Expression Profiling of Liver Cell Lines Expressing Entire or Parts of Hepatitis C Virus Open Reading Frame

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Although hepatitis C virus (HCV) is a causative agent of liver diseases, its mechanism of pathogenesis is still unclear, mainly because of the lack of adequate cell culture systems to support HCV infection and replication. In this report, we describe development and characterization of human hepatoma cell lines constitutively expressing entire (Hep394) or parts (Hep352, Hep3294) of the HCV open reading frame (ORF). The viral and cellular proteolytic machinery involved in the viral precursor processing was consistently functional, and processed HCV proteins were synthesized in these established cell lines. By using a cDNA microarray analysis coupled with semiquantitative reverse-transcription polymerase chain reaction (RT-PCR), we identified 12 genes up-regulated and 4 genes down-regulated in Hep394 cells. With regard to genes related to cell growth regulation, we found up-regulation of forkhead transcription factor FREAC-1, poly (A) binding protein PABP2, and Ras suppressor Rsu-1. Another category of changes in gene expression includes MHC antigens, which play an important role in the T-cell-mediated immune reaction in the liver. In conclusion, functional genomic approaches comparing expression among the different cell lines expressing parts of the HCV genome may promote our understanding of the molecular basis of pathogenicity of HCV infection. (HEPATOLOGY 2002;36:1431-1438.)

Hepatitis C virus (HCV) infection generally takes a chronic course, which is linked to development of cirrhosis and hepatocellular carcinoma (HCC).[1-3] Inflammation associated with hepatitis C and the repeated bouts of liver injury and hepatic regeneration are implicated as causes leading to these liver diseases. It has also been suggested that HCV itself has direct pathogenic potential. In particular, HCV core protein, which is a structural component of the virion, is now known to have a variety of other biologic functions. These include inhibition or stimulation of apoptosis, cooperation with oncogenes to transform fibroblasts, and modulation in expression of several genes.[4] Furthermore, transgenic mice persistently expressing HCV core protein develop hepatic steatosis and subsequent HCC.[5] However, the precise mechanisms by which HCV infection results in cirrhosis and HCC are still unclear because of the lack of adequate tissue culture systems and small animal models permissive for HCV infection and replication.

Stable transfectants that constitutively express the HCV cDNAs are useful in analyzing the biologic roles of the HCV gene products. The HCV genome, which is a single-strand positive-sense RNA of approximately 9,600 nt, encodes a polyprotein with a single open reading frame (ORF) of 3,008 to 3,033 aa.[6] This polyprotein precursor is then cleaved by cellular and viral proteases to yield functional proteins. So far, some human hepatoblastoma-derived HepG2 cell lines constitutively expressing parts of HCV polyprotein, such as core[7] and nonstructural (NS)

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ORF, open reading frame; NS, nonstructural; SDS, sodium dodecyl sulfate; neoR, neomycin phosphotransferase gene.

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proteins,[8,9] have been described. Recently, it has been reported that selectable dicistronic HCV RNA replicons containing NS regions or full-length genome replicate in human hepatoma Huh-7 cells.[10-13] However, not much is yet understood regarding the overall effects of entire HCV proteins on liver cells. In this report, we characterized a human hepatoblastoma cell line constitutively expressing the whole HCV polyproteins, which could to some extent mimic a long-term, persistent HCV infection and be used to assess a variety of biologic features of HCV proteins. We also analyzed gene expression profiling of the cell line and identified differentially regulated genes by cDNA microarray and reverse transcription-polymerase chain reaction (RT-PCR).

Materials and Methods

Cell Lines and Culture Condition. We constructed expression vectors, which carry HCV genotype 1b cDNA[14] encoding the entire ORF (pCAG394), core to NS3 protein (pCAG352),[15] or the C-terminus of NS2 to NS5B protein (pCAG3294) under the transcriptional control of CAG promoter.[16] HepG2 cells, differentiated human hepatoblastoma cells,[17] were cotransfected with pEF321swxneo[18] and the other constructs. As described previously,[19] G418-resistant colonies were clonally isolated and screened for abilities to express HCV proteins by indirect immunofluorescence staining with monoclonal and polyclonal antibodies to HCV structural and nonstructural proteins. Established cell lines, Hep394, Hep352, and Hep3294 were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Gaithersburg, MD) containing 600 μg/mL G418 (GIBCO), 2 mmol of L-glutamine, penicillin (50 IU/mL), streptomycin (50 mg/mL), and 10% fetal bovine serum. Core protein-expressing cell line Hep39[7] and control cell line HepSWX,[8] which were transfected with pcEF39neo and pEF321swxneo, respectively, were also maintained under the same condition and used in this study.

Antibodies. Anticore monoclonal antibody and anti-NS3 polyclonal antibody used for Western blotting were described previously.[20,21] Monoclonal antibodies raised against NS5A, NS5B were gifts from Dr. M. Kohara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), and anti-NS4A antibody was a gift from Dr. A. Takamizawa (Research Foundation for Microbial Disease of Osaka University, Osaka, Japan).

Western Blotting. Cells grown in 35-mm dishes were harvested and lysed in sodium dodecyl sulfate (SDS) sample buffer. Lysates were separated by 15% or 12.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described previously.[22] The blots of immobilon transfer membranes were blocked with nonfat milk, followed by reacting with monoclonal or polyclonal antibodies against HCV proteins diluted 1:100 to 1:200. Membranes were then incubated with biotinylated anti-mouse or anti-rabbit IgG and peroxidase-conjugated avidine. Antigen-antibody complexes were visualized by 4-chloro-1-naphthol.

Northern Blotting. Total cellular RNA was extracted as described previously.[23] The RNA (20 μg) was fractionated on a 1.2% formaldehyde agarose gel and transferred to a Hybond-N membrane filter (Amersham Life Science, Buckinghamshire, United Kingdom), followed by fixing on the membrane with UV light (UV Crosslinker, Funakoshi, Tokyo, Japan). [^{32}P]-labeled HCV cDNA fragment of core, NS3, or NS5B region was used for Northern blot hybridizations.

Quantification of HCV Core Antigen. The expression level of HCV core protein in cell lines was determined by the fluorescence enzyme immunoassay (FEIA) method as reported previously.[24] Cells were homogenized in a solution containing 0.3% Triton X-100, 1.5% propane sulfonic acid, and 15% SDS. After incubating at 56°C for 30 minutes, the samples were centrifuged at 1,000 rpm for 5 minutes, and the supernatants were subjected to FEIA. BCA protein assay kit (Pierce Chemical Company, Rockford, IL) was used to quantify total protein.

Cell Growth Rate Analysis. To examine the cell growth, 1×10^4 cells were seeded into 35-mm dishes in the medium containing G418, which was changed daily. Cells from triplicate dishes were harvested, and viable cells were counted every day for 10 days by trypan blue dye exclusion.

Microarray Procedures. The preparation procedure of our in-house microarray consisting of 2,304 named, human cDNAs was described previously.[25] The fluorescence-labeled probe was prepared as follows: Total RNA from harvested cells were isolated using an ISOGEN Reagent (Nippon Gene, Tokyo, Japan), and poly (A)$^+$ RNA was obtained from this total RNA using the Oligotex-dT30 messenger RNA (mRNA) purification kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer's instructions. Fluorescent nucleotide Cy3-dUTP or Cy5-dUTP was incorporated during reverse transcription of poly (A)$^+$ RNA from Hep394 and HepSWX as previously described.[26] The probes were purified and concentrated by passing through Centricon-30 microconcentrators (Millipore, Bedford, MA). The different fluorescent-labeled probes were mixed and applied onto microarray slides. Hybridization was carried out at 65°C overnight under a humidified condition. The array was washed, centrifuged, and then scanned with a fluores-

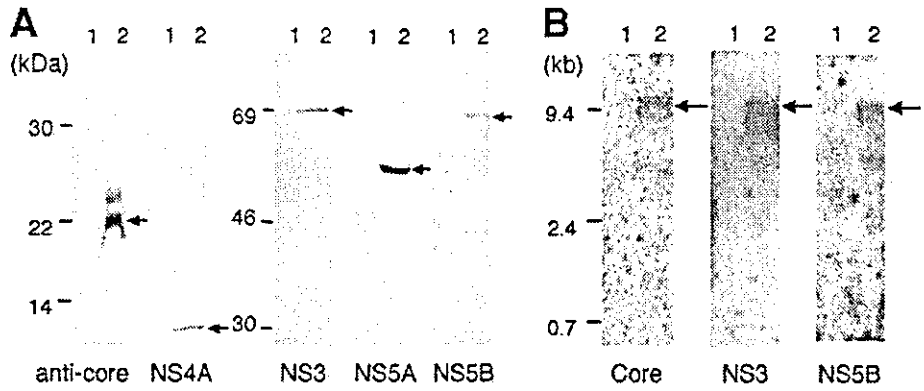

Fig. 1. HCV gene expression in HepG2 cell transformants integrated with HCV cDNAs covering entire or parts of the viral ORF. (A) Western blot analysis. HepSWX (**lanes 1**) and Hep394 (**lanes 2**) cells were lysed and separated by 15% (left) or 12.5% (right) SDS-PAGE. The blots of Immobilon transfer membranes were incubated with anticore, NS3, NS4A, NS5A, or NS5B monoclonal or polyclonal antibodies. The molecular mass markers are indicated at the left of each panel. Each HCV protein is noted by **arrows**. (B) Northern blot analysis. Total RNAs prepared from HepSWX (**lanes 1**) and Hep394 (**lanes 2**) cells were used for the analysis. HCV-specific RNA was detected by 5′-labeled probes. The DNA probes were derived from the sequence of core, NS3, or NS5B. The size markers are indicated at the left of each panel. The HCV RNAs are denoted by **arrows**.

cence laser confocal slide scanner (Scan Array 4000, GSI lumonics, Tokyo, Japan). Images were analyzed, and fluorescence intensities were measured by Quant Array (GSI lumonics). Two independent clones of Hep394 cells were compared with HepSWX cells, in which labeling with the fluorescent dyes was switched during synthesis to control labeling difference. Genes that differentially expressed by 1.5-fold or more in the average of 4 measurements were considered as differentially expressed genes.

RT-PCR. The levels of mRNAs differentially expressing between Hep394 and HepSWX cells were investigated by semiquantitative RT-PCR. The first strand of cDNA was synthesized from 500 ng of poly $(A)^+$ RNA as described previously.[26] PCR amplification was performed using various paired primers shown in Table 1. The reaction mixture contains 200 μmol/L of each dNTP, 1.0 μmol/L of 5′ upstream and 3′ downstream primers, 2.5 units of Taq polymerase, and cDNA corresponding to 500 ng of poly $(A)^+$ RNA. Amplification procedure was as follows: After 3 minutes at 96°C, the reaction of 96°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute was repeated for 20 to 40 cycles and, finally, 10 minutes at 72°C for 1 cycle. PCR cycles were limited to analyze the products in the linear phase of amplification. PCR products were electrophoresed on 2.0% agarose/ethidium bromide gels.

