

Epigenetic Silencing of Antiviral Genes Renders Clones of Huh-7 Cells Permissive for Hepatitis C Virus Replication

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Hepatitis C virus (HCV) does not replicate efficiently in wild-type human hepatoma Huh-7 cells, but it replicates robustly in certain subclones of Huh-7 cells. Previously, we demonstrated that silencing of cyclic AMP (cAMP) response element binding protein 3-like 1 (*CREB3L1*), a cellular transcription factor that inhibits HCV replication, allows HCV to replicate in HRP1 cells, a subclone of Huh-7 cells permissive for HCV replication. Here we show that silencing of myxovirus resistant 1 (*MX1*), a known interferon-induced antiviral gene, is responsible for HRP4 cells, another subclone of Huh-7 cells, being permissive for HCV replication. Both *CREB3L1* and *MX1* are epigenetically silenced through DNA methylation in HRP1 and HRP4 cells, respectively. We further demonstrate that Huh-7 cells exist as a mixed population of cells with distinct patterns of gene methylation and HCV replicates in subpopulations of Huh-7 cells that have antiviral genes epigenetically silenced by DNA hypermethylation. Our results demonstrate that understanding the mechanism through which subclones of Huh-7 cells become permissive for HCV replication is crucial for studying their interaction with HCV.

A powerful approach to study innate antiviral response is to compare wild-type Huh-7 cells, which do not support robust replication of hepatitis C virus (HCV), and certain subclones of Huh-7 cells that are permissive for HCV replication. The subclones of Huh-7 cells that support HCV replication can be selected by an HCV subgenomic replicon that consists of HCV RNA engineered to express a selectable marker gene, *neo*, in place of a portion of the viral RNA that is not required for viral replication (1). When Huh-7 cells were transfected with the HCV replicon RNA, followed by G418 selection, only a few cells survived the selection, an observation suggesting that most Huh-7 cells are not permissive for HCV replication (1, 2). When HCV RNA was eliminated from the surviving cells through interferon treatment, the cured cells showed dramatically enhanced permissiveness for HCV RNA replication, as demonstrated by the large number of cells that survived G418 selection following retransfection with the HCV replicon RNA (2). Huh-7.5 cells are a line of these cured cells (2). Unlike parental Huh-7 cells, Huh-7.5 cells failed to produce type 1 interferon in response to viral infection as a result of a dominant negative mutation in the *RIG-I* gene (3).

However, the mutation in *RIG-I* alone is not sufficient to make Huh-7 cells permissive for HCV replication (4, 5). We recently observed that cyclic AMP (cAMP) response element binding protein 3-like 1 (*CREB3L1*) is expressed in parental Huh-7 cells but not in Huh-7.5 cells and another subclone of Huh-7 cells supporting HCV replication, namely, HRP1 cells (5). *CREB3L1* is synthesized as a membrane-bound precursor. It is proteolytically activated in virus-infected cells so that the NH₂-terminal domain of the protein is able to enter the nucleus to activate transcription of genes encoding proteins that block the cell cycle (5). Inasmuch as active division of host cells is required for efficient HCV replication (6, 7), activation of *CREB3L1* not only blocks proliferation of HCV-infected cells but also inhibits viral replication (5). As a result, expression of *CREB3L1* must be silenced in cells highly permissive for HCV replication so that they are able to divide while supporting efficient viral replication. However, how expression of *CREB3L1* is silenced in these cells remains a mystery.

Cytosine methylation is a major epigenetic mechanism to reg-

ulate gene expression (8). In mammalian cells, cytosine methylation occurs at CpG dinucleotides, which are concentrated in small CpG-rich regions. These regions, which are designated CpG islands, are often associated with gene promoters. Methylation of the cytosines in CpG islands results in transcriptional repression of the gene (8).

In the current study, we determine that *CREB3L1* in HRP1 cells is silenced through gene methylation. Hypermethylation of myxovirus resistant 1 (*MX1*), an interferon-induced antiviral gene, also allows HCV to replicate in HRP4 cells, another subclone of Huh-7 cells permissive for HCV replication. We further demonstrate that Huh-7 cells are epigenetically unstable in that they exist as a heterogeneous population of cells with distinct patterns of DNA methylation. This instability may allow HCV to replicate in subpopulations of Huh-7 cells in which antiviral genes are epigenetically silenced by DNA hypermethylation.

MATERIALS AND METHODS

Materials. We obtained 5-azacytidine (5-azaC) and alpha interferon (IFN- α) from Sigma and PBL Interferon Source, respectively.

