) and then incubated at 30°C for 1 week.

Transfection, immunoblotting, and immunoprecipitation. Liposome-mediated DNA transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was described previously (38). Transfected cells were cultured at 2×10^5 cells/well in a six-well plate, harvested 30 to 48 h posttransfection, washed twice with phosphate-buffered saline (PBS), and incubated at 4°C for 30 min in 0.25 ml of lysis buffer (20 mM Tris-HCl [pH 7.4], 135 mM NaCl, 1% Triton X-100, and 10% glycerol supplemented with 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 5 mM Na_3VO_4). After freezing and thawing, lysed cells were centrifuged at $20,000 \times g$ for 5 min. The resulting cleared lysate was stored at −80°C prior to use for immunoprecipitation and blotting. Immunoprecipitation was carried out according to the method described previously (44). Briefly, lysates were preincubated at 4°C for 5 h in the lysis buffer with or without 1 mM $MgCl_2$ and 0.1 mg/ml of yeast tRNA (Sigma) prior to immunoprecipitation. The resulting lysates (0.2 ml) were gently rotated with 1.0 μg of anti-FLAG, anti-HA, or mixed mouse monoclonal anti-HCV core antibodies or mouse monoclonal antibody to the E1 protein at 4°C for 3 h with or without 1 mM $MgCl_2$ and 0.1 mg/ml of yeast tRNA. The immunocomplex was gently rotated at 4°C for 3 h with 10 μl of 50% (vol/vol) protein G-Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ) with or without 1 mM $MgCl_2$ and 0.1 mg/ml of yeast tRNA and then centrifuged at $20,000 \times g$ for 30 s. The precipitated beads were washed five times with 0.5 ml of lysis buffer containing or lacking 1 mM $MgCl_2$ and 0.1 mg/ml of yeast tRNA and then boiled in 50 μl of the loading buffer. The boiled samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins in gels were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA) and then blotted with primary antibody and secondary horseradish peroxidase-conjugated antibody. The immunocomplexes on membranes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

Protease protection assay of HCV proteins synthesized by in vitro transcription/translation. A plasmid encoding a FLAG-core-E1-HA protein was transcribed under the control of a T7 promoter by using the RiboMax large-scale RNA production system with Ribo m^7G cap analog (Promega, Madison, WI). In vitro translation was carried out in the presence of [^{35}S]methionine-cysteine (Amersham, Piscataway, NJ) by using rabbit reticulocyte lysate and canine pancreatic microsomal membrane (Promega). Translated sample was diluted sevenfold with PBS and then mixed with tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Sigma) at a final concentration of 2 μg/ml. The mixture was incubated at 30°C for 60 min with or without 0.5% Nonidet P-40, and then soybean trypsin inhibitor (Sigma) was added at a final concentration of 20 μg/ml. Digestion products were immunoprecipitated with anti-FLAG antibody.

Indirect immunofluorescence analysis. 293T cells were washed with PBS at 40 h after transfection and fixed with 3% paratormaldehyde in PBS for 20 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 for 3 min at room temperature and blocked with nonfat milk solution. Cells were incubated with the anti-E1 antibody for 60 min at 37°C and then with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG; TAGO, Burlingame, CA). HCV E1 protein was visualized by fluorescence microscopy (TE300; Nikon, Tokyo, Japan).

Velocity sedimentation with sucrose gradients. Transfected 293T cell were suspended in MNT buffer (20 mM 2-morpholinoethanesulfonic acid, 100 mM NaCl, 30 mM Tris-HCl [pH 8.6], and 0.1% Triton X-100) and then incubated at 4°C for 5 h with or without 0.1 mg/ml of yeast tRNA and 1 mM $MgCl_2$. Each sample was layered on top of 12 ml of sucrose with a 20 to 60% gradient and then centrifuged in a Beckman SW 41Ti rotor (Beckman Coulter, Tokyo, Japan) at 30,000 rpm for 3 h at 4°C. Centrifuged lysates were collected from the bottoms of the tubes and then concentrated with trichloroacetic acid. After washing with ethanol, concentrated proteins were subjected to SDS-PAGE and immunoblotting.

