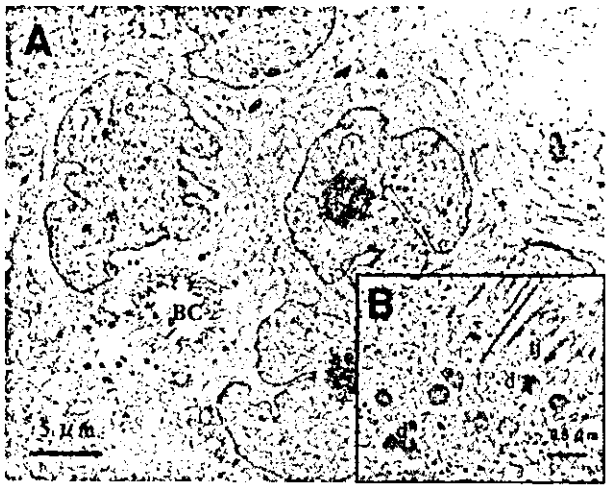

Fig. 3. Fine structural observation of FLC-5 cells incubated in the radial-flow bioreactor under TEM. (A) The cultured cells assume a spherical or cubical form. BC, Bile canaliculi-like structures. Tight junctions (tj) and desmosome (d) are visible in the intercellular spaces.

densely on the side of the viable cells facing the medium flow tract (Fig. 2B). Under a transmission electron microscope, the cells were distributed at a high density, and bile-canaliculi-like structures and desmosome and tight junction were visible in the intercellular spaces (Fig. 3). The cells were spherical or cubical in shape, indicating that a 3-dimensional culture had been obtained.

mRNA and Protein Expression of CYP3A4 in FLC-5 Cultured in the RFB. We compared mRNA expression of CYP3A4 in FLC-5 cultured in the RFB with that in FLC-5 cultured in monolayer. In the absence of rifampicin, the amount of CYP3A4 expressed in the RFB system was 7.7 times higher than that in monolayer culture. The amount expressed in a monolayer culture with rifampicin was 2.4 times that in a monolayer culture without rifampicin. In the RFB system, the amount of CYP3A4 expressed with rifampicin was 34 times higher than that without rifampicin. The amount expressed in the RFB system with rifampicin was 108 times higher than that in a monolayer culture with rifampicin (Fig. 4A). The microsomal fraction of the rifampicin-treated cells exhibited a markedly increased CYP3A expression level, even at the protein level (Fig. 4B). These results demonstrated that FLC-5 cultured in the RFB with rifampicin increased mRNA and protein expression of CYP3A4.

Metabolism of Testosterone in FLC-5 Cultured in the RFB With or Without Rifampicin. Next, we investigated whether a testosterone as one of the substrates for CYP3A4 might be metabolized obviously or not. FLC-5 cells incubated in a 15-mL volume RFB were combined with 50 μmol/L of testosterone and subjected to a 6-hour testosterone metabolism experiment in a closed system. In the control group, which was not pretreated with rifampicin, the amount of 6β-hydroxy testosterone released in the medium was significantly smaller than that in the rifampicin-pretreated condition (Fig. 5A). In cases in which CYP3A4 had been induced by pretreatment with rifampicin, the culture medium showed peaks of 6β-hydroxy testosterone (Fig. 5B). These results demonstrated that the RFB was useful for the *in vitro* metabolic system of substrate of CYP3A4.

Induction of CYP3A4 by PXR and Regulation of PXR by HNF-4α. We hypothesized that PXR, transcriptional factor for CYP3A4, and HNF-4α, transcriptional factor for PXR, effectively regulated the induction of CYP3A4 in RFB culture. To investigate, we first estimated the mRNA of PXR and HNF-4α by the TaqMan real-time PCR. In the absence of rifampicin induction, the amount of PXR mRNA expressed in the RFB system was 2 times higher than that in the monolayer culture but RXRα was almost of the same level. In the RFB system, the amount of PXR mRNA expressed with rifampicin was 15.5 times higher than that without rifampicin but RXRα was half of the lower level. The amount of PXR mRNA expressed in the RFB system with rifampicin was 22 times higher than that in a monolayer culture with rifampicin but of RXRα was 0.4 times the lower level (Fig. 6A).

On the other hand, in the absence of rifampicin, the amount of HNF-4α expressed in the RFB system was 1.23 times higher than that in the monolayer culture. The amount expressed in a monolayer culture with rifampicin was 1.25 times that in a monolayer culture without rifampicin. In the RFB system, the amount of HNF-4α mRNA expressed with rifampicin was 1.7 times higher than that without rifampicin. The amount expressed in the RFB

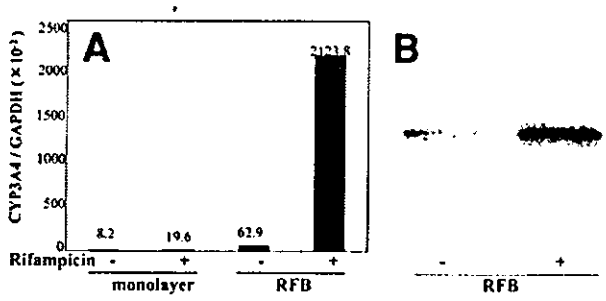

Fig. 4. (A) Comparison of the expression of human CYP3A4 mRNA in FLC-5 cells incubated under different conditions, as assessed by TaqMan 1-step RT-PCR. Each RNA concentration was calculated from the average of triplicate measurements and divided by the average of the corresponding GAPDH values obtained in the same way to yield a corrected RNA concentration. (B) Comparison of protein expression of human CYP3A4 in FLC-5 cells cultured in RFB with or without rifampicin, as analyzed by Western blotting. A 57-kd-specific band was observed corresponding to CYP3A4.

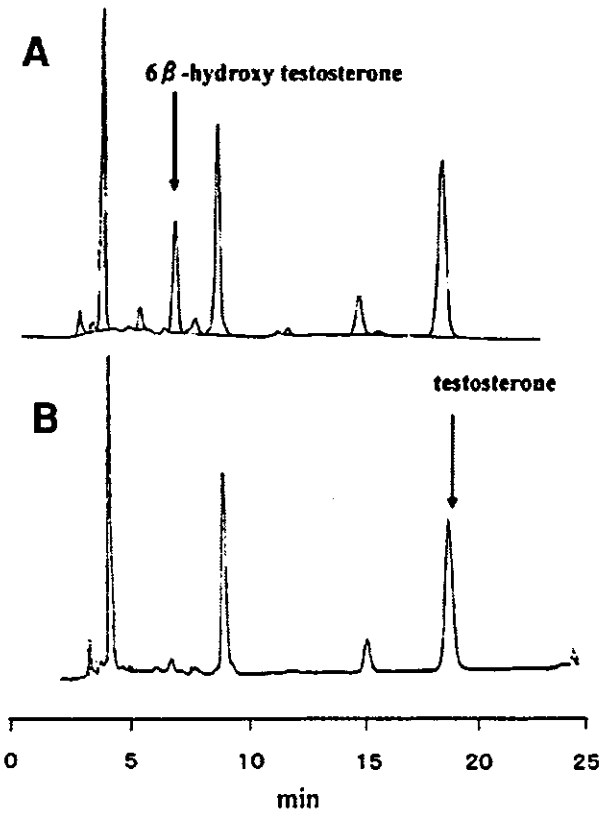

Fig. 5. Formation of 6β-hydroxy testosterone in FLC-5 cells. Testosterone was added to the medium used for the incubation of FLC-5 cells pretreated without and with rifampicin in the radial-flow bioreactor. (A) In the medium under rifampicin pretreatment condition, 6β-hydroxy testosterone was clearly detected. (B) 6β-hydroxy testosterone (a metabolite of CYP3A4) was also detectable but traced level in the medium without pretreatment of rifampicin.

system with rifampicin was 1.67 times higher than that in a monolayer culture with rifampicin (Fig. 6B).

