Inhibition of the Interferon-Inducible Protein Kinase PKR by HCV E2 Protein

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Most isolates of hepatitis C virus (HCV) infections are resistant to interferon, the only available therapy, but the mechanism underlying this resistance has not been defined. Here it is shown that the HCV envelope protein E2 contains a sequence identical with phosphorylation sites of the interferon-inducible protein kinase PKR and the translation initiation factor eIF2α, a target of PKR. E2 inhibited the kinase activity of PKR and blocked its inhibitory effect on protein synthesis and cell growth. This interaction of E2 and PKR may be one mechanism by which HCV circumvents the antiviral effect of interferon.

Hepatitis C virus (HCV) is an emerging virus of great medical importance and almost always causes chronic infections. The only available therapy is interferon (IFN), but most HCV isolates are resistant (*1*). IFN normally acts by inducing transcription of several antiviral genes, including the double-stranded RNA-activated protein kinase (PKR), which inhibits protein synthesis by phosphorylation of the translation initiation factor eIF2α. Many viruses have evolved strategies to overcome the antiviral effects of PKR (*2*). One of the HCV nonstructural proteins, NS5A, has been shown to bind to and inhibit PKR (*3*); however, the NS5A-PKR interaction can explain only those cases of IFN resistance that exhibit NS5A quasispecies divergence after IFN therapy (*4*). Such divergence has not been observed in most patients.

Our investigation of the IFN resistance of HCV focused on E2, an outer protein of the viral envelope that may participate in virus binding to target cells. E2 shows considerable sequence variation among different HCV isolates, and it induces neutralizing antibodies. E2 from most HCV isolates contains a 12–amino acid sequence that is similar to the PKR autophosphorylation site (*5*) and the eIF2α phosphorylation site, a target of PKR. The sequences of the PKR-eIF2α phosphorylation homology domain (PePHD) (Fig. 1A) of the more IFN-resistant HCV genotypes (1a and 1b) more closely resemble the sequences of PKR and eIF2α than do the corresponding sequences of the less resistant HCV genotypes (2a, 2b, and 3a) (Fig. 1B). This sequence is highly conserved within each genotype.

To determine if E2 physically interacts with PKR, we performed an in vitro binding assay with histidine (His)–tagged PKR and [^{35}S]methionine-labeled E2, synthesized from a construct derived from the HCV genotype 1a strain (*6*) (Fig. 2A). E2 bound to both wild-type PKR (lane 2) and a mutant PKR (K296R) that has a mutation in the adenosine triphosphate (ATP)–binding domain and is catalytically inactive (*7*) (lane 3), suggesting that autophosphorylation is not required for the E2-PKR interaction. Under the same conditions, only a trace amount of an E2 deletion mutant E2ΔC, which lacks the PePHD, bound to PKR (six times less as compared with the wild-type E2), suggesting that the PePHD region is the major, if not the only, PKR-binding sequence in E2. The cellular protein hnRNPK, which has a strong protein-protein interacting domain (*8*), did not bind to any of the proteins tested, and none of the proteins bound to the GFP-His control (lane 4).

To assess the effect of E2 on PKR activity in vitro, we performed kinase assays with immunopurified PKR from transfected human embryonic kidney 293 (HEK-293) cells. The immunoprecipitates were incubated with purified glutathione S-transferase (GST) or GST-E2 fusion proteins, using histone H2a as a substrate (*9*) (Fig. 2C). GST-E2, but not GST, inhibited histone phosphorylation by 90 to 98% (lanes 7 to 9), but PKR autophosphorylation was only slightly inhibited. The lack of robust inhibition of PKR autophosphorylation by GST-E2 was probably due to the highly activated state of the transfected human PKR even in the absence of poly(I·C) (Fig. 2C, compare lane 1 to lane 2). We also used recombinant His-tagged PKR purified from *Escherichia coli*, which exhibits only a low level of activation in the absence of double-stranded RNA (Fig. 2D, compare lane 1 to lane 2), in the kinase assay (*10*). GST-E2, but not GST, almost completely inhibited PKR autophosphorylation induced by the double-stranded RNA activator (lanes 5 and 6). The presence of GST-E2 (66 kD, similar to PKR) did not result in the appearance of any additional phosphorylated proteins, indicating that GST-E2 was not phosphorylated. These results suggest that E2 may serve as a pseudosubstrate of PKR and inhibit the function of PKR in vitro.

