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Phyllanthus amarus suppresses hepatitis B virus by interrupting interactions between HBV enhancer I and cellular transcription factors

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Abstract. The *Phyllanthus amarus* plant suppresses HBV mRNA transcription *in vitro* and exhibits therapeutic potential in chronic HBV carriers, although further work is necessary to define its mechanism of action. Analysis in HuH-7 cells with transfected plasmids using a luciferase reporter showed that *P. amarus* specifically inhibited HBV enhancer I activity. To identify the mechanism of this HBV enhancer I inhibition, liver-enriched cellular transcription factors were co-expressed in HuH-7 cells. The C/EBPα and β, as well as HNF-3α and β transcription factors, significantly up-regulated the HBV enhancer I activity. In contrast, co-transfection of HNF-1α or β had no effect upon the HBV enhancer I activity. Exposure to *P. amarus* inhibited C/EBPα- and β-mediated up-regulation of HBV enhancer I activity in a dose-dependent manner, whereas HNF-3α- and β-mediated up-regulation of HBV enhancer I was unaffected. *In vitro* gel shifts showed that *P. amarus* inhibited complexing of C/EBP transcription factors to a consensus oligonucleotide sequence, whereas DNA binding of AP-1 and SP-1 transcription factors was unaffected. As *P. amarus* down-regulates HBV mRNA transcription by a specific mechanism involving interactions between HBV enhancer I and C/EBP transcription factors, purification and further analysis of the active *P. amarus* component will advance insights into its antiviral activity.

Keywords. Hepatitis B virus, liver, medicinal plant, treatment.

Introduction

Hepatitis B virus (HBV) afflicts approximately 300 million people worldwide, with the spectrum of liver disease encompassing hepatitis, cirrhosis and hepatocellular carcinoma. Despite significant mechanistic insights into HBV replication and gene expression, satisfactory therapies are lacking [1]. Several years ago, crude extracts of the *Phyllanthus amarus* plant were found to inhibit HBV polymerase activity and to clear HBsAg in chronic HBV carriers [2,3]. Our own studies using the HepG2 2.2.15 cells, which support HBV replication, and G26 HBV transgenic mice, which express HBsAg independently of other viral proteins or HBV replication, showed that *P. amarus* extracts decreased virion production and down-regulated HBsAg mRNA transcription, including suppression of the viral glucocorticoid-responsive element [4]. These findings raised important questions concerning whether *P. amarus* regulated the HBV enhancers and whether this was a direct vs. an indirect effect.

The HBV enhancer I (HBEnI) is the better characterized of the two known HBV enhancers and regulates all four open reading frames of the virus [5,6]. The HBEnI contains binding sites for multiple transcription factors, including AP-1, nuclear factor-1 (NF-1), hepatocyte nuclear factor (HNF)-3, CCAAT/enhancer-binding proteins (C/EBPs) and members of the steroid/thyroid nuclear receptor superfamily (e.g. HNF-4), but not for others, such as HNF-1 [7–10]. The presence of multiple transcription factor-binding domains in DNA sequences is most consistent with their regulatory roles as subunits, although the precise contribution of individual transcription factors is often difficult to determine because unique inhibitors are lacking. The hepatotropism of HBV is directed by cell surface receptors regulating viral entry and also by cellular transcription factors governing viral gene expression, because HBV expression is limited to specific epithelial cells in liver and other organs, such as the kidneys, pancreas and stomach [11,12]. As cellular transcription factors are developmentally regulated in specialized epithelia, the lineage relationship between cells and specific transcription factors probably determines the permissivity for HBV gene expression in tissues [13,14].

We hypothesized that the mechanism as to how *P. amarus* regulated the HBEnI could be determined in cell culture using plasmid transfection assays. As a paradigm, we cloned an expression plasmid containing the HBEnI and the pre-S1 HBV promoter. Additional promoters regulating other HBV genes, such as core and X genes,

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are also recognized, although the pre-S1 promoter exhibits significant tissue type specificity [15]. The goals of our studies were to define whether the down-regulatory effect of *P. amarus* was specific to the HBEnI, to examine whether *P. amarus* interfered with HBEnI and cellular transcription factor interactions and to demonstrate whether *P. amarus* exerted its activity by either interrupting transcription factor binding to HBEnI or decreasing the availability of specific transcription factors.

Materials and methods

P. amarus

Plants harvested in Tamilnadu, India, by S.P.T., were solubilized in dimethyl sulphoxide (DMSO) as reported previously [4]. Briefly, the plant (4 g) was crushed with a blender in 45 mL of deionized water and stirred overnight at room temperature. Undissolved material was pelleted in glass tubes at $1000 \times \boldsymbol{g}$ for 20 min at 25 °C and solubilized in 5 mL of DMSO (Sigma Chemical Co., St Louis, MO, USA). The supernatant and DMSO-solubilized material were pooled, diluted fourfold in normal saline, passed through a 0·45-μm filter and stored at 4 °C until use. The final extract was estimated to contain 5% DMSO and approximately 20 mg mL^{-1} *P. amarus.*

Cells

The HuH-7 cells were derived from a human hepatocellular carcinoma and belong to the hepatocyte lineage [16]. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. For analysis, cells were exposed to 50, 100 or 200 μg mL^{-1} *P. amarus* or to equivalent amounts of the vehicle, DMSO.