RESULTS

Prediction of the topology of the E1 protein in the membrane. Although a small fraction of the HCV envelope glycoproteins expressed in 293T cells is translocated onto the plasma membrane (3), the vast majority of E1 is retained in the ER membrane (6). Previously, we showed that both a central hydrophobic region of E1 (residues 260 to 288) and the C-terminal transmembrane domain (residues 360 to 383) are important for ER retention (29). As in the C-terminal hydrophobic region, the amino acid sequence of the central hydrophobic region is highly conserved among HCV isolates (4). To investigate the role of these two hydrophobic regions in the biogenesis of the E1 glycoprotein, we utilized the TMHMM algorithm (19), a computer program trained to identify potential transmembrane helical regions. The algorithm identified both hydrophobic regions as having a high probability of transmembrane helix (Fig. 1A). To examine the function of the hydrophobic regions as transmembrane domains, we constructed a series of deletion mutants in the E1 protein in which one or the other of the hydrophobic segments was absent (Fig. 1B). Mutant E1 glycoproteins were expressed in 293T cells, and the cellular localization of E1 proteins was determined by indirect immunofluorescence analysis (Fig. 1B). The full-length E1 (383) was detected only in permeabilized cells, consistent with its retention in the ER. The 383d2 mutant, which contains both hydrophobic regions but lacks the intervening hydrophilic region (residues 289 to 340), was also detected in the cytoplasm but not on the cell surface as the full-length E1. By contrast, deletion mutants lacking the central (383d1) or C-terminal (288 and 360) hydrophobic domains were detected on the cell surface in nonpermeabilized cells, suggesting that both the central and the C-terminal hydrophobic domains are required for retention of the E1 protein on the ER membrane. If the central hydrophobic domain traverses the ER membrane as predicted by the TMHMM program, the region between positions 288 and 360 would be expected to lie in the cytoplasmic space. Based on this model and on the results with E1 deletion mutants, we suggest that the E1 protein might be able to retain two membrane topologies: the conventional type I topology and a polytopic topology that spans the membrane twice with N and C termini in the ER lumen and an intervening cytoplasmic loop, as reported previously (35) (Fig. 1C). Recently, a similar polytopic form of the fusion glycoprotein of Newcastle disease virus was identified (31).