We next investigated whether E2 inhibits cellular PKR activity in vivo, which would lead to enhanced protein synthesis. We cotransfected a reporter plasmid encoding the chloramphenicol acetyl transferase (CAT) gene under control of a cytomegalovirus (CMV) promoter and a plasmid encoding E2L (E2 with the leader peptide) or an empty vector (pcDNA3) into HeLa cells and measured CAT enzyme activity to determine the amount of protein synthesis (*11*). CAT activity was a factor of 5 higher in the presence of E2L (Fig. 3A). The enhancement of CAT activity correlated with the amount of transfected E2L plasmid (Fig. 3B).

To establish that this enhanced CAT activity was due to increased translation rather than transcription, we measured CAT RNA levels in the transfected cells. The cells transfected with various amounts of E2L plasmid contained equal amounts of CAT RNA (about 20 pg of RNA per sample, as determined by comparing with lanes 2 through 6 in Fig. 3C), indicating

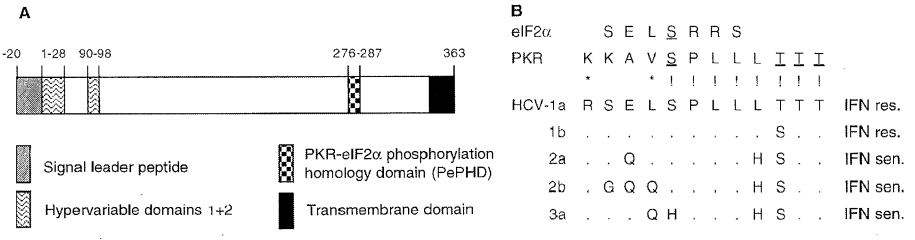

Fig. 1. HCV E2 shares sequence identity with eIF2α and PKR. (**A**) Structural diagram of HCV E2. (**B**) The sequences surrounding the PKR phosphorylation site in eIF2α (amino acids 48 to 54) and surrounding the autophosphorylation sites (amino acids 79 to 90) in the RNA-binding domain of PKR are shown (*24*). E2 sequences from the various HCV genotypes are aligned. Symbols: *, similar amino acid to PKR; !, identical to PKR; underlined, autophosphorylation sites in PKR and eIF2α phosphorylation site by PKR; and •, sequence identity with HCV-1a. IFN res. and IFN sen. designate IFN resistant and sensitive, respectively.

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that the E2L plasmid did not enhance transcription. Furthermore, CAT activity was enhanced only when E2 contained the leader sequence (E2L, Fig. 3D), suggesting that E2 needs to be associated with the endoplasmic reticulum (ER) to inhibit PKR. Truncation of the COOH-terminus of E2 (E2L-ΔC), which removes the PePHD, abolished the enhancement of CAT activity. Furthermore, mutation of PePHD to a sequence derived from HCV genotypes 2 and 3 (E2L-clu1) abolished enhancement of CAT activity. Finally, HCV NS5A, which also inhibits PKR activity (*3*), enhanced CAT activity by a factor of 2.5. These results suggest that E2 can enhance the translation of cellular mRNAs, most likely through its PePHD.

We next determined whether the translational enhancement by E2L was mediated through reduction of PKR activity. We transfected a pcDNA3 plasmid encoding E2L into HEK-293 cells. Expression of PKR was induced by the addition of IFN-α, and the kinase was activated by infection with adenovirus mutant dl331, which does not express the PKR inhibitor VA RNA_I (*12*). The results showed that the amount of phosphate incorporated into PKR was reduced by almost 50% in E2L-transfected cells (Fig. 3F, compare lanes 2 and 4), whereas the amount of PKR protein (as determined by [^{35}S]methionine labeling) was largely unaffected. Because slightly less than 50% of cells were transfected with this plasmid, and 100% were infected with adenovirus dl331,

