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Replication of Hepatitis C Virus (HCV) RNA in Mouse Embryonic Fibroblasts: Protein Kinase R (PKR)-Dependent and PKR-Independent Mechanisms for Controlling HCV RNA Replication and Mediating Interferon Activities

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Hepatitis C virus (HCV) infection causes chronic hepatitis and is currently treated with alpha interferon (IFN-α)-based therapies. The underlying mechanisms of chronic HCV infection and IFN-based therapies, however, have not been defined. Protein kinase R (PKR) was implicated in the control of HCV replication and mediation of IFN-induced antiviral response. In this report, we demonstrate that a subgenomic RNA replicon of genotype 2a HCV replicated efficiently in mouse embryonic fibroblasts (MEFs), as determined by cell colony formation efficiency and the detection of HCV proteins and both positive- and negative-strand RNAs. Additionally, the subgenomic HCV RNA was found to replicate more efficiently in the PKR knockout ($PKR^{-/-}$) MEF than in the wild-type ($PKR^{+/+}$) MEF. The knockdown expression of PKR by specific small interfering RNAs significantly enhanced the level of HCV RNA replication, suggesting that PKR is involved in the control of HCV RNA replication. The level of ISG56 (p56) was induced by HCV RNA replication, indicating the activation of PKR-independent antiviral pathways. Furthermore, IFN-α/β inhibited HCV RNA replication in $PKR^{-/-}$ MEFs as efficiently as in $PKR^{+/+}$ MEFs. These findings demonstrate that PKR-independent antiviral pathways play important roles in controlling HCV replication and mediating IFN-induced antiviral effect. Our findings also provide a foundation for the development of transgenic mouse models of HCV replication and set a stage to further define the roles of cellular genes in the establishment of chronic HCV infection and the mediation of intracellular innate antiviral response by using MEFs derived from diverse gene knockout animals.

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family, with a single-stranded and positive-sense RNA genome of approximately 9.6 kb in length (12, 51). The viral RNA genome contains a single open reading frame flanked by untranslated regions (UTR) at both the 5′ and 3′ ends. A large viral polyprotein precursor of about 3,010 amino acids is translated and is subsequently cleaved by both cellular and viral proteases into at least 10 different structural (C, E1, E2, and p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (42, 50). Replication of HCV RNA occurs in the cytoplasmic membrane-bound multiple-protein complex containing the HCV RNA and nonstructural proteins NS3 to NS5B (15, 42). Owing to the lack of a proofreading activity, the virally encoded RNA-dependent RNA polymerase (RdRp) catalyzes an error-prone RNA replication and thereby results in a mixture of HCV variants (quasispecies) in the infected individual (6). Based on genome sequence similarity, HCV is grouped into six major genotypes and numerous subtypes (55).

HCV is a common cause of chronic viral hepatitis, affecting approximately 170 million people worldwide (9, 68). The vast majority (~85%) of individuals exposed to HCV become chronically infected and carry a higher risk of developing cirrhosis and hepatocellular carcinoma (9, 13). The pegylated alpha interferon (PEG–IFN-α) in combination with ribavirin is currently the optimal therapy to treat HCV infection (13). However, the sustained virologic response (SVR) to IFN-based therapy varied significantly among patients infected with different HCV genotypes (19, 30, 44). In general, HCV genotypes 2 and 3 are sensitive to PEG–IFN-α and ribavirin combination therapy, with an SVR of more than 80%. By contrast, genotypes 1 and 4 are refractory to IFN-based therapy, with an SVR of only about 40 to 50%. The underlying mechanisms of chronic HCV infection, IFN-induced antiviral state against HCV replication, and IFN resistance have not been defined.

Many studies have demonstrated that virus infection, replication, and double-stranded RNA (dsRNA) can activate cellular antiviral pathways and induce IFN production (53, 54). IFN stimulates a large number of cellular genes (IFN-stimulated genes [ISGs]) (14, 25), many of which possess direct or indirect antiviral activities, including the well-characterized protein kinase R (PKR), 2′,5′-oligoadenylate synthetase/RNase L, and Mx (53, 54, 67). PKR is implicated in controlling HCV replication and mediating the IFN-induced antiviral state against HCV replication (21, 52). Several independent studies have found that PKR was induced by HCV RNA replication or

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dsRNA in the HCV-infected chimpanzee liver and in the HCV replicon RNA-harboring Huh7 cells (3, 56, 74). The activated PKR phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α), by which the translation of HCV proteins was inhibited (65). Additionally, other ISGs such as ISG6-16, ISG15, and ISG56 (p56) were also activated by HCV RNA replication and induced by IFN in Huh7 cells in which HCV RNA was persistently replicated (58, 59, 65, 74). P56 binds the e subunit of human eIF3 (eIF3e) and the c subunit of mouse eIF3 (eIF3c), leading to the blockage of protein translation initiation (31, 62). Therefore, the activation of both PKR and p56 can lead to the inhibition of HCV replication by disrupting the translation initiation of HCV proteins (21, 65). On the contrary, findings derived from other independent studies suggest that HCV replication can be suppressed by antiviral pathways independently of PKR (18, 28, 29). However, the roles of PKR-dependent and -independent antiviral pathways in cellular defense against HCV replication are still not well understood.

In an effort to better understand the role of PKR in the establishment of persistent HCV RNA replication and the mediation of IFN-induced antiviral action, we developed mouse cell culture systems of HCV RNA replication. The replication of a subgenomic genotype 2a (JFH1) HCV RNA in PKR knockout ($PKR^{-/-}$) and wild-type ($PKR^{+/+}$) mouse embryonic fibroblasts (MEFs) was determined by cell colony formation assay and the detection of high levels of HCV proteins and both positive- and negative-stranded RNAs. Findings derived from our studies provide a foundation for the development of transgenic mouse models of HCV replication as well as a powerful in vitro system to study the virus-host interactions in MEFs derived from diverse gene knockout mice. Additionally, the JFH1 RNA was found to replicate more efficiently in the $PKR^{-/-}$ MEFs than in wild-type ($PKR^{+/+}$) MEFs but failed to induce cell colony formation in MEFs that constitutively overexpressed a human PKR. In $PKR^{+/+}$ MEFs, the knockdown expression of PKR by specific small interfering RNAs (siRNAs) significantly enhanced the level of HCV RNA replication. Collectively, these findings demonstrate that PKR plays an important role in controlling HCV replication. Furthermore, IFN was able to inhibit HCV RNA replication in the $PKR^{-/-}$ MEF as efficiently as in the $PKR^{+/+}$ MEF, suggesting that PKR-independent antiviral pathways are evolved for the control of HCV replication and the mediation of IFN-induced antiviral response against HCV infection. Moreover, our studies set a stage to determine the roles of cellular genes in the establishment of a chronic HCV infection and the mediation of IFN-induced intracellular innate antiviral response.

MATERIALS AND METHODS

Cell culture. A human hepatoma cell line, Huh7, and mouse embryo fibroblasts (MEFs) were grown in Dulbecco's modified essential medium (DMEM) (Invitrogen) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, nonessential amino acids, and 10% fetal bovine serum (FBS) (Invitrogen). The HCV replicon RNA-harboring Huh7 cells and MEFs were maintained in DMEM containing 10% FBS and 5 μg/ml blasticidin (A.G. Scientific).

