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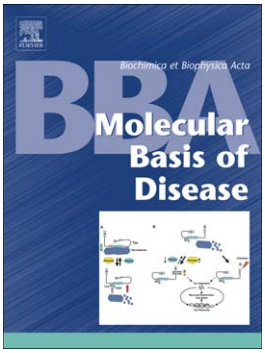

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Cellular Transcription Modulator SMARCE1 Binds to HBV Core Promoter Containing Naturally Occurring Deletions and Represses Viral Replication

Hong Pan[3], Dan Dan Niu[1], Huixing Feng[1], Lisa Ng [2], Ee Chee Ren[2] and Wei Ning Chen[1]

[1] School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 639798, [2] Genome Institute of Singapore, 60, Biopolis Street, Genome, #02-01, Singapore 138672, [3]School of Biological Sciences, Nanyang Technological University, Singapore 637551

* Corresponding Author:
Tel: (65) 63162870
Fax: (65) 62259865
Email:WNChen@ntu.edu.sg

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Abstract

Suppression of hepatitis B virus (HBV) replication, a causative agent for chronic hepatitis, is an effective approach to controlling disease progression. Host factors have a significant effect on viral replication efficiency and need to be better characterized. We have reported association between clinical virus load and deletions in HBV viral promoter. We showed here that HBV genome with such deletions led to decreased replication compared with wild type virus. Consistently, the promoter with deletion showed lower activity. A cellular transcription regulator recognizing the promoter with deletion was revealed in gel shift assay and subsequently identified as SMARCE 1 through DNA-protein array assay. The ability of SMARCE 1 in modulating the replication efficiency of HBV was further demonstrated. Taken together, our studies show a direct dependence of HBV on a host factor to modulate its replication efficiency, and provided a new platform for molecular characterization of mechanisms of disease outcome as a result of binding of new transcription factors to rearranged promoter sequences.

Keywords: HBV; Core Promoter and Deletion; Replication; DNA-Protein Interactions; SMARCE1

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Introduction

Hepatitis B virus (HBV) infection is a serious public health problem in many parts of world that results in complications such as cirrhosis and hepatocellular carcinoma [1]. Globally, despite effective vaccination against HBV and available antiviral treatments [2], there remain an estimated 350 million hepatitis B carriers with a lifetime risk for developing cirrhosis and hepatocellular carcinoma. Current antiviral therapies for HBV carriers include treatment such as alpha-interferon or lamivudine, but the long-term resolution of disease is disappointing due to low seroconversion rates and the development of drug-resistant viral mutants [3].

HBV belongs to a family of viruses known as hepadnaviridae and encodes only four genes in a highly compact viral genome: the surface gene (S), the core gene (C), the X gene (X), and the polymerase gene (P). Viral replication has been shown to occur via an RNA intermediate in the cytoplasm, but, unlike retroviruses, integration of HBV DNA into the host genome is not required. Despite a wealth of information on the virus itself and the possible role that host factors may play in the viral infectious life cycle [4], the direct relationship between chronic infection and host–pathogen interactions is poorly understood.

The core promoter (CP) of HBV plays a central role in HBV replication and morphogenesis. The core promoter controls the transcription of two core gene products: core and precore RNA. The core RNA is essential for viral replication, because it encodes the major capsid protein and the viral polymerase. Additionally, it also serves as the pregenomic RNA [5]. The nonessential precore RNA encodes the precore protein, which is processed in the endoplasmic reticulum to produce the secreted HBeAg [6]. Previous studies have shown that deletion in the core promoter can lead to suppression of viral replication and HBV DNA [7,8]. Conversely, our previous investigation in clinical serum samples has revealed that deletions in the viral core promoter may result in an increase in viral replication as indicated by the high level of HBV DNA [9]. In this case, high serum

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HBV DNA level (~ 228.16 pg/ml) as well as HBsAg level (~140 μg/ml) have been found in three patients in whom the infecting HBV contain deletions within the same region (nucleotide 1758-1777, 1749-1768) of the viral core promoter.

It is likely that cellular transcription factors recognize the altered viral promoter and modulate level of viral replication, as evidenced in our recent investigation on the role of heterogeneous ribonucleoprotein K (hnRNP K) in HBV promoter with single nucleotide mutation [10]. Analysis of virus-host interaction at gene expression level should not only provide new insights on the underlying mechanism of differential regulation of viral replication, but more importantly identify new targets for antiviral strategies.

In this study, a host protein—SMARCE 1 has been isolated by direct binding to a viral DNA fragment corresponding to HBV promoter region with deletion. The cotransfection experiment and gel shift assay further confirmed the binding affinity of SMARCE 1 to the DNA fragment. SMARCE 1 was found to bind and modulate HBV replication. Taken together, our studies show a direct dependence of HBV on a host factor to modulate its replication efficiency, and provided a new platform for molecular characterization of mechanisms of disease outcome as a result of binding of new transcription factors to rearranged promoter sequence.

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Result

Deletions in HBV Core Promoter Lead to Decreased Extracellular HBV Virion

Based on clinical samples, we have previously reported a possible role of deletions within HBV core promoter in viral replication [9]. To investigate the molecular mechanism of HBV replication in this context, we have recently established a cell-based HBV replication in which a replicative viral genome was reconstructed with the same reported deletions in the core promoter [11]. The amount of secreted HBsAg in cells transfected with deletion mutants was significantly lower than those transfected with the wild type

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genome [11]. In order to further validate the influence of core promoter deletions on the HBV replication capacity, the extracellular HBV DNA level was measured. Newly synthesized HBV virions released into supernatant were collected 2 days after transfection and HBV DNA level measured using a quantitative real-time PCR approach. The result shows deleted mutants displayed decreased levels of released HBV virions in the supernatant (Fig1). Our results were consistent with earlier report on the correlation between the amount of HBsAg and that of HBV DNA level which is indicative of HBV replication [10]. This in turn suggested that deletions in core promoter led to a decreased viral replication, in line with other reported investigations [7,8].

Deletions in HBV Core Promoter Lead to Decreased Intracellular HBV Replication

While the extracellular amounts of HBsAg and HBV DNA are indicative of HBV replication, a more direct measurement would be the level of intracellular viral RNA [4], real time PCR was used to measure the level of intracellular HBV core mRNA.

HepG2 cells were transiently transfected with different constructs including wild type HBV, HBV genome with deletion type 1 (DEL1), and HBV genome with deletion type 2 (DEL2), and mRNA was extracted from each type of HepG2 cells and used as template for reverse transcriptase (RT)-PCR using primer covering the coding region of HBV core. Then, the RT-PCR core gene product was used as template to perform the Real-Time PCR experiment. Conversely, the cellular housekeeping gene GAPDH was included as an internal control. Quantification of the PCR products in the calculated linear area of amplification showed significantly decreased HBV core mRNA level in the HepG2 cells transfected with HBV genome containing deletions in their core promoter (Fig. 2). Compared with the wild type, the mRNA level in the cells transfected with either DEL1 or DEL2 was much lower. As the coding region of HBV core was contained on the pregenomic RNA, our results indicated that the amount of pregenomic RNA was reduced in deleted mutants and thus HBV replication level was lower in these mutants.