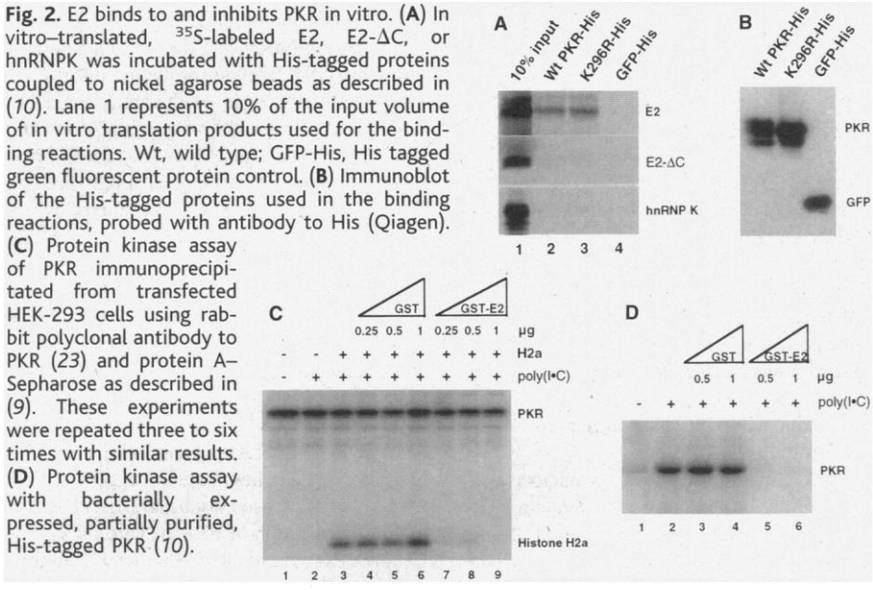

Fig. 2. E2 binds to and inhibits PKR in vitro. (**A**) In vitro–translated, ^{35}S-labeled E2, E2-ΔC, or hnRNPK was incubated with His-tagged proteins coupled to nickel agarose beads as described in (*10*). Lane 1 represents 10% of the input volume of in vitro translation products used for the binding reactions. Wt, wild type; GFP-His, His tagged green fluorescent protein control. (**B**) Immunoblot of the His-tagged proteins used in the binding reactions, probed with antibody to His (Qiagen). (**C**) Protein kinase assay of PKR immunoprecipitated from transfected HEK-293 cells using rabbit polyclonal antibody to PKR (*23*) and protein A–Sepharose as described in (*9*). These experiments were repeated three to six times with similar results. (**D**) Protein kinase assay with bacterially expressed, partially purified, His-tagged PKR (*10*).

