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VIRAL HEPATITIS

Gene expression analysis of primary normal human hepatocytes infected with human hepatitis B virus

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Abstract

AIM: To find the relationship between hepatitis B virus (HBV) and hepatocytes during the initial state of infection by cDNA microarray.

METHODS: Primary normal human hepatocytes (PNHHs) were isolated and infected with HBV. From the PNHHs, RNA was isolated and inverted into complement DNA (cDNA) with Cy3- or Cy5- labeled dUTP for microarray analysis. The labeled cDNA was hybridized with microarray chip, including 4224 cDNAs. From the image of the microarray, expression profiles were produced and some of them were confirmed by RT-PCR, immunoblot analysis, and NF-κB luciferase reporter assay.

RESULTS: From the cDNA microarray, we obtained 98 differentially regulated genes. Of the 98 genes, 53 were up regulated and 45 down regulated. Interestingly, in the up regulated genes, we found the TNF signaling pathway-related genes: LT-α, TRAF2, and NIK. By using RT-PCR, we confirmed the up-regulation of these genes in HepG2, Huh7, and Chang liver cells, which were transfected with pHBV1.2×, a plasmid encoding all HBV messages. Moreover, these three genes participated in HBV-mediated NF-κB activation.

CONCLUSION: During the initial state of HBV infection, hepatocytes facilitate the activation of NF-κB through up regulation of LT-α, TRAF2, and NIK.

Key words: cDNA microarray; Primary normal human hepatocytes; LT-α; TRAF2; NIK; NF-κB

INTRODUCTION

Human hepatitis B virus (HBV) is a causative agent for liver diseases such as cirrhosis and hepatocellular carcinoma (HCC)[1]. Chronic infection of HBV affects approximately 800 million people and is the principal cause of chronic liver diseases[2]. Moreover, HBV carriers have a much higher frequency of developing liver cancer than uninfected people[3].

HBV has a small, partially double-stranded DNA genome. After viral infection of hepatocytes, the partially double-stranded DNA genome converts into covalently closed circular DNA (cccDNA) in nuclei[4-7]. Several kinds of viral transcripts are then produced by the host RNA polymerase. The transcripts encode for viral polymerase, viral oncogene HBx protein, and viral structural proteins such as surface proteins and core proteins[3].

Many efforts have been made to investigate the process of liver disease by HBV. Traditional techniques such as Northern blot and reverse transcription polymerase chain reaction (RT-PCR) for identification of genes differentially expressed by HBV infection have shown limited success, because only one gene or at best a handful of genes can be studied in one experiment. However, complementary DNA (cDNA) microarray allows the study of several thousands of genes at one time. To evaluate the relationship between HBV infection and liver diseases, recent studies have analyzed the gene expression profiles at tissue level. In these studies, the effects of HBV infection are analyzed by cDNA microarray analysis of HCC tissue samples[8-10]. Through the analyses, many differentially expressed genes can be identified[11]. The analyses, however, have mainly focused on the gene expression profiles of already transformed cells or long-term infected HBV hepatocytes. Therefore, these analyses mostly stem from the analysis of the end result of pathogenesis of HBV in hepatocytes rather than the analysis of ongoing pathogenesis of HBV infection in hepatocytes. In this report, however, we focused on the gene expression profile analysis of the early stage HBV infection, thereby excluding factors such as responses to host immune surveillance. To mimic the early

stage HBV infection of hepatocytes, we isolated primary normal human hepatocytes (PNHHs) and the cells were infected with HBV in culture. These conditions were chosen as they could represent the most similar conditions to those *in vivo*, except for the absence of other types of cells such as immunocytes. Therefore, gene expression profiles in this report could show the result of interaction only between HBV and PNHHs. In this study we have identified 45 down-regulated genes and 53 up-regulated genes.

MATERIALS AND METHODS

Construction

pHBV1.2×, a plasmid which provides all HBV transcripts, was used to infect PNHHs as previously described[12]. This construct was similar to that as described by Guidotti *et al*[13]. Mammalian expression vector for NIK and NIK DN (aa 624-947) was provided by DV Goeddel (Turarik Inc.)[14], for TRAF2 and TRAF2 DN (aa 241-501) by Dr. SY Lee[15].

HBV production and infection

We transfected HepG2 cells with pHBV1.2× constructs for generation of HBV using Fugene 6 transfection reagent (Roche) as instructed by the manufacturer. After transfection, the cells were cultured for 5 d and harvested. HBV particles in the harvested media were cleared and concentrated through ultracentrifugation using PST55Ti rotor (Hitachi) for 1 h at 220 000 g with 3 mL cushion buffer containing 20 g/L sucrose, 50 mmol/L Tris-HCl pH 7.5, and 30 mmol/L NaCl. After ultracentrifugation, the pellet was resuspended with 1 × PBS. The resuspended viral solution was filtered with a 0.2 μm pore filter (Millipore). The titer of HBV solution was adjusted to 10^9 virus genome equivalent (v.g.e.) per mL. PNHHs were infected with the above virus solution at about 100 v.g.e. per cell. Using this method, the efficiency of HBV infection to PNHHs was generally 50%[16].

Isolation and culture of PNHHs

Healthy parts of a liver from a patient who underwent hepatic resection for an intrahepatic stone at the Inje University Paik Hospital, Pusan, Korea was obtained and used as the source of hepatocytes. The removed tissue was immediately placed in Hank's balanced salt solution (HBSS) and processed for cell culture. Isolation of hepatocytes was performed using a two-step collagenase perfusion technique[17,18]. The isolated hepatocytes were resuspended in a nutrient medium containing 90 mL/L Williams' E and 10 mL/L Medium 199, supplemented with 10 μg/mL insulin, 5 μg/mL transferrin, 10^{-7} mol/L sodium selenite, and 50 mL/L FBS.

