Site-specific mutation of the hepatitis B virus enhancer II B1 element: effect on virus transcription and replication

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The hepatitis B virus (HBV) enhancer II (EII) is highly liver-specific and plays an important role in regulating the transcription of all HBV genes. In this report, mutational analysis on the B1F-binding site in the major functional unit of HBV EII is described. The activity of HBV EII in EII–CAT reporter plasmids was significantly decreased when the sequence of the B1F-binding site in EII was mutated. Furthermore, a single point mutation in the B1 element that aborted the binding of B1F caused a dramatic decrease in viral gene transcription initiated from the HBV core promoter, which resulted in a reduction of the production of the HBV e antigen and pregenomic RNA, the template for viral DNA replication. In conclusion, the interaction of B1F with its target binding sequence in the EII region is crucial for liver-specific transcription and DNA replication of the virus.

With more than 350 million people worldwide chronically infected with hepatitis B virus (HBV) and at a high risk of developing a hepatocellular carcinoma, HBV infection represents a major health problem (reviewed by Kew, 1981). HBV is a hepatotrophic virus with a partially double-stranded DNA genome of approximately 3.2 kb in length. This genome contains four major open reading frames (ORFs), encoding the surface antigens, the core antigen and its secretable derivative HBsAg, the polymerase and the HBx transactivator (reviewed by Ganem & Varmus, 1987; Tognoni et al., 1985). Transcription of these genes is regulated by four promoters and two enhancers in the viral genome (Shaull et al., 1985; Tognoni et al., 1985; Yee, 1989; Schaller & Fischer, 1991).

The remarkable liver tropism of HBV can be attributed to at least two mechanisms: (i) the preferential attachment and entry of the virus into the host cell via an as yet unknown hepatocyte-specific receptor(s), and (ii) liver-specific virus regulatory elements in the HBV genome. One such regulatory element is the HBV enhancer II/core promoter region (EII/Cp), which is located immediately upstream from the core ORF and regulates the transcription of a set of viral RNAs of about 3.5 kb in length. These 3.5 kb RNAs encode not only the nucleocapsid proteins and the viral polymerase, but the shortest form of this 3.5 kb set of RNAs also serves as the pregenomic RNA, which is encapsidated and reverse transcribed during HBV replication and, thus, represents the template for HBV DNA synthesis (Summers & Mason, 1982; Will et al., 1987). Furthermore, another 3.5 kb RNA is translated to generate HBeAg. Although its biological function remains largely uncertain, HBeAg is an important diagnostic marker: clinical studies have shown that the level of HBeAg correlates with viral DNA replication and virus propagation (Hollinger, 1996).

Transcription of the 3.5 kb RNAs occurs in well-differentiated hepatocytes but not in either dedifferentiated hepatocytes or cells of nonliver origin. This liver-specific regulation of the EII/Cp region is contributed to by the binding of several liver-specific transcription factors within this regulatory region (Lopez-Cabrera et al., 1990, 1991; Yuh & Ting, 1991; Guo et al., 1993; Zhang et al., 1993; Li et al., 1995; Raney et al., 1995). HBV EII has been mapped to a 148 bp DNA fragment ranging from nt 1497 to 1644 (HBV subtype adr4) (Wang et al., 1990), which is located upstream from and partly overlaps Cp. Because of different binding regions for trans-acting factors, EII was initially divided into two fragments, A (nt 1497–1556) and B (nt 1557–1644), with almost 70% of EII activity retained by fragment B. Therefore, fragment B was thought to be the major functional unit of EII and, upon further characterization, was subdivided into three elements, B1 (nt 1557–1575), B2 (nt 1576–1606) and B3 (nt 1607–1644) (based on HBV subtype adr1; Wu et al., 1992).

Since its identification, many efforts have been made to investigate the interaction between EII and liver-enriched (Lopez-Cabrera et al., 1991; Yuh & Ting, 1991; Guo et al., 1993; Li et al., 1995; Raney et al., 1995) as well as ubiquitous transcription factors (Zhang et al., 1993). In our previous report (Li et al., 1998), a novel liver-specific transcription activator, which is an orphan nuclear receptor named B1F, was identified. In transfection assays with nonhepatic cells, i.e. HeLa cells, B1F

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could significantly stimulate EII/Cp activity, which was barely detectable without B1F stimulation. B1F has been shown to bind specifically to the B1 element of EII. In order to gain further insight into how this interaction may contribute to virus biological functions at the molecular level, mutation analysis of the B1F-binding site was performed and its influence on virus transcription in HepG2 cells was investigated.