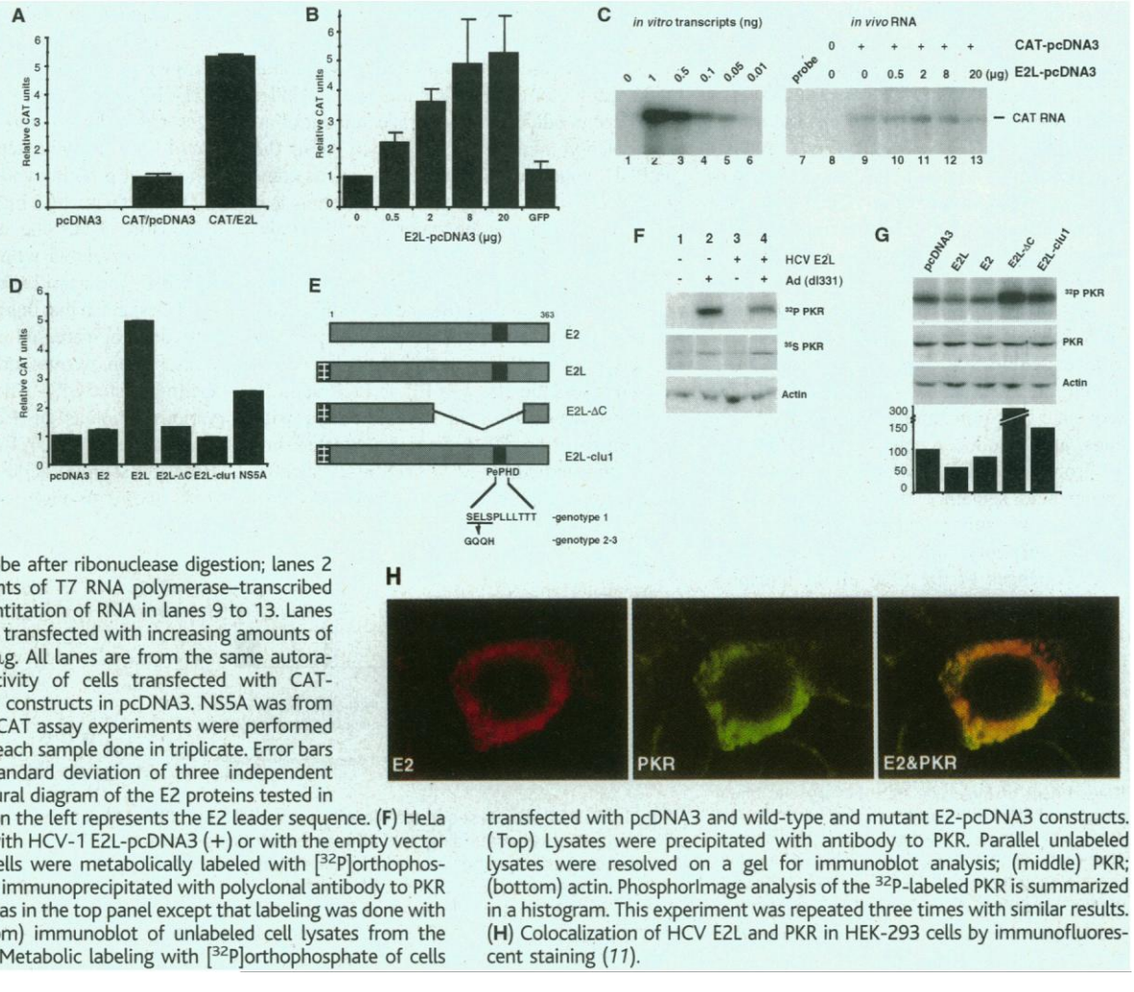

Fig. 3. HCV-1 E2 enhances protein synthesis in mammalian cells. (**A**) HeLa cells were transfected with CAT-expressing plasmids (20 μg) and pcDNA3 or E2L-pcDNA3 (20 μg). (**B**) CAT assays were performed on lysates from human hepatocellular carcinoma (Huh7) cells that were cotransfected with 15 μg of CAT-pcDNA3 and increasing amounts of E2L-pcDNA3, or GFP (2 μg). (**C**) Ribonuclease protection analysis (Hybspeed RPA; Ambion) was performed on RNA (50 μg per sample) prepared from cells in (B). Lanes 1 and 7, CAT probe after ribonuclease digestion; lanes 2 to 6, decreasing amounts of T7 RNA polymerase–transcribed CAT RNA used for quantitation of RNA in lanes 9 to 13. Lanes 9 to 13, RNA from cells transfected with increasing amounts of E2L-pcDNA3, 0 to 20 μg. All lanes are from the same autoradiogram. (**D**) CAT activity of cells transfected with CAT-pcDNA3 and various E2 constructs in pcDNA3. NS5A was from the HCV-1a strain (*6*). CAT assay experiments were performed three to six times with each sample done in triplicate. Error bars display average and standard deviation of three independent experiments. (**E**) Structural diagram of the E2 proteins tested in (D) (*24*). The hatching on the left represents the E2 leader sequence. (**F**) HeLa cells were transfected with HCV-1 E2L-pcDNA3 (+) or with the empty vector (−; pcDNA3). (Top) Cells were metabolically labeled with [^{32}P]orthophosphate, and lysates were immunoprecipitated with polyclonal antibody to PKR (*23*); (middle) the same as in the top panel except that labeling was done with [^{35}S]methionine; (bottom) immunoblot of unlabeled cell lysates from the same transfection. (**G**) Metabolic labeling with [^{32}P]orthophosphate of cells transfected with pcDNA3 and wild-type and mutant E2-pcDNA3 constructs. (Top) Lysates were precipitated with antibody to PKR. Parallel unlabeled lysates were resolved on a gel for immunoblot analysis; (middle) PKR; (bottom) actin. PhosphorImage analysis of the ^{32}P-labeled PKR is summarized in a histogram. This experiment was repeated three times with similar results. (**H**) Colocalization of HCV E2L and PKR in HEK-293 cells by immunofluorescent staining (*11*).

this result suggests that PKR autophosphorylation was almost completely inhibited in E2L-expressing cells. Examination of E2 mutants (Fig. 3G) revealed that E2 (without the leader peptide) reduced PKR activation by 20%, whereas—for reasons that remain to be determined—E2L-ΔC and E2L-clu1 activated PKR activity. Thus, only E2L efficiently inhibits PKR activation. HCV E2 is localized to the perinuclear ER region of the cytoplasm (*13*). Immunofluorescence studies showed that E2L colocalized with the endogenous PKR in the cells (Fig. 3H), consistent with the requirement for the E2 leader sequence in PKR inactivation. PKR has previously been reported to be associated with ribosomes (*14–16*), including most likely ER-bound ribosomes.

