Gene-expression profiles of a hepatitis B small surface antigen-secreting cell line reveal upregulation of lymphoid enhancer-binding factor 1

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The genome of hepatitis B virus (HBV) consists of four open reading frames, encoding the envelope proteins (Pre-S/S), the core proteins (Pre-C/C), the polymerase (P) and the transactivating X protein (X). In the sera of HBV-infected patients, hepatitis B surface antigen (HBsAg) particles without the viral genome can outnumber virions by more than 1000-fold. To analyse the interactions between HBsAg and host cells, global gene-expression profiles of a small HBsAg (SHBs)-secreting stable cell line (HepG2-S-G2) and its counterpart control cell line (HepG2-Neo-F4) were compared. Marked upregulation of lymphoid enhancer-binding factor 1 (LEF-1), a transcription factor in the Wnt pathway, was found in SHBs-expressing cells and was confirmed by interference experiments with small interfering RNA. However, compared with the control cells, HepG2-S-G2 did not show higher proliferative competence in culture or increased tumorigenesis in nude mice. A possible mechanism to explain the discrepancy between the upregulation of LEF-1 and the lack of increased tumorigenesis is SHBs expression resulting in altered expression and distribution of LEF-1 protein in cell compartments and upregulation of LEF-1 isoforms that could suppress, rather than enhance, the Wnt pathway.

INTRODUCTION

Hepatitis B virus (HBV) infects more than 350 million people worldwide and is a major cause of chronic viral hepatitis (Ocama et al., 2005). The genome of HBV is circular, double-stranded DNA with a single-stranded gap, and contains four open reading frames: the S/pre-S gene, which encodes the envelope proteins, the core/pre-core gene encoding the core and e protein, the P gene encoding the polymerase and the X gene encoding the X protein, which is a transactivator (Seeger & Mason, 2000). In the sera of infected patients, three morphological forms of viral particle can be detected: namely, 22 nm diameter spherical particles, tubular particles and 42 nm diameter spherical virions (Lee, 1997). The 22 nm diameter spherical particles are composed of the hepatitis B surface antigen (HBsAg), whilst the 42 nm virion particles consist of the viral genome encapsidated within the icosahedral core, which is surrounded by the viral envelope containing pre-S/S proteins. Uniquely, the 22 nm spherical particles, which do not contain viral DNA, usually outnumber the virions in patient serum by a factor of 100- to 1000-fold or more (Ganem & Prince, 2004). However, the interactions between HBsAg and host cells, and the mechanisms and biological significance of the high levels of HBsAg production and secretion have not been clarified fully.

HBsAg has been used as a prophylactic vaccine to protect healthy people from being infected. It has been suggested that the excess HBsAg particles could absorb anti-HBs, the neutralizing antibody for HBV, to help the mature virions escape from host immune responses and to establish persistent infection (Milich, 1997; Rehermann & Nascimbeni, 2005). It was further hypothesized that HBsAg acts as an apoptotic-cell mimic and interacts with the innate apoptotic-cell removal system in order to prevent the emergence of adaptive immunity (Vanlandschoot & Leroux-Roels, 2003). However, experimental evidence is needed to support this hypothesis. Recently, it has been reported that HBsAg inhibited the release of lipopolysaccharide-induced cytokines by interfering with the NF-κB pathway (Cheng et al., 2005), showing that HBsAg interferes with host immune responses. To date, although persistence of HBsAg in patient serum has been recognized as a high risk factor for development of hepatocellular carcinoma (Beasley et al., 1982; Lupberger & Hildt, 2007) and some groups have examined the possible roles that large and middle HBsAgs (LHBs and MHBs) play in the development of hepatocellular carcinoma (HCC) (Chisari et al., 1989; Hildt et al., 2002), the association of SHBs with tumorigenesis has not been elucidated.
To study the effects of persistent expression of HBsAg on host-cell functions, the overall effects of HBsAg on the expression of cellular genes were examined by using microarray assays. A head-to-head comparison was made between two cell lines that were cultured under the same conditions. One was an SHBs-secreting stable cell line (HepG2-S-G2), which was transfected with a plasmid containing the small S gene; the other was the corresponding control cell line (HepG2-G2-F4), transfected with the vector only. Cellular genes involved in cell metabolism, growth and death, signal-transduction pathways, cytoskeleton and extracellular matrix formation showed altered transcription in HepG2-S-G2 cells. Among these genes, lymphoid enhancer-binding factor 1 (LEF-1), a key component in the Wnt pathway, was induced consistently and markedly in cells expressing SHBs. Our data showed that, instead of the full-length LEF-1, truncated isoforms were upregulated by the expression of SHBs, which indicated functional suppression of the Wnt pathway.