Cell culture. Huh-7, Huh-7.5, and HRP1 cells were maintained in medium A (Dulbecco's modified Eagle's medium with 4.5 g/liter glucose, 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate, and 10% fetal calf serum). HRP4 HCV cells are a clone of Huh-7 cells transfected with the con1 HCV subgenomic replicon, followed by selection with 700 μ g/ml G418. HRP4 cells were generated after HRP4 HCV cells were treated with IFN- α for 2 weeks to remove the HCV replicon inside the cells. They were maintained in medium A. Huh-7-GL cells, a line of Huh-7 cells that contain a chromosomally integrated JFH-1 strain of HCV cDNA and consti-

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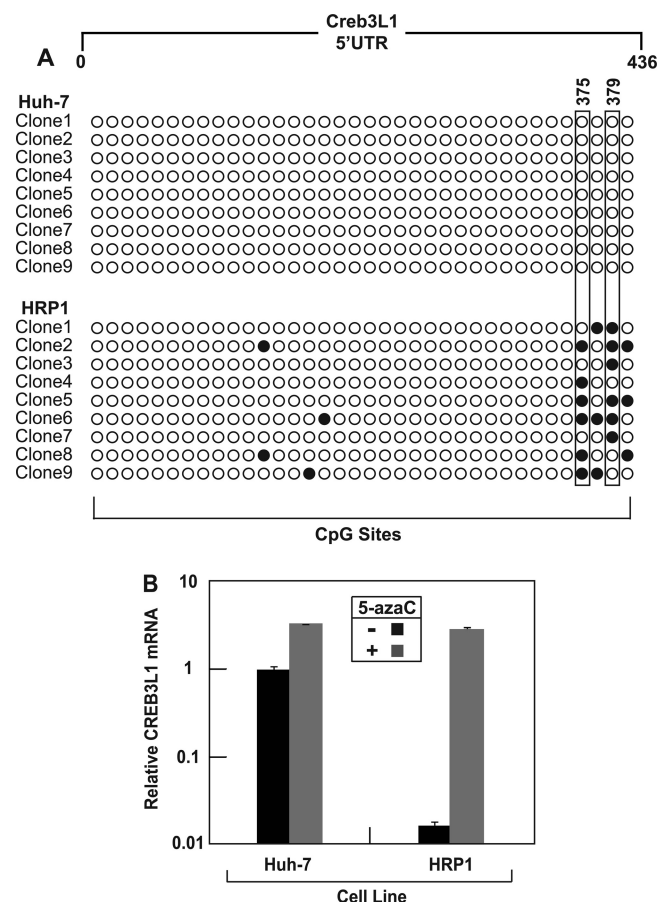


FIG 1 CREB3L1 is hypermethylated in HRP1 cells. On day 0, the indicated cells were seeded at $4 \times 10^5/60$ -mm dish. (A) On day 1, cells were harvested and genomic DNA was extracted for bisulfite sequencing as described in Materials and Methods. The closed and open circles denote methylated and unmethylated CpGs, respectively. The position is numbered according to the transcriptional initiation site, which is set at 1. Cytosines that were not methylated in Huh-7 cells but methylated in more than 50% of PCR clones amplified from HRP4 cells are boxed. (B) On day 1, cells were treated with 30 μ M 5-azaC. On day 4, cells were harvested, and the amount of CREB3L1 mRNA was quantified by RT-qPCR, with the value in Huh-7 cells untreated with 5-azaC set to 1. Results are reported as means and standard deviations (SDs) from three independent experiments.

tively produce infectious virus (9), were maintained in medium A supplemented with 5 μ g/ml blasticidin. HRP4/pMX1 and HRP4/pControl cells were generated by transfecting HRP4 cells with pCMV-MX1 (a plasmid encoding human MX1 driven by the cytomegalovirus promoter) and a control empty plasmid, respectively, followed by selection in medium A supplemented with 500 μ g/ml hygromycin. Huh-7/shControl and Huh-7/shMX1 cells were generated by transfecting Huh-7 cells with a control short hairpin RNA (shRNA) that does not target any human genes (SA Biosciences) and an shRNA targeting *MX1* (SA Biosciences, Clone ID 4), respectively, followed by selection in medium A supplemented with 500 μ g/ml hygromycin. Huh-7/shMX1/pMX1 cells were generated by transfecting Huh-7/shMX1 cells with pCMV-MX1, followed by selection in medium A supplemented with 700 μ g/ml of Zeocin. Huh-7/pNeo1 and Huh-7/pNeo2 cells were generated by transfecting Huh-7 cells with pcDNA3.1-neomycin, followed by selection in medium A supplemented with 500 μ g/ml G418. All cells were cultured in monolayers at 37°C in 5% CO₂.

Virus infection. The JFH-1 strain of HCV was produced from Huh7-GL cells as previously described (10). The HCV HP replicon RNA