DNA construction. pSGR-JFH1 containing the cDNA of a genotype 2a (JFH1) subgenomic HCV RNA was kindly provided by Takaji Wakita, as described previously (34). It was used as a vector for the construction of a subgenomic HCV cDNA that carries a blasticidin resistance gene as a selective marker. The neomycin phosphotransferase gene in the pSGR-JFH1 vector was replaced with a blasticidin resistance gene by two-step PCR and cloning. The T7 promoter and the HCV 5′untranslated region (UTR) was amplified by PCR using synthetic oligonucleotides JFH1-EcoRI (5′-GGAATTCTAATACGACTCACTATAG-3′) and JFH1-BLAST (5′-AGGCTTGGCCATTTTGGTTTTTCTTTGAGG-3′) as primers and pSGR-JFH1 as a template, while the blasticidin resistance gene was amplified by PCR using BLAST-ATG (5′-ATGGCCAAGCCTTTGTCTC-3′) and BLAST-T (5′-TTAGCCCTCCCACACATAAC-3′) as primers and pcDNA6/TR (Invitrogen) as a template. The two PCR DNA fragments were subsequently used as templates for the second round of PCR to combine the 5′UTR with the blasticidin resistance gene into one DNA fragment by using JFH1-EcoRI and BLAST-T as primers. The second PCR product was digested with EcoRI and ligated with the pSGR-JFH1 vector that was digested by EcoRI and PmeI (blunt end), resulting in a DNA construct designated pSGR-JFH1-BLAST. As a negative control, the GDD motif of the HCV RdRp active site was deleted. Deletion of the GDD motif was accomplished by PCR using synthetic oligonucleotides and was inserted into pSGR-JFH1-BLAST between the restriction enzyme sites SfiI and HindIII, resulting in a construct designated pSGR-JFH1-BLAST/ΔGDD.

RNA preparation, transfection, and cell colony selection. The pSGR-JFH1-BLAST and pSGR-JFH1-BLAST/ΔGDD DNAs were linearized by XbaI digestion and blunted by treatment with Mung bean nuclease (NEB). The subgenomic HCV RNAs were transcribed in vitro from the XbaI-digested DNAs by using a RiboMax T7 RNA transcription kit (Promega). The DNA templates were subsequently removed by a thorough treatment with RNase-free DNase I. Subgenomic HCV RNAs were then purified by using an RNeasy RNA purification kit (QIAGEN), and their concentrations were determined by spectrophotometry. Resulting RNA replicons were designated JFH1-BLAST and JFH1-BLAST/ΔGDD, respectively. The JFH1-BLAST and JFH1-BLAST/ΔGDD RNAs were transfected into Huh7 cells, $PKR^{+/+}$ MEFs, and $PKR^{-/-}$ MEFs by electroporation, as described previously (7, 43). Cell colony formation was selected by the addition of 5 to 10 μg/ml blasticidin to culture medium, which was changed every 3 to 4 days. Cell colonies were visualized by staining with a crystal violet solution. Individual colonies were picked up and amplified for the determination of HCV proteins and RNAs as well as for IFN sensitivity studies.

PKR knockdown by small interfering RNAs (siRNAs). PKR-specific siRNA and a nonspecific control siRNA were synthesized by Dharmacon (Lafayette, CO). The PKR SMARTpool siRNAs are a mixture of four individual siRNAs that target different regions of the mouse PKR mRNA. siRNAs were mixed with a transfection reagent (DharmaFECT) that was purchased from Dharmacon. The mixture of siRNAs and lipid was serially diluted to the indicated concentrations with medium and then transferred onto JFH1-BLAST RNA-harboring $PKR^{+/+}$ or $PKR^{-/-}$ MEFs in a 6-well culture plate. After 3 days of incubation, cells in one well were lysed in a radioimmunoprecipitation assay (RIPA) buffer (10) and cells in the other well were used for total RNA extraction with Trizol reagent (Invitrogen). The levels of HCV and cellular proteins were determined by Western blotting, and RNAs were determined by RNase protection assay (RPA).

Interferon sensitivity assay. The JFH1-BLAST replicon RNA-harboring MEFs in 6-well cell culture plates were treated with mouse IFNs at varying concentrations. Mouse IFN-α was purchased from Sigma, and mouse IFN-β was obtained from Pierce Biotechnology. At different time points (indicated) after treatment with mouse IFN-α/β, the levels of cellular and HCV proteins and positive-stranded HCV RNA were determined by Western blotting and RPA, respectively.

Western blot analysis. The levels of HCV NS3 and NS5B proteins as well as cellular proteins were determined by Western blot analysis. Proteins were separated by electrophoresis in a sodium dodecyl sulfate–10% polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was blocked with a Superblock dry blend buffer (Pierce) containing 5% skim milk overnight and then incubated with primary antibodies at room temperature for 1 h. Monoclonal antibodies specific to genotype 2a HCV NS3 and NS5B proteins were raised by using purified recombinant NS3 and NS5B proteins (71). Monoclonal antibody against mouse PKR was purchased from Santa Cruz Biotechnology. The RNase L-specific and mouse p56-specific polyclonal antibodies were prepared by immunizing rabbits with purified recombinant proteins (31, 73). Monoclonal antibody against β-actin was from Sigma. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Sigma) and goat anti-rabbit immunoglobulin G (Pierce) were used as secondary antibodies. Proteins were visualized by staining with chemiluminescent substrate (Roche). The protein levels were quantified by densitometry and analysis with the KODAK 1D software program (Kodak).

RNA quantification by RNase protection assay (RPA). The levels of positive- and negative-strand HCV RNAs were determined by RPA using radiolabeled negative-strand HCV 3′UTR and positive-strand 5′UTR RNA as probes, respectively (7, 43). The positive-strand HCV 5′UTR and negative-strand 3′UTR

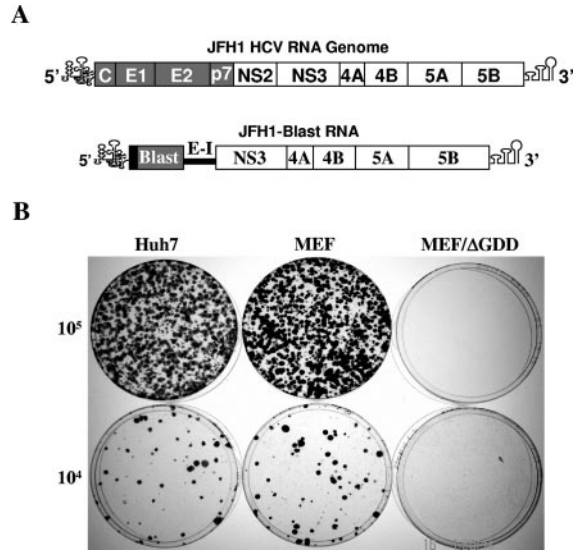

FIG. 1. A. Schematic of HCV RNA genome organization and the subgenomic JFH1-BLAST RNA. The JFH1-BLAST RNA consists of the 5′UTR, the core N-terminal 12-amino-acid coding sequence fused in frame with the blasticidin resistance gene (BLAST), the encephalomyocarditis virus internal ribosomal entry site (E-I), the NS3-NS5B coding region, and the 3′UTR. B. Cell colony formation induced by JFH1-BLAST RNA replication in Huh7 and MEF cells. Two micrograms of JFH1-BLAST or JFH1-BLAST/ΔGDD was transfected into 8×10^6 Huh7 cells or MEFs by electroporation (7). Cells were seeded onto 100-mm culture dishes at different densities (as indicated). At 24 h posttransfection, cell colony formation was determined by selection with the addition of 5 to 10 μg/ml blasticidin for about 2 to 3 weeks. The blasticidin-containing medium was changed every 3 to 4 days. Cell clones were visualized by staining with a crystal violet solution and photographed (7).