METHODS

Construction of plasmids. The small S gene of a full-length genotype C HBV isolate C8 (GenBank accession no. AF461363) from a chronic hepatitis B patient was used to construct plasmid pCMV-S. The small S gene (nt 157–837) was cloned into the BamHI/EcoRI site of the vector pCMV-Script (Stratagene). The E gene (nt 1826-2454) was cloned into the HindIII/XbaI site of the vector pcDNA3.1/myc-His A (Invitrogen).

Cell cultures. HepG2 and HuH7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 U penicillin ml\(^{-1}\), 100 μg streptomycin ml\(^{-1}\), 2 mM glutamine, 25 mM HEPES solution, 1 mM sodium pyruvate at 37 °C under 5% CO\(_2\). For the SHBs-expressing cell lines, the vector control cell lines and HepG2.2.15 cells, 250 μg G418 ml\(^{-1}\) was added to the above medium.

PLC/PRF/5 cells (ATCC) were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 100 U penicillin ml\(^{-1}\), 100 μg streptomycin ml\(^{-1}\), 2 mM glutamine, 25 mM HEPES solution, 1 mM sodium pyruvate and 0.1 mM MEM non-essential amino acids at 37 °C under 5% CO\(_2\) as recommended by ATCC.

All cell-culture reagents were purchased from Gibco.

Construction of SHBs-expressing cells. pCMV-S or pCMV-Script was transfected separately into HepG2 cells by using the calcium phosphate precipitation method. G418 (1 mg ml\(^{-1}\)) was added to the medium on the first day after transfection for selection. The medium was replaced every 2 days. After 2–3 weeks, drug-resistant cell clones were picked up and expanded. The medium from each cell clone was replaced every 2 days. After 2–3 weeks, drug-resistant cell clones were selected and assayed for the expression of SHBs by ELISA (Kehua). SHBs-positive cell clones and vector-control cell clones were selected and passaged in parallel for 15 passages before being used for the experiments described below.

Southern and Northern blots. Genomic DNA from cell cultures was extracted by the conventional SDS–proteinase K method. After digestion with BamHI, 15 μg genomic DNA was subjected to 0.7% agarose-gel electrophoresis and transferred onto positively charged nylon membranes (Schleicher & Schuell). Southern blots were hybridized with small S gene or neomycin-resistance gene fragments labelled with \(^{32}\)P by using a random-primer labelling kit (Roche).

Total RNA was isolated from cells by using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (15 μg) was subjected to Northern blotting and hybridized with \(^{32}\)P-labelled small S gene or neomycin-resistance gene probes.

Microarray analysis. HepG2-S-G2 or HepG2-Neo-F4 cells (2.5 × 10\(^7\) cells per sample) were collected separately on the fourth and eighth days after seeding. Global gene-expression profiles were analysed by using Affymetrix Human Genome U133 Plus 2.0 array chips, which contained over 47 000 transcripts and variants, including 38 500 well-characterized human genes. All microarray services (including experiments and data analysis) were provided by Gene Company (Shanghai).

Reverse transcription and real-time PCR. After treatment with 10 U DNase I (Takara), at 37 °C for 30 min, 2 μg total RNA was reverse-transcribed into cDNA at 42 °C for 1 h by using StrataScript reverse transcriptase (Stratagene) with random hexamers (Takara) according to the manufacturer’s protocol. Semiquantitative real-time PCR was carried out by using specific primer pairs designed by PrimerBank (Wang & Seed, 2003). Primers used to detect LEF-1 were designed as follows: for the β-catenin binding domain of LEF-1, primer P1: 5′-AATCATCCGGCGACAG-3′ and primer P2: 5′-TGTCGTGGTAGGGCTCCTC-3′ were used (Wang et al., 2005). For the 3′ untranslated region (UTR) of LEF-1, primer P3: 5′-CATATGCTTCTCCTGCCCTGTAAAG-3′ and primer P4: 5′-TTCAACTGTGGCCCTTTCCTAAGAGGCGAG-3′ were used. For real-time PCR, 2 μl of 10-fold dilutions of the cDNA products were assayed by using a Takara Ex-Tag R-PCR kit (Takara) with annealing for 5 min at 94 °C, followed by 40 cycles of 94 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. All experiments were performed twice independently.

