

Hepatitis C Virus Core Protein Cooperates with *ras* and Transforms Primary Rat Embryo Fibroblasts to Tumorigenic Phenotype

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We have previously demonstrated that hepatitis C virus (HCV) core protein regulates cellular proto-oncogenes at the transcriptional level; this observation implicates core protein in the alteration of normal hepatocyte growth. In the present study, the transforming potential of the HCV core gene was investigated by using primary rat embryo fibroblast (REF) cells which were transfected with or without cooperative oncogenes. Integration of the HCV core gene resulted in expression of the viral protein in REF stable transformants. REF cells cotransfected with HCV core and *H-ras* genes became transformed and exhibited rapid proliferation, anchor-independent growth, and tumor formation in athymic nude mice. Results from these studies suggest that the core protein plays an important role in the regulation of HCV-infected cell growth and in the transformation to tumorigenic phenotype. These observations suggest a possible mechanism for this viral protein in the pathogenesis of hepatocellular carcinoma in HCV-infected humans.

Hepatitis C virus (HCV) accounts for most cases of acute and chronic non-A, non-B liver disease (1, 10). The persistence of HCV in infected humans despite the presence of antibodies reactive to the putative envelope glycoproteins has previously been observed (9, 31). Isolate-specific neutralizing antibody responses (14) and serotypic variations among viral isolates have been suggested to be important features of HCV (30). The high degree of genetic heterogeneity of HCV in vivo, manifested both in the generation of viral quasispecies and in the continuous emergence of neutralization escape mutants, poses an obstacle to the development of a broadly reactive HCV vaccine based on antibody reactivity to HCV envelope glycoproteins (14). The most important feature of persistent HCV infection is the development of chronic hepatitis in half of the individuals infected with the virus and the potential for disease progression to hepatocellular carcinoma (5, 12, 20, 40). The viral genome has been detected in liver cells (25) with pathological changes.

The proto-oncogenes play an important role in cell proliferation and differentiation (3). Hepatocarcinogenesis involves alterations in the concerted action of proto-oncogenes, growth factors, and tumor suppressor genes (37). The gene corresponding to the core protein is relatively conserved among HCV strains (6). The presence of two conserved potential nuclear localization signals and a DNA binding motif in HCV core protein suggests its functional role as a gene regulatory protein (6, 46). Our earlier observation (35) suggests that the HCV core protein interacts with cellular proto-oncogenes at the transcriptional level, and this may implicate promotion of cell proliferation, thus affecting normal hepatocyte growth. The pathogenesis of hepatocellular carcinoma, therefore, may partly be due to the regulation of hepatocyte growth by HCV core protein. In this study, we have investigated the transforming potential of HCV core protein upon introduction into

primary rat embryo fibroblast (REF) cells. Results from this study suggested that HCV core protein in association with cellular oncogenes plays a major role in the immortalization and malignant transformation of cells.

MATERIALS AND METHODS

Cells and plasmids. Fisher rat primary embryo fibroblast cells (BioWhittaker, Walkersville, Md.) were grown in Eagle's minimum essential medium supplemented with 10% heat inactivated fetal calf serum. A partial cDNA clone of HCV-1 containing the 5' untranslated region; the C, E1, and E2 regions; and a portion of the NS2 region (kindly provided by Michael Houghton, Chiron Corporation, Emeryville, Calif.) was used as a template for amplification of the core region (amino acid residues 1 to 191) by PCR by a procedure similar to that described previously (21, 35). An HCV core gene construct was made by inserting the PCR-amplified genomic region into the mammalian expression vector pBabe/puro (34) bearing the Moloney murine leukemia virus long terminal repeat. The orientation of the recombinant gene construct was verified by digestion of the plasmid DNA with suitable restriction enzymes. Two plasmid DNAs expressing the human *c-myc* (33) and activated *H-ras*^{Val-12} (American Type Culture Collection) were used as cooperative oncogenes in the cotransfection of primary cells with the HCV core gene.