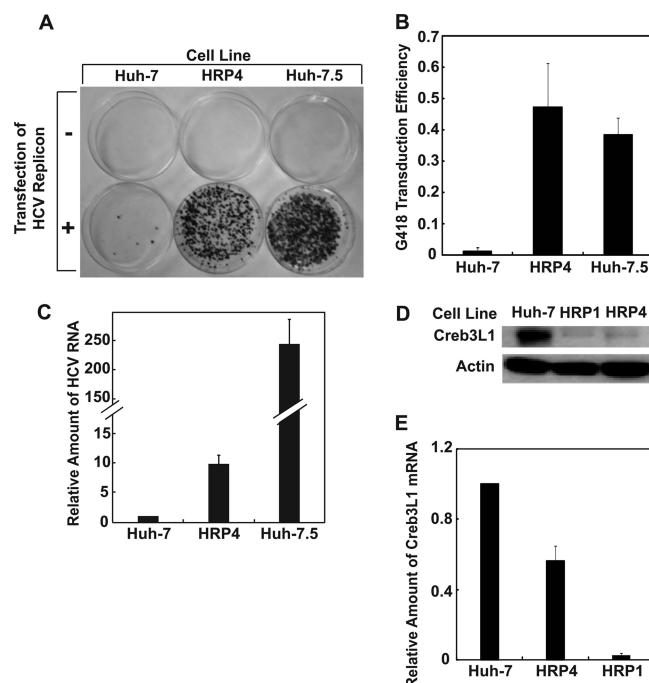


FIG 2 HRP4 cells are permissive for HCV replication. On day 0, the indicated cells were seeded at $7 \times 10^5/60$ -mm dish. (A and B) On day 1, cells were transfected with an HCV replicon (HP; 0.5 μ g/dish). On day 3, cells were treated with 700 μ g/ml G418. After 2 weeks of G418 selection, the cells were stained with crystal violet. A representative picture of the staining result (A) and quantification of G418 transduction efficiency (B) are presented. (C) On day 1, cells were infected with the JFH-1 strain of HCV. On day 5, cells were harvested, and the amount of intracellular HCV RNA was quantified by RT-qPCR, with the value in Huh-7 cells set at 1. (D) Quantification of the CREB3L1 protein in indicated cells by immunoblot analysis. (E) Quantification of CREB3L1 mRNA in indicated cells by RT-qPCR, with the value in Huh-7 cells set at 1. (B, C, and E) Results are reported as means and SDs from three independent experiments.

was *in vitro* transcribed as previously described (11) and transfected into Huh-7-derived cells using TransMessenger reagent (Qiagen). Sendai virus (Cantell strain) was purchased from Charles River Laboratories. The virus was added to cells after it was diluted 50-fold in cell culture medium (3 ml/60-mm plate).

Immunoblot analysis. Cell lysates were analyzed by 10% SDS-PAGE followed by immunoblot analysis with the indicated antibodies (1:2,000 dilution for anti-CREB3L1 [5] and 1:10,000 dilution for anti-actin). Bound antibodies were visualized with a peroxidase-conjugated secondary antibody using the SuperSignal enhanced chemiluminescence (ECL)-horseradish peroxidase (HRP) substrate system (Pierce).

Real-time qPCR. Real-time quantitative PCR (RT-qPCR) was performed as previously described (12). The relative amounts of RNAs were calculated through the comparative cycle threshold method by using human 36B4 mRNA as the invariant control.

Luciferase assays. Luciferase activity in the cell lysate was assayed with the dual-luciferase reporter assay system (Promega) using the Synergy 4 plate reader and Gen5 1.10 software (BioTek). Promoter activity was determined by firefly luciferase activity normalized against *Renilla* luciferase activity to control for transfection efficiency.

G418 transduction efficiency assays. Quantification of the percentage of cells that survived G418 selection following transfection with a HCV replicon was performed exactly as previously described (2).

Microarray analysis. Microarray analysis was performed exactly as previously described (13).

Bisulfite sequencing. Genomic DNA was purified using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol.

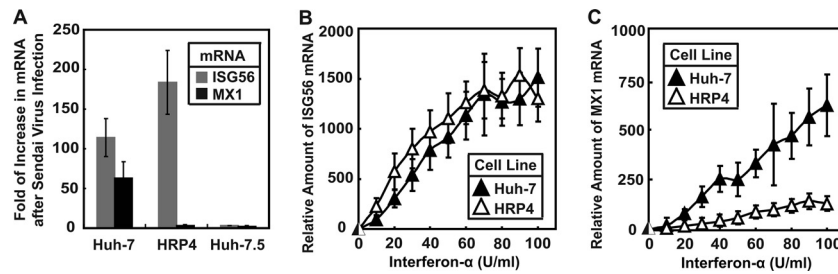


FIG 3 HRP4 cells are defective in inducing MX1 expression in response to interferon. On day 0, indicated cells were seeded at 4×10^5 /60-mm dish. (A) On day 1, some of these cells were infected by Sendai virus. On day 2, cells were harvested and the indicated mRNA was determined by RT-qPCR. Fold induction of the indicated mRNA by Sendai virus infection is presented. (B and C) On day 1, cells were treated with the indicated concentration of alpha interferon for 6 h. Cells were harvested, and the amount of ISG56 (B) and MX1 (C) mRNA was quantified by RT-qPCR, with the value in untreated HRP4 cells set at 1. (A to C) Results are reported as means and SDs from three independent experiments.

DNA was then subject to bisulfite C→U conversion using the EZ DNA Methylation Gold kit (Zymo) according to the manufacturer's protocol. Treated and untreated DNA was used for PCR using ZymoTaq Premix (Zymo). Amplified fragments were ligated into a plasmid using the TOPO TA cloning kit (Invitrogen) for sequencing analysis.

DNA methylation microarray. Genomic DNA was purified using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol. Genomic DNA (30 µg) was sonicated three times at 15% amplitude to break it into 300 bp to 1,000 bp (Brandson). The MeDIP kit (Diagenode) was used to immunoprecipitate methylated DNA. Both immunoprecipitated (IP) and input DNA was amplified with the WGA2 kit (Sigma). Amplified products were purified through the QIAquick PCR purification kit (Qiagen) and subjected to human DNA methylation 2.1 M deluxe promoter arrays analysis, performed by Roche NimbleGen. The signal intensity of methylation of a specific segment of DNA was determined by its log₂ IP/input ratio.