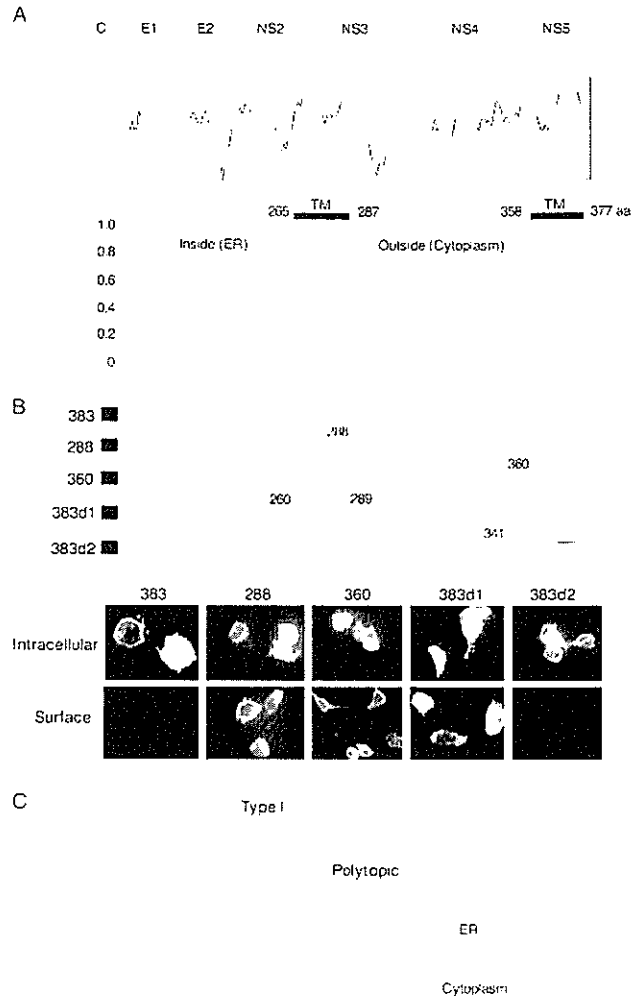

FIG. 1. Prediction of the membrane topology of the E1 protein. (A) Genome structure of HCV and a hydrophobic profile of the amino acid sequence of the E1 protein are shown at the top. The transmembrane helices in the E1 protein were predicted by the TMHMM program (19), and regions of high probability (amino acid residues 265 to 287 and 358 to 377) are indicated. (B) 293T cells transfected with the wild type (383) and deletion constructs were fixed with paraformaldehyde and permeabilized with Triton X-100 (intracellular) or not permeabilized (surface). E1 proteins were visualized with an anti-E1 monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG. (C) Possible topologies of the E1 protein on the ER. (Left) Type I topology model possessing a C-terminal transmembrane region; (right) a polytopic topology that spans the membrane twice, with both N and C termini in the ER lumen and with an intervening cytoplasmic loop.

Mutational analysis of putative N-glycosylation sites of the E1 glycoprotein. To explore the membrane topologies of E1, we examined the utilization of potential glycosylation sites. The E1 protein of HCV strain J1 (1) contains seven N-glycosylation sequence motifs (Asn-X-Ser/Thr) at amino acid positions 196, 209, 233, 234, 250, 305, and 325 (Fig. 2A). The Asn residues at these possible N-glycosylation sites were individually replaced with Gln, and the mutant E1 glycoproteins were expressed as a core-, E1-, or E2-containing polyprotein in 293T cells. In all cases, the mutant polyproteins were expressed and properly processed by signal peptidase and signal peptide peptidase to generate the core, E1, and E2 proteins (Fig. 2B). The mutant E1 proteins displayed distinct glycoforms consistent with changes in glycosylation. The wild-type E1 glycoprotein exhibited a major band of 34 kDa and a minor band of 32 kDa. The 325 mutant was unchanged from the wild-type E1, suggesting that the 325 position is not utilized, presumably due to an unfavorable NWSP motif in the genotype 1a protein (33). The 209, 233/234, and 250 mutants migrated faster than the authentic E1 protein and exhibited two bands of 32 and 30 kDa. The E1 of the 196 mutant was apparently not recognized by the monoclonal antibody directed to the N-terminal region of E1. In the 233 and 234 mutants, glycosylation occurred at the remaining Asn (234 or 233, respectively). These mutants comigrated with the wild-type E1 glycoforms, suggesting that