We next expressed E2 in a GCN2-deficient strain of the yeast *Saccharomyces cerevisiae*. GCN2 is an eIF2α kinase and a functional homolog of PKR (*17*). In *gcn2* (−) yeast, expression of PKR stimulates eIF2α phosphorylation, which in turn inhibits protein synthesis and retards cell growth (*17*). To study the effects of E2 on PKR in yeast, we used strain RY1-1, which lacks *gcn2* but contains two chromosomally integrated copies of *pkr* under the control of the galactose-inducible *gal1-cyc1* promoter (*18*). We transformed a DNA construct containing the E2 gene under the control of the *gal1* promoter (E2-pYES2) into this yeast strain. Yeast transformed with the vector alone (pYES2) grew well on synthetic dextrose

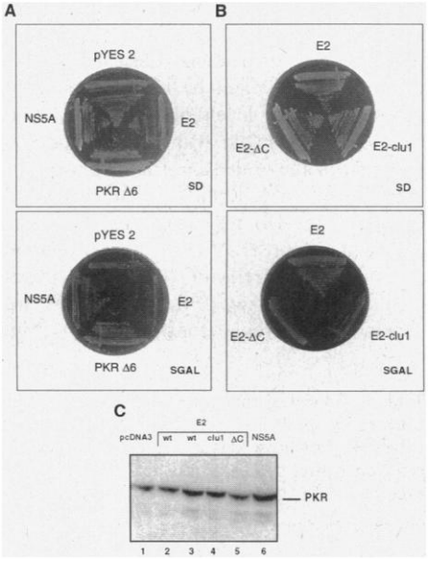

Fig. 4. HCV-1 E2 inhibits PKR in yeast. pYES 2 plasmids were introduced into yeast strain RY1-1 (*25*). (**A** and **B**) Yeast were transformed with the indicated plasmids, and transformants were streaked on SD or SGAL plates and grown at 30°C for 3 to 5 days. (**C**) Expression of PKR from RY1-1 strains transformed with various plasmids and monitored by protein immunoblotting with monoclonal antibody to PKR (*26*). pYES 2, vector alone. PKRΔ6, a dominant-negative mutant of PKR.

minimal (SD) medium but poorly on synthetic galactose (SGAL) medium (Fig. 4A). This was expected because gal-induced PKR expression would result in eIF2α phosphorylation, leading to inhibition of translation and cell growth (*17, 18*). In contrast, yeast that express the dominant-negative PKR mutant, PKRΔ6, grew well on both SD and SGAL (Fig. 4A). Cells transformed with E2 grew on both SD and SGAL, indicating that E2 inhibits PKR activity in this yeast model (Fig. 4, A and B). In contrast, yeast transformed with E2-clu1 and E2-ΔC, both of which failed to stimulate translation in mammalian cells (Fig. 3D), did not grow on SGAL, suggesting that the E2 PePHD is responsible for PKR inhibition in yeast. As previously demonstrated (*3*), yeast transformed with NS5A also grew well on both SD and SGAL media. PKR protein levels were comparable in all transformants (Fig. 4C); thus, the E2-mediated stimulation of cell growth was due to the inhibition of PKR kinase activity rather than the inhibition of PKR synthesis. When E2 was expressed in yeast strain H1816, which encodes wild-type eIF2α but no PKR, or strain H1817, which encodes a nonphosphorylatable mutant of eIF2α (S51A), neither E2 nor the E2 mutants altered the growth of yeast on SD or SGAL (*19*), indicating that E2 did not alter the growth of yeast cells in the absence of PKR.

Our results suggest that the E2 protein of HCV genotype 1 binds to and inhibits PKR in vitro and does so in mammalian cells and yeast because of sequence homology to the PKR and eIF2α phosphorylation sites. These effects correlate with the relative resistance of HCV genotype 1 to IFN. Thus, HCV may have evolved a two-level attack, namely NS5A and E2, on PKR to interfere with IFN action. Although the NS5A-PKR interaction may contribute to development of resistance during IFN therapy (*20*), the E2-PKR interaction may account for the intrinsic IFN resistance of HCV genotypes 1a and 1b. The combined effects of NS5A and E2 may explain why most HCV infections become persistent. Another potential outcome of PKR inhibition is the promotion of cell growth, which may contribute to HCV-associated hepatocellular carcinoma.