Expression plasmids

HBV luciferase plasmids. The plasmid, pCP10, which contains a head-to-tail HBV dimer [17], provided the sequence containing the HBV enhancer I (HBEnI, 912-bp *Bam*HI fragment spanning basepairs 490–1402) and the pre-S1 promoter (HBpreS1P, 514-bp *Bgl*II fragment spanning basepairs 2425–2839) for cloning into pGL2-luc vectors (Promega, Madison, WI, USA). The plasmids designated *pGL2.HBpreS1P/HBEnI-luc* and *pGL2.SV40P/HBEnI-luc* contained the HBEnI upstream to HBpreS1P or the simian virus 40 promoter (SV40P). The plasmid *pGL2.HBpreS1P/SV40En-luc* contained the SV40 enhancer (SV40En) upstream of HBpreS1P.

Control luciferase plasmids. The plasmid *pGL2.control* containing SV40 promoter and enhancer was a positive control, and the plasmid *pGL2.basic* with no regulatory elements was a negative control.

Other reporter plasmids. The plasmid palb-CAT contains a chloramphenicol acetyltransferase gene driven by the mouse albumin promoter and enhancer [18]. The plasmid pSVβgal contains the SV40 promoter and enhancer driving the *Escherichia coli lacZ* gene (Promega).

Transcription factors. Plasmids expressing HNF1α and β, HNF3α and β and C/EBPα and β were kindly provided by either Dr G. Crabtree, Stanford University or Dr R. Costa, University of Illinois [19,20]. The HNF-3α and β and C/EBP α and β plasmids were regulated by the CMV promoter and HNF1α and β by the SRα promoter.

Cell transfections

One day after plating 5×10^4 HuH-7 cells cm^{-2} in medium containing FBS, cells were washed twice with phosphate-buffered saline (PBS), pH 7·4, before adding 1 μg of plasmid cDNA ($\approx$ 90% supercoiled) and 5 μl of Lipofectin (Gibco BRL, Gaithersburg, MD, USA), which were preincubated in serum-free Optimem medium (Gibco BRL). Transcription factor plasmids and reporter plasmids were co-transfected in 1:5 or 1:10 ratios. After incubating cells with plasmids for 6 h, fresh RPMI-1640 medium containing *P. amarus* was switched and cells cultured for an additional 48 h before analysis. At least five separate experiments were performed for each condition.

Reporter assays

Luciferase activity. Cells were lysed and processed using a commercial kit (Promega). Cellular protein in the lysates was measured using the Bio-Rad assay (Bio-Rad, Hercules, CA, USA), and the results expressed as arbitrary light units mg^{-1} protein.

CAT activity. Transfected cells were lysed by three freeze–thaw cycles in 0·25 mol L^{-1} Tris-hydrochloride, pH 7·8. After pelleting cell debris, CAT activity was assayed in the supernatant as described previously [21].

LacZ activity. Cell monolayers were fixed with 0·5% glutaraldehyde in PBS and incubated for 6 h with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution at 37 °C [22]. Cells staining blue were counted in 10 randomly selected high-power fields and expressed as a percentage of the total cell count. LacZ activity was enzymatically assayed by incubating cell lysates in 120 mmol L^{-1} Na_2HPO_4, 80 mmol L^{-1} NaH_2PO_4, 2 mmol L^{-1} MgCl and 100 mmol L^{-1} β-mercaptoethanol with 1·33 mg mL^{-1} o-nitrophenyl β-D-galactopyranoside (Sigma) followed by spectrophotometry at 420 nm [23]. The data were normalized to total protein content in cell lysates.

Cell viability

The cell number and exclusion of 0·2% Trypan blue dye were manually determined using a haemocytometer. Use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

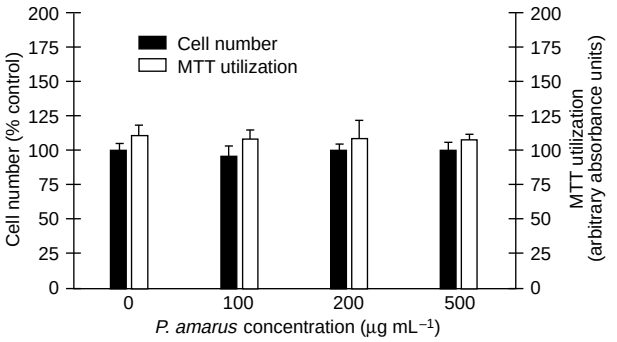

Figure 1. Effect of *P. amarus* upon cell viability. When HuH-7 cells were incubated with various concentrations (up to 500 μg mL^{-1}) of *P. amarus* for 72 h as shown here, or longer, there was no change in either the cell number or MTT utilization by cells, indicating an absence of cytotoxicity.

bromide dye (MTT) was analysed after exposing cells to 1 mg mL^{-1} MTT in phenol red-free minimal essential medium (MEM) for 90 min at 37 °C (Gibco BRL). The blue formazan product was solubilized in propanol and optical density measured at 560/690 nm [24].