Furthermore, to investigate the functional activity of PXR/RXRα proteins in our culture systems, we performed EMSA, with nuclear extracts prepared from FLC-5 cultured by monolayer or by RFB and radiolabeled oligonucleotide containing CYP3A4 ER6. As shown in Fig. 7A, specific protein-DNA complexes were observed both in nuclear extracts from monolayer- and in RFB-cultured FLC-5. However, the complex in RFB culture (shown as LC) exhibited a retarded mobility compared with that in monolayer culture (shown as C). This result further suggests that nuclear factor(s) was interacted with either PXR/RXRα heterodimer or ER6 motif in the RFB-cultured cells. Competition experiments with unlabeled oligonucleotides of wild-type CYP3A4 ER6-binding and NF-κB-binding site further demonstrated the specificity of the binding as exemplified in Fig. 7A. In addition, the complex in RFB culture was retarded further with anti-PXR antibodies (Fig. 7A, lanes 7 and 8, shown as SS).

To characterize the nature of protein-ER6 complex in the nuclear extracts from the RFB culture treated with rifampicin, we performed a long-time electrophoresis in the EMSA. As shown in Fig. 7B, lanes 5 and 6, we found a further retarded mobility of the protein-ER6 complex present in rifampicin-treated RFB culture (shown as SLC), which was hardly recognized in the standard condition of polyacrylamide gel electrophoresis (Fig. 7B, lanes 1-4). Such a super-shifted band was not observed in the monolayer culture in the presence of rifampicin (Fig. 7B, lane 3). Thus, it seemed possible that rifampicin induced formation of nuclear protein-DNA complex with higher molecular weight in the RFB culture.

On the other hand, we investigated the functional activity of HNF-4α proteins by the same method with nuclear extracts prepared from FLC-5 cultured by monolayer or by the RFB and radiolabeled oligonucleotide containing HNF-4α-binding site. As shown in Fig.

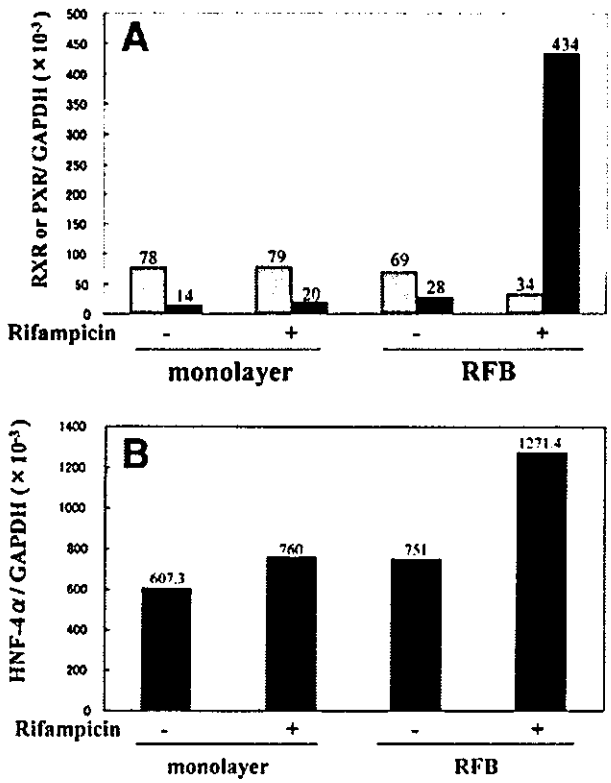

Fig. 6. Comparison of the expression of RXRα, PXR, and HNF-4α mRNA in FLC-5 incubated under different conditions, as assessed by TaqMan 1-step RT-PCR. Each RNA concentration was calculated from the average of triplicate measurements and divided by the average of the corresponding GAPDH values obtained in the same way to yield a corrected RNA concentration. (A) Data represent mean PXR (**solid bars**), and RXRα (**grey bars**). (B) Data represent mean HNF-4α.

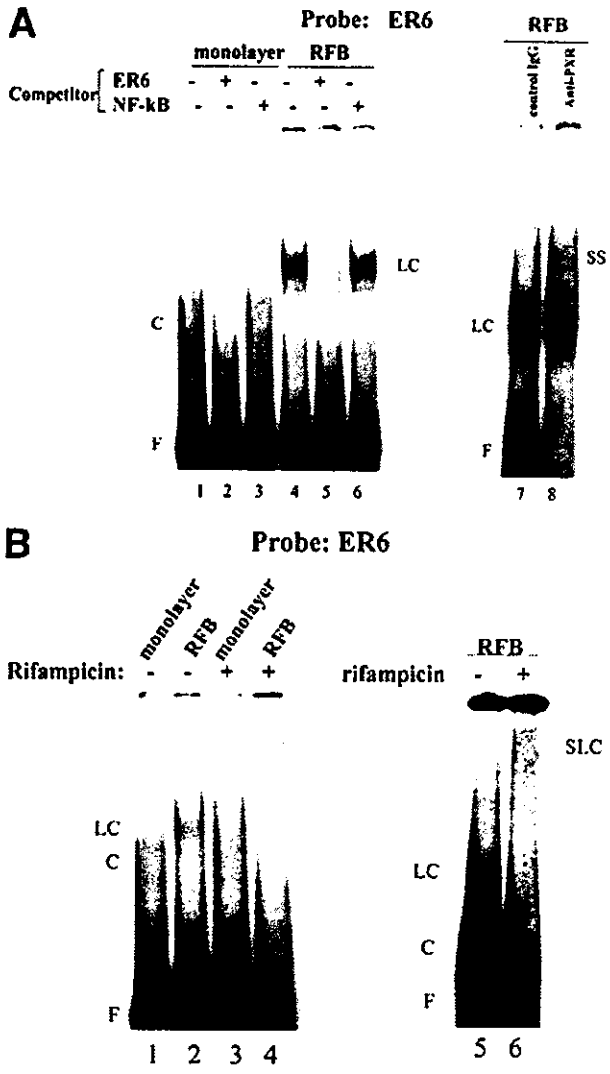

Fig. 7. Three-dimensional culture forms the large DNA nuclear factor complex, including RXRα/PXR heterodimer, their binding site, and the other factors. Ten micrograms of nuclear extracts from FLC-5 cultured by monolayer or RFB were used for EMSA with a ^{32}P-labeled CYP3A4 ER6 oligonucleotide in the presence of either a 50-fold excess of unlabeled oligonucleotide containing the CYP3A4 ER6 or an NF-κB-binding site as indicated. (A) **Lanes 1-8**, ER6 PXRE probe with FLC-5 nuclear extracts. **Lanes 1-3**, monolayer culture. **Lanes 4-8**, RFB culture. **Lanes 1 and 4**, no unlabeled oligonucleotide. **Lanes 2 and 5**, 50-fold excess of an unlabeled ER6 PXRE oligonucleotide. **Lanes 3 and 6**, 50-fold excess of an unlabeled NF-κB-binding site oligonucleotide. **Lane 7**, with the addition of a control goat IgG. **Lane 8**, with the addition of an antibody against PXR. (B) **Lanes 1-6**, ER6 PXRE probe with FLC-5 nuclear extracts. **Lane 1**, monolayer culture without rifampicin. **Lane 2**, RFB culture without rifampicin. **Lane 3**, monolayer culture with rifampicin. **Lane 4**, RFB culture with rifampicin. **Lanes 5 and 6**, long-time EMSA assay. **Lane 5**, no rifampicin. **Lane 6**, containing of rifampicin. C, LC, SLC, and SS indicate specific, large, super-large protein-DNA complexes, and antibody supershift, respectively, and F represents unbound probe.

