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Hepatitis C Virus Core Protein Promotes Immortalization of Primary Human Hepatocytes

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Hepatitis C virus (HCV) core protein has many intriguing properties as a viral factor and is implicated in cell growth regulation. In this study, the cell growth regulation potential of HCV core protein was investigated by introduction of the core genomic region into primary human hepatocytes, a natural host for virus replication and tropism. Core-transfected primary human hepatocytes displayed altered cell morphology resembling that of low-differentiated epithelial cells. Those cells retained an immortalized phenotype and exhibited continuous growth after more than 50 passages over 2 years. Stable hepatocyte transfectants exhibited albumin secretion and HCV core protein expression. Telomerase activity, a characteristic of immortalized or transformed cells, was evident in the transfected hepatocytes immediately after senescence. Anchorage-independent growth of the immortalized hepatocytes provided further evidence for a transformed phenotype. Results from these studies suggest that the HCV core protein promotes primary human hepatocytes to an immortalized phenotype, which may predispose cells over an extended period of time to undergo a transforming event. Thus, HCV core protein appears to contribute to virus-mediated pathogenesis in a persistently infected host.

INTRODUCTION

Hepatitis C virus (HCV) is an important cause of morbidity and mortality worldwide, causing a spectrum of liver disease ranging from an asymptomatic carrier state to end-stage liver disease. The most important feature of persistent HCV infection is the development of chronic hepatitis and the potential for disease progression to hepatocellular carcinoma (Saito *et al.,* 1990; Alter, 1996; Idilman *et al.,* 1998). The genomic region encoding a protein from the amino terminal (amino acids 1–191) of HCV is believed to be a core protein due to homology with related viruses. Growing evidence suggests that the core protein has many intriguing functional properties and has been implicated as a viral factor in pathogenesis (Shih *et al.,* 1993, 1995; D. W. Kim *et al.,* 1994; Lo *et al.,* 1995; Ray *et al.,* 1995, 1997, 1998; Matsumoto *et al.,* 1997; Hsieh *et al.,* 1998; Yasui *et al.,* 1998; Shrivastava *et al.,* 1998; You *et al.,* 1999; Mamiya and Worman, 1999; Owsianka and Patel, 1999; Lu *et al.,* 1999). Transfection of HCV core gene together with H-ras as a cooperative oncogene has been observed to transform primary rat embryo fibroblasts to a tumorigenic phenotype (Ray *et al.,* 1996b). A similar observation has been also noted after transfection of the core gene into murine fibroblasts (Chang *et al.,* 1998; Tsuchihara *et al.,* 1999). Expression of HCV core protein leads to the development of progressive hepatic steatosis (fatty change) and hepatocellular carcinoma in two independent lines of transgenic mice (Moriya *et al.,* 1997, 1998).

HCV is a typical RNA virus, and there is no evidence of integration of the viral genome into host chromosomes. Oncogenesis is a multistep process and thus makes it difficult to understand the precise mechanism of transformation at the cellular level. HCV gene expression that is unique to permissive natural host cells may influence the magnitude or spectrum of core protein activity. In this study, the core genomic region was introduced into primary human hepatocytes to study its ability to affect cell growth regulation.

RESULTS

Growth properties and morphology of HCV core gene-transfected primary human hepatocytes

The immortalization potential of the HCV core protein was investigated by transfection of primary human hepatocytes, a natural host for HCV infection, with a core expression plasmid. Cells from three different donors were transfected with a plasmid construct of the genomic region under the control of a CMV early promoter or an antisense orientation of the plasmid DNA or empty vector DNA as negative controls. Transfected cells were maintained in serum-free culture medium at 32°C in a CO_2 incubator and passaged at a 3- to 4-day intervals for the first 20 days. Cells transfected with the antisense orientation of the HCV core or empty vector

