A novel expression selection approach allows precise mapping of the hepatitis B virus enhancer

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ABSTRACT

We have used a novel approach called expression selection to precisely define the hepatitis B virus (HBV) enhancer. Expression selection is based on a shuttle vector containing an enhancerless SV40 T antigen gene, the SV40 origin of replication and a plasmid replicon. This vector is linearized, ligated with the sonicated DNA to be analyzed and transfected into eukaryotic cells, where only plasmids which have incorporated an enhancer can express T antigen and therefore replicate. Vectors amplified by replication are selectively rescued in E.coli and their inserts analyzed. When we performed this protocol with HBV DNA we rescued two overlapping fragments of 166 and 214 bp which in HBV DNA map about 500 bp upstream of the core antigen mRNA initiation site and 1150 bp downstream of the surface antigen mRNA initiation site. These results were confirmed by conventional deletion mapping. When compared to the SV40 enhancer in nonhepatic cell lines, the HBV enhancer is only 5 to 10% as active; nevertheless, it also acts in an orientation-independent manner and in a position downstream of a gene. The HBV enhancer is situated in the coding region of the potential reverse transcriptase, and thus is the first enhancer identified to map in a protein-coding region.

INTRODUCTION

Hepatitis B virus infection is a major world health problem and is also closely linked to liver cancer (1). The investigation of the HBV life cycle has been hampered by the lack of a system for HBV replication in vitro. Recent cloning and sequencing (2,3), the use of animal model systems (4,5), and eukaryotic expression systems (6,7), however, have brought some insight into the HBV gene organisation and life cycle. As a result of these studies, the structure of the major transcripts of the HBV-like viruses has been determined (8-10), and three promoter regions (11-15) and one polyadenylation site (8,11,16,17) have been identified. Since transcriptional...
enhancers (18,19; for review see 20) have now been defined in the genome of many viruses (21-35), we have screened the HBV genome (subtype ayw; 36) for sequences with enhancer activity. Here we define a region of HBV DNA capable of stimulating transcription of foreign genes in cis independent of its orientation and also in a downstream position. Similar results have recently been reported for HBV DNA of another subtype (37). Furthermore, we describe a new "expression selection" protocol which allows rapid functional identification of enhancer sequences and could be applied to other potential enhancers active in a wide variety of cell types.

MATERIALS AND METHODS

Plasmids

pSVXB-pl consists of a 2.81 kb fragment of SV40 DNA between nucleotides 2533 (BamHl site, convention of Tooze; 39) and 99 and including the SV40 origin of replication and an enhancerless T antigen gene. This SV40 DNA fragment is flanked by a polylinker, derived from plasmid pUC18 (40) and by the 1.85 kb EcoRl-AvaI fragment of pBR327 (41) harboring the ampicillin resistance gene and the origin of replication. During subcloning the AvaI site at the pBR-SV40 junction was conserved but the BamHl site was lost.

pSVXBenh was constructed by inserting a 1.5 kb BglII fragment originating from plasmid pBGXS512 (42) and containing the SV40 enhancer into the BamHl site of pSVXB-pl.

The four HBV DNA fragments originally inserted in pSVXB-pl (Fig.2) were obtained from a vector containing a tandem duplication of a HBV genome (strain ayw; 36,43) which was restricted with BamHl and EcoRl. The fragments were separated on a low melting agarose gel and inserted in the EcoRI and/or BamHI site of the pSVXB-pl polylinker. The resulting vectors were named according to the length of the HBV DNA fragments inserted (e.g. plasmid pSVXB912 harbors a 912 bp HBV DNA insert, Fig.2). The other, smaller HBV DNA fragments were obtained by restriction of pSVXB912 or of other vectors, and when necessary their sticky ends filled in with E.coli polymerase I. Fragments were then separated on low melting agarose gels and subcloned in
pSVXB-pl using standard techniques (44). Plasmid pSVX241di contains a dimer of the 241 bp HBV DNA fragment (Fig. 3A).