8A, specific protein-DNA complexes were observed both in nuclear extracts from monolayer- and RFB-cultured FLC-5. However, the complex in the RFB culture (shown as LC) exhibited the retarded mobility compared with that in monolayer culture (shown as C). This result suggests that nuclear factor(s) was interacted with either HNF-4α or HNF-4α-binding motif in the RFB-cultured cells. Competition experiments with unlabeled oligonu-

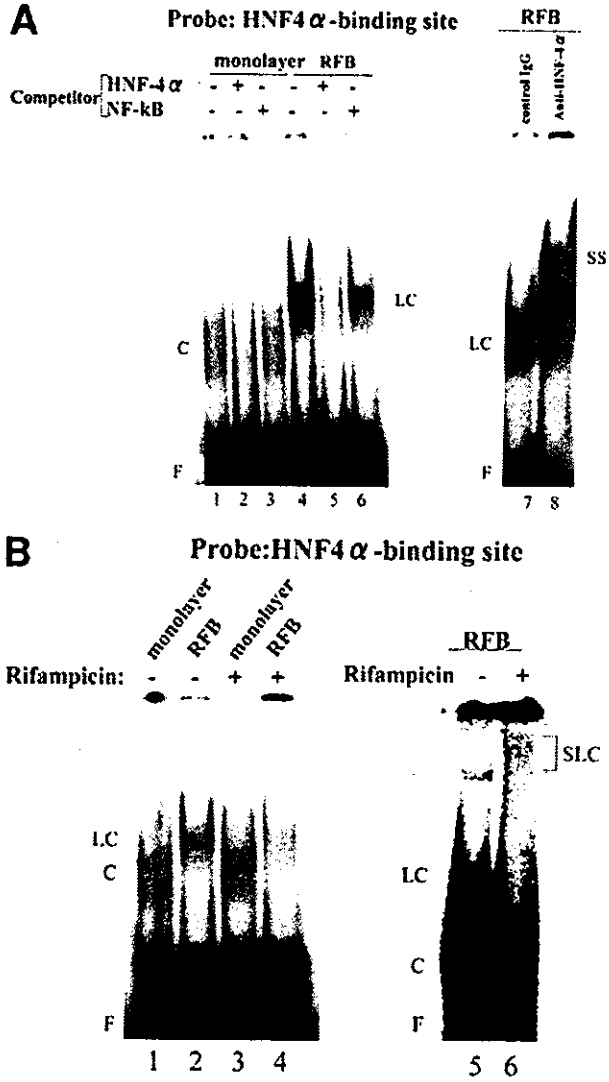

Fig. 8. Three-dimensional culture forms the large DNA-nuclear factor complex including HNF-4α, their binding site, and the other factors. Ten micrograms of nuclear extracts from FLC-5 cultured by monolayer or RFB were used for EMSA with a ^{32}P-labeled HNF-4α oligonucleotide in the presence of either a 50-fold excess of unlabeled oligonucleotide containing the HNF-4α or an NF-κB-binding site as indicated. (A) **Lanes 1-8**, HNF-4α probe with FLC-5 nuclear extracts. **Lanes 1-3**, monolayer culture. **Lanes 4-8**, RFB culture. **Lanes 1 and 4**, no unlabeled oligonucleotide. **Lanes 2 and 5**, 50-fold excess of an unlabeled HNF-4α-binding oligonucleotide. **Lanes 3 and 6**, 50-fold excess of an unlabeled NF-κB-binding site oligonucleotide. **Lane 7**, with the addition of a control goat IgG. **Lane 8**, with the addition of an antibody against HNF-4α. (B) **Lanes 1-6**, HNF-4α probe with FLC-5 nuclear extracts. **Lane 1**, monolayer culture without rifampicin. **Lane 2**, RFB culture without rifampicin. **Lane 3**, monolayer culture with rifampicin. **Lane 4**, RFB culture with rifampicin. **Lanes 5 and 6**, long-time EMSA assay. **Lane 5**, no rifampicin. **Lane 6**, containing of rifampicin. C, LC, SLC, and SS indicate specific, large, super-large protein-DNA complexes, and antibody supershift, respectively, and F represents unbound probe.

cleotides of wild-type HNF-4α-binding site and NF-κB-binding site demonstrated the specificity of the binding as exemplified in Fig. 8A. In addition, the complex in RFB culture was retarded further with anti-HNF-4α antibodies (shown as SS) (Fig. 8A, lanes 7 and 8).

To characterize the nature of protein-HNF-4α-binding site complex in the nuclear extracts from RFB culture treated with rifampicin, we performed a long-time electrophoresis in the EMSA. As shown in Fig. 8B, lanes 5 and 6, we found a retarded mobility of the protein-HNF-4α-binding site complex present in rifampicin-treated RFB culture (shown as SLC), which was hardly recognized in the standard condition of polyacrylamide gel electrophoresis (Fig. 8B, lanes 1-4). Such a super-shifted band was not observed in the monolayer culture in the presence of rifampicin (Fig. 8B, lane 3). Thus, it seemed possible that rifampicin induced formation of nuclear protein-DNA complex with higher molecular weight in the RFB culture.

Discussion

We have established 7 human HCC cell lines, and 5 of these can be incubated in serum-free medium. Analysis of CYP isoforms is being conducted in 3 cell lines. In FLC-4, FLC-5, and FLC-7, we have confirmed the expression of CYP3A as well as CYP1A1, CYP1B1, and CYP2E1, which are important phase-1 reaction enzymes, responsible for approximately 50% of therapeutic drug-metabolizing activity in the liver.[14,16] The present study investigated enzyme induction in FLC-5 cells cultured in the RFB, focusing on CYP3A4.

First, we confirmed the induction of CYP3A4 by rifampicin in FLC-5 cells, as reflected by the increase in the CYP3A4 mRNA and protein. Thus, an enzyme-inducing system has been established for human CYP3A4. It remains to be clarified as to why rifampicin caused more intense CYP3A4 induction in the 3-dimensional perfusion culture. It is possible that expression of CYP3A4 at the transcription level requires maintenance of the 3-dimensional form of the cells, the shear stress caused by perfusion, and the development of intercellular adhesion structures, such as junctional complexes. It was recently suggested that PXR/RXRα could be transcriptional factors related to CYP3A4 induction due to binding at the ER6 site. It would be of interest to clarify whether or not these transcriptional factors are also involved in FLC-5 cells. One of the recognized interspecies differences is that, whereas pregnenolone induces CYP3A4 in rat hepatocytes, it does not do so in human hepatocytes.