Cell transfection. REFs (5×10^5 cells per 10-cm-diameter plate) were transfected with the HCV core gene construct with or without the plasmid DNA containing the oncogenes by a procedure similar to that described earlier (33). Briefly, cells were fed with fresh culture medium 2 to 4 h before transfection. The HCV core gene (1 μ g) was transfected with or without 1 μ g of the *H-ras* gene or the *c-myc* gene and 18 to 19 μ g of carrier salmon sperm DNA by calcium phosphate coprecipitation (Bethesda Research Laboratories, Gaithersburg, Md.). The cells were washed with phosphate-buffered saline (PBS) and fed with fresh medium at 20 h posttransfection. The cells were refed every 4 to 6 days, and the number of transformed foci was determined at 2 to 3 weeks posttransfection (4). Morphologically transformed cells were visible within 2 weeks following transfection. For negative control, a plasmid containing the oncogene alone and the vector or antisense orientation of the HCV core plasmid were used for comparison. Adenovirus E1A and *H-ras* genes were used in cotransfection of REF cells as a positive control. Several foci from each set of transfections were picked and grown separately or together to study the transformation properties.

Soft agar assay. Cell colonies recovered after transfection were tested for proliferative focus formation and anchorage-independent growth in soft agar by a procedure similar to that described earlier (36). Each cell type was tested in triplicate for growth in soft agar. For the determination of cloning efficiency in agar, 10^3 cells were plated in a 35-mm-diameter dish. Liquid medium (0.2 ml) was layered over the agar cultures periodically to prevent desiccation. The number of large colonies (>0.1 mm in diameter) with dense centers was counted for each plate after 3 weeks.

Tumorigenicity in nude mice. To assay for tumor formation in vivo, stable transfectants were grown, washed with PBS, and resuspended in serum-free

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TABLE 1. Transformation of REF cells by transfection with the HCV core gene

Gene construct used in cotransfection	Oncogene(s) used in cotransfection	No. of foci obtained from expt ^a :		
		I	II	III
pBabe-HCV ₁₋₁₉₁	H- <i>ras</i>	139	117	125
pBabe-HCV ₁₋₁₉₁	<i>c-myc</i>	45	65	ND ^b
pBabe-HCV ₁₋₁₉₁	None	0	ND	2
None	H- <i>ras</i>	0	0	ND
None	<i>c-myc</i>	0	0	ND
None	H- <i>ras</i> and <i>c-myc</i>	7	26	ND
pUC-E1A	H- <i>ras</i>	178	ND	ND
pBabe-puro	None	ND	0	ND
pBabe-HCV ₁₋₉₁ (antisense orientation)	H- <i>ras</i>	ND	0	2
pBabe-HCV ₁₋₉₁ (antisense orientation)	<i>c-myc</i>	0	1	ND

^a Each primary plate was transfected as described in Materials and Methods, and numbers of foci obtained from three independent experiments are shown. Foci obtained from transfection of cells with H-*ras* and *c-myc* or pBabe-HCV₁₋₁₉₁ alone were flat and did not survive after the second or third passage.

^b ND, not determined.

medium. Cells (10⁷ per injection) were implanted subcutaneously into the flanks of female athymic nude mice. Four- to six-week-old female athymic nude mice (*nu/nu*) were procured from Taconic Farms (Germantown, N.Y.). Mice were housed in microisolator cages and provided with sterile food and water. Tumor formation was assessed twice a week for 10 weeks. Animals were sacrificed when tumor size reached ~2.5 cm in diameter. Tumors were collected and processed for the recovery of cells in culture and for extraction of DNA by the established procedures (33). Viable cells recovered from tumors were tested for protein expression by indirect immunofluorescence. The nucleic acids extracted from tumors were used for the detection of HCV core genomic sequence by Southern hybridization.

