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RAPID COMMUNICATION

Genotype-dependent activation or repression of HBV enhancer Ⅱ by transcription factor COUP-TF1

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Abstract

AIM: To study the expression of HBV enhancer Ⅱ by transcription factor COUP-TF1.

METHODS: In order to study the regulation of HBV variants in the vicinity of the NRRE we cloned luciferase constructs containing the HBV enhancer Ⅱ from variants and from HBV genotypes A and D and cotransfected them together with expression vectors for COUP-TF1 into HepG2 cells.

RESULTS: Our findings show that enhancer Ⅱ of HBV genotype A is also repressed by COUP-TF1. In contrast, two different enhancer Ⅱ constructs of HBV genotype D were activated by COUP-TF1. The activation was independent of the NRRE because a natural variant with a deletion of nt 1763-1770 was still activated by COUP-TF1.

CONCLUSION: Regulation of transcription of the HBV genome seems to differ among HBV genomes derived from different genotypes. These differences in transcriptional control among HBV genotypes may be the molecular basis for differences in the clinical course among HBV genotypes.

Key words: Hepatitis B virus; Hepatitis B virus x protein; COUP-TF1; Genotype; Enhancer Ⅱ

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INTRODUCTION

Hepatitis B virus (HBV) is a major health burden for the world[1]. In an otherwise immune competent host, HBV concentrations of 10^{12} to 10^{13} genome equivalents (GE)/l are frequently found. Thus, in a chronic carrier up to 10^{13} virions are produced per day[2]. Due to the high replication capacity[2,3] and the high error rate of the viral polymerase, HBV genomes with all possible single mutations and double mutations of every nucleotide of the HBV genome are produced every day[3]. Variants of HBV with point mutations, deletions or insertions have been described all over the genome of HBV[4-7]. In addition to viral variants the variability of the virus is increased by the divergence of HBV into 8 genotypes A-H that differ by at least 8% when comparing whole genomes[8-10]. The transcription of the hepadnaviral pregenomic (pG) and precore (pC) RNA is regulated by transcription factors binding to a region containing the enhancer Ⅱ (EII) and the core promoter (Figure 1A)[11]. The expression of both pG and pC RNA is tightly coupled[11], however, natural variants[12] and point mutations[13] located in this transcriptional element can uncouple the transcription of these RNAs. The variants and artificial point mutations that uncouple the transcription of the pG and pC RNA affect a site in this transcriptional element called the nuclear receptor responsive element (NRRE)[13,14]. COUP-TF, a member of the nuclear receptor family[15], binds to the NRRE and has been found to repress transcription from HBV enhancer Ⅱ/pC/pG promoter[13].

We have previously characterised an outbreak of HBV in children in a department of oncology[16,17]. In 1992 we observed a deletion of nt 1763 to 1770 affecting the c-terminus of HBx, enhancer Ⅱ/pC- and pG promoter[18] in serum from a single patient. Later on when we examined serum from 20 patients by amplification of the whole genome from different time points after infection, three

additional patients were found to have the deletion of 8 bp in enhancer Ⅱ[19]. This deletion was found mainly in sera from time points late after infection. The deletion of nt 1763-1770 was found to increase the ratio of pG to pC RNA, the replication of HBV *in vitro*, and to generate a new binding site for the transcription factor HNF-1[12]. However, the deletion of nt 1763-1770 also deletes large parts of the binding site for COUP-TF, which was mapped to 1755-1768[20-22]. We thus analysed whether enhanced replication from variant enhancer Ⅱ/pC/pG promoter may also be caused by the deletion of the binding site for the repressor COUP-TF1. In contrast to previous studies we used HBV genome isolates with different genotype backgrounds and observed significant differences.