To test whether the B1F-binding site within the B1 element is important for the activity of HBV EII, four point mutations were introduced into the B1 element by PCR-based site-directed mutagenesis. Firstly, bandshift assays were performed as described previously (Li et al., 1998) to verify that the mutated fragment was no longer capable of binding B1F. As shown in Fig. 1(a), fragment B1m4 (AACGACCCGACCTGAGGCA, lane 2), in contrast to the wild-type B1F-binding site (lane 1), was no longer able to bind B1F. Next, the functional significance of such a mutation was investigated in CAT assays. For these CAT assays, two each of wild-type and B1m4 CAT reporter plasmids were constructed (Fig. 1(b)). These reporter plasmids contain the HBV EII region in one of two locations, either upstream or downstream from an HBV Cp–CAT gene construct. In general, there was a significant decline in the activity of EII if the wild-type B1 sequence was replaced by B1m4 (Fig. 1(c)). Interestingly, there was an approximately 15-fold decline in CAT activity when reporter plasmids pENII.B1CpCAT and pCpCATENII.B1m4 were compared, whereas the reduction in CAT expression was only about threefold if EII was located downstream from the CAT gene. These data indicated that the stimulatory effect of B1F on the transcription of the CAT gene is much more significant if EII is located upstream from Cp, which is the natural arrangement in the viral genome. Therefore, the location of EII upstream from Cp is an important positional factor for B1F to fully exert its stimulatory effect. It is very likely that transcription from Cp as well as its tissue specificity is carefully co-ordinated through factors binding to several sites in EII.

The position of EII and Cp relative to each other should greatly influence the transcription of pregenomic RNAs. Since HBV EII partly overlaps Cp, it is quite difficult to clearly separate one from the other either functionally or physically. In the case of the B1F-binding site, its location-dependent activity may also reflect its role as an upstream regulatory element for Cp rather than that of a classical enhancer element. Taken together, these observations indicate that, especially in the natural order of the...
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E\textsubscript{II}/C\textsubscript{P} region, the interaction between B1F and its target binding sequence is critical for the activity of the HBV E\textsubscript{II}/C\textsubscript{P} element.

Because of the significant implication of the interaction between B1F and its binding site within the E\textsubscript{II} region, it was necessary to investigate how this interaction affected the liver-specific viral gene transcription and virus replication in the context of the whole viral genome. In our previous experiments, it has been shown that the HBV expression plasmid pSPT1.2xHBV is capable of generating all virus transcripts and produces hepatitis B virions after transient or stable transfection of HepG2 cells (Weiss et al., 1996). Plasmid pSPT1.2xHBV contains a terminally redundant HBV genome which is exclusively under the control of autologous virus regulatory elements. The terminal redundancy is required for the construct to generate correct 3'–5' kb virus transcripts. However, because of its terminal redundancy, there are two E\textsubscript{II} regions in pSPT1.2xHBV, one at the 5'-end and the other at the 3'-end of the HBV genome (Fig. 2a). Since CAT assay experiments demonstrated that mutations in the B1F-binding site reduced the activity of E\textsubscript{II} much more significantly if E\textsubscript{II} was located upstream from C\textsubscript{P} (Fig. 1c), we introduced several mutations into the 5'-end of the E\textsubscript{II} region within pSPT1.2xHBV. Fig. 2(b) shows two representative point mutations. The B1F-binding sequence in B1um, CGACCTTGAG, was changed to CTACCTTGAG and introduced into the B1 element of E\textsubscript{II}. This G to T transversion was used because of the bandshift experiment (Fig. 2b, lane 3) that revealed that B1F could not bind to the CTACCTTGAG sequence, while the B1a mutation (CTACCGACCTTGAG), for example, was not able to abolish the binding of B1F.