Microarray data accession numbers. Microarray analyses comparing gene expression between Huh-7 and HRP4 cells treated with interferon or untreated were deposited at Gene Expression Omnibus (GEO) under accession number 31903. DNA methylation microarray analyses comparing gene methylation profiles among various subclones of Huh-7 cells were deposited at GEO under accession number 31960.

RESULTS

To determine how expression of *CREB3L1* is suppressed in HRP1 cells, we performed extensive sequencing analysis but were unable to identify any mutation in the coding region or in the 5' regulatory element of the gene in HRP1 cells. We thus considered the possibility that the gene was silenced through an epigenetic mechanism in these cells. Methylation of cytosines in CpG islands is a well-characterized epigenetic mechanism to suppress transcription of cellular genes (8). *CREB3L1* contains a CpG island in its 5' untranslated region (UTR) located in the first exon (Fig. 1A). Bisulfite sequencing revealed that two cytosines in this region were methylated in more than 50% of the PCR clones amplified from the genomic DNA obtained from HRP1 cells (Fig. 1A). Neither cytosine was methylated in the parental Huh-7 cells (Fig. 1A). To confirm that hypermethylation of *CREB3L1* leads to inhibition of its expression, we treated the cells with 5-azaC, an inhibitor of DNA methylation (14). While having little impact on *CREB3L1* expression in Huh-7 cells, 5-azaC completely restored *CREB3L1* expression in HRP1 cells by increasing its mRNA by ~100-fold (Fig. 1B). The result shown in Fig. 1 suggests that *CREB3L1* is epigenetically silenced in HRP1 cells through DNA methylation. To determine whether hypermethylation of an antiviral gene is unique in HRP1 cells or a common mechanism to generate sub-

clones of Huh-7 cells permissive for HCV replication, we needed another line of Huh-7 cells also permissive for HCV replication but different from HRP1 cells. For this purpose, we transfected Huh-7 cells with an HCV subgenomic replicon RNA to obtain a line of Huh-7 cells that harbor the HCV replicon. These cells were treated with interferon to generate a clone of cured Huh-7 cells that no longer contained HCV RNA. These cells were designated HRP4 cells. HCV replicon RNA was then retransfected into these cells to determine their permissiveness for HCV replication. Judged by the number of colonies that contained the HCV replicon carrying the *neo* gene, HRP4 cells were more permissive for HCV replication than their parental Huh-7 cells (Fig. 2A and B). Quantification of the percentage of the cells that survived G418 selection indicated that HRP4 cells were as permissive as Huh-7.5 cells for replication of the HCV replicon (Fig. 2B). HRP4 cells were also susceptible for infection by the JFH-1 strain of HCV, although the cells were not as supportive as Huh-7.5 cells for infection by this strain of HCV (Fig. 2C). The observation that HRP4 cells are susceptible to infection by the JFH-1 strain of HCV suggests that they are different from HRP1 cells, because HRP1 cells are resistant to infection by the JFH-1 strain of HCV even though they are as permissive as Huh-7.5 cells for replication of the HCV subgenomic replicon (5). There is also a difference in *CREB3L1* expression between the two lines of cells permissive for HCV replication: while the *CREB3L1* protein was barely detectable by immunoblot analysis in both HRP1 and HRP4 cells (Fig. 2D), the amount of *CREB3L1* mRNA in HRP4 cells was much higher than that in HRP1 cells (Fig. 2E).

We then analyzed the interferon response in HRP4 cells. For this purpose, we analyzed expression of *ISG56* and *MX1*, two well-characterized interferon-induced antiviral genes (15, 16). Infection with Sendai virus, which stimulates production of interferon (3), activated expression of both *ISG56* and *MX1* in Huh-7 cells (Fig. 3A). Sendai virus failed to induce these genes in Huh-7.5 cells (Fig. 3A) because of a mutation in *RIG-I* that makes these

TABLE 1 mRNA increased in Huh-7 but not HRP4 cells in response to interferon treatment

Gene	NCBI nucleotide accession no.	Fold increase in Huh-7 cells	Fold increase in HRP4 cells
<i>MX1</i>	NM_002462	6.93	Absent
<i>C1orf53</i>	BI832220	6.34	1.75