MATERIALS AND METHODS

Transfection and luciferase-assay

All experiments were performed with the differentiated human hepatoblastoma line HepG2[23] cultivated in RPMI1640/10% FCS. The different expression constructs were used in luciferase assays. Briefly, 1-1.5 × 10^6 seeded cells were transfected with 1.5 μg luciferase construct and 3 μg of the COUP-TF1 or HNF-1 expression constructs using Lipofectamine (Life Technologies) according to the manufacturers description. All plasmids used were endotoxin free purified (EndoFree, Quiagen). After 48 h the transfected cells were harvested and lysed in Tris-buffer (250 mmol/L, pH 7.8) by 3 cycles of freezing and thawing. Protein concentration of lysates was determined by BCA-assay (Pierce). 15 μg of lysate protein adjusted to 50 μL with Tris-buffer were used in the luciferase activity assay[24]. The results are shown as factors relative to basal luciferase expression and represent the mean values of three independent transfections.

Plasmids

All constructs for reporter assays were cloned into pLuci3 (Promega). The derived constructs were controlled by sequencing. pLEII-A-991 and pLEII-D-Ari: contain the enhancer II/core promoter sequence of genotype A (GenBank: X51970) or D (GenBank: Y07587) from nt 1400 to 1903 and have been described in[25]. For pLEII-D-2.2.15 nt 1400 to 1903 were amplified from supernatants of the HBV expressing cell line HepG2.2.15[26] and cloned accordingly. The sequence of this fragment was identical to GenBank U95551. HBV enhancer Ⅱ, genotype D, with a corresponding truncated construct pL-EII (1730-1822). Derived from this sequence, constructs with an 8bp deletion of nt 1763-1770 or the frequently observed double mutation of 1762 (A/T), 1764 (G/T) have been cloned respectively (pL-EII(1730-1822)d8bp and pL-EII(1730-1822)A/T). For cotransfection expression vectors for HNF1[27] or COUP-TF1[28] were employed.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described by[24] using nuclear extracts from HepG2 cells. For EMSAs the following oligonucleotides with the corresponding binding sites were used: wt (HBV-1755-1805-bs-s): 5'-GAT CCT TAG GTT AAA GGT CTT TGT ATT AGG AGG CTG TAG GCA TAA ATT GGT CTG CGA-3'; wt (HBV-1755-1805-bs-as) 5'-GAT CTC GCA GAC CAA TTT ATG CCT ACA GCC TCC TAA TAC AAA GAC CTT TAA CCT AAG-3'; 8bp-Deletion (bs-HBVd8-1755-1805-s) 5' -GAT CTT AGG TTA AAT ATT AGG AGG CTG TAG GCA TAA ATT GGT CTG CGC A-3'; 8bp-Deletion (bs-HBVd8-1755-1805-as 5'-GAT CTG CGC AGA CCA ATT TAT GCC TAC AGC CTC CTA ATA TTT AAC CTA A-3'; 8bp-Deletion (HBVd8-dTBP-1755-1805-s) 5' -GAT CTT AGG TTA AAT ATT AGG AGG CTG TAG GCA TAA ATT GGT CTG CGC A-3'.

RESULTS

To analyse the effect of COUP-TF1 on a frequently found natural variant of the HBV EII/pC/pG promoter we cotransfected expression constructs for HNF1 and COUP-TF1 together with a luciferase reporter construct containing nt 1730-1817 (pL-pC-D-Ari) or nt 1730-1902 (pL-preG-D-Ari) (Figure 1A-C) into HepG2 cells as described[25].

HNF1 showed a very weak transactivation of both promoter constructs irrespective if wt or the deletion construct were employed. COUP-TF1 showed a strong transactivation of all constructs. It appeared as if the transactivation was higher when constructs that contained the deletion of nt 1763-1770 were used. Two aspects of the latter results were surprising: COUP did not repress transcription from the wt-pG promoter as expected from previously published data[13] and the deletion of a large part of the binding site for COUP in the Δ8bp-pG and -pC construct had no effect on the activation by COUP.