We assumed that enhancement of CYP3A4 expression during culture in the RFB is attributable to either increased expression of PXR/RXRα or to enhancement of their DNA-binding activity. Based on this assumption, we measured the mRNA levels of PXR/RXRα by quantitative PCR and evaluated their DNA binding potentials by EMSA.

RXRα mRNA expression in the RFB culture did not differ from that in monolayer culture, but the expression of PXR showed an approximately 2-fold increase; when the culture was treated with rifampicin, the increase was 22-fold (Fig. 6). When evaluated by EMSA, culture in the RFB caused retardation of the electrophoretic activity of ER6 and the PXR/RXRα complex. Retardation in the EMSA assay of the electrophoretic activity of ER6 and PXR/RXRα following 3-dimensional culture or rifampicin treatment has not been previously reported. The result obtained in the present study is probably attributable to the increase in molecular weight of the DNA-protein complex because of further binding of cofactors to the PXR/RXRα heterodimer (Fig. 7). This higher molecular weight complex, which is presumably accompanied by its conformational change, may bind to the ER6 motif with higher affinity and up-regulate its transcriptional activity. We still cannot rule out the possibility that the PXR/RXRα-independent pathway involves in increasing CYP3A4 expression. These findings suggest that 3-dimensional culture in the RFB results in the formation of a transcriptional control complex with a greater capacity to induce transcription.

It is known that HNF-4α may be involved in the control of PXR expression and that HNF-4α knockout mice are completely deficient in PXR.[17] In our study, the level of HNF-4α mRNA expression was elevated only slightly (by about 20%) in RFB culture, but the mobility of HNF-4α and its DNA-binding site were markedly retarded following culture in the RFB. Also, retardation of the electrophoretic activity of HNF-4α in EMSA following 3-dimensional culture or treatment with rifampicin has not been reported previously. However, because both complexes were found to be competitive in the competition assay, it seems likely that HNF-4α formed a complex with other factors during 3-dimensional culture in the RFB. The formation of this complex probably led to enhancement of the transcription control capability. During 3-dimensional culture, rifampicin evidently enhanced the induction of CYP3A4, and this correlated well with PXR induction. Furthermore, during 3-dimensional culture with RFB, rifampicin treatment retarded mobility of the complex of PXR/RXRα and CYP3A4 ER6 and that of HNF-4α and their respective binding sites. Interestingly, subsequent analysis revealed that prolonged EMSA resulted in the formation of a complex having a much larger molecular weight (Figs. 7 and 8). Although the relationship between the larger complex formation including

PXR or HNF-4α and the induction of CYP3A4 is still not clear, this suggests that during 3-dimensional culture in the RFB, rifampicin, which serves as a PXR ligand, induces the formation of a complex that allows more efficient transcription, leading to a more than 100-fold increase in CYP3A4 induction. From these results, we may say that 3-dimensional culture in the RFB enhances the transcriptional control capability of HNF-4α, and, with the induction of transcription of PXR in the downstream region, the expression of CYP3A4 eventually increases. Our results indicated that the induction of CYP3A4 in FLC-5 cells incubated in the RFB is akin to that seen in normal human livers.

In the experiment conducted to study actual drug metabolism in the bioreactor, induction of CYP3A by rifampicin resulted in degradation of testosterone to 6β-hydroxy testosterone. It was shown at the cell level that induction of CYP3A was associated with enhancement of the drug-metabolizing activity. As shown in Fig. 1, this system can therefore be utilized for the screening of unknown drugs that can induce CYP3A, *i.e.*, for examining drug interactions mediated by CYP3A. If induction of CYP3A4 expression by various drugs were studied using the RFB system and FLC-5 cells, it would become possible to establish a simulation of drug metabolism in a setting closely resembling that prevailing in the human body. Drug metabolism in the liver is determined not only by CYP expression but also by many other factors, such as the affinity of the drugs for binding proteins and membranous components. Drugs that can be nonselectively taken up by the liver can affect drug metabolism mediated by CYP, and they are usually deemed as being dangerous for clinical use. The bioartificial liver is therefore useful for conducting studies of drug metabolism in the liver.

In the present study, we confirmed that the expression of CYP3A4, specific to human liver, is enhanced in an HCC cell line derived from human liver, at the mRNA level, protein level, and at the level of functional activity in cultured cells by incubation in the RFB. PXR/RXRα regulates the inductivity of CYP3A4 in human HCC cells under 3-dimensional perfusion culture. The bioartificial liver composed of the human functional HCC cell line was useful in studying drug interactions during induction of human CYP3A4.

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Differential cellular gene expression induced by hepatitis B and C viruses

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Abstract

Hepatitis B virus (HBV) is a hepatotropic virus that causes acute and chronic hepatocellular injury and hepatocellular carcinoma. To clarify how HBV proteins regulate host cellular gene expression, we used our in-house cDNA microarray and HepG2.2.15 cells, which are derived from HepG2 cells and produce all HBV proteins. Of 2304 genes investigated, several genes were differentially expressed in HepG2.2.15 cells compared with HepG2 cells. These genes included insulin-like growth factor II and α-fetoprotein, consistent with previous reports. Furthermore, we previously performed similar microarray analyses to clarify the effects of hepatitis C virus (HCV) proteins on host cells, using a HepG2-derivative cell line, which produces all HCV proteins. Using these two microarray results, we compared the differences in cellular gene expression induced by HBV and HCV proteins. The expression of the majority of genes investigated differed only slightly between HBV and HCV protein-producing cells. However, HBV and HCV proteins clearly regulated several genes in a reciprocal manner. Combined, these microarray results shed new light on the effects of HBV proteins on cellular gene expression and on the differences in the pathogenic activities of these two hepatitis viruses.

Hepatitis B virus (HBV) is a hepatotropic virus consisting of a partially double-stranded circular DNA genome that causes acute and chronic hepatocellular injury and hepatocellular carcinoma. In HBV-infected hepatocytes, three major classes of HBV-specific messages are detected. A 3.5-kilobase (kb) RNA, which is slightly larger than the 3.2-kb unit length of the HBV genome, serves as mRNA for expressing the core protein and reverse transcriptase. Two other mRNAs are subgenomic in size: a 2.5-kb RNA encodes the large envelope protein HBV surface antigen and a 2.1-kb RNA encodes the middle and major HBV surface antigens [1].

Acs et al. derived cell line HepG2.2.15 from HepG2 cells transfected with a plasmid containing HBV DNA. It produces polyadenylated RNA with lengths of 3.5, 2.5, and 2.1 kb, and also secretes infectious HBV particles [2–4]. This cell line is a valuable model for investigating the effects of HBV proteins on host cells [5].

A breakthrough technology for functional research into human genome-based science, the cDNA microarray is the most powerful technique that can be applied to both basic and clinical researches. This technique has been used successfully to observe the altered expression of thousands of genes in interactions between pathogenic organisms and their host cells [6–8].