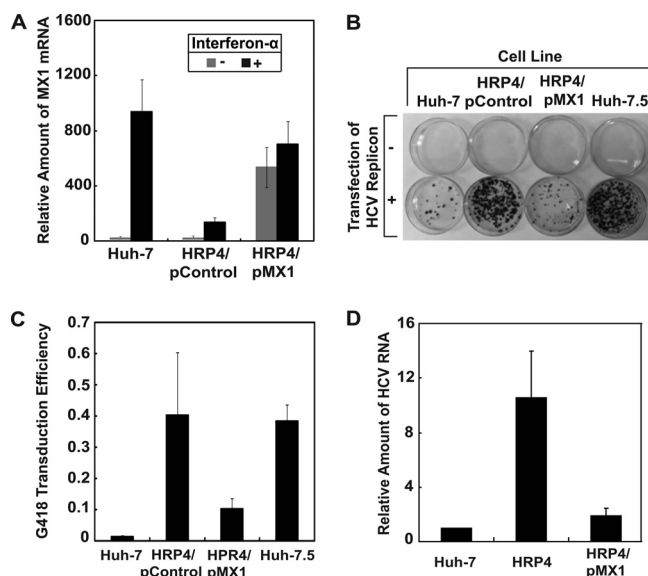


FIG 4 Restoration of MX1 expression reduces the permissiveness of HRP4 cells for HCV replication. On day 0, indicated cells were seeded at 7×10^5 /60-mm dish. (A) On day 1, cells were treated with 100 U/ml of alpha interferon for 6 h. The amount of MX1 mRNA was quantified by RT-qPCR, with the value in untreated Huh-7 cells set at 1. (B and C) The indicated cells transfected with the HCV replicon were selected by G418, stained with crystal violet, and analyzed as described in Fig. 2A and B. (D) The indicated cells were infected with the JFH-1 strain of HCV and analyzed as described in Fig. 2C. (A to D) Bar graphs show the means and SDs from three independent experiments.

cells defective in producing interferon in response to viral infection (3). Unlike Huh-7 or Huh-7.5 cells, Sendai virus stimulated expression only of *ISG56* and not of *MX1* in HRP4 cells (Fig. 3A). We then examined the effect of direct addition of IFN- α on expression of these genes. While IFN- α was equally potent in activating expression of *ISG56* in both Huh-7 and HRP4 cells (Fig. 3B), it was much less effective in stimulating expression of *MX1* in HRP4 cells (Fig. 3C). To determine whether HRP4 cells are defective in activating expression of genes other than *MX1* in response to IFN- α , we performed microarray analysis to compare the effects of IFN- α on gene expression in Huh-7 and HRP4 cells. This analysis revealed that there were only two genes activated by interferon by more than 5-fold in Huh-7 cells that were not activated to the same degree in HRP4 cells (Table 1). Remarkably, *MX1* was one of the two genes. The other gene, *C1orf53*, has never been characterized.

To investigate whether the lack of *MX1* expression accounts for the permissiveness of HRP4 cells for HCV replication, we first determined whether restoration of *MX1* expression in these cells inhibited HCV replication. For this purpose, we stably transfected HRP4 cells with a plasmid encoding *MX1* and selected a clone of the cells expressing *MX1* mRNA at a level similar to that in Huh-7 cells treated with interferon (Fig. 4A). These cells, which were designated HRP4/pMX1, were transfected with an HCV subgenomic replicon RNA. Compared to HRP4 cells transfected with a control plasmid (HRP4/pControl), HRP4/pMX1 cells were much less permissive for HCV replication, as measured by the number of colonies that contained the HCV replicon (Fig. 4B and C). These cells were also much more resistant to infection by the

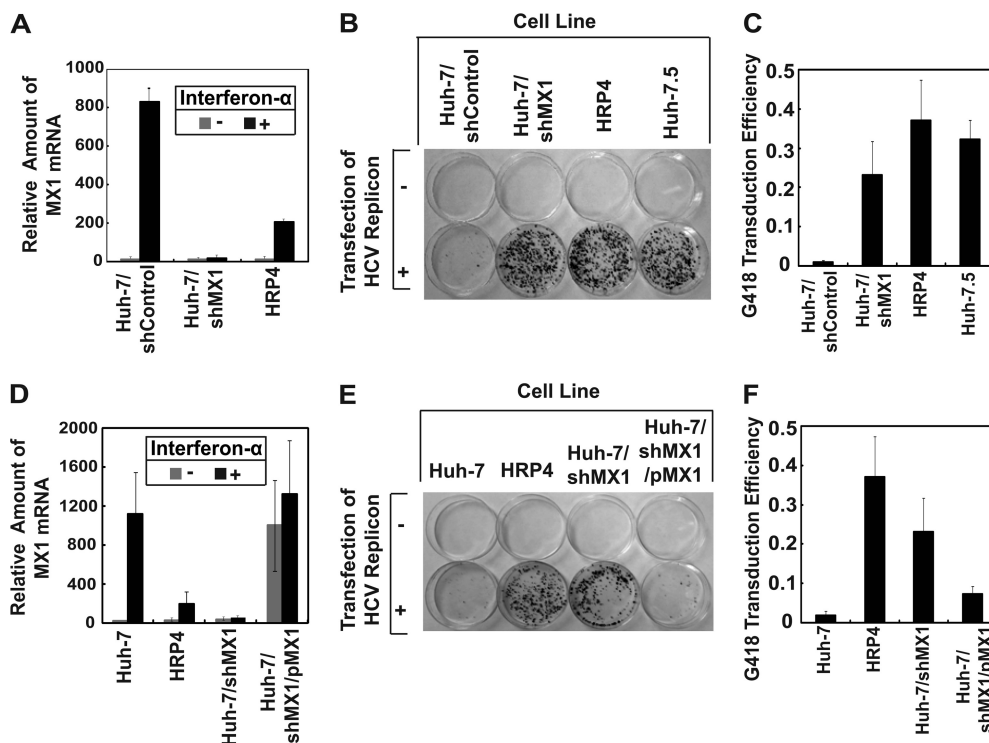


FIG 5 Silencing of *MX1* expression in Huh-7 cells increases their permissiveness for HCV replication. (A and D) Quantification of MX1 mRNA was performed as described in Fig. 4A, with the value in untreated Huh-7/shControl (A) or Huh-7 (D) cells set at 1. (B, C, E, and F) The indicated cells transfected with the HCV replicon were selected by G418, stained with crystal violet, and analyzed as described in Fig. 2A and B. (A to F) Bar graphs show the means and SDs from three independent experiments.

