CREB/PKA sensitive signalling pathways activate and maintain expression levels of the hepatitis B virus pre-S2/S promoter

F Tacke, C Liedtke, S Bocklage, M P Manns, C Trautwein

Background and aims: CREB (cAMP response element binding protein) transcription factors are key regulators of homeostatic functions in the liver, and CRE binding is increased in hepatic inflammation. During chronic hepatitis B virus (HBV) infection, mutations or deletions in the pre-S region are frequently observed. These mutations can affect the pre-S2/S promoter controlling HBV envelope protein expression (hepatitis B surface antigen (HBsAg)) and have been associated with worsened clinical outcome. We aimed to test if CREB activation impacts on HBsAg expression.

Methods: The effect of the CREB inducer protein kinase A (PKA) was tested by coexpression with HBV wild-type vector in vitro. Luciferase reporter gene constructs were cloned to identify novel regulatory regions for the HBV pre-S2/S promoter. Electrophoretic mobility shift assay (EMSA) gelshift and supershift experiments were conducted to confirm DNA transcription factor binding.

Results: Coexpression of HBV and PKA resulted in HBV-S mRNA induction and enhanced small envelope protein expression. We identified a CREB binding motif in the transcribed part of the pre-S2 region, contributing to basal S promoter activity via binding of activating transcription factor 2 (ATF2). A second CREB motif closely linked to the S-ATG showed a similar binding pattern involving ATF2 and CREB1, without appearing essential for basal promoter activity. Moreover, a sequence in the pre-S2 region is responsible for further transcriptional induction via CREB activators such as PAK and forkolin. EMSA experiments indicate that CREB1 and ATF4 are involved in complex formation conferring PAK dependent promoter activation.

Conclusions: Our data suggest a novel mechanism by which HBV may utilise CREB/PKA signal transduction pathways of hepatocytes to enhance its HBsAg expression during homeostasis and hepatic inflammation.
extremely high levels. They are not only essential for virion formation and part of the mature virion, but are also released as spheroid or filamentous subviral particles alone. 26 SHBs carry the most important antigenic epitope in the immuno-dominant “a-determinant” loop, and excess release of HBsAg particles has been associated with viral persistence as it may allow escape of the humoral anti-HBs response. 27

In this study, our aim was to test if CREB transcription factors enhance HBs expression. Coexpression of HBV wild-type plasmid and the CREB activator PKA increased HBs transcript and protein expression in vitro. Further analysis led to characterisation of CREB binding motifs in the transcribed pre-S2 region supporting basal pre-S2/S promoter activity by binding of CREB1/activating transcription factor 2 (ATF2) and of an Ets-like binding motif mediating transcriptional activation to CREB and its activators PKA and forskolin.

METHODS

Cell culture and transfection experiments

Human Huh7 hepatoma cells, shown to be negative for any HBV marker, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum under 5% CO2 at 37°C. DNA was transfected using the calcium phosphate coprecipitation method. 28 To test the potential effect of CREB/PKA on HBV-S expression, cotransfection experiments of 3 μg of replicon competent HBV wild-type 1.28 plasmid (genotype A, subtype adw2) and increasing concentrations of PKA expression plasmid were performed. 31

Northern and western blot analysis

Extracts were collected 48 and 72 hours after transfection, and 34 Northern and western blot analysis

1.28 plasmid (genotype A, subtype adw2) and increasing concentrations of PKA expression plasmid were performed. 31

Cloning of luciferase expression plasmids, luciferase, and beta-galactosidase reporter assays

A computer based search for potential transcription factor binding sites was performed using MatInspector software. 35 For construction of luciferase reporter plasmids, predicted promoter sequences were amplified by polymerase chain reaction (PCR) (Hotstart Herculease; Stratagene) using the HBV 1.28 wild-type plasmid as a template. 36 Oligonucleotides used for PCR (table 1) were purchased from MWG Biotech (Ebersberg, Germany) and flanked by XhoI and SacI restriction sites, respectively.

PCR fragments were cloned into the XhoI and SacI sites of the pGL3-basic vector (Promega, Madison, Wisconsin, USA). Constructs were checked by direct sequencing using an ABI prism 310 (Applied Biosystems Perkin Elmer, Foster City, California, USA) and the primer GL1 (5’TGT ATC TTA TGG TAC TGT AAC TG 3’).