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Deletions in HBV Core Promoter Lead to Decreased Promoter Activity

As the above-mentioned deletions were in the HBV enhancer element, the effect of these deletions on transcriptional efficiency was analysed. A 131-base-pair Enh II fragment covering wild type region, the same region without nt 1749-1768 (DEL1) and the same region without nt 1758-1777 (DEL2) were separately cloned upstream of a SV40 promoter–luciferase reporter gene vector. HepG2 cells were transiently transfected with these constructs and the respective luciferase activity was measured. The results shown in Fig. 3 indicated that DEL1 and DEL2 displayed levels of luciferase that were significantly lower compared with that of the wild type enhancer promoter. As a positive control, the vector containing both the SV40 promoter and enhancer sequences resulting in optimal luciferase expression was used (positive column 1, Fig. 3).

In addition, the cloning vector containing only the SV40 promoter–luciferase gene but without any enhancer element (negative column, Fig. 3) was used as the negative control. To determine if these results were specific to HepG2 cell line, the above-mentioned transfections were carried out in two cell lines: Chang liver cells and 293T cells. The results shown in Fig. 3 indicated that the promoter activity of either DEL1 or DEL2 was lower than that of the wild type HBV core promoter which was consistent with the results in HepG2. Our results therefore suggested that deletions spanning nt 1749-1768 and/or nt 1758-1777 had a significant effect on the transcriptional efficiency of Enh II. In addition, our assay on the promoter activity provided further support for the effect of deletions on the decreased HBV replication.

To determine if these results were specific to HepG2 cell line, the above-mentioned transfections were carried out in two cell lines: Chang liver cells and 293T cells. The results shown in Fig. 2b and 2c indicated that the promoter activity of either DEL1 or DEL2 was lower than that of the wild type HBV core promoter which was consistent with the results in HepG2. Our results therefore suggested that deletions spanning nt 1749-1768 and/or nt 1758-1777 had a significant effect on the transcriptional efficiency

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of Enh II. In addition, our assay on the promoter activity provided further support for the effect of deletions on the decreased HBV replication.

Cellular Protein SMARCE1 Binds to HBV Core Promoter with Deletion In Vitro

To further analyze the biological significance of DEL1 and/or DEL2 with deletions in Enh II region of HBV core promoter, the presence of direct physical HBV DNA–host interaction at the deleted region was investigated. DEL1 was chosen in this study by its more drastic decreased promoter activity compared to DEL2.

Non-radioactive DEL1 oligonucleotide probe was designed to contain the region surrounding nt 1749-1768, whereas the control probes was designed based on the wild type sequence which include the deleted region. Electrophoretic mobility shift assay was performed using HepG2 nuclear extracts in the presence of either the DEL1 or the wild type DNA probes. Using nuclear extracts from HepG2 cells, four DNA-protein complexes were detected and referred to as complexes I, II, III and IV respectively (Fig. 4). The complex I may be a result of nonspecific binding as it appeared for both wild type and DEL1 probes. The intensity of signals of complex IV was decreased by an unlabeled specific competitor. Significantly, the signal of complex III was removed by an unlabeled specific competitor. This result indicated that a distinct DNA binding protein was associated with the DEL1 probe in complex III (lane 6, Fig. 4). These data suggested that host DNA-binding proteins interacted directly with the DEL1 probe and may be involved in our observed decrease in the correlated viral replication.

To identify transcriptional factors which bind to this specific DEL1 probe, a protein-DNA array (Table I) was used. The commercially available array contained immobilized transcription factors, and incubation with DNA probes of interest would provide direct indication of protein-DNA interaction. Either wild type or DEL1 biotinylated probes were used in the analysis with the protein-DNA array. To assess the significance of any positive signal, a duplicate blot was then used in competition analysis using an excess non-biotinylated DNA probes. The absence or drastically reduced intensity of the positive

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signal in the duplicated blot would suggest that the respective the transcription factor interacted with DNA probe being analysed. Results shown in Fig. 5a and 5b indicated that five transcription factors recognized DEL1 but not the wild type probe.

The samples were also compared with the blot of protein-DNA incubated with the wild type DNA probe. Results shown in Fig. 5c and 5d indicated a different pattern compared to those obtained from DEL1 (Fig. 5a and 5b). Proteins that were recognized by DEL1 probe included PTTG1, PTTG2, PXR2, SIX2, SMARCE1 and TFE3 (Fig. 5a), while binding to wild type probe was seen with REVERB and SP3 (Fig. 5c). Significantly, all these detected protein-DNA interactions were reduced in a competitive manner in the presence of the respective non-biotinylated probe. This was shown in Fig. 5b for DEL1 DNA probe and in Fig. 5d for wild type DNA probe respectively.

Among the positive DNA-protein interactions detected in DEL1 DNA probe, the interaction between DEL1 probe and SMARCE1 protein (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1-related protein) was completely removed in the presence of non-biotinylated DEL1 probe (Fig. 5b). SMARCE 1 was therefore selected for further *in vitro* analysis for its role in HBV replication mediated by viral promoter containing DEL1 deletion.

To investigate whether the full length SMARCE1 protein interacts *in vitro* with DEL1 but not the wild type, the coding region of SMARCE1 was amplified, cloned in mammalian expression vector pXJ40 and the protein produced in 293T cells. The 293T cell line was selected as the decrease in deletion promoter activity (Fig. 3c) was less drastic compared with that in HepG2 or Chang cell lines (Fig. 3a and Fig. 3b). This would be more useful when the effect of SMARCE1 on DEL1 was subsequently analysed, as the relatively higher promoter activity for DEL1 in 293T cells (45.92%) compared with that in HepG2 cells (6.95%) would allow more accurate measurement of

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the changes either by activation or by repression. The production of full length SMARCE1 protein in 293T cells was further indicated by Western blot analysis using HA tag monoclonal antibody (Fig. 6). The presence of 46kDa protein, corresponding to the expected size of SMARCE1 protein, in 293T cells transfected with pXJ40-SMARCE1 construct (lane 1, Fig. 6) but not 293T cells transfected with the empty pXJ40 plasmid (lane 2, Fig. 6) suggested that full length SMARCE1 protein was produced in these cells.

