Activation of the CKI-CDK-Rb-E2F Pathway in Full Genome Hepatitis C Virus-expressing Cells*

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Hepatitis C virus (HCV) causes persistent infection in hepatocytes, and this infection is, in turn, strongly associated with the development of hepatocellular carcinoma. To clarify the mechanisms underlying these effects, we established a Cre/loxP conditional expression system for the precisely self-trimmed HCV genome in human liver cells. Passage of hepatocytes expressing replicable full-length HCV (HCR6-Rz) RNA caused up-regulation of anchorage-independent growth after 44 days. In contrast, hepatocytes expressing HCV structural, nonstructural, or all viral proteins showed no significant changes after passage for 44 days. Only cells expressing HCR6-Rz passed for 44 days displayed acceleration of CDK activity, hyperphosphorylation of Rb, and E2F activation. These results demonstrate that full genome HCV expression up-regulates the CDK-Rb-E2F pathway much more effectively than HCV proteins during passage.

Hepatitis C virus (HCV)† causes the persistent infection chronic hepatitis in most infected patients. This disorder eventually progresses to cirrhosis and hepatocellular carcinoma (HCC). Numerous studies have provided evidence supporting a link between chronic HCV infection and HCC (1, 2). However, exactly how HCV infection could be directly involved in the development of HCC remains unclear because of the lack of an efficient in vitro infection system.

HCV is a member of the Flaviviridae family and has a positive-strand RNA genome (—9.6 kb). Viral proteins are synthesized as a single polyprotein, which is then cleaved into structural (core, E1, and E2) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. In vitro expression of HCV core protein reportedly influences cell growth regulation, and the protein can interact with the cytoplasmic tail of the lymphoid-specific β2 microglobulin receptor (3), the death domain of tumor necrosis factor receptor-1 (4), and NF-κB (5), resulting in enhancement of inhibition or induction of apoptosis. HCV structural protein inhibits proapoptotic Fas-mediated apoptosis by inhibiting cytochrome c release from mitochondria in mice (6). Cellular transformation has been shown to be caused by HCV core protein in the presence of the ras gene (7, 8) and by loss of function of LZI9 (9) and the presence of STAT3 (signal transducer and activator of transcription-3) (10). NS3 (11) and NS4B and NS4B (12) proteins reportedly display tumorigenicity in the presence of ras. These results indicate that expression of individual HCV proteins does not cause cellular transformation in vitro.

HCV core protein transgenic mice reportedly show induced steatosis and, after 16 months of age, develop HCC (13). In contrast, HCV structural protein transgenic mice do not display neoplastic or cancerous lesions in the liver by 20 months of age (14). Moreover, conditional expression of an HCV structural protein region (nucleotides 294–3435) causes hepatic injury in transgenic mice (15), but HCC is not observed by at least 16 months of age (data not shown). The frequency of HCC occurrence is reportedly higher in full-length HCV polyprotein transgenic mice than in those with the structural protein only (16). However, whether HCV proteins can represent direct triggers of transformation in hepatocytes remains unclear.

Cirrhosis and irregular regeneration have been reported as risk factors for HCC (17). During persistent HCV infection, hepatic injury and regeneration repeatedly occur in the liver. An efficient system of HCV infection is required to clarify the effects of HCV on cell growth. Efficient replication systems have recently been established using HCV replicons and HuH-7 cells (18, 19). HuH-7 cells display a point mutation at codon 220 of p53 (20), and chemosensitivity is decreased compared with other cell lines with wild-type p53 (21). To clarify how HCV infection modifies hepatocyte growth, we established an expression cassette of replicable full genome HCV, as