RNA probes were prepared by in vitro T7 RNA polymerase transcription in the presence of [α-^{32}P]UTP (MP Biochemicals) from the HindIII-linearized pUC19/T7-2a5UTR and pUC19/T7-2a(−)3′UTR DNAs, respectively (8). RPA was performed by using an RPA III kit (Ambion) following the manufacture's instructions. RNA products were analyzed in a 6% polyacrylamide–7.7 M urea gel, visualized by autoradiography, and quantified with PhosphorImager analysis.

RESULTS

Construction of subgenomic HCV RNAs carrying a blasticidin resistance gene. Previous studies of HCV RNA replication in cell culture were primarily performed by using HCV replicon RNAs that contain a neomycin phosphotransferase gene (Neo) as a selective marker (4, 40, 43). In this study, we constructed a subgenomic HCV RNA of genotype 2a (JFH1) that carries a blasticidin resistance gene (BLAST) rather than Neo as a selective marker for the study of HCV RNA replication in MEFs. BLAST was chosen here because blasticidin kills cells much faster than G418 sulfate, resulting in a quicker selection of cell colony formation, which is particularly important for the fast-growing mouse fibroblasts (data not shown). Additionally, blasticidin can be used for the selection of cell colony formation in a variety of gene knockout mouse cells, considering that most gene knockout mice already contain Neo as a selective marker. Therefore, the subgenomic JFH1 RNA was constructed by replacing the coding region of C, E1, E2, p7, and NS2 with a blasticidin resistance gene (BLAST) and inserting an internal ribosomal entry site derived from encephalomyocarditis virus, which controls the translation of the HCV NS3 to NS5B proteins (Fig. 1A). Consistent with findings reported by others (16), the cell colony formation efficiency induced by the BLAST-containing HCV replicon was comparable to that induced by Neo-containing replicons (data not shown). Thus, the replication of the subgenomic HCV RNA was unaffected by the replacement of Neo with BLAST as a selective marker.

Replication of the JFH1-BLAST RNA in MEFs. Previous studies found that the subgenomic JFH1 HCV RNA was able to replicate in human hepatic as well as nonhepatic HeLa and 293 cells (35), suggesting that the genotype 2a HCV RNA replication is not restricted by hepatic factors (nonhepatropic). However, the replication of JFH1 HCV RNA in mouse cells has not been reported. Thus, we performed cell colony formation experiments to determine whether the JFH1 RNA could replicate in MEFs. Results in Fig. 1B show that JFH1-BLAST RNA replication induced cell colony formation in both Huh7 and MEF cells (Fig. 1B). The cell colony formation efficiency induced by the JFH1-BLAST RNA in PKR$^{+/+}$ MEFs is compatible with that obtained in Huh7 cells (Fig. 1B; Table 1). As a control, a GDD deletion (ΔGDD) in the active site of the HCV RdRp (NS5B) ablated the ability of the JFH1-BLAST RNA to induce cell colony formation (Fig. 1B). To further confirm that cell colony formation in MEF was induced by HCV RNA replication, a number of cell colonies were picked up and amplified. The levels of HCV NS3 and NS5B proteins were determined by Western blot analysis, while the levels of both positive- and negative-stranded HCV RNAs were quantified by RPA. The NS3 and NS5B proteins as well as both positive- and negative-stranded HCV RNAs were detected by Western blot analysis and RPA, respectively, in all cell colonies examined (Fig. 2). Similar to findings with Con1 RNA replication in Huh7 cells, the JHF1 replicon RNA resulted in varying levels of HCV proteins and RNAs among different MEF cell clones (Fig. 2). Taken together, these results demonstrate that cell colony formation in MEFs was induced by JFH1-BLAST RNA replication.

Effect of PKR expression on HCV RNA replication. To determine the role of PKR in the control of HCV RNA replication, we compared the efficiency of cell colony formation and the levels of HCV proteins and RNAs in PKR$^{+/+}$ and PKR$^{-/-}$ MEFs. The replication of JFH1-BLAST RNA induced efficient cell colony formation in PKR$^{-/-}$ MEFs. It is estimated that the efficiency of cell colony formation induced by JFH1 RNA replication in PKR$^{-/-}$ MEFs was about fivefold higher than that in PKR$^{+/+}$ MEFs (Table 1). Unlike PKR$^{+/+}$ MEFs, however, PKR$^{-/-}$ MEFs have a propensity to detach from culture plates once grown to confluence. Also, PKR$^{-/-}$ MEFs

TABLE 1. Efficiency of cell colony formation induced by JFH1-BLAST RNA

Cell type	No. of cell colonies/μg RNA
Huh7	$(6.4 \pm 0.4) \times 10^4$
PKR$^{+/+}$ MEF	$(5.8 \pm 1.4) \times 10^4$
PKR$^{-/-}$ MEF	$(3.3 \pm 0.4) \times 10^5$