Electrophoretic mobility shift assay was then carried out with DEL1 and wild type DNA probes separately, using nuclear protein extracted from 293T cells producing full length SMARCE1. Nuclear proteins extracted from 293T cells transfected with cloning vector were used as negative control. After electrophoresis and membrane transfer, both DNA-protein complexes and free probes were identified with streptavidin-horseradish peroxidase and substrate. As shown in Fig. 7, DNA-protein complex II was seen when DEL1 probe was incubated with nuclear proteins from SMARCE1 transfected cells (lane 2, Fig. 7B), but not when wild type probe was incubated with the same nuclear protein (lane 5, Fig. 7B). Addition of excessive non-biotinylated DEL1 probe was able to compete off the binding of biotinylated DEL1 probe to complex II, as shown by its reduced intensity (lane 1, Fig. 7B), suggesting that complex II was formed by DEL1-protein interaction. The involvement of SMARCE1 in complex II was supported by its absence in the electrophoretic mobility shift assay using nuclear proteins from 293T cells transfected with pXJ40 plasmid (lane 1 and 2, Fig. 7A). Furthermore, the complex II was not detected in other conditions where either DEL1 probe or SMARCE1 protein was not present: including the absence of nuclear proteins (lane 3 and 6, Fig. 7A; lane 3 and 6, Fig. 7B), incubation of nuclear protein with the wild type probe (lane 4 and 5, Fig. 7A; lane 4 and 5, Fig. 7B) and incubation with nuclear proteins from cells transfected with the empty pXJ40 (Fig. 7A). Our results therefore suggested that DEL1 DNA fragment interacted *in vitro* with SMARCE1 protein.

To further support that the complex III was indeed formed by DEL1 probe and SMARCE1, the supershift assay was carried in the presence of the anti-HA antibody

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which should recognize the SMARCE1 expressed as a HA-tag protein (see Fig. 6). Results suggested that partial supershift was detected for complex III in the presence of anti-HA andibody (arrowhead, lane 4, panel C, Fig. 7). This partial supershift band was not detected in the absence of the anti-HA antibody (lane 2, panel C, Fig. 7). As a control, the unlabeled specific probe was able to compete off the DNA-protein complexes (including complex III) detected in lane 2 and 4. Our results therefore indicated that complex III was formed by DEL1 probe and SMARCE1.

SMARCE1Modulates Replication of Mutant HBV with Deletion in Viral Promoter

To investigate whether SMARCE1 bind to HBV core promoter with DEL1 deletion, the above-mentioned transcription reporter system for promoter activity assay was used. In this case, HepG2 cells were co-transfected with either DEL1 reporter construct/pXJ40-SMARCE1 or wild type reporter construct/pXJ40-SMARCE 1 and effect of SMARCE1 analyzed by luciferase activity. Results shown in Fig. 8 suggested that SMARCE1 exerted a stronger repression effect on DEL1 promoter, as the DEL1 promoter activity was repressed from 41.25% to 26.2%. As a control, the repression effect of SMARCE1 was not significant towards the wild type promoter with the wild type activity at 85.35% repressed slightly to79.65% (Fig. 8). This was expected as our blot binding and in vitro analyses indicated no interaction between SMARCE1 and the wild type DNA probe. These results indicated that SMARCE1 not only interacted with DEL1 DNA fragment, but also influence the promoter activity.

To determine the functional significance of SMARCE1 on HBV replication, the amount of HBsAg secreted into culture medium was analysed as previously reported [11]. This was based on the simplicity of HBsAg measurement, and more importantly on the close correlation between the amount of HBsAg and HBV replication as demonstrated in this study (Fig. 2). pXJ40-SMARCE1 plasmid was co-transfected into 293T cells with either the wild type replicative HBV genome or the replicative viral genome with DEL1 deletion in the core promoter (Pan et al., 2005). It is interesting to note that both the wild type or DEL1 replicative genomes had been cloned upstream of the CMV promoter of

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pcDNA3.1 such that the replication would be driven by the respective viral core promoter rather than the CMV promoter.

Consistently with our in vitro analysis indicating a repression effect by SMARCE1, the amount of HBsAg from cells transfected with DEL1 replicative genome decreased in the presence of SMARCE1 (26.37% with SMARCE1 as opposed to 36.8% without SMARCE1, Fig. 9). T test was used for the statistic analysis. A value of $P<0.05$ indicates that the difference is significant. We concluded that the replicative genome with deletion in the core promoter had lower replication efficiency. In addition, cells transfected with 5 μg of SMARCE1 had lower amount of secreted HBsAg compared with those transfected with 1 μg of the same plasmid, suggesting that SMARCE1 was indeed involved in the repression of HBsAg secretion (*data not shown*). As a control, the repression effect of SMARCE1 was not significant towards the wild type replicative genome with the amount of HBsAg at 50.89% reduced slightly to49.94% (Fig. 9). This was expected as our blot binding and *in vitro* analyses indicated no interaction between SMARCE1 and the wild type viral promoter. These results indicated that SMARCE1 not only interacted with DEL1 promoter, but also influence replication of DEL1 genome.

In summary, our results provide evidence on the interaction between cellular protein SMARCE1 and HBV promoter with DEL1 deletion, and more importantly demonstrate the function correlation between DNA-protein recognition and HBV replication.

Discussion

The interesting observation that chronic HBV carriers have different serum viral loads prompted us to investigate host proteins involved. To understand whether the natural deletions at 1749-1768 and 1758-1777 had any functional impact, reporter constructs were developed to test effects of such deletions on the ability to drive reporter gene

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transcription. It was evident that both deletions had lower activity than the wild type constructs. Transcription factors bind specifically to promoter sequence. Such binding will be affected if the binding site is removed by deletion for example. On the other hand, the rearranged sequence in the promoter from a deletion may create binding sites for new transcription factors. For HBV, it has been reported that even one base pair change in promoter region (1752 G/A) has resulted in the binding of hnRNP K, and leading to the suppression of HBV replication [10]. In addition, some of the mutations in the core promoter have resulted in binding sites for hepatocyte nuclear factor 1 and HNF3 [12]. It is therefore not surprising that the 20bp deletion in the core promoter may lead to binding of new transcription factors including SMARCE1 as demonstrated in our investigation.

To investigate the underlying basis for this enhanced transcriptional activity, a new approach based on directly probing for physical DNA-protein interactions was developed. Using an initial electrophoretic mobility shift assay followed by a DNA-protein array assay, a deletion (1749-1768) oligonucleotide fragment (DEL1) was found to bind to a host binding factor. The binding factor was identified as SMARCE1, a known protein that has been shown to be involved in a number of cellular functions. The full name of SMARCE 1 is SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1-related (SMARCE 1-related protein) (HMG domain protein HMG20B) (Structural DNA-binding protein BRAF35) (BRCA2- associated factor 35) (Sox-like transcriptional factor). Its subcellular localization is in both nuclear and cytosol. Members of the Swi/Snf family of chromatin-remodeling complexes play critical roles in transcriptional control. These complexes can be divided into three classes on the basis of the similarities of their ATPase subunits to the Swi2/Snf2, Isw1, and Mi-2 proteins [13]. The human SWI/SNF complexes contain either BRG1 or Brm as the catalytic ATPase subunit and approximately 10 BRG1-associated factors (BAFs). The BAF170 and/or BAF155, BAF60, BAF57, BAF53, and BAF47 (hSNF5/Ini1) subunits are present in all mammalian SWI/SNF complexes and conserved from yeast to humans, except for BAF57 [14].