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1 The abbreviations used are: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; DHFR, dihydrofolate reductase; IFN, interferon; CDK, cyclin-dependent kinase; FACS, fluorescence-activated cell sorter; dsRNA, double-stranded RNA; kb, kilobase pair; CKI, cyclin-dependent kinase inhibitor.
firmed in IMY cells and *Tupaia belangeri,* and HepG2 cells originating from human hepatoblastoma and exhibiting characteristics of differentiated hepatocytes in that the response to growth factors and p53 functions is retained (22, 23). Liver cell lines in which HCV genomes can be conditionally expressed using the CreloxP switching system (2) and precisely self-switched at the 5′ and 3′ termini using ribozyme sequences were thus established in this study. HCV-expressing cells were passaged and characterized according to changes in cell growth regulation.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning and Plasmid Construction—Full genome HCV cDNA (nucleotides 1–9611; DDBJ/GenBank†/EBI accession number AJ045702) was cloned from the serum of a chronic hepatitis patient (HCV genotype 1b; 50% chimpanzee infectious dose, 10⁻⁵⁵ copies/ml; PCR titer, 10⁻⁵³ copies/ml). Complementary DNA clones with a consensus sequence in more than three clones were utilized for construction of a full-length clone. This was then subcloned under the control of a CAG promoter (24) and the CreloxP conditional expression cassette using a neomycin resistance gene with a polyA signal as a stuffer (see Fig. 1A) (2). The precise HCV RNA can be exactly excised by the presence of hammerhead ribozyme (Fig. 1B) at the 5′ terminus and the hepatitis D virus ribozyme (26) at the 3′ terminus (see Fig. 1A). Mutant HCR6-Fse was constructed by digestion of the HCR6-Rz clone with FseI (at nucleotide 3379; Takara), deleted using T4 polymerase (T4 Polyf.I.) (27). This mutation introduces a stop codon at nucleotide 3606. Mutant HCR6-Age was constructed by digestion of the HCR6-Rz clone with AgeI (at nucleotide 155; Nippon Gene) and NotI (at nucleotide 1967; Takara) and filled in with T4 polymerase (see Fig. 1A). HCR6-CN5 was constructed by removal of the 5′-untranslated region (nucleotides 1–155).

Additional expression was performed by further transfection of the pCAG-PURO-Mer-Cre-Mer vector (see Fig. 1B), the addition of 4-hydroxynamoxifen (100 nM), or infection with the AxCanCre virus (2). AxCanCre was prepared by inserting the structural genes of Cre recombinase into adenovirus E1A- and E1B-deleted regions under the control of the CAG promoter. The adenoviral vector AxCanW1 lacks these inserted genes.

The pCAG-PURO-Mer-Cre-Mer vector was constructed from pAN-Mer-Cre-Mer (27) and pCAG-PURO, for which the puromycin gene was deleted from dBase-PURO under the control of the CAG promoter (28). The dihydrofolate reductase (DHFR)-luciferase reporter plasmid has the DHFR promoter in the pGL3 vector (Promega).

Cells and Reagents—HepG2 cells were transfected with HCR6-Rz, HCR6-Fse, HCR6-Age, and HCR6-CN5 clones using a modified calcium phosphate method and selected with G418 (800 μg/ml; Invitrogen). Rz2-9, Rz2-18, Rz2-22, Rz24, Rz26, Rz27, Age8, and CN5-1 were established (Table I). These cells were further transfected with pCAG-PURO-Mer-Cre-Mer DNA and selected with puromycin (1.0 μg/ml). Rz26 and Rz2M13 were derived from Rz2-18, and Rz2M24 was derived from Rz24. Rz2M2-8 and Rz2M2-9 were derived from Rz2-S and Rz2-B, respectively. FseM28 and AgeM8 were derived from Fse28 and Age8, respectively. Switching expression was performed by the addition of 4-hydroxynamoxifen (100 nM) for 4 days or by infection of CN5-1 with the AxeCanCre virus (multiplicity of infection of 20). For characterization of cell growth speed, 2 × 10⁴ or 4.7 × 10⁴ cells were plated onto 75- or 175-cm² Falcon tissue culture bottles and passaged every 4 days for 44 days. Cell numbers were counted at every point of passage, and averages of three experiments were calculated.

Immunoblot Analysis and Core Protein Quantitation—HCV-expressing cells (1 × 10⁶) were lysed with 100 μl of lysis buffer A (1% SDS, 0.5% Nonidet P-40, 0.15 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 10 mM Tris, pH 7.4), and 30 μg of total protein was electrophoresed on SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). HCV proteins were detected using the originally established anti-core (515) (29), anti-E1 (384), anti-E2 (544), anti-NSSA (C14II-2-1, anti-NSSB (AG52), anti-NSSA (5A32), and anti-NSSB (SB4) monoclonal antibodies or rabbit anti-NS3 (R212) and anti-NS4/B (RR10) polyclonal antibodies. HCV core protein was quantitated in cell lysates using the fluorescent enzyme-linked immunosorbent assay (EIA) (30). Antibodies against p53 (Novo Castra); phospho-CDC2 (New England Biolabs Inc.); actin (Roche Applied Science); and Rb (Pharmingen) were purchased and utilized according to the manufacturers’ protocols.