Confirmation of PNHH infection with HBV

A test was performed with the isolated DNA to determine whether the HBV- infected PNHHs formed cccDNA. Amplification with primers specific for both outside regions was performed with the isolated DNA because the region was specific for cccDNA rather than partially double-stranded DNA found in viral particles. The primers used are (5'-CTATGCTGGGTCTTCCAAATT-3') which anneals to near the codon for amino acid 80 in human HBc open reading frame (ORF) and (5'-TTTCTGTGTA-AACAATATCTG-3') which anneals to near the codon for amino acid 680 in HBV Pol ORF. Therefore, if cccDNA was present in the isolated DNA, an amplified 1 kb product could be obtained. In this test, DNA isolated from mock-infected PNHHs was used as the negative control. A PCR test was performed with RNA to confirm whether HBV transcripts were produced. Reverse transcription amplification was performed with primers specific for the epsilon regions: (5'-CAACTTTTTTTCACCTCTGCC-TA-3') which anneals to DR1 and the reverse primer (5'-GATCTCGTACTGAAAGGAAAGA-3'). In addition, to detect HBV genome, real-time PCR was also performed as previously described[12].

cDNA array analysis

RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. With the total isolated RNA, reverse transcription was performed with Cy5-labeled dUTP in the experimental sample and Cy3-labeled dUTP in the control. Fifty micrograms of RNA, 1.5 ng oligo dT primer, and 1 ng control RNA containing lambda DNA sequences with a poly A sequence at the 3' ends for reverse transcription, were mixed and volume of the mixture was adjusted to 20 μL. The mixture was incubated at 70℃ for 5 min. After incubation, the mixture was quickly cooled on ice. With this whole reaction mixture, the labeling reaction was performed under the following conditions: 1 × reverse transcription buffer, 0.6 mmol/L Cy3- or Cy5-dUTP, 40 U of RNase inhibitor (Roche), 50 U of AMV-RT (Roche) and a dNTP mix containing 1 mmol/L dATP, 1 mmol/L dGTP, 1 mmol/L dCTP, and 0.4 mmol/L dTTP at 42℃ for 1 h. After 1 h, 50 U of AMV-RT was added to the reaction mixture and the mixture was further incubated for 1 h for complete reverse transcription. Reverse transcription was stopped by the addition of 5 μL 0.5 mol/L EDTA. The synthesized cDNA was purified using a chromaspin column (Clonetech) as instructed by the manufacturer and precipitated with ethanol. Both Cy3- and Cy5- labeled cDNAs were resuspended with 100 μL hybridization buffer, containing 6 × standard saline citrate (SSC), 2 g/L sodium dodecyl sulfate (SDS), 5 × Denhardt solution, and 1 mg/mL salmon sperm DNA. The labeled cDNA was used for hybridization to the cDNA microarray chip at 62℃. The chip was arrayed using a GMS417 arrayer (Genetic MicroSystems Inc., Woburn, MA) with 4224 cDNAs and internal standards such as tubulin and actin and external standards such as lambda DNA. After 16-18 h of hybridization, the hybridized array was washed twice at 58℃ for 30 min with washing buffer I containing 2 × SSC and 2 g/L SDS and washed once with washing buffer Ⅱ containing 0.05 × SSC at room temperature for 5 min.

Analysis of chips

For quantification of the signals, the chips were scanned using an array scanner generation Ⅲ (Molecular Dynamics) followed by image analysis using ImaGene ver. 3.0 software (BioDiscovery Ltd., Swansea, UK). The signal intensity

of each spot was adjusted to obtain more accurate data by subtracting the background signals from the immediate surroundings. In this analysis, a difference in the ratio of more than two folds was considered significant.

Cells and transfection

HepG2, Huh7, and Chang liver cells were maintained in minimum essential media (Sigma) supplemented with 100 mL/L fetal bovine serum. For reverse transcription-polymerase chain reaction and luciferase reporter assay, cells were seeded in 12- well plates at a density of 0.2×10^6 cells per well and transfected on the following day with the aperopriate DNA and fugene 6 (Roche) as described by the manufacturer. To normalize the total DNA, pUC119 and backbone DNA of pHBV1.2× were used. The transfection efficiency for HepG2 with fugene 6 was usually 10%-20%.

RT-PCR analysis

Cells were transfected with pUC119 and pHBV1.2×. After 48 h of transfection, total RNA was extracted with TRIzol reagent (Life Technologies) as described by the manufacturer. cDNA was produced by reverse transcription using the same procedure as cDNA microarray analysis. Following reverse transcription, the synthesized cDNA was amplified with 2.5 U Hot start Taq polymerase (Takara), GAPDH specific primer set, and appropriate primer set. The sequences of the primer set are as follows: TRAF2 specific forward(5'-AGGGGACCCTGAAAGAATAC-3'), TRAF2 specific reverse(5'-CAGGGCTTCAATCTTGTCTT-3'), NIK specific forward(5'-TACCTCCACTCACGAAGGAT-3'), NIK specific reverse(5'-CAAGGGAGGAGACTTGTTTG-3'), LT-α specific forward(5'-AGCTATCCACCCACACAGAT-3'), LT-α specific reverse(5'-GTTTATTGGGCTTCATCGAG-3'), GAPDH specific forward(5'-ATCATCCCTGCCTCTACTGG-3'), and GAPDH specific reverse (5'-TGGGTGTCGCTGTTGAAGTC-3'). PCR amplification was performed using Gene mp PCR system 2400 (Perkin-Elmer) with 5 min initial denaturation at 95℃ and 35 cycles of 50 s at 95℃, 50 s at 60℃, and 50 s at 72℃, followed by 7 min of extension at 72℃. To separate the PCR fragments, 15% agarose gel was used.