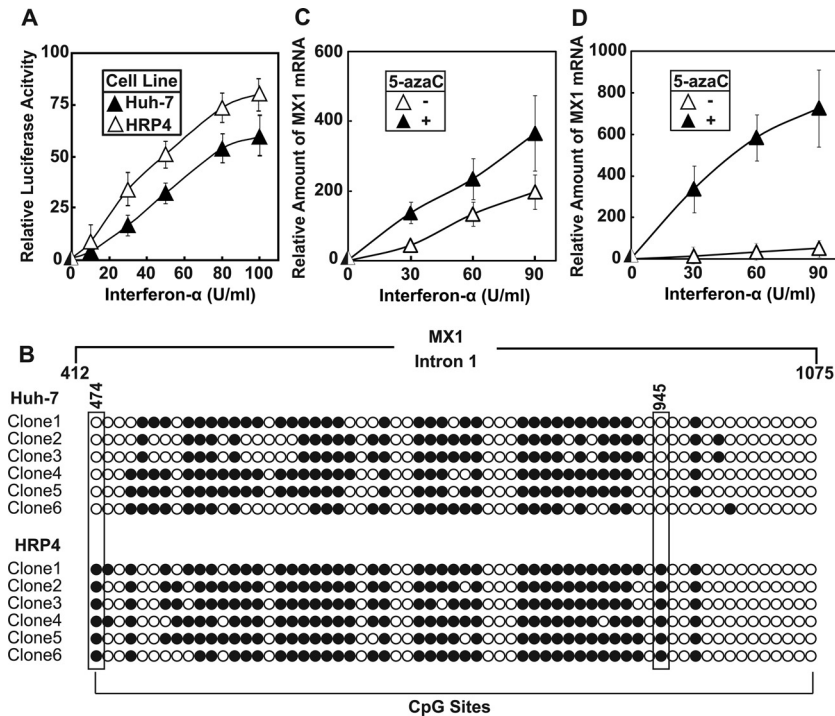


FIG 6 *MX1* is hypermethylated in HRP4 cells. On day 0, indicated cells were seeded at 7×10^5 cells/60-mm dish. (A) On day 1, cells were transfected with a firefly luciferase reporter plasmid driven by the *MX1* promoter (1 μ g/dish) and a plasmid encoding *Renilla* luciferase that was driven by the constitutive cytomegalovirus (CMV) promoter (0.5 μ g/dish). On day 2, cells were treated with the indicated amount of alpha interferon for 6 h. Luciferase activity was measured, and the promoter activity was determined by firefly luciferase activity normalized against *Renilla* luciferase activity to control for transfection efficiency. Results are the means and SDs from triplicate incubations of a representative experiment. (B) Methylation of CpG sites in the first intron of *MX1* was analyzed as described in Fig. 1A. (C and D) On day 1, Huh-7 (C) and HRP4 (D) cells were treated with 4 μ M 5-azaC or untreated. On day 4, cells were treated with the indicated concentration of alpha interferon for 6 h. The amount of *MX1* mRNA in these cells was quantified by RT-qPCR, with the value in Huh-7 cells untreated with either interferon or 5-azaC set at 1. Results are the means and SDs from three independent experiments.

JFH-1 strain of HCV than HRP4/pControl cells, as determined by the amount of intracellular viral RNA following the viral infection (Fig. 4D).

We also determined whether knockdown of *MX1* expression in Huh-7 cells rendered these cells more permissive for HCV replication. For this purpose, we generated a line of Huh-7 cells (Huh-7/sh*MX1* cells) in which *MX1* was knocked down by more than 90% by a stably transfected shRNA in cells treated with interferon (Fig. 5A). Judging by the number of colonies that contained a transfected HCV subgenomic replicon, these cells were much more permissive for HCV replication than Huh-7 cells stably transfected with a control shRNA (Huh-7/shControl) but not so permissive as HRP4 cells (Fig. 5B and C). To determine the specificity of the shRNA-mediated knockdown of *MX1*, we stably transfected Huh-7/sh*MX1* cells with a plasmid overexpressing *MX1* to generate a line of cells (Huh-7/sh*MX1*/p*MX1*) in which expression of *MX1* was restored to the level found in Huh-7 cells treated with interferon (Fig. 5D). Restoration of *MX1* expression in Huh-7/sh*MX1* cells markedly reduced their permissiveness for HCV replication (Fig. 5E and F).

