Transcriptional Repression of p53 Promoter by Hepatitis C Virus Core Protein*

(Received for publication, December 4, 1996, and in revised form, March 7, 1997)

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Our previous results have suggested that the putative core protein of hepatitis C virus (HCV) transcriptionally regulates cellular and viral genes, inhibits cisplatin and c-myc-mediated apoptotic cell death under certain conditions, and transforms primary rat embryo fibroblast cells with a cooperative oncogene. Because HCV appears to cause hepatocellular carcinoma, we evaluated the regulatory role of the HCV core protein on p53, a well known tumor suppressor gene, by an in vitro transfection assay. HCV core protein repressed transcriptional activity of the p53 promoter when tested separately in COS7 and HeLa cells. Deletion mutational analysis of the HCV core gene indicated that the regulatory domain involved in the repression of p53 transcriptional activity is located around amino acid residues 80–122 encompassing a putative DNA binding motif and two major phosphorylation sites. Results from this study suggest that the putative core protein may have an important biological role in the promotion of cell growth by repressing p53 transcription, and this appears to be consistent with certain earlier observations about HCV core moving into the nucleus.

The mechanism of HCV replication at the molecular level so far has depended upon comparative analysis and expression of its partial or entire genome with those of distantly related flaviviruses or pestiviruses (4). The virus genome encodes a single polyprotein precursor of ~3,000 amino acids (5) that is cleaved by both host and viral proteases (6, 7) to generate three putative structural proteins (core, E1, and E2) and at least six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The structural proteins are located in the amino-terminal one-fourth of the polyprotein. The genomic region encoding the putative core protein is relatively conserved (8). The carboxyl terminus of the core protein has been established at amino acid position 191. The HCV core protein demonstrates diverse biological functions including the regulation of cellular and unrelated viral genes at the transcriptional level (9), suppression of cisplatin and c-myc-induced apoptosis (10), and transformation of REF cells in cooperation with the H-ras oncogene (11).

Hepatocellular carcinoma accounts for over 90% of primary liver cancers and may progress through the inactivation of the p53 gene via mutations (12, 13). Because HCV appears to cause hepatocellular carcinoma, in this study we evaluated the ability of the HCV core protein to regulate the expression of p53 in a cotransfection system. Our results suggested that the HCV core protein caused down-regulation of p53, and this appeared to be consistent with the controversial observations about HCV core moving into the nucleus.

Experimental Procedures

Plasmids—A partial cDNA clone (Blue4/C5p-1) of strain HCV-1a containing the 5′-untranslated region, C, E1, E2, and a portion of the NS2 region kindly provided by Michael Houghton (Chiron Corporation, Emeryville, CA) was used as a template for amplification and cloning of the core genomic region (encapping amino acids 1–191) into mammalian expression vectors following a similar procedure described recently (11, 14). Briefly, two synthetic oligonucleotide primers, sense (5′-GTGGTCTGGGAATCCCCGGGA-3′) and antisense (5′-CGTGG-GAATTGGCACTTGAAG-3′), containing EcoRI restriction enzyme sites were used for PCR amplification. The amplified DNA was inserted into the EcoRI site of the pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA) under the control of the cytomegalovirus (CMV) early promoter (CMV Core1–191). Alternatively, the PCR amplified genomic region was inserted into the mammalian expression vector pBabe/puro bearing the Moloney murine leukemia virus long terminal repeat (MuLV LTR). A 2.4-kilobase human p53 promoter sequence (15) fused with the chloramphenicol acetyltransferase (p53-CAT), kindly provided by David Reisman (University of South Carolina), was used as a reporter plasmid to study the role of the HCV core protein on the p53 promoter.

Cell Culture and Transfection—COS7 and HeLa cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were transfected with the reporter (p53-CAT) and an effector (HCV core) plasmid using lipofectamine (Life Technologies, Emeryville, CA) was used as a template for amplification and cloning of the core genomic region (encapping amino acids 1–191) into mammalian expression vectors following a similar procedure described recently (11, 14). Briefly, two synthetic oligonucleotide primers, sense (5′-GTGGTCTGGGAATCCCCGGGA-3′) and antisense (5′-CGTGG-GAATTGGCACTTGAAG-3′), containing EcoRI restriction enzyme sites were used for PCR amplification. The amplified DNA was inserted into the EcoRI site of the pcDNS3 mammalian expression vector (Invitrogen, San Diego, CA) under the control of the cytomegalovirus (CMV) early promoter (CMV Core1–191). Alternatively, the PCR amplified genomic region was inserted into the mammalian expression vector pBabe/puro bearing the Moloney murine leukemia virus long terminal repeat (MuLV LTR). A 2.4-kilobase human p53 promoter sequence (15) fused with the chloramphenicol acetyltransferase (p53-CAT), kindly provided by David Reisman (University of South Carolina), was used as a reporter plasmid to study the role of the HCV core protein on the p53 promoter.