Nuclear protein isolation and gel mobility shift assay

The probes were: C/EBP oligonucleotides, consensus, 5′-TGCAGATTGCGCAATCTGCA; mutant, 5′-TGCAGAGACTAGTCTCTGCA (Santa Cruz Biotechnology, Santa Cruz, CA, USA); AP-1 consensus oligonucleotide, 5′-CGCTTGATGAGTCAGCCGGAA; and SP-1 consensus oligonucleotide, 5′-ATTCGATCGGGGCGGGGCGAGC (Promega). The oligonucleotides were labelled with [γ-^{32}P]-ATP using polynucleotide kinase to specific activities of 5×10^{7} cpm μg^{-1} (Boehringer Mannheim, Indianapolis, IN, USA). Nuclear extracts were prepared from isolated rat hepatocytes by the method of Dignam *et al.* [25], and the concentration of nuclear proteins was measured using the Bio-Rad assay with bovine serum albumin standards. Gel shifts with AP-1 and SP-1 oligonucleotides used HeLa cell nuclear extracts (Promega). Radiolabelled oligonucleotides were incubated with 6 μg of nuclear extract each in the presence of 1 μg of poly[dI–dC] for 20 min at room temperature. DNA–protein complexes were resolved in 4% polyacrylamide gels in 0·5×Tris borate–EDTA buffer. After electrophoresis at 100 V for 2 h, the gel was dried and autoradiographed at −70 °C.

Statistical analysis

Data are expressed as means ± SEM with significance determined by Student's *t*-tests. $P<0{\cdot}05$ was considered significant.

Results

P. amarus *is non-toxic to cells*

To demonstrate whether changes in gene expression or regulation could simply be accounted for by cell toxicity,

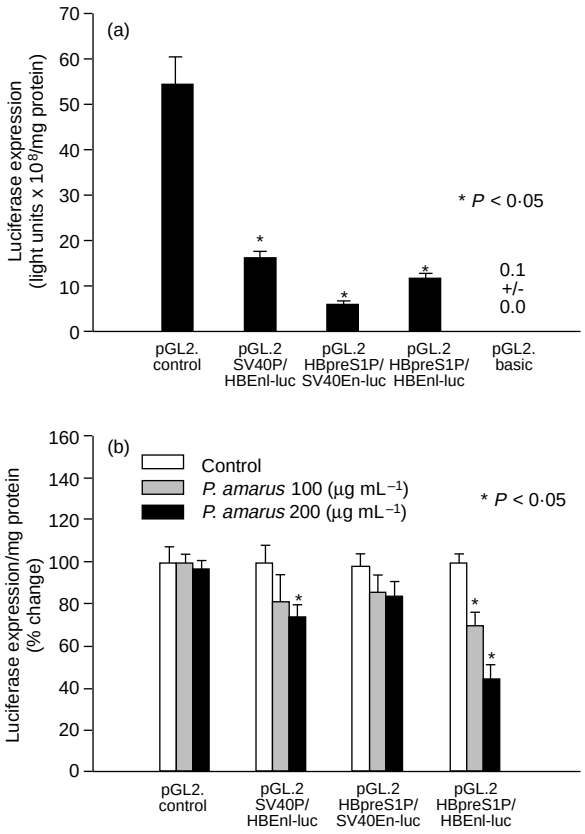

Figure 2. Gene expression in transient transfection assays. (A) Analysis of the relative transcriptional efficacy of the HBEnI and pre-S1 promoter and SV40 enhancer and promoter in HuH-7 cells showed that luciferase activity was most efficiently expressed by the homologous SV40 regulators (*pGL2.control* plasmid), followed by the heterologous unit containing the HBEnI and SV40 promoter, which was several-fold more potent than the *pGL2.HBpreS1P/HBEnI* sequence. The SV40 enhancer and pre-S1 promoter combination was the least efficient and *pGl.2.basic* devoid of any promoters showed no luciferase expression. (B) In the presence of *P. amarus*, luciferase expression driven by the pre-S1 promoter/HBEnI combination was most affected, with suppression by up to 50%. *P. amarus* also suppressed luciferase expression driven by the SV40 promoter and HBEnI combination, although the magnitude of suppression was less. In contrast, the pre-S1 promoter/SV40 enhancer containing plasmid or the pGL2.control plasmid were unaffected by *P. amarus.*

HepG2 2.2.15 cells were incubated for 72 h with medium containing 100, 200 or 500 μg mL^{-1} *P. amarus* (Fig. 1). The cell viability was also unchanged in response to *P. amarus* treatment in additional experiments conducted after exposing cells to up to 500 μg mL^{-1} *P. amarus* for 5 days, along with unchanged cell morphology (not shown). In addition, *P. amarus* did not change *lacZ* expression after transfection of HuH-7 cells with pSVβgal, which resulted in similar numbers of X-gal-positive blue cells in control and test conditions (2·4 ± 0·4% vs. 2·2 ± 0·5%, P = NS). The *lacZ* activity measured by enzymatic assays was also similar in cells exposed to 500 μg mL^{-1} *P. amarus* (control, 0·13 ± 0·01 lacZ units mg^{-1} protein h^{-1}; test, 0·10 ± 0·01 lacZ units mg^{-1} protein h^{-1}, P = NS). Use of more than one parameter for cell viability provided rigorous evidence of no