Transient transfection. HepG2 cells (3 × 10\(^5\) per well) or HuH7 cells (1 × 10\(^5\) per well) were seeded separately into 24-well plates and cultured for 24 h, when cells were 90% confluent. Plasmid DNA (0.75 μg per well) was transfected into cells by using Lipofectamine 2000 reagent (Invitrogen). In all transfection experiments, pSEAP2-control vector (0.25 μg per well; BD Biosciences) was co-transfected to normalize transfection efficiency. Cell-culture supernatants and cells were collected 48 h after transfection for further experiments.

Small interfering RNA (siRNA) synthesis and transfection. To confirm that the upregulation of LEF-1 genes was associated with expression of SHBs, siRNAs were synthesized, based on reported sequences (Li et al., 2004). S1 siRNA targeted a region within HBsAg to block its expression, whilst Scr was a scrambled control sequence (synthesized by Shanghai GeneChem). The sequences are as follows: S1 siRNA, 5′-GGUAUGUUGCCCGUUGUGTdTdT-3′, 3′-dTGTC-CAUACAACGGGACCAAAG-5′; Scr siRNA, 5′-UCUCGGCAACUGUCACGTdtdTdT-3′, 3′-dTGCAUGAGGCUUCAGACAGCA-5′. To study the effect of blocking SHBs secretion on LEF-1, 5 × 10\(^4\) PLC/PRF/5 cells were seeded into each well of a 24-well plate. When the cells were 50% confluent 100 nM siRNA was transfected into them by using Lipofectamine 2000.

Cell-proliferation assay. HepG2-S-G2 cells and HepG2-Neo-F4 cells (2 × 10\(^5\) per well) were seeded separately into each well of a 96-well plate. Twenty microlitres of AQueous One Solution cell proliferation assay reagent (Promega) was added to each well every 24 h after seeding for a period of 4 days. After incubation at 37 °C for
2 h, the OD_{490} was measured. All assays were performed three times independently, each in triplicate.

**Evaluation of tumorigenesis of HepG2-S-G2 cells in nude mice.** Nude mouse inoculation was performed as described previously (Lee et al., 2005). Male, athymic BALB/c nu/nu mice (5 weeks old) were obtained from the Liver Cancer Institute of Zhongshan Hospital, Fudan University. All mice were bred in laminar-flow cabinets under specific-pathogen-free conditions. Approximately $1.5 \times 10^7$ cells in 0.2 ml PBS were injected subcutaneously into the right flanks of the mice, which were then observed daily for signs of tumour development.

**Cellular fractionation and Western blot.** Cells were detached by scraping and harvested by low-speed centrifugation (900 g). Cell pellets were resuspended in hypotonic buffer (10 mM Tris/HCl, pH 7.8; 10 mM NaCl), allowed to swell for 15 min on ice and disrupted by 50 strokes with a tight-fitting pestle. Nuclei were collected by centrifugation (900 g for 5 min at 4 °C). Post-nuclear homogenates were centrifuged at 15,000 g for 20 min at 4 °C to separate cytoplasm and cell membranes.

Protein samples were separated by SDS-PAGE in 12 % polyacrylamide gels and transferred onto a nitrocellulose membrane (Amersham Biosciences). The membranes were incubated for 2 h at room temperature in blocking buffer (PBS containing 0.05 % Tween 20 and 5 % non-fat milk powder) followed by overnight incubation at 4 °C with anti-LEF-1 antibody (rabbit polyclonal antibody; Abcam) diluted 1:500 in blocking buffer. After being washed with PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$ and 0.05% Tween 20, adjusted to pH 7.4), the membranes were incubated at room temperature for 2 h with the corresponding horseradish peroxidase-conjugated secondary antibody diluted 1:1000 in blocking buffer. After washing, signals were detected by enhanced chemiluminescence (ECLplus; Amersham Biosciences) on Kodak Biomax Light films.