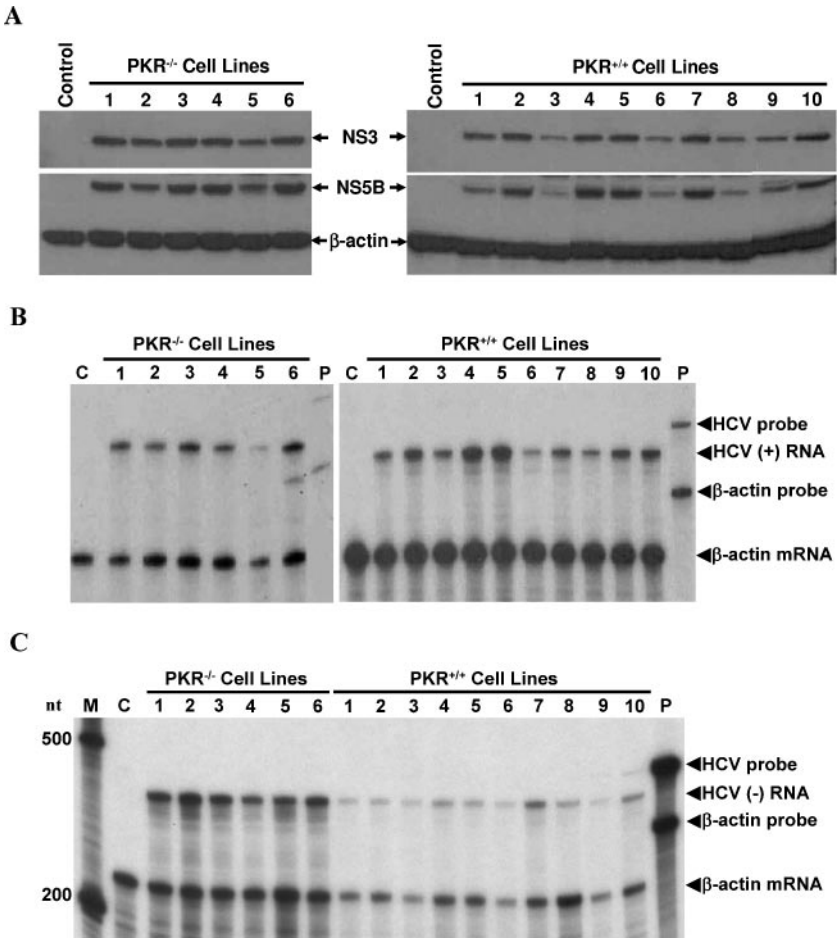

FIG. 2. A. Determination of HCV NS3 and NS5B proteins by Western blot analysis. Upon selection with blasticidin, the $PKR^{+/+}$ and $PKR^{-/-}$ MEF cell lines (numbered on the top) resistant to blasticidin were picked up and expanded. Cells were lysed in a RIPA buffer as described previously (8). Twenty-five micrograms of each cell lysate was electrophoresed in a sodium dodecyl sulfate–10% polyacrylamide gel and then transferred onto a nitrocellulose membrane. The HCV NS3 and NS5B proteins were detected by Western blotting using monoclonal antibodies specific to NS3 and NS5B, respectively (8). β-Actin was used as an internal control and was detected by using a monoclonal antibody against β-actin (Sigma). Proteins were visualized by chemiluminescent staining (Roche). Parental cells without HCV RNA were used as controls, as indicated on the top. B. Determination of positive-stranded HCV RNA by RPA. Total cellular RNAs were extracted with Trizol reagent (Invitrogen) from $PKR^{-/-}$ and $PKR^{+/+}$ cell lines that contain the JFH1-BLAST RNA. A total of 10 μg RNA was used to hybridize with 3×10^4 cpm of [^{32}P]UTP-labeled mouse β-actin RNA probe (Ambion) and 10^5 cpm of the negative-stranded HCV 3′UTR RNA-containing probe. The RPA was performed by following the manufacturer's procedures for RPA III kits (Ambion). Upon digestion with RNase A/T1, the RNA products protected from RNase digestion were analyzed in 6% polyacrylamide–7.7 M urea gels. C. Quantification of negative-stranded (−) HCV RNA by RPA. The radiolabeled positive-stranded HCV 5′UTR RNA was used as a probe. Cell lines are the same as those numbered on the tops of panels A and B. The levels of HCV RNAs were quantified with a PhosphorImager using the levels of mouse β-actin mRNA as controls to normalize amounts of total RNAs used in the assay. Both (−)3′UTR and (+)5′UTR RNA probes contains about 40 nucleotides (nt) of unpaired region so that the protected HCV RNA products migrated faster than undigested HCV RNA probes. Sizes of RNA markers are indicated on the left by numbers (nucleotides), and arrows on the right highlight the probes and protected RNA products. HCV (−) RNA, negative-stranded HCV RNA products; and HCV (+) RNA, positive-stranded HCV RNA products. Total RNAs from parental $PKR^{-/-}$ and $PKR^{+/+}$ MEFs without HCV RNA replication were used as negative controls, respectively (indicated by the letter C). P, probe only.

appeared to grow faster than wild-type ($PKR^{+/+}$) MEFs, an observation consistent with previous findings that PKR is a negative regulator of cell growth (26, 70). Therefore, it is not clear whether higher cell colony-forming efficiency observed in $PKR^{-/-}$ MEFs was due to differences in growth phenotypes of $PKR^{-/-}$ MEFs. Alternatively, we determined and compared the levels of HCV proteins and positive- and negative-stranded RNAs between $PKR^{+/+}$ and $PKR^{-/-}$ MEFs (Fig. 2). Although the levels of HCV proteins and RNAs in $PKR^{+/+}$ MEFs vary between different cell lines, the overall levels of HCV NS3 and NS5B proteins as well as both positive- and negative-strand HCV RNAs in the $PKR^{-/-}$ cell clones were higher than those in the $PKR^{+/+}$ MEFs upon quantification (Fig. 2). It is noteworthy that the positive-stranded HCV RNA in $PKR^{+/+}$ MEFs was exposed longer than that in $PKR^{-/-}$ MEFs, as can be seen by the intensity of the β-actin mRNA and the ratios between HCV and β-actin RNAs (Fig. 2). In order for a comparison of HCV RNA levels between $PKR^{-/-}$ and $PKR^{+/+}$ MEFs that contain varying levels of HCV RNAs, the average levels of HCV RNAs were calculated upon nor-