We then investigated the mechanism through which expression of *MX1* is suppressed in HRP4 cells. IFN- α was equally effective in activating a luciferase reporter gene driven by the 5' flanking region of *MX1* in both Huh-7 and HRP4 cells (Fig. 6A). This observation suggests that there is no defect in the interferon-stimulated signaling pathway leading to activation of *MX1* in HRP4

cells. We thus speculated that the defect must be in the *MX1* gene itself. Since we were unable to identify any mutations in the coding region or in the 5' regulatory element of the gene in HRP4 cells, we suspected that the gene was epigenetically silenced in these cells. *MX1* was known to contain multiple CpG islands and silenced in several tumor cells through hypermethylation (17, 18). We identified two cytosines in a CpG island located in the first intron of *MX1* that were methylated in HRP4 cells but not in their parental Huh-7 cells through bisulfite sequencing analysis (Fig. 6B). While 5-azaC only slightly induced expression of *MX1* in Huh-7 cells treated with IFN- α (Fig. 6C), the compound markedly raised the amount of *MX1* mRNA in interferon-treated HRP4 cells and completely restored its induction by IFN- α in these cells (Fig. 6D).

Our results with HRP1 and HRP4 cells indicate that different anti-HCV genes are hypermethylated and silenced in different subclones of Huh-7 cells permissive for HCV replication: in HRP1 cells, *CREB3L1* is hypermethylated but expression of *MX1* is not affected (5), whereas in HRP4 cells, *MX1* is hypermethylated but production of *CREB3L1* mRNA is only slightly reduced (Fig. 2E). In contrast to these cells, neither gene is methylated in parental Huh-7 cells. These results suggest that genes methylated in these cells may be different. To test this hypothesis, we performed a methylated DNA microarray analysis (19). We immunoprecipitated methylated genomic DNA fragments with an antibody reacting against methylated cytosines and hybridized these fragments with probes covering all annotated promoters and CpG

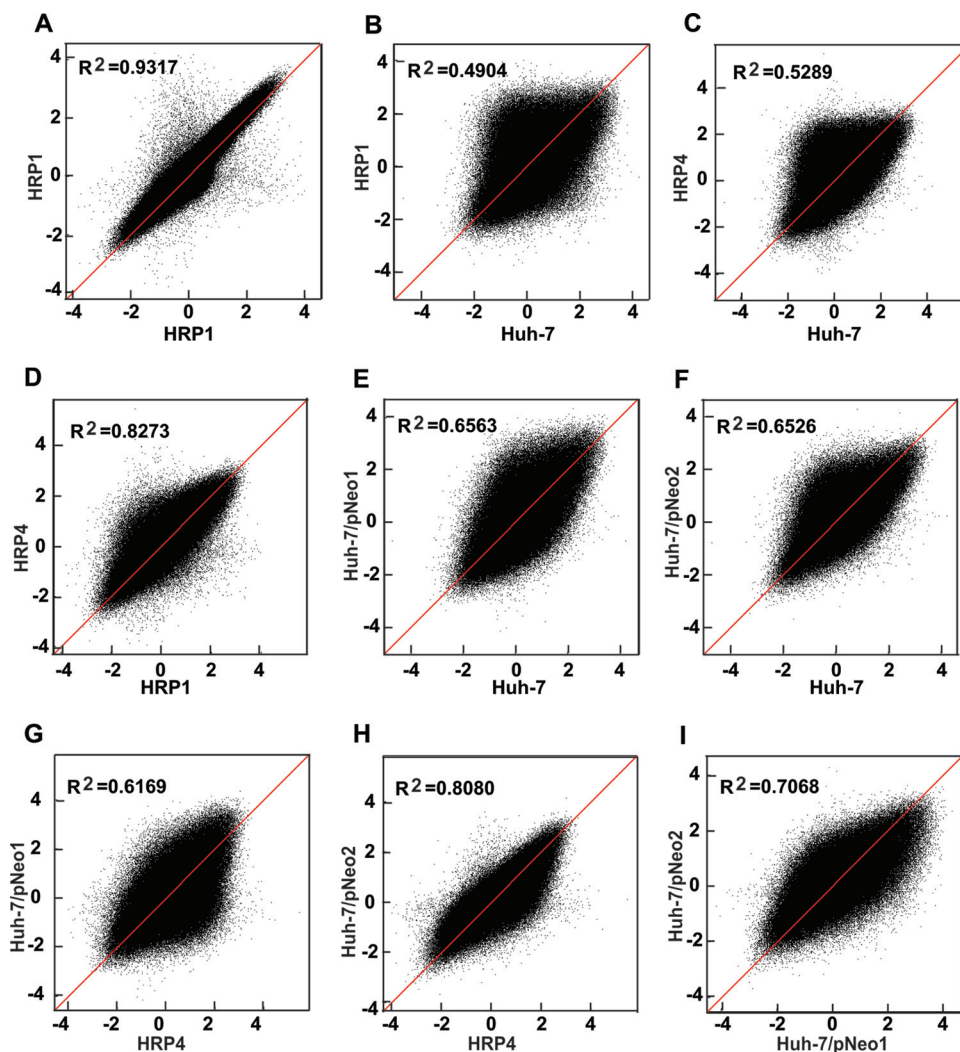


FIG 7 DNA methylation profiles are different among subclones of Huh-7 cells. The DNA methylation microarray was performed as described in Materials and Methods. The coordinates of each point show the signal intensity of methylation of a specific segment of DNA in cells indicated on the x and y axes. The diagonal line $y = x$ is highlighted in red. The R^2 value for each comparison is presented.