**ATLAS cDNA Array Assay**—Using 2–5 μg of total RNA from Rz2-18, Rz2M6, and M13 cells with or without 4-hydroxynamoxifen treatment, total RNA was isolated; cDNA probes were synthesized; cultured for 0, 12, and 44 days, respectively; and hybridized to filters (ATLAS human cancer cDNA expression array, Clontech) according to the manufacturer’s protocol. The population of cells in each cell cycle phase was characterized using Modifit LT software in FACSCalibur (BD Biosciences) by counting 10,000 cells.

**Fluorescence-activated Cell Sorter (FACS) Analysis**—Flow cytometry was performed using a FACSCalibur (BD Biosciences) by counting 10,000 cells.

**Antibodies against Core and Other HCV Proteins**—The anti-Core antibody (515) was prepared by Dr. H. Sato. The anti-E1 (384) antibody was prepared by Drs. S. Hasegawa and S. Tanaka. The polyclonal antibody against E2 (544) was provided by Dr. M. Yamanaka. Anti-NS5B (5B14) monoclonal antibody was a kind gift from Dr. M. Kohara. Anti-NS3 (AG52) was a gift from Dr. T. Endo. Anti-NS4A (C14II-2-1) and anti-NS4B (SB4) monoclonal antibodies were provided by Dr. H. Sato.

**CDK Assay**—HCV-expressing cells were transfected with the pGL3 vector that includes CDK4 promoter (Promega) and selected with G418. The dihydrofolate reductase (DHFR)-luciferase reporter plasmid has the DHFR promoter in the pGL3 vector (Promega).

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When extensive washing, hybridization intensities were quantitated using a Fuji BAS 2000 image intensifier. Values for each regulatory gene were standardized to the average for nine housekeeping genes.

**CDK Assay**—HCV-expressing cells were transfected with the pGL3 vector that includes CDK4 promoter (Promega) and selected with G418. The dihydrofolate reductase (DHFR)-luciferase reporter plasmid has the DHFR promoter in the pGL3 vector (Promega).

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**Fluorescence-activated Cell Sorter (FACS) Analysis**—Flow cytometry was performed using a FACSCalibur (BD Biosciences) by counting 10,000 cells.
**FIG. 1. Structure of HCR6-Rz vectors, conditional expression of full genome HCV RNA and proteins, and chronological changes in cell growth properties upon HCR6-Rz gene expression.**

**A**, the HCR6-Rz clone contains full-length HCV cDNA surrounded by ribozyme (Rz; gray box) and hepatitis D virus ribozyme (HDV-Rz; white box) sequences under the control of the CAG promoter (CAG-Pro.; cytomegalovirus IE enhancer (gray box) and chicken β-actin promoter (white box)) with a Cre/(loxP) switching expression cassette comprising the neomycin resistance gene (Neo) as a stuffer surrounded by loxP sequences. The region for the probe used in genomic Southern blotting is also indicated (black box). UTR, untranslated region; C, core. **B**, shown is the structure of pCAG-PURO-Mer-Cre-Mer. The Cre enzyme (gray box) is surrounded by tamoxifen-binding domains (TBD; black arrows) (27). **C**, switching expression of full genome HCV RNA was detected by Northern blotting in RzM6 cells. The molecular size markers are shown in an RNA ladder (Invitrogen). **D**, HCV protein before (−) and after (+) tamoxifen treatment was detected by Western blotting in RzM6 cells. **E**, DNA from serially passaged RzM6 cells was subjected to genomic Southern blot analysis. Rz-Hep cells were treated with tamoxifen (100 μM), and XbaI- and SalI-digested genomic DNA was probed using the HCV core to the E1 (KpnI-SalI) region (A), yielding an unrecombined 2.9-kbp fragment (day 0) and a recombined 1.8-kbp fragment after 8, 16, 28, and 44 days. **F**, RzM6 cells were treated with tamoxifen and passaged for 44 days at an interval of 4 days. Expression of core protein was quantitated by fluorescent enzyme-linked immunosorbent assay (30), and the results were divided by the total protein concentration at each time point.
TCTGGGTG-3' (antisense primer), and 5'-CTACGTGGATCTCCCAAAGCAAC-3' (probe). The primers and probes used for quantitation of HCV negative-strand RNA, IFN, and glyceraldehyde-3-phosphate dehydrogenase will be described elsewhere. A reporter dye (6-carboxyfluorescein) was covalently attached to the 5'-end, and a quencher dye (6-carboxytetramethylrhodamine) was joined to the 3'-end of the probe sequence using an amino-linked method.