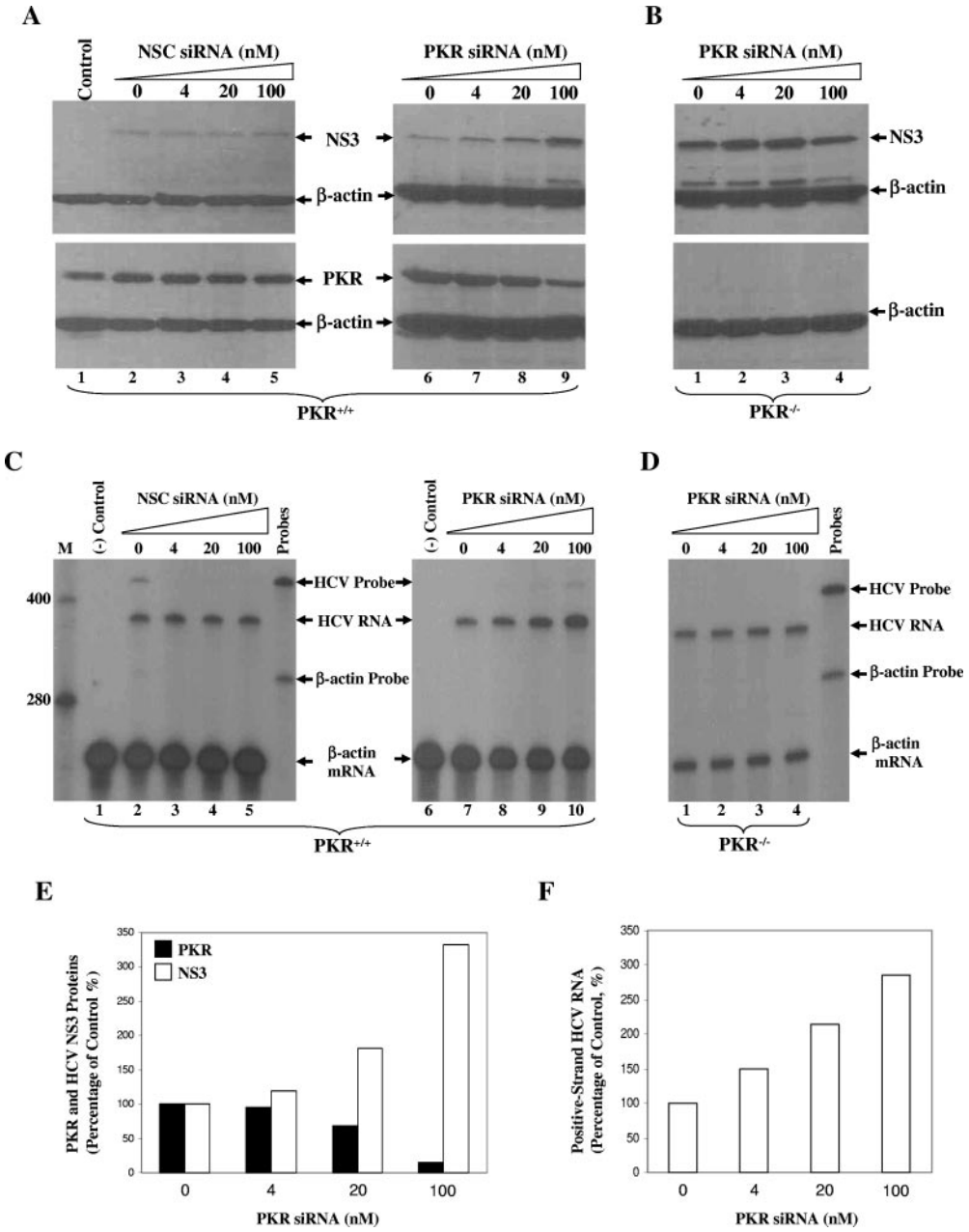

FIG. 3. Effects of PKR knockdown expression by siRNAs on HCV RNA replication. JFH1-BLAST RNA-harboring $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were transfected with increasing concentrations of PKR SMARTpool siRNAs or a nonspecific control siRNA, as indicated on the top. A concentration of 100 nM of siRNAs was mixed with DharmaFECT lipid reagent and then diluted with medium to 20 and 4 nM. The mixtures of siRNAs and lipid were transferred onto cells in a 6-well culture plate, with incubation for 3 days. The levels of PKR and HCV NS3 proteins were determined by Western blot analysis (A and B), and positive-stranded HCV RNAs were determined by RPA, as described in the legend to Fig. 2 and in Materials and Methods (C and D). The JFH1-BLAST RNA-harboring $PKR^{-/-}$ MEFs were used as controls for nonspecific effects of PKR siRNAs on HCV RNA replication (B and D). Parental cells without HCV RNA replication were used as negative controls as indicated on the top. Numbers on the top indicate the concentrations (in nanomolars) of siRNAs. $PKR^{+/+}$ and $PKR^{-/-}$ MEFs are shown at the bottom. Arrows highlight corresponding proteins, RNA probes, and RNA products. E. Relative levels of PKR and HCV NS3 proteins in $PKR^{+/+}$ MEFs upon PKR siRNA treatment. The levels of PKR and HCV NS3 proteins in panel A were determined by densitometry. The levels of PKR and NS3 proteins in cells without PKR siRNA treatment were used as controls and were considered 100%. The relative levels of PKR and NS3 proteins in PKR siRNA-treated $PKR^{+/+}$ MEFs (A) were calculated as percentages of control (*y* axis) and plotted against the concentrations (in nanomolars) of PKR-specific siRNA (*x* axis). F. Correlation of the relative levels of positive-stranded HCV RNA and PKR siRNA concentrations. The percentage of control of positive-stranded HCV RNA was calculated in the same way as that described for panel E. The relative levels of HCV RNA are plotted against PKR siRNA concentration (in nanomolars). NSC, nonspecific control.

malization with the level of β-actin mRNA. The levels of HCV RNAs in PKR$^{-/-}$ MEFs are, on average, about 2.5-fold higher than that in PKR$^{+/+}$ MEFs (Fig. 2 and data not shown). These results suggest that PKR is inhibitory to HCV RNA replication. This finding was further verified by transfection of the JFH1-BLAST RNA into a PKR-null MEF cell line, which constitutively overexpressed a human PKR (45). It turned out that the JFH1-BLAST RNA did not induce cell colony formation, suggesting that it failed to persistently replicate in MEFs that overexpressed a human PKR (data not shown). To provide more direct evidence to demonstrate the role of PKR in the control of HCV RNA replication, efforts were made to determine the effects of PKR knockdown expression by specific siRNAs on HCV RNA replication. Synthetic mouse PKR siRNAs were used to specifically knock down PKR expression in the JFH1-BLAST RNA-harboring PKR$^{+/+}$ MEFs. Results are shown in Fig. 3. The SMARTpool PKR siRNAs efficiently reduced PKR expression in a dose-dependent manner (Fig. 3A). The level of PKR was decreased by 5%, 32%, and 84% upon treatment with 4, 20, and 100 nM concentrations of PKR siRNA, respectively (Fig. 3A and E, black bar). On the contrary, the level of PKR expression was unaffected by a nonspecific control siRNA (Fig. 3A). The effects of PKR knockdown expression on HCV RNA replication were subsequently determined by quantifying the levels of HCV NS3 protein and positive-stranded RNA (Fig. 3). The levels of NS3 protein (Fig. 3E) as well as positive-stranded RNA (Fig. 3F) were inversely proportional to the decrease of the PKR level. The level of NS3 protein was increased by a factor of 1.2-, 1.8-, and 3.3-fold when treated with 4, 20, and 100 nM of PKR siRNA, respectively (Fig. 3E). Likewise, the level of positive-stranded HCV RNA was enhanced by a factor 1.5-, 2.1-, and 2.8-fold at 4, 20, and 100 nM of PKR siRNA, respectively (Fig. 3F). As controls, the levels of NS3 protein and positive-stranded HCV RNA in the JFH1-BLAST RNA-harboring PKR$^{-/-}$ MEFs were not significantly changed by PKR siRNAs (Fig. 3B and D). The PKR siRNA knockdown experiments were performed in three PKR$^{+/+}$ cell clones and two PKR$^{-/-}$ cell clones in which the JFH1-BLAST RNA was persistently replicating. Findings similar to those shown in Fig. 3 were obtained for all cell clones tested, suggesting that the effects of PKR expression on HCV RNA replication are not cell clone specific (data not shown). Taken together, these findings demonstrate that PKR is a cellular inhibitor of HCV RNA replication.

Activation of intracellular antiviral pathways by HCV RNA replication in MEFs. Findings derived from previous studies demonstrate that the replication of genotype 1b HCV RNA triggered an activation of the intracellular antiviral pathways, as shown by increased levels of PKR, p56, and ISG15 expression in Huh7 cells (58, 59). To determine whether the replication of the subgenomic JFH1 HCV RNA induced intracellular antiviral response, we measured the levels of PKR and p56 expression in PKR$^{-/-}$ and PKR$^{+/+}$ MEFs. As expected, PKR was detected in all PKR$^{+/+}$ cell lines but not in PKR$^{-/-}$ cells (Fig. 4). In PKR$^{+/+}$ MEFs, the levels of both PKR and p56 expression were markedly increased by HCV RNA replication, consistent with the findings derived from Con1 RNA replication in a human hepatoma cell line, Huh7 (58). In the JFH1 RNA-replicating PKR$^{-/-}$ MEFs, p56 expression was considerably up-regulated (Fig. 4). It will be interesting to determine