Results

Stable Expression of HCV Proteins in Human Hepatoblastoma-Derived Cell Lines. Expression and processing of HCV proteins in Hep394 cells, which chromosomally integrate HCV cDNA covering the entire ORF in HepG2 cells, were analyzed by Western blotting (Fig. 1A). With the use of specific monoclonal antibodies, HCV structural and nonstructural proteins of the expected sizes were observed in Hep394 cells (Fig. 1A, lanes 2) but not in the empty-vector control HepSWX cells (Fig. 1A, lanes 1).[8] All results were confirmed in at least 2 additional independent clones. We detected Core, NS3, NS4A, NS5A, and NS5B proteins by immunofluorescence analysis as shown.[19] Similarly, E1, E2, and NS4B proteins were observed with monoclonal antibodies against each HCV protein (data not shown). Expression and proper processing of these HCV proteins were confirmed in the immunoprecipitation analysis of insect cells infected with a recombinant baculovirus Ac394, which harbors the same HCV expression cassettes.[27] We then performed Northern blot analyses to exclude the possibility that processed HCV proteins shown above were generated from aberrantly spliced RNAs in cells. As shown in Fig. 1B, a single unspliced RNA of approximately 9 kb covering the entire HCV ORF was found in Hep394 cells by using probes either of 5′-labelled core, NS3, or NS5B cDNA fragment. This full-length RNA is present, suggesting that the HCV proteins found in our expression system are produced from the polyprotein precursor by proteolytic processing.

As reported previously, we have also established other independent HepG2-based cell lines that constitutively express HCV core to NS3 protein (named Hep352)[15] and the C-terminus of NS2 to NS5B protein (Hep3294).[19] Expression and processing of the HCV proteins in these cell lines had been confirmed by immunofluorscence staining and Western blot analyses. The cell growth of

Hep394, Hep352, and Hep3294 was apparently slower than that of control cells; 7 days after the passage, the number of these cells was decreased by between 41% and 65% as compared with that of either HepSWX or parental HepG2 cells (Fig. 2). We found that Hep394, Hep352, and Hep3294 cells were maintained in continuous cultures for more than 30 passages over 6 months without loss of their characteristics, such as expression levels of HCV proteins and cell-growth rates.

To compare the level of expression of HCV proteins among cell lines established, we quantified protein concentrations of the core proteins in Hep394 and Hep352 cells as well as Hep39 cells[7] by FEIA. As shown in Fig. 3, the expression levels of core protein varied, whereas the core protein level in Hep394 cells was approximately 3 ng/mg total protein and was lower than those in Hep352 and Hep39 cells by 20- and 3-fold, respectively.

Identification of Differentially Expressed Genes in Hep394 Cells. The identification of genes with altered expression patterns is important to the future understanding of the pathogenic effects of HCV. mRNA expression profiling was therefore carried out. A cDNA microarray consisting of 2,304 genes was applied to identify differentially expressed genes. Two independent clones of Hep394 cells were compared with control HepSWX cells. The scatterplot of fluorointensities of Cy3 versus Cy5 for all genes was examined to assess the accuracy of each experiment, and we observed high correlation in all paired samples (data not shown). Hybridization image analyses revealed that 16 genes were up-regulated and 5 genes were down-regulated (Table 1).

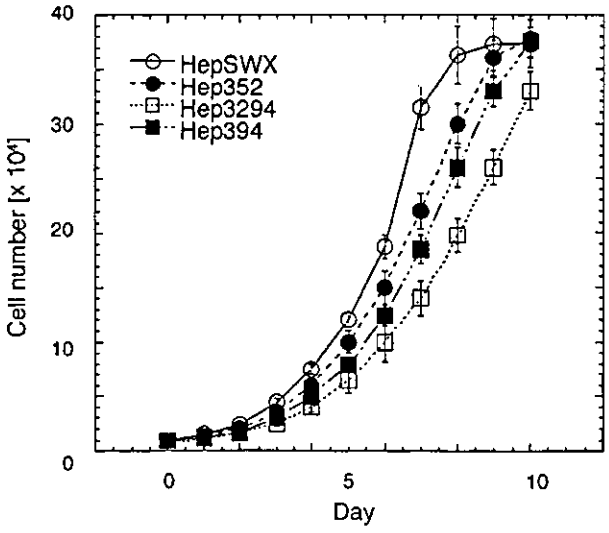

Fig. 2. Cell growth rate analysis. HepSWX (○), Hep352 (●), Hep3294 (□), and Hep394 (■) cells were grown in 35-mm dishes and counted daily as described in Materials and Methods section. Results show the mean cell number ± SD obtained from triplicate dishes.

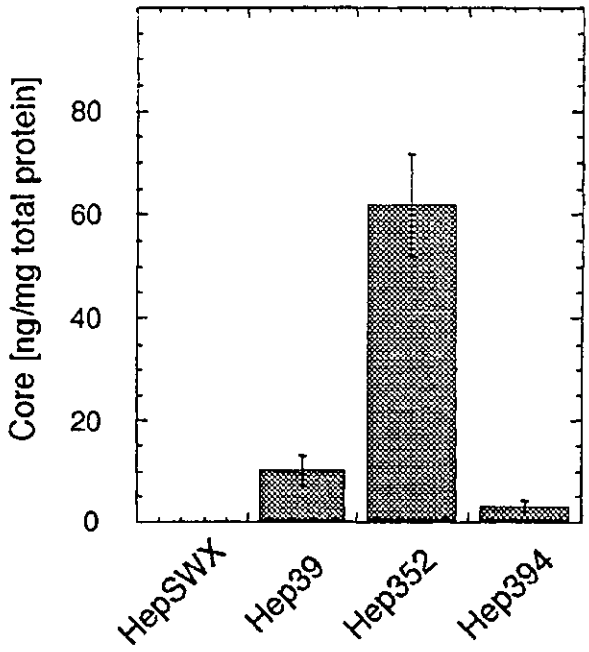

Fig. 3. Quantitation of HCV core protein in cells. The level of HCV core protein in HepSWX, Hep39, Hep352, and Hep394 was determined by the fluorescence enzyme immunoassay method. Results represent the mean ± SD of values obtained in triplicate experiments.

We examined the differential expression of these 21 genes by semiquantitative RT-PCR analysis of an additional 1 clone of Hep394 compared with HepSWX cells. Primer pairs used are summarized in Table 1. As shown in Fig. 4, we found the differential expression of 12 genes that were up-regulated and 4 genes that were down-regulated in Hep394 cells. GAPDH expression was examined in the RNA samples to confirm equal quantification. Furthermore, we determined the expression levels of these 16 genes in Hep352 and Hep3294 and compared it with the pattern of Hep394. Among 12 genes up-regulated in Hep394 cells, we observed that 5 genes (PABP2, amyloid β precursor protein, Ras suppressor Rsu-1, metallothioneine II, and archain) were expressed at similar or higher levels in Hep3294 cells but at a lower level in Hep352 compared with Hep394 cells (group I as shown in Fig. 4). Although we should consider the possibility that different expression levels of viral proteins in cell lines used may account for the variation of gene regulation, factor(s) responsible for up-regulation of these genes possibly reside in NS3 through NS5B proteins. A gene of HLA-A exhibited a greater expression in Hep352, but not in Hep3294 cells (II), suggesting that protein(s) in core through NS2 region may regulate its expression. FREAC-1 and HLA-C were up-regulated only in Hep394 cells and were not, little if any, detectable in Hep352 and Hep3294 (III). This suggests that 2 or more HCV proteins may attribute

Table 1. Genes Differentially Expressed Between Hep394 and HepSWX Cells Identified by Microarray Analysis

Genes	Accession No.	Average Ratio (range)	Sense Primer	Antisense Primer
Up-regulation				
Cyr61	AA777187	2.7 (2.1-3.3)	TAAATGGTTAATTCCATA	TGTAGCTTTTGGGGAGGG
Tumor rejection antigen gp96	AA598758	2.4 (2.3-2.5)	ACCCACTTTTTCCTGTGACCCA	CACCATTTGGATCCTGTGTGGA
Inter α-trypsin inhibitor	R06580	1.5 (0.9-2.1)	CATTGCTCCGGAATGTTCAGTT	TCCTGCAAAGTCTCCCATCTG
Amyloid β precursor like protein 2	H89517	1.9 (1.3-2.5)	GCGATCTTTGTTCTCAGCACG	GCTTTAGAGAAGGAAGCAGCCC
Metallothionein 1-B	M13485	2.1 (1.1-3.2)	GCTGTGTCTGCAAAGGCTCAT	GAATGTAGCAAACCGGTCAGG
Orosomucoid 1	AA700876	2.0 (1.2-2.5)	TCTCTGTCCTGATCCAAGGCTG	ATGCTGACAAGCCAGAGACGA
Cystatin C	AA599177	1.7 (0.9-2.1)	AGCCCTTGCTGAGCAACAAA	GGCCTGTGCTATTCACCTCTTA
PABP2	AA040742	1.6 (1.2-1.9)	TACCATGATGTCGCTCTAGCCC	CCTTGGCCTTAGATGAGTCCC
TGF β superfamily protein	AA450062	1.9 (1.7-2.1)	AAGTGACCATGTGCATCGG	TCTTGATCTGCGCGTGCAT
Ras suppressor protein, Rus 1	L12535	2.0 (1.7-2.3)	AACCCAATGCTGCGTGTGA	AAGCCAGAGCCCACTATGGAA
Human forkhead protein FREAC-1	AA112660	1.9 (1.6-2.3)	GTCACAAATGCTGCTGCACTCTAGCA	TAATCAAAAACACCGCGTAGG
MHC class 1 HLA 1C	AA464246	2.3 (1.3-3.7)	AGCGCACCATGAAGTTGAGAC	GACCTCTGGCATCTCTTTCTGC
Metallothionein-1E	AA872383	2.0 (1.1-2.9)	GCAAATGGCTCAGTGTTG	GAACAGCTCTTCTCCCAGAT
Cardiac gap junction	AA487623	2.1 (1.7-2.8)	CACATGATCTGATGGACTAGG	AAACTACGGTCATGTTCAGC
Archain	AA598401	1.9 (1.5-2.2)	TTACCCCAAAAGTAATCTCA	GGACAAGAAGTCTGTCATGT
MHC class 1 HLA-A	AA644657	1.7 (1.6-1.8)	TCATCAACCTCTCATGGCAAG	ATGTCCACCATGACCCTCTTC
Down-regulation				
Lysozyme	M19045	0.5 (0.3-0.7)	GGAATCAGCCTAGCAAACTGGA	CAGCGATGTTATCTTGCAGCA
Glutathione S transferase θ 2	AF240786	0.5 (0.4-0.7)	GGCAAGTCTTAAGCAAGCCATT	AGGTCATTCTGAAGGCCAAGG
GTR2-2	AA775872	0.5 (0.3-1.0)	TCTACTTCATGGCTGGAGGAGG	TCATTCCCCGCTGAAGCTT
Ubiquitinol cytochrome c reductase complex subunit VI requiring protein	AL136663	0.6 (0.5-0.7)	GGTGCTGCTTCTGCAAAGA	CTTTCTCCCCTGTCTGGAATT
Asialoglycoprotein receptor 2	R98050	0.3 (0.2-0.5)	CTCTCTTTGCTCAGCTCTTCCC	TGGAACGATGACTTCTGCCTG
Control				
GAPDH	M33197		ACCACAGCTCATGCCATCAC	TCCACCACCCTGTTGCTGTA