**Immunofluorescent staining for studying the distribution of LEF-1 in cell compartments.** Cells were seeded on poly-L-lysine-coated coverslips and cultured for 48 h. After being washed twice with PBS, cells were fixed in 4 % paraformaldehyde in PBS for 15 min at room temperature, and were quenched with PBS containing 30 mM glycine for 5 min. After being permeabilized with PBS containing 0.1 % Triton X-100, cells were blocked with 3 % BSA in PBS for 2 h at room temperature followed by overnight incubation at 4 °C with rabbit polyclonal anti-LEF-1 antibody diluted 1:25 in blocking buffer. Fluorescein isothiocyanate-labelled anti-rabbit secondary antibody (Chemicon) was added and incubated for 2 h at room temperature. After staining with 4′,6-diamidino-2-phenylindole (DAPI), cells were examined under a confocal laser-scanning microscope (TCS-NT; Leica).

**RESULTS**

**Characterization of an SHBs-expressing stable cell line and its control counterpart**

After transfection of HepG2 cells with pCMV-S, followed by selection with G418, four stable cell lines secreting SHBs were established. Four counterpart control cell lines were established in parallel. Among these cell lines, HepG2-S-G2, which expressed the highest level of SHBs, was selected for the subsequent experiments and HepG2-Neo-F4 was used as the control cell line. Total RNA and genomic DNA from both cell lines were isolated and assayed by Northern and Southern blotting to confirm the integration and transcription of the HBV small S gene (Fig. 1a, b). The dynamic secretion of SHBs by HepG2-S-G2 cells is shown in Fig. 1(c). The level of SHBs in cell supernatant increased from the second day after seeding and reached the stationary phase on day 7. Therefore, day 4, when cells were in the exponential phase, and day 8, when cells were in the stationary phase, were chosen as the time points to study the global gene-expression profiles of HepG2-S-G2 and HepG2-Neo-F4 cells. To evaluate the secretion efficiency of SHBs in HepG2-S-G2 cells, $1 \times 10^7$ cells were harvested and lysed by sonication in 0.5 ml PBS buffer. Cell-culture supernatant and cell lysate (50 μl per sample) were collected to determine secretory and intracellular SHBs by ELISA. When the corresponding volumes of supernatant (7 ml) and cell lysate (0.5 ml) were calculated, the majority of SHBs produced by HepG2-S-G2 cells was secreted extracellularly (Fig. 1d).

**Microarray analysis and global gene-expression profiles of HepG2-S-G2 and HepG2-Neo-F4 cells**

The global gene-expression profiles of HepG2-S-G2 and HepG2-Neo-F4 cells cultured for 4 and 8 days, respectively, were compared and analysed by using Affymetrix Human Genome U133 Plus 2.0 array chips, which contained over 47,000 transcripts and variants, including 38,500 well-characterized human genes. The cut-off value for significance was set as a 2-fold change. Results showed that, in HepG2-S-G2 cells, 340 genes were suppressed and 673 genes were induced in association with the expression of SHBs on day 4, whereas 219 genes were downregulated and 683 genes were upregulated on day 8 (the details of microarray data are available in the GEO database under accession no. GSE4549). In total, 132 genes were found to be upregulated reproducibly and 60 were found to be downregulated in HepG2-S-G2 cells on both day 4 and day 8. Many cellular genes involved in lipid and carbohydrate metabolism, cell growth and death, signal-transduction pathways and cytoskeleton and extracellular matrix formation were affected in association with SHBs expression (summarized in Table 1). Twenty-one genes involved in different cellular functions were selected and studied by semiquantitative real-time PCR to check the reliability of microarray data. Most of the real-time PCR results were consistent with the microarray data (Table 2).

The HepG2.2.15 cell line harbours head-to-tail dimers of the HBV genome integrated into its chromosome and secretes HBV virions as well as HBsAg particles (Sells et al., 1987). To examine whether the altered gene expression detected in HepG2-S-G2 cells was related to specific chromosomal integration sites or to specific HBV strains, the genes with altered expression profiles were determined by comparing their mRNA levels in HepG2.2.15 and its parental cell line HepG2. By real-time PCR, 11 of the 21 selected genes showed changes in HepG2.2.15 cells similar
to those found in HepG2-S-G2 cells (Table 2), suggesting that changes in the expression profiles of these 11 genes were probably associated with HBsAg expression or virus replication. Among these 11 genes, lymphoid enhancer-binding factor 1 (LEF-1) was upregulated consistently and markedly in cells expressing SHBs, and was upregulated by 42-fold in HepG2-S-G2 cells and by 8-fold in HepG2.2.15 cells.