Although various mechanisms, such as activation of host immune responses, have been suggested as being significant in the pathogenesis of HBV infection, the initial effects of HBV infection on hepatocytes remain unclear. An extensive study of the HBV transactivator X protein (HBx) has revealed its contribution to host gene alterations [9,10]. However, little is known of the effects of all HBV proteins or HBV replication in host cells on the function of HBV-infected cells. Therefore, this study investigated the initial alteration of gene expression in HBV-infected cells by examining the differences in the gene expression profiles of HepG2.2.15 and HepG2 cells, using our in-house cDNA microarray. In addition, we recently established HepG2 cells that integrate the full

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hepatitis C virus (HCV) (Hep394) and compared the expression profiles in Hep394 and HepG2 cells [11]. This paper compares the results of experiments using HepG2.2.15 and Hep394, and reveals differences in the effects of HBV and HCV proteins on gene expression in host cells.

Materials and methods

Cell lines. Human hepatoblastoma cells (HepG2) were obtained from the Riken cell bank (Tsukuba Science City, Japan). HepG2.2.15, derived from HepG2 transfected with a plasmid carrying four 5′–3′ tandem copies of the HBV genome, was provided by Dr. George Acs. This cell line produces not only several of the replicative intermediates of HBV DNA, but also Dane-like particles. These two cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum.

RNA extraction. Total cellular RNA was extracted using an acid guanidinium thiocyanate–phenol–chloroform method according to the manufacturer's instructions (Isogen Reagent; Nippon Gene, Tokyo, Japan). Poly(A) mRNA was obtained from this total RNA using an Oligotex-dT30 mRNA Purification Kit (TaKaRa Shuzo, Kyoto, Japan). Purified message was routinely quality-controlled on formaldehyde agarose gels.

Preparation of the cDNA microarray. A cDNA microarray consisting of 2304 cDNAs was made as previously described [7,12,13]. Briefly, human cDNAs were purchased from Research Genetics. PCR-amplified cDNA products were mixed with nitrocellulose in dimethyl sulfoxide just before printing and then spotted onto carbodiimide-coated glass slides using robotics (SPBIO-2000, Hitachi Software Engineering, Yokohama, Japan). In this study, we spotted 2280 sequence-validated cDNAs on the array. This array includes cDNAs of housekeeping genes, such as human β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to serve as internal controls, and the luciferase gene from *Photinus pyralis* was used as a negative control.

Microarray procedures. All the cDNA microarray analyses followed our published protocols [13]. In each experiment, fluorescent cDNA probes (Cy5 or Cy3-labeled) were prepared from a 2-μg mRNA sample isolated from HepG2.2.15 cells and a 2-μg control mRNA sample (Cy3 or Cy5-labeled) isolated from HepG2 cells. The different fluorescent-labeled probes were mixed and applied to a microarray following incubation at 65 °C overnight under humidified conditions. Fluorescent images of the hybridized microarrays were scanned with a fluorescence laser confocal slide scanner (Scan Array 4000, GSI Lumonics, Ottawa, Canada). The images were analyzed using Quant Array (GSI Lumonics) according to the manufacturer's instructions.

Analysis of mRNA expression by RT-PCR. RT-PCR quantification was used to verify the microarray data. The cDNA templates for RT-PCR were synthesized from 500 ng poly(A) RNA, using SuperScript II reverse transcriptase and oligo(dT) primer (Invitrogen, Carlsbad, CA). PCR amplification was performed as described previously [13]. The PCR schedule consisted of incubation for 5 min at 96 °C, followed by 20–30 cycles of 96 °C for 20 s and 62 °C for 1 min. The PCR products were analyzed by running the reaction products on 1.2% agarose/ethidium bromide gels.

Cluster analysis. We performed cluster analysis of all 2304 genes to clarify the relationships between HepG2.2.15 and Hep394, using GeneMaths software (Applied Math, Sint-Martens-Latem, Belgium). The data were log transferred, normalized, mean centered, and applied to average linkage clustering. The gene cluster data were presented graphically as colored images and the genes analyzed were arranged as ordered by the clustering algorithm, so that genes with the most similar expression patterns were placed adjacent to each other.

Results

Analysis of altered mRNA expression in HepG2.2.15 cells by cDNA microarray

To examine the effects of HBV proteins on gene alteration in host cells, microarray analyses were performed, using HepG2.2.15 and HepG2 cells. cDNA

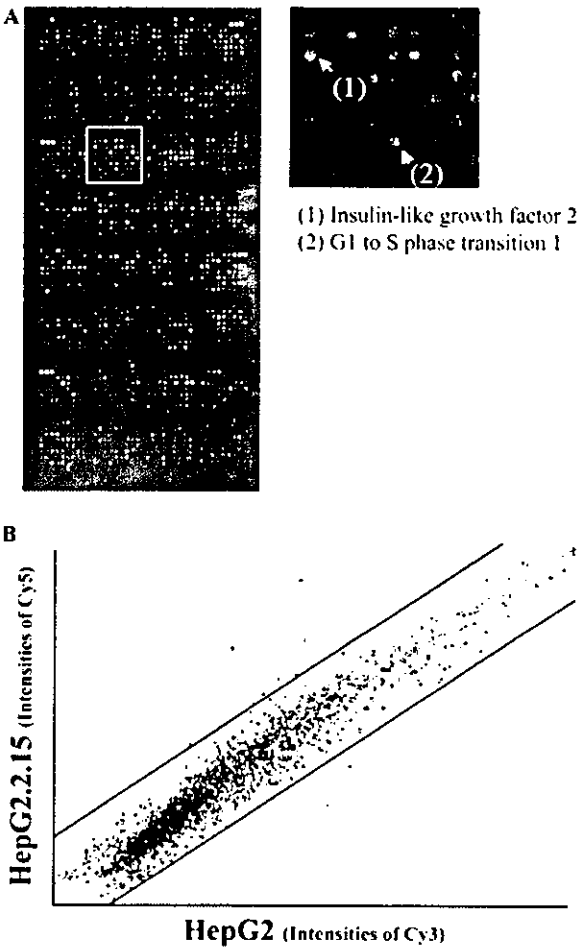


Fig. 1. (A) A typical false-color image of the microarray. mRNA was extracted from HepG2 and HepG2.2.15 cells. Each RNA sample was used as a template for synthesizing cDNA probes, which were incorporated with either Cy3- or Cy5-dUTP, respectively. The probes were mixed and hybridized to a microarray slide. The slides were scanned with a fluorescence laser-scanning device (Scan Array 4000). Red and green spots show relative high and low expression in HepG2.2.15 compared with HepG2 cells, respectively. A close-up image of a representative portion is also shown. Arrows indicate typical red or green signals. (B) A scatter plot of all 2304 genes spotted on our microarray. The two lines parallel to the diagonal in the graph represent 1.8- and 0.55-fold changes in expression.

prepared from mRNA from each cell was labeled with different fluorescent dyes to visualize the differences in mRNA expression. Fig. 1A is a false-color image, where hybridization with cDNAs derived from HepG2.2.15 and HepG2 cells is indicated by Cy-5 fluorochrome (red) and Cy-3 fluorochrome (green) signals, respectively. Red indicates that expression of the corresponding gene was greater in HepG2.2.15 cells than in HepG2 cells and green indicates the opposite. Yellow indicates similar mRNA expression levels. The majority of genes investigated showed only small differences, with ratios ranging between 1.8 and 0.55, as shown in Fig. 1B. However, seven genes were upregulated more than 1.8-fold and three genes were downregulated 0.55-fold or less in HepG2.2.15 cells compared with HepG2 cells (Table 1). These genes included insulin-like growth factor II and α-fetoprotein, the expression of which is reported to be altered by HBx protein.

