Volume 229, number 2, 349–354 FEB 05678 March 1988

Binding of tissue-specific factors to the enhancer sequence of hepatitis B virus

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Received 22 January 1988

We have identified tissue-specific factors, in human hepatoma cells, that bind specifically to the transcriptional enhancer sequence of the human hepatitis B virus (HBV). Two different types of protein factor were found in nuclear extracts of hepatoma cells by the gel mobility shift assay. One factor was observed in human hepatoma cells but not in human kidney, lung, or vein cells, or in embryonic mouse cells. The other was discovered in both human hepatoma cells and human vein cells. DNase I footprint analysis, using the enhancer fragment (164 bp, *Acc*I-*Sph*I) from HBV, revealed that two specific sites are recognized by the nuclear factors. These sites contain consensus octamer sequences which have been found in many other enhancer elements. These results strongly suggest that the two nuclear factors found in hepatoma cells play key roles in the function of the HBV enhancer.

Gene regulation; Enhancer sequence; Trans-acting factor; (Hepatitis B virus)

1. INTRODUCTION

Human hepatitis B virus (HBV) is the etiologic agent of acute and chronic liver disease [1]. Recent epidemiological studies [2,3] have also demonstrated that HBV is associated with hepatocellular carcinoma.

The genome of HBV is a partially single-stranded circular DNA with a long or L(−) strand of 3200 bases and a short or S(+) strand with varying degrees of incompleteness [4,5]. Upon infection by HBV, two major transcripts are produced referred to as the 2.1 kb and 3.5 kb RNAs [6,7]. Both transcripts are derived from the L(−) strand. The 2.1 kb RNA encodes the envelope protein (HBsAg), the 3.5 kb RNA being the mRNA for the core protein (HBcAg) and, possibly, for the DNA polymerase [5]. The 3.5 kb RNA is also believed to serve as a template for the replication of the viral genome by the virally encoded reverse transcriptase [8]. Little is known about the mechanisms of control of viral transcription. However, it appears that the mechanisms that control synthesis of these two viral transcripts are different [5]. There is evidence that the 2.1 kb RNA is constitutively expressed, whereas the 3.5 kb RNA is inducible [9].

Recently, Shaul et al. [10] identified an enhancer element in the HBV genome which is located about 450 bases upstream from the promoter for the 3.5 kb RNA. It is assumed that the enhancer plays a key role in the regulation of gene expression of HBV [10]. Furthermore, studies in vivo of the expression of HBV using the 'CAT' system revealed that the enhancer has strict host specificity and functions only in liver cells of human origin [11]. Therefore, it has been suggested that the enhancer requires trans-acting tissue factor(s), present in liver cells, for its activity [11].

Here, we describe the identification of tissue-specific factors in nuclear extracts prepared from human hepatoma cells. These factors bind specifically to the HBV enhancer sequence.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2. MATERIALS AND METHODS

2.1. *Materials*

Human hepatoma cells (HepG2), human kidney cells (A-498), human lung cells (CCD-11lu) and embryonic mouse cells (NIH 3T3) were obtained from the American Type Culture Collection (ATCC). Endothelial cells of human vein (passage 10) were a gift from Dr R.P. Tewari (Southern Illinois University). Plasmid pAM6 [12], which contains the entire genome of HBV linked to pBR322, was obtained from the ATCC. Restriction enzymes were purchased from US Biochemical Corp. Bacterial alkaline phosphatase and T_4 polynucleotide kinase were obtained from Bethesda Research Laboratories. [γ-^{32}P]ATP was purchased from New England Nuclear.

2.2. *Preparation of the DNA probe*

The probe for the 'gel mobility shift assay' was prepared from plasmid pAM6 digested with restriction endonucleases *Acc*I and *Sph*I. The digested DNA was treated with bacterial alkaline phosphatase and labelled with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The labelled *Acc*I-*Sph*I fragment, which spans 164 bases and contains the entire HBV enhancer sequence (see fig.1) [10], was recovered, after electrophoresis, from a 6% polyacrylamide gel by electroelution.