Development of chronic hepatitis and the potential for disease progression to hepatocellular carcinoma (1) are important features of hepatitis C virus (HCV) infection in humans. Successful purification of HCV from either infected tissues or cultured cells has not been reported. It is possible that HCV is stringently hepatotropic being restricted to replicate and release only in differentiated hepatocytes, which may have HCV receptors on the cell surface and specific functions that support viral growth (2). Thus it is difficult to study the biological properties and pathogenicity of the virus in the absence of unequivocal evidence for common ancestry of HCV (3).
HCV Core Protein Represses p53 Transcription

FIG. 1. Transcriptional repression of human p53 promoter activity by the HCV core protein under the control of CMV early promoter (A) or MuLV-LTR promoter (B). COS7 cells were transfected with 2 μg of the p53-CAT reporter plasmid and HCV core expression plasmid in a dose-dependent manner (A, lanes 1–5; B, lanes 1 and 2). Cell extracts were analyzed for CAT activity after 48 h of transfection.

was calculated as the percentage of the two acetylated forms of chloramphenicol relative to the total amount of [14C]chloramphenicol. Duplicate transfection experiments were repeated at least three times to verify the reproducibility of the results.

Semi-quantitative RT-PCR—p53 mRNA synthesis, from the transformed primary REF, was studied by RT-PCR as described previously (16). Transformed REF cells were prepared by cotransfection with the core gene construct and the cooperative H-ras oncogene as described earlier (11). Briefly, cells were fed with fresh culture medium 2–4 h before transfection. Cells were transfected with HCV core gene alone or together with H-ras gene (1 μg) and carrier salmon sperm DNA (18–19 μg) by calcium phosphate coprecipitation (Bethesda Research Laboratories, Gaithersburg, MD). The cells were washed with phosphate-buffered saline, fed with fresh medium at 20 h post-transfection and refed every 4–6 days. Morphologically transformed cells were separated after 3 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. Transformed cells were treated with acid guanidinium thiocyanate-phenol-chloroform for extraction of the RNA. First strand cDNA was synthesized using random primer and avian myeloblastosis virus reverse transcriptase. Sense (5′-GAGGCGCTGGCCCACTAGA-3′), nucleotide positions 734–753, and antisense (5′-AGCTCTCGGAACATCTC-3′, nucleotide positions 1224–1245) synthetic oligonucleotide primer corresponding to the nucleotide sequence of the p53 gene (17) were used in the RT-PCR for the amplification of a specific genomic sequence. These selected primers used in the RT-PCR amplification represent a conserved sequence of both human and rat p53. A p53 cDNA, kindly provided by Arnold Levine (Princeton University, NJ), was used as a positive control in PCR amplification. A PCR amplified ~490-base pair fragment from the p53 gene plasmid DNA was isolated by 1.2% agarose gel electrophoresis. The DNA band from p53 plasmid was excised, eluted using an ultra-free MC column (Millipore Corporation, Bedford, MA), and radiolabeled with [α-32P]dCTP by random priming with a commercially available kit (Boehringer Mannheim) for use as a probe. Reaction products from different cycles of PCR were analyzed by 1.2% agarose gel electrophoresis followed by Southern blot hybridization. The autoradiogram was densitometrically scanned (Molecular Dynamics), and the level of p53 expression in experimental and negative control cells was compared.

RESULTS AND DISCUSSION

A cotransfection assay was used to investigate the role of the HCV core gene product on the transcription of the human p53 gene. COS7 cells were cotransfected with the plasmid encompassing the human p53 promoter linked to a reporter CAT gene (p53-CAT) and increasing amounts of CMV Core1–191 plasmid DNA. The vector alone was also used as a negative control in the cotransfection assay. A representation of the CAT assay result is shown in Fig. 1A. Results from this experiment suggested that the core protein inhibits p53 promoter activity in a dose-dependent manner. The CAT activity decreased (20–85%) as the quantity of CMV Core1–191 plasmid DNA was increased in the transfection of cells (Fig. 1A, lanes 1–5). A similar effect was observed utilizing the MuLV LTR-driven HCV core gene in a transfection with the human p53 promoter reporter construct in the transient transfection assay (Fig. 1B). An in vitro transient assay using a mouse p53-CAT construct and the CMV Core1–191 plasmid DNAs was also performed. HCV core protein displayed repressor activity on the mouse p53 promoter (data not shown). Taken together, these results suggested that repression of p53 promoter activity may be attributed to the exogenous expression of the HCV core protein.