NOTE. Two independent clones of Hep394 cells were compared with Hep SWX cells, in which labeling with the fluorescent dyes was switched during synthesis to control labeling difference. Genes that differentially expressed by 1.5-fold or more in the average of 4 measurements are listed. Their accession numbers of GenBank, Cy5/Cy3 ratios, and primer pairs used in RT-PCR analyses are also shown.

to their transcriptional regulation. The expression of tumor rejection antigen gp96, inter-α trypsin inhibitor, orosomucoid 1, and cystain C were enhanced similarly or even higher both in Hep352 and in Hep3294 cells (IV). The down-regulated genes were grouped in 3 classes as follows: Reduced expression was found in Hep394 and Hep3294 (GTR2-2 and asialoglycoprotein receptor; V), Hep394 and Hep352 (glutathione S transferase θ 2; VI), and Hep394 only (lysozyme; VII). As in the cases of up-regulated genes, several mechanisms modulated by the viral factor(s) may contribute to the down-regulation of genes identified in this study.

Discussion

Here, we have established and characterized several human hepatoma cell lines in which expression and processing of whole or parts of HCV polyproteins can be monitored for a prolonged period of time. So far, a lack of well-defined and reproducible cell culture systems permissive for HCV infection and full replication has limited the rational study of the viral life cycle and pathogenesis against hepatocytes. In this context, stable cell lines expressing selectively part of or the entire HCV cDNA are useful in assessing the biologic features of HCV proteins. Regarding cell lines expressing whole HCV proteins, only a limited number of culture systems have been reported. Moradpour et al.[28] described a panel of human osteosarcoma-derived, tetracycline-regulated cell lines expressing the entire HCV ORF. Recently, it has been shown that dicistronic HCV RNAs encoding the full-length viral polyprotein replicate and give rise to G418-resistant clones following transfection of Huh7 cells.[12,13] Although this RNA replicon system is a robust, cell-based system for functional analysis of the viral replication, there has so far been no evidence for virus particle assembly in the system, suggesting that Huh7 cells lack some host cell factors important for HCV production. We report here a stable transfectant of the gene covering the entire HCV ORF in human hepatoblastoma-derived HepG2 cells, which is termed Hep394. The viral and cellular proteolytic machinery involved in precursor processing was shown to be consistently functional in Hep394 cells. Sizes and subcellular localization of the viral proteins are in agreement with the data obtained from transient expression and *in vitro* translation studies. HCV proteases have generally been thought to play a pivotal role in the viral life cycle, and the NS2/3 cysteine and NS3 serine proteases are now major targets for developing anti-HCV therapeutic agents. Our expression system, therefore, should be useful not only for the systematic study of HCV protein matu-

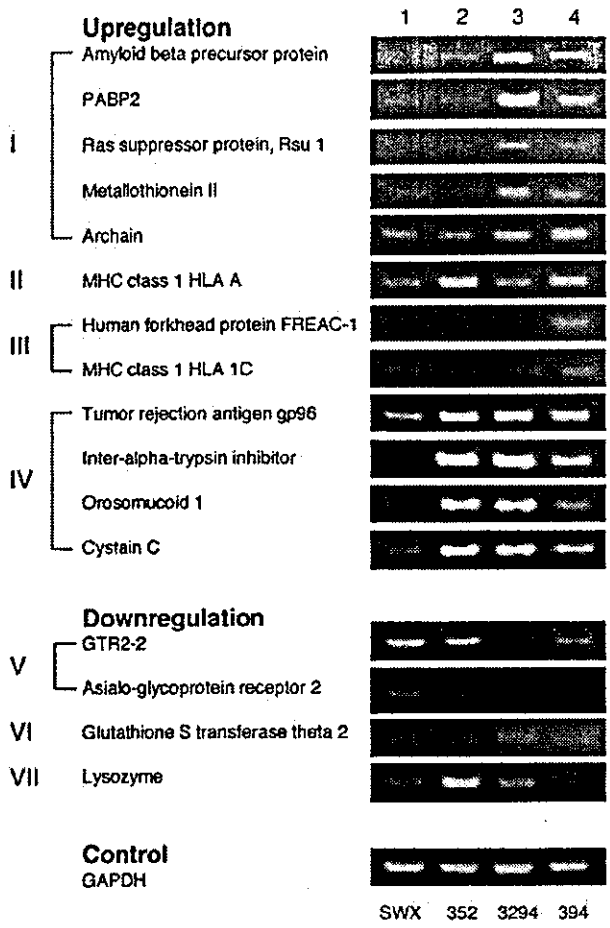

Fig. 4. Semiquantitative RT-PCR analysis of differentially expressed genes in Hep394 cells. Total RNAs were extracted from HepSWX (lanes 1), Hep352 (lanes 2), Hep3294 (lanes 3), and Hep394 cells (lanes 4), and analyzed genes were isolated from cDNA microarray by RT-PCR. Primers used and sizes of PCR products are shown in Table 1. GAPDH was amplified in parallel as a control.

ration but also for testing novel inhibitors against viral proteases in human liver cells. HepG2 cells used in this study express certain hepatocyte markers and produce several hepatic compounds like albumin.

Large-scale analysis to search for differentially expressed genes is an important tool to study the viral pathogenesis as well as virus-host interaction. cDNA microarray technique is now available to reveal the mRNA expression status of thousands of genes simultaneously and to identify disease-related gene expression profiles in a variety of biologic samples. Thus far, the cDNA microarray has been applied to the studies involving HCV infection with use of liver tissues from chronic hepatitis C patients[29] and from a chimpanzee during an acute resolving infection.[30] Until now, a large number of differentially expressed genes have been identified through such studies. However, it is not easy to evaluate the direct significance of their altered expression by HCV proteins. Heterogeneity in clinical samples and some secondary effects of cellular pathways such as immune response may be involved. It is therefore possible to identify differentially regulated mRNAs in liver cell lines constitutively expressing the whole HCV polyprotein. In this study, high-density cDNA microarray was used to analyze gene expression profiles of Hep394 cells and control HepSWX cells. Fluorescent cDNA probes were prepared by forward and reverse labeling to each sample pair to validate the results of the paired experiments. We also analyzed the mRNA levels of selected genes by semiquantitative RT-PCR and identified various genes whose expression was commonly altered in all 3 Hep394 cell clones.

Among the subset of differentially expressed genes was a number of transcriptional or translational regulators such as FREAC-1 and PABP2, which were up-regulated in Hep394 cells. The forkhead transcription factor FREAC-1 is a potent transcriptional activator, which is involved in many aspects of embryonic development and participates in cellular transformation.[31] Forkhead proteins may be implicated as nuclear targets for TGF-β and insulin-like signalling.[32,33] PABP2 is an RNA-binding protein exhibiting a preferential affinity for poly (A) stretches and is implicated in initiation of translation, mRNA stability, and regulation of poly (A) tail length during the polyadenylation reaction.[34,35] With regard to genes related to cell growth regulation, we found up-regulation of Ras suppressor Rsu-1, which is thought to have dual activities: inhibiting Ras-dependent transformation and enhancing Ras-dependent differentiation.[36,37] These 3 genes (FREAC-1, PABP2, and Rsu-1), which have not been reported to be altered in expression in livers infected with HCV and cell lines expressing HCV proteins, could be candidates for induction of cell growth abnormality.

Another category of changes in gene expression includes MHC antigens. Although human hepatocytes normally little express MHC class I antigen, its expression in cells is induced under certain circumstances, including viral infection, hepatocellular carcinogenesis, and liver allograft rejection.[38,39] It has been shown that hepatocyte expression of HLA is enhanced in livers of patients with chronic hepatitis C in association with increased $CD8^+$ T-cell infiltration.[40,41] By contrast, it has been recently reported that cell-surface expression of MHC class I is not affected by HCV proteins in the tetracycline-regulated cell line.[42]

In this report, we found up-regulation of HLA-A and -C in Hep394 cells. HLA-C gene has recently been reported to be up-regulated in cancerous tissues of HCC patients compared with noncancerous tissues.[43] HLA

molecules play an important role in the T-cell-mediated immune reaction and have been suggested to be strongly associated with the clinical profile or course of liver diseases. Hep394 thus provides an excellent model to study the mechanisms of expression of MHC antigens on hepatocytes infected with HCV.

In addition, we found that gene expression of inter-α trypsin inhibitor and tumor rejection antigen gp96 are up-regulated not only in Hep394 but in Hep352 and Hep3294. Inter-α trypsin inhibitor is a plasma serine protease inhibitor and contributes to stability of extracellular matrix. Overexpression of inter-α trypsin inhibitor, therefore, may modify remodeling of the extracellular matrix, leading to abnormalities in cellular processes such as tissue morphogenesis and differentiation.[44] The ER molecular chaperone gp96, a member of the heat-shock protein family, is known to elicit $CD8^+$ CTL responses against antigenic viral or tumor peptides.[45,46] gp96 Could assist folding and structural maturation of nascent polypeptides in the ER and involve in MHC class I-restricted antigen presentation as peptide-binding chaperones in a peptide-transporting relay line of chaperones. Thus, it is of interest to consider that differential expression of gp96 may influence the immune response pathway as well as protein folding in HCV infection.

In summary, we established human hepatoma cell lines constitutively expressing entire or parts of the HCV polyproteins. A cDNA microarray analysis coupled with semiquantitative RT-PCR allowed us to identify differentially expressed genes in the cell lines and to address mapping HCV protein(s) responsible for their regulations. Functional analysis of the genes detected in this study may help to define the molecular mechanisms of pathogenic effects of HCV.