Our results for the pG and pC construct (nt 1730-1900) were seemingly in conflict with the data of[13] who reported a repression of nt 1443 to 1990 of HBV by COUP. Thus, we cloned nt 1400 to 1902 into luciferase constructs. Figure 1C shows that COUP-TF1 also activated expression from the complete enhancer Ⅱ of the isolates described in[18,19,29]. No upstream element seemed to influence the activation of enhancer Ⅱ by COUP-TF1 because all deletion constructs of pLEII-D-Ari and pLEII-D-Ari-Δ8bp were activated by COUP-TF1 (Figure 1).

Because pLEII-D-Ari was cloned from serum of patients infected during massive immune suppression and HBV genomes from these patients are known to contain many variants[19], we analysed the effect of COUP-TF1 on enhancer Ⅱ from an assumed wt-HBV genotype D genome. Figure 2 shows that this construct was also activated by COUP-TF1 even stronger than pLEII-D-Ari. In comparison we analysed a similar construct cloned from genotype A[25]. Quite in contrast to the activation of pLEII-D-Ari, the genotype A construct pLEII-A991 was repressed by COUP-TF as reported for a construct containing enhancer Ⅱ of HBV genotype C[13].

COUP-TF1 is known to exert its repressive effect on HBV enhancer Ⅱ of HBV genotype C through the NRRE around nucleotide 1755-1768[13,14]. However, activation of HBV EII by COUP-TF1 also was observed when we tested constructs with a deletion of nt 1763-1770.