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Ample experimental evidence has suggested that the SWI/SNF complexes play important roles in fundamental cellular processes such as transcription, replication, and the repair of chromatin [13,15]. As a result, mammalian SWI/SNF complexes have been implicated in diverse physiological and pathological processes, including cell proliferation and differentiation, retrovirus infection, and carcinogenesis [14]. It has been reported that both recombinant BAF57 and the whole complex bind double strand DNA [16].

In this study, the cotransfection experiment and gel shift assay further confirmed the binding affinity of SMARCE1 to DEL1 probe. An important next step was to show if SMARCE1 acts on a full-length HBV genome containing the same DEL1 deletion in its promoter. To this end, cells were co-transfected with the full-length replicative HBV genome constructed [11] carrying such a deletion (DEL1) and pXJ40-SMARCE1 plasmid. Compared with similar transfection where the replicative genome was used instead, the replication of wild type genome as measured by the extracellular amount of HBsAg was higher than DEL1 genome. Interestingly, no significant difference in replication was seen in cells transfected with the wild type genome with or without pXJ40-SMARCE1. Consistently with the *in vitro* analyses including the promoter activity (Fig. 8) and gel shift assay (Fig. 7), the presence of SMARCE1 protein led to a decrease in the amount of HBsAg from cells transfected with DEL1 genome.

It appears that SMARCE 1 could downregulates the transcription. In several recent studies, chromatin immunoprecipitation (ChIP) experiments demonstrated that Swi/Snf components were physically present at repressed promoters. Targeted repression by Swi/Snf appears to require its recruitment by regulatory proteins. In one study, the potential tumor suppressor prohibition, which represses E2F activation, was shown to recruit Swi/Snf to particular E2F-dependent promoter where they inhibit E2F-mediated transcription [17]. In another study, Swi/Snf was shown to be recruited to neuronal genes by the CoREST corepressor, which itself interacts with the DNA binding repressor [18]. In this case the CoREST–Swi/Snf interaction is dependent upon BAF57. Besides its role in chromatin remodelling, SMARCE1 (also known as BAF57) has recently been shown

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to bind directly to the promoter of the cylindromatosis tumor suppressor gene, and induces apoptosis by stimulating its expression [19].

Taking these known functions of SMARCE1 into the context of its new findings shown in this study, it is possible that other cellular protein factors, some of which may have been identified using our DNA-protein blot binding approach (Fig. 5), could act in concert to support the replication activity of HBV in the host. The mechanistic aspect of SMARCE 1 on HBV replication needs to be further explored which should provide insights into defining virulence and fitness of a virus.

It is interesting to note that our *in vitro* analysis showing an overall reduction in activity (either in HBsAg or promoter activity) for HBV genome carrying naturally occurring deletions, while the opposite situation with high level of HBV DNA has been observed in clinical samples [9]. This paradox could be explained by the more heterogeneous nature of clinical samples, as these may contain not just the HBV variant with deletions in the core promoter but also other mutant strains. Furthermore, natural mutations occurring on other parts of HBV genome could also influence the overall replication. Nevertheless, the *in vitro* analysis reported in our study provides a valuable platform to study the underlying mechanism of regulation in a homogeneous condition.

In addition to our earlier report [9], deletions within HBV core promoter have been identified in other clinical samples [20]. Similarly to our findings in this study, such deletions have been found to reduce the amount of extracellular HBsAg [21]. However, no cellular proteins recognizing HBV core promoter with deletions have been characterized to date. Therefore, the identification and characterization of SMARCE1 reported in this study not only provides new approach in analyzing DNA-protein interactions, but also provide a starting point for further molecular understanding of regulation of HBV replication by host proteins.

In summary, a host protein SMARCE1 immobilized on a membrane was isolated by direct binding assay using a DNA fragment corresponding to HBV core promoter with

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deletion. Furthermore, SMARCE1 was found to bind to and repress the replicative efficiency of HBV genome containing the same deletion in viral promoter. Our approach has opened a new way to study DNA-protein interaction and more significantly hold promise in identifying a new class of targets for the intervention of chronic hepatitis B infection.

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Materials and methods

Construction of Plasmids

Construction of the replicative HBV genome containing deletions in the viral core promoter was carried out using pcDNA3.1+ mammalian expression vector (InvitroGen, USA), as described previously (Pan et al., 2005). Plasmids pGL3-Control (a Luciferase plasmid with SV40 enhancer and promoter) and pGL3-Promoter (an enhancerless luciferase plasmid with SV40 promoter upstream of luciferase gene) were from Promega (USA). Plasmid pGL3-Promo/wt was constructed by amplifying the basic functional unit of Enh II by PCR using primers LucF5'-GCACGCGTCAACGACCGACCTTGAGG-3' LucR5'-GCAGATCTACCAATTTATGCCTACAGCCTC-3' comprising HBV nucleotide positions 1686–1801. The 131-base-pair PCR fragment was MluI/BglII-digested and ligated with MluI/BglII digested pGL3-Promoter. The other deletion

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constructs were constructed using pcDNA3.1+/DEL1 and pcDNA3.1+/DEL2 as template to introduce the HBV Enh II deletions. The first deletion was at nucleotide position 1749-1768, the second deletion was at nucleotide position 1758-1777. All constructs were confirmed by sequencing. SMARCE1 expression construct was carried out by cloning a 1.2-kb RT-PCR fragment corresponding to its coding region (amplified from total RNA extracted from HepG2 cells) into the pXJ40 plasmid (kindly provided by Dr. CG Koh), in-frame with a HA tag.

Cell Culture and Transfection

Three cell lines (HepG2, Chang liver cells, and 293T) were used in this study. They were maintained at 37ºC in a humidified atmosphere of 5%CO_2 in DMEM supplemented with penicillin (100IU/ml) and Streptomycin (100µg/ml) and 10% fetal calf serum. Transfection of individual construct was carried out using Effectene (Qiagen, Germany) , and culture medium and/or cells were harvested 48 hrs after transfection.

Measurement of Extracellular HBsAg

2 µg of each of the constructs with deletions in the viral core promoter, as well as the wild type HBV genome in pcDNA3.1 vector were transfected separately into cells (HepG2, Chang or 293T) using Effectene transfection reagent (Gibco BRL, Life Technologies, USA). Each transfection was carried out in three independent experiments. Two days after transfection, culture medium was collected and 200 µl were used for the measurement of HBsAg (Auszyme kit, Abbott Laboratories, USA).

Quantification of HBV DNA from Supernatant and Intracellular mRNA by Real-Time PCR

QIAamp DNA Blood Mini Kit (Qiagen, Germany) was used to isolate HBV particles from culture medium of transfected cells. Conversely, mRNA from transfected cells was

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isolated using Oligotex mRNA mini kit (Qiagen, Germany). Relative HBV DNA level was measured by real-time PCR (ICycler Instrument, Bio-Rad, USA) using primers specific to HBV core gene. Experiments were done in triplicate.