For analysis of reporter gene expression, cells were harvested 24 hours after transfection. Luciferase activity and β-galactosidase activity (for transfection efficiency control) were measured from cellular extracts, as previously described. 37 38 Luciferase activity was assessed relative to β-galactosidase activity to normalise for variations in transfection efficiency. All transfection experiments were carried out in triplicate.

Cloning of luciferase expression plasmids, luciferase, and beta-galactosidase reporter assays

Table 1

<table>
<thead>
<tr>
<th>Primer sequences used for construction of luciferase expression constructs</th>
<th>5’-CAGATGGACACTCTAACCCCCCTGC-3’</th>
<th>5’-CGTCGGGATCCCTACAGG-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- as</td>
<td>5’-TACGAGGGAAGCCAAATGCGGAGCC-3’</td>
<td>5’-CAGATGGACACTCTAACCCCCCTGC-3’</td>
</tr>
<tr>
<td>2- as</td>
<td>5’-TACGAGGGAAGCCAAATGCGGAGCC-3’</td>
<td>5’-CGTCGGGATCCCTACAGG-3’</td>
</tr>
<tr>
<td>3- as</td>
<td>5’-TACGAGGGAAGCCAAATGCGGAGCC-3’</td>
<td>5’-CAGATGGACACTCTAACCCCCCTGC-3’</td>
</tr>
<tr>
<td>4- as</td>
<td>5’-TACGAGGGAAGCCAAATGCGGAGCC-3’</td>
<td>5’-CGTCGGGATCCCTACAGG-3’</td>
</tr>
<tr>
<td>5- as</td>
<td>5’-CACTGACAGATCCGGCAAGATGCT-3’</td>
<td>5’-CAGATGGACACTCTAACCCCCCTGC-3’</td>
</tr>
<tr>
<td>6- as</td>
<td>5’-TACGAGGGAAGCCAAATGCGGAGCC-3’</td>
<td>5’-CGTCGGGATCCCTACAGG-3’</td>
</tr>
<tr>
<td>7- as</td>
<td>5’-TACGAGGGAAGCCAAATGCGGAGCC-3’</td>
<td>5’-CAGATGGACACTCTAACCCCCCTGC-3’</td>
</tr>
<tr>
<td>8- as</td>
<td>5’-TACGAGGGAAGCCAAATGCGGAGCC-3’</td>
<td>5’-CGTCGGGATCCCTACAGG-3’</td>
</tr>
</tbody>
</table>

Mutated (mut) sequences are underlined.
Supershift experiments were performed as described using antibodies against CREB1, ATF1, ATF2, ATF4, activator protein 1 (AP-1), and Ets-1/2 family members (all Santa Cruz Biotechnology). For the supershift experiments with the Ets wild-type oligonucleotide, nuclear extracts were first preincubated with cold mutated Ets oligo to reduce the unspecific signal (top arrow in fig 7C).

**RESULTS**

**PKA stimulates expression of HBV-S RNA and HBsAg**

CAMP dependent signalling pathways are pivotal in regulating homeostatic functions in the liver and are activated during hepatic inflammation. We tested if CREB transcription factors influence HBV-S gene and protein expression. Replication competent HBV 1.28 wild-type plasmid was cotransfected with an expressing vector for the catalytic subunit of PKA, a strong endogenous CREB inducer. Cotransfection of HBV wild-type and 1 µg PKA in Huh7 hepatoma cells resulted in a significant increase in HBV-S RNA in northern blot analysis 24 and 48 hours after transfection (fig 1A, B). Levels of pregenomic/precore RNA were not significantly affected. After cotransfection with PKA, SHBs expression was markedly induced, as evidenced by western blot analysis 48 and 72 hours after transfection (fig 1C).