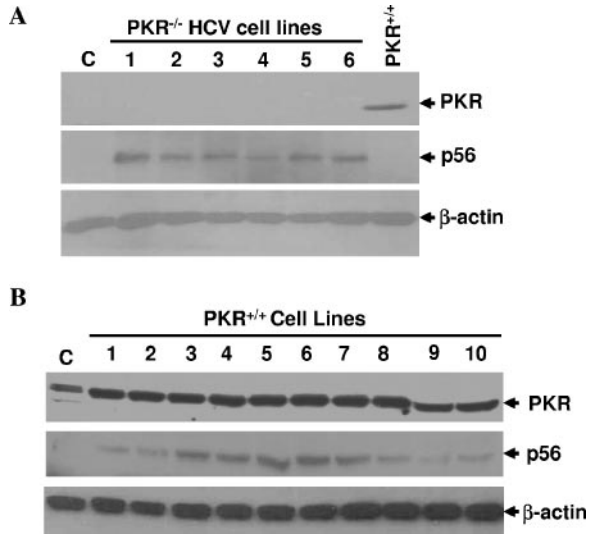

FIG. 4. Detection of cellular proteins PKR and p56 by Western blot analysis. Western blot analysis is described in Materials and Methods. The JFH1-BLAST RNA-harboring PKR$^{-/-}$ (A) and PKR$^{+/+}$ (B) MEF cell lines are the same as those shown in Fig. 2 (numbered on the top). Arrows on the right highlight the cellular proteins PKR and p56. β-Actin was used as an internal control to normalize amounts of proteins used between samples. C, negative control.

what other intracellular antiviral factors are affected by HCV RNA replication. Nevertheless, these results demonstrate that HCV RNA replication in MEFs can activate both PKR-dependent and PKR-independent antiviral pathways.

Inhibition of HCV RNA replication by IFN in PKR$^{+/+}$ and PKR$^{-/-}$ MEFs. PKR is a major mediator of IFN-induced antiviral response and is also implicated in the IFN-induced suppression of HCV RNA replication (21, 67). To compare the IFN sensitivity of genotype 2a HCV RNA replication in PKR$^{+/+}$ MEFs to that in PKR$^{-/-}$ MEFs, the JFH1-BLAST RNA-harboring PKR$^{+/+}$ and PKR$^{-/-}$ MEFs were treated with increasing concentrations (in units/milliliter) of mouse IFN-α and IFN-β, respectively. At 48 h after IFN treatment, the levels of positive-stranded HCV RNA were determined by RPA. Similar to human IFNs for inhibition of Con1 RNA replication in Huh7 cells (27, 52), both mouse IFN-α and IFN-β potently inhibited the replication of the JFH1-BLAST RNA in either PKR$^{+/+}$ or PKR$^{-/-}$ MEFs. The inhibition of HCV RNA replication by both IFN-α and IFN-β was dose dependent (Fig. 5). Interestingly, both IFN-α and IFN-β inhibited HCV RNA replication in PKR$^{-/-}$ MEFs as efficiently as in PKR$^{+/+}$ MEFs (Fig. 5C and D). Similar results were also observed in two other PKR$^{+/+}$ and PKR$^{-/-}$ MEF cell lines in which JFH1-BLAST RNA replicated (data not shown). These findings demonstrate that the IFN-induced antiviral effect in PKR$^{-/-}$ MEFs was mediated by PKR-independent antiviral mechanisms.

To compare the activation of intracellular antiviral pathways by IFN in PKR$^{-/-}$ MEFs with those in PKR$^{+/+}$ MEFs, we treated the JFH1-BLAST RNA-harboring PKR$^{+/+}$ and PKR$^{-/-}$ MEFs with 100 U/ml of mouse IFN-α. The levels of PKR and p56 were determined by Western blot analysis at different time points after IFN treatment (Fig. 6A). PKR ex-