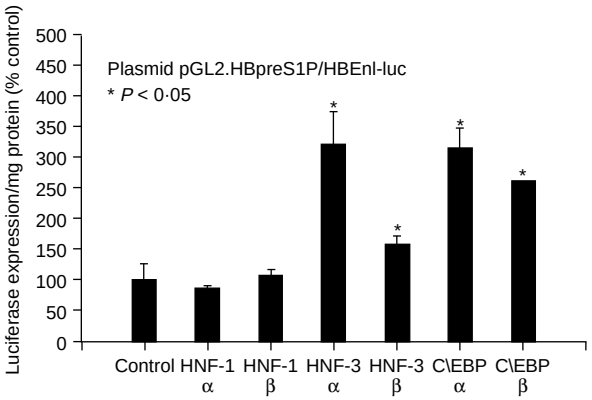

Figure 3. Regulation of HBEnI activity by cellular transcription factors. Luciferase activity 48 h after transfection of the *pGL2.HBpreS1P/HBEnI-luc* in HuH-7 cells was $1{\cdot}1 \pm 0{\cdot}3 \times 10^8$ light units mg^{-1} protein (100%). The *pGL2.HBpreS1P/HBEnI-luc* plasmid was maximally up-regulated by HNF-3α ($323 \pm 51\%$ of control), followed by C/EBPα ($314 \pm 33\%$), C/EBPβ ($260 \pm 0\%$) and HNF-3β ($158 \pm 14\%$), whereas neither HNF-1α nor β showed any up-regulation.

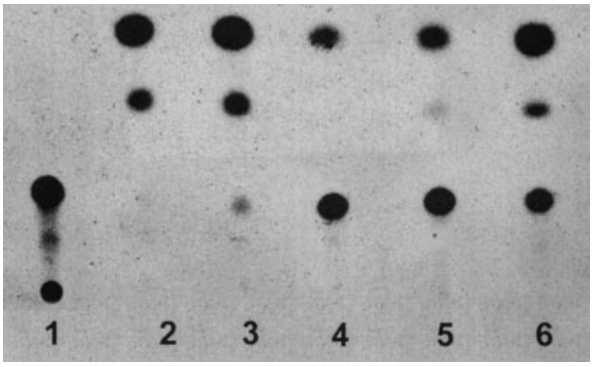

Figure 4. Regulation of the mouse albumin promoter and enhancer in HuH-7 cells. The cells were transfected with the palb.CAT plasmid, and CAT activity was assayed as described in Materials and methods after culture for 48 h. Lane 1, chloramphenicol alone; lane 2, co-transfection with HNF-1β in 1:5 molar ratio; Lane 3, co-transfection with HNF-1β at a 1:10 molar ratio; lane 4, palb.CAT alone; lane 5, co-transfection with HNF-3β at 1:10 molar ratio; and Lane 6, co-transfection with C/EBPα at molar ratio of 1:10. Note that gene expression was up-regulated by co-transfection with HNF-1 and C/EBP plasmids.

change in cell membrane integrity, metabolic capacity and transcriptional activity after exposure to *P. amarus.* Our previous studies showed that *P. amarus* did not interfere with cell proliferation mechanisms during log-phase cell growth [4].

P. amarus *shows specificity for HBEnI compared with SV40 enhancer in HuH-7 cells*

The SV40 enhancer and promoter most efficiently expressed luciferase, followed by the HBEnI and SV40 promoter combination, which was several-fold more potent than the HBEnI and pre-S1 promoter combination, as well as the SV40 enhancer/pre-S1 promoter, although the activities of the last two combinations were also readily apparent (Fig. 2A). The findings indicated that homologous regulatory sequences were more effective than heterologous ones and that the SV40 sequences were more potent than the HBV sequences in HuH-7 cells. Vehicle alone containing equivalent concentrations of DMSO, by itself, had no effect upon the expression of reporter plasmids (data not shown).

P. amarus dose dependently inhibited HBEnI and pre-S1 promoter by up to approximately 50% of the controls, $P < 0{\cdot}001$ (Fig. 2B). In addition, *P. amarus* significantly suppressed the HBEnI/SV40 promoter combination ($P < 0{\cdot}03$), although the magnitude of suppression was less, most possibly because of differences in the regulation of pre-S1 and SV40 promoters. In contrast, luciferase plasmids driven by the SV40 enhancer were unaffected by *P. amarus*. These results demonstrated that the down-regulatory effect of *P. amarus* was specific to the HBEnI, as the SV40 sequences or the HBV pre-S1 promoter were spared. However, whether *P. amarus* affected the HBV sequences alone, altered the binding of cellular transcription factors with HBV sequences or decreased the availability of cellular transcription factors required further studies.

Interactions between HBV enhancer, cellular transcription factors and P. amarus

To test the hypothesis that *P. amarus* inhibited HBEnI by modulating transcription factor interactions, co-transfection experiments with *pGL2.HBpreS1P/HBEnI-luc* and HNF-3 α or β or C/EBP α or β were performed in HuH-7 cells. The ability of these transcription factor plasmids, particularly HNF3 α and C/EBPs, which are liver enriched, to up-regulate the HBV enhancer I activity was clearly shown (Fig. 3). HNF-1 α and β had no significant effect upon luciferase activity, although a specific binding site is thought to exist in the pre-S1 promoter sequence cloned in our *pGL2.HBpreS1P/HBEnI-luc* plasmid [26]. To eliminate the possibility that our HNF-1 plasmid was not functionally intact in HuH-7 cells, we analysed up-regulation of the mouse serum albumin promoter and enhancer, which contain HNF-1-binding sites [27]. When the palb-CAT plasmid was co-transfected with transcription factor plasmids, the mouse serum albumin enhancer and promoter were up-regulated by HNF-1, as well as HNF-3α and C/EBPα (Fig. 4).