Plasmid pUC18/HBV912 was constructed by inserting the 912 bp BamHI-BamHI HBV DNA restriction fragment (Fig. 2) in the BamHI site of vector pUC18 (40).

**Expression selection**

Plasmid pUC18/HBV912 was used as source of HBV DNA for the expression selection experiments. It contains about 0.9 kb of HBV DNA including the HBV enhancer and about 2.7 kb of vector sequences. pUC18/HBV912 DNA was vigorously sonicated such that the majority of the fragments was 200-300 bp in length. The ends of the sonicated fragments were repaired with the Klenow fragment of E.coli polymerase I, and 0.8 μg of sonicated DNA were blunt end ligated with 0.8 μg of SmaI linearized pSVXB-pl DNA. The resulting plasmid pool was cut with SmaI to linearize self-ligated plasmid and transfected into E.coli HB101, which were grown in ampicillin-containing medium. 2 μg of plasmid DNA (derived from the amplification of a pool of about $10^5$ independent clones, as determined by plating an aliquot of the bacteria on ampicillin plates immediately after transfection), were used to transfect about $10^7$ CV-1 cells with the DEAE-Dextran mediated transfection (45,46) which can be performed with relatively crude plasmid preparations. For T antigen staining (18,42) cells were split one day after transfection; low molecular weight DNA extracted from CV-1 (47) cells was restricted with DpnI (49) and the replicated plasmids rescued in E.coli by transfection (44). Colony hybridization was as described (44) and the test for expression in CV-1 cells was performed with plasmid DNA extracted from 10ml minicultures. Sequencing of the inserts of pHB23 and pHB31 was done from the XbaI and EcoRI sites of the polylinker using the chemical degradation protocol (48).

**RESULTS**

A novel vector for expression selection of enhancer sequences

Preliminary results obtained with the SV40 enhancer trap (38) indicated that an enhancer, which was weakly active in monkey kidney CV-1 cells, was encoded on the HBV genome (R.C. 7459)
and W.S., unpublished). Since the enhancer trap approach relies on rescue of infectious SV40 virus, which can take many weeks in the case of a weak enhancer, and requires the subcloning of the incorporated piece of DNA in plasmid vectors, we decided to try to define the HBV enhancer with a new protocol, involving the biological selection of plasmid DNA rather than viral DNA. For this we constructed a shuttle vector containing a plasmid replicon, the SV40 origin of replication and an enhancerless T antigen gene (plasmid pSVXB-pl, Fig.1). Foreign DNA inserted in the polylinker of this plasmid can be selected for enhancer function because only the plasmids carrying a functional enhancer produce sufficient T antigen to replicate in eukaryotic cells. In addition to this novel expression selection approach, we also performed a conventional screening of the HBV genome for enhancer sequences, which we will describe first.

**Mapping of the HBV enhancer by fragment subcloning**

The conventional approach involved the introduction of defined, progressively smaller pieces of HBV DNA into the polylinker of pSVXB-pl and test for induction of T antigen expression as measured by immunofluorescence. We started by inserting four fragments covering the whole 3.2 kb HBV genome into pSVXB-pl (Fig.2 and Materials and Methods). The resulting clones, called pSVXB1504, pSVXB912, pSVXB490 and pSVXB276 (numbers corresponding to the length of the HBV DNA fragments inserted) were used to transfect CV-1 cells. Two days after transfection these cells were stained for T antigen. Only pSVXB912 transfected cells showed bright T antigen immunofluorescence.

