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Distinctive gene expression profiles associated with Hepatitis B virus x protein

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Hepatitis B virus (HBV) is a major risk factor for the development of hepatocellular carcinoma (HCC). HBV encodes the potentially oncogenic HBx protein, which mainly functions as a transcriptional co-activator involving in multiple gene deregulations. However, mechanisms underlying HBx-mediated oncogenicity remain unclear. To determine the role(s) of HBx in the early genesis of HCC, we utilized the NCI Oncochip microarray that contains 2208 human cDNA clones to examine the gene expression profiles in either freshly isolated normal primary adult human hepatocytes (Hhep) or an HCC cell line (SK-Hep-1) ecotopically expressing HBx via an adenoviral system. The gene expression profiles also were determined in liver samples from HBV-infected chronic active hepatitis patients when compared with normal liver samples. The microarray results were validated through Northern blot analysis of the expression of selected genes. Using reciprocally labeling hybridizations, scatterplot analysis of gene expression ratios in human primary hepatocytes expressing HBx demonstrates that microarrays are highly reproducible. The comparison of gene expression profiles between HBx-expressing primary hepatocytes and HBV-infected liver samples shows a consistent alteration of many cellular genes including a subset of oncogenes (such as c-*myc* and c-*myb*) and tumor suppressor genes (such *as APC, p53, WAF1* and *WT1*). Furthermore, clustering algorithm analysis showed distinctive gene expression profiles in Hhep and SK-Hep-1 cells. Our findings are consistent with the hypothesis that the deregulation of cellular genes by oncogenic HBx may be an early event that favors hepatocyte proliferation during liver carcinogenesis. *Oncogene* (2001) **20,** 3674–3682.

Keywords: hepatitis B virus; cDNA microarray; liver cancer; chronic active hepatitis

Introduction

The hepatitis B virus (HBV) has been clearly recognized as an etiological factor for hepatocellular carcinoma (HCC) (reviewed in Beasley, 1988; Bradley, 1999; Butel, 2000; Feitelson, 1999; Murakami, 1999; Robinson *et al*., 1999). Overwhelming evidence indicates a direct link between chronic infection with HBV and the development of HCC (Bova *et al*., 1991; Brechot *et al*., 1998; Harris and Sun, 1984). These studies support the hypothesis that HCC is a viral-mediated disease and indicate that study of the mechanisms related to HBV-mediated oncogenicity is the key to understanding liver carcinogenesis.

Among the four gene products encoded by HBV, HBx is considered to be potentially oncogenic based on the following body of evidence: (1) HBx can either induce liver cancer directly in certain strains of transgenic mice (Kim *et al*., 1991; Ueda *et al*., 1995; Yu *et al*., 1999) or sensitize other strain of transgenic mice to chemical- or oncogene-mediated liver carcinogenesis (Slagle *et al*., 1996; Terradillos *et al*., 1997); (2) HBx can induce neoplastic transformation in cultured cells (Hohne *et al*., 1990; Kim *et al*., 2001; Koike, 1995; Shirakata *et al*., 1989); and (3) HBx is integrated preferentially and also expressed in some cases during the development of HCC (Kobayashi *et al*., 1997; Paterlini *et al*., 1995; Su *et al*., 1998; Unsal *et al*., 1994). However, a direct role of HBx mediated oncogenicity has yet to be determined. In addition, HBx is essential for viral infection *in vivo* (Chen *et al*., 1993; Zoulim *et al*., 1994). Although HBx does not bind to DNA, it exerts a pleiotropic effect on diverse cellular functions as a transcriptional co-activator (reviewed in Murakami, 1999; Yeh, 2000). The significance of HBx-mediated transcriptional activation in hepatocarcinogenesis has been recognized (Andrisani and Barnabas, 1999; Caselmann, 1995). HBx modulates several cellular processes, including the stimulation of cell proliferation, reduction of DNA repair, abrogation of p53-mediated apoptosis, activation of mitogen activation protein kinase (MAPK) pathways, and the induction of apoptosis by altering the TNFα and NF-κB signaling pathways (reviewed in Brechot *et al*., 2000; Kew, 1997; Murakami, 1999; Yeh, 2000). These

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findings are consistent with the hypothesis that a persistent alteration of multiple cellular genes by HBx may result in a growth stimulus for hepatocytes and thus, contribute to HCC development.

cDNA microarrays are powerful tools to identify disease-related gene expression profiles in biological samples (DeRisi *et al*., 1996). A cDNA microarray consists of thousands of different cDNA clones spotted onto a glass microscope slide. Each microarray is hybridized with two fluorescently labeled cDNA populations made from one experimental and one reference RNA sample. Each RNA is reverse-transcribed in the presence of Cy5- or Cy3-coupled dUTP in red or green, respectively (Shalon *et al*., 1996). The intensity data (red/green, R/G) extracted after scanning of the hybridized microarray represents the ratio of concentrations of the two different fluorescent-labeled mRNA samples. Under the assumption that there is a logical relationship between the function of a gene and its pattern of expression, the correlation of such patterns with cellular phenotype can provide essential clues that may lead to the discovery of novel markers for a particular diseased state (DeRisi *et al*., 1996; Young, 2000). Thus far, the microarray technique has been applied recently to the study of human breast (Perou *et al*., 2000), lymphoid (Alizadeh *et al*., 2000), and ovarian tumors (Ono *et al*., 2000). It is expected that the information provided by molecular profiling of human tumors may lead to a better classification of cancers and to the detection of new oncogenic molecules. However, data on precancerous and cancerous liver samples are currently unavailable.