**Upregulation of LEF-1 is associated with SHBs expression**

The LEF-1 gene belongs to the LEF/TCF family of the high-mobility group (HMG) of transcription factors, and functions as the crucial downstream mediator of the Wnt signal-transduction pathway (Clevers & van de Wetering, 1997; Hovanes et al., 2000; Giles et al., 2003). Activation of LEF-1 promotes the expression of dozens of downstream effector proteins, such as cyclin D1 and c-myc, which subsequently regulate the progress of cell proliferation and the cell cycle (Shtutman et al., 1999; Tetsu & McCormick, 1999). The role of LEF-1 in the Wnt signalling pathway suggested that this gene might be implicated in the development of HBV-induced HCC.

To assess whether the association of SHBs and LEF-1 upregulation was a ubiquitous phenomenon, LEF-1 expression levels in the other three SHBs-expressing stable cell lines and three control cell lines established in our lab were determined by real-time PCR. As shown in Fig. 5(b), LEF-1 was upregulated in all four SHBs-expressing cell lines compared with the four control cell lines, indicating that induction of LEF-1 was associated closely with SHBs expression.

To verify further that the upregulation of LEF-1 was associated with SHBs expression, pCMV-S was transiently transfected into HepG2 and Huh7 cell lines. As shown in Fig. 2, induction of LEF-1 was observed in cells transiently expressing SHBs. When the levels of LEF-1 mRNA were compared by real-time PCR between cells transfected with pCMV-S and cells transfected with the vector pCMV-Script, LEF-1 mRNA was increased by 1.9 ± 0.4-fold in.
Table 1. Cellular functions affected by the expression of SHBs

<table>
<thead>
<tr>
<th>Cellular function</th>
<th>Upregulated</th>
<th>Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid metabolism</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Cell growth and death</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Cytoskeleton and extracellular matrix</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Energy and other metabolism</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Immunological and acute-phase response</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Lipid and sterol metabolism</td>
<td>1</td>
<td>0</td>
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<tr>
<td>Protein modification</td>
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</tr>
<tr>
<td>Transcriptional regulation</td>
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<td>3</td>
</tr>
<tr>
<td>Transporter</td>
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<td>2</td>
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<tr>
<td>Ubiquitination</td>
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<td>3</td>
</tr>
<tr>
<td>Xenobiotic and aromatic compound metabolism</td>
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</tr>
<tr>
<td>Unknown genes</td>
<td>74</td>
<td>12</td>
</tr>
<tr>
<td>Other</td>
<td>12</td>
<td>5</td>
</tr>
</tbody>
</table>

HepG2 cells and by 2.2 ± 0.1-fold in Huh7 cells (Fig. 2a, b). Considering the relatively low transfection efficiency (approx. 5–10% in HepG2 cells and 30% in Huh7 cells), the magnitude of increased LEF-1 expression (around 2-fold) was considered significant.

To study whether blocking the expression of SHBs could inhibit the upregulated expression of LEF-1, HBsAg-specific siRNA S1 was used to treat PLC/PRF/5 cells. This cell line is a hepatoma cell line isolated from an HCC patient, and secretes a substantial amount of HBsAg that can be monitored easily. Also, HBsAg is the sole viral protein produced by PLC/PRF/5 cells (Alexander et al., 1976; Wen et al., 1981). Reduction of HBsAg secretion was observed 1 day after siRNA transfection, with maximal inhibition on the second day, consistent with previous studies (Li et al., 2004). Compared with the control scrambled siRNA (Scr)-transfected cells, S1 siRNA transfection resulted in an 86.2 ± 1.2% reduction of HBsAg, and the expression of LEF-1 was reduced by 40.6 ± 15.6% (Fig. 2c, d). A similar phenomenon was observed in transiently transfected Huh7 cells. When S1 siRNA was co-transfected with pCMV-S into Huh7 cells, the upregulation of the LEF-1 gene in SHBs-expressing Huh7 cells reverted to the normal level. In comparison with Scr siRNA co-transfected cells, SHBs expression decreased by 60.0 ± 5.3% in the S1 siRNA co-transfected cells, and the expression of LEF-1 was reduced by 55.1 ± 9.0% (Fig. 2c, d).

To assess whether the induction of LEF-1 was SHBs-specific or simply because of overexpression of a secretary protein, HBeAg (another secretory viral protein) was transfected into HepG2 or Huh7 cells. No significant changes in LEF-1 mRNA levels were observed in HBeAg-overexpressing cells, which indicated that LEF-1 was induced specifically by SHBs (Fig. 3).