Nucleic acid analysis. DNA was isolated from the transformed REF cells or from mouse tumors by a procedure similar to that described earlier (33). HCV core genomic sequence was amplified (amino acids 1 to 154) from a similar quantity of the isolated DNA by PCR using HCV core sequence-specific primers (sense, 5'GTGCTTGCGAATTCCCGGGA3', and antisense, 5'CTTCCAGAAATTCGGACGCCAT3'). The sequences of the primers were adopted from the published sequence of HCV-1 (11). Minor nucleotide changes as underlined in the primer sequences reflect the creation of *EcoRI* restriction sites for convenient cloning whenever necessary. PCR amplification was performed by a procedure similar to that previously described (21). The amplified DNA was analyzed by 1.5% agarose gel electrophoresis. For detection of HCV core genomic sequences in mouse tumors, DNA isolated from tumors was digested with *EcoRI* and electrophoresed on 1.5% agarose gel. Separated DNA was transferred onto nitrocellulose and hybridized with a randomly primed ³²P-labeled HCV core DNA by a procedure similar to that described earlier (32).

Expression of HCV core protein. Stable transfectants or cells recovered from mouse tumors were tested for expression of HCV core protein by indirect immunofluorescence using specific antibodies. Briefly, cells were treated with 1% formaldehyde for 30 min at room temperature. After washing, cells were further incubated with ethanol-acetic acid (90:10) for 1.5 min at 4°C. Cells were washed and treated with the primary antibody. The anti-C22 antibody (43) (kindly provided by M. Houghton, Chiron Corporation) was used as a primary antibody. A second antibody, anti-rabbit immunoglobulin conjugated to rhodamine isothiocyanate, was used for detection of the core protein by immunofluorescence. For immunoprecipitation of HCV core protein, ~10⁶ cells were incubated for 1 h at 37°C in methionine-free medium and metabolically labeled for 4 h with the addition of 250 µCi of [³⁵S]methionine-cysteine mix (Dupont, NEN). Labeled cells were rinsed with cold PBS and incubated with lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.4% Nonidet P-40, 0.5% aprotinin) on ice for 40 min. Cell lysates were mixed by vortexing and centrifuged at 14,000 × *g* for 10 min at 4°C. Clarified lysates were mixed with pooled HCV-infected human sera (31) from three different patients (038, 063, and 093) on a rocker at 4°C overnight. Protein A immobilized on Sepharose 4B (Pharmacia) beads was added to this mixture, and the resulting mixture was incubated under similar conditions for an additional 2 h. Beads were washed extensively with the lysis buffer and suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Samples were heated at 100°C for 3 min, and immunoprecipitated proteins were resolved on SDS-15% polyacrylamide gels for analysis by autoradiography.

RESULTS

Transfection and cloning efficiency. The HCV core gene was used to determine its transformation potential by introduction of this gene into primary REF cells with or without the cooperative oncogenes. As a negative control, the HCV core gene

was substituted with the vector DNA or the antisense HCV core gene for transfection. Several foci were obtained from experimental transfections (Table 1), and immortalized cell lines were established. Similar cotransfection experiments using primary baby rat kidney cells were also performed, and several focus-forming colonies were obtained following cotransfection with HCV core and H-*ras* or *c-myc* genes (data not shown), supporting results obtained with the REF cells. On the other hand, transfection with the negative control did not show any focus formation or immortalization of cells. A number of foci randomly isolated from each transfection set of REFs were grown separately. HCV core gene- and H-*ras*-transfected foci showed higher levels of cell immortalization (71%) than did HCV core gene- and *c-myc*-transfected cells (57%). Immortalized cells were grown as a pool of five individual foci from each set of transfections. HCV core and H-*ras* or *c-myc* gene-cotransfected cells have remained immortalized for at least 30 passages tested to date. Pooled immortalized cells from each transfection set were analyzed to establish their respective growth rates by plating equal numbers of cells and counting cell numbers by trypan blue exclusion at 24-h intervals. The two types of REF transfectants showed similar growth patterns up to 48 h. However, HCV core gene- and H-*ras*-cotransfected cells gave evidence of accelerated growth after 48 h compared with the cells cotransfected with HCV core and *c-myc* genes (Fig. 1). A similar observation was also made when at least three individual clones were tested for cell growth.

