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A human hepatitis B viral enhancer element

Yosef Shaul[1], William J.Rutter[3] and Orgad Laub[2]

Departments of [1]Virology and [2]Genetics, The Weizmann Institute of Science, Rehovot, Israel, and [3]Hormone Research Institute, University of California, San Francisco, CA 94143, USA

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Fragments of the cloned hepatitis B virus (HBV) genome were assayed *in vivo* for the presence of a transcriptional enhancer element. We demonstrate that sequences positioned ~450 bp upstream from the HBcAg gene promoter are required for its efficient activity. These HBV stimulatory sequences activate transcription when inserted upstream to a heterologous SV40 early promoter. Like other known enhancer elements, this HBV sequence acts in an orientation-independent manner. Furthermore, the HBV enhancer element exhibits a preferred activity in a human hepatoma cell line.

Key words: human hepatitis B virus (HBV) enhancer element/CAT assay/PLC/PRF/5 cells

Introduction

Human hepatitis B virus (HBV) is a partially single-stranded DNA virus whose host range is restricted to humans and chimpanzees. The host specificity and the lack of an *in vitro* system for HBV propagation have hampered the study of the molecular biology of this virus. Information on the genetic organization of the virus has been provided by cloning and sequencing of the HBV genome (Galibert *et al.*, 1979; Pasek *et al.*, 1979; Valenzuela *et al.*, 1980; Ono *et al.*, 1983; Fujiyama *et al.*, 1983; Kobayashi and Koike, 1984). The viral gene promoters have been detected and mapped by *in vitro* transcription experiments (Rall *et al.*, 1983), by the expression of cloned HBV DNA in chimpanzee liver cells (Will *et al.*, 1982; Cattaneo *et al.*, 1983, 1984) by expression in alternate host cells using a viral vector (Laub *et al.*, 1983) and by using DNA-mediated gene transfer techniques (Pourcel *et al.*, 1982; Malpiece *et al.*, 1983; Standring *et al.*, 1984). However, nothing is known about the regulation of these promoters.

In the SV40 genome, an enhancer element was identified which is required for efficient expression of early viral genes (Benoist and Chambon, 1981; Gruss *et al.*, 1981). These elements increase the level of transcription of an adjacent gene from its promoter in a fashion that is relatively independent of position and orientation (Moreau *et al.*, 1981; Banerji *et al.*, 1981; Fromm and Berg, 1982, 1983). Subsequent studies revealed similar elements in a number of other DNA viruses including polyoma virus (de Villiers and Schaffner, 1981; Tyndall *et al.*, 1981) papilloma virus (Lusky *et al.*, 1983) adenovirus (Weeks and Jones, 1983; Sassone-Corsi *et al.*, 1983) and BK virus (Rosenthal *et al.*, 1983). Retroviruses also contain enhancer elements in their long terminal repeats (Luciw *et al.*, 1983). Furthermore, the retrovirus enhancer element may play a role in the activation of host proto-oncogenes, thereby inducing tumor formation (for review, see Bishop, 1983). The striking correlation between the integration of HBV DNA in liver cells and the incidence of hepatoma formation might suggest similar mechanisms. Accordingly,