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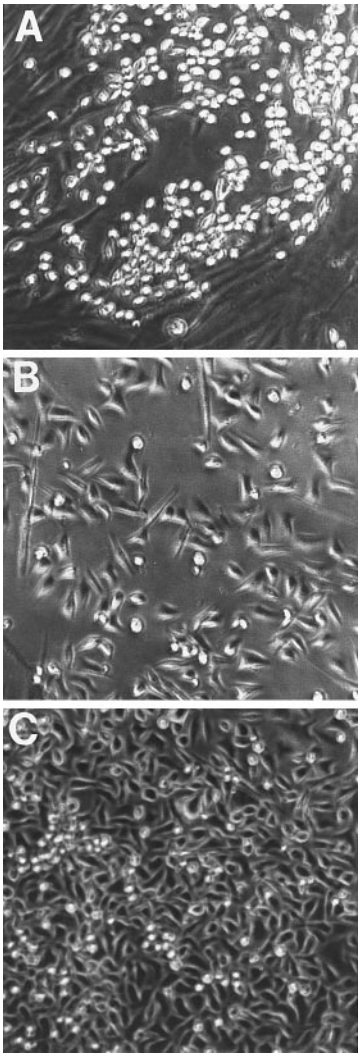

FIG. 1. Phenotypic changes of HCV core gene-transfected primary human hepatocytes after senescence. Transfected cells grown on collagen-coated plates displayed a dramatically altered phenotype at the end of the senescence period and had a slightly elongated morphology surrounded by a number of small round dead cells under phase-contrast microscopy (A). The immortalized cells then exhibited a change in phenotype to epithelial-like cells within 7 days (B) and were able to achieve greater confluency within another 1–2 weeks (C).

DNA as a negative control failed to exhibit growth after senescence (crisis period), which continued for about 12–16 weeks, at which time the cells began to degenerate. In contrast, hepatocytes transfected with core plasmid DNA replicated autonomously in primary culture at a frequency of $1.0–1.5 \times 10^{-4}$ after a senescence period of $\geq$16 weeks. A rapid phenotypic change of the hepatocytes at the beginning of replication from senescence or crisis period was observed (Block *et al.,* 1996). Under these conditions, some of the hepatocytes from senescence culture developed a slightly elongated morphology and began to form colonies which were initially surrounded by a number of small round dead cells (Fig. 1A). Within a week, as some of the hepatocytes continued to degenerate, 20–50 small colonies exhibiting a morphology more closely resembling epithelial cells were observed (Fig. 1B). These cells (passage 8) gradually increased in confluency and grew within 1–2 weeks as stable cell lines (Fig. 1C). In our hands, the transfected hepatocytes of two donors survived, while cell lines from one donor were lost due to contamination of the cell culture. Illustrations of negative control cells were not included as they did not recover from the senescence period and did not progress further. Cellular senescence is characterized by a decline in sensitivity to growth factors resulting in the cessation of cell growth. Replicative senescence is believed to represent a tumor suppression mechanism that the aspiring tumor cell must overcome in order to achieve an immortal state. Growth beyond the replicative senescence checkpoint correlates well with genetic lesions that interfere with one or more of the key cellular mortality pathways (Bishop, 1991). The expression of HCV core protein appears to result in the establishment of hepatocytes with an unlimited proliferative potential or an immortalization phenotype as the negative control plasmid DNAs failed to exhibit a recovery of cell growth. Cells transfected with HCV core alone exhibited focus-forming morphology at a frequency of $5–8 \times 10^{-4}$. Colonies of cells which survived after senescence were trypsinized, pooled to avoid artifact arising from clonal analysis, and subcultured to develop cell lines. The majority of these cells (>70%) survived serial passage. HCV core-transfected hepatocytes exhibited a population doubling time of between 0.4 and 0.6 per day after passage 8.

Secretion of liver-specific protein by hepatocyte transfectants

To determine whether the hepatocytes retained functions under the culture conditions after transfection with HCV core gene, we examined the expression of genes whose protein products are cell/tissue type-specific and are considered hallmarks of the hepatocyte phenotype. Adult hepatocytes synthesize a variety of serum proteins such as albumin, glucose-6-phosphatase, and α_1-antitrypsin (A1AT), whereas α-fetoprotein is a major product of fetal hepatocytes (Derman *et al.,* 1991; Isom and Strom, 1992). Albumin secretion by the immortalized hepatocytes was confirmed by Western blot analysis (Fig. 2). An SV40 T-antigen-immortalized human hepatocyte cell line (THLE5B) was used as a positive control, and cells of nonhepatocyte origin, such as human embryonic lung epithelial (L-132) and baby hamster kidney (BHK) cells, were used as negative controls in this assay. Glucose-6-phosphatase activity was also observed in HCV core-transfected hepatocytes (data not shown). Immortalized hepatocytes under similar conditions, however, did not exhibit a detectable expression of A1AT mRNA or α-fetoprotein from an early stage of establish-