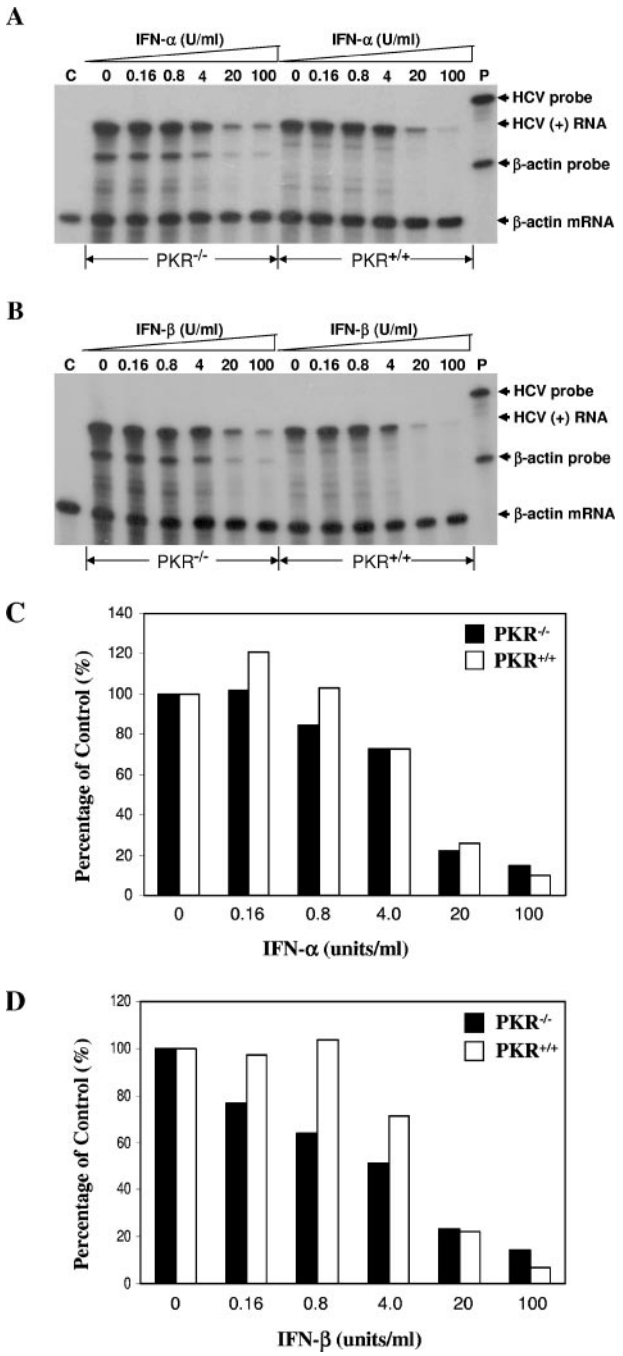

FIG. 5. Inhibition of HCV RNA replication by IFN-α/β in $PKR^{-/-}$ and $PKR^{+/+}$ MEFs. The JFH1-BLAST RNA-containing $PKR^{-/-}$ and $PKR^{+/+}$ MEFs were treated with increasing concentrations of either mouse IFN-α (A) or mouse IFN-β (B). At 48 h after the addition of IFN, total cellular RNAs were extracted with Trizol reagent (Invitrogen). The levels of positive-stranded HCV RNA were determined by RPA, as described in the legend to Fig. 2. IFN concentrations (in units/milliliter) are shown by numbers on the top. The HCV RNA-harboring $PKR^{-/-}$ and $PKR^{+/+}$ MEFs are indicated at the bottom. Total RNAs extracted from parental $PKR^{-/-}$ and $PKR^{+/+}$ MEFs were used as negative controls, as indicated by C on the top. The RNA probes and protected positive-stranded HCV RNA products are highlighted by arrows on the right. C. Comparison of the relative levels of positive-stranded HCV RNA in response to mouse IFN-α treatment between $PKR^{-/-}$ and $PKR^{+/+}$ MEFs. The data shown in panel A were quantified by PhosphorImager analysis. The relative level of positive-stranded HCV RNA was calculated as a percentage of control, considering the level of HCV RNA without IFN treatment (control) as 100%. The relative levels of HCV RNA (in percentages) are plotted against IFN-α concentrations (in units/milliliter). D. Comparison of mouse IFN-β inhibitory activity against HCV RNA replication between $PKR^{-/-}$ and $PKR^{+/+}$ MEFs. The data in panel B were quantified with a PhosphorImager and converted to percentages of control in the same way as that described for panel C. The relative levels (in percentages) of HCV RNA (*y* axis) are plotted against IFN-β concentrations (in units/milliliter; *x* axis).

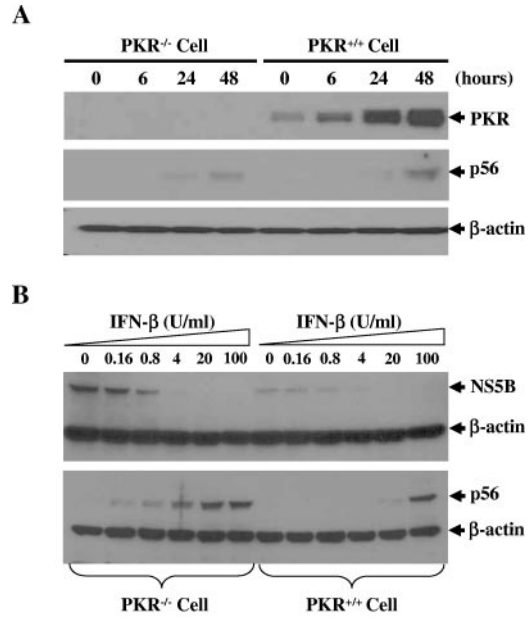

FIG. 6. Induction of cellular gene products by IFN-α/β treatment in $PKR^{-/-}$ and $PKR^{+/+}$ MEFs. (A) Induction of PKR and p56 by mouse IFN-α. The JFH1-BLAST RNA-harboring $PKR^{-/-}$ and $PKR^{+/+}$ MEF cell lines were treated with 100 U/ml of mouse IFN-α. At different time points (shown on the top), IFN-α-treated cells were lysed in a RIPA buffer. The levels of cellular proteins PKR, p56, and β-actin were determined by Western blot analysis, as described in Materials and Methods. (B) Correlation of p56 expression and suppression of HCV NS5B protein expression. The JFH1-BLAST RNA-harboring $PKR^{-/-}$ and $PKR^{+/+}$ MEFs were treated with increasing concentrations of mouse IFN-β for 48 h. The levels of NS5B and p56 were determined by Western blot analysis.

pression was remarkably induced by IFN in the JFH1-BLAST RNA-replicating $PKR^{+/+}$ MEFs. Likewise, p56 expression was induced by IFN treatment in both $PKR^{+/+}$ and $PKR^{-/-}$ MEFs, suggesting that PKR-independent antiviral pathways were induced by IFN treatment. The level of p56 expression was also induced by mouse IFN-β in a concentration-dependent manner (Fig. 6B). The reduction of HCV NS5B protein is inversely correlated with the increase of p56 expression (Fig. 6B). However, the exact role of p56 in the suppression of HCV replication has not been determined. Collectively, our findings suggest that the inhibition of HCV RNA replication by IFN was mediated by PKR-independent antiviral pathways, at least in the $PKR^{-/-}$ MEFs. P56 is a major negative regulator of eIF3 and thereby inhibits the translation initiation of HCV proteins leading to suppression of HCV replication (65). Con-

sidering that the level of p56 was significantly enhanced by HCV RNA replication in both $PKR^{+/+}$ and $PKR^{-/-}$ MEFs (Fig. 4), there must be other ISGs evolved for the inhibition of HCV RNA replication which remain to be determined.

DISCUSSION

The study of HCV replication and virus-host interaction during HCV infection has been hampered by our inability to develop efficient cell culture systems and small-animal models of HCV replication (42). Recently, several groups, including us, have successfully developed reliable and robust cell culture systems of infectious HCV production and propagation in human hepatoma cells using the cDNA-derived genotype 2a HCV RNA (8, 39, 63, 72). These advances allow the study of molecular aspects of HCV infection and discovery of antiviral drugs that inhibit each step of the entire HCV infectious cycle. In this report, we provide substantial evidence to demonstrate for the first time that HCV RNA can replicate in MEFs. The JFH1 RNA was able to induce efficient cell colony formation in MEFs. Also, high levels of HCV proteins and both positive- and negative-stranded HCV RNAs were detected in the persistently JFH1 RNA-replicating MEFs. The significance of these findings is severalfold. First, our findings suggest that the hepatropic replication of HCV in humans is not due to the permissiveness or restriction at the step of RNA replication. This was initially suggested by the detection of HCV RNA in lymphocytes derived from the HCV-infected patients (11, 60). Further evidence for the nonhepatic replication of HCV RNA came from the observations that subgenomic RNAs of genotype 1b and 2a HCV could replicate in HeLa and 293 cells, albeit at extremely low efficiency (2, 35, 75). Now, findings obtained from our studies demonstrate that HCV RNA is able to replicate efficiently in MEFs, suggesting that HCV RNA replication is species and cell type independent. The hepatropism of HCV replication in humans is most likely determined by HCV receptors/coreceptors expressed on the cell surface or defects in HCV assembly and/or budding. Additionally, our findings demonstrate a proof of concept that HCV RNA replication in MEFs is an alternative and powerful system to study HCV and host interactions and to determine the underlying mechanisms of chronic HCV infection and IFN-based therapy. Like Huh7, MEF is not only highly permissive to the JFH1 HCV RNA replication but also is very responsive to IFN-induced antiviral pathways leading to the inhibition of HCV RNA replication (Fig. 5). Thus, the HCV RNA replication in MEFs opens up new avenues to define the roles of cellular genes in the control of HCV replication and the mediation of IFN-based therapy inhibiting HCV replication. The advantage of studying cellular genes controlling HCV RNA replication in MEFs lies in the facts that numerous mouse genes have already been well defined for their roles in the host innate immunity and IFN signaling and that a variety of MEFs derived from diverse gene knockout mice are readily available. More importantly, our studies also set the stage for the development of transgenic mouse models of HCV replication. We have recently demonstrated that the JFH1 HCV RNA derived from the chromosomally integrated cDNA was expressed and replicated efficiently in Huh7 cells (8). The cDNA-derived JFH1 RNA by cellular PolII polymerase transcription was also expressed and replicated in mouse hepatocytes and MEFs (unpublished results). Our success in the construction of an infectious HCV cDNA has led us to generate transgenic mice that contain the subgenomic or full-length JFH1 cDNA (G. Luo, unpublished data). The determination of cDNA-derived HCV RNA replication in transgenic mice is now in progress.