Anchorage-independent growth. Anchorage-independent growth in a semisolid medium of soft agar is a strong indicator of the transformed phenotype (15). Thus, we tested whether the cells immortalized following transfection with the HCV core gene might take on any characteristics associated with anchorage-dependent growth in a soft agar assay. To assess relative anchorage independence, for each cell type the number of colonies that grew in soft agar was determined and normalized to the frequency of colony formation in liquid medium. The average number of colonies counted, represented as percentages of growth, were as follows: 70% for cells cotransfected with gene constructs pBabe-HCV₁₋₁₉₁ and H-*ras* and <10% for cells cotransfected with gene constructs pBabe-HCV₁₋₁₉₁ and *c-myc* (results are means of those from three independent experiments). Cells cotransfected with HCV core and H-*ras* genes showed anchorage-independent colonies (Fig. 2). Cells immortalized following cotransfection with HCV core and *c-myc* genes did not show growth in the soft agar assay. These results suggested that anchorage independence is asso-

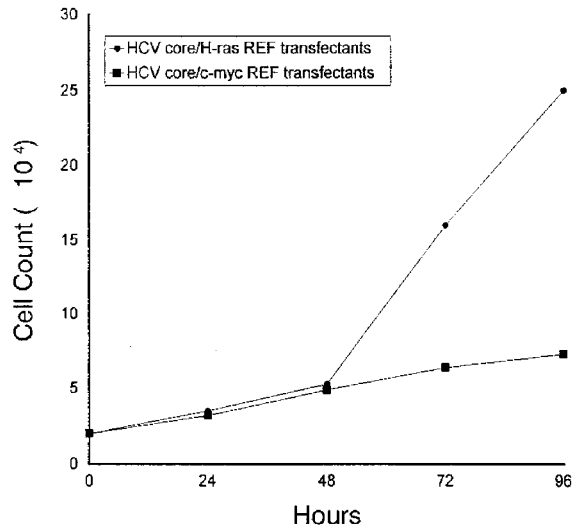


FIG. 1. Comparison of the growth rates of REF cells transformed by cotransfection with HCV core and *c-myc* or *H-ras* genes.

ciated with the HCV core gene when it is cotransfected with *H-ras*.

Tumorigenicity of HCV core gene transfectants in nude mice. To assess the effect of the HCV core gene in immortalizing primary cells, 10^7 cells were injected subcutaneously into nude mice. Six of six mice injected with HCV core (pBabe-HCV₁₋₁₉₁) and *H-ras* gene-transformed cells developed a tumor(s) within ~2 weeks after inoculation (transfected cells showing tumor formation were ~2.5 cm in diameter). Three

individual clones picked from the transformed cells also formed tumors in nude mice when tested separately. On the other hand, zero of six mice injected with the immortalized cells obtained following cotransfection with HCV core (pBabe-HCV₁₋₁₉₁) and *c-myc* genes showed tumor formation following a 10-week observation period. Results from this study indicated that tumorigenicity, like anchorage independence, is specific for HCV core and *H-ras* gene-cotransfected REF cells.

Nucleic acid analysis for integration of the HCV core gene in transformed cells. In order to further characterize the immortalized nontumorigenic or tumorigenic cell lines, the integration of the HCV core gene was examined. DNA extracted from transformed cells was tested by PCR for amplification of the integrated HCV genomic sequences. The typical amplification of the HCV core gene from REF transfectants is shown in Fig. 3A. Cells transformed with HCV core and *H-ras* or *c-myc* genes suggested the integration of the core gene (lanes 2 and 3). DNA from E1A- and *H-ras*-transfected cells (lane 1) and HCV core plasmid DNA (lane 4) were used as the negative and positive controls, respectively. Minor nonspecific bands with smaller molecular sizes were observed with all the samples tested.