Immunoblot analysis

To confirm NIK and TRAF2 protein expression, we performed immunoblot analysis with anti-NIK rabbit polyclonal antibody (Santa Cruz) and anti-TRAF2 mouse monoclonal antibody (Santa Cruz). HepG2 cells were seeded in 6- well plates at a density of 0.4×10^6 cells per well. Cells were lysed with RIPA buffer containing 25 mmol/L Tris-HCl (pH 7.4), 150 mmol/L KCl, 5 mmol/L EDTA, 10 mL/L Nonidet P-40 (NP-40), 5 g/L sodium deoxycholate, and 1 g/L SDS and centrifuged at 12000 g for 10 min. The supernatants were separated by 12% SDS-PAGE protein gel for immunoblot analysis.

Luciferase reporter assay

After seeded on 12-well plates, HepG2 cells were co-transfected with appropriate DNA (Figure 5), 0.1 μg pNF-κB-luciferase and 0.1 μg pCMV-β-galactosidase. After 48 h of transfection, cell extracts were prepared and luciferase reporter assay was performed using a luciferase assay system (Promega) as described by the manufacturer. The transfection efficiency was normalized by its galactosidase activity. The assay was triplicated and repeated at least twice.

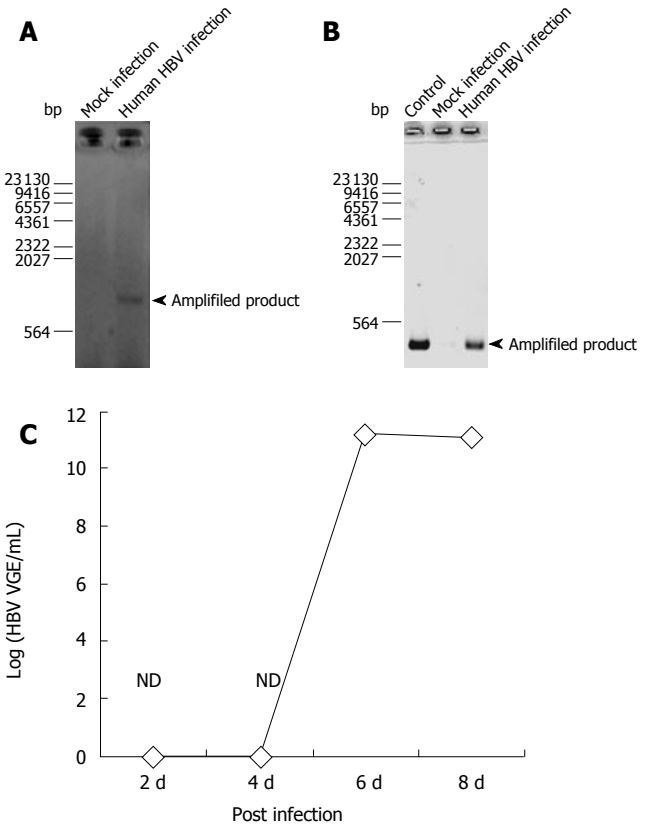

Figure 1 Confirmation of HBV infection for PNHHs using PCR for cccDNA formation and RT-PCR for production of transcripts. From PNHHs infected with HBV, RNA and DNA were extracted using TRIzol reagent as described in Materials and Methods. As a negative control, mock-infected PNHHs were used. **A**: With the extracted DNA, cccDNA was confirmed by a PCR analysis as described in Materials and Methods; **B**: With the extracted RNA, a transcript of HBV was confirmed by RT-PCR analysis as described in Materials and Methods; **C**: With the extracted RNA, a tanscript of HBV was confirmed by real-time PCR as previously described[19].

RESULTS

Confirmation of PNHH infection with HBV

To confirm HBV infection to PNHHs, DNA was isolated during the RNA purification step with TRIzol reagent as instructed by the manufacturer. Infection was confirmed through PCR-based amplification specific only for cccDNA in nuclei of the infected cells. Figure 1 shows that the amplified product appeared in HBV- infected cells, indicating that HBV did infect PNHHs and that the nucleocapsid was transported into nuclei of the infected hepatocytes (Figure 1A). RT-PCR analysis of the HBV transcripts amplified with primers specific for epsilon and polymerase regions as described in Materials and Methods confirmed the presence of HBV RNA transcripts in the infected cells (Figure 1B). Real time PCR showed that HBV was not detected until 6 d after infection (Figure 1C).