To analyze if the 912 bp HBV DNA fragment could enhance T antigen expression independent of its orientation, we transfected CV-1 cells with pSVXB912 and pSVXB912- (the latter containing the HBV fragment in the opposite orientation; Fig.2). Both transfections yielded a similar number of T antigen positive cells, showing that the HBV enhancer identified on the 912 bp HBV DNA fragment works in both orientations. To delimit further the enhancer activity we cut the 912 bp fragment with HindII, and separately cloned the 501 and 411 bp fragments (Fig.2). Only pSVXB411 transfected cells showed bright
Figure 1. The shuttle vector pSVXB-pl for expression selection of enhancer sequences.

pSVXB-pl encodes a plasmid replicon and an ampicillin-resistance gene as well as the SV40 origin and an enhancerless T antigen gene. Its construction is described in Materials and Methods. The polylinker present upstream of the T antigen gene allows easy introduction of foreign sequences, which can then induce T antigen expression, and thus replication, in eukaryotic cells. Enhancer-containing plasmids can then selectively be rescued by transfection in E.coli.

fluorescence upon T antigen staining. Finally we constructed six clones with deletions of the 411 bp fragment at one, the other or both ends (Fig.3A). We quantitated the activity of these

Figure 2. Delimitation of the HBV enhancer by subcloning of fragments of the HBV genome in vector pSVXB-pl.

A linear map of the circular HBV genome, arbitrarily opened at nucleotide 1004 (BamHI site, standardized HBV numbering system; 58) is shown. The restriction sites used for subcloning are indicated on the top. The subcloned fragments are indicated with their length in bp (e.g. 912 corresponds to the fragment cloned in vector pSVXB912). Induction of T antigen immunofluorescence in CV1 cells are indicated by a + and lack of induction by a −.
Figure 3. Fine mapping of the HBV enhancer by deletion analysis and by expression selection.

For quantitative tests of T antigen immunofluorescence we took great care that the plasmid DNAs to be tested were prepared using precisely the same protocol; the cells to be transfected were split from a large pool one day before transfection. For these transfections plasmid DNA purified through CsCl gradients was introduced into CV-1 cells by a modified calcium phosphate procedure (38,63,64). T antigen immunofluorescence staining was as described (18,42). For each transfection a field corresponding in average to 35,000 cells was evaluated. Vector pSVXBenh, with the SV40 enhancer, was taken as 100% enhancer activity and resulted in 11.6% positive cells in the first experiment and in 19.7% positive cells in the second experiment. Vector pSVXB-pl, the negative control, yielded no bright fluorescent cells in either experiment.

fragments for their ability to enhance T antigen expression in comparison with the SV40 enhancer (pSVXBenh, Materials and Methods) and the entire HBV 411 bp fragment. The results of two separate sets of transfections done in parallel are shown in Fig.3A. They were confirmed by several transfections of subgroups of the plasmids, using different DNA preparations. In summary, the data presented in Fig.3A allow the assignment of the HBV enhancer around the center of the 411 bp fragment: Short deletions at both ends of this fragment do not affect its
strength (compare pSVXB321, pSVXB274 and pSVXB241di with pSVXB411). Larger deletions, however, drastically affect enhancer strength, indicating that a critical region of the HBV enhancer is situated around HBV nucleotide 2500. (For example, pSVXB166 with a deleted righthand side is down by almost two orders of magnitude, and pSVXB245 with a deleted lefthand side is down by one order of magnitude). In agreement with the above conclusion a short fragment covering only the left part of this apparently critical sequence also displays significantly reduced enhancer activity (pSVXB151, covering nucleotides 2361 to 2512).

Mapping of the HBV enhancer by expression selection

In parallel to this conventional method for enhancer mapping, we tested a new protocol based upon the fact that replication of viral recombinant plasmids in eukaryotic cells depends on an enhancer sequence (65-67; J. de Villiers and J. Banerji, personal communication). The novelty of our approach was the selective rescue of such amplified plasmids in E.coli, thus allowing for the isolation of fragments with enhancer activity from a random mixture of DNA. For this, we constructed a vector containing the SV40 origin of replication and the early region but not expressing T antigen because the enhancer region is deleted (Fig.1). The insertion of foreign DNA with enhancer function into this vector is expected to allow T antigen expression, and thus replication, and the amplified enhancer-containing plasmids are expected to be selectively rescued by transfection in E.coli.