Genomic DNAs extracted from tumors of nude mice were analyzed by Southern hybridization for detection of the core gene. A typical hybridization of the core sequence is shown in Fig. 3B. DNA from all three mouse tumors tested showed strong hybridization of bands and migrated at ~573 bp in a manner similar to that of an ethidium bromide-stained positive control DNA band from *Eco*RI-digested core plasmid DNA (not shown). On the other hand, DNA from E1A- and *H-ras*-transfected negative control cells failed to show any detectable reactivity in Southern hybridization.

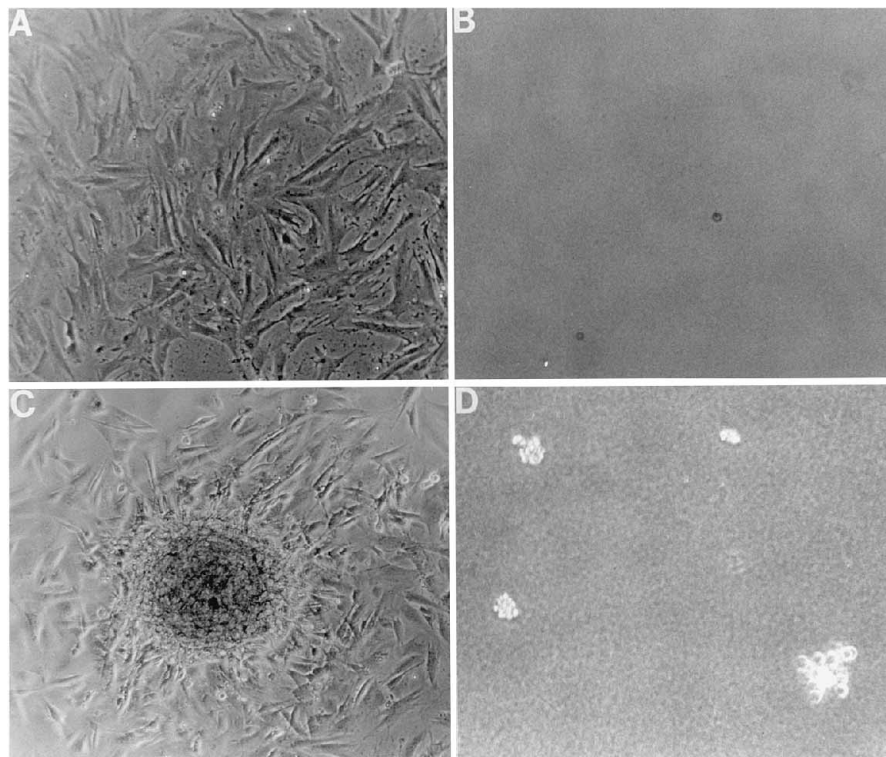


FIG. 2. Morphology of REF cells transformed by HCV core and *c-myc* (A) or *H-ras* (C) genes. Colony formation by HCV core gene- and *c-myc* (B)- or *H-ras* (D)-cotransfected REF cells in a soft agar assay is also shown.