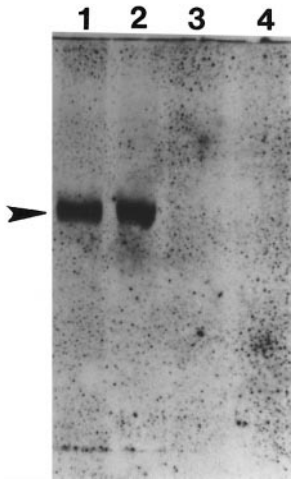

FIG. 2. Albumin secretion in the culture fluid from immortalized human hepatocytes by Western blot analysis. Results from THLE5B positive control (lane 1), HCV core-transfected immortalized hepatocytes (lane 2), and L-132 (lane 3) and BHK (lane 4) cells of nonhepatic origin as the negative controls are shown. An arrow on the left shows the position of the ~65-kDa albumin band detected from the culture supernatant. Molecular weight of the protein band was ascertained from positions of prestained molecular weight markers (BRL).

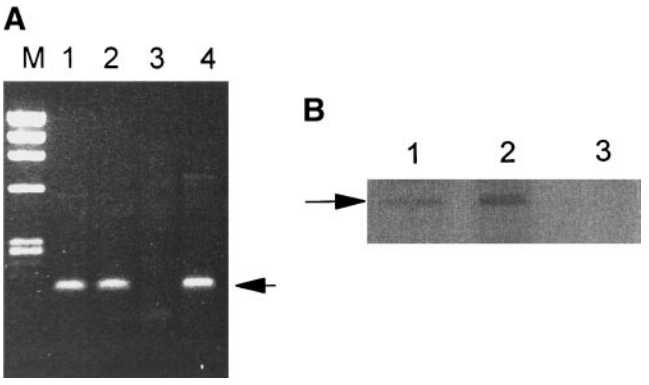

FIG. 3. (A) RT-PCR amplification of HCV core-specific mRNA from HCV core-transfected immortalized primary human hepatocytes of two different sets (lanes 1 and 2), SV40 T-antigen-immortalized THLE5B hepatocytes as a negative control (lane 3), and core plasmid DNA (lane 4) as a positive control is shown. The position of the ϕX174HaeIII DNA marker (M) is shown on the left. (B) Detection of HCV core protein expression by Western blot analysis using two different pools of immortalized human hepatocytes (lanes 1 and 2) and THLE5B as a negative control (lane 3). All lanes were edited from the same blot. HCV core-immortalized cells exhibited a major polypeptide of ~22 kDa as indicated by the arrow on the left. Molecular weight of the protein band was ascertained from positions of prestained molecular weight markers (BRL).

ment. The absence of A1AT may be due to the culture medium conditions (Wu *et al.,* 1994) or the development of a transformed phenotype, as suggested earlier with the loss of differentiated markers in SV40-transformed hepatocytes (Woodworth *et al.,* 1988). The lack of α-fetoprotein expression is not unexpected from an established cell line, as this indicates that they may be derived from differentiated hepatocytes (Pfeifer *et al.,* 1993). HCV core gene-transfected stable cell lines did not exhibit the presence of smooth muscle actin, suggesting the absence of stellate cells in the immortalized cell population.

