Hepatitis C virus (HCV)\(^1\) was discovered by cDNA cloning in 1989 and shown to cause chronic liver disease (1, 2). Approximately 4 million Americans and 150 million individuals worldwide are infected with HCV and at risk for cirrhosis and hepato-cellular carcinoma (3–6). Because development of a robust vaccine is unlikely, the emphasis is on the management of chronic HCV infection. Approximately 4 million Americans and 150 million individuals worldwide are infected with HCV and at risk for cirrhosis and hepatocellular carcinoma (3–6). Because development of a robust vaccine is unlikely, the emphasis is on the management of chronic HCV infection.

**EXPERIMENTAL PROCEDURES**

*Yeast Two-Hybrid Screening—* The Matchmaker Two-Hybrid System 2 was used to screen human liver Matchmaker cDNA library HL4002AB (CLONTECH) with the cytoplasmic domain (amino acids 1 to 123, which precede the first predicted transmembrane segment) of HCV core protein bait in the yeast two-hybrid assay (17). Library screening was performed using previously described methods (18, 19). To construct the bait plasmid, DNA encoding amino acids 1–123 of HCV core protein (numbering as in Ref. 8) was amplified by PCR with pHCV-1 (13), provided by M. Houghton (Chiron Corp.) as template. The HCV sequences in pHCV-1 derive from a library made from the plasma of an infectious chimpanzee (13). The amplified DNA was cloned into the GAL4 DNA binding domain fusion vector pAS2–1 (CLONTECH) and pAS2–1 to confirm the specificity of the reactions.

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DNA sequencing of isolated library plasmid inserts and the bait constructs was performed on a 373A Sequencer (Applied Biosystems) at the Columbia University Cancer Center DNA core facility. Sequence analysis was performed using the Wisconsin Package (Genetics Computer Group).
and applications available via the internet at the National Center for Biotechnology Information World Wide Web site. 2

In Vitro Binding Assays—A PCR product encoding the cytoplasmic domain of HCV core protein (amino acids 1–123) was cloned into pBFT4 for in vitro transcription-translation (21). DBX cDNA encoding amino acids 409 to 662 was expressed from pBluescript II-DBX digested and cloned into pGEX2T (Amersham Pharmacia Biotech) to yield pGEX2T-DBX 409–662, which expressed a glutathione S-transferase (GST) fusion protein in Escherichia coli. Plasmid construction was confirmed by DNA sequencing. In vitro transcription-translation was performed with the TNT T7 Coupled Reticulocyte Lysate System (Promega) using T7 Lmethionine (NEN Products). Binding assays were performed as described previously (21).

Cell Transfection and Confocal Immunofluorescence Microscopy—A PCR product encoding full-length HCV core protein (amino acids 1–191) obtained using pHCV-1 (13) as template was cloned in-frame into pBFT4, which contains an initiation codon and FLAG tag 5’ to the cloning site. A DNA fragment was isolated by restriction endonuclease digestion at sites-flanking the initiation and termination codons and cloned into pSVK3 (Amersham Pharmacia Biotech) to obtain pSVK3-FLAG-core for expression of HCV core protein with a FLAG tag at its amino terminus. To obtain full-length DBX cDNA, PCR was performed using a Marathon-ready cDNA human liver library (CLONTECH) as template to amplify the first 1439 nucleotides of DBX cDNA, which was ligated into pGEX2T-DBX 409–662. The DNA fragment was subsequently used to construct DBX plasmids for in vitro transcription and cloning into pBluescript II SK+. The coding region of pGEX2T-DBX was isolated by restriction endonuclease digestion and cloned into pBluescript II SK+ (Stratagene) to produce pBluescript-DBX. A cDNA containing the 3’ 686 nucleotides of DBX, excluding the stop codon, was amplified by PCR and ligated into pBluescript II-DBX to replace the corresponding nucleotides. The entire DBX coding region was then excised by restriction endonuclease digestion and ligated into pcDNA3.1 (+)/Myc-His A (Invitrogen) to produce DBX, excluding the stop codon, was amplified by PCR and ligated into pBluescript II SK+, which was excised by restriction endonuclease digestion and cloned into pGEX2T-DBX, which expressed a glutathione S-transferase (GST) fusion protein in Escherichia coli. Plasmid construction was confirmed by DNA sequencing. In vitro transcription-translation was performed with the TNT T7 Coupled Reticulocyte Lysate System (Promega) using T7 Lmethionine (NEN Products). Binding assays were performed as described previously (21).