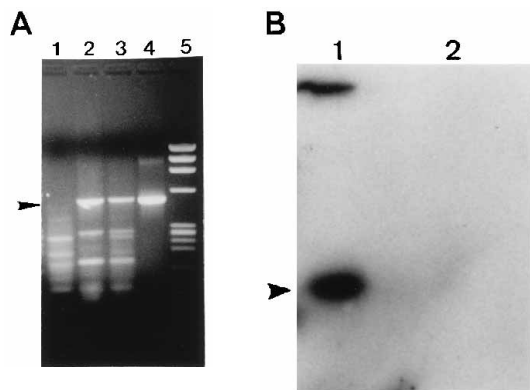


FIG. 3. Integration of the HCV core gene in transformed cells. (A) PCR amplification for the detection of integrated HCV core genes in immortalized or tumorigenic REF cells. PCR-amplified DNA from E1A- and *H-ras*-transformed cells as a negative control (lane 1), HCV core gene- and *H-ras*-transformed cells (lane 2), HCV core gene- and *c-myc*-transformed cells (lane 3), and HCV core plasmid DNA as a positive control (lane 4) were separated by 1.5% agarose gel electrophoresis. The positions of ϕ X174-*Hae*III digest (Bethesda Research Laboratories) molecular size markers are shown (lane 5). The arrow on the left shows the position of an ~462-bp amplified HCV core sequence. (B) Southern hybridization of mouse tumor DNA with an HCV core-specific probe. Genomic DNA from a mouse tumor (lane 1) or from E1A- and *H-ras*-transformed REF cells as a negative control (lane 2) was digested with *Eco*RI and separated by 1.5% agarose gel electrophoresis. Transferred DNA on the nitrocellulose blot was hybridized with randomly primed 32 P-labeled HCV core DNA as a probe. The arrow on the left shows the position of the HCV core gene.

Expression of HCV core protein in stable transfectants.

Expression of HCV core protein in the immortalized REF cells or in cells recovered from mouse tumors was examined by indirect immunofluorescence. Cells immortalized following cotransfection with HCV core and *c-myc* or *H-ras* genes showed intracellular expression of the HCV core protein as granular inclusion bodies in the nucleus. A similar pattern of immunofluorescence was also observed in cells recovered from tumors following inoculation of HCV core gene and *H-ras* transfectants (Fig. 4). On the other hand, primary REF cells transiently transfected with vector DNA as a negative control showed a complete absence of reactivity. Nuclear localization of core protein in the absence of downstream E1 envelope protein sequence has been reported previously for CV1 and HuH-7 cells (27, 46).

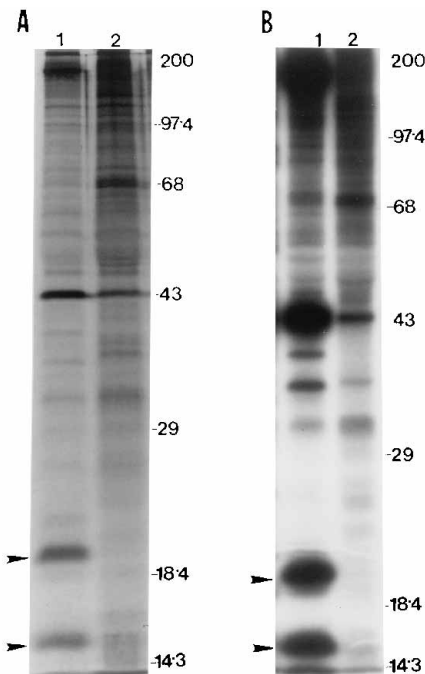


FIG. 5. Immunoprecipitation of HCV core protein from transformed REF cells. Cells cotransfected with HCV core gene- and *H-ras* (A)- or *c-myc* (B)-transformed REF cells were labeled with [35 S]methionine-cysteine. Transformed REF cell lysates were immunoprecipitated with pooled HCV-infected human sera (lanes 1). Similarly, transiently mock-transfected REF cells were used as the negative controls (lanes 2). Immunoprecipitates were analyzed by SDS-15% PAGE under reducing conditions. Arrows on the left indicate the positions of HCV core protein. Approximate positions of the low-molecular-weight prestained markers (Bethesda Research Laboratories) are shown on the right.