Luciferase Assay

For the luciferase assays, 1μg of plasmid DNA together with 1 μg of control/promoter luciferase plasmid-DNA pBind was used for each transfection mix, and, after incubation for 48 h at 37 ºC, cells were harvested with Cell Culture Lysis Reagent (CCLR; Promega). 20 μl of cell lysates was mixed with 100 μl of Luciferase Assay Reagent (Promega), and luciferase activity was measured as relative light units determined with a Turner 20/20 luminometer (Promega, USA). pBIND luciferase activity was used to normalize the transfection efficiency. Relative luciferase activity was expressed as fold increase over vector without the enhancer element. Experiments were performed in triplicate. For analysis of SMARCE1, luciferase assay was also applied as follows: 293T cells were first transfected with luciferase reporter constructs and SMARCE1 expression vector. After removing the culture medium and rinsing twice with PBS, 200 μl of cell lysis buffer was added to the cells, which were then shaken at room temperature for 15–20 min. The cells were dislodged by scraping or pipetting and then transferred to a 1.5-ml microcentrifuge tube before being centrifuged at 14,000 rpm at room temperature for 1 min to remove cellular debris. 10 μl of the cell extract was mixed with 50 μl of substrate (Promega, USA), and luminescence measured.

Preparation of Nuclear Protein Extracts and Gel-Shift

Cultures were trypsinized, rinsed twice with ice-cold PBS, and incubated on ice. Nuclear extraction from cells was conducted using the NE-PER Extraction kit (PIERCE, USA) according to the manufacturers' instruction. Nuclear extracts were then aliquoted and stored at -80 ºC. Protein concentration was quantitated with the Protein Assay kit (Bio-Rad Laboratories, Hercules, USA) using Ig G as standard. Gel shift assay was conducted using the Gel shift assay kit (PIERCE, USA) according to the manufacturers' instruction.

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Binding reaction procedures were performed at 37 ºC for containing 3 μg of HepG2 nuclear extracts, free DNA and DNA–protein complexes were resolved on 6% non-denaturing polyacrylamide gels. The sequences of the oligonucleotide probes were as follows:

WT forward: 5'-(biotin) gggaggagct gggggaggag attaggttaa aggtctttgt attaggaggc tgta-3'

WT reverse: 5'-taca gcctcctaat acaaagacct ttaacctaat ctcctccccc agctcctccc-3'

DEL 1 forward: 5'-(biotin) gggaggagctgggggag tgtattaggaggctgta-3'

DEL1 reverse: 5'-tacagcctcctaataca ctcccccagctcctccc -3'

Protein-DNA Array Analysis

For DNA-protein interaction assay, the procedure was conducted using the protein-DNA array kit (Panomics, USA) according to the manufacturers' instruction. Briefly, annealed biotinylated probes were incubated with the membrane with immobilized transcription factors for 30 min at room temperature and after wash, the membrane was incubated with the diluted strptavidine-HRP for 30min at room temperature.

Confirmation of DNA-Protein Interaction

For gel shift assays, the nuclear extracts from 293 T cells which overexpress pXJ40-SMARCE1 were incubated with individual biotin-labeled probes in binding buffer for 20 min at room temperature. The negative control consisted of free probe and without nuclear extracts. For the competition control, an excess of unlabeled cold probes (wild type cold probes and deletion 1 cold probes respectively) was added to the sample containing nuclear extract and biotin-labeled probe. The samples were separated on a 6% polyacrylamide gel in 0.5% Tris-borate-EDTA, transferred onto a nylon membrane, and fixed on the membrane by UV cross-linking. The biotin-labeled probe was detected with streptavidin-horseradish peroxidase (Pierce, USA). For supershift assay, in addition to the procedures as described above, with the exception that 2 μg of anti-HA (Santa Cruz) antibodies was incubated with the nuclear cell extract for 30 min on ice before the

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addition of the biotin-end-labeled DEL1 probe. The samples were then treated as described above.

Western Blot Analysis

The protein extracted from 293T cells transfected with pXJ40-SMARCE1 vector and from normal 293T cells was separated on a 7.5% SDS gel and electroblotted onto nitrocellulose membranes. The membrane was then incubated with a monoclonal antibody against HA (Santa Cruz, USA). After incubation with a secondary antibody (PIERCE, USA), the membrane was overlaid with luminol enhancer and substrate for 5 min. The image was acquired using a fixer and developer (Kodak Corp., USA).

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Table 1 Identity of Transcription Factors Immobilized on TF Array

TF Protein Array Version III

	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18	19 20	21 22	23 24
A	PFDN4	POU2AF1	PPARα	PPARβ	PPARγ1	PPARγ2	PSMC5	PTGER2	PTTG	PTTG2	PURA	pos
B	PXR-2	REL	ETS1	REVERB	RFXANK	RXR1B	RXRG	RYBP	SCML1	SHP	SIX2	pos
C	SMARCB1	SMARCE1	MEF2A	SP1	SP3	SP4	STAT1	STAT3	STAT4	STAT6	SURB7	pos
D	TCEA1	TCF7L2	PXR-2	TF2D	TFE3	TFEC	TLX	TRb1	TRCP	TRIP13	v-Myc	pos
E	YY1											pos
	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos

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Legend to Figures

Fig. 1 Quantification of HBV DNA by Real-Time PCR. Two days after transient transfection with HBV wild type or deleted constructs, HBV DNA was measured in culture medium. Mean values and standard deviations from three independent experiments were shown. Values were normalized to HBV copy number of the wild type construct (100%).

Fig. 2 Quantification of HBV Core gene Relative to GAPDH Expression. Quantification of intracellular HBV Core gene expression in cells transfected with replicative HBV geome with deletions in core promoter, in relation to that of cells transfected with wild type HBV genome. Means and standard deviations were based on more than three independent experiments.

Fig. 3 Promoter activity assay in three cell lines. (a) HepG2, (b) Chang liver cell line, and (c) 293T. Each type of cells was transfected with the respective Enh II constructs (wild type, DEL1 or DEL2). For each cell type, the first column represented the ratio of the luciferase activity of internal positive control (promoter and enhancer). The second column (labeled as 'negative') represented the ratio of activity of the negative control (promoter but no enhancer). The other columns of each cell line represented the ratio of activity between vectors with the promoter and the Enh II (wild type, DEL1 or DEL2) to that of pBIND. Results of the luciferase assay were normalized to the level of the internal positive control set at 100%. (For each cell line, the difference between wild type and DEL1 was significant as indicated by a $p<0.05$. The difference between wild type and DEL2 was also significant by a $p<0.05$.).

Fig. 4 Analysis of Cellular Protein Binding to Probe Covering Deletion nt 1749-1768. Electrophoretic mobility shift assays were performed using HepG2 nuclear extracts with two different biotinylated probes: the wild type and DEL1. These DNA fragment was end labeled with biotin, incubated with 5μg of HepG2 nuclear extract, and electrophosed on a 6% nondenaturing polyacrylamide gel. Lane 1, labeled wild type probe alone without

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nuclear extract. Lane 2, labeled wild type probe and HepG2 nuclear extract with 200 fold of unlabeled specific competitor. Lane 3, labeled wild type probe with $HepG_2$ nuclear extract. Lane 4, labeled DEL1 probe alone without nuclear extracts. Lane 5, labeled DEL1 probe and HepG2 nuclear extract with 200 fold of unlabeled specific competitor. Lane 6, labeled DEL1 probe with $HepG_2$ nuclear extract probe. I, II, III, and IV marked the locations of DNA-protein complexes I, II, III, and IV respectively. FP indicated free labeled DEL1 probe.