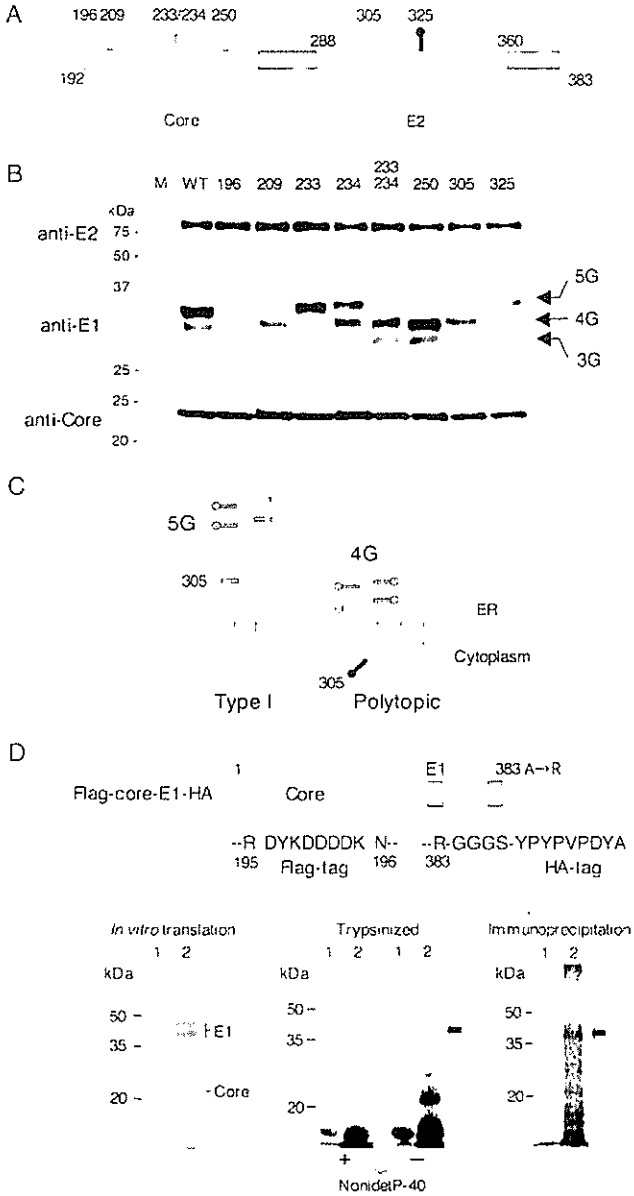

FIG. 2. Mutational analysis of N-glycosylation sites and protease protection assay of the E1 protein. (A) Positions of potential N-glycosylation sites (gray and black spikes) in the E1 protein are shown. (B) Asn residues in the possible N-glycosylation sites in the E1 protein were individually replaced with Gln. Mutant plasmids encoding the core, E1, and E2 polyproteins (A) were expressed in 293T cells, and processed core, E1, and E2 proteins were detected by immunoblotting. (C) Type I and polytopic topology models of E1 proteins bearing carbohydrates at positions of 196, 209, 234, 250, and 305 (5G) and 196, 209, 234, and 250 (4G), respectively. The 305 mutant would exhibit a single band of 4G irrespective of the topologic models. (D) Structure of the FLAG-core-E1-HA construct encoding the HCV core and E1 polyprotein carrying FLAG and HA tags in the N- and C-terminal regions of the E1 protein (top). (Bottom, left) In vitro translation of capped RNA transcribed from the FLAG-core-E1-HA (lane 2) and without RNA (lane 1) in the presence of [^{35}S]methionine-cysteine using rabbit reticulocyte lysate and canine pancreatic microsomal membrane. (Bottom, middle) Translated products of FLAG-core-E1-HA (lane 2) and without RNA (lane 1) were digested with trypsin in the presence (+) or absence (−) of 0.5% Nonidet P-40. (Bottom, right) Digestion products were immunoprecipitated with control (lane 1) and anti-FLAG (lane 2) antibody. Black and white arrows indicate protected and digested E1 protein, respectively.

only one or the other of the overlapping motifs can be utilized in the wild-type molecule. Glycosylation in this region was absent in the double mutant (233/234). The existence of two glycoforms of E1 may reflect incomplete and stochastic use of the available glycosylation sites or, alternatively, the presence of two discrete topological forms of E1 protein. For instance, the major band of 34 kDa in the wild-type glycoprotein might correspond to the type I topology form, with glycosylation at 196, 209, 234, 250, and 305 (5G), whereas the minor band of 32 kDa might correspond to the polytopic form of E1, bearing glycans at positions 196, 209, 234, and 250 (4G). In this regard, it is noteworthy that the 305 mutant of E1 exhibited only a single band of 32 kDa. The absence of a second glycoform is consistent with the putative cytoplasmic localization of Asn305 in a polytopic form of E1 (Fig. 2C). Taken together, this mutational analysis provides support to the model in which the HCV E1 glycoprotein is able to exist in either the type I or polytopic form. In the latter form, an extended cytoplasmic domain in E1 would be available to interact with the core protein in the virion.