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Interaction of Hepatitis C Virus Core Protein With Retinoid X Receptor α Modulates Its Transcriptional Activity

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Hepatic steatosis and hepatocellular carcinoma (HCC) are common and serious features of hepatitis C virus (HCV) infection, and the core protein has been shown to play distinct roles in the pathogenesis. Here we report the direct interaction of HCV core protein with retinoid X receptor α (RXRα), a transcriptional regulator that controls many aspects of cell proliferation, differentiation, and lipid metabolism. The core protein binds to the DNA-binding domain of RXRα, leading to increase the DNA binding of RXRα to its responsive element. In addition, RXRα is activated in cells expressing the core protein as well as in the livers of the core-transgenic mice that would develop hepatic steatosis and HCC later in their lives. Using promoter genes of cellular retinol binding protein II (CRBPII) and acyl-CoA oxidase as reporters, we also show that the expression of the core protein enhances the transcriptional activity regulated by the RXRα homodimer as well as by the heterodimer with peroxisome proliferator activated receptor α. Furthermore, expression of the CRBPII gene is also up-regulated in the livers of HCV core-transgenic mice. In conclusion, these results suggest that modulation of RXRα-controlled gene expression via interaction with the core protein contributes to the pathogenesis of HCV infection. (HEPATOLOGY 2002;35:937-946.)

Hepatitis C virus (HCV) is one of the major causes of post-transfusion and community-acquired hepatitis. Overwhelming lines of epidemiological evidence have indicated that persistent HCV infection poses a major risk toward development of hepatocellular carcinoma (HCC), but its molecular mechanism has not been clearly elucidated.

HCV contains a positive-stranded RNA genome of ~10 kb that encodes a single, large polyprotein of about 3,000 amino acids (aa). The core protein, derived from the N-terminus of the polyprotein, localizes mainly in the cytoplasm. However, it is also found in the nucleus; three independent nuclear localization signals have been identified.[1,2] Besides forming a viral nucleocapsid by interacting with its viral RNA,[3] the core protein has various properties that modulate cellular processes in a number of ways, including binding to cellular proteins, suppressing or enhancing apoptosis, and modulating the transcription of some genes.[4,5] These findings suggest that the core protein is deeply involved in hepatocarcinogenesis. In addition, transgenic mice expressing the core protein have been shown to develop HCC, indicating the direct contribution of the core protein to the development of HCC.[6]

Retinoid X receptor (RXR) is a member of the nuclear hormone receptor superfamily of ligand-controlled transcription factors. These receptors modulate gene expression by binding cooperatively as dimers to sequences called hormone responsive elements.[7] RXR forms ho-

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; aa, amino acid; RXR, retinoid X receptor; 9cRA, 9-cis retinoic acid; RAR, all-trans retinoic acid receptor; PPAR, peroxisome proliferator activated receptor; GST, glutathione S-transferase; CRBPII, cellular retinol binding protein II; ACOX, acyl-CoA oxidase; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-polymerase chain reaction; CBB, Coomassie Brilliant Blue; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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modimers and is involved in 9-*cis* retinoic acid (9cRA)-mediated gene activation. It also forms heterodimers with other receptors, including all-*trans* retinoic acid receptor (RAR) and peroxisome proliferator activated receptor (PPAR).[7,8] There are three RXR isotypes, RXRα, β, and γ; among them, RXRα is abundantly expressed in the liver and plays important roles in regulating cell proliferation and differentiation. In addition, PPARα-RXRα heterodimer is deeply involved in lipid metabolism. It is therefore important to determine whether or not RXRα function is altered in cells infected with HCV.

In this study, we show that HCV core protein interacts with RXRα *in vitro* as well as *in vivo*. We also show that the binding of the core protein to the DNA-binding domain of RXRα increases the DNA binding activity of RXRα in a dose-dependent manner, in addition to increasing the transcriptional activity regulated by both the RXRα homodimer and the PPARα-RXRα heterodimer. As described above, RXRα is associated with the expression of the genes implicated in various biological programs. Therefore, the modulation of its transcriptional activity by the core protein may contribute to the disturbance of cell proliferation, differentiation, and lipid metabolism in the liver, leading to oncogenic potential and perturbation of lipid metabolism. We propose that the interaction of RXRα with the core protein constitutes one of the mechanisms by which hepatic steatosis and HCC are developed in HCV infection.

Materials and Methods

Plasmid Constructions. The complementary DNA clone of HCV genotype Ib, NIHJ1, was used in this study.[9] pBlue39, used to synthesize the core protein *in vitro*, was constructed by insertion of the *Pst*I-*Bam*HI fragment from pSR39[2] into pBluescriptII (Stratagene, La Jolla, CA). pCAG39 and pCAG38, which express the core protein of aa 1-191 and 1-153, respectively, were constructed by inserting the core-coding genes into pCAGGS vector.[10] Genes encoding RXRα and PPARα were derived from complementary DNA of HepG2 cells by polymerase chain reaction (PCR) and cloned into pCAGGS (pCAG-RXRα, pCAG-PPARα). The primers were 5′-CATGAGTTAGTCGCAGCAATG-3′ and 5′-CTAAGTCATTTGGTGCGGCGC-3′, 5′-CAGCACCATCTGGTCGCGATG-3′ and 5′-TGGCTGATCTGAAGGAACTCA-3′, respectively. The plasmid used to express glutathione *S*-transferase (GST)–RXRα fusion protein in *Escherichia coli* was constructed by inserting RXRα gene into pGEX-4T-1 vector (Amersham Pharmacia, Uppsala, Sweden). Genes encoding the deletion mutants of RXRα, namely R135 (aa 1-135), R200 (aa 1-200), R(135-200) (aa 135-200), R221 (aa 1-221), R135Ndel (aa 135-462), and R200Ndel (aa 200-462) were also cloned into the same vector. R221-m1 and R221-m1m2, mutant forms of R221 protein, were made by the PCR-based method described previously.[11] The promoter gene of rat cellular retinol binding protein II (CRBPII) was derived by PCR from rat genomic DNA and cloned into pGL3-Basic (Promega, Madison, WI) (pGL-CRBPII). The primers were 5′-AAGATCTGTCTGAGCTGAAGACACAGGA-3′ and 5′-AAAGCTTGATGGCGGCCTCTGGTTTGAT-3′. The promoter region of the human acyl-CoA oxidase (ACOX) gene was a gift from Dr. R.A. Roberts,[12] and was inserted into the same vector (pGL-ACOX). The PPARα responsive element in this plasmid was mutated by the PCR method[11] from AGGTCAGCTGTCA to AGGTCACTGGTCA as reported previously.[13] pRL-TK (Promega) was used as an internal control for the luciferase assay. All these plasmids were verified by sequencing.

GST Pull-down Assay. GST fusion proteins were expressed in *Escherichia coli* and purified on glutathione Sepharose beads. *In vitro* synthesized [^{35}S] methionine-labeled proteins were generated using the TNT Coupled Reticulocyte Lysate System (Promega). Protein binding assays were performed as described.[14]

In Vivo *Coimmunoprecipitation.* The human embryonic kidney 293T cells were transfected with core- and RXRα-expressing plasmids with Lipofectamine (Gibco-BRL, Gaithersburg, MD), and staurosporine (Sigma, St. Louis, MO) was added to some dishes. After 24 hours, cells were lysed in lysis buffer (20 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 1 mmol/L sodium orthovanadate, 30 mmol/L sodium fluoride, 1% Triton X-100, 0.02% sodium dodecyl sulfate [SDS] and Protease Inhibitor Cocktail [Complete; Roche, Indianapolis, IN]). Lysates were incubated for 3 hours at 4°C with protein A-Sepharose-bound anti-RXRα antibody (D-20 and ΔN197; Santa-Cruz Biotechnology, Santa-Cruz, CA), or anti-FLAG M5 antibody (Kodak, New Haven, CT). Beads were washed three times with lysis buffer and mixed with SDS sample buffer (4.6% SDS, 125 mmol/L Tris-HCl [pH 6.8], 10% 2-mercaptoethanol, and 0.2% bromophenol blue). Immunoblots were developed using anti-RXRα (D-20) and anti-HCV core antibody (Anogen, Mississauga, Canada) as described previously.[15]

Electrophoretic Mobility Shift Assay. GST fusion proteins bound to the beads were purified with elution buffer (10 mmol/L reduced glutathione, 50 mmol/L Tris-HCl [pH 8.0]). Nuclear proteins were extracted from HepG2 cells stably[16] or transiently expressing the core protein, and the liver of the transgenic mice,[6] using Nu-

clei Isolation Kit (Sigma). HepG2 cells transiently expressing the core protein were prepared by transfecting cells with pCAG39. ^{32}P-labeled oligonucleotides containing the RXRα-binding site (5′-AGCTTCAGGTCAGAGGTCAGAGAGCT-3′) were incubated on ice for 30 minutes, along with 10 μg of proteins, in binding buffer (10 mmol/L Tris [pH 8.0], 6% glycerol, 1 mmol/L dithiothreitol [DTT], and 1 μg of poly[dI-dC]). For competition or supershift experiments, a 50-fold of unlabeled, identical, or unrelated oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′), or anti-RXRα antibody (D-20) was added 20 minutes before the addition of labeled oligonucleotide. HCV core protein used was purified as described previously.[17] The protein used as a control was prepared from cells infected with the wild-type baculovirus (AcNPV).

Luciferase Assay. Chinese hamster ovary cells were transfected with 0.5 μg of pCAG-RXRα (for PPARα assay, plus 0.5 μg of pCAG-PPARα). After 24 hours, cells were transfected with 0.2 μg of a reporter plasmid, 0.01 μg of pRL-TK, and 0.5 μg of pCAG39 or pCAGGS vector, and then 9cRA (ICN, Costa Mesa, CA) or WY-14643 (Calbiochem, La Jolla, CA) was added to some dishes. After 24 hours, cells were harvested, and luciferase activities were determined by the Dual-Luciferase Reporter Assay System (Promega) as described previously.[18]

Reverse Transcription-PCR. Total RNA was extracted from the livers of HCV core gene transgenic mice and nontransgenic littermates using RNAzolB (TEL-TEST, Friendswood, TX). Three μg of total RNA were reverse-transcribed by Superscript II (Gibco-BRL) using oligo(dT) primer. Detection of the mouse CRBPII gene was performed using the following primers: 5′-GGTGGAGTTTGACGAACACAC-3′ and 5′-TTTGAACACTTGTCGGCACAC-3′. As an internal control, the β-actin gene was amplified. The primers were 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TGGAATCCAGGGGAGCCTGTAGTGCAG-3′.

Statistical Analysis. Results are expressed as the means ± SE. The significance of the difference in means was determined by Student's *t* test.