We tested this protocol with random HBV DNA fragments 200-300 bp in length obtained by sonication of plasmid pUC18/HBV912 (Materials and Methods). These fragments were blunt-end ligated into pSVXB-pl DNA linearized with SmaI. To obtain enough DNA for transfection of eukaryotic cells we amplified the mixture of plasmids in E.coli (Fig.4 and Materials and Methods; this step is not required if larger quantities of plasmid and sonicated DNA are used). Plasmid DNA from the E.coli mass culture was then used to transfect CV-1 cells. From these an aliquot was stained for T antigen 48 hours after transfection and they were found to yield some brightly positive cells. At the same time low molecular weight DNA was extracted, digested with DpnI to
Figure 4. Expression selection protocol for the isolation of functional enhancer sequences. For details see text and Materials and Methods.

eliminate input plasmids that did not replicate in CV-1 cells, and transfected into E.coli. (Dpnl cuts only methylated DNA, and thus does not restrict DNA which has replicated in CV-1 cells, but cuts several times residual input plasmids which have
replicated only in E.coli HB101; 49). Thirty-three plasmids were rescued in that way. Because of the possibility of incomplete DpnI digestion and since plasmids without enhancer sequences might also replicate as "parasites" (see Discussion), we hybridized these colonies with nick-translated HBV DNA. Four out of thirty-three colonies turned out to be positive. Plasmid DNA from these four colonies was used to transfec CV-1 cells, which were assayed for T antigen immunofluorescence two days after transfection. Cells transfected with two of the four plasmids were positive, and thus we examined the insert of the two corresponding clones, pH23 and pH31, by sequencing. As diagrammed in Fig.3B, the HBV inserts of these plasmids were 166 and 214 bp long, and were centered in both cases around HBV position 2500. Thus two different experimental approaches identified the region between HBV nucleotides 2400 and 2600 as the HBV enhancer.

Finally to analyze whether the HBV enhancer can stimulate transcription of a gene when situated downstream of the promoter, we constructed a vector containing the HBV enhancer (274 bp fragment, Fig.3A) located about 3 kb downstream of the cap site of a rabbit β-globin mRNA. As positive and negative controls we used vectors containing the SV40 enhancer and no enhancer in the same position, respectively. We then monitored β-globin transcription after transfection of simian CV-1 and human HeLa cells. In both cell lines β-globin transcription was stimulated by a factor of 3-5 by the HBV enhancer and by a factor of about 100 by the SV40 enhancer (data not shown). Thus we conclude that the HBV enhancer, albeit with low efficiency, can also act from a position downstream of a gene.

DISCUSSION

We have defined an about 200 bp fragment of HBV DNA which stimulates expression of T antigen and transcription of β-globin in CV-1 and HeLa cells. This HBV promoter element can work in either orientation and when situated downstream of a gene, and thus has all the characteristics of an enhancer. The HBV enhancer is situated between nucleotides 2400 and 2600, that is, within the very long open reading frame apparently coding for
the viral reverse transcriptase (50,51), and thus constitutes the only enhancer known to reside in a protein-coding region.

To define the HBV enhancer we adopted two strategies, the first based on classical deletion mapping and the second on biological selection for enhancer sequences. The results of the two strategies agreed, defining the same region of HBV DNA as a weak enhancer. The fact that we could define by expression selection an enhancer only 5-10% as active as the SV40 enhancer indicates that this novel protocol can be applied to a variety of potential enhancer sequences including very weak ones. Another advantage of the expression selection protocol is that it is fast: Plasmid molecules having trapped enhancers can be recovered from CV-1 cells 48 hrs after transfection. The majority of colonies rescued in E.coli, however, did not contain an enhancer. We interpret these plasmids to be residual input DNA escaping DpnI digestion and/or "parasites", i.e. replicating in CV-1 cells due to the T antigen produced by a cotransfected recombinant containing an enhancer. A second round of functional selection in CV-1 cells would, most likely, lower the background of negative colonies, and experiments are in progress to test this. Expression selection using a shuttle vector has another advantage over the previously used viral enhancer trap approach: The latter is restricted to cells fully permissive for SV40 (old world monkey cells), whereas expression selection can be applied to any cell line in which T antigen can drive SV40 DNA replication. This includes the many human cell lines semi-permissive for SV40, which should allow for selection of cell type-specific enhancers. In addition, functional promoter sequences could also be selected for by a similar protocol involving a slightly modified vector.