In this study, we used the NCI human Oncochip cDNA microarray containing 2208 human cDNA elements to determine systematically which cellular genes were altered in freshly isolated normal human hepatocytes expressing HBx and in liver samples from patients with HBV-associated chronic active hepatitis, a precancerous condition. Moreover, we also compared gene expression profiles in primary human hepatocytes and in an HCC cell line expressing HBx. Our data are consistent with the hypothesis that increased expression of oncogenes and decreased expression of tumor suppressor genes are associated with HBx tightly. Further determination of these differentially expressed genes may have diagnostic and prognostic value for patients with HCC.

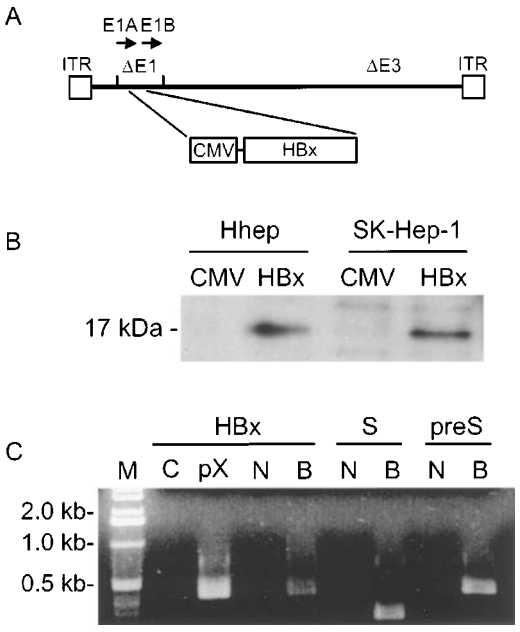

Figure 1 (**a**) A schematic representation of a replication defective adenoviral vector encoding an HBx cDNA under the control of the cytomegalovirus later promoter (CMV). This vector has deletions of the E1 and E3 regions. ITR; inverted terminal repeat. (**b**) Western blot analysis of HBx. Cell lysates from either freshly isolated primary human hepatocytes (Hhep) or SK-Hep-1 cells were prepared 2 days after infection with Ad-HBx (HBx) or control adenovirus (CMV) and analysed with anti-HBx monoclonal antibodies. HBx was shown as a single 17 kDa band. (**c**) PCR detection of the presence of HBV-encoded HBx, S and pre-S genes in HBV-infected liver samples. C, negative control without templates; pX (a plasmid containing the HBx cDNA), positive control for HBx; M, 1-Kb DNA ladder; N, normal liver samples; B, HBV-infected liver samples

Results

Determination of the expression of HBx in cultured liver cells and in frozen liver samples

Freshly isolated human hepatocytes have a very low proliferative capacity. To ensure high efficiency of HBx expression in these cells, we constructed an adenoviral vector expressing HBx (Figure 1a). Infection of Ad-HBx resulted in a comparable and efficient expression of HBx in SK-Hep-1 cells and in freshly isolated hepatocytes (Hhep), as determined by Western blotting with anti-HBx monoclonal antibodies (Figure 1b). No visible cytopathic effect was observed in these cells after infection with either Ad-HBx or Ad-CMV at this MOI (data not shown). To determine the presence of viral genes in frozen liver samples of HBV-associated chronic active hepatitis cases, we used the PCR assay with primers specific to HBx, pre-S and S genes. HBV-associated liver samples (B), but not control liver samples (N), showed bands corresponding to the expected sizes, demonstrating that these samples contain HBx (X), pre-S and S genes (Figure 1c).

Reciprocal labeling experiment

To examine the reproducibility of the cDNA microarray technique, we performed both forward and reverse labeling reactions for every RNA sample. In the forward reaction, the sample of interest was labeled with Cy5 (producing a red signal when scanned) and the control was labeled with Cy3 (producing a green signal when scanned). The reverse reaction was carried out in exactly the same way as the forward reaction, except the sample of interest was labeled with Cy3 and the control with Cy5. Representative quadrants from

forward and reverse labeling microarrays are shown (Figure 2a). The forward and reverse labeling complex probes were hybridized to separate but identical arrays. Results from each sample set consisted of data from forward and reverse paired experiments. One example of the correlation between forward and reverse labeling hybridizations is shown in Figure 2b. This scatterplot represents ratios of hybridization intensities from freshly isolated normal primary human hepatocytes infected with Ad-HBx to those with Ad-CMV, detected by the forward and reverse labeling (Pearson linear correlation coefficient, $r=0.8254$, $P<0.005$). While more than 80% of 2208 cDNA clones analysed remained unchanged by HBx in primary human hepatocytes, less than 20% of the genes were differentially expressed by greater than twofold.