islands in the entire genome. The signal intensity of methylation of a specific segment of DNA was determined by its hybridization signal normalized against its input amount before immunoprecipitation. We then compared the global gene methylation profiles of two lines of cells by plotting their methylation signal intensities against each other. If two lines of cells shared a similar pattern in gene methylation, all dots in the plot should be closely fitted into a straight line, $y = x$. This was exactly the case when we compared gene methylation in HRP1 cells from two independent experiments (Fig. 7A). This pattern was not observed when we compared gene methylation of Huh-7 and HRP1 cells (Fig. 7B), Huh-7 and HRP4 cells (Fig. 7C), or HRP1 and HRP4 cells (Fig. 7D). Thus, the global gene methylation pattern is different among these cells.

Inasmuch as both HRP1 and HRP4 cells were selected from Huh-7 cells with an HCV subgenomic replicon, the above-mentioned results suggest that HCV infection may induce global changes in gene methylation in Huh-7 cells in an attempt to create a cellular environment more suitable for the virus to replicate. Alternatively, Huh-7 cells may exist as a heterogeneous popula-

tion of cells with distinct patterns of gene methylation. This heterogeneity allows HCV to select a small population of the cells that have antiviral genes hypermethylated for replication. To test which one of these hypotheses was correct, rather than transfecting an HCV subgenomic replicon RNA carrying *neo*, we transfected Huh-7 cells with a plasmid carrying *neo* and selected two independent clones of the stably transfected cells (Huh-7/pNeo1 and Huh-7/pNeo2). Since these cells had never been infected by HCV, their gene methylation profiles were not expected to be different from that in Huh-7 cells if the alteration in gene methylation was induced by the virus. However, DNA methylation microarray analysis revealed that gene methylation profiles in these cells were also different from those in Huh-7 (Fig. 7E and F) and HRP4 (Fig. 7G and H) cells. The gene methylation profiles of these two lines of cells were also different (Fig. 7I). Thus, the difference in gene methylation among subclones of Huh-7 cells is most likely caused by epigenetic instability of Huh-7 cells that allows them to exist as a mixed population of cells with distinct gene methylation profiles.

DISCUSSION

The current study reveals a critical role of gene methylation in generating subclones of Huh-7 cells permissive for HCV replication. We show that Huh-7 cells are a heterogeneous population of cells with distinct profiles in gene methylation. Most of these cells express genes that inhibit HCV replication so that they are resistant to the viral infection. A small number of the cells have certain anti-HCV genes hypermethylated and inactivated so that they are permissive for HCV replication. For example, *CREB3L1* and *MX1*, two anti-HCV genes, are silenced through gene methylation in our permissive subclones, HRP1 and HRP4 cells, respectively. Methylation of interferon-induced genes other than *MX1* has been reported to be responsible for generation of other subclones of Huh-7 cells permissive for HCV replication (20). This clonal variation in gene methylation may also explain why expression of so many genes is different between parental Huh-7 cells and subclones of Huh-7 cells permissive for HCV replication (5, 7). The difference among subclones of Huh-7 cells permissive for HCV replication may also explain conflicting results regarding host proteins that control HCV infection in the literature. One such example is *MX1*. In the current study, we demonstrate that *MX1* plays an important role in inhibiting HCV replication by showing that cells deficient in *MX1* were permissive for HCV replication and that restoration of *MX1* expression in these cells abolished their ability to support HCV replication. However, a previous study showed that overexpression of *MX1* failed to inhibit HCV replication (21). The most likely explanation for the discrepancy is that there was no defect in *MX1* expression in subclones of Huh-7 cells harboring the HCV replicon in the earlier study (21). These results suggest that while silencing *MX1* makes Huh-7 cells more permissive for HCV replication, overexpression of *MX1* in cells already expressing normal amounts of the protein may not necessarily make the cells more resistant to HCV infection. Thus, identifying the mechanism through which subclones of Huh-7 cells become permissive for HCV replication is crucial for understanding their interaction with HCV.

An important observation in the current study is that HRP4 cells also express much less *CREB3L1* protein than their parental Huh-7 cells. This result reaffirms our previous conclusion that *CREB3L1* plays a critical role in limiting HCV replication in Huh-7-derived cells. While both HRP1 and HRP4 cells express very little *CREB3L1* protein, the amount of *CREB3L1* mRNA is much higher in HRP4 cells than in HRP1 cells. This result suggests that epigenetic silencing of *CREB3L1* observed in HRP1 cells is unlikely to be the mechanism through which *CREB3L1* expression is inhibited in HRP4 cells. Instead, the result suggests that *CREB3L1* expression is inhibited at a posttranscriptional step in HRP4 cells. Identification of the mechanism through which *CREB3L1* expression is inhibited in HRP4 cells may reveal novel pathways that regulate this antiviral factor.

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