Results

Binding of RXRα With HCV Core Protein. We examined whether HCV core protein could bind to RXRα by GST pull-down assay. As shown in Fig. 1A, GST-RXRα pulled down the core protein, whereas GST did not. Coomassie Brilliant Blue (CBB) staining indicated that the amounts of GST and GST-RXRα protein used were similar. We also observed that GST-RXRα pulled down C-terminal truncated core proteins (aa 1-111 and aa 1-153) (data not shown). Similarly, the core protein (aa 1-111) fused to GST (GST-core) as well as GST-RXRα, but not GST alone, pulled down RXRα (Fig. 1B). These results indicate that the core protein directly binds to RXRα *in vitro*. We then determined which region of RXRα binds to the core protein. RXRα is composed of four domains: the A/B, C (DNA-binding domain), D (hinge domain), and E/F (dimerization and ligand-binding domain).[8] We constructed the plasmids that express one or more than two domains of RXRα fused to GST and used them for the binding assay. As shown in Fig. 1C, R(135-200), R200, R221, and R135Ndel, but not R135 and R200Ndel, pulled down the core protein. All the fusion proteins that bound to the core protein contained the DNA-binding domain of RXRα. To exclude the possibility that the core protein binds indirectly to RXRα via a small amount of contaminating DNA, R(135-200) was incubated with the core protein in the presence of DNaseI. We confirmed that DNaseI could work in this condition (data not shown). As shown in Fig. 1D, R(135-200) pulled down the core protein even in the presence of DNaseI. Thus, the results show that the core protein directly binds to the DNA-binding domain of RXRα.

There are two zinc finger motifs in the DNA-binding domain of RXRα, which play a critical role in mediating the DNA-binding ability. These zinc finger structures are composed of a zinc ion surrounded by four cysteines.[19] By mutating these cysteines, a zinc finger structure is theoretically disrupted. We therefore constructed plasmids encoding R221-m1 and R221-m1m2 protein, mutant forms of RXRα, in which the first zinc finger structure (R221-m1) or both (R221-m1m2) are disrupted by introducing the three cysteine to alanine mutations (Fig. 2A). Using these bacterially expressed proteins, we determined the DNA binding activity of these mutant forms of RXRα by electrophoretic mobility shift assay (EMSA). As expected, R221-m1 and R221-m1m2 as well as GST alone lost their DNA binding activity, whereas wild-type R221 bound to the oligonucleotides containing the RXRα binding site (Fig. 2B). We then used these mutant proteins to examine whether the core protein binds to RXRα via the zinc finger motifs. As shown in Fig. 2C, mutant RXRα as well as the wild-type proteins bound to the core protein. These results suggest that zinc finger structures in the DNA-binding domain of RXRα are not essential for its binding to the core protein.

Interaction of RXRα With HCV Core Protein in Cells. To determine whether HCV core protein can interact with RXRα in cells, we performed coimmunoprecipitation on cells expressing RXRα and the core protein (Fig. 3). Previous studies indicated that the core protein

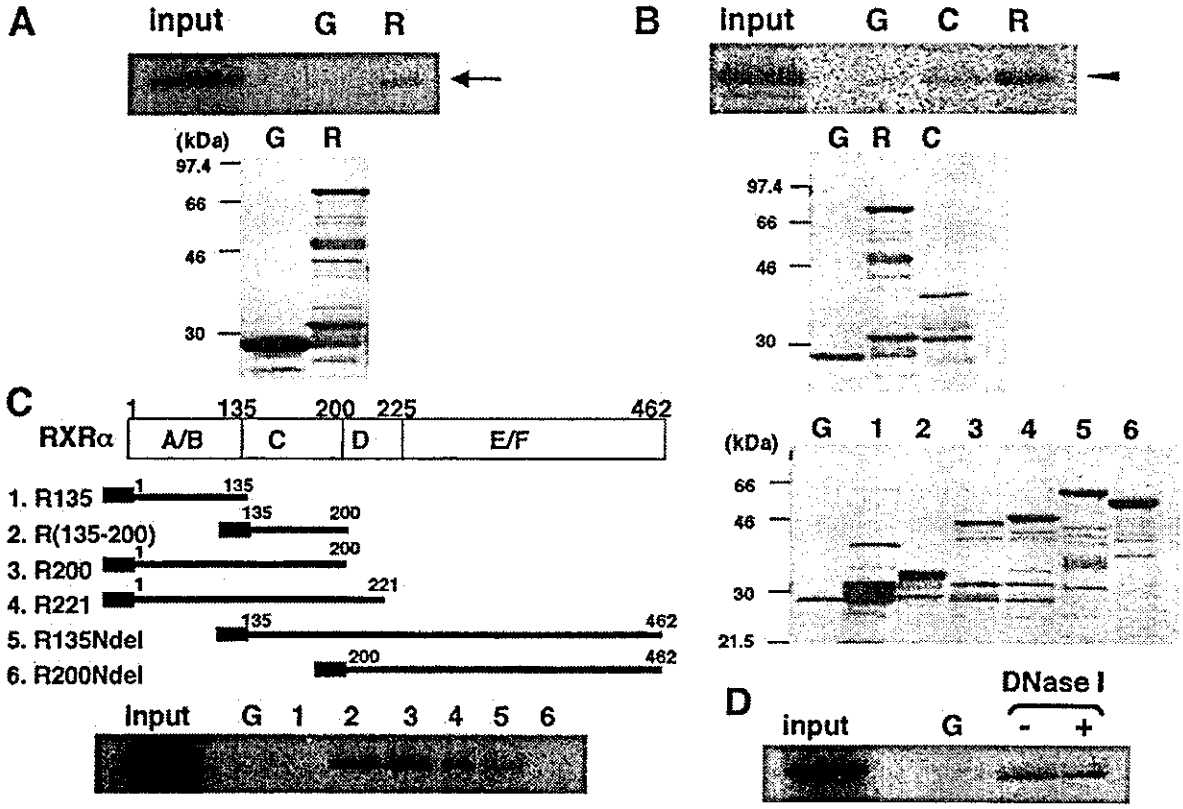

Fig. 1. Interaction of HCV core protein with RXRα in its DNA-binding domain *in vitro*. (A) *In vitro* synthesized [^{35}S]-labeled HCV core protein was incubated with GST (G) or GST-RXRα fusion (R) proteins bound to glutathione-Sepharose beads. Bound proteins were then analyzed by SDS-PAGE. The core protein is indicated by the **arrow**. The **lower panel** shows CBB staining of GST and GST-fusion proteins used. (B) *In vitro* synthesized RXRα was incubated with GST (G), GST-RXRα (R), or GST-core (C) proteins bound to the beads, and bound proteins were analyzed as well. RXRα is indicated by the **arrowhead**. (C) RXRα is composed of the A/B, C (DNA-binding), D (hinge), and E/F (ligand-binding and dimerization) domains. Various deletion mutants were constructed and used for the binding assay. The boundaries of amino acid residues are indicated above the boxes. The **closed square** is GST protein. The **right panel** shows CBB staining of GST-fusion proteins used. (D) Twenty units of DNaseI were added to the binding buffer and incubated under the same condition.

expressed from the full-length core construct was partly localized in the nucleus both in cultured cells[20,21] and in transgenic mice,[6] although it was mostly localized in the cytoplasm. RXRα was usually localized in the nucleus, so we first used a nuclear form of the core protein (aa 1-153) in this assay.

Lysates of cells expressing RXRα and the core protein were subjected to immunoprecipitation using two kinds of anti-RXRα antibodies, one of which recognizes the N-terminus of RXRα (D-20), while the other recognizes the C-terminus (ΔN197). Because it has been shown that RXRα is phosphorylated by mitogen-activated protein kinase[22,23] and that anti-RXRα (ΔN197) antibody cannot recognize the phosphorylated form of RXRα,[23] cells were incubated in the presence or absence of protein kinase C inhibitor staurosporine. By Western blot analysis, we confirmed that cells transfected with the core-expression plasmid expressed similar amount of the core protein irrespective of the staurosporine treatment (data not shown).

In cells cotransfected with the core- and RXRα-constructs in the presence of staurosporine, the core protein was coimmunoprecipitated by anti-RXRα (ΔN197) (lane 2) but not by anti-RXRα (D-20) (lane 3) or anti-FLAG (lane 4) antibodies. In contrast, the core protein was not almost precipitated by anti-RXRα (ΔN197) antibody in the absence of staurosporine (lane 1), probably because of poor recognition of phosphorylated RXRα by this antibody. It remains unknown why the core protein was not coimmunoprecipitated even in the presence of staurosporine by anti-RXRα (D-20) antibody, which recognizes the N-terminal region. One possible reason is that the epitope that this antibody recognizes is near the domain necessary for the interaction with the core protein, and thus this antibody could not recognize RXRα interacting with the core protein in cells. We also performed the same

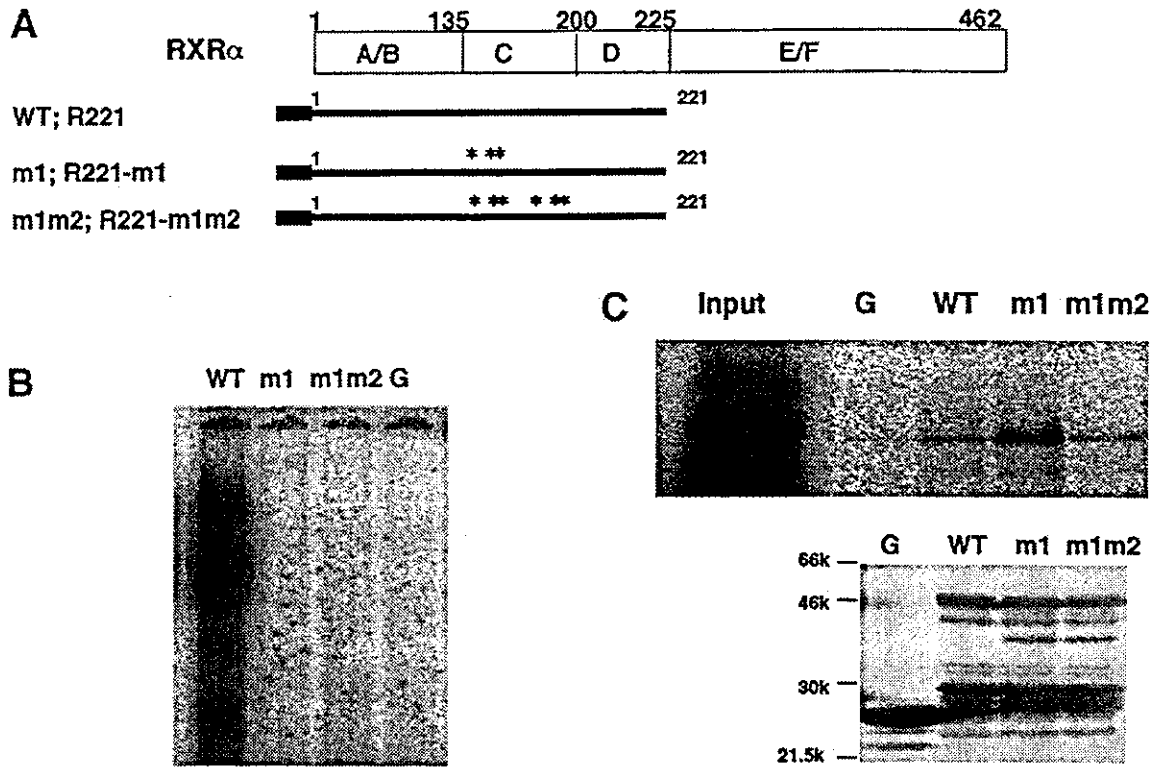

Fig. 2. Characterization of RXRα having disrupted zinc finger structure in its DNA-binding domain. (A) Mutant forms of RXRα (aa 1-221; R221) whose first (m1) and both (m1m2) of the zinc finger motifs were disrupted were constructed. The **asterisks** indicate a mutation from cysteine to alanine. (B) Proteins expressed from the constructs shown in (A) were incubated with ^{32}P-labeled DNA containing RXRα binding site and then analyzed by EMSA. (C) *In vitro* synthesized [^{35}S]-labeled core protein was mixed with the proteins and bound proteins were analyzed by SDS-PAGE. The **lower panel** shows CBB staining of proteins used in EMSA and pull-down assay.