**Known pre-S2/S promoter is flanked by putative transcription factor binding sites for CREB, CEBP/β, Ets, and Stat1**

The HBV pre-S2/S promoter (‘S promoter’) is a TATA-less promoter and controls transcription of the 2.1 kb S-mRNA generating the middle and small surface protein. The promoter was shown to be located in the coding region of the pre-S gene between nucleotides 3045 and 3180 (EcoRI = 1) (see fig 2A). Known pre-S2/S promoter is flanked by putative CREB, CEBP/β, Ets, and Stat1 binding sites. Therefore, a fragment covering the pre-S1/pre-S2 region, including the identified regions of interest, was amplified by PCR and cloned into the luciferase expression vector pGL2. The resulting construct was referred to as construct 1 and is depicted in fig 2A. Of note, the predicted Ets site was partially overlapping with an AP-1 motif. As it is well known that regulatory elements outside of the ‘core’ S promoter sequence constitute part of the S promoter activity, such as upstream binding motifs for HNF3 and NF1 or a downstream Sp1 binding site, we then aimed to test whether these putative binding sites may contribute to S promoter activity. Therefore, a fragment covering the pre-S1/pre-S2 region, including the identified regions of interest, was amplified from a HBV wild-type plasmid and cloned into the luciferase expression vector pGL2. The resulting construct was referred to as construct 1 and is depicted in fig 2A. For a detailed analysis we generated further reporter plasmids by introducing several 3’ deletions of different lengths into construct 1 (construct 1B, 2–5), as shown in fig 2A.

**CREB binding motif contributes to basal S promoter activity**

The reporter gene constructs (fig 2A) were transfected in Huh7 human hepatoma cells, and luciferase activity was measured and normalised to β-galactosidase activity as a marker for transfection efficiency. As shown in fig 3A, constructs 1 and 1B were of similar length but differed with respect to predicted CREB binding sites at positions 157 and 140 (compare with fig 2A), respectively. For constructs 2–5, no CREB binding site was detected by computer analysis.

The highest reporter gene expression was detected in construct 1 where luciferase was under the control of the 388 bp sequence upstream from the S-ATG that includes the consensus oligonucleotide corresponded to the sequence 5’-AGA GAT TGC CTG ACG TCA GAC AGC TAG-3’, and the CREB mutated consensus oligonucleotide to the sequence 5’-AGA GAT TGC CTG TGG TCA GAC AGC TAG-3’.
two putative CREB binding sites (fig 3B, compare constructs 1 and 2–5). In order to determine the functional relevance of the two neighbouring putative CREB motifs in construct 1, additional 3’ deletion constructs were generated and tested for promoter activity (fig 3B). Deletion of the downstream CREB site (CREB I, construct 1B) had no significant effect on basal promoter activity. In contrast, by also deleting the upstream CREB site (CREB II) as in construct 2, we observed a strong decrease in luciferase activity compared with construct 1 or 1B. These results suggest an essential role for the upstream putative CREB site (CREB II) in maintaining basal promoter activity. Further 3’ deletions as in constructs 3–5 were only correlated with a minor reduction of the basal transcriptional activity compared with construct 2, indicating that basal promoter activity is predominantly mediated by CREB II.

To assess complex formation to the CREB II motif, EMSA experiments were performed using oligonucleotides representing either the CREB II sequence, as found in the promoter or a related sequence comprising a mutation in the CREB core recognition site (fig 4A). When the CREB II oligonucleotide was incubated with Huh7 nuclear extracts, complex formation was detected (fig 4B, left panel). To test for specificity to CREB, competition experiments were performed using wild-type (wt) or mutated (mut) CREB consensus oligonucleotides, as described previously. Binding of the two slow migrating complexes could be readily reduced using low amounts of cold consensus wild-type CREB oligo, but not with mutated CREB oligo (fig 4B, left panel). In contrast, incubation with a mutated CREB II oligonucleotide did not reveal specific complex formation in gelshift analysis (fig 4B, right panel).

As the CREB/ATF family of transcription factors comprise a variety of different proteins able to interact with CREB binding motifs and each other, supershift experiments were carried out to define the factors involved in the binding to the CREB II motif. Complex formation with the CREB II site could be modified using an ATF2 antibody (fig 4C) resulting in formation of a new slow migrating complex. These data suggest that the upstream CREB site (CREB II) mediates basal promoter activity via ATF2 binding. To further support this finding we introduced the (non-sense) mutation represented by the “CREB II mutated” oligonucleotide into reporter construct 1B, resulting in construct 1Bmut, and performed reporter assays in Huh7 cells. As shown in fig 4D, mutation of the CREB II element resulted in strong reduction of luciferase activity (to 10% of construct 1B).