Effect of HCV Core Protein on Growth of Yeast Strains—The coding region for full-length HCV core protein (amino acid 1–191) was excised from pBFT4 by restriction endonuclease digestion and ligated into p423GPD (ATCC) to produce p423GPD-core. The coding region for the cytoplasmic domain of HCV core protein (amino acid 1–123) was also ligated into p423GPD to yield p423GPD-core1–123. Constructs were confirmed by DNA sequencing. Yeast strains YTC83, YNM1DX, and YNM1D were transformed with p423GPD, p423GPD-core, and p423GPD-core1–123 using the lithium acetate-mediated method (24) and grown on histidine-leucine dropout plates for 7 days. Plates were photographed to record colony growth.

RESULTS

HCV Core Protein Binding to DBX—Screening of 8 × 105 recombinants of a human liver cell cDNA library with the cytoplasmic domain of HCV core protein as bait in the yeast two-hybrid assay led to the isolation of 5 positive clones, 3 of which encoded portions of DBX, the longest from amino acid 409 to amino acid 662. The 2 other positive clones encoded portions of epsilon 14-3-3, a member of the 14-3-3 family of proteins that has numerous proposed functions, including activities in signal transduction. DBX is the human orthologue of the mouse DEAD box protein PL10 (25–27). PL10 is the functional orthologue of S. cerevisiae Ded1p, an ATP-dependent RNA helicase for capped mRNA (20). DBX is 95% identical in primary structure to PL10 and 54% identical to Ded1p (Fig. IA). In the yeast two-hybrid assay, HCV core protein interacts with DBX and PL10 but not with Ded1p (Fig. IB).

We confirmed the interaction between HCV core protein and DBX in an in vitro binding assay. The cytoplasmic domain of HCV core protein was synthesized by in vitro translation and incubated with GST or a GST fusion protein containing DBX from amino acid 409 to amino acid 662. Proteins were precipitated with glutathione-Sepharose, and HCV core protein bound to GST but did bind to GST-DBX fusion protein in buffers containing NaCl concentrations as high as 1 M (Fig. 2A). Binding also occurred in buffers containing 1% of the nonionic detergent Nonidet P-40 (Fig. 2B).

Co-localization of HCV Core Protein and DBX in Cells—An interaction between HCV core protein and DBX in mammalian cells was further supported by their intracellular co-localization. Indirect immunofluorescence microscopy of transfected HeLa cells showed that full-length HCV core protein, which contains the cytoplasmic domain and a single transmembrane segment, was localized to the endoplasmic reticulum in discrete foci (Fig. 3A). A similar localization in the endoplasmic reticulum has been reported by others (13). Focal aggregates of HCV core protein likely arise because this
Fig. 1. Primary structures of DBX, PL10, and Ded1 and their interactions with HCV core protein in the yeast two-hybrid assay. A, alignment of deduced amino acid sequences of DBX (GenBank™ accession number AF000982), PL10 (GenBank™ accession number J04847), and Ded1p (GenBank™ accession number X57278) is shown. Identical amino acids are shown as white on cyan. Conserved substitutions are shown as black on magenta. Dots represent gaps to optimize alignments, which were obtained using the Pileup program. B, two-hybrid assays showing interaction of HCV core protein with DBX and PL10 but not with Ded1p. Yeast strain Y190 was co-transformed with a plasmid expressing the cytoplasmic domain of HCV fused to the GAL4 DNA binding domain and plasmids expressing either a portion of DBX or the corresponding portions of PL10 or Ded1p fused to the GAL4 transcriptional activation domain. Transformants giving β-galactosidase activity (positive interactions) are blue. Control reactions of DBX, PL10, and Dep1p with GAL4 DNA binding domain alone were negative (data not shown).