RESULTS

Establishment of Conditional Full Genome HCV-expressing Hepatocytes—The conditional expression system of the full genome HCV cDNA clone (HCR6-Rz) was established using the Cre/loxP system (Fig. 1A). The precise HCV RNA was trimmed using the ribozyme sequence at the 5' and 3' termini (Fig. 1, A and C). Infectivity of the HCR6-Rz cDNA clone was observed in susceptible human liver cell lines and animals. Four independent HCR6-Rz-expressing HepG2 cell lines (Rz2-8, Rz2-18, Rz2-22, and Rz2-24) were established. HCV expression was induced using Cre recombinase from a modified Mer-Cre-Mer expression cassette (Fig. 1B) (27) in the presence of 4-hydroxytamoxifen (100 nM; referred to as tamoxifen below). These tamoxifen-inducible Rz-Hep cells were designated RzM6 and M13 (parental strain Rz2-18), RzM2-8 (parental strain Rz2-8), RzM2-9 (parental strain Rz2-9), and RzM24 (parental strain Rz24) (Table I). In Rz-Hep cells, expression of full-length HCV RNA (9.6 kb) was induced, and full-length HCV RNA was trimmed precisely at the 5' and 3' termini by the ribozyme sequence, which was obtained by Northern blotting (Fig. 1C) and 5'-34A tailing, the 3'-oligonol-adenate tailing cloning method, and sequencing (data not shown). All HCV protein expression was induced by tamoxifen treatment (Fig. 1D).

Cell Growth Properties of HCV-expressing Hepatocytes during Passage—To examine the modification of cell growth properties by HCV, we passaged full genome HCR6-Rz-expressing HepG2 cells (RzM6, RzM13, RzM24, and the respective parental strains) every 4 days for >40 days (Fig. 1, E and F; and Fig. 2, A and B). After tamoxifen treatment on day 8, Cre-mediated transgene recombination occurred (Fig. 1E). The 2.9-kbp DNA fragment on day 0 and the already recombined 1.8-kbp fragment in response to tamoxifen treatment after day 8 (Fig. 1E, 8, 16, 28, and 44 days) were detected with XbaI- and SalI-digested genomic DNA by Southern blot analysis (Fig. 1, A and E). HCV protein was persistently expressed in RzM6 cells up to at least 44 days (Fig. 1F), and this expression was quantitated by fluorescent enzyme-linked immunosorbent assay and Western blotting (data not shown). The cell growth ratio of RzM6 cells was examined for 44 days (Fig. 2B, panel a); it was reduced to ~50% in 8–12 days after the onset of HCV expression by tamoxifen (core protein expression of 3.4 ng/mg of total protein) (Fig. 1F) and then unexpectedly recovered to 100% by 20 days and exceeded 100% at 44 days of passage. In contrast, the cell growth of the parental cell line (Rz2-18) did not show any significant change during passage after tamoxifen treatment (Fig. 2B, panel a). RzM24 cells with 1.2 ng/mg core protein expression on day 8 showed suppression of cell growth to 50% on day 8, but recovered to 100% on day 16, which was faster than the recovery seen for RzM6 cells (Fig. 2B, panel b). Suppression of cell growth by HCR6-Rz on day 8 was also observed in RzM13, RzM2-8, and RzM2-9 cells (data not shown). To define the essential HCV genome region for cell growth modification, truncated 5'-untranslated region-to-NS2 protein (HCR6-Fse), E2-to-3' untranslated region (HCR6-Age), and core-to-NS5 protein (HCR6-CN5) expression cassettes

TABLE I

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<th>DNA</th>
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<td>Rz2-18</td>
<td>RzM6</td>
<td>RzM13</td>
<td>3.5</td>
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<td></td>
<td>Rz24</td>
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<td></td>
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<tr>
<td></td>
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<td></td>
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<td>FseM28</td>
<td>3.0</td>
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* Nanograms of core protein/mg of total protein after 4 days of induction.

were introduced into HepG2 cells (Fig. 2A). HCV core protein expression levels in FseM28 and CN5-1 cells were similar to that observed in RzM6 cells. MCM, Mer-Cre-Mer, ND, not detected.

To further characterize the process of cell growth modification by HCV, we performed FACS analysis (Table II). Passaged cells were analyzed on days 0, 8, and 44, as these time points occurred prior to HCV expression, at the time of the most retarded cell growth, and when the growth ratio had returned to >100% (Fig. 2A), respectively. Expression of HCR6-Rz and HCR6-Fse retarded the cell cycle at the G0/G1 phase after 8 days. At 44 days of passage, the percentage of cells in the G0/G1 phase returned to the level seen prior to HCV expression (Table II). Neither parental nor other cell lines showed any significant change in cell cycle on days 0, 8, and 44.