HCV core gene expression in hepatocyte transfectants

Total RNA was extracted from HCV core gene-transfected hepatocytes and from negative control THLE5B cells. Extracted RNA was tested for HCV core-specific mRNA expression by RT-PCR using specific primers. Stable hepatocyte transfectants exhibited HCV core-specific mRNA expression, and the negative control RNA did not show a detectable HCV core-specific amplified product (Fig. 3A). GAPDH was used as an internal control and revealed the presence of RNA in all samples tested (not shown). Western blot analysis of cell lysates using a specific monoclonal antibody, C7-50, followed by chemiluminescence also exhibited core protein expression as a major band of ~22 kDa. This polypeptide band was present only in stable core transfectants established from the primary hepatocytes (Fig. 3B). HCV core gene transfectants demonstrated distinct periplasmic localization of the core protein by indirect immunofluorescence using a core-specific monoclonal antibody, C7-50 (Fig. 4). Core gene expression in the immortalized hepatocytes was detected at a number of passage levels by RT-PCR, Western blot analysis, and indirect immunofluorescence starting from passage 8 (when cells were stable after senescence) up to passage 40. We cannot conclude at this time whether persistent production of core protein is necessary for the maintenance of the immortalized state of these cells and will await further in-depth analysis.

Telomerase activity in immortalized hepatocytes

Telomerase is a ribonucleoprotein that synthesizes telomeric DNA repeats onto the ends of chromosomes. More than 85% of human cancers express telomerase

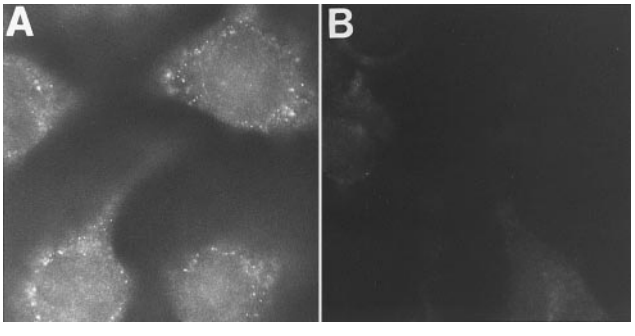

FIG. 4. Predominant periplasmic expression of HCV core protein in immortalized human hepatocytes (A) and untransfected primary hepatocytes as a negative control (B) was detected by immunofluorescence using a monoclonal antibody to the core protein.

activity and a large proportion of human hepatocellular carcinomas are positive. Either oncogene activation or a loss of tumor suppressor function is the probable mechanism by which the strict repression of the telomerase reverse transcriptase component in primary somatic cells is overridden (Greenberg *et al.,* 1999). Cell extracts from human epithelial cervical carcinoma (HeLa) cells as a positive control and human foreskin fibroblasts (HFF) as a negative control, along with the HCV core gene-transfected immortalized human hepatocytes, were used to measure the telomerase activity (Fig. 5A). Stable hepatocyte transfectants with HCV core gene at passage 8 exhibited distinct telomeres as observed with other positive control HeLa cells. These telomeres were observed to be elongated as the passage number of the transfected cells was increased from 8 to 15 to 40 (Fig. 5B). Each passage number corresponds to ~7 days culture after passage 8. However, telomerase activity was not detected in the mock-transfected primary human hepatocytes (PH), collected after 7 days of transfection. Since PH did not replicate for longer time, cell extracts from the early stage of the culture were used in this assay. These results suggested that telomerase is reactivated in core protein-immortalized primary human hepatocytes immediately after senescence (passage 8).

Anchorage-independent growth

Anchorage-independent growth in a semisolid medium of soft agar is a strong indicator of the transformed phenotype (Hamburger and Salmon, 1980). Thus, we tested whether cells immortalized following transfection with the HCV core gene might take on any characteristics associated with anchorage-independent growth in a soft-agar assay. Immortalized hepatocytes from passage 8 did not exhibit a strong growth on soft agar, exhibiting a small number of colonies. In contrast, the immortalized cells when tested at passages 15 and 40 grew on soft agar as microscopically visible colonies within 4 weeks of seeding. The typical morphology of cells grown on soft agar is shown in Fig. 6. These results suggest that anchorage independence is associated with cell lines having an established replicative turnover.