Cell lines immortalized by cotransfection with HCV core and *c-myc* or *H-ras* genes were separately tested for core protein expression by immunoprecipitation. Two major polypeptide bands of ~16 and ~20 kDa were immunoprecipitated by HCV-infected pooled human sera (Fig. 5). A similar experiment using transiently mock-transfected REF cells as the negative control did not show precipitation of these polypeptides. Additional polypeptide bands precipitated by HCV-infected human sera from transformed cell lysates probably represent

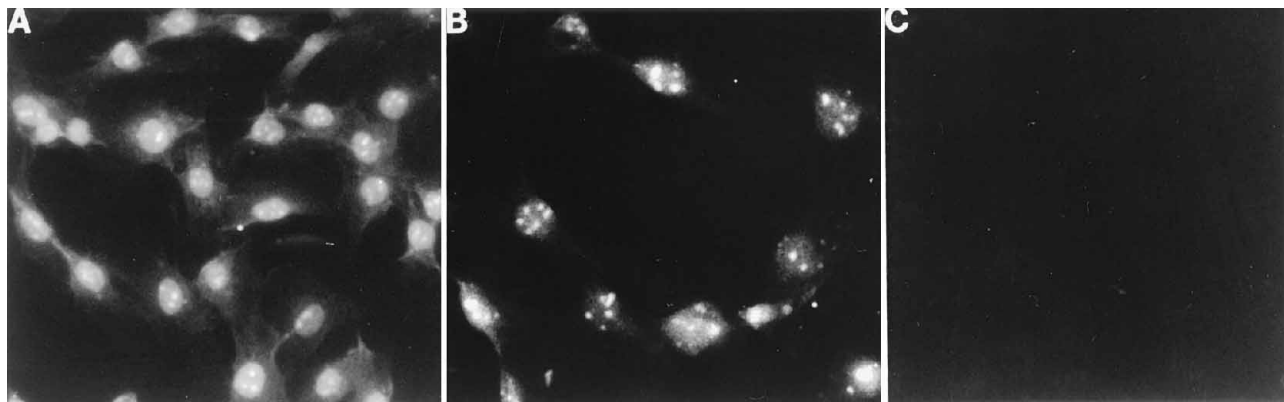


FIG. 4. Indirect immunofluorescence using transformed REF cells. Cells cotransfected with HCV core and *c-myc* genes (A) and cells recovered from tumors generated by inoculation of HCV core gene- and *H-ras*-transformed REF cells (B) were reacted with anti-C22 antibody to HCV core protein and stained with anti-rabbit immunoglobulin-rhodamine isothiocyanate conjugate. Results from a similar experiment using vector DNA-transfected REF cells as a negative control are also shown (C).

homopolymeric or heteropolymeric complexes under these experimental conditions and require further characterization. An earlier report (27) suggested that in the presence of downstream E1 envelope protein sequence, polypeptides of ~19 and ~21 kDa appear as the major core gene products. However, in the absence of the envelope protein sequence a polypeptide of ~16 kDa becomes the major HCV-1 core gene product. Under our experimental conditions, HCV-infected human sera recognized two distinct polypeptides of ~16 and ~20 kDa from the transformed cell lines.