2.3. *Preparation of nuclear extracts*

Cells were grown in Eagle's minimum essential medium with Earle's balanced salt solution, 2 mM L-glutamine, 10% fetal bovine serum, and 100 U/ml each of penicillin G and streptomycin. Nuclear extracts were prepared as described by Dignam et al. [13] and Prywes and Roeder [14], with slight modifications. Approx. 5×10^7–1.5×10^8 cells were harvested and washed with phosphate-buffered saline (PBS, pH 7.4). The cells were then suspended in 10 ml buffer A [10 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT] and allowed to swell at 0°C for 10 min. The cells were collected by centrifugation, resuspended in 2 ml buffer A and homogenized with a glass Dounce homogenizer (type B pestle). The cell nuclei were sedimented by centrifugation at $1000 \times g$ for 12 min. The nuclei were suspended in 1 ml buffer B [20 mM Tris-HCl (pH 7.5), 20% glycerol, 1.5 mM $MgCl_2$, 0.5 mM DTT]. KCl solution (4 M) was added to the suspension to give a final concentration of 0.3 M. The suspension was rocked at 4°C for 30 min, and then subjected to centrifugation for 20 min at $20000 \times g$. The supernatant was dialyzed for 2 h at 4°C vs buffer C [20 mM Tris-HCl (pH 7.5), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT]. The dialysate was centrifuged at 11000 $\times g$ for 15 min and the supernatant stored at −70°C.

2.4. *Gel mobility shift assay and DNase I footprint analysis*

^{32}P-labelled probe was mixed with various amounts of nuclear extract in 20 μl of a reaction mixture that contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 40 ng sonicated salmon sperm DNA. The mixture was incubated at room temperature for 30 min, and then applied to a 6% polyacrylamide gel (30:0.8, acrylamide/bisacrylamide). The gel was run at 10 V/cm in 0.25 × TBE (25 mM Tris-HCl, 25 mM boric acid, 1 mM EDTA), dried and autoradiographed.

For footprint analysis, bands detected by the gel mobility shift assay were cut out from the gel and electroeluted. Samples were treated with 1 μg/ml of DNase I for 60 s on ice in the presence of 10 mM $MnCl_2$. DNase I was inactivated by the addition of 10 mM EDTA, 0.6 M NaCl, 0.2% SDS followed by phenol extraction and ethanol precipitation. As a control, the 'G+A' reaction, described by Maxam and Gilbert [15], was performed using the 5'-end-labelled probe used for the mobility shift assay. The DNA samples were analyzed on denaturing 8% polyacrylamide gels.

3. RESULTS

3.1. *Nuclear factors that bind to the HBV enhancer*

In order to identify specific cellular factors that interact specifically with the HBV enhancer, the gel mobility shift assay was performed. The assay system is based on the observation that fragments of DNA that are bound to proteins migrate through acrylamide gels more slowly than free fragments of DNA. This system has been successfully used by several investigators to detect various nuclear factors required for the regulation of transcription [16,17].

The ^{32}P-labelled fragment which contains the enhancer, and which spans 164 bases (*Acc*I-*Sph*I, fig.1) was mixed with various concentrations of nuclear extracts prepared from human hepatoma cells (HepG2), and analyzed on polyacrylamide gels. As shown in fig.2, two distinct bands (I,II) were observed when the gel was subjected to autoradiography. Inclusion of SDS and proteinase K in the reaction mixture disrupted the formation

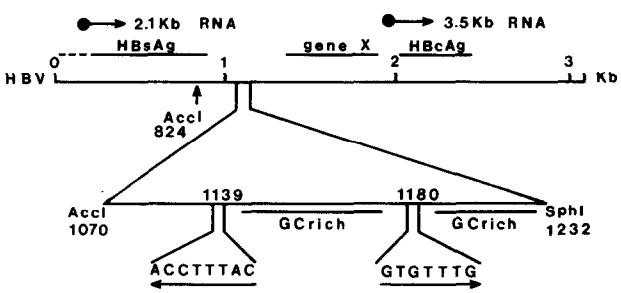

Fig.1. Structure of the genome of HBV (adw). The numbering of the nucleotide sequence is based on the data of Onda et al. [21]. The enhancer spans the region between nucleotides 1070 and 1232. This region contains two stretches of the sequences which are similar to the consensus sequence of enhancers, namely $GTGG^{TTT}_{AAA}G$.