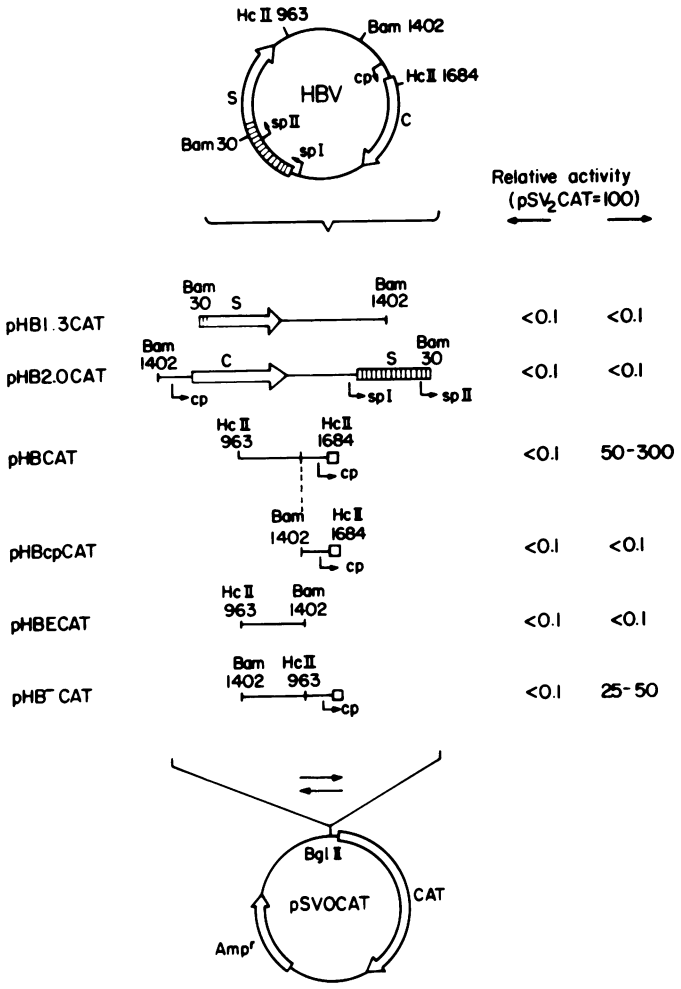

Fig. 1. The structures of plasmid constructs containing the HBV fragments. The map of the HBV genome (Valenzuela *et al.*, 1980), partial cleavage sites of restriction endonuclease *Hinc*II (HCII) and *Bam*HI (Bam) are indicated at the top of the figure. The HBcAg gene (C) and HBsAg gene (S) are represented by the white arrows. The pre-surface antigen region is marked by the dashed area 5′ to S. The HBV fragments which were inserted in the pSVOCAT plasmid are shown. The *Bam*HI fragments were ligated directly into the unique *Bgl*II site in the pSVOCAT plasmid (Gorman *et al.*, 1982). The *Hinc*II sites were converted into *Bal* ends using synthetic DNA linkers. All fragments were inserted in two opposite orientations indicated by small arrows within the *Bal*II site of pSVOCAT. Alexander cells were transfected with the plasmids and cell extracts were prepared after 40 h and assayed for CAT activity. In each experiment the pSV2CAT was used as a control DNA. The maximal and the minimal values of the relative CAT activity, as compared with that of pSV2CAT, are indicated at the right hand of the figure. Every plasmid was assayed 2–3 times with at least two independent DNA preparations.

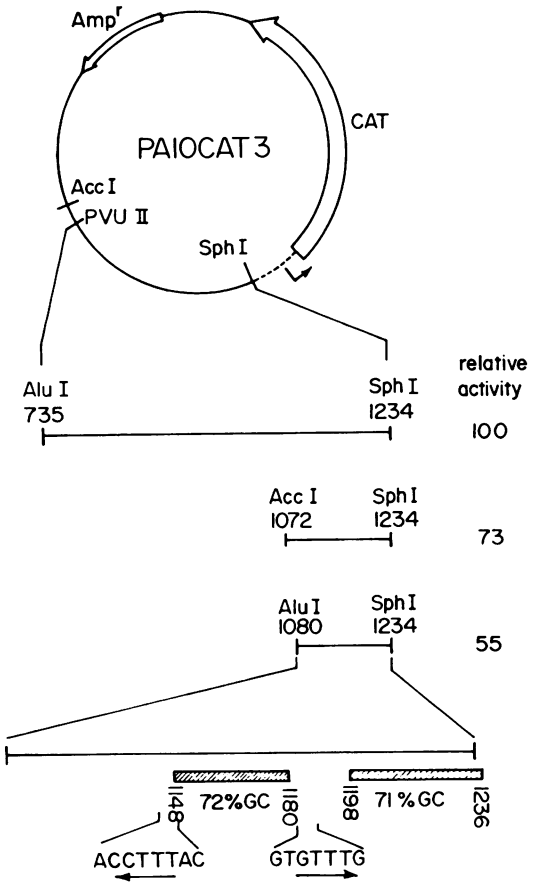

Fig. 2. Activation of SV40 early gene promoter by HBV enhancer. pA10CAT3 plasmid contains the CAT gene with the SV40 small t-antigen intron, the SV40 early polyadenylation addition signal at the 3′ end, and the SV40 early promoter, *Hind*III 5171 – *Sph*I 128, at the 5′ end of the CAT coding sequence. The direction of transcription from the SV40 promoter is indicated by small black arrows. HBV DNA fragments were inserted into the pA10CAT3 plasmid at the *Pvu*II-*Sph*I sites or at the *Acc*I – *Sph*I sites. The plasmids were introduced into COS cells by calcium phosphate co-precipitates. The relative CAT activity of different inserts are indicated. The sequence composition of the *Alu*I 1080 – *Sph*I 1234 fragment is shown.