The PKR-inhibitory activity of HCV E2 protein in mammalian cells is enhanced by the presence of the leader sequence, suggesting that E2 and PKR likely interact with each other at the ER. An ER-resident eIF2α kinase (PERK) has recently been described, which shares homology with PKR and recognizes the same substrate, eIF2α (*21*). This may provide another target for E2.

References and Notes

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9. PKR was immunoprecipitated from wild-type PKR-transfected HEK-293 cells as described in (*22*). Briefly, immunoprecipitates were incubated in the presence of increasing amounts of GST or GST-E2 (0.25, 0.5, and 1 μg of soluble protein). Kinase reactions contained poly(I·C) (0.1 μg/ml; Pharmacia Biotech) and [γ-^{32}P]ATP in kinase buffer (*22*) and were incubated for 20 min at 30°C. Histone H2a (3 μg; Sigma) was the substrate. The ^{32}P-labeled proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to autoradiography.
10. BL21(DE3) bacterial cells were transformed with plasmids expressing His-tagged wild-type and mutant (K296R) PKR (pET-14b; Novagen) or green fluorescent protein (pRSET-GFP; Invitrogen), and were induced for 3 hours in the presence of 0.5 M isopropyl-β-D-thiogalactopyranoside. Proteins were extracted by sonication, and equal amounts of His-tagged proteins were incubated with nickel nitrilotriacetic acid agarose (Qiagen) for 18 hours at 4°C. The beads were washed with phosphate-buffered saline (PBS) containing 0.02 M imidazole (Amresco) and 0.3% NP-40 and were then incubated for 2 hours at 4°C in binding buffer [0.04 M Hepes-K (pH 7.5), 0.1 M KCl, 0.1% NP-40, 0.02 M β-mercaptoethanol]. The beads were washed seven times with binding buffer plus 0.02 M imidazole and 0.85% NP-40. Bound radiolabeled proteins were eluted with SDS sample buffer and boiled before SDS-PAGE analysis.
11. Cell lysates were assayed for CAT enzyme activity 2 days after transfection. Transfections were performed in triplicate and the results represent three separate experiments. CAT activity is expressed in relative units, and error bars were calculated on the basis of standard deviation of the mean. Cells that were infected with adenovirus dl331 strain (multiplicity of infection of 10) were also treated with IFN-α (+; 1000 U/ml). Immunoblots were performed with antibody to actin (Santa Cruz Biotechnology) and polyclonal antibody to PKR (*23*) and were developed by chemiluminescence (Amersham; ECL). For immunofluorescent staining of E2 and PKR, HEK-293 cells plated in eight-well chamber slides were transfected with 1 μg of COOH-terminal Flag-tagged E2L and treated with IFN-α 18 hours before fixation. Three days after transfection, the cells were fixed in 4% formaldehyde and permeabilized in acetone. The cells were then double-stained with a rabbit polyclonal antibody to Flag (Zymed) (1:300) and a monoclonal antibody to PKR (1:5) (Ribogene). Confocal microscopy was performed on a Nikon PCM2000 confocal microscope.
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transformed into these strains to determine if yeast growth was affected in the absence of PKR. No retarded or stimulated growth phenotype was observed in these strains in the presence of E2.
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24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
25. RY1-1 carries two chromosomal copies of PKR at the Leu2 locus (*18*) and has wild-type eIF2α.
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Replication of Subgenomic Hepatitis C Virus RNAs in a Hepatoma Cell Line

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An estimated 170 million persons worldwide are infected with hepatitis C virus (HCV), a major cause of chronic liver disease. Despite increasing knowledge of genome structure and individual viral proteins, studies on virus replication and pathogenesis have been hampered by the lack of reliable and efficient cell culture systems. A full-length consensus genome was cloned from viral RNA isolated from an infected human liver and used to construct subgenomic selectable replicons. Upon transfection into a human hepatoma cell line, these RNAs were found to replicate to high levels, permitting metabolic radiolabeling of viral RNA and proteins. This work defines the structure of HCV replicons functional in cell culture and provides the basis for a long-sought cellular system that should allow detailed molecular studies of HCV and the development of antiviral drugs.