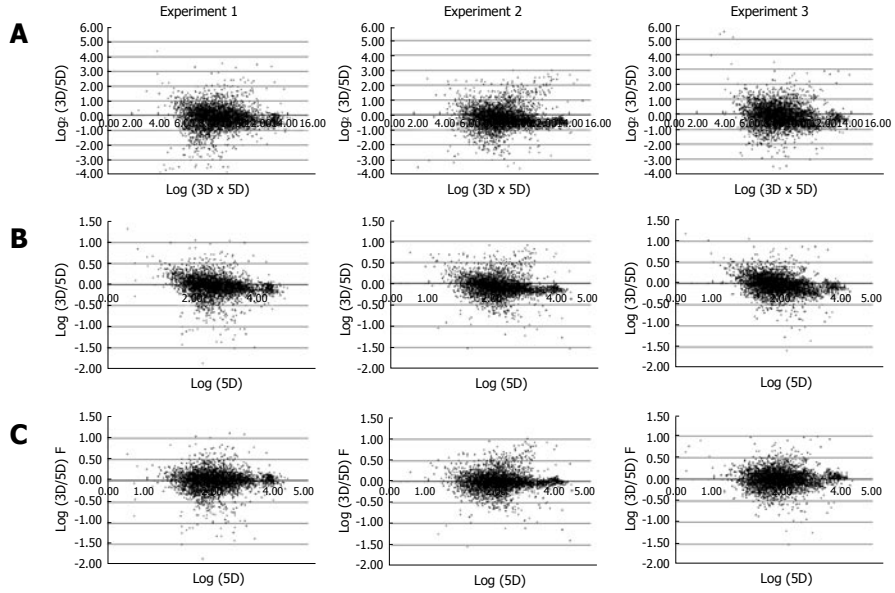

Figure 2 Scatter plot analysis. For normalization of the Cy3 (3D) and Cy5 channel signal (5D) channels, data obtained from the raw image scanning were plotted in a scatter plot using Excel software (Microsoft). **A**: The X-axis represents Log_2 (3D/5D) and the Y-axis Log_2 (3D/5D); **B**: The X-axis represents Log (5D) and the Y-axis Log (3D/5D); **C**: The X-axis represents Log (5D) and the Y-axis Log (3D/5D) F, in which "F" is the function for normalization. The bottom panel shows data with signals fitted to an exponential decay curve.

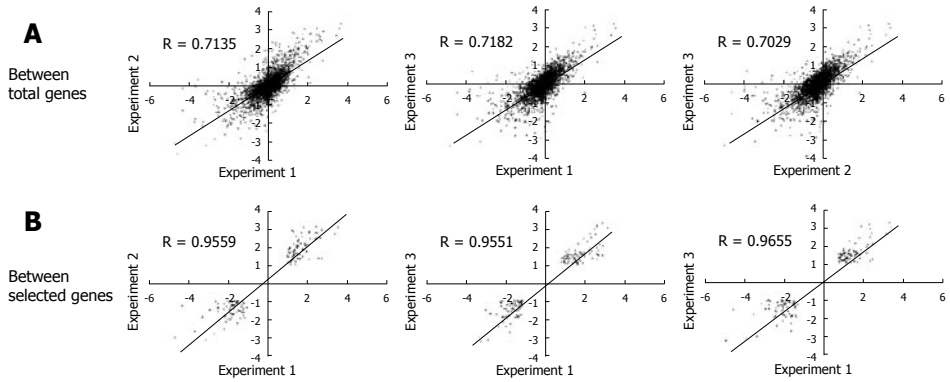

Figure 3 Correlation between three sets of PNHHs infected for eight days. **A**: With the reliable signals in obtained signals, the correlation efficient was calculated between each experiment; **B**: In addition, another correlation efficient was also calculated with only the selected genes, which were differentially expressed more than 2 folds.

Raw data analysis

The experiments were carried out in triplicate at the infection step for more certain identification of genes differentially expressed by HBV infection. From each of the HBV infected cells for over 8 d, RNA was isolated and analyzed by microarray. As a result, three sets of cDNA array images were obtained. We analyzed the intensity of the raw image through scatterplot analyses. Figure 2A shows scatterplot analyses of log (Cy3 signal × Cy5 signal) *vs* log2 (Cy3 signal/Cy5 signal). This showed that each plot tended to divert from the general small curve (Figure 2A). But, each scatterplot analysis of Log (Cy3 signal/Cy5 signal) *vs* Log (Cy5 signal) showed a curve closer to the exponential decay (Figure 2B). Therefore, the data were fitted to an exponential decay curve for Cy3 per Cy5 channel correction (Figure 2C). Through these steps, we obtained a higher confidence ratio of the Cy3 signal compared to the Cy5 signal for each chip. With the ratios obtained, we analyzed the correlation coefficient between the data of the three chips. The correlation coefficient turned out to be more than 0.7 (Figure 3A), suggesting that the relationship between each chip was significant. The correlation coefficient for genes that were differentially expressed more than two folds was more than 0.95 (Figure 3B). Selected genes that were differentially expressed more than two folds, showed a high reproducibility among the triplicate microarray analyses.

Analysis of differentially regulated genes

Through a microarray analysis of PNHHs infected with HBV, we obtained the profile of 45 genes that were down regulated more than two folds compared to the control. The 45 down-regulated genes were analyzed classified by function (Table 1). Table 1 shows that many transcription factors related to RNA polymerase II, were down-regulated by HBV infection. In contrast, transcription factors such as C/EBP, which is used for transcription of HBV genes[19,20], were not differentially expressed. That is, the C/EBP expression level was changed less than two folds.

From the analysis by cDNA microarray, 53 up-regulated genes were identified by an increase of more than two folds in their differential expression. Table 2 shows that growth- and tumor-related molecules comprised a proportion of the up-regulated genes. The positive effector genes for tumor and proliferation have found to be GDF11[21] and NOL1[22] and the negative effector genes EXTL3[23] and RAD50. The most interesting genes have found to be the TNF signaling pathway- related genes. LT-α is an inflammatory cytokine and induces the TNF signaling pathway as a ligand for TNF receptor (TNFR). LT-α binds to TNFR and recruits TRAF2. MAP3K14 (NF-κB inducing kinase, NIK) binds to TRAF2 and activates NF-κB[24].