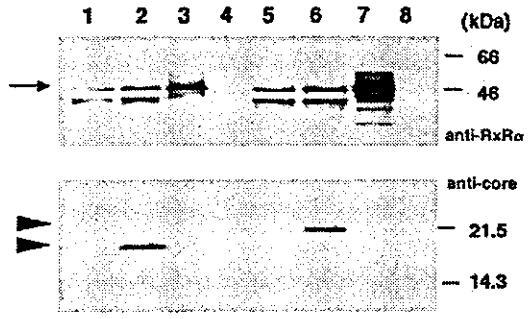

Fig. 3. Interaction of RXRα with HCV core protein in cells. Cells were transfected with truncated (lane 1-4) or full-length (lane 5-8) core- and RXRα-expression plasmids, and incubated in the presence (lane 2-4, 6-8) or absence (lanes 1 and 5) of 50 nmol/L staurosporine. Cells were harvested 24 hours after transfection, lysed, and subjected to immunoprecipitation. Lysates were immunoprecipitated with anti-RXRα (ΔN197) antibody (lanes 1, 2, 5, and 6), anti-RXRα (D-20) antibody (lane 3 and 7), anti-FLAG (M5) antibody (lanes 4 and 8). Immunoprecipitates were then analyzed by SDS-PAGE followed by Western blot analysis with antibodies indicated on the right side. The **arrow** and **arrowheads** indicate RXRα and the core protein, respectively.

experiment using cells expressing the full-length core protein (aa 1-191). In these cells, the core protein was mostly localized in the cytoplasm and nuclear core protein was detected in only a small portion of cells. So we used a large amount (about 20-fold) of cells in this assay and detected a specific interaction between the core protein and RXRα in the same condition (lane 6). We further performed immunofluorescence staining of RXRα and the core protein (aa 1-153) coexpressed in cells. Confocal microscopic analysis showed that RXRα and the core protein colocalized partially in the nucleus (data not shown). When the full-length core construct was used, colocalization was observed in some cells expressing the core protein in the nucleus. Together, these experiments indicated that the core protein and RXRα form a complex *in vivo*.

Enhancement of the DNA Binding Activity of RXRα by HCV Core Protein. Because the above data indicated that HCV core protein interacts with RXRα, we next examined whether this interaction influences the DNA binding activity of RXRα. R221 protein, which contains a DNA-binding domain but lacks a ligand-binding domain, was prepared (Fig. 4A) and used for the fol-

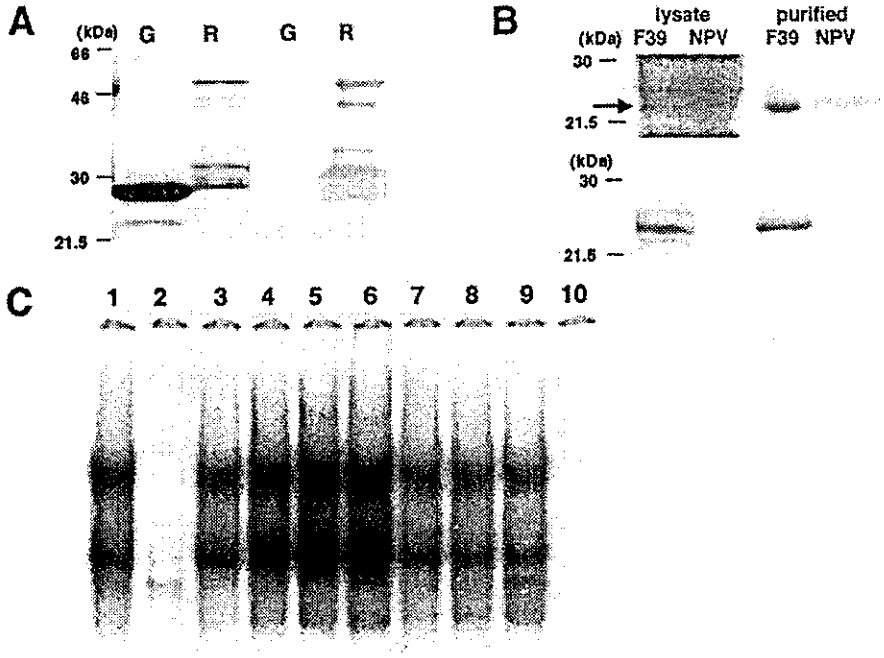

Fig. 4. The effect of HCV core protein on the DNA binding activity of RXRα. (A) CBB staining (**left**) and Western blot analysis with anti-RXRα antibody (**right**) of partially purified GST (G) and R221 (R). (B) CBB staining (**upper**) and Western blot analysis with anti-core antibody (**lower**) of lysate and purified core protein. The lysate (lysate) of Tn-5 cells infected with AcF39 (F39) or AcNPV (NPV) was separated by SDS-PAGE and stained with CBB, then purified. Purified protein (purified) was also separated and subjected to CBB staining and Western blot analysis. The **arrow** indicates FLAG-tagged core protein. (C) R221 protein was incubated with ^{32}P-labeled oligonucleotides in the presence or absence of the purified core protein, and analyzed by EMSA. Lane 1, mixture of R221 with ^{32}P-labeled oligonucleotides containing RXRα binding site; lane 2, addition of 50-fold amount of the same, unlabeled oligonucleotides to the mixture in lane 1; lane 3, addition of 50-fold amount of unrelated, unlabeled oligonucleotides to the mixture in lane 1; lanes 4-6, addition of the core protein (lane 4, 0.5 μg; lane 5, 1.0 μg; lane 6, 1.5 μg) to the mixture in lane 1; lanes 7-9, addition of control protein (lane 7, 0.5 μg; lane 8, 1.0 μg; lane 9, 1.5 μg) derived from AcNPV-infected cells to the mixture in lane 1; lane 10, mixture of the core protein with ^{32}P-labeled oligonucleotides.

lowing experiment. The core protein tagged with FLAG epitope was expressed by recombinant AcNPV (AcF39) and partially purified as described previously (Fig. 4B).[17] We confirmed that this purified core protein bound to R221 protein (data not shown). The protein of the same molecular mass (approximately 23 kd) similarly prepared from wild-type AcNPV-infected cells was used as a control. Using these proteins, we determined the effect of the core protein on the DNA binding activity of RXRα by EMSA (Fig. 4C). In the absence of the core protein, shifted bands corresponding to RXRα-bound DNA were observed (lane 1). Not one but two shifted bands were detected, presumably because of the presence of parasitic cleavage in RXRα as observed by CBB staining (Fig. 4A). These bands disappeared by the addition of a 50-fold amount of the same nonlabeled oligonucleotides (lane 2) but not by a 50-fold amount of nonlabeled, unrelated oligonucleotides (lane 3), confirming that both bands reflected the specific binding of RXRα to DNA. In the presence of the core protein, the intensity of these shifted bands increased in a dose-dependent manner (lanes 4-6). No supershifted bands corresponding to the complex of RXRα and the core protein were observed in these lanes. In addition, no shifted bands were detectable in the presence of the core protein only (lane 10), and CBB staining of the nondenaturing gel used in this study showed no migration of the core protein into the gel (data not shown). These results suggest that the core protein enhances DNA binding activity of RXRα, even after the dissociation of the core protein from RXRα. The enhancement of the DNA binding activity of RXRα was not observed when proteins derived from AcNPV-infected cells were added (lanes 7-9), indicating that the increased intensity of the shifted bands was caused by expression of the core protein, but not because of AcNPV- or cell-derived factors.

We next examined whether this enhancement of RXRα DNA binding activity by the core protein actually occurred *in vivo*. We extracted nuclear proteins from HepG2 cells stably or transiently expressing the core protein and performed EMSA. As shown in Fig. 5A, DNA binding activity of RXRα was increased in cells expressing

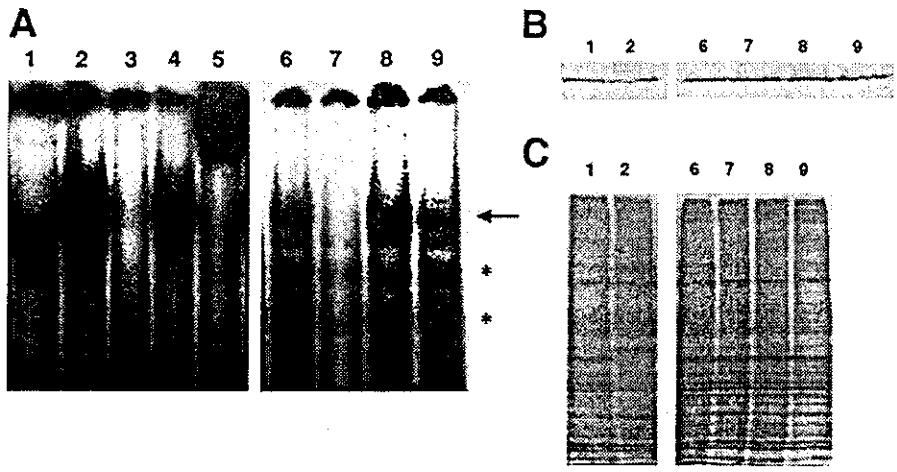

Fig. 5. RXRα activity in cells expressing HCV core protein. (A) Nuclear proteins were extracted from HepG2 cells stably or transiently expressing the core protein and analyzed by EMSA as shown in Fig. 4. Lane 1, HepG2 cells transfected with empty vector (Hepswx cells); lane 2, HepG2 cells stably expressing the core protein (Hep39); lane 3, addition of 50-fold amount of the same, unlabeled oligonucleotides to the mixture in lane 2; lane 4, addition of 50-fold amount of unrelated, unlabeled oligonucleotides to the mixture in lane 2; lane 5, addition of anti-RXRα antibody to the mixture in lane 2; lane 6, HepG2 cells transiently expressing the core protein; lane 7, parental HepG2 cells; lane 8, core-expressing HepG2 cells incubated with 9cRA; lane 9, parental HepG2 cells incubated with 9cRA. The **arrow** indicates the band corresponding to DNA-RXRα complex. **Asterisks** are nonspecific bands. (B) The same amount of nuclear proteins used for EMSA were separated by SDS-PAGE and Western blot analysis was performed with anti-RXRα antibody. (C) Nuclear proteins used for the assay were separated by SDS-PAGE and stained with CBB.

the core protein. This activation was also observed in cells stimulated by 9cRA, a ligand of RXRα. Compared with cells transiently expressing the core protein, RXRα was more activated in a stable cell line, because of the number of cells expressing the core protein. By Western blot analysis, RXRα expression was similar between these cells (Fig. 5B), indicating that the core protein did not enhance RXRα expression. These results suggest that the core protein increases DNA binding activity of RXRα *in vivo* as well as *in vitro*.