The addition of *P. amarus* to HuH-7 cells transfected with *pGL2.HBpreS1P/HBEnI-luc* plasmid effectively suppressed the HBEnI activity to only $20 \pm 4\%$ of controls with a dose of 200 $\mu g\,mL^{-1}$ (Fig. 5). Moreover, *P. amarus* also markedly inhibited the up-regulation of HBEnI activity by co-expressed C/EBPα or β transcription factors. In fact, the effect of *P. amarus* upon C/EBP-mediated HBEnI up-regulation was selective because *P. amarus* decreased HBEnI activity only marginally when the *pGL2.HBpreS1P/HBEnI-luc* plasmid was co-transfected with either HNF3α or β. These findings suggested that C/EBPs play a predominant role in regulating the HBEnI, which would also be consistent with the epithelial cell-type restriction of HBV expression, as shown previously [12]. We expected no change in HuH-7 cells

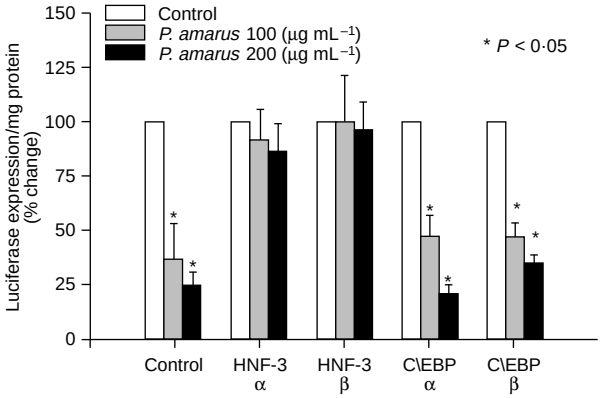

Figure 5. Effect of *P. amarus* upon the regulation of HBEnI by cellular transcription factors. C/EBPα and β and HNF-3α and β were co-expressed along with *pGL2.HBEn/HBsP1-luc* plasmid in HuH-7 cells. *P. amarus* inhibited luciferase expression by the plasmid without co-transfection of transcription factors to $37 \pm 16\%$ and $25 \pm 6\%$ of controls respectively, $P < 0{\cdot}001$. *P. amarus* also significantly inhibited the expression of *pGL2.HBEn/HBsP1-luc* plasmid when co-transfected with C/EBPβ to $47 \pm 10\%$ ($100\,\mu g\,mL^{-1}$) or $20 \pm 4\%$ ($200\,\mu g\,mL^{-1}$), and in cells co-transfected with C/EBPβ to $46 \pm 7\%$ ($100\,\mu g\,mL^{-1}$) or $34 \pm 4\%$ ($200\,\mu g\,mL^{-1}$) of controls. In contrast, *P. amarus* did not significantly down-regulate luciferase expression upon co-transfection with either HNF-3α or HNF-3β plasmids.

exposed to *P. amarus* after co-transfection with *pGL2.HBpreS1P/HBEnI-luc* and HNF-1 plasmids, as the latter were ineffective in up-regulating HBEnI. Although these experiments clearly demonstrated that *P. amarus* could interfere with interactions between HBV and cellular transcription factors, it was unclear whether the mechanism involved the occupation of DNA-binding sites by *P. amarus* components or the decreased availability of transcription factors, e.g. by degradation or inactivation, transcriptional down-regulation or altered post-transcriptional processing. One way to investigate these possibilities was to perform gel shift assays to show whether, in the presence of preformed transcription factors, *P. amarus* interfered with transcription factor binding to specific consensus DNA sequences. The general principle is that the electrophoretic mobility of DNA is retarded by complexing with protein.

P. amarus *inhibited binding of C/EBPs to a consensus sequence*

Gel-shift assays used nuclear extracts from hepatocytes, which contain abundant C/EBPs, or HeLa cells, which offer an excellent system for analysing general transcription factor activity, including for AP-1 and SP-1. The assays were performed in the presence of either *P. amarus* or the vehicle alone. The studies showed that C/EBPs in hepatic extracts could no longer bind and shift their consensus DNA sequence in the presence of *P. amarus* (Fig. 6A). This effect of *P. amarus* was dose dependent. In contrast, there was no C/EBP binding to a mutant oligonucleotide, which served as a negative control.