Lack of effects on cell proliferation in SHBs-expressing cells

LEF-1 has been reported to regulate transcription of its downstream effector genes, such as cyclin D1 and c-myc, which are involved in cell proliferation (Shutman et al., 1999; Tetsu & McCormick, 1999; Coyle-Rink et al., 2002). When the cell-proliferation efficiency of HepG2-S-G2 and HepG2-Neo-F4 cells was compared, no significant difference was found (Fig. 4a). The expression levels of c-myc and cyclin D1 in HepG2-S-G2 cells were studied by real-time PCR; no marked changes in the expression of these genes were detected in HepG2-S-G2 cells compared with HepG2-Neo-F4 cells (data not shown).

To evaluate the tumorigenesis of SHBs-expressing cells further, HepG2-S-G2 cells and HepG2-Neo-F4 cells (1.5 × 10^7 cells) were injected into nude mice (three mice in each group). Signs of tumour development were observed daily for 2 months. No tumours formed in either group, which implied no increased tumorigenesis in HepG2-S-G2 cells.

Cellular distribution pattern of LEF-1 is altered in SHBs-expressing cells

By immunofluorescence staining, a higher concentration of LEF-1 protein was detected in the cytoplasm of HepG2-S-G2 cells, whilst in HepG2-Neo-F4 cells, LEF-1 was located mainly in the nucleus (Fig. 4b). To verify this altered pattern of LEF-1 cellular distribution, cytoplasm and nuclei were isolated separately and levels of LEF-1 protein were determined by Western blot. Consistent with the results of the immunofluorescence staining, the LEF-1 protein level was increased markedly in the cytoplasm of HepG2-S-G2 cells compared with HepG2-Neo-F4 cells (Fig. 4c). Results revealed that, compared with HepG2-Neo-F4 cells, higher levels of LEF-1 isoforms were observed in the nucleus of HepG2-S-G2 cells, whereas full-length LEF-1 did not show any obvious change in the nucleus. This change in cellular distribution and expression pattern of LEF-1 isoforms in SHBs-expressing cells could probably lead to a change of function of the LEF-1 gene.

LEF-1 isoforms were induced in SHBs-expressing cells

Whilst no significant changes in cell cycle or cell proliferation were found in HepG2-S-G2 cells (Fig. 4a), the 55 kDa full-length LEF-1, 38 kDa LEF-1 dominant-negative isoform (LEF-1DN) and other truncated isoforms were all detected in the SHBs-expressing cells by Western blot (Fig. 4c). To assess the association between SHBs expression and LEF-1 isoforms, two pairs of primers were...
designed to detect the expression of LEF-1 isoforms in all four SHBs-expressing cell lines and four vector control lines by real-time PCR (Fig. 5b). Primers (P3 and P4) used in previous studies were targeted to the 3’ UTR of LEF-1 mRNA, and thus could detect both the full-length form and the isoforms. Another pair of primers (P1 and P2) was designed to target the β-catenin-binding domain (Fig. 5a) and could differentiate the 38 kDa LEF-1 DN from the 55 kDa full-length LEF-1. All four SHBs-expressing cell lines and four vector control cell lines were tested with these two pairs of primers to study the expression pattern of LEF-1. Results showed that the truncated isoform of LEF-1 was induced markedly in SHBs-expressing cells, whereas full-length LEF-1 did not show a significant change (Fig. 5b). A similar phenomenon was observed in HepG2.2.15 cells compared with HepG2 cells (Fig. 5c). Considering the suppressive effect of LEF-1DN on the Wnt pathway (Hovanes et al., 2001), these results implied functional suppression, rather than activation, of the Wnt pathway by SHBs expression.

**DISCUSSION**

HBsAg has been studied extensively with regard to its antigenicity, mutations, synthesis, secretion and binding to putative receptors. An epidemiological study has indicated that long-term persistence of HBsAg in chronic hepatitis B patients is a risk factor for the development of HCC (Lupberger & Hildt, 2007). In this study, we concentrated on studying the effects exerted by persistent SHBs expression on cell functions, and used a stable cell line that was transfected exclusively with the small S gene. To date, there have been reports on global gene-expression profiles in cells expressing full-length HBV, the HBV X gene and the pre-core/core gene (Wu et al., 2001; Ng et al.,

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**Table 2.** Up- and downregulation of 21 selected genes in cells expressing SHBs

Numbers represent fold changes in gene expression in HepG2-S-G2 cells compared with HepG2-Neo-F4 cells.