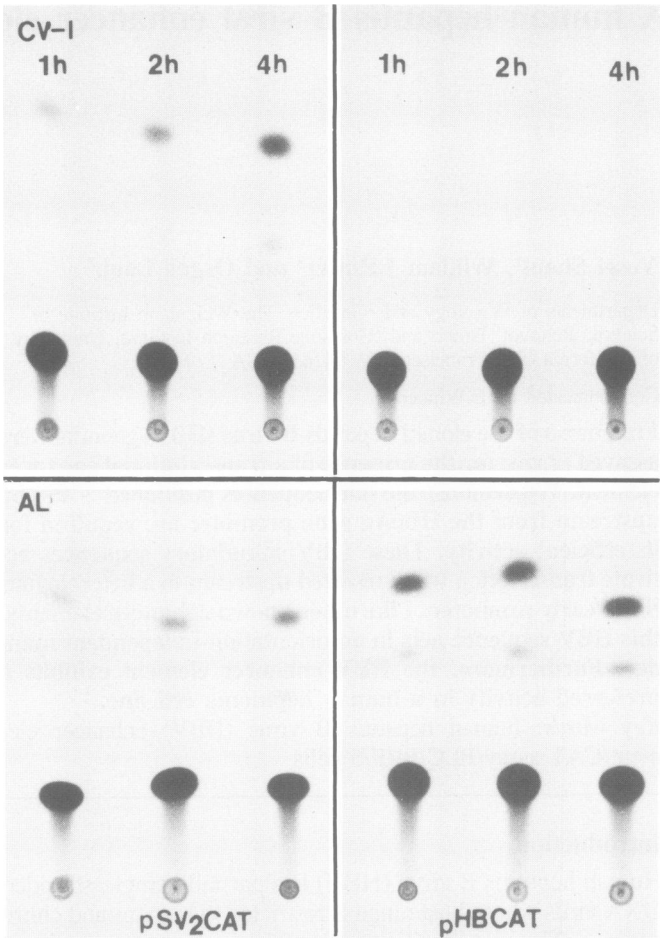

Fig. 3. Cell preference of the HBV enhancer. pSV2CAT and pHBCAT were transfected in CV-1 or Alexander cells (AL) using the calcium phosphate co-precipitation method. Cell extracts were prepared after 44 h and assayed for CAT activity. The CAT reactions were incubated for 1, 2 and 4 h.

we have searched for an enhancer element in the HBV genome. In this report we describe experiments using the 'CAT system', recently developed by Gorman *et al.* (1982) which indicate that an HBV enhancer element is located ~450 bp upstream of the HBcAg gene promoter. This enhancer element is required for efficient expression of the HBcAg gene promoter *in vivo*.