Our data implied that the CREB II site mediates basal promoter activity via ATF2 binding whereas the CREB I motif appeared to be dispensable for transcriptional activation. However, due to the close proximity of both elements, functional compensation of CREB II by CREB I could not be excluded. In EMSA experiments, CREB I showed the same
Hepatitis B virus pre-S2/S promoter regulation by CREB/PKA

In constructs 2 and 3, CREB/PKA strongly induced luciferase activity compared with the basal level. This indicates that the PRE elements contribute to basal promoter activity.

In constructs 4 and 5, we observed a further increase in luciferase activity induced by CREB/PKA. This suggests that certain CREB/PKA responsive elements are present in these constructs.

Our analysis identified CREB binding motifs in the pre-S2/S core sequences of construct 1. A CREB II element was detected in the downstream region of the pre-S2/S core sequence.

The reporter plasmids were transfected into Huh7 cells, and luciferase activity was measured. The results showed that constructs 1 and 1B had the highest luciferase activity, while constructs 2–5 had lower activity. Deletion of the CREB II element in construct 1B resulted in a significant decrease in luciferase activity.

The CREB specific complex formation was detected using EMSA. A specific complex was formed with CREB II, as shown in the figure. This suggests that CREB II is a key factor in the regulation of the pre-S2/S promoter.

**Figure 3**

**Figure 4**

Our analysis identified CREB binding motifs in the pre-S2/S core sequences. These motifs contribute to basal promoter activity. The full length promoter construct 1 (see fig 2A) was used to identify these CREB binding sites.

This suggests that CREB/PKA responsive elements are present in these constructs. The CREB II element is a key factor in the regulation of the pre-S2/S promoter.

**S promoter activity is inducible via CREB, PKA, and forskolin**

Our analysis identified CREB binding motifs in the pre-S2/S core sequence. These motifs contribute to basal promoter activity. Thus, we next investigated whether cAMP signalling pathways also mediate inducible S promoter activation. As indicated by the cotransfection experiment of HBV wild-type and the PKA expression plasmid, the reporter gene expression was quantified.

We observed a significant increase in luciferase activity when constructs 1 and 1B were transfected with CREB and/or PKA. This suggests that CREB/PKA responsive elements are present in these constructs.

Our analysis identified CREB binding motifs in the pre-S2/S core sequences of construct 1. A CREB II element was detected in the downstream region of the pre-S2/S core sequence. The CREB specific complex formation was detected using EMSA. A specific complex was formed with CREB II, as shown in the figure. This suggests that CREB II is a key factor in the regulation of the pre-S2/S promoter.
constructs 1–3 carry a putative Ets binding motif which was deleted in constructs 4 and 5.

Forskolin is a chemical inductor of PKA/CREB signalling pathways. It increases endogenous PKA transcription and subsequently activates CREB.39,44 In order to test if extracellular signals or exogenous activators may also enhance S promoter activity, construct 3 was either cotransfected with PKA and/or cells were stimulated with 10 μM forskolin. Stimulation with forskolin resulted in maximal activation of relative luciferase expression that could not be further stimulated by cotransfecting the PKA expressing construct (fig 6B).

These experiments indicated that the putative Ets binding motif might be involved in mediating PKA dependent promoter activation. We thus mutated the putative Ets sequences in this part of the promoter region (constructs 3-mut-I and 3-mut-II, fig 7A). The mutant Ets constructs were then cotransfected with the catalytic subunit of PKA. These experiments showed that the intact Ets motif was necessary to confer full PKA mediated gene activation of the S promoter (fig 7B).