Anchorage-independent Growth Activity of HCV-expressing and Passaged Cells—To clarify changes in the character of HCV gene-expressing cells during passage for 44 days, we examined the anchorage-independent growth of these cells (Table III). RzM6 cells formed an average of 7.5 colonies on day 0, a value that had decreased to 2.5 colonies after 8 days of HCV genome expression. Notably, cells passaged for 44 days with HCR6-Rz expression increased colony numbers to 65.3 (Table III). Tamoxifen-treated Rz2-18 cells did not show any significant change in colony formation after 44 days of passage. Tamoxifen-treated RzM24 cells (another HCR6-Rz-expressing cell line) also showed significantly elevated colony numbers after 44 days of passage (Table III). We attempted to further characterize the anchorage-independent growth of Fse-Hep, Age-Hep, and CN5-Hep cells, but did not observe any significant increase in anchorage-independent growth activity during passage in comparison with RzM6 cells (Table III).

Target Host Factors of HCR6-Rz during Passage—To clarify the molecular mechanisms underlying this modification of anchorage-independent growth by HCR6-Rz, ATLAS cDNA array

Fig. 2. Structure of the truncated HCV gene and cell growth properties of Rz-Hep cells during passage. A, structures of HCR6-Fse, HCR6-Age, and HCR6-CN5 expression vectors. CAG-Pro, CAG promoter; Neo, neomycin resistance gene; Rz, ribozyme; UTR, untranslated region; C, core; HDV-Rz, hepatitis D virus ribozyme. B, panel a, growth of Rz2-18 (white bars) and RzM6 (black bars) cells. The growth ratio was determined by dividing the number of cells with tamoxifen by the number of cells without tamoxifen based on the average of three independent experiments. Panel b, growth properties of Rz2-24 (white bars) and RzM24 (black bars) cells. Panels c and d, serial passage of Fse28 (white bars) and FseM28 (black bars) cells and Age8 (white bars) and AgeM8 (black bars) cells, respectively. Cell growth properties are indicated by the multiplicity ratio of cell growth (division of cell numbers with tamoxifen by those without tamoxifen). The results represent the average of two independent experiments. Panel e, HepG2 (white bars) and CN5-1 (black bars) cells. The growth ratio was determined by dividing the number of cells infected with AxCANCre (cre) by the number of cells infected with AxCaw1 (swa).
Full Genome HCV Up-regulates the CKI-CDK-Rb-E2F Pathway

The percentages indicate the population of cells in each cell cycle phase. The results represent the means of three experiments performed in duplicate. The results represent the means of three experiments performed in duplicate.

<table>
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<tr>
<th>Passage</th>
<th>G0/G1</th>
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<th>G2/M</th>
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<tr>
<td>Day 0</td>
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<td>35.7 ± 7.1</td>
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<tr>
<td>Day 8</td>
<td>57.9 ± 2.9</td>
<td>32.9 ± 4.3</td>
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<tr>
<td>Day 44</td>
<td>60.1 ± 2.9</td>
<td>31.6 ± 8.2</td>
<td>8.3 ± 5.3</td>
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<tr>
<td>RzM24</td>
<td>57.8 ± 0.3</td>
<td>36.2 ± 1.7</td>
<td>6.0 ± 1.4</td>
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<td>Day 4</td>
<td>57.8 ± 3.1</td>
<td>34.0 ± 0.1</td>
<td>8.2 ± 3.2</td>
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<tr>
<td>Day 8</td>
<td>57.8 ± 3.0</td>
<td>32.8 ± 2.3</td>
<td>9.4 ± 0.8</td>
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<tr>
<td>Day 44</td>
<td>59.3 ± 1.4</td>
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<td>6.8 ± 2.5</td>
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<table>
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<tr>
<th>Passage</th>
<th>Day 0</th>
<th>Day 8</th>
<th>Day 44</th>
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<tr>
<td>Rz2-18</td>
<td>10.0 ± 0.1</td>
<td>10.0 ± 0.8</td>
<td>10.4 ± 0.5</td>
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<td>FseM28</td>
<td>55.8 ± 1.0</td>
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<td>AgeM8</td>
<td>55.5 ± 7.6</td>
<td>36.3 ± 7.7</td>
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<tr>
<td>Day 44</td>
<td>56.5 ± 6.9</td>
<td>35.4 ± 7.2</td>
<td>8.1 ± 0.3</td>
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</table>