DISCUSSION

Human cells are refractory to establishment and spontaneous immortalization of normal human cells *in vitro* is an extremely rare event. Primary hepatocytes have a limited life span in culture. Immortalization of primary human hepatocytes following the introduction of HCV core gene and an associated telomerase activity immediately after senescence, characteristic of an immortalized phenotype, was evident from our studies. It is possible that the presence of core protein is necessary at least up to passage 8 for immortalization. The mechanism by which cells bypass senescence and become

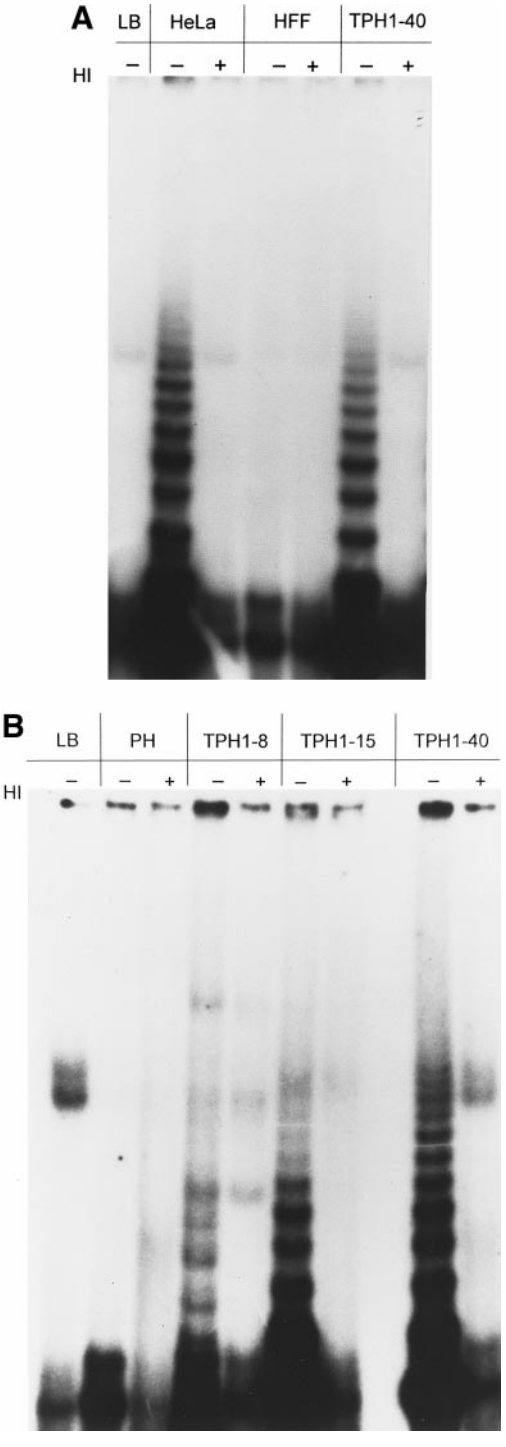

FIG. 5. Telomerase activity in positive and negative control cells of human origin and in HCV core-transfected primary human hepatocytes (TPH1). Lysis buffer (LB), in the absence of cell extracts, was also used as a negative control. The enzyme activity in positive (HeLa) and negative (HFF) control cells (A) and at different stages of cell passage (B) is shown. Lanes are marked on top for cells (HeLa, HFF, TPH1) or hepatocyte cell passage number (TPH1 passage 8, 15, and 40). PH indicates mock-transfected primary human hepatocytes after 7 days of transfection and HI indicates heat-inactivated samples analyzed for telomerase activity. "+" denotes heat-inactivated samples and "−" denotes samples without heat treatment.

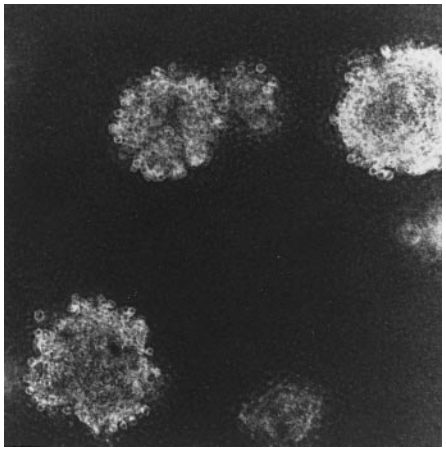

FIG. 6. Phenotypic properties of the immortalized primary human hepatocytes following transfection with core gene. Immortalized hepatocytes from passage 15 were grown on soft agar. The typical anchorage-independent growth and colony formation of cells from passage 15 after 4 weeks on soft agar are shown.