Enhancement of the Transcriptional Activity of RXRα by HCV Core Protein. We next examined whether HCV core protein would affect the transcriptional activity of RXRα. RXRα forms homodimers and activates the transcription of genes having RXRα binding sequence in their promoter regions. One of the genes is a CRBPII gene, whose promoter region contains five nearly perfect tandem repeats of the sequence AGGTCA.[24] We therefore made a plasmid encoding the luciferase gene under the promoter region of the CRBPII gene, and used it to determine the modulation of RXRα transcriptional activity by the core protein. As shown in Fig. 6A, luciferase activity in Chinese hamster ovary cells transiently expressing the full-length core protein increased approximately 2-fold compared with those not expressing the core protein irrespective of the presence or absence of 9cRA ($P < .01$). This enhancement by the core protein was also observed in human hepatoma HepG2 and FLC4 cells (data not shown). In addition, this increase of luciferase activity was not observed when the promoter of the SV40 early region was used (data not shown), indicating that the core protein selectively transactivated the CRBPII gene in cooperation with RXRα. This was also shown by EMSA showing RXRα activation in core-expressing cells (Fig. 5A). In addition, we observed the increase of luciferase activity in cells expressing both the core, E1, and E2 of HCV proteins ($P < .01$), but not in cells expressing only E1 and E2 (Fig. 6B).

To examine whether this enhanced transcription of CRBPII gene by the core protein is physiologically significant, we next determined the CRBPII gene expression in the liver of transgenic mice constitutively expressing the core protein. As reported previously, these transgenic mice developed hepatic steatosis and some finally developed HCC.[6,25] Total RNA was extracted from the liver of 2- to 3-month-old, transgenic- and nontransgenic mice and reverse transcription-PCR (RT-PCR) of the CRBPII gene was performed. As shown in Fig. 7A, expression of CRBPII gene in the liver of the transgenic mice was clearly detectable whereas no or slight expression was observed in nontransgenic littermates. This observation is consistent with the previous reports showing that expression of CRBPII messenger RNA is detected early in fetal development and peaks at 3 to 4 days after birth, but then becomes quiescent in the adult liver.[26,27] A similar tendency was observed when we changed cycles of the PCR, reflecting the real gene expression. We also performed EMSA using nuclear proteins extracted from the liver of

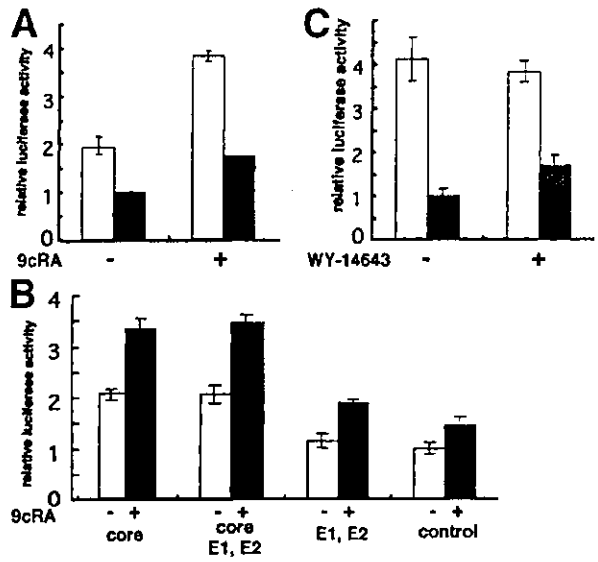

Fig. 6. The effect of HCV core protein on transcriptional activity of RXRα in cells. (A) Chinese hamster ovary cells were transfected with the reporter plasmid pGL-CRBPII, *Renilla* luciferase-expression plasmid (pRL-TK), and the core-expression plasmid (pCAG39) or empty vector (pCAGGS). Cells were incubated with or without 1 μmol/L 9cRA, lysed after 24 hours, and luciferase activities were determined and normalized on the basis of *Renilla* luciferase activities. (B) Luciferase activities were determined in the cells expressing the core protein, the core and envelope proteins of HCV (E1, E2), only E1 and E2 proteins. (C) Chinese hamster ovary cells were transfected with the reporter plasmid pGL-ACOX instead of pGL-CRBPII shown in A, incubated with or without 100 μmol/L WY-14643, and luciferase activities were determined. Luciferase activity was normalized by taking the activity of pCAG empty vector-transfected cell (without ligand) lysate as 1 (relative luciferase activity). Results are expressed as the mean (**bar**) ± SD (**line**) of at least 3 experiments. (A and C) **Open bars**, pCAG39-transfected cells; **closed bars**, pCAG empty vector-transfected cells. (B) **Open bars**, without the treatment of 9cRA; **closed bars**, with the treatment of 9cRA.

the mouse, and found that RXRα was more activated in the liver of the transgenic mouse (Fig. 7B). These results suggest that RXRα activation leads to enhanced expression of CRBPII gene in the livers of core-transgenic mice that would develop steatosis and possibly HCC in their later life.

RXRα also forms a heterodimer with PPARα, which is related to lipid metabolism. We thus determined the effect of the core protein on the transcriptional activity of the PPARα-RXRα heterodimer. Because the ACOX gene is regulated by PPARα,[13] we determined the promoter activity of this gene in cells expressing the core protein. As shown in Fig. 6C, luciferase activity in cells expressing the core protein increased approximately 2- to 4-fold compared with those not expressing the core protein regardless of the absence or presence of the PPARα ligand WY-14643. Thus, these data show that the core protein has an ability to enhance the transcriptional activation mediated not only by the RXRα homodimer but also by the PPARα–RXRα heterodimer.

Discussion

In this report, we show that HCV core protein interacts with RXRα in its DNA-binding domain. We also showed that a target disruption of zinc finger structures in the DNA-binding domain, which abolishes the binding of RXRα to DNA, did not affect the binding of these proteins *in vitro*. Therefore, the zinc finger structures may not be essential for this interaction. Nevertheless, the DNA-binding domain of nuclear receptors is generally highly conserved, suggesting a potential ability of other nuclear receptors to interact with the core protein. In this regard, there have been only a few reports showing the interaction of viral proteins and retinoic acid–related receptors. Adenovirus E1A interacts with RARβ in its AF-2 domain and functions as a cofactor.[28] The Epstein-Barr virus BZLF-1 was shown to interact with the ligand-binding domain of RXRα and RARα, and to repress the transcriptional activity of RXRα.[29] To our knowledge, however, this is the first report to show the direct interaction of a viral protein with the DNA-binding domain of RXRα.

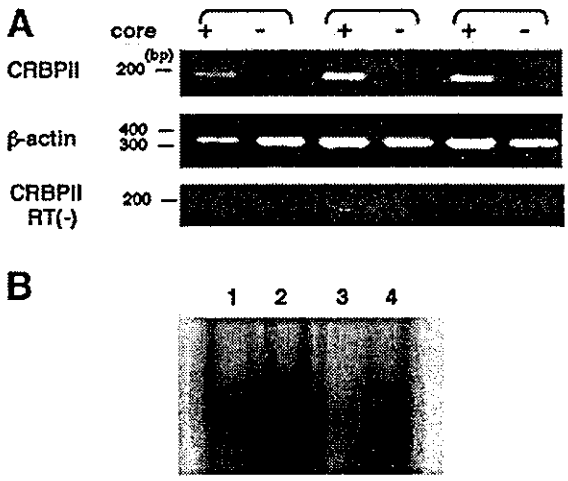

Fig. 7. Expression of the CRBPII gene and RXRα activity in HCV core-transgenic mice. (A) Total RNA was extracted from the livers of the core-transgenic mice (core +) and their littermates (core −) and RT-PCR was performed using the primers that amplify mouse CRBPII gene (35 cycles). PCR products were visualized by ethidium bromide staining after agarose (2%) gel electrophoresis. The β-actin gene (25 cycles) was used as an internal control (**middle panel**). PCR products without RT are also shown in the **bottom panel**. (B) EMSA was performed in the same procedure as Fig. 5 using nuclear proteins extracted from the liver of the mice corresponding to the **right pair** in A. Lane 1, nontransgenic mouse; lane 2, core-transgenic mouse; lane 3, addition of 50-fold amount of the same, unlabeled oligonucleotides to the mixture in lane 2; lane 4, addition of 50-fold amount of unrelated, unlabeled oligonucleotides to the mixture in lane 2.

We further showed that core-RXRα interaction leads to enhancement of the DNA binding and transcriptional activity of RXRα in core-expressing cells as well as in the liver of the core-transgenic mice, although the mechanism of its enhancement remains unknown. Because the transcriptional activity of RXRα homodimer and heterodimer is enhanced in core-expressing cells, the core protein may function as a cofactor of RXRα. Otherwise, the core protein may directly induce a conformational change in the DNA-binding domain. A recent report showed that certain conformational changes can occur within the DNA-binding domain of RXRα in the presence of RAR and leads to an increased affinity of RXRα to DNA.[30] It is possible, however, that an alternative mechanism works for the enhancement of the transcriptional activity of RXRα by the core protein. Further study is necessary for elucidating the mechanism of RXRα activation by the core protein.