Comparison of the microarrays of HepG2.2.15 and Hep394

Recently, we established a HepG2-derivative cell line, Hep394, which integrates the full HCV genome and produces all HCV [11]. We previously performed a similar microarray analysis to clarify the effects of HCV proteins on host cells using this cell line [11]. Since this study examined the effects of HBV proteins on gene expression in host cells, we compared the results of the two experiments, to compare the effects of HBV and HCV proteins on cellular gene expression. Fig. 2 shows that the majority of genes investigated differed only slightly in HBV protein-producing cells and HCV protein-producing cells. However, several genes clearly showed reciprocal regulation by HBV and HCV proteins (representative results are indicated by arrows in Fig. 2). These results suggest that HBV and HCV are both hepatitis viruses with differing biologic and pathogenic activities.

RT-PCR confirmed the modification of gene expression by HBV

To confirm the results of our microarray selection, we analyzed the levels of several mRNAs by semi-quantitative RT-PCR. Of 10 genes selected in the microarray analyses using HepG2.2.15, four genes that also had altered expression in Hep394 were analyzed and their changes in expression were all confirmed by RT-PCR (Fig. 3). Fatty acid binding protein, thioltransferase, and asialoglycoprotein receptor are upregulated in HepG2.2.15 cells, but downregulated in Hep394 cells. GTR2-2 (glypican) is downregulated in both HepG2.2.15 and Hep394 cells. This altered mRNA expression is thought to be involved in the pathogenesis of HBV and may also explain the differences and similarities in the pathogenesis of HBV and HCV.

Discussion

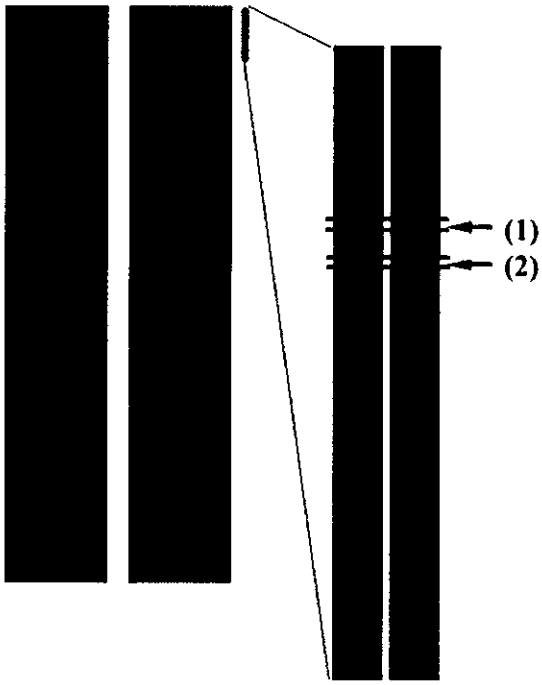
This study demonstrated alterations in gene expression induced by HBV, using HepG2.2.15 cells and our in-house cDNA microarray consisting of 2304 known genes. Although this is only a small fraction of the human genes, new information on the pathogenesis of HBV was revealed and similarities and differences in gene expression in host cells induced by HBV and HCV were identified.

Our microarray analyses showed that gene expression in the HBV-transfected cell line HepG2.2.15 differed from that in parental HepG2 cells. Since this cell line is a purified liver cell model that produces HBV particles, we believe that it can reveal the initial effects induced by HBV proteins after HBV infection, without considering the in vivo effects of other cells, such as lymphocytes.

Of the 2304 genes investigated, seven were upregulated and three were downregulated in HepG2.2.15 cells. Of these, HBx protein is already reported to modulate

Table 1
Genes whose expression levels were changed in comparing HepG2.2.15 with HepG2 cells

	Accession No.	Relative mRNA expression ratio (HepG2.2.15/HepG2)
Upregulation		
Insulin-like growth factor 2	N74623	4.8
α-Fetoprotein	T59043	4.0
Kruppel-related zinc finger protein	AA455657	3.4
Phosphoglycerate kinase	AA599187	2.7
Fatty acid binding protein	T53220	2.7
Thioltransferase	AA291163	2.2
Asialoglycoprotein receptor	H58255	1.8
Downregulation		
GTR2-2 (Glypican)	AA775872	0.28
MDM-2	XM_017531	0.38
G1 to S phase transition 1	AA486233	0.55



(1) Fatty acid binding protein
(2) Asialoglycoprotein receptor

Fig. 2. The molecular signature of HepG2.2.15 and Hep394 cells. The gene cluster data are presented graphically as colored images, and all 2304 genes analyzed in the microarray are arranged so that genes with the most similar expression patterns are placed adjacent to each other. Data are the ratio of hybridization of the fluorescent cDNA probe prepared from HepG2.2.15 or Hep394 to the parental HepG2 cells and are a measure of the relative gene expression in each sample. Red and blue represent up- and downregulation, respectively, relative to HepG2 cells. Black bars on the side indicate the portion of the enlarged image. Arrows in the enlarged section show typical examples of differential gene expression between HepG2.2.15 and Hep394 cells.

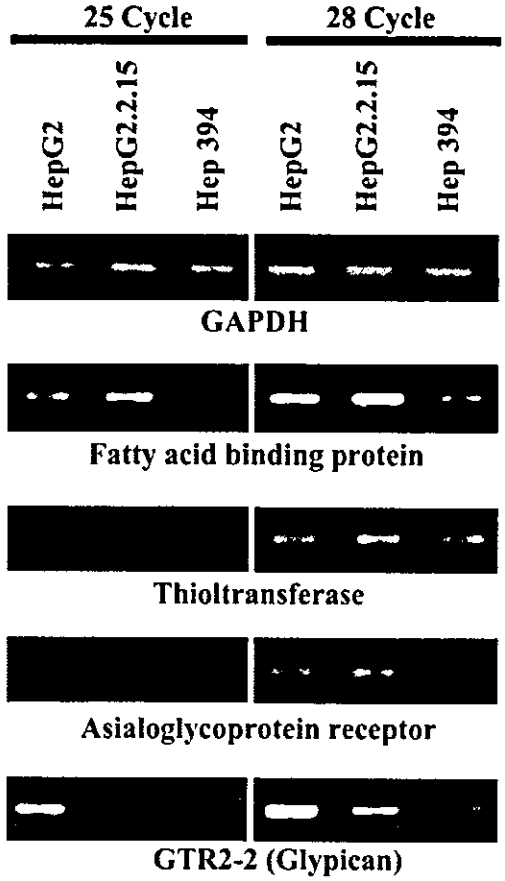


Fig. 3. RT-PCR analysis of mRNA expression of selected genes based on the microarray results. mRNA was extracted from HepG2.2.15 and HepG2 cells, and then the mRNA expression of arbitrarily selected genes showing typical expression changes in HepG2.2.15 and Hep394 cells in the microarray analyses was analyzed by RT-PCR. The differential expression of selected genes was confirmed by RT-PCR. GAPDH was amplified as an internal control.

insulin-like growth factor II and α-fetoprotein, confirming previous findings [14,15]. The expression of the other eight genes was newly revealed to be modulated by HBV proteins. One was the asialoglycoprotein receptor, which is thought to specifically bind HBV-particles and mediate their hepatic endocytosis [16]. We showed that HBV upregulates the expression of asialoglycoprotein receptors, which might cause positive feedback of the hepatic endocytosis of HBV. Thioltransferase, another upregulated gene, is a glutaredoxin reported to be essential for virion morphogenesis of vaccinia virus [17,18]. Although this redox protein is not known to be associated with the HBV life cycle, its function should be further examined.