immortalized is still an open question (Sedivy, 1998). Immortalization of human cells requires the reactivation of the telomere-lengthing enzyme, telomerase (Greider and Blackburn, 1985; Harley, 1991), as the maintenance of telomeres is required for cells to escape from replicative senescence and proliferate indefinitely (Counter *et al.,* 1992). It is possible that the cells will be irretrievably committed to growth once the telomerase is activated. Normal cells have a strictly limited growth potential and remain senescent after a defined number of population doublings. In contrast, tumor cells often exhibit an apparently unlimited proliferative potential and are termed immortalized. A variety of viral proteins have been reported to immortalize cells, suggesting that multiple mechanisms may lead to an escape from senescence. It is likely that some components in the signal transduction pathway(s) are not expressed or are not functioning in the senescent cell. Immortalization is an early event in malignant transformation. The introduction of immortalizing genes may provide functions that are inactive in the senescent cells or possibly overcome in some manner the effects of growth-suppressing activities in senescent cells (Linder and Marshall, 1990).

A recent finding of *de novo* hepatocellular carcinoma with recurrent HCV and cirrhosis, 7 years after orthotropic liver transplantation, reiterates the oncogenic potential of HCV (Saxena *et al.,* 1999). Hepatic steatosis occurs at a high rate in chronic hepatitis C patients, even in those with cirrhosis, and a close relationship between the presence of steatosis and intrahepatic core protein has been noted (Fujie *et al.,* 1999). A defective viral genome has been observed to arise from deletions or mutations in HCV persistently infected cells (Ruster *et al.,* 1996; Shimizu *et al.,* 1997; Yeh *et al.,* 1997). HCV often causes persistent infection and there is no evidence that the HCV genome integrates in infected cells. Cell cycle progression is inhibited in productively infected cells and most infected cells are eventually killed as the virus completes its replication cycle. However, if cell growth is induced by a nonproductive infection, the continued presence of viral gene products would likely be detrimental for host cells.

A virus can initiate potentially oncogenic events by a number of different mechanisms. The ability of the core protein to alter the growth properties of primary human hepatocytes is consistent with the results from our and other studies which examined the effect of core protein upon the growth of cells of nonhepatic origin (Ray *et al.,* 1996b; Chang *et al.,* 1998; Tsuchihara *et al.,* 1999). Our earlier experiment with primary rat embryo fibroblasts (Ray *et al.,* 1996b) suggested that core protein along with a cooperative H-ras oncogene may transform cells into an immortalized and tumorigenic phenotype. A similar observation was noted using a subclone of an established murine fibroblast (BALB/3T3 A31-I-1) which is relatively resistant to ras-induced transformation and requires the presence of another oncogene (Tsuchihara *et al.,* 1999). Chang *et al.* (1998) also performed similar work, as we reported earlier, using primary REF cells. Under their experimental conditions, primary REFs did not exhibit focus formation and failed to survive. The reason for this discrepancy is unclear and may arise from a number of factors as already discussed (Chang *et al.,* 1998), including minor amino acid changes in the HCV core protein from different virus strains used. It is also important to note that in our earlier study a predominant nuclear localization of the core protein was observed upon cotransfection of REF cells with core and a cooperative oncogene. However, in this study or in an earlier study using core-transfected HeLa cells (Ray *et al.,* 1996a), a predominant perinuclear localization of the core protein was observed. Use of two distinct cell types (REFs and epithelial cells) and different experimental conditions could be possible reasons for this difference and will need further investigation. The possibility that the core protein functions may arise from overexpression of the transfected gene cannot be ruled out. However, overexpression does not appear to be a determining factor in this case as REF cells transfected with the same plasmid and a c-myc cooperative oncogene did not generate immortalize phenotype even though a similar level of core protein expression was apparent (Ray *et al.,* 1996b). The core protein expression level in transfected cells may have an impact on the magnitude of the effect without altering the functional activity of this viral protein.