To determine whether HCV core protein-mediated p53 transcriptional regulation is cell type-specific or may be influenced by the endogenous expression of integrated E6 from human papilloma virus 18, we chose to use human cervical carcinoma (HeLa) cells. A CAT assay was performed by transfection of p53-CAT and different doses of the HCV core gene. The mean results from at least three independent experiments are shown in Table I. The results indicated that the inhibition of p53 promoter activity by the HCV core protein was not limited to a specific function in COS7 cells. The inhibition of p53 promoter activity by the core protein might be due to a phenomenon called squelching (18), where high levels of cotransfected activator proteins have been shown to repress expression of certain genes through sequestration of coactivators away from the promoter. To exclude the possibility of squelching in the inhibition of p53 promoter activity by HCV core protein, the core-mediated activation of the c-myc promoter was tested. Under the same conditions, the core protein stimulated the human c-myc promoter (>400% as the index) and inhibited p53 promoter (68% as the index) activity in COS7 cells (data not shown). Additionally, the dose-dependent regulation of p53 promoter activity (Fig. 1A) suggested that the suppression is not due to squelching.

To identify the domain responsible for suppression of p53 promoter activity, a full-length clone and deletion mutants of the core gene encompassing different lengths of amino acids (amino acids 1–55, 1–80, 1–122, and 80–191) were used in a transient expression. The CAT assay results of transcriptional regulation of the p53 promoter by the full-length and HCV core gene deletion mutants and vector DNA are shown in Fig. 2. The results indicated that the core protein domain required for the majority of p53 repression is located between amino acid positions 80 and 122.

To determine whether the endogenous p53 gene is repressed by the HCV core gene in HCVcore/H-ras transformed REF cells, the p53 mRNA level was measured by semiquantitative RT-PCR. The level of p53 mRNA present in the primary REF cells and HCV core transformed REF cells appeared to be similar. This observation is inconsistent with the CAT assay results presented here. However, it is possible that the level of the HCV core promoter expression is different in HCV core/ ras transformed REF cells than in an in vitro transient assay. HCV core protein-mediated repression of p53 may be an early event that precedes cellular transformation. Therefore p53 expres-

| Amount of CMV Core (μg) | Cell type used | p53 status | CAT expression% | Number of experiments |
|-------------------------|----------------|------------|-----------------|----------------------|
| 2                       | COS7           | Wild type  | 32.8 ± 2.1      | 5                    |
| 4                       | COS7           | Wild type  | 39.8 ± 0.6      | 5                    |
| 2                       | HeLa           | Bound by HPV6* | 37.2 ± 1.8      | 3                    |
| 4                       | HeLa           | Bound by HPV6* | 3.4 ± 0.9       | 3                    |

* Percentages of CAT activity presented as the means ± S.D. indicate the extent of conversion with respect to the basal level (100%) of p53 promoter-CAT gene construct.
HCV Core Protein Represses p53 Transcription

Inhibition of p53 transcription by the core protein of HCV may serve as an important factor in this multi-step process. In addition, it is conceivable that the inappropriate expression of core protein (in the absence of viral morphogenesis) may contribute to malignancy by activating specific target genes, as well as by inactivating other inhibitory genes in addition to p53.

Cancer is a multi-step process that requires a cumulative effect altering both positive and negative regulators of cell proliferation and cell survival (34, 35). Inactivation of p53 is an important event in human cancer (19, 36). However, with the exception of functional inactivation of the p53 protein by viral proteins (33, 37), products of the MDM2 gene (38, 39) and PAX4 (40) and all previously reported examples of p53 inactivation in human tumors have been due to mutations within the p53 gene or gross chromosomal rearrangement within the p53 locus. Inhibition of p53 transcription by the core protein of HCV may serve as an important factor in this multi-step process. In addition, it is conceivable that the inappropriate expression of core protein (in the absence of viral morphogenesis) may contribute to malignancy by activating specific target genes, as well as by inactivating other inhibitory genes in addition to p53.

Acknowledgments—We thank Robert B. Belshe for helpful discussions, Michael Houghton for providing the HCV cDNA (Blue4/C5p-1), and Michael Houghton for providing the HCV cDNA (Blue4/C5p-1), respectively.
Arnold Levine for p53 cDNA, David Reisman for the p53-CAT construct, and Kathy Banker for preparation of the manuscript.

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