HepG2 cells were transfected with either pSPT1.2xHBV or pSPT1.2xHBV/B1um and the amounts of HBeAg and HBV s antigen (HBsAg) in the tissue culture supernatant were quantified with a commercial ELISA kit (Murex Diagnostics). As summarized in Fig. 3(a), the single point mutation in the binding site of B1F caused a fourfold decline in HBeAg expression and about a twofold reduction of HBsAg expression. This is in accordance with findings from other groups who have shown that E\textsubscript{II} stimulates a much stronger
transcription from $C_p$ than that from $S$ promoters, which are mainly influenced by the HBV enhancer I (reviewed by Schaller & Fischer, 1991).

Since the amount of HBeAg typically correlates with the amount of virions in the tissue culture supernatant, the fourfold reduction of HBeAg expression after transfection of HepG2 cells with pSPT1.2xHBVB1um, as compared with pSPT1.2xHBV, can be taken as evidence that binding of B1F to its target sequence within the $E_{II}$ region is important for regulating the transcription of the 3-5 kb pregenomic RNA. To confirm the importance of B1F-binding, the level of virus transcription was investigated with semi-quantitative RT–PCR. In RT–PCR, two pairs of HBV-specific primers were designed to specifically detect transcription initiated from $C_p$ and also total viral mRNAs (Fig. 2a). The first pair of primers (p1983 and p2711) generates a 729 bp fragment, which is specific for the 3-5 kb mRNA initiated from $C_p$. The other pair of primers (p1261 and p1660) should generate a fragment of 399 bp, which, due to a single common poly(A) signal sequence used by all HBV transcripts, should be present in all virus transcripts. As shown in Fig. 3(b), the level of the transcripts initiated from $C_p$, i.e. precore mRNA and pregenomic mRNA, was dramatically (14-fold, quantified with Aida evaluation software from Raytest) diminished in HepG2 cells that had been transfected with plasmid pSPT1.2xHBVB1um as compared with the level in cells transfected with the wild-type HBV expression plasmid pSPT1.2xHBV (Fig. 3b, lanes 2 and 4 in comparison with lanes 3 and 5). In contrast, the amount of total virus transcripts was only slightly reduced (10%) if HepG2 cells were transfected with plasmid pSPT1.2xHBVB1um instead of pSPT1.2xHBV (Fig. 3b, lane 6 in comparison with lane 7). These data indicated that the single point mutation (G to T transversion) in the binding sequence of B1F significantly affects the transcription of the 3-5 kb mRNAs. The decrease in 3-5 kb pregenomic RNA synthesis is in accordance with the reduction in HBeAg levels described above. This demonstrates that B1F is one of the critical factors regulating liver-specific gene transcription and, especially, the synthesis of the 3-5 kb viral RNAs. Since the 3-5 kb pregenomic RNA serves as the template for reverse transcription, which is an essential step in HBV replication, the significant decline of the pregenomic mRNA should also affect viral DNA replication. Therefore, B1F not only plays a crucial role in liver-specific viral gene transcription, but it is also assumed to have a major impact on viral DNA replication and
might be important in co-ordinating the regulation of HBV transcription during infection.

In conclusion, binding of B1F to its target sequence within the B1 element of the HBV EII region is crucial for the activity of EII and also plays an important role in liver-specific transcription initiated from HBV Cp with respect to virus antigen expression (especially HBeAg) and pregenomic RNA generation.

In the past few years, several liver-specific and liver-enriched factors such as HNF1, HNF3, HNF4 and C/EBP, as well as ubiquitous factors such as SP1, have been shown to bind to the EII region (Lopez-Cabrera et al., 1991; Yuh & Ting, 1991; Guo et al., 1993; Zhang et al., 1993; Li et al., 1995; Raney et al., 1995). The action of a combination of transcription factors binding to the EII/Cp region and other regulatory regions of the HBV genome permits modulation of HBV transcription in such a way that all of the virus transcripts are synthesized in a balanced ratio, as required for normal HBV propagation. The data presented in this report indicate that B1F plays a critical role in this interplay of different transcription regulators. Undoubtedly, the investigation of such an interplay among various transcription factors is not only critical for a detailed understanding of EII function, but is also of great importance for understanding the cell-specific transcription machinery (Raney et al., 1997).

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References


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