When we defined upregulation as a more than 1.8-fold increase in mRNA expression and downregulation as a 0.55-fold reduction or less, HBV proteins modulated only the expression of 10 of the 2304 genes (0.43%) studied. As we described previously, microarray analysis can yield false-negative results because of its limited sensitivity. HBV proteins might modulate other genes and it is possible that we underestimated the modulation in this microarray study. Even a relatively small difference in the array analysis could be physiologically significant.

Another main outcome of this study was the discovery of similarities and differences in the altered gene expression induced in host cells by HBV and HCV. Using chronic viral hepatitis tissue lesions, microarrays recently showed that gene expression differs in hepatitis B and C lesions, and suggested that different molecular mechanisms underlie the pathogenesis of hepatitis caused by HBV and HCV [19]. In this study, we identified characteristic differences in the expression of some genes by HBV and HCV in vitro, including asialoglycoprotein receptor and fatty acid binding protein. This altered in vitro expression is thought to be induced by

the viral proteins themselves after the initial infection in vivo. This differential gene expression might be linked to differences in the pathogenesis of HBV and HCV. The significance of the differential expression of these genes should be examined further.

HBV proteins, especially HBx protein, transactivate viral and cellular genes via various signaling cascades [20]. In this study, we revealed several genes whose expression is modulated by HBV proteins. To unify the mechanism involving these genes, we believe that the promoter regions of these genes should be analyzed using the genome sequence to reveal new signaling pathways that are affected by HBV proteins.

In conclusion, we demonstrated altered gene expression induced by HBV proteins using our in-house cDNA. Similarities and differences in gene expression in host cells induced by HBV and HCV proteins were also identified. These results should provide new insight into the molecular mechanisms of hepatitis B virus and improve our understanding of differences in the molecular pathogenesis of hepatitis B and C.

Acknowledgments

We thank Dr. G. Acs for providing cell lines. We are also grateful to many colleagues for helpful discussion during the course of this work.

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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-

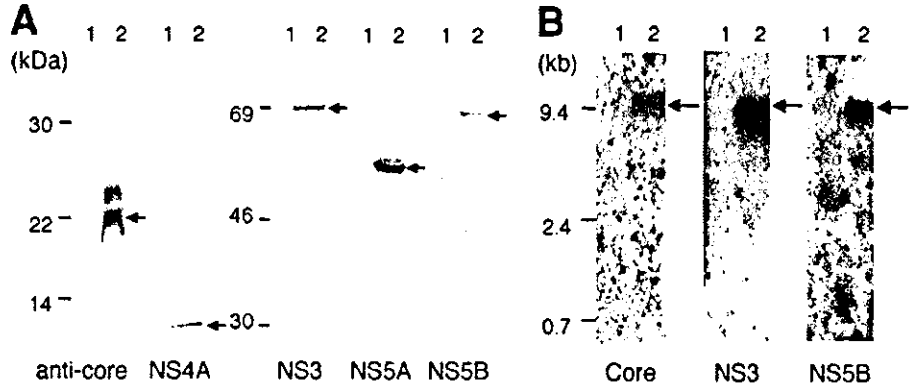

Fig. 1. HCV gene expression in HepG2 cell transformants integrated with HCV cDNAs covering entire or parts of the viral ORF. (A) Western blot analysis. HepSWX (**lanes 1**) and Hep394 (**lanes 2**) cells were lysed and separated by 15% (left) or 12.5% (right) SDS-PAGE. The blots of Immobilon transfer 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).

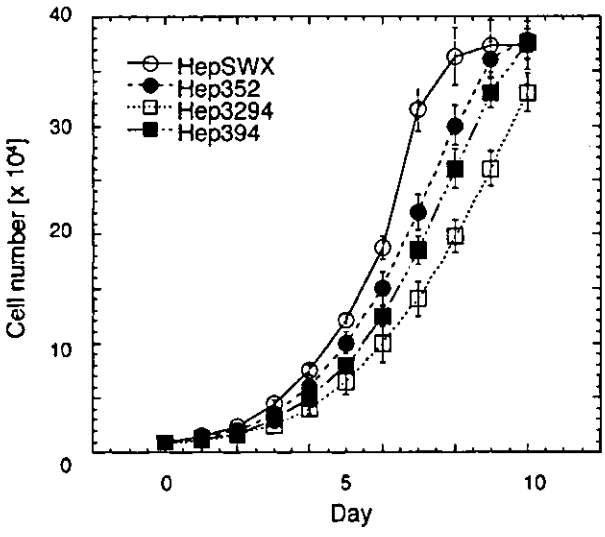

Fig. 2. Cell growth rate analysis. HepSWX (○), Hep352 (●), Hep3294 (□), and Hep394 (■) cells were grown in 35-mm dishes and counted daily as described in Materials and Methods section. Results show the mean cell number ± SD obtained from triplicate dishes.

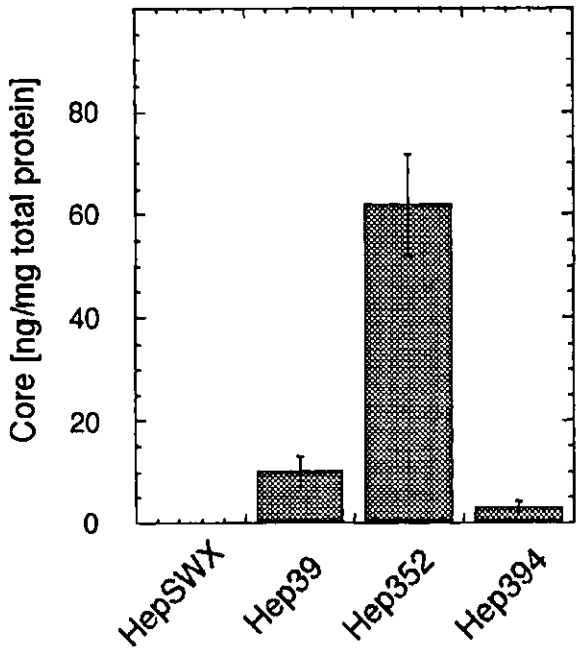

Fig. 3. Quantitation of HCV core protein in cells. The level of HCV core protein in HepSWX, Hep39, Hep352, and Hep394 was determined by the fluorescence enzyme immunoassay method. Results represent the mean ± SD of values obtained in triplicate experiments.