Table 1 Forty-five differentially down-regulated genes obtained and categorized by their function

Categoty	UniGene	Gene name	Symbol	Locus	Function	Control/ HBV infection	*P*-value
Transcription/ RNA Pol Ⅱ	Hs.442675	Thyroid hormone receptor interactor 8	TRIP8	10	Transcription co-activator of Pol Ⅱ promoter	3.469	0.030
Transcription	Hs.57475	Sex comb on midleg homolog 1	SCMH1	1p34	Pol Ⅱ transcription	3.315	0.019
Transcription	Hs.119014	Zinc finger protein 175	ZNF175	19q13.4	C2H2 zinc-finger protein 175	2.942	0.017
Transcription/ RNA Pol Ⅱ	Hs.437905	Spi-B transcription factor (Spi-1/PU.1 related)	SPIB	19q13.3-q13.4	RNA polymerase Ⅱ transcription factor	2.743	0.017
Transcription/ RNA Pol Ⅱ	Hs.148427	LIM homeobox protein 3	LHX3	9q34.3	RNA Pol 2 transcription factor and activate pituitary hormone genes	2.492	0.006
Signal	Hs.17154	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4	DYRK4	12p13.32	Dual-specificity protein kinase 4	3.938	0.005
Signal	Hs.262886	Inositol polyphosphate-5-phosphatase, 145kD	INPP5D	2q36-q37	Modulating cytokine signaling within the hemopoietic system	3.587	0.009
Signal	Hs.75249	ADP-ribosylation factor-like 6 interacting protein	ARL6IP	16p12-p11.2	Activator of phospholipase D (PLD)	2.801	0.010
Tumor/Suppress	Hs.77793	c-src tyrosine kinase	CSK	15q23-q25	Downregulate the tyrosine kinase activity of the c-src oncoprotein	3.377	0.021
Tumor/Induce	Hs.89839	EphA1	EPHA1	7q34	Overexpression of EPH mRNA was found in a hepatoma	3.039	0.022
Tumor/Induce	Hs.79070	V-myc avian myelocytomatosis viral oncogene homolog	MYC	8q24.12-q24.13	Promotes cell proliferation and transformation	2.357	0.011
Immune response	Hs.118354	Human MHC Class I region proline rich protein mRNA	CAT56	6p21.32	Immune response	2.588	0.024
Miscellaneous	Hs.180610	Splicing factor proline/ glutamine rich	SFPQ	1p34.3	Pre-mRNA splicing factor required for pre-mRNA splicing	10.471	0.006
Miscellaneous/ Cytoskeleton	Hs.75064	Tubulin-specific chaperone c	TBCC	6pter-p12.1	Cofactor in the folding pathway of beta-tubulin	10.2	0.020
Miscellaneous	Hs.438683	BCM-like membrane protein precursor	SBB142	1q23.1	BCM-like membrane protein precursor	3.751	0.004
Miscellaneous	Hs.8203	Endomembrane protein emp70 precursor isolog	LOC56889	10q24.2	Low similarity to human endosomal protein P76	3.502	0.038
Miscellaneous	Hs.311609	Nuclear RNA helicase, DECD variant of DEAD box family	DDXL	19p13.13	Member of the DEAD/H box ATP-dependent RNA helicase family	2.868	0.026
Miscellaneous/ Energy	Hs.150922	BCS1 (yeast homolog)-like	BCS1L	2q33	Function in the assembly of complex III of the respiratory chain	2.682	0.007
Miscellaneous	Hs.6679	hHDC for homolog of Drosophila headcase	LOC51696	6q23-q24	hHDC for homolog of Drosophila headcase	2.611	0.020
Miscellaneous	Hs.5300	Bladder cancer associated protein	BLCAP	20q11.2-q12	Appears to be down-regulated during bladder cancer progression	2.459	0.028
Miscellaneous	Hs.179526	Upregulated by 1, 25-dihydroxyvitamin D-3	VDUP1	1q21.2	Upregulated by 1, 25-dihydroxyvitamin D-3	2.394	0.043
Miscellaneous	Hs.440961	Calpastatin	CAST	5q15-q21	Inhibitor of the cysteine (thiol) protease calpain	2.276	0.000
Miscellaneous	Hs.275775	Selenoprotein P, plasma, 1	SEPP1	5q31	An oxidant defense in the extracellular space	2.186	0.007
EST	Hs.371233	ESTs		Xp22.3	Moderately similar to T08795 hypothetical protein DKFZp586J1822.1	7.826	0.025
EST	Hs.229338	ESTs		X		4.687	0.007
EST	Hs.212957	ESTs		3q26.1	Moderately similar to ZN91_HUMAN ZINC FINGER PROTEIN 91	4.611	0.019
EST	Hs.211823	ESTs		2q37.1		4.519	0.030
EST	Hs.57836	ESTs		17		3.323	0.029
EST	Hs.87912	ESTs		14q24.1		3.314	0.013
EST	Hs.12429	ESTs	FLJ22479	4q26-q27	Hypothetical protein FLJ22479	3.217	0.041
EST	Hs.213586	ESTs		7		2.759	0.044
EST	Hs.2755711	ESTs		22	Weakly similar to T20379 hypothetical protein	2.723	0.019
EST	Hs.191435	ESTs		8p23.1-p22	Weakly similar to S65657 alpha-1C-adrenergic receptor splice form 2	2.638	0.023
EST	Hs.31293	ESTs		9p13.1		2.286	0.035
Predicted protein	Hs.414464	Hypothetical protein	HSD3.1	14q31.3		7.314	0.008