Protease protection assay of the E1 protein. To confirm the presence of the cytoplasmic domain in the E1 protein, in vitro translation products of the HCV core and E1 polyprotein carrying FLAG and HA tags in the N- and C-terminal regions of the E1 protein, respectively, were digested with trypsin, and the protected portion of the E1 glycoprotein was immunoprecipitated by anti-FLAG antibody. As shown below in Fig. 4D, treatment of the translation products with trypsin in the presence of Nonidet P-40 resulted in complete digestion, and a 22-kDa band (major) and several <35-kDa faint bands were detected in the absence of the detergent. When in vitro-translated HCV core protein was treated similarly, no band was detected, irrespective of the presence of detergent (data not shown); therefore, the protected bands from trypsin digestion were derived from the E1 protein. Although the 22- to 35-kDa bands were specifically immunoprecipitated with anti-FLAG antibody but not with control antibody, the 35-kDa protein corresponding to the type I topology of the E1 protein resistant to trypsin digestion was dominant. This might be due to the difference in the reactivity of the anti-FLAG antibody, which recognizes the intact E1 protein more efficiently than digested ones. These results further support the presence of the polytopic form of HCV E1 glycoprotein, which has a cytoplasmic region together with a type I topology in the ER.

HCV core protein binds to the E1 protein in the presence of tRNA. The HCV core protein undergoes extensive conformational changes upon binding to nucleic acid and self-assembling into nucleocapsid-like particles (20). To investigate the effects of nucleic acid on oligomerization of the core protein, lysates of 293T cells expressing HCV core protein were incubated in the presence or absence of yeast tRNA (20) and subjected to velocity sedimentation in a sucrose gradient. Oligomerized core protein was detected in fractions 1 to 4 in the presence of tRNA but not in those in the absence of tRNA (Fig. 3A). To specifically examine the interaction between HCV core and E1 proteins in the assembly of the nucleocapsid-like particles, we coexpressed the core protein with an E1 protein possessing a FLAG tag near its N terminus and an HA tag at the C terminus (Flag-E1-HA) (Fig. 3B, left). The transfected cells were lysed with Triton X-100, and the E1 protein