Retinoid and its derivative, retinoic acid, play important roles in development, differentiation, and homeostasis, and RXRs and RARs are engaged in the control of gene expression by retinoid signals. Although some reports have suggested that retinoids/RXRs may be implicated in carcinogenesis,[31,32] as far as we know, none have ever shown the effects of up-regulation of RXRα expression and/or transcriptional activity on the pathogenesis in liver tissues. RXRα is possibly associated with the transcription of a variety of genes in hepatocytes, as indicated in a study showing that the metabolic pathways of adult mice having a hepatocyte-specific disruption for the RXRα gene were compromised.[33] In this study, we showed that RXRα activity as well as expression of an RXRα-regulated gene was enhanced in the livers of transgenic mice expressing the core protein that had almost normal livers but would possibly in the future develop steatosis and HCC. So it is possible that RXRα activation by the core protein may contribute to these pathogeneses by affecting the gene expression regulated by RXRα. Because hepatic steatosis and HCC are also commonly observed in the liver of HCV-infected patients,[34] these findings in this study can be a clue to elucidating the mechanism of these pathogeneses and developing a therapeutic agent in humans.

The core protein has been shown to modulate NF-κB activation; depending on the experimental conditions, the core protein can either inhibit or enhance its activation.[35-38] There are several reports showing a physical association of RXRα with members of the NF-κB family and their inhibitors, the IκB proteins.[39-41] In particular, the NF-κB components p50 and p65 have been shown to interact with the N-terminal ABC domains of RXRα,[41] which are close to the binding site for the core protein shown in this study. Considering these findings, the core protein could possibly compete with p50 and p65 for interaction with RXRα and influence the NF-κB signaling pathway.

In conclusion, we have shown that HCV core protein is able to interact directly with RXRα, thereby enhancing DNA-RXRα binding and also the transcriptional activity regulated by RXRα homo- and heterodimers. These findings should initiate a new line of research into the mechanistic functions of the core protein in various pathogeneses, including hepatic steatosis and HCC by HCV infection.

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Structural Analysis of Vaccinia Virus DIs Strain: Application as a New Replication-Deficient Viral Vector

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DIs is a restrictive host range mutant of vaccinia virus strain DIE that grows well only in chick embryo fibroblast cells but is unable to grow in most mammalian cells. In this study, we identified one major deletion (15.4 kbp) which results in the loss of 19 putative open reading frames in the left end of the genome. We then established a system to express foreign genes by inserting them into the deleted region of DIs. We constructed rDIs to express the bacteriophage T7 polymerase (T7pol) gene and showed the expression in various mammalian cell lines by reporter luciferase gene expression under the T7 promoter. We also expressed the full-length human immunodeficiency virus (HIV)-1 NL432 *gag* gene. The expressed *gag* gene product induced high levels of cytotoxic T lymphocytes in immunized mice. These data suggest that DIs is useful as an efficient, transient replication-deficient viral vector.

Key Words: attenuated vaccinia virus; transient viral vector; new vaccine.

INTRODUCTION

Vaccinia virus is the prototype member of the *Orthopoxvirus* genus in the family *Poxviridae.* It contains many genes that are required for replication and expression of the linear, double-stranded DNA genome within the cytoplasm of the host cells (Moss, 2001). A notable feature of vaccinia virus is its ability to multiply in many cell types from various species. However, deletion of some specific genes of vaccinia virus results in high attenuation with restricted host range, as with MVA (modified vaccinia Ankara) (Mayr *et al.,* 1975) and NYVAC (Tartaglia *et al.,* 1992). MVA can no longer replicate or replicates very poorly in a variety of mammalian cell lines and is nonpathogenic even for immunodeficient animals (Mayr *et al.,* 1975; Stittelaar *et al.,* 2001). The genome of MVA is 178 kbp in length, containing seven large deletions and one large insertion compared with wild-type vaccinia virus (Antoine *et al.,* 1998). In nonpermissive cells, MVA macromolecular synthesis is unperturbed but assembly of virus particles is interrupted at an immature and noninfectious stage (Carroll and Moss, 1997; Sutter and Moss, 1992). In contrast, NYVAC was constructed as a highly attenuated vaccinia vector generated by the precise deletion of 18 open reading frames (ORFs), including a number of viral gene functions associated with virulence (Tartaglia *et al.,* 1992).

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Recombinant vaccinia viruses expressing foreign genes are proposed as new vaccines (see review, Moss, 2001). However, previously used vaccine strains are all still potentially virulent and the recombinant viruses thereof would be inadequate for actual use in live vaccines. Therefore there is every reason to expect to have highly attenuated vaccinia strains. In this context, MVA have been used for the expression of various kinds of foreign genes (Belyakov *et al.,* 1998; Seth *et al.,* 1998; Sutter and Moss, 1992, 1994; Wyatt *et al.,* 1995, 1996). Some of these recombinant viruses were studied as recombinant vaccines and appeared to be even more effective than comparable replication-competent vaccinia virus-based vaccines (Belyakov *et al.,* 1998; Nam *et al.,* 1999; Ourmanov *et al.,* 2000a,b; Seth *et al.,* 1998, 2000; Stittelaar *et al.,* 2000; Sutter *et al.,* 1994). NYVAC has also been used for the expression of foreign genes and the development of new vaccines is ongoing (Brockmeier *et al.,* 1993; Hel *et al.,* 2000; Kazanji *et al.,* 2001; Konishi *et al.,* 1998, 1992).

The DIs strain is a highly restricted host range mutant of vaccinia virus that has been isolated by successive 1-day egg passage of DIE, an authorized strain for smallpox vaccine and actually used in Japan until 1981. When inoculated onto the chorioallantoic membrane (CAM) of 12-day eggs, it produces vaccinia-common, complement-fixing antigen and hemagglutinin. Moderate cytopathic effects (CPEs) were observed in primary monkey kidney cells infected with DIs, whereas no CPE were observed when HeLa cells were infected with this mutant (Tagaya

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et al., 1961). DIs does not replicate and is not pathogenic for mice, guinea pigs, or rabbits (Kitamura and Kitamura, 1963). Moreover, this strain does not replicate in various mammalian cell lines, including BHK, RK13, and CV-1 (Fujii and Minamitani, personal communication). In this study, we first compared the physical map of DIs with the parental virus DIE and determined its deleted regions. We then determined the regions responsible for its highly restrictive host range of DIs. Moreover, we have established a system for expressing foreign genes by inserting them into the deleted region. We showed the usefulness of this system by expressing (i) bacteriophage T7 polymerase and (ii) the full-length HIV-1_{NL432} *gag* gene. The recombinant DIs that expressed HIV-*gag* induced high levels of cytotoxic T lymphocytes in immunized mice. The replication-deficient DIs strain is thus useful as a viral expression vector and expected to be a safe live vaccine.

RESULTS

Mapping of the deleted region of DIs

We first compared the *Hin*dIII restriction profile of DIs with that of DIE (Fig. 1A). Compared to DIE, fragment C of DIs migrated faster, while fragments K, M, and N disappeared. *Pst*I restriction profiles revealed that fragment A of DIs migrated faster but other fragments migrated similarly to those of DIE (data not shown). Taken together, a major deletion in the left end of the DIE genome resulted in the generation of DIs. To determine the exact size of the deletion, a set of primers was synthesized for amplification of this region (Fig. 1B). The amplified fragment was inserted into the *Eco*RI site of pUC19 and the sequence was determined. The junction sequence TCATCAATCATCATTT (Fig. 1C) corresponds to nt 17145–17161 (TCATCAATCATACATTT, one additional A is shown as a bold character) and nt 32546–32662 (TCATCAATCATCATTT) of the vaccinia virus Copenhagen strain. This result suggests that the 15.4-kbp deletion in DIs arose as a result of homologous recombination between these sequences (nt 17145–17161 and 32546–32662). This deletion resulted in a partial or complete loss of 19 putative ORFs, including 2 known host range genes (C7L and K1L) (Gillard *et al.*, 1986; Perkus *et al.*, 1990); 17 other genes were also partially or completely deleted, but they are thought to be nonessential for viral replication (Goebel *et al.*, 1990; Johnson *et al.*, 1993; Nam *et al.*, 1999).

Establishment of the system to insert foreign genes into DIs

Because of rDIs's highly restrictive host range for replication, rDIs may become a unique expression vector. Initial attempts to insert the *Escherichia coli lacZ* gene into the tk locus of DIs by conventional methods (Mackett *et al.*, 1982; Sutter and Moss, 1992) were unsuccessful (data not shown). This may be due to destruction of the tk gene as has been reported for recombinant MVA (Scheiflinger *et al.*, 1996). Therefore, we selected the region flanking the deletion site in DIs for the insertion of foreign genes using the transfer vector pUC/DIs (Fig. 2A). We initially obtained the recombinant virus containing the *E. coli lacZ* gene (rDIsLacZ) by using transfer vector pUC/DIsLacZ and selection from blue virus plaques stained with X-Gal. Purified virus was propagated in CEF cells and used as the parental virus for generating recombinant DIs. We obtained recombinant viruses by using transfer vectors that harbored target genes in the cloning site of pUC/DIs. Recombinant viruses were selected from colorless virus plaques stained with X-Gal, purified four times by repetitive selection, and propagated in CEF cells (Fig. 2B). The establishment of this system allowed us to construct several recombinant viruses, including fully or partially complemented viruses of the deleted region of DIs.

Complementation analysis of the deleted region

We characterized the deleted region of DIs in more detail. This region in the parental DIE was divided into four parts (d1, d2, d3, and d4) and 10 recombinant viruses were constructed which fully or partially complemented regions within the deletion (Fig. 2C). Complementation was confirmed by PCR using Vac H-C and Vac H-F primers to amplify the inserted regions (data not shown). Replications of these complemented viruses were compared (Table 1). The recombinant d1234, in which the entire deleted region was re-inserted, grew in several mammalian cell lines, with the exception of CHO cells. Recombinant d123, which contained both host range genes (K1L and C7L) reached titers comparable to those of DIE. Recombinants d23 and d234, which contained one host range gene (K1L), could grow in several mammalian cell lines (Table 1). By comparison, recombinants d1, d2, d3, d4, d12, and d34 were restricted for replication in CV-1 and HeLa cells (Table 1). These results indicate the presence of a gene(s) in the d2 region of the deletion that supports extension of the host range by K1L.

Recombinant DIs expressing bacteriophage T7 RNA polymerase (rDIs-T7pol)

rDIs-T7pol was generated by homologous recombination in CEF cells using rDIsLacZ and the transfer vector pUC/DIsT7pol. Anti-T7 RNA polymerase rabbit serum was used to screen the recombinant viruses. To determine whether mammalian cells infected with DIsT7pol could express a foreign gene by transfection of a plasmid carrying the cDNA under the T7 promoter, we used the firefly luciferase gene as a reporter. A previous report showed that the translatability of the uncapped RNA was improved by inserting the EMCV untranslated region between the T7 promoter and the target gene (Elroy-