Predicted protein	Hs.100914	Hypothetical protein FLJ10352	FLJ10352	18p11.21		6.239	0.006
Predicted protein	Hs.181112	HSPC126 protein	HSPC126	13q14.12		3.322	0.045
Predicted protein	Hs.306711	KIAA1081 protein	ELKS	12p13.3		3.122	0.036
Predicted protein	Hs.101891	KIAA1193 protein	KIAA1193	19p13.3	Weakly similar to RPB1_HUMAN DNA-directed RNA Pol II largest subunit	3.029	0.030
Predicted protein	Hs.272759	KIAA1457 protein	KIAA1457	12q24.31		2.98	0.020
Predicted protein	Hs.172089	Homo sapiens mRNA; cDNA DKFZp586I2022		11q22.1		2.784	0.036
Predicted protein	Hs.7049	Hypothetical protein FLJ11305	FLJ11305	13q34		2.65	0.028
Predicted protein	Hs.445255	KIAA0368 protein	KIAA0368	9q32		2.423	0.016
Predicted protein	Hs.192190	KIAA0782 protein	KIAA0782	11q13.3		2.332	0.009
Predicted protein	Hs.169910	KIAA0173 gene product	KIAA0173	2p24.3-p24.1	Similar to S72482 hypothetical protein	2.171	0.014

Table 2 Fifty-three differentially up-regulated genes obtained and categorized by their function

Categoty	UniGene	Gene name	Symbol	Locus	Function	Control/HBV infection	*P*-value
Signal	Hs.82887	Protein phosphatase 1, regulatory (inhibitor) subunit 11	PPP1R11	6p21.3	Soluble protein phosphatase inhibitor(reppresor)	3.623	0.026
Signal	Hs.437575	TNF receptor-associated factor 2	TRAF2	9q34	Required for activation of NFkappaB	3.23	0.026
Signal	Hs.6527	G protein-coupled receptor 56	GPR56	16q13	Member of the G protein-coupled receptor family	2.817	0.009
Signal	Hs.29203	Homo sapiens G protein beta subunit mRNA, partial cds	GBL	16p13.3	G protein-linked receptor protein for signalling pathway	2.76	0.009
Signal/ Cytoskeleton	Hs.2157	Wiskott-Aldrich syndrome	WAS	Xp11.4-p11.21	Involved in transduction of signals from receptors on the cell surface to the actin cytoskeleton	2.446	0.018
Signal	Hs.440315	Mitogen-activated protein kinase kinase kinase 14	MAP3K14	17q21	Binds to TRAF2 and stimulates NF-kappaB activity	2.185	0.043
Tumor/ Induce	Hs.15243	Nucleolar protein 1 (120kD)	NOL1	12p13.3	Transforms NIH3T3 cells when overexpressed	5.273	0.004
Tumor/ Supress	Hs.9018	Exostoses (multiple)-like 3	EXTL3	8p21	Tumor suppressor, glycosyltransferase activity	5.07	0.093
Tumor/ Supress	Hs.41587	RAD50 (S. cerevisiae) homolog	RAD50	5q31	Associates with MRE11, nibrin (NBS1) and the tumor suppressor BRAC1	2.443	0.013
Growth/ Positive	Hs.511740	Growth differentiation factor 11	GDF11	12q13.13	Regulators of cell growth and differentiation in both embryonic and adult tissues	2.969	0.023
Cell cycle/ Negative	Hs.76364	Allograft inflammatory factor 1	AIF1	6p21.3	Involved in negative regulation of growth of vascular smooth muscle cells	3.573	0.031
Cell cycle/ Positive	Hs.25313	Microspherule protein 1	MCRS1	12q13.12	Involved in cell-cycle-dependent stabilization of ICP22 in HSV1-infected cells	3.273	0.044
Cell cycle/ Positive	Hs.371833	Nuclear receptor binding factor-2	NRBF-2	10	A possible gene activator protein interacting with nuclear hormone receptors	2.568	0.018
Cell cycle/ Positive	Hs.440606	Centrosomal protein 2	CEP2	20q11.22-q12	Regulate centriole-centriole cohesion during the cell cycle	2.422	0.022
Enzyme/ Glycosylation	Hs.4814	Mannosidase, alpha, class 1B, member 1	MAN1B1	9q34	N-linked glycosylation	3.097	0.008
Enzyme/ lysophospholipase	Hs.889	Charot-Leyden crystal protein	CLC	19q13.1	Phospholipid metabolism and anti-pathogen	3.065	0.035
Enzyme/Protease	Hs.75890	Site-1 protease	MBTPS1	16q24	A sterol-regulated subtilisin-like serine protease	2.873	0.023
Immune response	Hs.2014	T cell receptor delta locus	TRD@	14q11.2	T-cell antigen receptor, delta polypeptide	3.69	0.023
Immune response	Hs.465511	Granzyme M	GZMM	19p13.3	Serine protease for anti-pathogen response	3.201	0.023