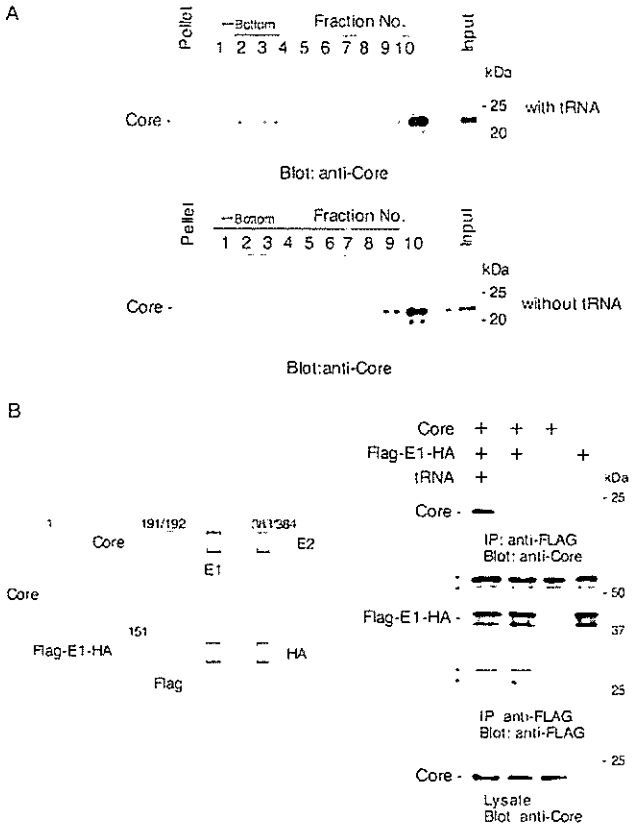

FIG. 3. HCV core protein binds to E1 protein in the presence of tRNA. (A) Cell lysates of 293T cells expressing HCV core protein were subjected to velocity sedimentation with a sucrose gradient in the presence or absence of tRNA. Oligomerized core protein was detected in fractions 1 to 4 in the presence of tRNA but not in those in the absence of tRNA. (B, left) cDNAs used for expression. FLAG-E1-HA encodes FLAG tag after the signal peptide and HA tag after the transmembrane region. (Right) Immunoprecipitation analyses. Cell lysates of 293T cells expressing core and FLAG-E1-HA proteins were immunoprecipitated by anti-FLAG antibody in the presence or absence of tRNA. The asterisks indicate nonspecific bands.

was immunoprecipitated by using an anti-FLAG antibody. Coprecipitation of core protein with E1 was assessed by Western blot analysis using a core-specific monoclonal antibody. Although HCV core protein was clearly coprecipitated with FLAG-E1-HA in the presence of tRNA, little association was seen in the absence of tRNA (Fig. 3B, right). Nonspecific precipitation of the core protein with tRNA was not observed (data not shown). Although a small amount of the intracellular core protein may already associate with viral RNA under the intracellular conditions, a large amount of RNA may be required for oligomerization that is detectable by the sedimentation assay. Together, our results suggest that tRNA facilitates oligomerization of the HCV core protein and potentiates the interaction between the core protein and E1

The region spanning amino acid residues 72 to 91 in the HCV core protein is crucial for binding to the E1 protein in yeast. The interaction between the HCV core and E1 proteins likely occurs on the cytosolic side of the cell membrane and, thus, presumably involves the posited cytoplasmic loop region in the polytopic form of the E1 glycoprotein. To investigate the possibility for this specific interaction in cells, core protein lacking the transmembrane region (Core1-173) was examined for interaction with the putative E1 cytoplasmic loop region in a yeast two-hybrid system (Table 1). When Core1-173 was expressed with the E1 cytoplasmic region (residues 288 to 346), the yeast was able to grow on the dropout plate lacking Trp, Leu, His, and Ade, suggesting that the core protein associates with the cytoplasmic loop of the E1 protein in yeast. To determine the region of the HCV core protein responsible for the interaction with the cytoplasmic domain of E1, deletion mutants of the core were tested. Association in the yeast two-hybrid system was seen with Core24-173, Core38-173, Core58-173, Core72-173, and Core1-151 mutants, but not with Core92-173 and Core1-25. Nonspecific interaction of the GAL4 activation domain with these core mutants was not observed. These results suggest that the region spanning from amino acid residues 72 to 91 in the HCV core protein is important for interaction with the cytoplasmic domain of the E1 protein in yeast.

Amino acid residues 72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein in mammalian cells. To examine the involvement of amino acid residues 72 to 91 of the HCV core protein in the interaction with the E1 protein in mammalian cells, FLAG-E1-HA was coexpressed with either a wild-type core or a deletion mutant lacking amino acid residues 72 to 91 (CoreΔ72-91) in 293T cells (Fig. 4A). Cell lysates were incubated with yeast tRNA, and FLAG-E1-HA was immunoprecipitated with anti-FLAG antibody. As shown in Fig. 4B (left), only the wild-type core protein, but not CoreΔ72-91, coprecipitated with E1. Self-oligomerization was also prevented by the deletion in CoreΔ72-91 (Fig. 4B, right). These results suggest that amino acid residues 72 to 91 in the HCV core protein play a crucial role in the interaction with the E1 protein and oligomerization of the core protein.