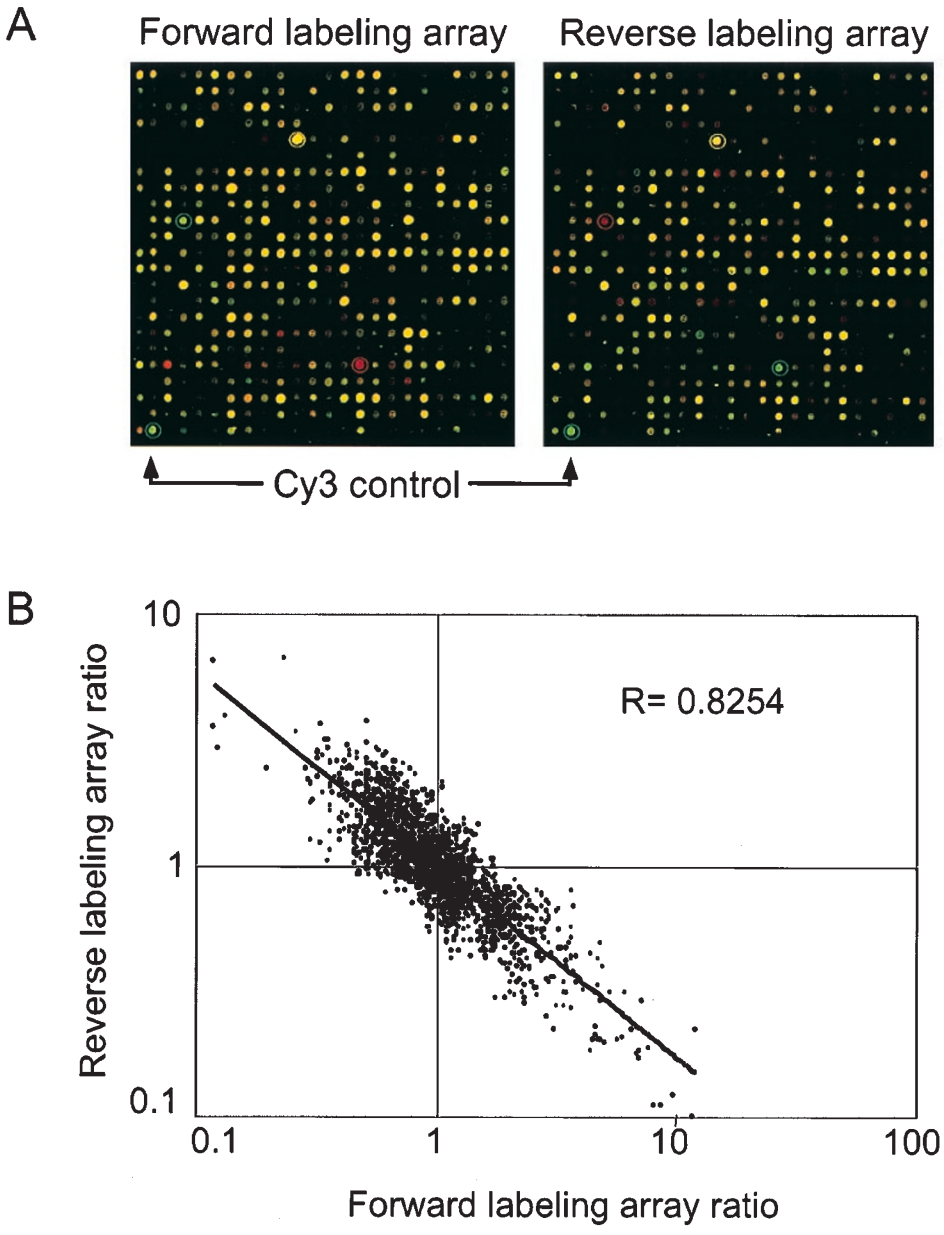

Figure 2 Reproducibility of cDNA microarrays with forward and reverse labeling. (**a**) Representative quadrant of two identical microarrays with reciprocal labeling of total RNA of Hhep infected with HBx. In the forward reaction, the HBx-Hhep RNA was labeled with Cy5 and the CMV-Hhep RNA with Cy3, whereas in the reverse reaction, the HBx-Hhep RNA was labeled with Cy3 and the CMV-Hhep RNA with Cy5. Therefore, the spots that appear green on the left panel should appear red on the right panel, and vice versa. Yellow spots indicate that the cDNA clones are relatively equal amounts in both the sample and the control. The circled Cy3 dots in the lower left corners are experimental controls. (**b**) Scatterplot analysis of the ratio of gene expression in forward and reverse labeling of samples from (**a**). The Pearson linear correlation coefficiency value (r) is indicated ($P<0.005$). The X- and Y-axes are in logarithmic scales

Distinctive gene expression pattern of SK-Hep-1 and Hhep infected with HBx vs control