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-

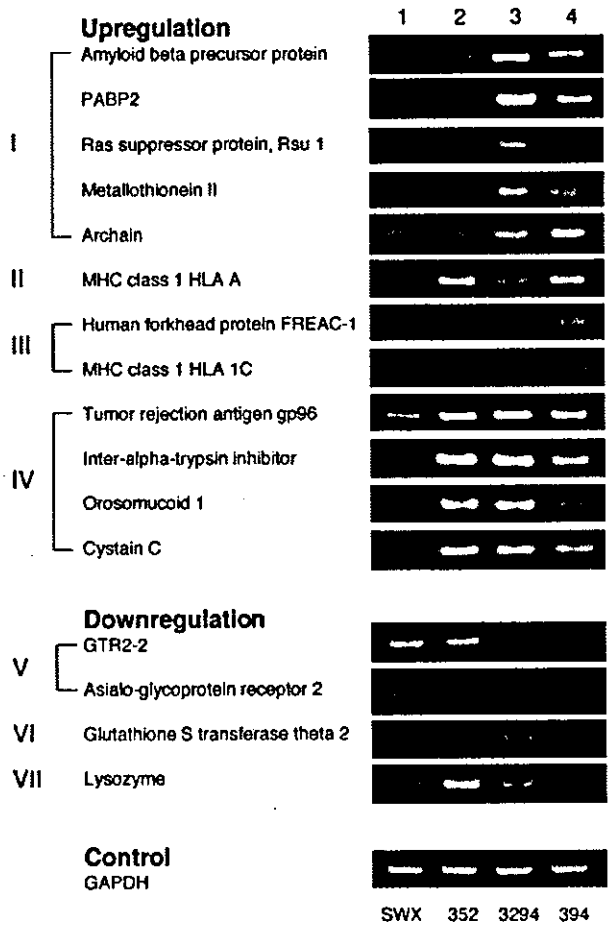

Fig. 4. Semiquantitative RT-PCR analysis of differentially expressed genes in Hep394 cells. Total RNAs were extracted from HepSWX (lanes 1), Hep352 (lanes 2), Hep3294 (lanes 3), and Hep394 cells (lanes 4), and analyzed genes were isolated from cDNA microarray by RT-PCR. Primers used and sizes of PCR 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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Serum lipid profile of patients with genotype 1b hepatitis C viral infection in Japan

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Abstract

Hepatitis C virus (HCV) infection is associated with the development of steatosis in the liver. Recently, infection with genotype 3a HCV has been reported to have a closer association with hepatic steatosis than that with genotype 1 or 2 HCV. Moreover, infection with genotype 3a HCV but not with genotype 1 has been shown to be associated with serum hypocholesterolemia or hypobetalipoproteinemia in European countries. We conducted a case control study to characterize the serum lipid profile in patients infected with genotype 1b HCV, which is the most prevalent HCV genotype in Japan. These patients had significantly lower serum cholesterol levels than those infected with HBV or genotype 2a HCV who had similar liver disease progression and body mass index. Further analysis of serum apolipoproteins revealed that not only apolipoprotein B but also apolipoprotein CII and apolipoprotein CIII levels were significantly reduced, while apolipoprotein AI, AII and E levels were similar in patients infected with genotype 1b HCV and those with HBV or genotype 2a HCV. These results indicate that, in Japan, infection with genotype 1b HCV is a cause of lipid metabolism disturbances, which may be associated with the pathogenesis of hepatitis C liver disease.

Keywords: Apolipoprotein; Hypocholesterolemia; Steatosis

1. Introduction

Infection with hepatitis C virus (HCV) is the cause of a sequence of liver diseases that finally leads to the development of hepatocellular carcinoma (HCC) worldwide [1,2]. Chronic hepatitis C, which precedes the development of HCC, is

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characterized by several histopathologic features: lymphocytic follicle formation, bile duct damage and steatosis (fatty change) [3–5]. In addition, an association between HCV infection and lipid metabolism has been extensively reported. For example, the low-density-lipoprotein (LDL) receptor [6,7] is suggested to be associated with HCV particles, and analysis using a cell culture system has revealed that the secretion of apolipoprotein AII from cells is modulated by the core protein of HCV [8,9]. In experimental animal models, the core protein or nonstructural protein(s) of HCV is steatogenic when expressed in the liver of mice [10,11]. Lipid analysis of fatty liver in mice transgenic for the HCV core gene revealed that the amount of carbon 18 mono-unsaturated fatty acids increased, being distinct from lipid accumulation in the fatty liver of simple obesity mice [12]. Importantly, those transgenic mouse strains develop HCC after the phase of hepatic steatosis [11,13]. Therefore, it is essential to elucidate the changes in lipid metabolism in patients with HCV infection.

Recently, several reports from European countries have pointed out that the degrees of hepatic steatosis are different among chronic hepatitis C patients depending on the genotype of the infecting virus: steatosis is more marked in patients with genotype 3a HCV infection, which is moderately prevalent in European countries such as France or Italy [14] than in those with genotype 1 or 2 infection [15–17]. In addition, hypocholesterolemia or hypobetalipoproteinemia was observed only in patients with genotype 3a HCV infection but not in those with genotype 1 HCV infection [17]. However, in Japan where the genotype 3 HCV infection is very rare, hepatic steatosis is also common in patients with chronic hepatitis of genotype 1b HCV infection, and the detection of the HCV core protein in liver tissue is an independent risk factor for steatosis in a specific liver tissue by multivariate analysis [18,19]. We, therefore, explored for dyslipidemia or alteration in lipid metabolism in patients with genotype 1b HCV infection in Japan by determining the levels of lipids and apolipoproteins in the serum.

2. Patients and methods

2.1. Patients

We studied 50 patients (male:female = 30:20) with histologically proven noncirrhotic chronic hepatitis C who were admitted to our hospitals from January 1999 to December 2000. Patients were selected according to the following criteria: (1) presence of HCV-RNA in serum; (2) absence of cirrhosis; (3) a body mass index (BMI) <25; (4) alcohol consumption <40 g/day; (5) absence of evident diabetes; and (6) not taking drugs influencing lipid metabolism (lipid-lowering agents, nonsteroidal anti-inflammatory drugs (NSAIDS)). Exclusion of cirrhotic patients was done by limiting the patients to only those who showed F1 or F2 [3,4] on liver biopsy that was performed within 1 year before serum lipid determination. Diagnosis of overt diabetes was done according to the guidelines of Japan Diabetes Society: at least two determinations of fasting blood glucose ≥ 126 mg/dl or casual blood glucose ≥ 200 mg/dl. Patients were regarded not to have overt diabetes if they did not meet this criterion and were not subject to insulin or oral hypoglycemic agents. The general characteristics of the 50 patients are shown in Table 1. All the patients were negative for the hepatitis B surface antigen (HBsAg) in serum. Informed consent was obtained from the patients, and human experimentation guidelines of the hospitals were followed in the conduct of this research.

As a control, 50 patients infected with hepatitis B virus (HBV) were selected according to the same criteria for selection of hepatitis C patients except for the criterion (1). Instead, the presence of HBsAg and absence of HCV-RNA in serum have been added as a criterion.

2.2. Viral serology

The levels of HBsAg, antibody to HBsAg (anti-HBs) and anti-HCV in the sera were determined using commercially available enzyme immunoassay kits (Dainabot, Tokyo, Japan) according to the manufacturer's instructions. HCV-RNA levels were determined using a commercially available