Results

The HBcAg gene promoter is activated by an upstream sequence

Recent *in vitro* and *in vivo* studies have revealed that the HBV genome contains at least three RNA polymerase II promoters. The pre-surface gene promoter (SPI) and the HBcAg gene promoter (CP) were mapped by *in vitro* studies using the cell-free extract system (Rall *et al.*, 1983). The surface gene promoter (SPII) was defined in an SV40 expression vector (Laub *et al.*, 1983) and by DNA-mediated gene transfer (Cattaneo *et al.*, 1983, 1984; Standring *et al.*, 1984). We have studied the ability of the HBV promoters to activate the expression of a heterologous gene; chloramphenicol acetyl transferase (CAT). The entire HBV genome was dissected by *Bam*HI into two fragments of 1.4 kb (from nucleotide 30 to 1402) and 2.0 kb (from nucleotide 1402 in a clockwise direction to nucleotide 30). The 1.3-kb fragment contains the HBcAg gene promoter (CP), the pre-surface gene promoter (SPI), and the surface gene promoter (SPII). The *Bam*HI fragments were inserted, in either of the two possible orientations, at the 5′ end of the CAT gene in the pSVOCAT plasmid (Figure 1). pSVOCAT contains the CAT gene, the SV40 small t antigen intron and the SV40 early polyadenylation addition signal at the 3′ end; it does not contain a promoter or enhancer element. Thus it can be used to test for these elements (Gorman *et al.*, 1982). Significantly, no expression of the CAT gene was detected. Since the putative HbcAg gene promoter was previously mapped at nucleotide 1682 (Rall *et al.*, 1983) close to the *Bam*HI (1402) site, it seemed plausible that upstream sequences were required for expression. We therefore constructed a new plasmid, pHBCAT, in which the HBcAg gene promoter, including 500 bp of upstream sequences (*Hinc*II 963 – 1684) was introduced into pSVOCAT in two possible orientations (Figure 1). A significant expression of the CAT gene was observed when the HBcAg gene promoter was inserted in the sense orientation to the CAT gene (Figure 1). Thus, the sequence upstream from the *Bam*HI nucleotide 1402 site is important for the activity of the HBcAg gene promoter. To verify this point, we digested the HBV *Hinc*II (nucleotide

963 – 1684) fragment with *Bam*HI. The resulting two fragments were inserted, in the two possible orientations, at the 5′ end of the CAT gene in the pSVOCAT plasmid vector. pHBCpCAT carries the *Bam*HI/*Hinc*II fragment (1402 – 1684) which contains the HBcAg gene promoter (Rall *et al.*, 1983). Plasmid pHBECAT carries the *Hinc*II/*Bam*II fragment (963 – 1402) upstream sequences. As was expected, neither of these constructs expresses the CAT gene. We concluded that the *Hinc*II/*Bam*HI (963 – 1402) fragment contains an HBV-specific enhancer element which is required for the HBcAg gene promoter activity. It is well documented that enhancer elements act independently of orientation and position with respect to the promoter (Khoury and Gruss, 1983). To demonstrate the orientation independence of the HBV enhancer, we constructed the pHB$^-$ CAT plasmid which carries the core promoter fragment (1402 – 1684) in the sense orientation while the enhancer fragment (963 – 1402) was inserted in the opposite orientation. As shown in Figure 1, significant CAT expression was observed when the pHB$^-$ CAT plasmid was assayed.

The HBV transcription enhancer element stimulates a heterologous promoter

An additional important feature of the transcriptional enhancer element is its ability to stimulate a heterologous promoter (Banerji *et al.*, 1981; Khoury and Gruss, 1983). We chose the SV40 early promoter (SV40 nucleotides 5171 – 128) without the SV40 enhancer element at the 5′ end of the CAT gene in the pA10CAT3 construct. This plasmid contains a unique *Sph*I site conveniently located 5′ to the SV40 promoter and a *Pvu*II site within the pBR portion of the plasmid. When this *Sph*I-*Pvu*II fragment was replaced with the *Alu*I 735 – *Sph*I 1234 HBV enhancer fragment (Figure 2) and tested in COS cells, it induced a 105-fold higher level of activity compared with the pA10CAT3 vector plasmid. 75% of this activity was still maintained when the HBV fragment was reduced in size to 172 bases, by the removal of the *Alu*I 735 – *Acc*I 1072 portion. Deletion of eight more bases (from *Acc*I 1072 to *Alu*I 1080) reduced the activity to 55% of that achieved by the *Alu*I 735 – *Sph*I 1234 HBV fragment (Figure 2). Sequence analysis of these 154 bases (*Alu*I 1080 – *Sph*I 1234) revealed a unique structural feature; two stretches of 31 and 32 nucleotides rich in GC residues (71 – 72%, preceded by eight bases which are very similar to the GTGG$^{TTT}_{AAA}$G consensus enhancer sequence (Neiher *et al.*, 1983; Khoury and Gruss, 1983). That this portion of HBV contains enhancer activity was also confirmed by insertion of *Bam*HI fragment (30 – 1402) at the 3′ end of the CAT gene in the pA10CAT3 plasmid vector. Insertion of this fragment resulted in a 5-fold induction of the CAT gene when the construct was transfected into human hepatoma Alexander cells (data not shown).