HCV is a plus (+) strand RNA virus that causes acute and chronic liver diseases (*1*). Although the acute phase of infection is usually associated with mild symptoms, most patients fail to clear the virus and contract persistent infection that frequently leads to chronic liver disease, including cirrhosis and hepatocellular carcinoma. Given the high prevalence of the virus, HCV has become a focus of intensive research.

Originally cloned in 1989 (*2*), the viral genome is now well characterized. It has a length of ~9.6 kb and its single, long open reading frame (ORF) encodes a ~3000–amino acid polyprotein (*3*) (Fig. 1A). The ORF is flanked at the 5′ end by a nontranslated region (NTR) that functions as an internal ribosome entry site (IRES) and at the 3′ end by a highly conserved sequence essential for genome replication (*4*). The structural proteins are in the NH_2-terminal region of the polyprotein and the nonstructural proteins (NS) 2 to 5B in the remainder. By analogy to related +strand RNA viruses, replication occurs by means of a minus (−) strand RNA intermediate and is catalyzed by the NS proteins forming most likely a cytoplasmic membrane–associated replicase complex.

Despite the availability of cloned infectious genomes (*5*), molecular studies of HCV replication and the development of antiviral drugs have been hampered by the low efficiencies of currently available cell culture systems and by the fact that the only animal model is the chimpanzee. Thus, to date, research on HCV replication has depended largely on the infection of cell lines or primary cell cultures with sera and the detection of viral replicative intermediates with the highly sensitive reverse transcriptase–polymerase chain reaction (RT-PCR) (*6*).

To overcome these limitations, we established an efficient cell culture system that is based on the transfection of cloned viral consensus genome sequences. Owing to the high amount of HCV RNA, we used as starting material for cloning total liver RNA isolated from a chronically infected patient who had undergone liver transplantation (*7*). Using long-distance RT-PCR, we amplified the complete ORF in two overlapping fragments. Several clones of each fragment were analyzed, and an isolate-specific consensus sequence was established, which belongs to the worldwide distributed genotype 1b. The 5′ and 3′ NTRs were amplified separately by standard RT-PCR and were assembled with the reconstituted consensus ORF. A 5′-flanking T7 RNA polymerase promoter and an engineered restriction site at the 3′ end allowed for production of run-off RNA transcripts with authentic 5′- and 3′-terminal sequences (*8*). As a negative control for all transfection experiments for each parental construct, a defective genome was generated carrying an in-frame 10–amino acid deletion (Δ) encompassing the NS5B RNA polymerase active site.

We initially transfected various cell lines and primary human hepatocytes with in vitro transcripts corresponding to the cloned full-length genome or the deletion mutant. We monitored RNA replication by comparing the amounts of +strand RNA detected by RT-PCR in cells transfected with the parental or the defective genome (*9*). In no case was a significant difference found between the genomes, suggesting that no replication occurred.

The failure of these experiments might be attributable to errors in the cloned genome, low transfection efficiencies, or cytopathogenicity of HCV, which would lead to a selective loss or growth disadvantage of cells supporting virus replication. Alternatively, the cell lines used might be nonpermissive or support only low levels of viral RNA replication not detectable with our method. To overcome some of these restrictions, we constructed selectable replicons that transduced neomycin (G418) resistance only to those cells that support HCV replication. Based on recent results with flavi- (*10*) and pestiviruses (*11*) and on mapping of the HCV-IRES, we generated bicistronic constructs (*12*) (Fig.

Table 1. G418-resistant cell clones obtained after transfection of Huh-7 cells with in vitro–transcribed HCV replicon RNAs. The number of viable cell clones after subpassage of clones obtained in experiment 1 is given in the right column.

Construct	Experiment			Subpassages (exp. 1)
	1	2	3	
I_{377}neo/NS2-3′/wt	12	20	38	1
I_{377}neo/NS2-3′/Δ	0	3	8	0
I_{377}neo/NS3-3′/wt	20	>60	40	2
I_{377}neo/NS3-3′/Δ	2	8	18	0
I_{389}neo/NS2-3′/wt	6	20	25	1
I_{389}neo/NS2-3′/Δ	1	10	4	1
I_{389}neo/NS3-3′/wt	30	15	17	5
I_{389}neo/NS3-3′/Δ	1	2	6	0

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