The E1 cytoplasmic domain interacts with the core protein in mammalian cells and inhibits the interaction with intact E1 protein in *trans*. To assess the involvement of the E1 cytoplasmic region in the interaction with core protein in mammalian

TABLE 1. Interaction between the core and the E1 cytoplasmic region in yeast

Bait	Growth with prey[a]			
	E1 cytoplasmic loop		No insert	
	Dropout	Control	Dropout	Control
Core1-173	+	+	−	+
Core24-173	+	+	−	+
Core38-173	+	+	−	+
Core58-173	+	+	−	+
Core72-173	+	+	−	+
Core92-173	−	+	−	+
Core1-151	+	+	−	+
Core1-25	−	+	−	+
No insert	−	+	−	+

[a] HCV core mutants were expressed as fusion proteins with the DNA binding region by using a bait plasmid. The HCV E1 cytoplasm region was expressed as a fusion protein with an activation domain by using a prey plasmid. Yeast growth was observed in dropout plates lacking Trp, Leu, Ade, and His (dropout) or plates lacking Trp and Leu (control). +, growth; −, no growth.

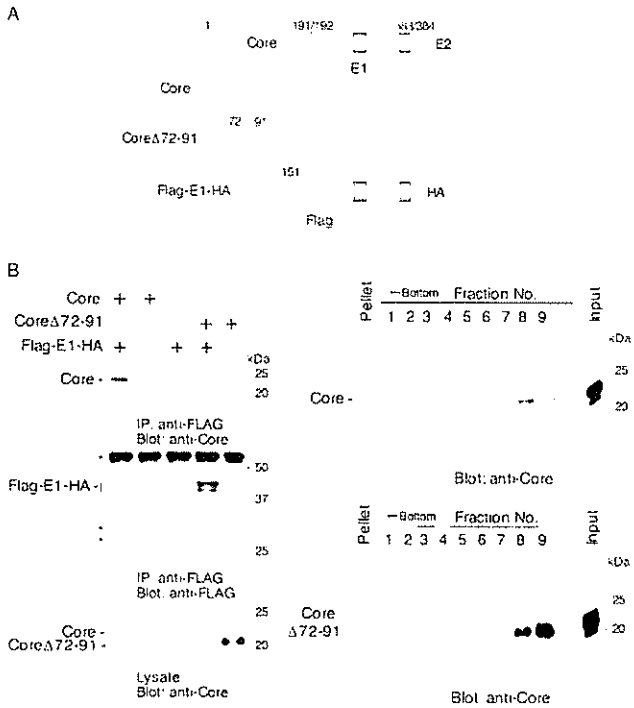

FIG. 4. Amino acid residues72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein. (A) cDNAs used for expression. CoreΔ72-91 is an HCV core protein carrying a deletion of amino acid residues 72 to 91. (B, left) FLAG-E1-HA was coexpressed in 293T cells with either a wild-type core or CoreΔ72-91, and the interaction was analyzed by immunoprecipitation in the presence of tRNA. The asterisks indicate nonspecific bands. (Right) Oligomerization of a wild-type core or CoreΔ72-91 in the presence of tRNA. Wild-type core protein was self-oligomerized, but CoreΔ72-91 was not.