DISCUSSION

Although it is likely that both virus-induced and immunologically mediated mechanisms play an important role in pathogenesis, the mechanisms responsible for HCV persistence and disease are not well understood (8, 19). Previously, we have shown that HCV core protein regulates cellular proto-oncogenes at the transcriptional level and that this implicates the core protein in alterations of normal hepatocyte growth (35). In the present study, we have demonstrated that the HCV core gene in cooperation with the *H-ras* or *c-myc* gene immortalizes REF cells. Additionally, cells cotransfected with the core and *H-ras* genes exhibit rapid proliferation, anchor-independent growth, and tumor formation in athymic nude mice. Transformation of primary REF cells appears to be due to the effect of HCV core protein, as we and others (2, 23, 38) have not obtained stable transformants with *H-ras* alone. The high frequency of transformation following cotransfection of cells with the *ras* oncogene and collaborating genes such as the adenovirus early region 1A gene (E1A) or relatives of the *myc* oncogene has been previously reported (23, 24, 39). Similarly, human papillomavirus type 16 and human papillomavirus type 18 DNAs were shown to readily transform primary rat cells in cooperation with an activated *ras* gene (13, 28), and the E6 and E7 gene products of human papillomavirus were identified as the major oncoproteins (16, 17, 26, 29, 47, 51). Although our earlier results suggested that HCV core protein transactivates the *c-myc* promoter, we did not observe tumorigenicity in cells cotransfected with the HCV core gene and the *c-myc* gene controlled by its own promoter, whereas the *c-myc* gene under the control of the Moloney murine leukemia virus long terminal repeat or the simian virus 40 early promoter is known to transform cells in concert with the *H-ras* oncogene (18). A possible reason for nontumorigenic transformation, unlike that exhibited with the *H-ras* cooperative oncogene, may be the weak nature of the *c-myc* promoter (33). The HCV core gene alone did not immortalize primary cells under the experimental conditions used in this study. However, established rodent cells (NIH 3T3) were readily transformed by the HCV core gene in the absence of a cooperative oncogene, showed anchor-independent growth, and showed tumorigenicity in nude mice (data not shown). Recently, transformation of NIH 3T3 cells to the tumorigenic phenotype by the nonstructural protein NS3 of HCV was demonstrated and the proteinase activity associated with this protein was suggested as the cause of transformation (41). However, results obtained with established cell lines are difficult to interpret because such lines may harbor mutant recessive oncogenes, such as p53, which may influence the transforming activity (44). Further studies of the oncogenic potential of NS3 in primary cells will provide additional evidence concerning the biological significance of this viral protein.

Earlier investigations suggested a cytoplasmic localization of the HCV core protein when the downstream sequences of the core in gene constructs are concurrently used (42, 43). How-

ever, other studies have shown nuclear localization of the core protein in the absence of E1 sequences (27, 46), and the reason for this discrepancy is not clear. Nuclear staining of the core protein was also observed in liver tissues from biopsy samples of patients with chronic HCV infection (48). Our results clearly demonstrate nuclear localization of the core protein in transformed REF cells and raise an important concern regarding its biological significance. The exact reason for predominant nuclear localization of the HCV core protein in transformed REF cells is not clear at this time and requires further examination. HCV core protein appears to be complex and may display independent activities in different intracellular locations. Besides being a component in the virion, the core protein is likely to act as a nuclear target at which other distinct signalling pathways may converge and/or cross-talk (45). Although the idea is speculative, the core protein by translocation into the nucleus may exert a direct role in the development of hepatocellular carcinoma, and study should be initiated to address this possibility.

Activation of certain oncogenes may be common to the process of hepatocarcinogenesis. Hepatitis B virus and HCV infections are likely to affect carcinogenic pathways by causing p53 abnormalities independently (50). In chronic hepatitis delta virus infection, the *c-myc* protein accumulates selectively in the livers of patients and in the same nuclei that contain the hepatitis D virus antigen (49). Although integration of the HCV genome into hepatocytes has not been demonstrated, the persistent nature of this viral agent in the hepatocytes raises a concern that interaction of the core protein with cellular oncogenes may affect normal cell growth. The mechanism of interaction of the HCV core, whether it be with a transcriptional factor in a signal cascade or as a direct interaction with the proto-oncogene, is not known at this time. However, transformation may be a multistep process (7), and whether the transcriptional regulation of cellular proto-oncogenes by HCV core protein plays an essential role in the transforming activity remains to be elucidated. In conclusion, on the basis of the persistent nature of HCV infection and the transforming property of the core protein, induction of specific cytotoxic T-lymphocyte responses (22) may be crucial for vaccine strategies designed to prevent infection or inhibit carcinogenesis.

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