We used a hierarchical clustering algorithm analysis tool (Perou *et al*., 2000) to determine gene expression patterns that are altered commonly by HBx in primary human hepatocytes and in SK-Hep-1 cells. The analysis yielded four distinctive gene expression patterns when comparing an HCC cell line with freshly isolated primary human hepatocytes from two donors expressing HBx (Figure 3). Genes that were differentially expressed by greater than twofold ($P<0.05$) in SK-Hep-1 cells or Hhep cells following HBx expression are shown (Figure 3). Among the genes analysed, 13 genes including *Fas, CIP2* and *CASPASE-4* were upregulated in both cell types (group A). A total of 11 genes including *TGFβ*1, *MEKK1* and *MAdCAM-1* were downregulated in both cells (group B). Six genes including *APR* and *IL-6* were upregulated in SK-Hep-1 cells, but down-regulated in Hhep cells (group C). On the contrary, 13 genes including *TGFβ*2, *Survivin and* $Bcl\text{-}X_L$ were upregulated in Hhep cells, but down-regulated in SK-Hep-1 cells (group D). These data indicate that freshly isolated primary hepatocytes respond differently to the expression of HBx when compared with an established hepatocellular carcinoma cell line.

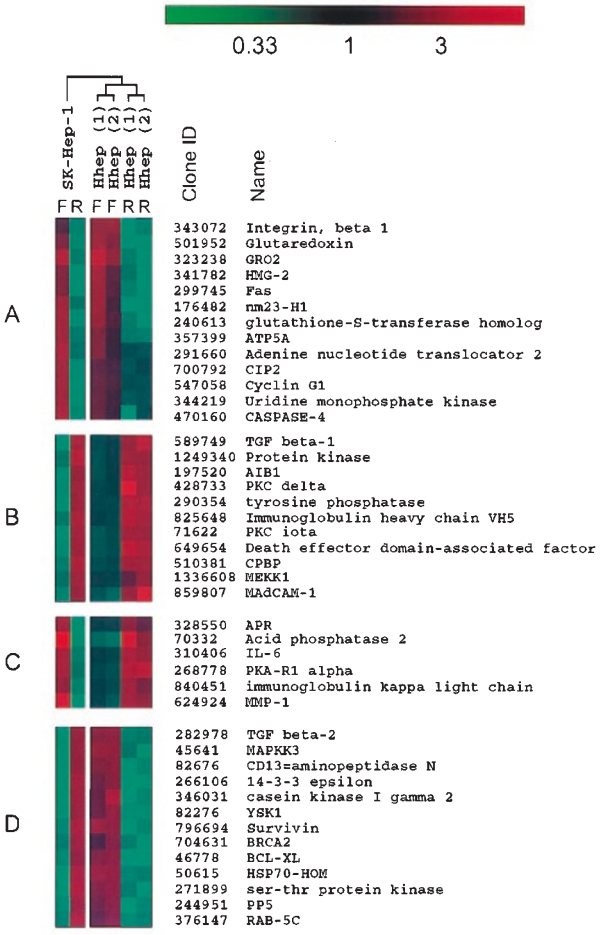

Figure 3 Cluster algorithm analyses of gene expression patterns between SK-Hep-1 cells and freshly isolated adult primary human hepatocytes (from two donors; Hhep-1, Hhep-2) infected with Ad-HBx vs Ad-CMV. In each sample set, data from both forward (F) and reverse (R) labeling were included in each analysis. Panels A and B represent genes that were upregulated (red in forward labeling, panel A) or downregulated (green in forward labeling, panel B). Panel C represents genes that were upregulated in SK-Hep-1 cells, but downregulated in Hhep cells. Panel D represents genes that were downregulated in SK-Hep-1 cells, but upregulated in Hhep cells. The I.M.A.G.E. Consortium clone ID is indicated. The upper color scale bar represents the degree of expression ratio.

Significant correlation between microarray and Northern blot analysis

On the basis of the microarray data, we selected a total of 18 cDNA clones, in which 16 were differentially expressed (twofold or more) in Hhep cells expressing HBx. Sequence analysis of the corresponding genes obtained from Research Genetics based on the I.M.A.G.E. Consortium cDNA clone ID revealed that 15 of 18 clones (83%) were matched to their identities. Then Northern blot analysis was conducted with the use of these 15 identified cDNA clones as probes. Three examples of the Northern blot data were shown in Figure 4a. MEKK1 was downregulated in both SK-Hep-1 and Hhep cells infected with Ad-HBx, whereas APR, a PMA-inducible gene, was upregulated by HBx in SK-Hep-1 cells, but was downregulated in Hhep cells. In contrast, Pdcd2 (programmed cell death 2 gene) was upregulated in Hhep cells, but remained unchanged in SK-Hep-1 cells expressing HBx. Quantitative results from Northern blot and microarray analyses of a total of 15 genes in Hhep cells expressing HBx were plotted in Figure 4b. A Pearson linear regression analysis indicates a significant correlation between Northern blot analysis and microarray analysis ($r=0.9226$, $P<0.005$). In summary, by comparison of data from the two methods, seven genes appeared to be upregulated, while six genes were downregulated, and two remained unchanged.