Shaul, Rutter and Laub (37) recently reported studies on the HBV enhancer, performed mostly in Alexander cells (a hepatoma cell line harboring several copies of the HBV genome), and using HBV DNA of another subtype and the CAT gene as a test system. They concluded that a fragment of about 160 bp mapping between HBV nucleotides 2361 and 2519 (Fig.5, sequence adw) harbored most of the HBV enhancer activity. Our results define a slightly different HBV enhancer region, centered about 65
Figure 5. Map of the HBV transcripts and proteins, position and sequence of the HBV enhancer.

The two major HBV mRNAs are represented as heavy lines, the HBV proteins as boxes with an arrow, indicating the direction of translation, the HBV enhancer region as a black box. Pre-S indicates the part of the HBsAg transcription unit which is not always expressed, P the potential reverse transcriptase and X an unassigned open reading frame (58,68). The sequence of the HBV DNA fragment identified by Shaul et al. (37; adw subtype) and the ayw sequences rescued in our shuttle plasmids pHB23 and pHB31 are shown; identical nucleotides are indicated by dashes. Screening of the HBV enhancer sequence for homologies with consensus sequences from other enhancers (26,30,31,66,69,70) did not reveal perfect matches; a run of 11 alternating purines and pyrimidines maps between HBV nucleotides 2513 and 2523.

nucleotides downstream of the region defined by Shaul et al. (Fig. 5, sequences ayw/23 and ayw/31). This difference could have several reasons, notably the very limited number of constructions tested by Shaul et al. and the fact that their
vector still contained 27 bp of the SV40 enhancer. In addition, Shaul et al. found that in hepatoma cells the HBV enhancer was stronger than the SV40 enhancer. The weak activity of the HBV enhancer in non-hepatic cells could imply that its activity is modulated by some liver-specific components and/or by a viral factor, similar to what was proposed for the Herpes simplex virus enhancer (28).

The HBV enhancer region is situated about 1150 bp downstream of the major initiation site for surface antigen mRNA and about 500 bp upstream of the major initiation site for core antigen mRNA (11; H. Will and H. Schaller, personal communication; Fig.5). Shaul et al. (37) have demonstrated that the HBV enhancer is essential for the activity of the core antigen promoter in Alexander cells. It is conceivable that the enhancer is also required for surface antigen transcription: Indications for this come from studies of HBsAg expression in monkey kidney Vero cells, in which the sequences between HBV nucleotides 2517 and 2967 were found to be essential for efficient HBsAg expression (52) and from the observation that in Alexander cells only those HBV DNA integrates having preserved the enhancer region efficiently transcribe the HBsAg gene (53-55).

The mapping of an enhancer on the HBV genome has also implications regarding the still unknown mechanism of induction of primary hepatocellular carcinoma by HBV. This liver carcinoma arises many (10-50) years after infection with HBV (1), suggesting a slow mode of oncogenesis by HBV. It is known that retroviruses, with respect to their mode of oncogenesis, can be classified into fast-transforming retroviruses carrying an oncogene, and slow-transforming retroviruses de-regulating transcription of cellular oncogenes by promoter/enhancer insertion (56,57). Very recently it has been demonstrated that enhancer activity directly correlates with the oncogenic potential of retroviruses (35) and since HBV-like viruses show several peculiarities of the retroviral life cycle including reverse transcription (4,58), it is tempting to speculate that they also induce oncogenesis by enhancer insertion. However, in spite of careful search (59-61) no known cellular oncogene could
be mapped in the vicinity of HBV DNA integrated in liver tumors or cell lines derived from them. Now it would be interesting to repeat this analysis with highly sensitive probes covering exclusively the HBV enhancer region.

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