Transcription	Hs.436871	Zinc finger protein 173	ZNF173	6p21.3	DNA/protein binding, transcriptional protein	3.411	0.002
Transcription	Hs.108139	Zinc finger protein 212	ZNF212	7q36.1	DNA/protein binding, transcriptional protein	2.341	0.045
Apoptosis	Hs.36	Lymphotoxin alpha	LTA	6p21.3	A member of the tumor necrosis factor family	14.912	0.019
Miscellaneous	Hs.434384	Titin	TTN	2q31	Large myofilament protein	4.087	0.012
Miscellaneous	Hs.58927	Nuclear VCP-like	NVL	1q41-q42.2	Member of the AAA family of ATPases	4.005	0.035
Miscellaneous	Hs.122552	G-2 and S-phase expressed 1	GTSE1	22q13.2-q13.3	Accumulates in late S/G2 phase, is phosphorylated in mitosis, and disappears in G1 phase	3.491	0.007
Miscellaneous/ Glycosylation	Hs.82921	Solute carrier family 35 (CMP-sialic acid transporter), member 1	SLC35A1	6q15	Important for normal sialylation of glycoproteins and glycolipids	3.352	0.013
Miscellaneous	Hs.410455	Unc119 (C.elegans) homolog	UNC119	17q11.2	May function in photoreceptor neurotransmission	3.328	0.030
Miscellaneous	Hs.55041	CGI-22 protein	MRPL2	6p21.3	Unknown	3.052	0.01
Miscellaneous/ Cytoskeleton	Hs.74088	Bridging integrator-3	BIN3	8q21.2	Related to actin assembly-competent state	2.677	0.018
Miscellaneous	Hs.25237	Mesenchymal stem cell protein DSCD75	LOC51337	8q24.3	Moderately similar to uncharacterized Drosophila CG4666	2.615	0.042
EST	Hs.95867	Homo sapiens EST00098 gene, last exon	EST00098	9q34.1		8.781	0.027
EST	Hs.98785	ESTs	KSP37	4p16		3.749	0.011
EST	Hs.136912	ESTs	MGC10796	3q13.13		3.435	0.003
EST	Hs.101774	ESTs	FLJ23045	20p11.23		3.387	0.032
EST	Hs.420262	ESTs		13		3.355	0.022
EST	Hs.124840	ESTs		11q13.1		3.114	0.021
EST	Hs.272299	ESTs	RP4-622L5	1p36.11-p34.2		3.008	0.034
EST	Hs.415048	ESTs		5		2.891	0.025
EST	Hs.531268	ESTs		16		2.889	0.013
EST	Hs.273830	ESTs	FLJ12742	1		2.739	0.020
EST	Hs.190162	ESTs		1p32.3		2.708	0.021
EST	Hs.303172	ESTs		18		2.578	0.04
EST	Hs.59203	ESTs		7		2.437	0.022
EST	Hs.231444	ESTs		1		2.343	0.005
Unknown sequence	Hs.284265	Homo sapiens pRGR1 mRNA, partial cds		6q27		2.966	0.043
Unknown sequence	Hs.291385	Homo sapiens clone 23664 and 23905 mRNA sequence		4p14-p12		2.439	0.041
Predicted protein	Hs.31718	Homo sapiens cDNA FLJ11034 fis, clone PLACE1004258	VRL			17.359	0.000
Predicted protein	Hs.61960	Hypothetical protein	FLJ20040	16p13.3		9.166	0.002
Predicted protein	Hs.274552	Homo sapiens cDNA FLJ10720 fis, clone NT2RP3001116	FLJ10720	5		4.751	0.015
Predicted protein	Hs.279761	HSPC134 protein	HSPC134	14q11.2		3.501	0.038
Predicted protein	Hs.283716	Hypothetical protein PRO1584	PRO1584	8p21.2		3.493	0.033
Predicted protein	Hs.464526	Homo sapiens clone 23649 and 23755 unknown mRNA, partial cds		18q11.2		3.198	0.032
Predicted protein	Hs.274412	Homo sapiens cDNA FLJ10207 fis, clone HEMBA1005475	UPF3A	17p11.2		3.076	0.012

LT-α, TRAF2, and NIK were also up-regulated in the experiment (Table 2). This means that HBV activates NF-κB through up-regulation of LT-α, TRAF2, and NIK.

RT-PCR analysis and immunoblot assay of selected genes

According to the cDNA microarray data, three genes related to the TNF signaling pathway, LT-α, TRAF2, and NIK, were up-regulated. Upregulation of these genes was confirmed by RT-PCR. For RT-PCR analysis, primer sets specific to LT-α, TRAF2, and NIK, were used and experiments were performed in hepatoma-derived cell lines, including HepG2, Huh7, and Chang liver cells. As a result, the mRNA levels of these three genes in each cell line were increased by pHBV1.2× transfection (Figure 4). In addition to RT-PCR, the expression of NIK and TRAF2 was confirmed at the protein level (Figure 5). The expression of LT-α, was confirmed by immunofluorescence staining analysis (data not shown).

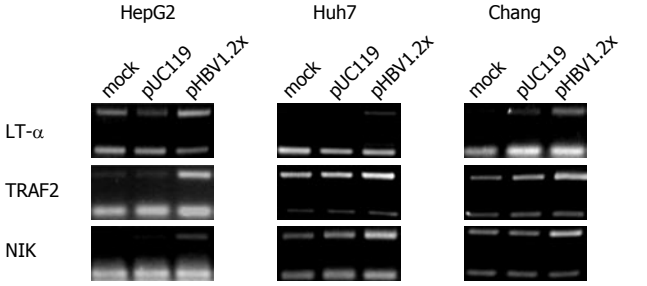

Figure 4 RT-PCR analysis of selected genes. Mock means untransfected cells. pUC119 is backbone of pHBV1.2x, so pUC119 transfected cells are the negative control for pHBV1.2x transfected cell. In the hepatoma-derived cell lines, HepG2, Huh7, and Chang liver cells, TRAF2, NIK, and LT-α mRNA level in pHBV1.2x transfected cells were up-regulated rather than mock and pUC119 transfected cells.

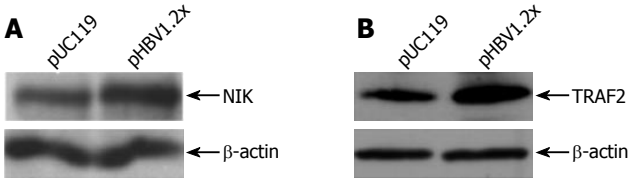

Figure 5 Immunoblot assay of TRAF2 and NIK. pUC119 is a backbone DNA about pHBV1.2x. In the HepG2 cells transfected with pHBV1.2x, the protein levels of NIK (**A**) and TRAF2 (**B**) were increased. β-actin was used to normalize total protein level.