Genes commonly deregulated by HBx in vitro *and HBV* in vivo

HBV-associated chronic active hepatitis is a precancerous condition in the development of HCC. To examine the physiological relevance of genes that were differentially regulated by HBx in Hhep cells, we compared gene expression profiles of HBV-infected liver samples from individuals with chronic active hepatitis. A total of seven liver samples from HBV-infected patients along with seven liver samples from controls were included in this analysis. Histological analyses were confirmed as chronic active hepatitis patients with grade 1–2 activity (data not shown). Among the seven HBV-infected patients, five were Caucasian males with ages ranging from 33 to 61 years. Among the seven control patients, four were Caucasian males with ages ranging from 23 to 54 years. Total RNA from seven HBV-infected liver tissues were pooled and compared

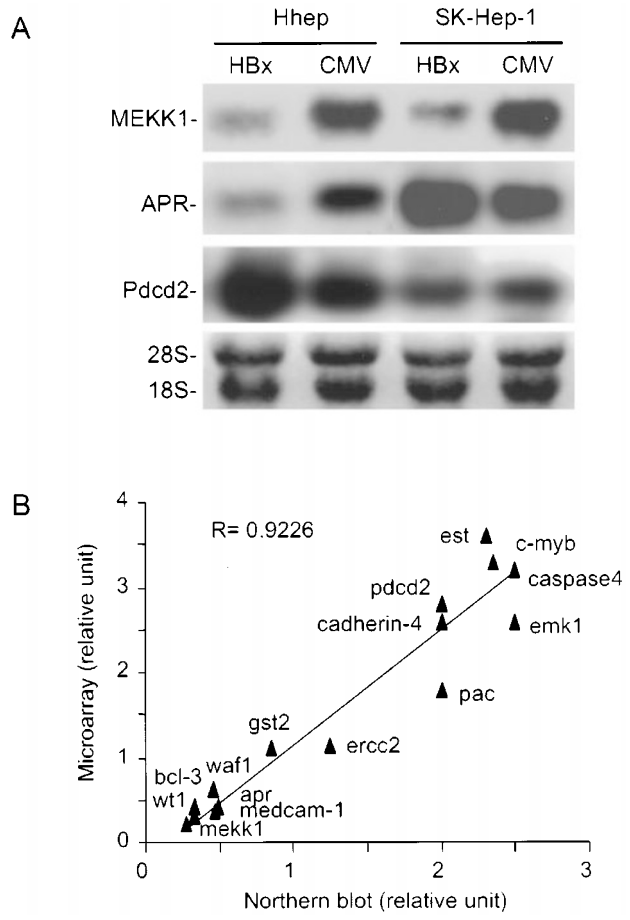

Figure 4 The correlation between Northern blot and cDNA microarray analyses. (**a**) Northern blot analysis of representative genes differentially expressed in both SK-Hep-1 cells and Hhep cells infected with Ad-HBx or Ad-CMV at MOI 5. (**b**) The correlation of the ratio of gene expression level of 15 cDNAs, as detected by cDNA microarray vs Northern blot analysis from Hhep cells infected with Ad-HBx in comparison to Ad-CMV

with total RNA pooled from seven non-HBV-infected normal liver tissues, by cDNA microarray analysis using the same reciprocal labeling approach as before. Then a hierarchical clustering algorithm analysis was used to compare gene expression profiles from Hhep cells expressing HBx from two different donors with profiles from HBV-infected liver samples. Genes that were altered consistently in all these profiles (two from two different primary Hhep and one from HBV liver samples) with expression greater than two fold were selected and the results are summarized in Table 1. Genes that appear to be consistently affected include genes involved in cell growth, apoptosis, metabolic enzymes and microtubule-associated proteins. For example, a number of cell cycle related genes and oncogenes, i.e., *cdk4, cyclin I, cyclin D3, CIP2*, c-*myc* and c-*myb*, whose products promote cell proliferation, were upregulated by HBx in Hhep cells and in HBV-infected liver samples. In contrast, genes favoring cell growth inhibition such as *p53, WAF1, WT1* and *APC* appeared to be downregulated in both. We also found that apoptosis-related genes such as *Bcl-X_L DAD1, Fas, Caspase4* and *pdcd2* were upregulated in these samples. In addition, cell adhesion protein (*vinculin*) and microtubule-associated proteins (*α4-tubulin and γ-tubulin*) were highly expressed.

Discussion

In general, HCC is considered to be a fatal disease because of its poor prognosis, with the exception of a few patients who received liver transplantation. This is largely because of the lack of a method for early diagnosis, and the lack of information on the phenotypic changes associated with the development of HCC. Changes in gene expression profiles during the genesis of HCC are largely unknown. In this study, we utilized the NCI Oncochip human cDNA microarrays in an attempt to identify and classify genes that are differentially altered in association with an HBV-mediated process. One of the major goals of this study was to categorize the expression of genes in freshly isolated adult normal human hepatocytes transiently expressing HBx and in liver samples from chronic hepatitis B virus carriers. By unsupervised cluster analysis, we found shared gene expression patterns in HBx or HBV-mediated processes, which caused the upregulation of a cluster of oncogenes and the downregulation of a cluster of tumor suppressor genes. These results are consistent with our hypothesis that HBx has oncogenic potential whose functions may be associated with increased expression of cell growth promoting genes and decreased expression of tumor suppressor genes to block negative growth regulation, thereby providing a growth advantage for hepatocytes during the early development of HCC.