Finally, assays were performed to determine whether

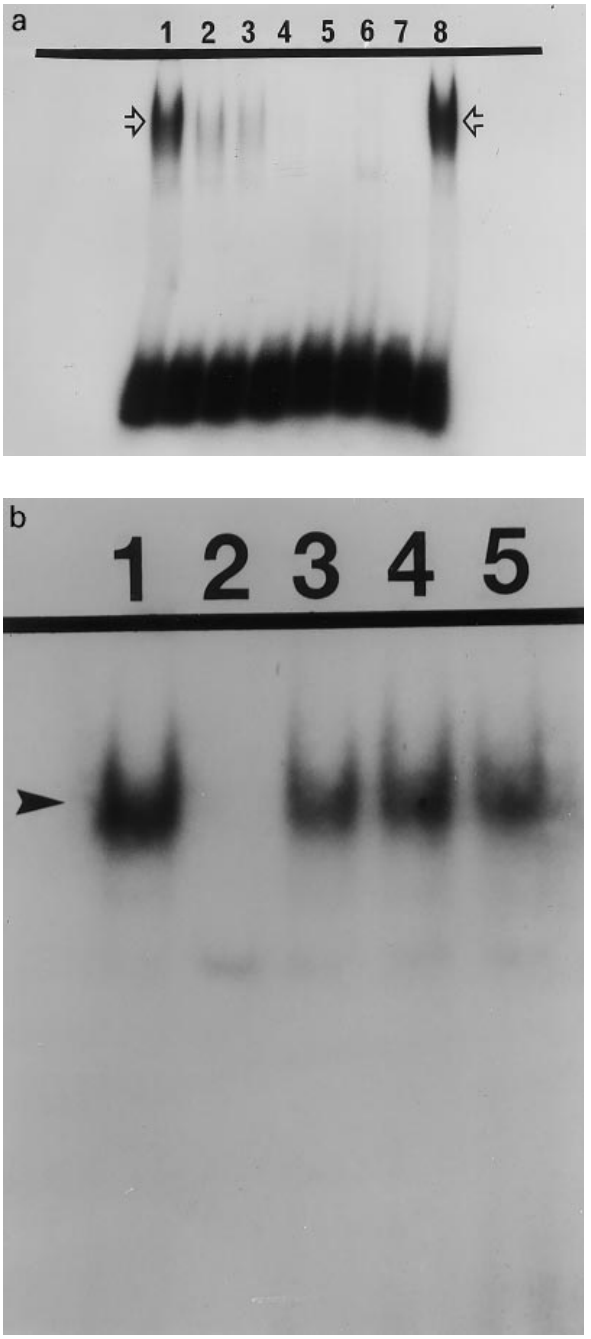

Figure 6. Mobility shift assay showing interference by *P. amarus* of DNA binding. (A) Gel shift assays showing inhibition by *P. amarus* of C/EBP binding in the presence of rat hepatocyte nuclear extracts. Lanes 1–5, 2, 10, 50, 100, and $200\,\mu g\,mL^{-1}$ *P. amarus* extract respectively; lane 6, mutated C/EBP binding consensus sequence served as a negative control; lane 7, consensus sequence alone also served as a negative control; lane 8, vehicle (0·05% DMSO) alone. *P. amarus* in concentrations of $10\,\mu g\,mL^{-1}$ or more inhibited C/EBP binding. (B) Gel shift assays showing the absence of inhibition by *P. amarus* of non-specific DNA binding using general transcription factors. Lane 1, AP-1 consensus sequence incubated with HeLa cell extract in the presence of 0·05% DMSO; lane 2, AP-1 consensus sequence alone serving as a negative control; lanes 3–5, 200, 100 and $50\,\mu g\,mL^{-1}$ *P. amarus* extract respectively. *P. amarus* inhibited C/EBP binding but had no effect upon either AP-1 binding or SP-1 binding (not shown).

the inhibitory effect of *P. amarus* was specific for C/EBPs. When binding of AP-1 or SP-1 to their consensus DNA sequences was tested with HeLa cells extracts, gel shifts were readily observed (Fig. 6B). AP-1 is an ubiquitous transcription factor and represents a heterodimer of the early activated cell cycle-regulated c-*fos*/c-*jun* gene product. SP-1 is also extensively distributed, with binding domains present on a wide variety of cellular and viral promoter elements, although its regulatory role may be dependent upon the presence or absence of additional transcription factor subunits. However, *P. amarus* showed no effect upon the binding of either AP-1 or SP-1, which was further consistent with a specific effect of *P. amarus* upon C/EBP binding.

Discussion

The data demonstrate in several ways that *P. amarus* down-regulates HBV by specifically inhibiting HBEnI. The initial evidence of such an inhibitory activity in *P. amarus* was generated by our experiments using HepG2 2.2.15 cells, which support HBV replication [28]. In these cells, exposure to *P. amarus* resulted in decreased steady-state HBV mRNA levels and abolition of dexamethasone-mediated up-regulation in HBV mRNA transcription, indicating an inhibitory effect upon the glucocorticoid-responsive element of the viral enhancer [4,29]. Secondly, our data here show that *P. amarus* inhibited HBEnI activity in transient plasmid-based expression systems, but did not affect the SV40 enhancer in independent reporter systems. Finally, we found that *P. amarus* interfered with the regulatory axis between HBEnI and cellular transcription factors by inhibiting DNA binding. Despite our use of crude *P. amarus* in enormous concentrations, there was no evidence of cell toxicity, as shown by Trypan blue dye exclusion, cell number changes or MTT utilization.

The relative specificity of the inhibitory effect of *P. amarus* on HBEnI activity may be caused, at least partly, by cell type-specific mechanisms regulating the HBEnI enhancer in liver cells [12]. Although the SV40 enhancer, as well as some other viral regulators, are broadly active in a variety of cell types, the HBEnI is extinguished in cells of non-epithelial origin and even in permissive epithelial cells, such as hepatocytes, renal tubular cells, gastric epithelial cells, pancreatic acinar cells, etc., significant differences are apparent in the magnitude of gene expression. In transient cell transfection assays, the HBEnI was appropriately regulated by HNF-3α and C/EBPα transcription factors in hepatocytes but not in non-parenchymal epithelial liver cells or fibroblasts, which indicates that transcription factors subserve critical roles in regulating the HBEnI activity. Our data suggest that, among various transcription factors capable of regulating HBV, C/EBPs are dominant. The C/EBPs, which are enriched in hepatocytes and participate in terminal differentiation programmes [14], serve as important transcriptional modulators [30]. Proteins of the C/EBP family accumulate preferentially in non-mitotic, terminally differentiated liver cells and regulate multiple liver-specific genes, including the serum albumin gene. This might well be why HBV is expressed in cells of epithelial origin in various organs that are presumably permissive for C/EBP activity [12].