Inhibition of in Vitro Translation of Capped mRNA by HCV Core Protein—We examined the effects of HCV core protein on the translation of capped and uncapped luciferase RNA in an in vitro reticulocyte lysate assay. If HCV inhibits the function of DBX as a RNA helicase, it should theoretically decrease the translation of capped RNA but not significantly affect the translation of uncapped RNA. In the in vitro translation assay, the cytoplasmic portion of HCV core protein significantly inhibited the in vitro translation of luciferase from capped but not uncapped RNA (Fig. 5). Capped RNA translation was approximately 4-fold higher than uncapped RNA translation in this assay (data not shown). This finding suggests that HCV apparently associates.

DBX Rescues Ded1-deletion Yeast Mutants and Rescue Is Prevented by HCV Core—DBX likely functions as an ATP-dependent RNA helicase for cellular mRNA, which can be inferred from its sequence similarity to mouse PL10 and yeast Ded1p (20, 27). To examine the effect of HCV core protein on DBX function, we took advantage of yeast genetics and the fact that S. cerevisiae has only one essential DBX-like protein, Ded1p (20). When driven by a yeast GPD promoter and carried on a centromere plasmid, mouse PL10 cDNA, as described previously (20), and DBX cDNA rescued the lethality of cells with a chromosomal ded1 deletion. This indicates that DBX can likely function as a RNA helicase as it can replace the function of the yeast DEAD box RNA helicase Ded1p. Expression of full-length HCV core protein severely inhibited the growth of DBX- and PL10-complemented ded1-deletion yeast but not ded1-deletion yeast complemented with DED1 cDNA driven by the same promoter on a centromere plasmid (Fig. 4). This is consistent with the observation that DBX and PL10, but not Ded1p, bind to HCV core protein. The cytoplasmic domain of HCV core protein that binds to DBX, without a transmembrane segment, did not significantly inhibit the growth of DBX- and PL10-complemented ded1-deletion yeast (data not shown), suggesting that inhibition of function may result from trapping of these proteins in aggregates at the endoplasmic reticulum membrane (see Fig. 3).

Fig. 2. Binding of DBX to HCV core protein in vitro. A, a standard amount of 35S-HCV core protein (amino acids 1–123), 10% of which is shown in the autoradiogram (lane 1), was used in each binding assay. 35S-HCV core protein was incubated with glutathione-Sepharose (lane 2); 20 μg of GST coupled to glutathione-Sepharose (lane 3) in binding buffer containing 0.15 M NaCl and 0.2 μg of GST-DBX fusion protein coupled to glutathione-Sepharose in buffers containing the NaCl concentrations indicated above each lane (lanes 4–8). Glutathione-Sepharose was then washed with buffer containing the indicated NaCl concentration, and the bound proteins were eluted with 4% SDS, subjected to SDS-polyacrylamide gel electrophoresis, and detected by autoradiography of dried slabs gels. B, binding assay similar to that shown in panel A in which GST-DBX fusion protein was incubated with 35S-HCV core protein in buffers containing 0.15 μM NaCl and 0.05 to 1.0% of Nonidet P-40 (lanes 4–6). Migrations of molecular mass standards are indicated in kilodaltons at the left of each panel.
core protein may inhibit the translation of capped mRNA in cells, presumably by inhibiting DBX function.

DISCUSSION

HCV core protein binds to the human DEAD box protein DBX. DBX rescues the lethal phenotype of ded1-deletion, demonstrating that it can function as a RNA helicase for capped mRNAs, replacing the essential yeast DEAD box RNA helicase Ded1p. Our findings that HCV core protein prevents DBX from rescuing ded1-deletion yeast and that it inhibits the translation of capped RNA in vitro strongly suggest that it may inhibit cellular mRNA translation in vivo. These results, however, cannot establish if translation inhibition occurs as a result of HCV core protein inhibiting DBX RNA helicase activity per se or by an interaction that results in “trapping” DBX at a location near the membrane of the endoplasmic reticulum where it cannot function properly. Inhibition of host cell mRNA translation could theoretically provide viral RNA molecules with enhanced access to ribosomes and the rest of the protein synthesis machinery of the cell, a phenomenon shared by several different viruses (29). A recent report has shown that high levels of expression of HCV structural and nonstructural proteins is toxic to mammalian cells (30); however, it is not clear if this toxicity results from inhibition of host cell translation. Because the development of a robust cell culture system to study HCV has remained elusive, it would be extremely difficult to directly investigate the effects of HCV infection on host cell mRNA translation. Despite these methodological constraints limiting the ability to directly test the hypothesis, our discovery that HCV core binds to DBX and inhibits capped mRNA translation in experimental assays suggests that it can similarly inhibit mRNA translation in infected human cells.