Fig. 5 DNA-protein Array Assay. The response elements on the array are spotted in duplicate. The right and bottom sides of the array indicate where biotinylated DNA were spotted. The transcription factor array was incubated with the biotinylated DEL1 probe (panel A) and both biotinylated and an excess of non-biotinylated DEL1 probe (panel B). Specific DNA-protein interactions (boxed) were competed out with non-biotinylated probe. Panel C and D: results of the array incubated with wild type probe. The array was incubated with the biotinylated wild type probe (panel C) and both biotinylated and an excess of non-biotinylated wild type probe (panel D). Specific Protein/DNA interactions (boxed) were competed out with non-biotinylated probe.

Fig. 6 Analysis of SMARCE1 Protein Level. Western blot analysis was carried out using HA tag monoclonal antibody. A 46kD protein corresponding to SMARCE1 was detected in lane 1 (293T cells transfected with pXJ40-SMARCE1). No protein was detected in lane 2 (293T cells transfected with pXJ40 plasmid).

Fig. 7 Gel Shifty Assay on DNA Binding Specificity of SMARCE1 Expressed in Mammalian Cells. Panel A, binding of probes (wild type or DEL1) to nuclear extracts from 293T cells transfected with pXJ40 plasmid. 200 fold in excess of non-biotinylated DNA probes were added as competitors in gel shift assays. I, II, III , IV and V mark the location of the complexes. Panel B, binding of probes (wild type or DEL1) to nuclear extracts from cells transfected with pXJ40-SMARCE1 vector and producing SMARCE1 protein. Panel C, supershift assay in the presence of anti-HA antibody. Lane 1, only the labeled DEL1 probe was added. Lane 2, the labeled DEL1 probe was incubated with

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nuclear extract from cells transfected with the SMARCE1 construct. Lane 3, 200 fold in excess of the unlabeled DEL1 probe was added with the labeled DEL1 probe and the SMARCE1 expressing nuclear extract. Lane 4, the anti-HA antibody was added to the SMARCE1 expressing nuclear extract before the addition of labeled DEL1 probe. Arrowhead indicated the partial shift of DEL1-SMARCE1 complex.

Fig. 8 Luciferase Activity with or without SMARCE1. 293T cells transfected with the respective Enh II constructs (wild type or DEL1) were assayed for the effects of SMARCE1. For each cell type, the first column represented the ration of the luciferase activity of internal positive control (promoter and enhancer) from pBIND plasmid. The second column represented the ratio of activity of the internal negative control (promoter only). The other columns of represent the ratio of activity between Enh II (wt, del1, del2) vectors to that of positive control. Cells cotransfected with pXJ40-SMARCE1 plasmid was in shaded column. Results of the luciferase assay were normalized to the level of the internal positive control (set at 100%).

Fig. 9 Semi-quantitative Measurement of Extracellular HBsAg. Two days after transient cotransfection with HBV replicative genome and SMARCE1 overexpression vector and empty vector, the culture medium was collected and HBsAg was measured by IMX (Abbott Laboratories, USA). Mean HBsAg values were obtained from three independent experiments.