<table>
<thead>
<tr>
<th>Affymetrix probe set ID</th>
<th>Gene name</th>
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<th>Real-time PCR data*</th>
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<td></td>
<td></td>
<td>S-G2/Neo-F4</td>
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<td>Day 8</td>
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*Results shown are the mean of two independent assays.
2004; Locarnini et al., 2005; Nakanishi et al., 2005). To our knowledge, the data presented here are the first report of global gene-expression profiles of a cell line producing SHBs only. The global changes shown in this study provide important information for further studies on other aspects of HBsAg–host cell interactions (Table 1).

The upregulation of LEF-1, in association with SHBs expression, was not only shown in the HepG2-S-G2 stable cell line, but also observed in three other stably SHBs-expressing cell clones established in our laboratory and in HepG2.2.15 cells compared with their parental cell line, HepG2. These studies excluded the possibility of artefacts arising from a specific integration site or due to a specific HBV strain. In addition, the microarray studies were carried out in both the exponential phase and the stationary phase of HepG2-S-G2 cells, and results were confirmed by real-time PCR assays at both time points. Thus, the results obtained were reproducible.

To verify that upregulation of LEF-1 in HepG2-S-G2 cells was indeed associated with SHBs expression, a transient SHBs expression cell-culture system was used to study the association between SHBs expression and upregulation of LEF-1. The results obtained were in accordance with those observed with HepG2-S-G2 cells. The association between SHBs expression and upregulation of LEF-1 was further implied by inhibition of SHBs expression by siRNA, which reduced the expression of LEF-1 to the basal level. Furthermore, overexpression of HBeAg, another secretory viral protein, did not induce LEF-1 upregulation significantly, which confirmed the specific contribution of SHBs to the induction of LEF-1.

We focused on this gene because LEF-1 is one of the key components of the Wnt pathway and functions as a transcription factor belonging to the LEF/TCF family. The Wnt signalling pathway is involved in various differentiation events during development and leads to

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**Fig. 2.** Association between expression of SHBs and upregulation of LEF-1. Plasmids pCMV-S and pCMV-Script were transiently transfected into HepG2 or Huh7 cells. For SHBs inhibition experiments, siRNA was co-transfected with pCMV-S into Huh7 cells or transfected alone into PLC/PRF/5 cells. All experiments were carried out three times. (a) Expression of SHBs in culture medium of pCMV-S-transfected HepG2 cells and Huh7 cells. White bars, HepG2; black bars, Huh7. (b) Real-time PCR showing that LEF-1 expression was increased by 1.9 ± 0.4-fold in pCMV-S-transfected HepG2 cells and by 2.2 ± 0.1-fold in Huh7 cells. The concentration of LEF-1 mRNA in mock-transfected cells (ie. blank) was set arbitrarily as 1 unit, the concentrations of LEF-1 mRNA in other experimental groups were compared with that of the blank and the fold changes are expressed as unit values. White bars, HepG2; black bars, Huh7. (c) By blocking with S1 siRNA, HBsAg was reduced significantly in PLC/PRF/5 cells and transiently transfected Huh7 cells. White bars, Huh7; black bars, PLC/PRF/5. (d) Expression of LEF-1 was reduced by 40.6 ± 15.6 % in association with the inhibition of HBsAg in PLC/PRF/5 cells. Similar results are shown in transiently transfected Huh7 cells. The upregulation of the LEF-1 gene in SHBs-expressing Huh7 cells reverted to the normal level when the expression of SHBs was blocked. Units of LEF-1 expression are as described for (b). White bars, Huh7; black bars, PLC/PRF/5. ‘Blank’ indicates mock-transfected cells; ‘pCMV’ indicates pCMV-Script-transfected cells; ‘pCMV-S’ indicates SHBs-transfected cells; ‘Scr’ indicates scrambled control siRNA-transfected cells; ‘S1’ indicates HBsAg-specific siRNA-transfected cells.
carcinogenesis when it is activated aberrantly (Ganem & Prince, 2004). It was reported that mutations in the canonical Wnt pathway were the principal cause of 90% of all colorectal cancers, although found less frequently in cancers at other sites, such as breast cancer and HCC (de La Coste et al., 1998; Kato et al., 2001; Roberts & Gores, 2005; Lee et al., 2006). Wnt pathway activation can lead to translocation of unphosphorylated β-catenin into the nucleus, and binding of LEF-1 to the activated β-catenin promotes the expression of downstream effector proteins, such as cyclin D1 and c-myc (Eastman & Grosschedl, 2004).