Hepatocarcinogenesis involves alterations in the concerted action of proto-oncogenes, growth factors, and tumor suppressor genes. The effects of HCV core protein might result either from direct or indirect activation of transgene expression or from an independent pathway which leads to an alteration in hepatocyte turnover. It is likely that the core protein contributes directly to the

proliferation of infected host cells and malignant cells may arise from immortalized hepatocytes. The lack of a suitable cell culture system and small animal model for HCV infection makes it difficult to understand virus replication and the pathogenesis of HCV-related disease. However, as other HCV proteins (NS3 and NS5A) have also been implicated in HCV persistence and pathogenesis (Sakamuro *et al.,* 1995; Gale *et al.,* 1999; Ghosh *et al.,* 1999), it is difficult to correlate the functional effect of core protein when expressed together with the other proteins of the virus. However, the results presented here clearly establish a cell growth regulatory role for the core protein in primary human hepatocytes, a natural host for HCV. The capacity to immortalize primary human hepatocytes will provide a valuable model for studying the regulation of hepatocyte proliferation-related phenomena by the HCV core gene and associated factors, as they may relate to multistage hepatocarcinogenic events.

MATERIALS AND METHODS

Cells and plasmids

Normal human hepatocytes prepared by a collagenase/dispase perfusion of three adult autopsy donors with no clinical evidence of cancer and an absence of antibodies to hepatitis A, B, and C viruses, and HIV, were obtained from two different sources (Human Cell Culture Center, Inc., Woodbine, GA, and Clonetics, San Diego, CA). Cells seeded on collagen type I-coated plates were delivered by a special carrier within 24 h of cell harvest. Medium of the cell culture plate was changed with hepatocyte maintenance medium (Clonetics, San Diego) and incubated overnight at 37°C. CMV-Core (amino acids 1–191), an antisense orientation of the core, or pcDNA3 empty vector plasmid was used for transfection of hepatocytes in this study. The plasmids were derived as described earlier (Ray *et al.,* 1996b) from a partial cDNA clone of HCV 1a (Blue4/C5p-1), kindly provided by Dr. Michael Houghton (Chiron Corp., CA). A nonneoplastic human hepatocyte cell line (THLE5B), generated by transfection of primary human liver epithelial cells with SV40 T antigen (Pfeifer *et al.,* 1993), was kindly provided by Dr. Curtiss C. Harris (National Cancer Institute, MD) and was used as a control cell line when needed.

Transfection and maintenance of hepatocytes

Cells were transfected with HCV core plasmid, the antisense orientation of the core, or an empty vector using strontium phosphate as described earlier (Brash *et al.,* 1987; Pfeifer *et al.,* 1993). Briefly, cells plated overnight were refed with fresh culture medium 2 to 4 h before transfection. HCV core gene (1 μg) was transfected with 18–19 μg of carrier salmon sperm DNA. For negative control, a plasmid containing the antisense orientation of the plasmid or empty vector DNA was used. The cells were washed and fed with fresh medium 1.5 h posttransfection, refed every 2 to 3 days, and passaged at a 3- to 4-day intervals for ~20 days. An enriched PFMR-4 medium (Biofluids, MD) supplemented with EGF, transferrin, retinoic acid, bovine pituitary extract, glutamine, insulin, hydrocortisone, T_3, P/E stock, and gentamicin or a defined culture medium supplemented with growth factors and antimicrobials for small airway epithelial cell growth (SAGM; Clonetics, CA) was tested for hepatocyte growth. In our hands, enriched PFMR-4 or SAGM exhibited similar hepatocyte growth profiles (as also observed in control THLE5B cells). The number of proliferative foci in transfected hepatocytes was determined 16–20 weeks after transfection. Pooled colonies from transfected cells were recovered by trypsinization (0.025% trypsin containing 0.01% EDTA in HBSS; Clonetics), followed by treatment with trypsin-neutralizing solution (Clonetics). Cells were centrifuged and transferred to fresh collagen-coated plates and incubated with culture medium for growth at 33°C in a CO_2 incubator.