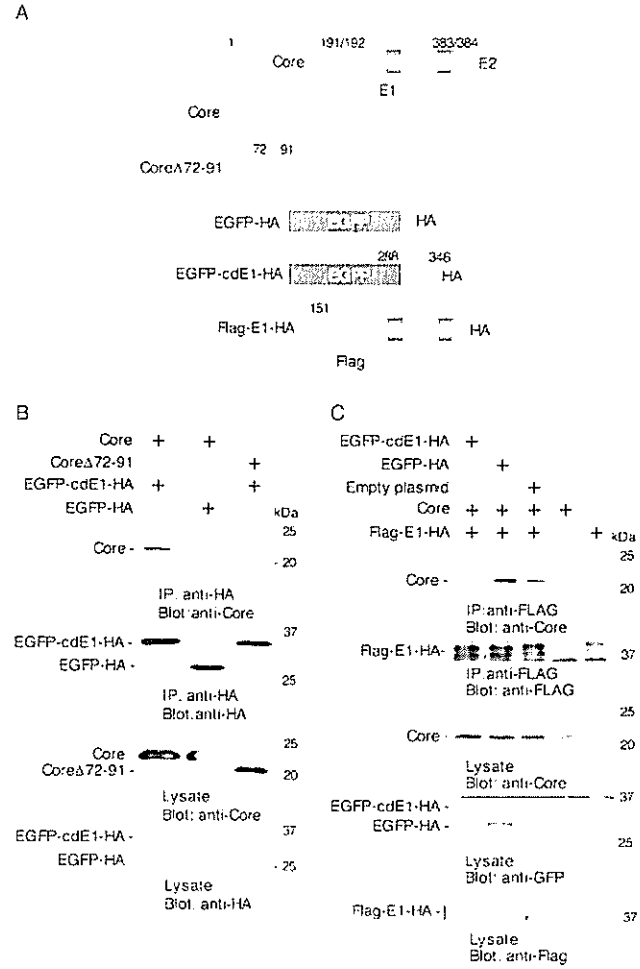

FIG. 5. Interaction of the E1 cytoplasmic loop with the core protein. (A) cDNAs used for expression. EGFP-cdE1-HA is an EGFP fusion protein carrying the E1 cytoplasmic region of amino acid residues 288 to 346 followed by an HA tag. (B) Wild-type core or CoreΔ72-91 was coexpressed with EGFP-cdE1-HA in 293T cells, and their interaction was analyzed by immunoprecipitation. EGFP-cdE1-HA coprecipitated with wild-type core protein, but not with CoreΔ72-91. (C) Inhibition of the interaction of the core protein with FLAG-E1-HA by expression of EGFP-cdE1-HA. Expression of EGFP-cdE1-HA but not of EGFP disrupted the interaction between core and E1 proteins.

cells, we constructed an enhanced green fluorescent protein (EGFP) fusion protein carrying the E1 cytoplasmic domain followed by an HA tag (EGFP-cdE1-HA) (Fig. 5A). Upon coexpression of EGFP-cdE1-HA with the wild-type core protein in 293T cells, the two proteins could be coprecipitated using anti-HA antibody (Fig. 5B). The mutant CoreΔ72-91 protein was unable to associate with EGFP-cdE1-HA (Fig. 5B). Together, these studies demonstrate that the cytoplasmic loop region of E1 is able to interact with the core protein and that core residues 72 to 91 are required for this association.

To further confirm the specificity of the interaction of the E1 cytoplasmic region with the core protein, we examined the ability of the EGFP-cdE1-HA protein to inhibit the association of the intact E1 protein (in Flag-E1-HA) with the wild-type core protein (Fig. 5C). Expression of EGFP-cdE1-HA but not EGFP-HA competed strongly with the interaction between core and the FLAG-tagged Flag-E1-HA protein. These results suggest that the cytoplasmic loop in the intact E1 glycoprotein can directly bind to HCV core protein. Interestingly, the EGFP-cdE1-HA protein was unable to inhibit this interaction in the context of the intact core and E1 and E2 polyproteins (data not shown), suggesting that expression of the core and E1 proteins in *cis* may prevent subsequent interaction with E1 expressed in *trans*.

Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein. Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes revealed that the region from Gln^{302} to Pro^{328} is highly conserved (Fig. 6A). To determine residues in the E1 cytoplasmic region that are critical for interaction with the core protein, blocks of four residues each in the conserved region were replaced with Ala in the polyprotein (core, E1, E2, and p7) (Fig. 6A). These mutant polyproteins were expressed in 293T cells and immunoprecipitated with anti-core antibody; coprecipitated E1 protein was detected by immunoblotting using an anti-E1 monoclonal antibody (Fig. 6B). The replacement of four amino acid residues, 304 to 307, with Ala in the conserved region of the E1