DEAD box RNA helicases unwind capped mRNA (20), and...
inhibition of their function should decrease translation of cellular mRNA. Inhibition of DBX function by HCV core protein may only partially inhibit host mRNA translation in mammalian cells because they contain other putative RNA helicases (31). In contrast, the translation of HCV RNA, which is not capped, utilizes internal ribosome entry sites (11, 12), and can be unwound by its own RNA helicase, which is part of the HCV NS3 protein (32, 33), and may proceed without DBX. This hypothetical mechanism is reminiscent of that used by poliovirus, which inhibits translation factor eIF-4F (34, 35) and also has RNA with internal ribosome entry sites (36). In cells, eIF-4F exists as a complex with eIF-4B, which has RNA binding activity, and eIF-4A, which is also a DEAD box RNA helicase (37). HCV and poliovirus infection may both therefore cause a decrease in the unwinding of capped mRNA in host cells.

In addition to inhibiting capped mRNA translation in infected host cells, the interaction between HCV core protein and DBX may play other possible roles, including the recruitment of DBX to participate in HCV replication itself. Recruitment of host cells proteins into virions to enhance viral replication has been demonstrated in other systems. For example, the principal structural protein of the human immunodeficiency virus HIV-1 binds to cyclophilins and recruits cyclophilin A into viral particles, which appears to be necessary for efficient viral replication (38, 39). In a similar fashion, recruitment of DBX into HCV particles by binding to core protein may enhance viral replication. This could theoretically occur by DBX altering viral genomic RNA structure in viral particles in newly infected cells. Testing of this hypothesis is limited at the present time because of the lack of an efficient cell culture system for HCV.

HCV core protein has also been shown to bind to lymphotoxin-β receptor and other tumor necrosis factor receptor family members (14, 15) as well as ribonucleoprotein K (16). In our yeast two-hybrid screen, we did not isolate clones for these proteins, possibly because of subtle differences in our bait construct and the different cDNA library we used. The demonstration that other proteins interact with HCV core protein suggests that its expression in cells may have myriad consequences. Other groups (40, 41) have also reported that HCV core protein represses transcription from the p53 promoter and other eukaryotic promoters. The overall effect of HCV core protein on cell physiology under natural conditions of infection is, however, difficult to assess at the present time because of lack of a cell culture system for HCV.

Finally, it should be noted that the best current treatment regimens for chronic hepatitis C are effective in only a minority of patients (42). If interactions between HCV and host cell proteins alter cell survival or enhance viral replication, they could be rational targets for antiviral drug design. Regardless of the physiological significance, the tight binding of any polypeptide to a structural or nonstructural protein of HCV may potentially interfere with viral replication. The identification of polypeptides such as DBX that bind to HCV proteins therefore has implications for the design of compounds which may be therapeutically useful in the treatment of patients with chronic hepatitis C.

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REFERENCES
1. Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Science 244, 359–362
2. Kuo, G., Choo, Q.-L., Alter, H. J., Gitnick, G. L., Redeker, A. G., Purrel, R. H., Miyamura, T., Dienstag, J. L., Alter, M. J., Stevens, C. E., Tegtmeier, G. E., Bonino, F., Colombo, M., Lee, W.-S., Kuo, C., Berger, K., Szuster, J. R., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) Science 244, 362–364
3. Alter, M. J. (1995) Semin. Liver Dis. 15, 5–14
4. Mansell, C. J., and Locarnini, S. A. (1995) Semin. Liver Dis. 15, 35–32
5. Miyamura, T., and Worman, H. J. (1997) Curr. Opin. Infect. Dis. 10, 3980–3997
6. National Institutes of Health Consensus Development Panel (1997) Hepatology 26, 15–1683
7. Kata, N., Hjihikata, M., Otsuyama, Y., Nakagawa, M., Okoshi, S., Sugimura, T., and Shimotohno, K. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9524–9528
8. Choo, Q.-L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallgecs, C., Cotti, G., Medina-Selby, R., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G., and Houghton, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2451–2455
9. Okamoto, H., Okada, S., Sugiyama, Y., Kurai, K., Iruko, H., Mochida, A., Miyakawa, Y., and Mayumi, M. (1991) J. Gen. Virol. 72, 2697–2704