Cell type preference of the HBV enhancer

Maximal activity of several viral enhancers is obtained in their natural host cells and a degree of cell specificity has been observed (Laimins *et al.*, 1982). To address the question of the cell type preference of the HBV enhancer, we compared the effect of the HBcAg gene promoter-enhancer complex with that of the SV40 element in two different cell lines: a primate kidney cell line (CV-1 cells), and the human PLC/PRF/5 hepatoma cell, the Alexander cell line. The Alexander cells contain at least seven inserts of HBV DNA (Shaul *et al.*, 1984), and express, constitutively, the HBV surface antigen (Macnab *et al.*, 1976). The results are summarized in Figure 3. The SV40 enhancer (pSV2CAT) stimulates the SV40-driven CAT gene expression more efficiently in CV-1 cells than in Alexander cells (ratio 2:1). The HBV enhancer on the other hand, exhibits a striking preferred activity (50-fold) in Alexander cells. Because this analysis is limited to two cell lines only, further experiments are needed to establish the suggested tissue or cell specificity of the HBV enhancer-activity. These studies are now in progress.

Discussion

We have shown that the HBV sequences positioned between nucelotides 1080 and 1234, ~450 bp upstream from the HBcAg gene promoter, influence the activity of the HBcAg gene promoter. Like the other viral enhancer elements, so far defined, this HBV enhancer induces a heterologous promoter and acts independently of orientation with respect to the promoter.

Only one RNA polyadenylation signal has been detected in the HBV genome; it is located in the coding portion of HBcAg (Standring *et al.*, 1984; Cattaneo *et al.*, 1984). The transcription of the full-length HBcAg sequence must involve mechanisms which override this termination/polyadenylation signal in the first round of transcription, but not in the second round. The RNA that initiates at the HBcAg promoter, under the regulation of HBV enhancer, is larger than a viral full-length transcript and thus seems to be the most plausible candidate mRNA for the HBcAg and the putative polymerase gene; it may also serve as the viral pre-genome RNA. It was proposed that such a pre-genome RNA may act as a template for reverse transcription in the HBV replication cycle (Cattaneo *et al.*, 1984). The HBV enhancer element, therefore, might also directly regulate the replication of the virus. Furthermore, the HBV enhancer overlaps with the probable coding region (open reading frame) of the putative polymerase gene. To our knowledge, this is the first indication that a coding region may serve as a transcriptional enhancer element. Finally, the presence of a strong enhancer within the HBV genome provides a possible explanation for the oncogenic activity of the virus. By analogy with the mechanism proposed for the transforming activity of oncogenic viruses that do not themselves contain oncogenes (Payne *et al.*, 1982), integration of HBV DNA, containing the enhancer, within the host genome may increase the expression of genes in the general vicinity of the integration site. In the event that such HBV sequences are integrated close to a gene regulating replication or differentiative function, transformation could occur.

Materials and methods

Cell growth

Cells were cultured in Dulbecco's modified Eagle's minimal essential medium (GIBCO) containing 10% fotal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were seeded for 1 day before transfection; at the time of transfection the cells were 40 – 80% confluent.

Construction of plasmids and DNA preparations

The cloned HBV genome used in our study is the 3.2-kb HBV of Valenzuela *et al.* (1980). All DNA constucts were made by standard recombinant DNA techniques (Maniatis *et al.*, 1982) and plasmid DNA was purified by centrifugation in CsCl gradients. The DNA samples were analysed on agarose gels before transfection and only samples with >80% form I DNA were used.

Cell transfection and CAT assay

Subconfluent cultures of cells were transfected with 10 μg/100 mm dish of supercoiled plasmid DNA by the calcium phosphate coprecipitate technique (Graham and van der Eb, 1973). Four hours after the addition of the

DNA, cells were exposed to 20% glycerol for 2 min. Cells were collected 44 h after the addition of DNA and extracts were prepared by sonication and centrifugation (Gorman *et al.*, 1982). CAT assays were essentially similar to those described by Gorman *et al.* (1982) with the modifications described by Walker *et al.* (1983). X-ray film was exposed to the chromatograms and activities quantitated by counting regions of the chromatograms in a liquid scintillation spectrometer.

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