We next attempted to further characterise possible transcription factors involved in binding to the Ets motif. The family of Ets transcription factors consists of more than 40 different proteins, and protein-protein interactions (for

Figure 5  Analysis of neighbouring cAMP response element binding protein (CREB), protein kinase A (PKA), and the exogenous PKA activator forskolin. (A) Deletion constructs 1–5 (see fig 2A) were cotransfected in Huh7 hepatoma cells with 50 ng CREB-plasmid and/or 1 μg PKA expression plasmid, as indicated. Construct 1 conferred the highest basal activity (as expected from figs 3–5). Luciferase expression was highly induced by cotransfection of CREB and even further enhanced by PKA in all constructs. However, the highest relative induction was seen in constructs 2 and 3 whereas this inducible activity was remarkably lower in constructs 4 and 5. Constructs 1–3 contain a putative Ets-1 binding motif, which was not present in constructs 4 and 5. (B) Forskolin is a chemical inductor of PKA/CREB signalling pathways. Construct 3 was either cotransfected with PKA and/or cells were stimulated with 10 μM forskolin, as indicated. Stimulation with forskolin resulted in maximal activation of relative luciferase expression.
example, between AP1 and Ets) are common.\textsuperscript{45 46} We therefore tested a variety of different factor specific antibodies in supershift analysis. Nuclear extracts from either unstimulated Huh7 cells (lane 2) or forskolin stimulated Huh7 cells (lanes 3–10) were incubated with a radioactive labelled double stranded oligonucleotide representing the Ets motif. In order to reduce an unspecific signal (top arrow), nuclear extracts were first preincubated with unlabelled mutated Ets oligonucleotide. Supershift EMSA analysis was performed with antibodies directed against Ets1/2 (lane 4), c-jun (lane 5), SP1 (lane 6), CREB1 (lane 7), activating transcription factor (ATF1) (lane 8), ATF2 (lane 9), and ATF4 (lane 10). Incubation with anti-CREB1 and anti-ATF4 reduced specific complex formation (bottom arrow, lower signal).

DISCUSSION

Transcriptional regulation via adenylate cyclase signalling pathways is mediated by a family of cyclic AMP responsive nuclear factors, including CREB. The activation function of CRE binding proteins is modulated by phosphorylation by several kinases (for example, PKA) and other cellular cofactors.\textsuperscript{4} CREB transcription factors play a pivotal role in the liver as they are essential for regulating hepatic metabolic functions, such as gluconeogenesis or lipid oxidation, as well as controlling hepatocyte proliferation.\textsuperscript{4, 14} In addition, CRE pathways are activated in experimental settings of hepatic inflammation via increasing c-jun.\textsuperscript{4} In this study, we identified a role for CREB/PKA sensitive signalling in maintaining basal promoter activity and inducible expression of the hepatitis B virus pre-S2/S promoter.

Regulation of HBV-S gene expression is crucial for viral pathogenesis, as the envelope proteins are essential for virus formation, infectivity, and immunogenicity. The strongest regulatory element within the HBV genome is the pre-S2/S promoter ("S promoter"), located within the pre-S region, subsequently causing the release of HBs particles.\textsuperscript{20} The extraordinary high prevalence of naturally occurring pre-S mutants during chronic HBV infection have led to identification of several pre-S mutations affecting the S promoter, thereby providing a molecular explanation for an unfavourable clinical outcome seen in many of these patient populations.\textsuperscript{21} The highest reduction in HBsAg expression can be detected when the CCAAT motif in the HBV pre-S2/S promoter is affected, although even introduction of nonsense mutations into the CCAAT motif into promoter constructs with otherwise normal sequence shows a residual promoter activity of approximately 25%,\textsuperscript{20} thereby suggesting alternative signalling pathways for maintaining basal viral promoter activity.

As CREB/PKA pathways are central in hepatocyte signal transduction, we tested whether HBV-S gene and envelope protein expression are upregulated on CREB/PKA activation. In fact, cotransfection of HBV wild-type and PKA expression plasmid in hepatoma cells resulted in increased HBV-S RNA and SHBs protein expression (fig 1). By applying reporter gene and DNA binding analysis experiments, we identified two novel regulatory elements in the pre-S2 region, possibly mediating this CREB/PKA sensitive S promoter activation—a CREB transcription factor binding motif in the pre-S2 region contributing to basal S promoter activity via binding of ATF2, and a predicted Ets site in the pre-S2 region mediating inducible activation of gene expression by PKA/forskolin, possibly involving CREB1 and ATF4. Interestingly, these regulatory regions are located within the transcribed (pre-S2) region of the HBV genome. Given the dense organisation of the circular HBV DNA genome, the contribution of regulatory elements within the transcribed region to the activity of the previously described upstream "core" S promoter is not unexpected. Similar promoter organisations with regulatory elements located in intronic regions have been shown for a variety of promoters, such as CD95 or caspase 8.\textsuperscript{37 41}