Test for hepatocyte markers

Albumin secretion by hepatocytes in the culture medium was examined by Western blot analysis. Culture fluid from cells grown in serum-free medium (3 ml from a 72-h culture of ~10^6 cells/ml) was mixed with 2× loading buffer and subjected to SDS–PAGE, followed by transfer onto nitrocellulose. Proteins on the nitrocellulose blot were analyzed with a goat anti-human albumin peroxidase conjugate (Cappel, PA) and the peroxidase signal was detected by chemiluminescence (ECL; Amersham). In a similar manner, expression of α-fetoprotein in the cell lysates was analyzed using a specific goat antibody (Cappel) by Western blot analysis. Expression of A1AT was analyzed by blot hybridization of cytoplasmic RNA extracted from nearly confluent cultures. A 540-nucleotide radiolabeled antisense RNA probe corresponding to the 3′ end of mouse A1AT mRNA, known to cross-hybridize with human A1AT RNA, was used for detection of mRNA. The activity of glucose-6-phosphatase in the cell homogenate was measured using a microtechnique modified from Bruchell *et al.* (1988). Western blot analysis of cell lysates with antibody to smooth muscle actin (Sigma Chemical Company, MO) was performed to examine the marker for cells of primary hepatic stellate origin as already described (Brown *et al.,* 1997). A positive control of hepatic stellate cells was included in this assay.

Detection of HCV core gene expression

Total RNA was isolated from hepatocytes and subjected to RT-PCR as already described (Ray *et al.,* 1998). HCV core-specific sequences were amplified (amino ac-

ids 102 to 160) from reverse-transcribed RNA (cDNA) by PCR using HCV core sequence-specific primers (sense 5′GGCTCTCGGCCTAGCTGG3′ and antisense 5′CTTCCAGAATTCGGACGCCAT3′). The amplified DNA was analyzed by 1.5% agarose gel electrophoresis. Core protein expression was separately examined by Western blot analysis (Ray *et al.,* 1996a) using a specific murine monoclonal antibody, C7-50 (Moradpur *et al.,* 1996), kindly provided by Dr. Jack R. Wands (Harvard Medical School, MA). An anti-mouse immunoglobulin coupled with horseradish peroxidase was used as the second antibody to detect HCV core protein by chemiluminescence (ECL; Amersham). Stable hepatocyte transfectants were also tested for expression of HCV core protein by indirect immunofluorescence. Briefly, cells were treated with 1% formaldehyde for 30 min at room temperature. After being washed, cells were incubated with ethanol/acetic acid (90/10) for 1.5 min at 4°C. Cells were washed and treated with a HCV core protein-specific monoclonal antibody, C7-50. A second antibody, anti-mouse Ig conjugated to fluorescein isothiocyanate, was used for detection of the core protein by fluorescence microscopy.

Telomerase assay

Telomerase activity was detected by a PCR-based telomerase-repeat amplification protocol (TRAP) as described earlier (Bryan *et al.,* 1995; N. W. Kim *et al.,* 1994). Cell lysates were prepared from 10^5 cells using CHAPS detergent buffer and the protein concentrations of the cellular extracts were determined using a commercially available assay kit (Bio-Rad). CHAPS cell extracts were incubated in the presence of four dNTPs at 50 μM each, 10× TRAP buffer (N. W. Kim *et al.,* 1994), [γ-^{32}P]ATP-labeled 0.1 μg TS primer (5′AATCCGTCGAGCAGTT3′), 1 μg of T4g32 protein (Roche), 0.1 μg of CX primer (5′[CCCTTA]$_3$ CCCTAA3′), and 2 U of *Taq* DNA polymerase in a 50-μl reaction volume. Lysis buffer without cell extracts or heat-inactivated cell extracts were used as the negative controls. After extension of TS primer by telomerase for 30 min at 30°C, tubes were transferred to a thermocycler for amplification of 27 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. One-half of the reaction was analyzed by electrophoresis on a 12.5% native polyacrylamide (19:1) gel in 0.5× TBE. The gel was exposed to X-ray film at −70°C for autoradiography.

Soft-agar assay

Transfected cells were tested for anchorage-independent growth and proliferative foci formation on soft agar as described earlier (Ray *et al.,* 1996b). Cells were examined over an incubation period of 6 weeks to determine colony formation. Colonies exceeding ~0.1 mm in diameter were considered positive.

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