The analysis of mRNA from RzM6 and Rz2-18 cells was performed on days 0, 12, and 44. Molecules for which expression levels showed significant changes during passage of RzM6 cells upon HCV expression and not during passage of Rz2-18 cells after tamoxifen treatment were identified as cyclin D1, p21WAF1/CIP1, RhoA, Rho guanine nucleotide dissociation inhibitor, and EB1. Protein expression levels were examined by Western blotting in these regulators and in other cell cycle regulators: p53, IFN regulatory factor-1, MDM2, p16INK4A, p27KIP1, cyclin A, cyclin E, phosphorylated CDC2, and Rb (Fig. 3A). Consistent with changes in cell cycle progression, the expression levels of p21WAF1/CIP1 were up-regulated after 8 days of passage and returned to base-line levels after 44 days (Table II). Expression of cyclin D1 was also up-regulated on day 8, whereas the Rb protein was dephosphorylated (Fig. 3A), which may indicate that cyclin D1 was inactivated by p21WAF1/CIP1 (34, 35). In particular, in RzM6 cells passaged to day 44, expression of hyperphosphorylated Rb protein was increased more dramatically than in cells on days 0 and 8 (Fig. 3A).

After the onset of HCV protein expression, the cell growth ratio decreased in FseM28 cells on day 8. In contrast, no significant changes were observed in AgeM8 cells (Fig. 2B). FACS analysis revealed that the cell cycle progression of FseM28 cells was decreased after 8 days of expression (Table II). However, the expression levels of the p21WAF1/CIP1 protein did not show any significant difference in either FseM28 or AgeM8 cells on day 8 (Fig. 3B). These results indicate that cell cycle retardation by HCV structural protein does not mediate p21WAF1/CIP1. Moreover, the phosphorylation status of Rb was not accelerated after passage for 44 days in FseM28 and AgeM8 cells compared with RzM6 cells (Fig. 3, A and C). Therefore, we further characterized the events that occurred within 44 days of passage in RzM6 cells.

Examination of HCV Replication in HCR6-Rz-expressing Cells—HCV possesses positive-strand RNA as a viral genome and produces negative-strand RNA as a mold only for replication. The quantity of negative-strand viral RNA is thus proportional to viral replication. Negative-strand RNA synthesis was detected by strand-specific real-time detection-PCR (as described under “Experimental Procedures”) in RzM6 cells on both days 8 and 44, but was not observed in Rz2-18 cells (Fig. 4A) or in FseM28 or AgeM8 cells (data not shown). IFN induction was then examined and was shown to be produced efficiently only in RzM6 cells on day 8 (Fig. 4B). Downstream 2′-5′-oligoadenylate synthetase and PKR mRNA expression levels increased on day 8 and decreased to base-line levels after 44 days in RzM6 cells (Fig. 4, C and D). The activity of PKR was initially increased on day 8 and decreased below base-line levels after 44 days in these cells (Fig. 4E). Thus, replication of HCV might produce negative-strand RNA for formation of dsRNA, with the subsequent production of IFN and activation of downstream signaling pathways in HCR6-Rz-expressing cells on day 8.

Activation of E2F and CDK by HCR6-Rz—Replication of HCV and activation of the IFN pathway were observed only in Rz-Hep cells. However, the pathways that HCR6-Rz might modify to cause acceleration of Rb hyperphosphorylation after passage for 44 days remained unclear. A previous study reported that CKI, CDK, and Rb regulatory pathways play significant roles in many types of tumor formation, including HCC (36). The downstream target of p21WAF1/CIP1 was therefore characterized. The p21WAF1/CIP1 protein inhibits the activity of
FIG. 3. Modification of cell growth regulatory proteins by HCV. A, protein expression levels of cell growth regulatory genes on days 0, 8, and 44 of passage in exponentially growing Rz2-18 and RzM6 cells. The results are representative data from three experiments. IRF-1, IFN regulatory factor-1; Rho-GDI, Rho guanine nucleotide dissociation inhibitor. B, expression of the p21^{WAF1/CIP1} protein in Fse28, FseM28, Age8, and AgeM8 cells in the exponential cell growth stage on days 0, 8, and 44 of passage as determined by Western blotting. The results are representative panels from two independent experiments. C, detection of the Rb protein by Western blotting in Rz-Hep, Fse-Hep, and Age-Hep cells in the exponential growth phase. The results from SDS-PAGE (6.5%) show representative data from two experiments.