The cDNA microarray technology has permitted systematic approaches to biological discovery that will impact the fields of biology, pharmacology and medicine. Microarray hardware with sensitive and highly reproducible performance characteristics are necessary for the development of valuable databases. This will be challenging especially with the large number of genes that exist in mammalian genomes (Young, 2000). In this report, we have applied forward and reverse labeling to each sample pair. This approach was used for two reasons: (1) it provided a duplicate experiment for each run; and (2) it served to mutually validate the results of the paired experiment. Our primary microarray analysis on gene expression patterns in freshly isolated human primary hepatocytes expressing HBx vs CMV control showed a good reproducibility between forward and reverse labeling. The reproducibility also was evident in microarrays between two independent experiments using hepatocytes derived from two different patients (data not shown). This approach provided a solid basis for further analysis. We also used Northern blot analysis to verify data from cDNA microarrays. Analyses of the 15 genes that were differentially expressed in Hhep cells

Table 1 Positive and negative transcriptional regulation of cellular genes by HBx in normal primary human hepatocytes and by HBV in liver samples with chronic active hepatitis

Up-regulated		*Down-regulated*	
Clone ID	*Gene description*	*Clone ID*	*Gene description*
Cell cycle regulators		Tumour suppressors	
740079	Cdk4	24415	p53
248295	Cyclin I	595474	WAF1
327182	Cyclin D3	594782	WT1
700792	CIP2	125294	APC
Oncogenes, growth factors and their receptors			
525825	IGF-1R	589749	TGFβ-1
812965	c-*myc*	486457	Gas6
248613	c-*myc*	511387	cysteine-rich FGF-R
Cytokines and their receptors			
366884	Interfron α/β receptor2	310406	IL-6
509641	Interferon-γ receptor α chain	768496	EB13
Metabolic enzymes			
713922	GST M4	840990	GST
416477	Thymidylate synthase	277507	GST M5
628828	glucose transporter protein5	504791	GST A4
Signaling molecules			
486399	A6 tyrosine kinase	713641	MEKK1
67185	Manic fringe	86189	CD4
649619	AP4	328550	APR
Apoptosis response proteins			
46779	Bcl-XL		
341699	DAD1		
303183	Pdcd2		
299745	Fas		
470160	Caspase 4		
Cell adhesion and microtubule associated proteins			
504248	Vinculin		
612273	α4-tubulin		
727614	γ-tubulin		

*Listed genes were differentially expressed in both primary hepatocytes expressing HBx and HBV-infected liver tissues with ratios greater than twofold

showed a linear correlation ($r = 0.9226$) between Northern blot and microarray. Our studies suggest that the cDNA microarray technique is a reliable approach.

HBx is a multi-functional protein (Brechot *et al*., 2000; Feitelson, 1999; Kew, 1997; Murakami, 1999; Yeh, 2000). However, most of the studies that involve the examination of HBx-mediated functions utilize tumor cell lines or fibroblasts. This can lead to contradicting results associated with HBx, largely because of the use of different cell types and transformed cells (Han *et al*., 2000). We chose freshly isolated adult normal primary human hepatocytes for our initial microarray studies, because they are natural host cells for HBV infection and their response to HBx expression would closely resemble human infection. To examine whether there is any difference between primary hepatocytes and tumor cell lines in response to HBx, we compared HBx-mediated gene expression profiles of primary hepatocytes from two healthy donors with those of an HCC-derived SK-Hep-1 cell line, because it contains wild-type p53 without endogenous HBx expression. Although the two cell types shared many common genes that were deregulated by HBx, there was a cluster of genes displaying an opposite response. These results indicate that HBx may behave differently with respect to its role as a transactivator to modulate cellular gene expression in primary hepatocytes when compared with a cultured liver tumor cell line. This may account for the contradicting reports that describe HBx-mediated functions. For instance, it has been shown that HBx may either sensitize or antagonize cells undergoing apoptosis (Chirillo *et al*., 1997; Elmore *et al*., 1997; Kim *et al*., 1998; Shih *et al*., 2000; Wang *et al*., 1995). Therefore, one should be cautious when interpreting the results obtained from using a tumorigenic cell line to study HBx-associated responses.

Given the fact that HBx overexpression in human primary hepatocytes is different from its expression in native HBV-infected hepatocytes, we addressed whether the differentially expressed genes detected *in vitro* could be representing those *in vivo* from liver tissue of chronic HBV-infected patients. Therefore, we performed cluster analysis of the differentially expressed genes in Ad-HBx-infected hepatocytes and pooled seven HBV-infected liver samples vs normal controls. We found that the multiple clustered genes of cell cycle regulators, tumor suppressors, oncogenes, growth factors and their receptors, cytokines and their receptors, metabolic enzymes, signaling molecules apoptosis response proteins cell adhesion, and microtubule associated proteins, as listed in Table 1, are consistently either induced or repressed more than

twofold. These data indicate that HBx may deregulate some genes, if not all, commonly in both *in vitro* and *in vivo*.