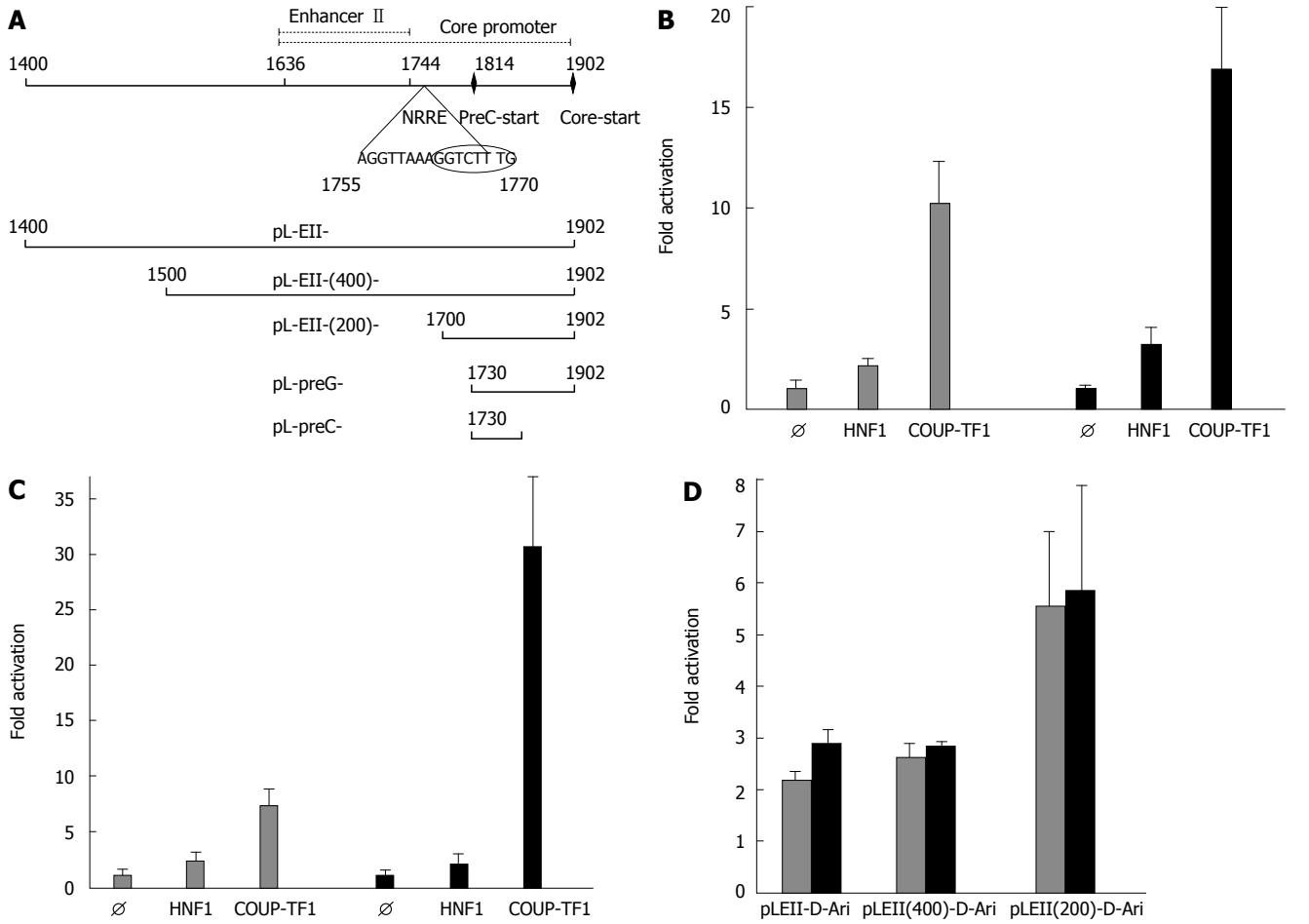

Figure 1 Structure (**A**) and activation of the precore (**B**), the pregenomic (**C**) and enhancer II (**D**) of HBV genotype D by COUP-TF1. **A**: Schematic structure of HBV enhancer II and the core promoter. The sequence of the nuclear receptor response element (NRRE) and the natural deletion of nt 1763-1770 (encircled) is indicated. Below the transcriptional elements the HBV fragments cloned into the luciferase reporter vector pGL3 are shown. For cloning the indicated nucleotides from two plasmids containing wt (GenBank: Y07587) and HBV with a deletion of nt 1763-1770[18] were amplified and cloned. HepG2 cells were transfected with luciferase reporter constructs containing pregenomic promoter (pL-preG-D-Ari, wt in grey); **B**: Precore promoter (pL-preC-D-Ari, wt in grey) **C**: The complete and truncated wt enhancer II **D**: (pLEII-D-Ari, in grey) corresponding variant constructs with a deletion of nt 1763-1770 (Δ8bp) in black. For cotransfection the empty expression vector (Ø) or expression vectors for HNF1[27] or COUP-TF1[28] were employed.

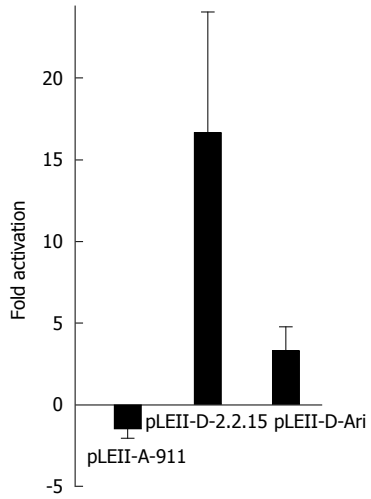

Figure 2 Influence of the nuclear receptor COUP-TF1 on enhancerII/core promoter constructs of different HBV genotypes. HepG2 cells were transfected with luciferase constructs for the complete enhancer II (1400-1902) of genotype A (pLEII-A-991) or two constructs (pLEII-D-Ari and pLEII-D-2.2.15) derived from genotype D and the expression vector for COUP-TF1.

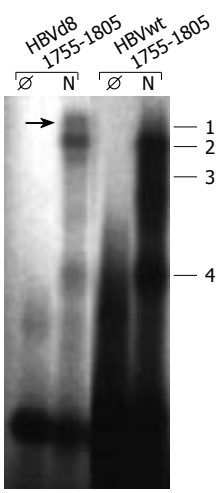

Figure 3 Deletion of nt 1763-1770 leads to altered band shifts in electrophoretic mobility shift assay with oligonucleotides nt 1755-1805. EMSA was performed as described by[25] with oligonucleotide nt 1755-1805 wt (HBVwt 1755-1805) and with a deletion of nt 1763-1770 (HBVd8 1755-1805) incubated with (N) or without (Ø) nuclear extract prepared from HepG2 cells with a method[25].