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CDK4/cyclin D and CDK2/cyclin E, resulting in hypophosphorylation of Rb and suppression of E2F activity (37, 38). To assess the possibility that these pathways are modified by HCR6-Rz expression, E2F activity was characterized using the DHFR-luciferase reporter plasmid. In RzM6 cells, E2F activity showed a 0.6-fold decrease on day 8 and a 1.8-fold increase after 44 days of passage, whereas in Fse-Hep and Age-Hep cells, no significant changes were observed (Fig. 5A). This Rh-E2F pathway was thus suppressed on day 8 and enhanced by 1.8-fold by day 44 in RzM6 cells (Figs. 3 and 5A).

To clarify the hyperphosphorylation mechanism of Rb, we characterized CDK4/6 and CDK2 activities during passage of RzM6 cells (Fig. 5B). The kinase activity of CDK4/6, which was quantitated using a Fuji BAS 2000 phospho-imager, was 69% on day 8 and 139% on day 44 compared with day 0. CDK2 activity was suppressed to 20% on day 8 and increased to 222% on day 44 compared with day 0. In contrast, in the HCV-non-expressing parental Rz2-18 cells, CDK4/6 and CDK2 activities did not show any significant change during passage (Fig. 5B). The protein expression levels of CDK4 and CDK2 likewise did not show any significant change in either RzM6 or Rz2-18 cells (Fig. 3A).

The stability of p21^{WAF1/CIP1} is reportedly decreased by expression of HCV proteins, and degradation of hyperphosphorylated Rb is apparently accelerated in HCC (39). The stability of the p21^{WAF1/CIP1} and Rb proteins was therefore characterized at the indicated times after cycloheximide treatment (Fig. 5C). The results for the half-life experiment with the p21^{WAF1/CIP1} and Rb proteins did not show any significant differences between HCV protein-non-expressing cells (0 day) and HCV protein-expressing cells passed for 44 days. Activation of E2F was therefore predominantly caused by activation of CDKs and the resulting hyperphosphorylation of Rb.

**DISCUSSION**

HCV-induced cell growth modifications have not yet been fully clarified because of the lack of an efficient in vitro infection system. This study therefore established a Cre/loxP conditional expression system for the full-length HCV genome, which is self-trimmed by a double ribozyme. This enabled us to reproduce the state of hepatocytes persistently infected with HCV. As a result, passage of HCR6-Rz was found to cause up-regulation of anchorage-independent growth and the CDK-Rb-E2F pathway.

The Rh protein became hyperphosphorylated (Fig. 3A), and anchorage-independent growth was accelerated in full genome HCV RNA-expressing hepatocytes that were passed for 44 days (Table III). Degradation of hyperphosphorylated Rb is reportedly accelerated and E2F is activated, and both correlate closely with hepatocyte transformation (39). In fact, RzM6 cells passed for 44 days displayed accelerated tumorigenicity in nude mice (data not shown).

In the case of RzM6 cells, the stability of p21^{WAF1/CIP1} and Rb did not change; and thus, the increased hyperphosphorylation of Rb was predominantly caused by activation of CDKs (Fig. 5). Recent findings have suggested that CDK4/6 is responsible only for phosphorylation of Rb and that CDK2 is necessary for hyperphosphorylation of Rb (35, 40). In HCR6-Rz-expressing cells, both CDKs were activated, particularly CDK2 (Fig. 5B). Acceleration of tumorigenicity by HCR6-Rz may be caused during passage of growth-repressed cells, which might induce the disruption of cyclin-CDK-CKI complexes, thereby resulting in the activation of CDKs. A previous study reported that the papilloma virus oncoprotein E7 abrogates the inhibitory effect of p21^{WAF1/CIP1} on cyclin E-CDK2 complex activity without influencing p21^{WAF1/CIP1} expression levels (41). The E8 protein from bovine papilloma virus reportedly accelerates anchorage-independent growth by activation of cyclin A and CDK2 and deregulation of p21^{WAF1/CIP1} (42). This was not observed in RzM6 cells after 44 days of passage (Fig. 3A).

Hyperphosphorylation of Rb results in the release of E2F, allowing the cell cycle to progress from G1 to S phase (43, 44) by overcoming restriction point and cellular transformation via cooperation with other oncogenes. Recent reports have noted the absence or down-regulation of CKI (p16^{INK4A}, p21^{WAF1/CIP1}, and p27^{KIP1}) expression in most HCCs (36) and have described a significant correlation between hyperphosphorylation of Rb and HCC (39, 45). These results shed light on the mechanism responsible for the high incidence of HCC in HCV-infected patients.