The most interesting changes are increased expression of (a) cell cycle regulators like *Cdk4, cyclin I and cyclin D3* and (b) oncogenes, growth factors and their receptors like *IGF-1R*, c-*myc* and c-*myb* with the exception of *TGFβ-1*, but decreased expression of tumor suppressors like *p53, WAF1 and APC1*. These suggest that HBx may alter cell proliferation through the induction of cyclin-dependent kinase and cyclin genes and the inhibition of negative cell cycle regulators, which support cell proliferation (Benn and Schneider, 1995). In particular, *p53*, as a tumor suppressor, has been shown in this study to be repressed about twofold in HBx-infected primary hepatocytes and 2.3-fold (data not shown) in HBV-infected liver tissues respectively, which is in agreement with previous observations by others (Lee and Rho, 2000). The down-regulation of *WAF-1* in both cases by HBx could be, in part, because of inactivation of *p53*. On the contrary, *IGF-1R*, an important growth factor in sustaining the tumor abnormal growth, is upregulated by HBx/HBV more than 2.5- and 2.2-fold, respectively, in both *in vitro* and *in vivo*, which is in line with a recent study (Tao *et al*., 2000). Likewise, c-*myc*, known as an oncogene, is induced in HBx-infected hepatocytes as well as in HBV-infected liver tissues with greater than twofold, which probably collaborates with HBx in a multistage transformation (Terradillos *et al*., 1997). In addition, our results showing a consistent increase in *CDk4, Cyclin I and Cyclin D3* related with HBx/HBV infection also continued previously published findings that HBx deregulates cell cycle checkpoint controls through its activation of *CDk2, CDC2* as well as *cyclins A, B*, and *E* (Benn and Schneider, 1995). This further supports the relevance of the genes identified by our cDNA microarray technique as bona-fide targets of HBx.

Moreover, we found that several genes involved in apoptosis were upregulated by HBx in primary hepatocytes and in HBV-positive liver samples. Examples include two anti-apoptotic genes (*BCL-XL, DAD1*) and three pro-apoptotic genes (*Fas, caspase4 and pdcd2*) (Boise *et al*., 1993). The physiological consequence of such unbalanced induction of apoptotic-response genes by HBx is unclear. However, such phenomena may explain this unusual property of HBx, being capable of either an anti- or pro-apoptotic inducer (Chirillo *et al*., 1997; Elmore *et al*., 1997; Kim *et al*., 1998; Shih *et al*., 2000; Su and Schneider, 1997; Terradillos *et al*., 1998; Wang *et al*., 1995).

HBx induces *Vinculin*, a cell adhesion molecule associated with actin, as well as α4-*tubulin* and γ-*tubulin*, which are microtubule-associated proteins. γ-*tubulin* is a centrosomal protein and is involved in the formation of mitotic spindle poles (Salisbury *et al*., 1999; Sluder and Hinchcliffe, 1999). It is plausible that increased expression of these microtubule-associated proteins would increase the activity of centrosomes, resulting in abnormal mitosis and genomic instability. Consistently, we found that HBx directly induced centrosome amplification and genomic instability (Forgues *et al*., 2001, manuscript in preparation). Moreover, HBx also may deregulate apoptotic-response genes, thereby allowing infected hepatocytes to escape from genomic surveillance networks. Further investigations of these HBx-regulated genes may provide further insight into the mechanisms associated with HBV-mediated liver carcinogenesis.

Materials and methods

Construction of an adenovirus expressing HBx

The recombinant adenoviruses were made by the Massey Cancer Center Virus Vector Shared Resource, Medical College of Virginia, Virginia Commonwealth University. A hemagglutanin (HA) epitope tagged HBx cDNA (adr subtype) was inserted into the adenovirus transfer plasmid, pZERO-TGCMV, between the *Hind*III and *Cla*I sites, which were then made into the adenovirus (E1-, E3-) as described (Valerie, 1999, 2000). The resulting adenoviruses (Ad-HBx, carrying HBx; and Ad-CMV, a control virus without an insert) were amplified in 293 cells, and high-titered viruses were made essentially as described previously (Valerie and Singhal, 1995). The Ad-HBx construct is shown schematically in Figure 1a.

Cell culture and tissue samples

Freshly isolated normal human primary hepatocytes were obtained through Bio Whittaker (Walkersville, MD, USA) from organ donors who died of trauma. These cells were plated in fibronectin-coated plates at a density of 1×10^5 cells per cm^2 and were maintained in HCM medium (Bio Whittaker) for 24 h prior to adenoviral infection. The HCC cell line, SK-Hep-1, was obtained from ATCC (www.ATCC.org) and was cultured at 50% confluence in EMEM medium plus 10% fetal bovine serum prior to viral infection. Primary hepatocytes and SK-hep-1 cells were infected with Ad-HBx or Ad-CMV at MOI 5 and incubated for 48 h prior to harvesting.

Frozen liver tissue samples obtained from seven patients who had received liver transplants for HBV infection, ranged in mass from 400 to 900 mg. Seven frozen normal liver samples were obtained through the Cooperative Human Tissue Network (CHTN) funded by the National Cancer Institute and the LHC resource contract at the University of Maryland. The status of HBV in these samples were verified by immunohistochemical analysis (data not shown) and PCR detection for the presence of HBx, S and pre-S genes, using primers as described previously (Hsu *et al*., 1993).