Although HCV RNA can replicate in a broad range of mammalian cells, the efficiency of cell colony formation induced by HCV RNA replication varied considerably between different HCV genotypes and cell types. In Huh7 cells, both genotype 1 and genotype 2 HCV RNAs were shown to replicate efficiently (4, 5, 32, 34, 40). In HeLa and 293 cells, however, the cell colony-forming efficiency induced by HCV RNA replication differed remarkably between genotype 1 and genotype 2 HCV. The subgenomic 1b RNA with mutations adapted in Huh7 cells was unable to induce cell colony formation in either HeLa or 293 cells unless the total cellular RNA extracted from the 1b replicon RNA-harboring Huh7 cells was transfected into HeLa or 293 cells (75). Even so, the Huh7 cell-derived 1b replicon RNA only resulted in low efficiency of cell colony formation in HeLa and extremely low efficiency (a few colonies out of millions of cells) in 293 cells (75). The low level of HCV RNA replication in 293 cells was independently confirmed by an observation that the subgenomic 1b RNA only replicated in 293 cells that were somatically fused with the replicon RNA-harboring Huh7 cells (2). By contrast, the subgenomic JFH1 RNA (genotype 2a) was able to induce cell colony formation in both HeLa and 293 cells, albeit at 10- and 400-fold lower efficiency, respectively, compared to that in Huh7 cells (35). Also, the present study demonstrates that the JFH1 RNA induced efficient cell colony formation in the mouse nonhepatic MEFs. Contrary to the cell colony-forming efficiency in human hepatic and nonhepatic cells, however, JFH1 RNA induced cell colony formation in MEFs as efficiently as in Huh7 cells (Fig. 1; Table 1). The molecular basis underlying the differential replication of genotype 1 and 2 HCV RNAs among different cell types remains to be determined. It is most likely that the efficiency of HCV RNA replication is modulated by cellular factors. This interpretation is consistent with the findings that adapted mutations were not identified in either genotype 1b or 2a replicon RNAs isolated from HeLa, 293, or Hepa1-6 cells (2, 35, 75), suggesting that HCV RNA replication observed in small numbers of HeLa, 293, or Hepa1-6 cells was determined by adaptations of cellular genes but not by viral evolution (adaptation). Several studies have identified cellular proteins modulating HCV RNA replication, including the polypyrimidine tract-binding protein (10), hVAP-A (17), FBL-2 (64), and cyclophilin B (66). Additionally, a recent study demonstrated that a micro-RNA (miR-122) expressed abundantly in the human liver was also required for HCV RNA replication (33). miR-122 was detected in Huh7 and Hepa1-6 cells but not in HeLa cells (33). Whether miR-122 was expressed in the HCV RNA-replicating HeLa and 293 cells is not yet known. Moreover, efficient HCV RNA replication could be due to the inactivation or deficiency of cellular genes that otherwise possess antiviral activities. This mechanism was elegantly demonstrated by the finding that HCV RNA replication was markedly increased in Huh7.5 cells in which the IFN-inducible gene, RIG-I, was inactivated by a mutation (58). It appears that HCV RNA replication is regu-

lated by multiple cellular pathways; which of them confers the permissiveness of hepatic and nonhepatic cells to HCV RNA replication remains to be determined.

Several lines of circumstantial evidence derived from our studies suggest that PKR is evolved for the control of HCV RNA replication. First, the replication of JFH1 RNA replicons resulted in higher efficiency of cell colony formation in $PKR^{-/-}$ MEFs than that in $PKR^{+/+}$ cells. Additionally, the level of JFH1 RNA replication in $PKR^{-/-}$ MEFs was two- to threefold higher than that in $PKR^{+/+}$ MEFs. Furthermore, the JFH1 RNA failed to induce cell colony formation in MEFs that constitutively expressed a human PKR. More significantly, the levels of HCV RNA and proteins were enhanced in the HCV RNA-harboring $PKR^{+/+}$ MEFs when PKR expression was down-regulated by PKR-specific siRNAs, demonstrating a direct role of PKR in the suppression of HCV RNA replication. These findings are consistent with those reported by others (52, 57, 58, 65). Wang et al. observed that the higher efficiency of HCV RNA replication correlated with the lower levels of PKR activity and eIF2α phosphorylation (65). Moreover, the HCV NS5A protein was found to enhance HCV RNA replication by binding and inhibiting PKR activity (47, 65). It is likely that PKR also plays an important role in the mediation of the IFN-induced suppression of HCV replication, since PKR expression was remarkably increased by mouse IFN-α/β treatment in the JFH1 RNA-replicating $PKR^{+/+}$ MEFs (Fig. 6A). It appears that IFN caused a slightly greater reduction of HCV RNA at higher concentrations (100 U/ml) in $PKR^{+/+}$ MEFs than in $MKR^{-/-}$ MEFs (Fig. 5C and D). It is known that PKR activation by dsRNA would lead to the phosphorylation of serine 51 of eIF2α. Therefore, the translation initiation of HCV proteins was inhibited. This mode of action was demonstrated by findings derived from studies by Wang and coworkers that the translation but not the proteolytical processing or stability of HCV proteins was inhibited by IFN-induced cellular antiviral pathways (65).

In addition to PKR, PKR-independent antiviral pathways are believed to play important roles in cellular defense against HCV replication (1, 28, 29, 45, 65). In the present study, we demonstrated that p56 was activated by HCV RNA replication in $PKR^{-/-}$ MEFs. Also, p56 was induced by IFN in a dose-dependent manner in both $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. It remains to be determined whether other ISGs were also activated by IFN treatment in $PKR^{-/-}$ MEFs. Several previous studies have demonstrated that ISG6-16 and ISG15 were stimulated by HCV RNA replication and IFN treatment in HCV RNA-harboring Huh7 cells (58, 59, 65, 74). Further investigations are warranted to determine PKR-independent cellular antiviral pathways against HCV RNA replication using $PKR^{-/-}$ MEF culture systems in which HCV RNA is replicated.

Numerous studies have demonstrated that HCV devises multiple mechanisms to evade cellular antiviral pathways (21). The HCV proteins E2, NS3/4A, and NS5A were all implicated in the blockage of IFN signaling and antiviral response (21). Earlier studies have revealed that NS5A binds PKR via the PKR-binding domain and thereby blocks the activated PKR-mediated phosphorylation of eIF2α (20, 22–24). Likewise, the unglycosylated form of E2 was able to inhibit the translation initiation of HCV proteins by interaction with PKR and PERK (48, 49, 61). These findings suggest that PKR plays a critical role in the establishment of chronic HCV infection and in the mediation of IFN-induced antiviral response. More recent studies by several independent groups demonstrated that the NS3/4A serine protease plays a key role in the evasion of cellular signaling pathways leading to IFN production (37, 38, 46, 58). The NS3/4A serine protease was found to cleave TRIF and MAVS/IPS-1/VISA/Cardif, which are important mediators of the IFN signaling triggered by viral infection (36–38, 41, 46, 69). However, their roles in HCV resistance to IFN-based therapy have not been illustrated. The MEF culture system of HCV RNA replication will be valuable for defining the roles and mechanisms of cellular genes mediating the viral evasion of host defense systems.

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