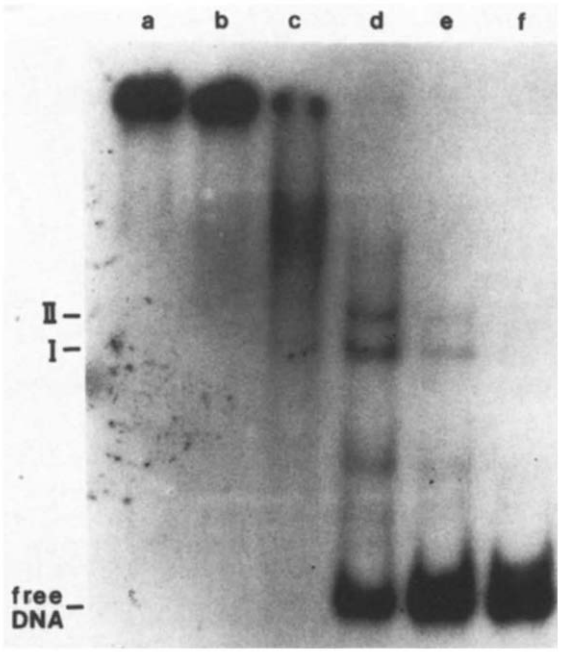

Fig.2. Detection of enhancer-binding proteins in nuclear extracts. ^{32}P-labelled enhancer fragment was mixed with various amounts of nuclear extract prepared from HepG2 as described in section 2 (a, 14 μg; b, 7 μg; c, 1.4 μg; d, 0.7 μg; e, 0.14 μg; f, 0.014 μg protein of nuclear extract). Mixtures were incubated for 30 min at room temperature and applied to a 6% acrylamide gel. The gel was dried and autoradiographed.

of bands I and II (not shown). This result suggests that these bands represent discrete protein-DNA complexes. The upper band, which appears around the position of the wells, is considered to represent non-specific binding of proteins to DNA, since this band is generally observed when any DNA probe and a high concentration of nuclear extract are allowed to react.

To determine whether these bands are specific to the HBV enhancer, we performed a competition assay as shown in fig.3. As competitors, plasmids pBR322 and pAM6, the latter consisting of the entire HBV genome carried by pBR322 [12], were used. When excess amounts of unlabelled DNA from plasmid pAM6 DNA, digested with *Acc*I and *Sph*I, were included in the reaction mixture, the intensity of bands I and II was dramatically decreased (fig.3a, lanes 4–6). However, when the same amount of pBR322 DNA, digested with *Acc*I and *Sph*I, was used, the intensity of the bands did not change (fig.3a, lanes 1–3). Furthermore, when the unlabelled restriction fragment, generated by digestion with *Acc*I and *Sph*I, that spans 164 bases and contains the enhancer sequence (see fig.1), was added to the reaction mixture as a competitor, the intensity of bands I and II decreased significantly (fig.3b, lanes 3,4). However, addition to the reaction mixture of a different restriction fragment, which spans 250 bp (*Acc*I-*Acc*I, see fig.1) and is adjacent to the 5′-end of the enhancer fragment, had little effect on the intensity of bands I and II (fig.3b, lane 2). These results strongly suggest that the binding of the proteins which, with DNA, generate bands I and II is specific for the HBV enhancer sequence.

3.2. *Tissue specificity of the nuclear factors*

Recently, Jameel and Siddiqui [11] investigated the function of the HBV enhancer using the CAT assay system and various cell lines. They found that the enhancer sequence functions only in human hepatoma cell lines. In order to examine the tissue specificity of the factors that are responsible for the formation of bands I and II, we prepared nuclear extracts from various cell lines, including human hepatoma, lung, kidney, vein cells and embryonic mouse cells. These extracts were mixed with the HBV enhancer fragment and the resultant protein-DNA complexes were analyzed by polyacrylamide gel electrophoresis, as described above. As shown in fig.4, only the nuclear extract of hepatoma cells contained the proteins that could generate both bands I and II. However, endothelial cells from human vein contained a protein factor which comprised only band I but not band II. These results suggest that the protein factors that are responsible for the formation of bands I and II are highly specific to human liver cells.

3.3. *DNase I footprint analysis*

In an attempt to identify the binding sites of the nuclear factors on the enhancer DNA, footprint analysis was performed. Bands I and II, as shown in fig.2, were excised from the gel and electroeluted. The samples were treated with DNase I and analyzed by electrophoresis on an 8% acrylamide gel.