**Fig. 3.** Association of LEF-1 induction and HBeAg expression. HBeAg was transiently transfected into HepG2 or Huh7 cells and LEF-1 induction was determined by real-time PCR. All experiments were carried out three times. (a) Expression of HBeAg in culture medium of HBeAg-transfected HepG2 cells and Huh7 cells. (b) LEF-1 expression did not show significant changes in HBeAg-transfected HepG2 cells and Huh7 cells. Units of LEF-1 expression are as described for Fig. 2(b). White bars, HepG2; black bars, Huh7. ‘Blank’ indicates mock-transfected cells; ‘pcDNA’ indicates empty vector pcDNA3.1-transfected cells; ‘HBeAg’ indicates HBeAg-overexpressing cells.

**Fig. 4.** Altered cellular distribution and expression pattern of LEF-1. (a) Cells (2 × 10⁴) were seeded into each well of a 96-well plate and a cell-proliferation assay was carried out as described in Methods. All experiments were carried out three times, each time in triplicate. The data were obtained from one experiment, as there was considerable variation in cell-culture conditions between experiments. No significant difference in cell proliferation was observed between HepG2-S-G2 cells and HepG2-Neo-F4 cells. The standard curve shows a linear correlation between cell number and OD₄₅₀. ■, HepG2-S-G2; □, HepG2-Neo-F4. (b) The cellular location pattern was altered in HepG2-S-G2 cells. LEF-1 was located in both the cytoplasm and the nucleus in HepG2-S-G2 cells, whereas in HepG2-Neo-F4 cells, LEF-1 was located predominantly in the nucleus. (c) Cell nuclei and cytoplasm were separated by cellular fractionation as described in Methods and levels of LEF-1 protein were determined by Western blotting. Lamin A/C is a marker of the nucleus used for normalization. GAPDH is a cytoplasmic marker for normalization.
The 3' UTR of LEF-1 was induced significantly (P<0.05, t-test) in four HBsAg-expressing cell clones compared with the four vector-control cell clones, whereas the β-catenin-binding domain of LEF-1 did not show a significant change (P>0.05, t-test) as measured by real-time PCR analysis. The housekeeping gene GAPDH was used as the internal control. The abundance of LEF-1 mRNA was determined as the ratio of LEF-1 mRNA to GAPDH mRNA. LEF-1 5' indicates the fragment containing the HMG DNA-binding domain. LEF-1 3' indicates the fragment containing the β-catenin-binding domain. LEF-1 5' were induced significantly in HepG2.2.15 cells compared with HepG2 cells, whereas the β-catenin-binding domain of LEF-1 did not show a significant change. GAPDH was used as the control.

To investigate mechanisms that might account for the discrepancy between upregulation of LEF-1 and the absence of changes in the transcriptional level of downstream genes and in the competence of cell proliferation, the cellular distribution and expression patterns of LEF-1 protein were studied. By immunofluorescence, LEF-1 was detected in both the cytoplasm and nucleus in HepG2-S-G2 cells, whereas it was located predominantly in the nucleus of the control HepG2-Neo-F4 cells. When full-length LEF-1 and truncated isoforms of LEF-1 in HepG2-S-G2 and control cells were compared, truncated isoforms (including the 38 kDa dominant-negative isoform) of LEF-1 were induced at a level higher than that of the 55 kDa full-length LEF-1 in HepG2-S-G2 cells. It was reported that the 55 kDa full-length LEF-1 were at higher risk of developing HCC than those who were only serum HBsAg-positive (Yang et al., 2002).
As SHBs is anchored in the endoplasmic reticulum (Clayton et al., 2001; Khan et al., 2004) and has not been shown to have a transcription-regulation activity, SHBs probably did not interact directly with LEF-1. Other cellular factors or viral proteins might also participate in this complex interaction. For instance, HBx has been reported to be essential for the activation of Wnt/β-catenin signalling in hepatoma cells (Cha et al., 2004), and reduced the phosphorylation level of β-catenin by suppressing GSK-3β function through the Erk pathway (Ding et al., 2005). The individual and combined effects of HBV proteins on host-cell functions can be explored by using models expressing one or more of the viral proteins, which may contribute to elucidation of the pathogenesis of HBV.

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REFERENCES


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