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Figures

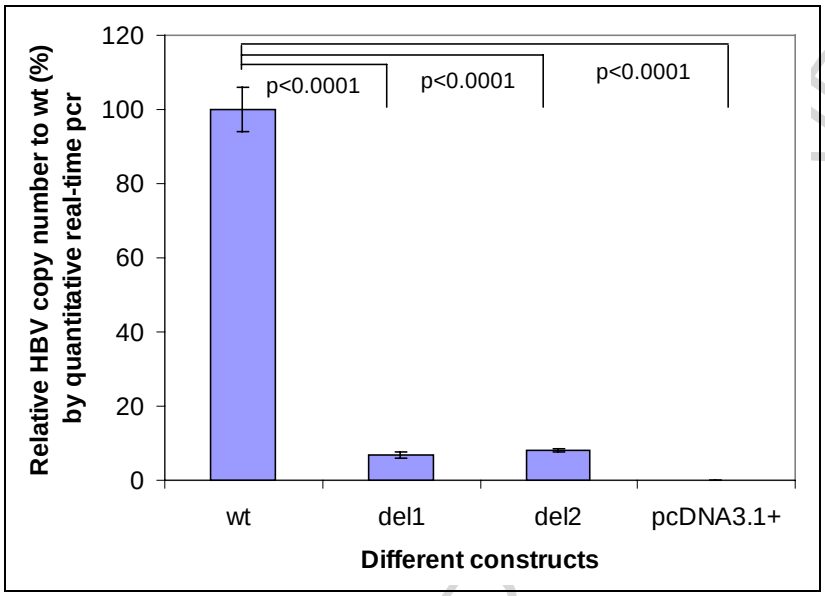

Figure 1

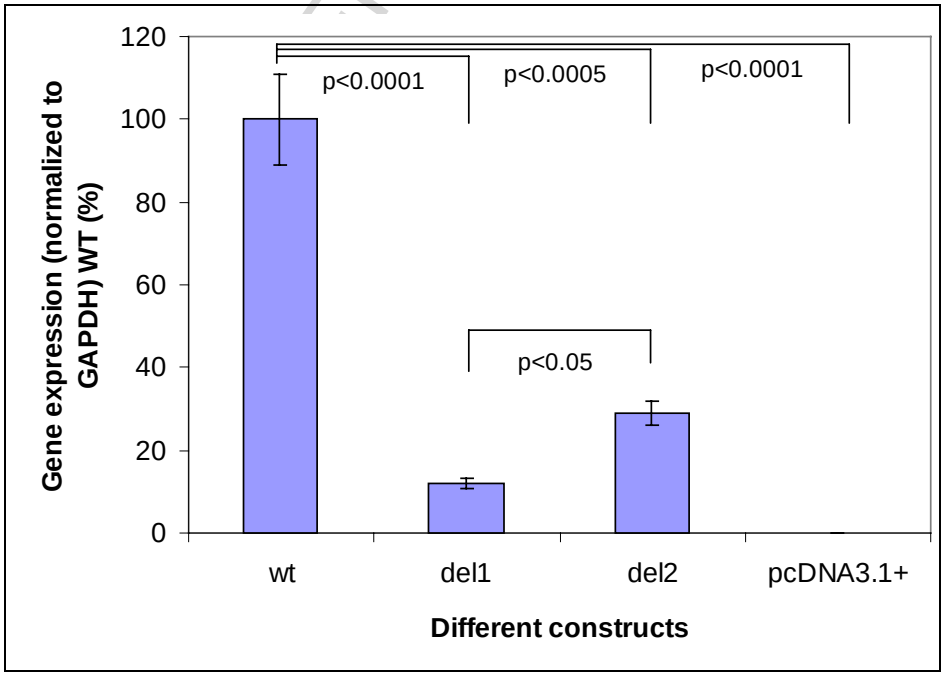

Figure 2

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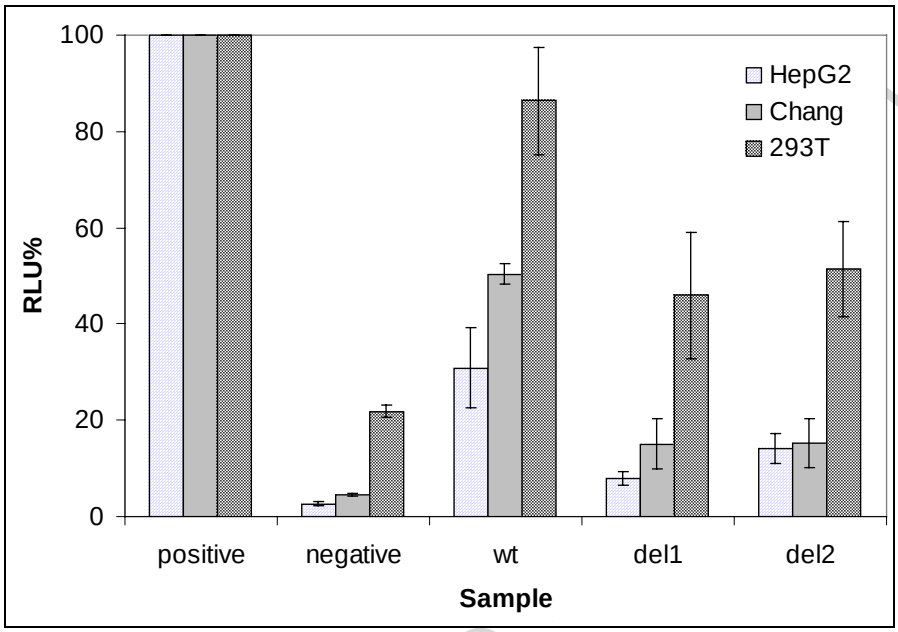

Figure 3

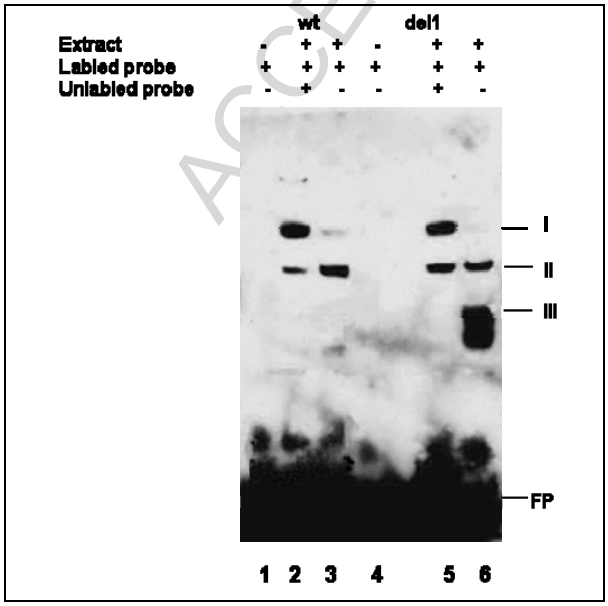

Figure 4

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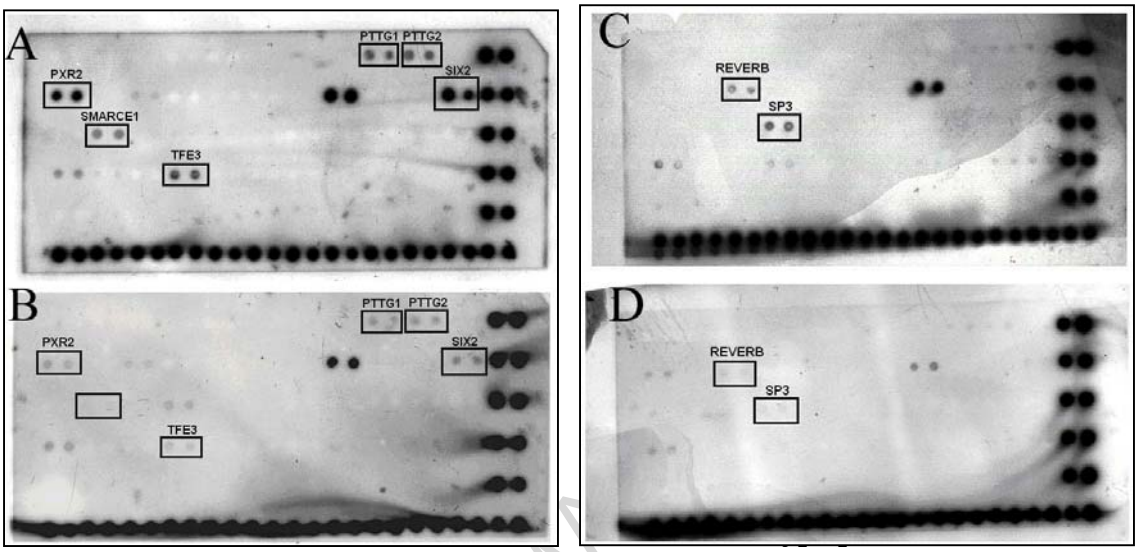