Western blot analysis

Total cellular extracts were prepared 2 days after Ad-HBx or Ad-CMV infection in RIPA buffer (50 mM Tris-HCl, pH 7.4/150 mM NaCl/1% Triton X-100/1% deoxycholate/1.0% SDS/1% aprotinin) as described previously (Forrester *et al*., 1996). In brief, protein samples (120 μg) were separated through a 16% SDS polyacrylamide gel and transferred to an Immobilin-P membrane (Millipore, Bedford, MA, USA). The membrane was probed with anti-HBx monoclonal antibody as reported previously (Wang *et al*., 1994). Blots were developed using Renaissance Western blot chemiluminescence

and exposed to reflection autoradiography film (DuPont/NEN, Boston, MA, USA).

RNA extraction

Frozen liver tissue samples were homogenized in 1 ml of Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA) per 100 mg tissues using Ultra-Turrax T8 homogenizer (Ika Works, Wilmington, NC, USA) at ~25 000 r.p.m. for approximately 1 min. Lysis of cells in culture was performed by adding Trizol reagent directly to the culture flask 2 days after infection. RNA extraction proceeded according to the manufacturer's protocol. The quality of extracted RNA was determined by spectrophotometry and by the appearance of characteristic 28S and 18S rRNA fragments on a 1% of agarose gel.

Northern blot analysis

Northern blot analysis was conducted as described previously (Wu *et al*., 1997). Total RNA (10 μg) was separated on a 1% agarose gel and stained with ethidium bromide. Then the RNA was transferred to a Hybond-N nylon membrane (Amersham Pharmacia, Piscataway, NJ, USA) and fixed at 80°C for 2 h. Northern blots were prehybridized for 2 h at 65°C in 6 × SSC (saline sodium citrate), 5 × Denhardt's solution, 0.5% SDS, 100 μg/μl of herring sperm DNA. Probes were labeled with α-^{32}P-dCTP (Amersham Pharmacia, Piscataway, NJ, USA) according to the hexamer-random primed method following the manufacturer's protocol (Promega Inc., Madison, WI, USA). Membranes were hybridized under the same conditions as stated for prehybridization and were washed four times afterwards for 15 min with 1 × SSC/0.1% SDS and once with 0.2 × SSC/0.1% SDS at 65°C. The membranes were exposed and scanned with a Phosphorimager radio-analytic scanning system (Fuji Photo Film Co., Ltd, Japan) to quantify the amount of radioactivity in individual bands, which was standardized by the intensity of 28S rRNA scanned with the EAGLE EYE™II (Stratagene, La Jolla, CA, USA).

Microarray analysis

DNA microarray analysis of gene expression was performed according to protocols available at (http://nciarray.nci.nih.gov). In brief, for each comparative array hybridization, a complex cDNA probe was synthesized by a single round of reverse transcription as follows: 50 μg of total RNA was mixed with 4 μg of oligo-dT primer, a nucleotide pool (2 μl; 10 mM each dATP, dCTP, dGTP, 4 mM dTTP, and 6 mM aa-dUTP; Sigma), 1 μl of RNAse inhibitor (20 – 40 U/μl, Promega Inc.), 8 μl of 5 × first-strand buffer and 2 μl of Superscript II reverse transcriptase (200 U/μl) (Gibco-BRL Kit) and the mixture was incubated at 42°C for 1 h. For the labeling, a vial of NHS-ester Cy3 and Cy5 dye (Amersham) was dissolved in 72 μl of 0.1 M $NaHCO_3$ (pH 9) and 9 μl of either Cy3 or Cy5 solution was added to the cDNA sample and incubated at room temperature for 1 h in the dark. After incubation, 9 μl of 4 M hydroxylamine was added to the sample and incubated at room temperature for 15 min in the dark to prevent cross-coupling. Test probe and control probe were combined and purified using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA, USA). In 17 μl of purified probe, 1 μl of Cot1 human DNA (10 μg/μl; Gibco-BRL), 1 μl of poly(A) RNA (10 μg/μl; Sigma), 1 μl of yeast tRNA (4 μg/μl, Gibco-BRL), 4.4 μl of 20 × SSC and 0.7 μl of 10% SDS were added to form the final probe mixture. The mixture was placed on the array under a 22 mm × 22 mm glass coverslip. The slides were incubated overnight at 65°C in a slide chamber. For each experiment, there were two identical microarrays on which reciprocally labeled cDNAs from test and control samples were applied. In the forward reaction, the test sample was labeled with Cy5 and the control with Cy3, whereas in the reverse reaction, the test sample was labeled with Cy3 and the control with Cy5. Clones with ratios that were consistently outside the twofold threshold for up or downregulation in both labeling experiments were included for further analysis.

Array quantification, data processing and cluster analyses were conducted as described at (http://nciarray.nci.nih.gov). Cellular genes, whose expression levels had a significant change in ratios with greater than a 95% confidence interval, were included in our cluster analysis. A correlation between forward and reverse labeling experiments as well as a correlation between microarray and Northern blot analysis were assessed using MS-Excel software.

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