Mapping of the pre-S1 promoter has shown binding sites for HNF-1, as well as HNF-3, which enhance its activity in hepatocytes [31,32], although we were unable to show an up-regulatory effect of HNF-1 upon our *pGL2.HBpreS1P/HBEnI-luc* plasmid. Our findings here are in agreement with non-deleterious down-regulation by *P. amarus* of serum albumin mRNA levels in HepG2 2.2.15 cells [4], which also probably resulted from interference with cellular transcription factor-mediated up-regulation, because the serum albumin gene is regulated by HNF-1, C/EBPs and other transcription factors [27]. Interestingly, *P. amarus* did not influence transcriptional regulation of the HBEnI by HNF-3α and β transcription factors, which indicated selectivity of the drug. However, the HNF-3s were less potent than C/EBPs in regulating the HBEnI because *P. amarus* did not affect HNF-3-mediated up-regulation of the viral enhancer but suppressed enhancer activity by virtually up to 70% of the controls, presumably through its inhibitory effects upon C/EBPs. It was helpful to show that interference by *P. amarus* of C/EBP binding to DNA was a specific effect because *P. amarus* did not interfere with the binding of SP-1 and AP-1 transcription factors. The HBEnI does contain an AP-1-binding site, but our data suggest that AP-1 must not play a significant modulatory role in HBV expression because *P. amarus* did not affect its binding to DNA and yet markedly decreased HBEnI activity. Similarly, the SP-1 transcription factor binds to a variety of viral and cellular promoters, but *P. amarus* had no effect on altering its DNA-binding capacity. Such clear demonstrations of transcription factor interactions *in vivo* have not been possible previously because specific inhibitors were not available. Therefore, abrogation by *P. amarus* of C/EBP binding to DNA offers specially intriguing possibilities for further dissection of general transcriptional mechanisms, as well as the regulation of HBV.

We excluded inadequate intracellular expression of the transfected HNF-1 plasmid in our system, because the HNF-1α and β plasmids could up-regulate the mouse serum albumin promoter/enhancer in HuH-7 cells. The HBEnI contains additional transcription factor-binding domains, such as the one capable of binding a liver-specific factor and a ubiquitous transcription factor [32], which is believed to be EF-C or RFX1 belonging to a dimerizing family of nuclear proteins [33]. These too were less important in HuH-7 cells. The gel shift assays strongly suggested to us that *P. amarus* blocked the availability of DNA-binding sites to C/EBPs. Such a mechanism will also help to reconcile why *P. amarus* has been found to suppress HBV DNA polymerase activity *in vitro* [2,4]. Our *in vitro* HBV polymerase assays were based upon the principle of primer extension using virion particles containing the entire genome [4]. If the primer DNA sequences were rendered unavailable after binding of *P. amarus* to HBV DNA, the assay would show

inhibition of DNA polymerase activity. This, rather than enzymatic mechanisms, most probably reconcile our findings concerning the inhibition of the HBEnI, as well as viral polymerase activity, because *P. amarus* remains active despite treatments, such as extensive heating, that eliminate the activity of most proteins, although further work will be necessary to prove this possibility. Of course, inhibition of the HBEnI may also down-regulate polymerase gene transcription and decrease viral replication *in vivo*.

In view of the ubiquitous regulatory roles of the HBEnI, inhibition by *P. amarus* of the enhancer should profoundly affect the viral life cycle. We believe that the findings have important translational connotations. In chronic HBV carriers, liver injury occurs during periods of viral replication or reactivations, during which specific viral proteins induce a host immune response [35,36]. If the viral load in treated patients were to diminish progressively or an imbalance in viral gene expression altered tolerance [37], the spontaneous rates of HBV clearance could possibly be augmented. Because effective treatments against HBV are lacking, the antiviral potential of *P. amarus* needs further attention and analysis. Our *in vitro* assays shall greatly facilitate the isolation of the active *P. amarus* component that interferes with HBV transcription. Purification of the active ingredient is necessary for the systematic and accurate analysis of the antiviral potential of *P. amarus.*