As shown in fig.5A, the nuclear factor that comprises band I recognizes a region of 27 bases which

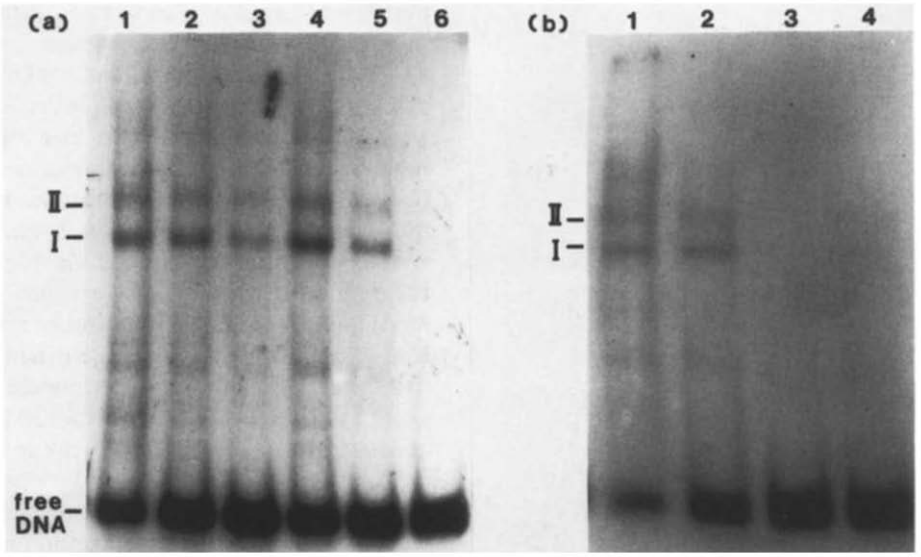

Fig.3. Competition for binding to enhancer sequences. (a) ^{32}P-labelled enhancer probe was mixed with 0.7 μg nuclear extract of HepG2 cells in the presence of either unlabelled pBR322 (1–3) or unlabelled pAM6 (4–6), both of which were digested with *Acc*I and *Sph*I, and used as competitors. Concentrations of competitor DNAs were 10 ng (1,4), 100 ng (2,5), and 1 μg (3,6). Mixtures were incubated for 30 min at room temperature and subjected to polyacrylamide gel electrophoresis. The gel was dried and autoradiographed. (b) A competition assay similar to that described in (a) was performed but purified fragments of DNA were used as competitors. (1) Control, no competitor; (2) 50 ng of 250 bp *Acc*I-*Acc*I fragment (see fig.1) as competitor; (3) 50 ng of 164 bp *Acc*I-*Sph*I enhancer fragment (see fig.1) as competitor; (4) 20 ng of 164 bp *Acc*I-*Sph*I enhancer fragment.

spans nucleotides 1176–1202. This region contains a sequence similar to the consensus sequence ($GTGG^{TTT}_{AAA}G$) of many other enhancers. Similar

nucleotides 1158–1154 (fig.3B). This region also contains a similar motif to the consensus sequence of enhancers. However, the orientation of the sequence is opposite to that found in the case of band I.

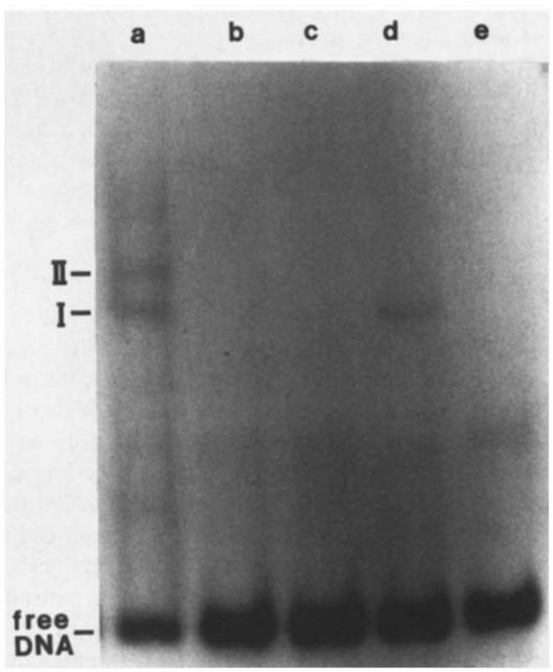

Fig.4. Tissue specificity of the nuclear factors. Nuclear extracts were prepared from human hepatoma (a), human kidney (b), human lung (c), human vein (d), and embryonic mouse cells (e). 0.7 μg protein of each nuclear extract was mixed with ^{32}P-enhancer probe and the gel mobility shift assay was performed as described in section 2.