Figure 5

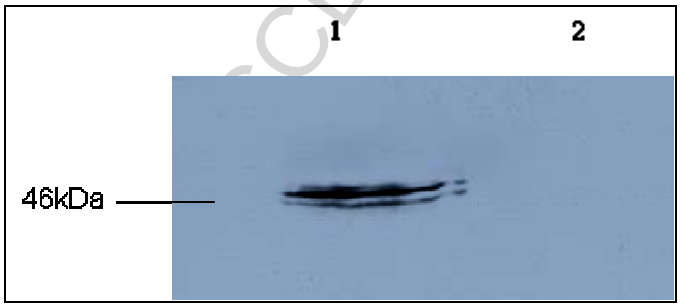

Figure 6

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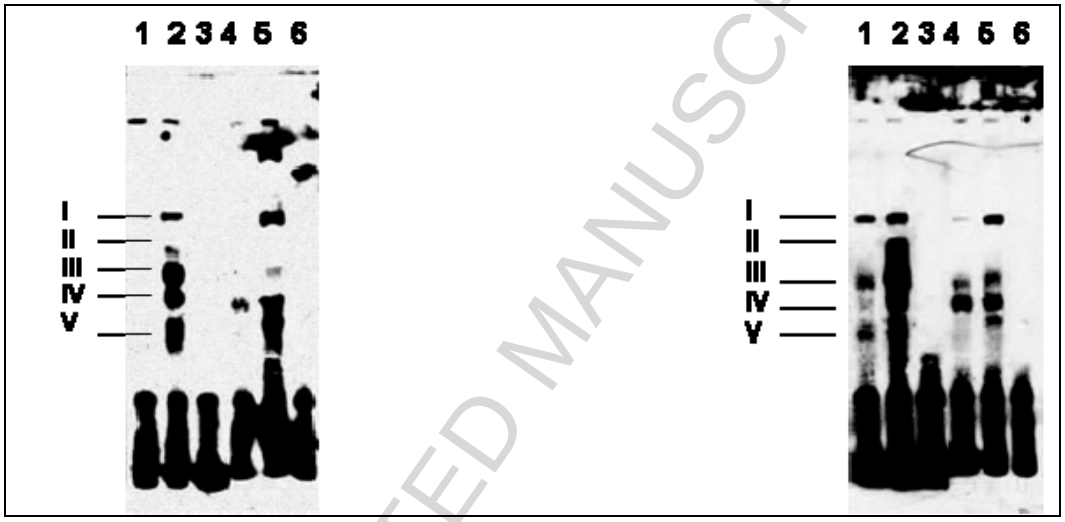
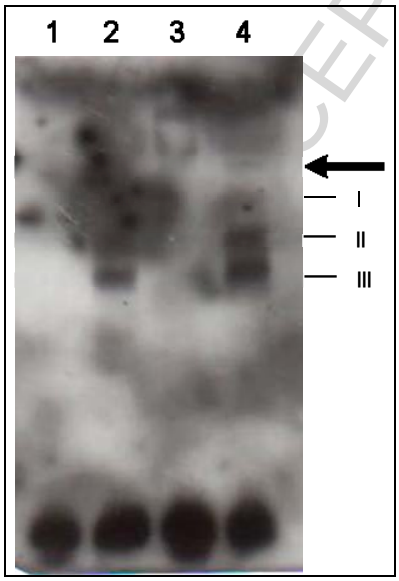
Lane No.	1	2	3	4	5	6
Cells Nuclear extracts	+	+	--	+	+	--
Labeled wt probe	--	--	--	+	+	+
Unlabeled wt probe	--	--	--	+	--	--
Labeled del1 probe	+	+	+	--	--	--
Unlabeled del1 probe	+	--	--	--	--	--



Panel A Panel B



Panel C

Figure 7

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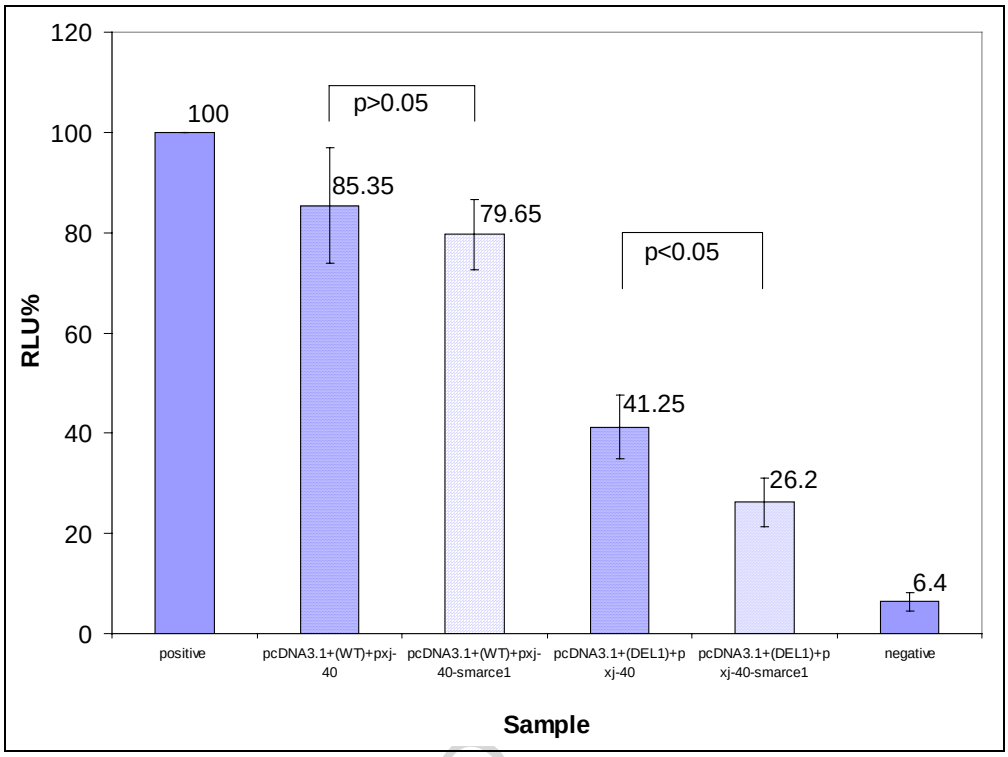

Figure 8

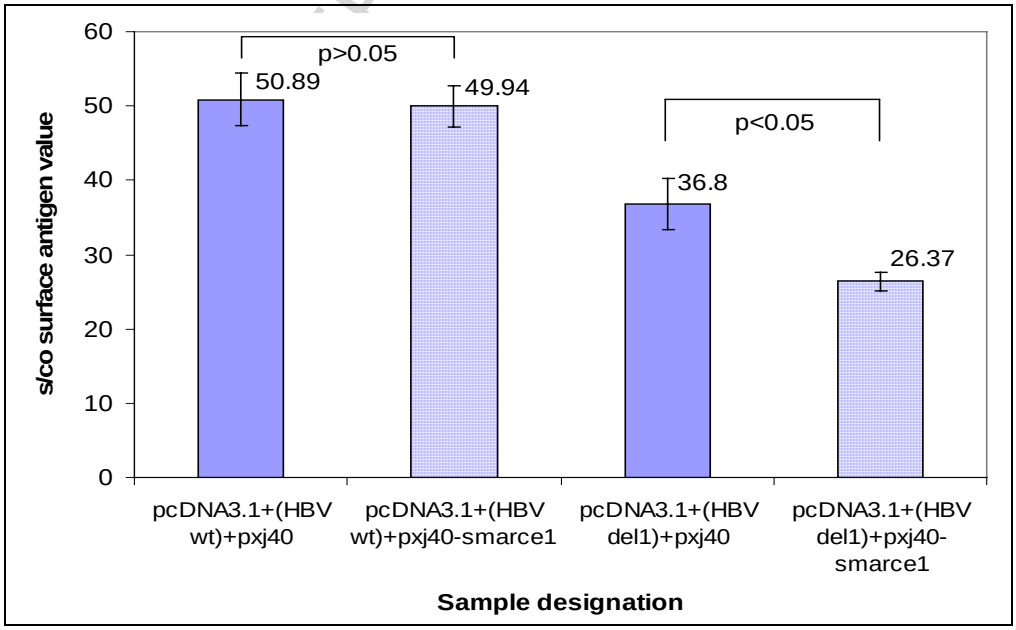

Figure 9