FIG. 4. Inhibition of DBX and PL10 but not Ded1p by HCV core protein. Yeast strains with chromosomal ded1 deletion complemented with either DBX, PL10, or Ded1p cDNAs driven by the yeast GPD promoter on centromeric plasmids were transformed with a plasmid that expressed full-length HCV core protein (top) or control plasmid p423GPD (bottom). The resulting transformants were spread on histidine, leucine drop-out plates and incubated at 30 °C for 7 days, and photographs (negatives are shown) were taken of each plate. Note colony growth of all yeast strains transfected with control plasmid (bottom panels). In contrast, DBX- and PL10- complemented ded1 deletion strains do not demonstrate significant colony growth when HCV core protein is expressed, whereas growth of the Ded1p-complemented strain is unaffected (top panels).

FIG. 5. Inhibition of translation of capped mRNA in vitro by HCV core protein. Rabbit reticulocyte lysates were incubated with glutathione-Sepharose beads loaded with either 300 ng of a GST-HCV core fusion protein or GST. In vitro synthesized capped or uncapped luciferase mRNAs were translated at 30 °C for 90 min, and luciferase activity was measured. Results are expressed as the relative luciferase activities produced in reticulocyte lysate-treated equal concentrations of GST-HCV core fusion protein (shaded bars) or GST (open bars, arbitrarily assigned 100% activity). Values shown are means ± S.E. (n = 6). *p < 0.0001; †, no significant difference.
10. Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I., and Okayama, H. (1991) J. Virol. 65, 1105–1113
11. Reynolds, J. E., Kaminski, A., Kettinen, H. J., Grace, K., Clarke, B. E., Carroll, A. R., Rowlands, D. J., and Jackson, R. J. (1995) EMBO J. 14, 6010–6020
12. Fukushi, S., Kurihara, C., Ishiyama, N., Hoshino, F. B., Oya, A., and Katayama, K. (1997) J. Virol. 71, 1662–1666
13. Selby, M. J., Choo, Q.-L., Berger, K., Kuo, G., Glazer, E., Eckart, M., Lee, C., Chien, D., Kuo, C., and Houghton, M. (1993) J. Gen. Virol. 74, 1103–1113
14. Matsumoto, M., Hsieh, T. Y., Zhu, N., VanArsdale, T., Hwang, S. B., Jeng K. S., Gorbalenya, A. E., Lo, S. Y., Ou, J. H., Ware, C. F., and Lai, M. M. C. (1997) J. Virol. 71, 1301–1309
15. Zhu, N., Khoshanan, A., Schneider, R., Matsumoto, M., Dennert, G., Ware, C., and Lai, M. M. C. (1998) J. Virol. 72, 3691–3697
16. Hsieh, T.-Y., Matsumoto, M., Chou, H.-C., Schneider, R., Hwang, S. B., Lee, A. S., and Lai, M. M. C. (1998) J. Biol. Chem. 273, 17651–17659
17. Fields, S., and Song, O. (1989) Nature 340, 245–246
18. Chien, C., Bartel, P. L, Sternglanz, R., and Fields, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9579–9582
19. Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., and Eldredge, S. J. (1993) Genes Dev. 7, 555–569
20. Chuang, R.-Y., Weaver, P. L., Liu, Z., and Chang, T.-H. (1997) Science 275, 1468–1471
21. Ye, Q., and Worman, H. J. (1996) J. Biol. Chem. 271, 14653–14656
22. Soullam, B., and Worman, H. J. (1995) J. Cell Biol. 130, 15–27
23. Boeke, J. D., Trueheart, J., Natoussis, G., and Fink, G. R. (1987) Methods Enzymol. 154, 164–175
24. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
25. Lahn, B., and Page, D. C. (1997) Science 278, 675–680