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References

1 Perrillo RP, Mason AL. Therapy for hepatitis B virus infection. Gastroenterol Clin North Am 1994;23:581–601.
2 Venkateswaran PS, Millman I, Blumberg BS. Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis B virus: *in vitro* and *in vivo* studies. Proc Natl Acad Sci USA 1987;84:274–8.
3 Thyagarajan SP, Subramanian S, Thirunalasundari T, Venkateswaran PS, Blumberg BS. Effect of *Phyllanthus amarus* in chronic carriers of hepatitis B virus. Lancet 1988;2:764–6.
4 Lee C-D, Ott M, Thyagarajan SP, Shafritz DA, Burk RD, Gupta S. *Phyllanthus amarus* downregulates hepatitis B virus mRNA transcription and replication. Eur J Clin Invest 1996;26:1069–1076.
5 Su H, Yee JK. Regulation of hepatitis B virus gene expression by its two enhancers. Proc Natl Acad Sci USA 1992;92:2708–712.
6 Antonucci TK, Rutter WJ. Hepatitis B virus (HBV) promoters are regulated by the HBV enhancer in a tissue-specific manner. J Virol 1989;63:579–83.
7 Ben-Levy RO, Faktor O, Berger I, Shaul Y. Cellular factors that interact with the hepatitis B virus enhancer. Mol Cell Biol 1989;9:1804–9.
8 Dikstein R, Faktor O, Shaul Y. Hierarchic and cooperative binding of the rat liver nuclear protein C/EBP at the hepatitis B virus enhancer. Mol Cell Biol 1990;10:4427–30.
9 Garcia AD, Ostapchuk P, Hearing P. Functional interaction of nuclear factors EF-C, HNF-4, and RXR alpha with hepatitis B virus enhancer I. J Virol 1993;67:3940–50.
10 Chen M, Hieng S, Qian X, Costa R, Ou JH. Regulation of hepatitis B virus ENI enhancer activity by hepatocyte-enriched transcription factor HNF3. Virology 1994;205:127–132.
11 Ishikawa T, Ganem D. The pre-S domain of the large viral envelope protein determines host range in avian hepatitis B viruses. Proc Natl Acad Sci USA 1995;92:6259–63.
12 Aragona E, Burk RD, Ott M, Shafritz DA, Gupta S. Cell-type specific mechanisms regulate hepatitis B virus transgene expression in liver and other organs. J Pathol 1996;180:441–9.
13 Kuo CJ, Conley PB, Chen L, Sladek FM, Darnell JE, Crabtree GR. A transcriptional hierarchy involved in mammalian cell-type specification. Nature 1992;355:457–61.
14 Vellanoweth RL, Suprakar PC, Roy AK. Transcription factors in development, growth, and aging. Lab Invest 1994;70:784–99.
15 Gupta S, Shafritz DA. Molecular biology of the hepatitis B virus. In: Walker WA, Durie PR, Hamilton JR, Walker-Smith JA, Watkins JB, eds. Pediatric gastrointestinal disease. pathophysiology, diagnosis, management. Philadelphia: BC Decker, 1991, 874–90.
16 Nakabayashi H, Taketa K, Miyano M, Yamane T, Sato J. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res 1982;42:3858–63.
17 Elfassi E. Broad specificity of the hepatitis B virus enhancer. Virology 1987;160:259–62.
18 Wu CH, Wilson JM, Wu GY. Targeting genes: delivery and persistent expression of a foreign gene driven by mammalian regulatory elements *in vivo*. J Biol Chem 1989;264:16985–7.
19 Mendel DB, Khavari PA, Conley PB *et al.* Characterization of a cofactor that regulates dimerization of a mammalian homeodomain protein. Science 1991;254:1762–7.
20 Samadani U, Porcella A, Pani L *et al*. Cytokine regulation of the liver transcription factor hepatocyte nuclear factor-3 beta is mediated by the C/EBP family and interferon regulatory factor 1. Cell Growth Diff 1995;6:879–90.
21 Gorman CM, Moffat LF, Howard BH. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 1982;2:1044–51.
22 Gupta S, Vemuru RP, Yerneni PR, Jagtiani RK, Roy Chowdhury N, Shafritz DA, Burk RD, Roy Chowdhury J. Hepatocyte transplantation: Prolonged cell survival following ex-vivo manipulation and release from culture matrix components. Transplant Proc 1993;25:2370–2374.
23 Rosenthal N. Identification of regulatory elements of cloned genes with functional assays. Methods Enzymol 1987;152:704.
24 Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986;89:271–7.
25 Dignam JD, Lebovitz RM, Roeder RG. Eukaryotic gene transcription with purified components. Nucleic Acid Res 1983;11:1475–89.
26 Chang HK, Wang BY, Yuh CH, Wei CL, Ting LP. A liver-specific nuclear factor interacts with the promoter region of the large surface protein gene of human hepatitis B virus. Mol Cell Biol 1989;9:5189–97.
27 Wu KJ, Wilson DR, Shih C, Darlington GJ. The transcription factor HNF1 acts with C/EBP alpha to synergistically activate the human albumin promoter through a novel domain. J Biol Chem 1994;269:1177–82.
28 Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci USA 1987;84:274–8.
29 Tur-Kaspa R, Burk RD, Shaul Y, Shafritz DA. Hepatitis B virus DNA contains a glucocorticoid responsive element. Proc Natl Acad Sci USA 1986;83:1627–31.
30 Raney AK, Easton AJ, McLachlan A. Characterization of the minimal elements of the hepatitis B virus large surface antigen promoter. J Gen Virol 1994;75:2671–9.
31 De-Medina T, Faktor O, Shaul Y. The S promoter of hepatitis B virus is regulated by positive and negative elements. Mol Cell Biol 1988;8:2449–55.
32 Gustin K, Shapiro M, Lee W, Burk RD. Characterization of the role of individual protein binding motifs within the hepatitis B virus enhancer I on X promoter activity using linker scanning mutagenesis. Virology 1993;193:653–60.

33 Reith W, Ucla C, Barras E *et al*. RFX1, a transactivator of hepatitis B virus enhancer I, belongs to a novel family of homodimeric and heterodimeric DNA-binding proteins. Mol Cell Biol 1994;14:1230–44.
34 Pei DQ, Shih CH. Transcriptional activation and repression by cellular DNA-binding protein C/EBP. J Virol 1990;64:1517–22.
35 Liaw YF, Tai D-I, Chu CM, Chen T-J. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology 1988;8:493–6.
36 Cerny A, Fowler P, Brothers MA, Houghton M, Schlicht HJ, Chisari FV. Induction *in vitro* of a primary human antiviral cytotoxic T cell response. Eur J Immunol 1995;25:627–30.
37 Nayersina R, Fowler P, Guilhot S *et al*. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. J Immunol 1993;150:4659–71.