Our results showed that the basal activity of the HBV pre-S2/S promoter is supported by a CREB dependent mechanism (figs 3–5). We identified two putative CREB motifs with similar binding patterns in the pre-S2 region, and supershift analysis suggested that ATF2 is involved in complex formation at these CREB binding motifs. Although the DNA binding assays implied a functional redundancy for both CREB sites, only the upstream CREB site appeared
essential for full basal promoter activity in our reporter gene experiments. Additional observations indicate the functional relevance of these CREB sites. In order to characterise pre-S2 protein function, Le Seyec et al generated HBV constructs with deletions in the pre-S2 region. They observed that deletion of any of the two CREB motifs (deletion L144/L153 = “CREB-II”), deletion L154/L163 = “CREB-I”) was associated with a reduction in HBsAg secretion of between 15% and 30% in vitro.44 Interestingly, deletion of the downstream CREB motif in HBV constructs was associated with HBsAg reduction. This would argue that the downstream CREB site may functionally compensate for loss of the upstream site in HBV deletion constructs, although this was not observed in our experiments applying the luciferase reporter system.

We also observed inducible upregulation of promoter activity by CREB and its activators PKA or forskolin (figs 6, 7). Our reporter gene experiments showed that a sequence in the pre-S2 region comprising an Ets motif was significantly involved in mediating cAMP dependent activation. The family of Ets transcription factors consists of more than 40 different proteins, and protein-protein interactions (for example, between AP1 and Ets) are common.45 46 Our DNA binding experiments pointed towards involvement of Ets factors and/or AP-1 as forskolin/PKA inducible complex formation could be detected towards these elements. However, supershift experiments with anti-CREB1 and anti-ATF4 antibodies reduced complex formation, indicating inducible heterodimer binding of two CREB family members to this element. Earlier results showed that ATF-1/2/4 can associate with a reduction in HBsAg secretion of between 15% and 30% in vitro.44 45 The pre-S2 region comprising an Ets motif was significantly associated with a reduction in HBsAg secretion of between 15% and 30% in vitro.44 45 Our supershift experiments suggested that CREB1 and ATF4 are involved in complex formation, thereby potentially activating transcription via the Ets motif. However, we cannot exclude the fact that more than the identified factors are involved in this process.

Taken together, our experiments show that CREB motifs in the pre-S2 region contribute to basal S promoter activity and that CREB/PKA can upregulate HBsAg expression. However, the true in vivo relevance of these in vitro findings needs to be further addressed (for example, by coinjecting HBV and PKA plasmids in a mouse model of acute HBV infection and monitoring HBsAg expression47 or by assessing HBsAg levels after acute infection in CREB knockout mice).48 Our experiments indicate a novel mechanism by which HBV is able to utilise the cellular signal transduction machinery of hepatocytes in order to enhance its HBsAg expression level.

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Conflict of interest: None declared.

REFERENCES


EDITOR’S QUIZ: GI SNAPSHOT .......................................................... 

Answer

From question on page 1278

The diagnosis was splenic infarction secondary to idiopathic splenic vein thrombosis. The patient was treated with intravenous heparin for six days. Following resolution of abdominal pain and tenderness, she was converted to oral warfarin therapy. Computed tomography scan three months later demonstrated a normal spleen with complete resolution of splenic oedema and pulmonary consolidation. The splenic vein was attenuated and ill defined, with evidence of collateral vessel formation. The patient made a full clinical recovery.

The principal challenge in this case was the choice of conservative treatment versus splenectomy to avert rupture. While splenectomy appears to be an effective treatment in splenic vein thrombosis, it incurs the cost of a lifelong requirement for prophylactic antibiotic therapy. Our patient represents successful management of splenic vein thrombosis by anticoagulation, of which there are only a few reported cases in the literature.

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