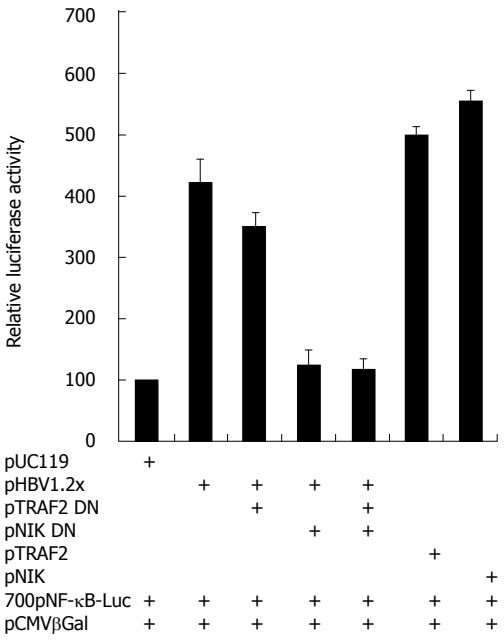

Figure 6 HBV-mediated NF-κB activation through TRAF2 and NIK. In pHBV1.2x transfected HepG2 cells, NF-κB activity was increased more than pUC119 transfected cells. But in cotransfected cells with pHBV1.2x and TRAF2 DN or NIK DN, NF-κB activity was decreased less than pHBV1.2x transfected cells.

NF-κB activation through TRAF2, NIK mRNA up regulation

According to cDNA microarray and RT-PCR analysis, mRNA expression of LT-α, TRAF2, and NIK was up-regulated by HBV. Since their expression was related to NF-κB activation. HBV-mediated NF-κB activation might be involved in the up regulation of these genes. To determine whether these genes actually are involved in HBV-mediated NF-κB activation, we performed a luciferase assay with a pNF-κB-luciferase vector as a reporter plasmid. To elucidate whether HBV-mediated NF-κB activation is dependent on TRAF2 and NIK of three genes, we cotransfected pTRAF2 DN or pNIK DN, the dominant negative form of pTRAF2 or pNIK, with pNF-κB-luciferase, pCMV β-galactosidase, and pHBV1.2× (Figure 6). The experiment for LT-α was performed with anti- LT-α, to neutralize LT-α. The pHBV1.2× produced about a 4.2 folds greater increase in NF-κB luciferase activity than pUC119. However, pHBV1.2× cotransfection with TRAF2 DN or NIK DN produced about a 3.5 or 1.2 fold relative increase (Figure 6) and treatment with anti- LT-α decreased the ratio to less than 4.2 folds (data not shown). These findings led us to think that HBV could activate NF-κB and that this HBV-mediated NF-κB activation might require LT-α, TRAF2, and NIK. DC.

DISCUSSION

In this report, we focused on the interaction between HBV and hepatocytes during the initial stage of infection. To mimic hepatocyte infection with HBV under *in vivo* conditions, we isolated PNHHs and infected them with HBV. We chose this method because cultured cell lines such as HepG2 are seldom infected with HBV[25,26], and transformed cultured cells have many physiological properties that are altered in the original state of hepatocytes[27,28]. In this experiment, the same hepatocytes were used as a control. Since they are produced under identical conditions, a pair of samples of the same genetic background could be obtained. With these samples, we were able to analyze differentially expressed genes. As a result, we obtained gene expression profiles and 98 consistently differentially expressed genes were identified by gene expression profiles. Of these genes, 53 were up-regulated and 45 down-regulated. It was reported that there are no genes uniformly correlated with HBV DNA profile during the initial host response to HBV infection[29]. However, because this study was performed on chimpanzees, there are some considerations in making a comparision between this study with our report. Our report analyzed the effect of HBV on PNHHs at cellular level without any other cell types, including immunocytes. So the influence of immunocytes was not included in this analysis. In addition, the difference in human beings and chimpanzees needs to be taken into consideration.

The results of our study showed that a proportion of the down-regulated genes was transcription factor-related genes and a proportion of the up-regulated genes was TNF signaling pathway-related genes. Down regulation of transcription factors may be helpful for the transcription of the HBV gene because the transcripts of the host cell can be repressed and the transcriptional machinery can be efficiently used for viral transcription. C/EBP, which is involved in viral genome transcription[19,20], had no substantial differential expression in this experiment. In addition to down regulation of transcription factor for virus transcription, up regulation of cell proliferation-related genes may help viral replication. Of the up-regulated genes,

LT-α, TRAF2, and NIK may induce cell proliferation *via* NF-κB activation.

In fact, LT-α is mainly related to the signal cascade for apoptosis and generally involves the host defense system[30]. However LT-α is also related to cell proliferation. Usually, TNF signaling including LT-α, can induce apoptosis and proliferation[31]. TNF signaling by LT-α has a signal cascade from TNFR to TRADD. In the case of apoptosis, TRADD-FADD interaction is needed to activate caspase 8[31]. In the case of proliferation, TRADD-TRAF2 interaction induces activation of NF-κB, a proliferation-inducing transcription factor[31]. After TRAF2 binds to TRADD, NIK binds to TRAF2 and activates NF-κB through IKK activation and IκB-α degradation[24,32-34]. In cDNA microarray data, among genes related to the two opposite effects initiated by LT-α, proliferation-related genes are upregulated. FADD is not differentially altered by more than two folds. Therefore, HBV infection may strengthen the TNF signaling pathway to cell proliferation through the induction of gene expression.

In conclusion, HBV induces NF-κB activation by upregulating LT-α, TRAF2, and NIK, and cell proliferation by activating NF-κB.

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