After 8 days of passage, p21^{WAF1/CIP1} expression was accelerated in full genome HCV RNA (HCR6-Rz)-expressing hepatocytes, and CDK4/6 and CDK2 activities and phosphorylation of Rb were suppressed (Figs. 3A and 5B). These events introduced cell growth retardation at the G1/G0 phase (Table II). Although HCR6-Fe expression induced suppression of cell growth after 8 days, no up-regulation of p21^{WAF1/CIP1} was observed during passage (Fig. 3B). HCR6-Age expression did not influence cell growth during passage. This may indicate that HCV structural protein modifies another pathway to suppress cell growth rather than up-regulation of p21^{WAF1/CIP1}. The precise pathway modified by HCR6-Fe is now under investigation.

In RzM6 cells, up-regulation of RhoA, Rho guanine nucleotide dissociation inhibitor, and EB1 mRNA transcription was observed, but no increase in protein expression levels was apparent (Fig. 3A). These inconsistencies might be due to the existence of other modifications in post-transcriptional steps, and the exact reasons are currently under investigation. Expression of p21^{WAF1/CIP1} was accelerated at the transcriptional level as detected by Northern blotting (1.3-fold) and p21^{WAF1/CIP1} promoter-luciferase reporter assay (3.5-fold) after 8 days of HCR6-Rz expression (data not shown). Modification of p21^{WAF1/CIP1} expression was not observed in HCR6-Fe- or HCR6-Age-expressing cells. The precise mechanism underlying modification of p21^{WAF1/CIP1} expression by HCR6-Rz is not known at present. One possible inducer is IFN, given that it inhibits cell growth by transcriptional up-regulation of p21^{WAF1/CIP1} (46–48). HCV negative-strand RNA is the replicative intermediate for HCV and was produced only in HCR6-Rz-expressing cells, an effect that was confirmed by strand-specific real-time detection-PCR (Fig. 4A). The HCV negative-strand can form dsRNA with positive-strand viral RNA and can stimulate production of IFN-α and IFN-β by activation of mitogen-activated protein kinases and IFN regulatory factor-3 (49). Consistent with these results, expression of IFN-α and IFN-β mRNAs was increased at 8 days of passage and returned to basal levels in RzM6 cells after 44 days of passage (Fig. 4B). HCV dsRNA still existed in RzM6 cells passed for 44 days (Fig. 4A), but IFN-β, PKR, 25'-oligoadenylate synthetase, and p21^{WAF1/CIP1} expression returned to basal levels (Figs. 3A and 4, B–E). The existence of dsRNA might thus play a significant role in activation of CDKs in these cells. The detailed mechanisms underlying the activation of the CDK-Rb-E2F pathway during passage of HCR6-Rz-expressing cells for 44 days are now under investigation.

Synthesis of HCV negative-strand RNA was detected in HCR6-Rz-expressing and passaged cells, but not in truncated HCV-expressing cells (Fig. 4A) (data not shown). The HCR6-Age and HCR6-CN5 constructs possess the NS5B protein, which is predicted to encode RNA polymerase. However, negative-strand RNA synthesis was not detected. Frieß et al. (50) recently reported that the 5'-untranslated region sequences of...
nucleotides 1–125 and 296–341 are important for replication of the HCV replicon. HCR6-Age lacks nucleotides 196–341, so production of replicative forms in Age-Hep cells might be below detectable levels.

The present results indicate that passage of full genome HCV-expressing cells activates the CKI-CDK-Rb-E2F pathway. Future studies on primary hepatocytes are necessary to delineate the effects of full genome HCV on the CKI-CDK-
Fig. 5. Activation of E2F and CDK occurs in RzM6 cells after 44 days of passage. A, DHFR promoter activity in RzM6, Fse-Hep, and Age-Hep cells. Each reaction was standardized using the Renilla luciferase reporter plasmid (relative luciferase activity). The ratio of the fold increase was calculated by the division of each relative luciferase activity in cells prior to HCV expression. Fold activation of relative luciferase activity is given as a ratio. The results represent the average of 3 wells in two independent experiments. B, CDK2 and CDK4/6 activities in Rz2-18 and RzM6 cells on days 0, 8, and 44. NRS, immunoglobulin derived from normal rabbit serum; NMS, immunoglobulin derived from normal mouse serum. The percentage of each kinase activity relative to day 0 as quantitated using a phospho-imager plate is shown. tr.Rb, truncated Rb. C, stability of the p21WAF1/CIP1 and Rb proteins characterized by Western blotting after cycloheximide treatment for the indicated times.
Rb-E2F pathway. This may represent a novel mechanism for the transformation of hepatocytes during persistent HCV infection.

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Activation of the CKI-CDK-Rb-E2F Pathway in Full Genome Hepatitis C Virus-expressing Cells
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