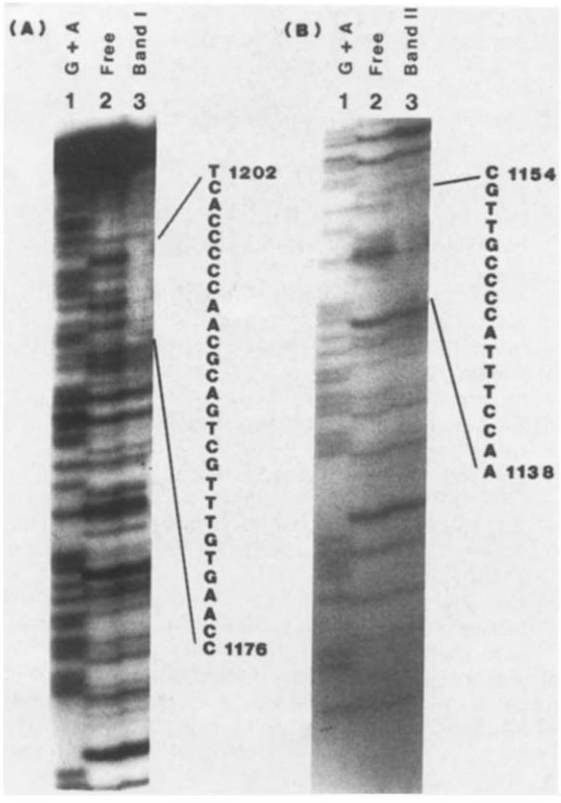

Fig.5. DNase I footprint analysis of DNA from bands I and II. Bands I, II and free form of probe detected by the gel mobility shift assay as shown in fig.2 were cut out from the gel, electroeluted and treated with DNase I as described in section 2. As a control, a G+A reaction was performed according to Maxam and Gilbert [15], using the 5′-end-labelled DNA probe. DNA samples were analyzed on 8% denaturing acrylamide gels. (1) G+A reaction as a control; (2) free form of DNA probe; (3) band I (A) and band II (B).

4. DISCUSSION

Here, we have described two different types of protein factor, which are present in nuclear extracts of human hepatoma cells and which bind specifically to the HBV enhancer sequence. We do not as yet know whether these factors mediate enhancer function directly. However, the binding of these factors to the enhancer is sequence-specific, and they are present only in a specific tissue, namely, the hepatoma cell. These results strongly suggest that the newly identified factors are responsible for the function of the enhancer. Similar systems have been used to identify several nuclear factors that interact with various enhancers, such as those of SV40 [18], cytomegalovirus [19], c-*fos* gene [14] and immunoglobulin [20]. While further experiments are necessary to determine the functional requirements of these factors for enhancer activity, the gel mobility shift assay and DNase I footprint analysis have proved to be useful for the identification of these factors.

The binding site of the nuclear factor to the DNA band in band I was identified by footprint analysis. The region protected by the protein stretches from nucleotides 1176 to 1202. This region consists of a GC-rich region and an octamer consensus sequence ($GTGG^{TTT}_{AAA}G$) which has been found in a variety of enhancer elements [18]. The binding site of the second factor on the DNA in band II spans nucleotides 1138–1154. This site also includes a GC-rich region and consensus octamer sequence. It is noteworthy that the orientations of the consensus sequence in the DNA from bands I and II are opposite (fig.1). This difference may partially explain the orientational independence of the enhancer function [11].

The nuclear factor that generates band I is present in both hepatoma and vein cells but was not detected in any of the other cell lines tested. By contrast, the factor comprising band II is present only in hepatoma cells. These results, together with those of the footprint analysis, suggest that the two nuclear factors described here are biochemically and, possibly, functionally distinct. However, we have recently partially purified these factors and found that both are biochemically distinguishable but have very similar molecular masses. Therefore, we cannot rule out the possibility that the two factors are identical but occur in two separate forms, possibly covalently modified.

Very little is known about the control of gene expression in HBV. It has been demonstrated that, after infection with HBV, the 2.1 kb and 3.5 kb RNAs are detectable at almost the same level [9]. However, in patients with integrated viral sequences, the amount of 2.1 kb RNA is at least 10-times greater than that of 3.5 kb RNA [9]. Thus, the 3.5 kb RNA appears to be inducible. The HBV enhancer is located about 450 bp upstream from the promotor for the 3.5 kb RNA [10]. Therefore, it is assumed that the HBV

enhancer and the tissue-specific nuclear factors described here play key roles in the regulation of gene expression, and that these factors and the enhancer may also be involved in the oncogenesis of hepatocarcinoma.

Acknowledgements: We thank Ms M. Watabe for excellent technical assistance and Ms B. Reichert for preparing the manuscript.

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