Thus, activation by COUP-TF1 may be exerted through other binding sites. To study the binding of the NRRE by cellular proteins we performed EMSAs with nuclear extracts prepared from HepG2 cells as described in[24]. Figure 3 shows that the wild type oligonucleotide was bound by several proteins purified from nuclear extracts of the human hepatocyte line HepG2. Four bands could be discerned in the EMSA with nuclear extract. Band 2 and 4 were common to wt and the 8 bp deletion. Band 1 was unique to the variant, band 3 to the wt.

DISCUSSION

Our results show that the ubiquitous transcription factor COUP-TF1 was a strong activator of wt and variant pG, pC promoter and enhancer Ⅱ constructs (Figure 1 and 2) derived from sera of a single source outbreak[18,19,29]. This result was surprising because Yu *et al*[13] reported that the wt-enhancer Ⅱ (nt 1443 to 1990) was repressed by COUP-TF1. The involvement of distal elements in enhancer Ⅱ that may be needed for activation by COUP-TF1 is unlikely because we did not observe a change in activation by deletion of upstream sequences (Figure 1D).

The NRRE seems to be dispensable for activation by COUP-TF1 in our genotype D constructs because all constructs containing a deletion of nt 1763-1770 were activated by COUP-TF1 as well as the wt-constructs (Figure 1). In addition, no binding of COUP-TF1 was observed by EMSA when we used an oligonucleotide containing a frequently found deletion of nt 1763-1770 (data not shown). These data support the findings of Yu and Mertz who reported that two frequently occurring natural point mutations of nt 1764 and 1766 also abolish binding by COUP-TF1[14].

Our data are compatible with the report from the group of Mertz if HBV genotype differences are taken into consideration. Yu *et al*[13] found that the NRRE from nt 1755-1768 was essential for the repression of transcription from HBV enhancer Ⅱ, pC and pG promoter of genotype C[13,14]. We have analysed activation of HBV enhancer Ⅱ, pC and pG promoter of genotype D by COUP-TF1. For activation by COUP-TF1 nt 1763 to 1770 of the NRRE seem to be dispensable. Thus, our data imply that activation of HBV enhancer Ⅱ, pC and pG promoter is regulated by other elements than the NRRE. We can not rule out the possibility that COUP-TF1 exerts its activation of enhancer Ⅱ of HBV genotype D by indirect mechanisms not involving direct binding to the promoter as in the activation of the vHNF1 promoter[30].

Further analyses with constructs containing HBV DNA from other sources showed that very similar constructs of HBV enhancer Ⅱ reacted differently to COUP-TF1: constructs derived from genotype A (Figure 3) or C [13] were repressed by COUP-TF1 whereas constructs of genotype D derived from two different sera were activated. A sequence analysis (data not shown) of these four constructs revealed no differences in the NRRE at bp 1755 – 1768, which has been shown to be essential for repression by COUP-TF1[13,14]. However, the sequences upstream of the NRRE showed sequence variability as expected for HBV isolates of different genotypes. However, we were not able to detect a sequence motif that may be indicative for activation or repression by COUP-TF1.

HBV genotypes influence the course and outcome of preventive and therapeutic measures[8-10,31-33]. Very limited data are available on the effect of the sequence variability on *in vitro* properties of HBV, which may explain the different outcome of HBV infections depending on the genotype of HBV. In a relatively large analysis using HBV constructs of HBV genotypes A, C, D and E, Sommer *et al* observed differences in splicing of the HBV pregenome[34]. Other groups observed higher repression of apoptosis by HBx derived from genotypes D compared to HBx of genotype C origin[35]. Using another system, HBx of genotype D also showed a higher activity than HBx of genotype A in the induction of apoptosis[36].

Thus, the variability induced by HBV genotypes may result in different molecular biology of HBV. However, there is more research to be done because our current results do not answer the question whether the differences described by several groups can be attributed to properties conserved in a given genotype or only represent certain variants that may occur in all 8 HBV genotypes.

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