MOLECULAR AND CELLULAR BIOLOGY, Jan. 1987, p. 545–548
0270-7306/87/010545-04$02.00/0

Vol. 7, No. 1

Identification of a Promoter Element Located Upstream from the Hepatitis B Virus X Gene

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Received 7 August 1986/Accepted 7 October 1986

We have analyzed a series of plasmids in which the sequences located upstream from the hepatitis B virus (HBV) X gene were linked to the chloramphenicol acetyl transferase (CAT) gene. Expression of the marker CAT gene in transfected cells clearly demonstrated that sequences preceding the X gene contain an active promoter. RNA mapping by primer extension indicated that the RNA encoded by the X gene promoter initiates at multiple sites spanning nucleotides 1250 to 1350 on the HBV genome. Deletion within the adjacent HBV enhancer element region significantly reduced the activity of the X gene promoter, suggesting that the X gene promoter requires the enhancer element for maximal activity.

Hepatitis B virus (HBV) is a small, human DNA virus which causes acute and chronic hepatitis and is strongly associated with the development of primary hepatocellular carcinoma (for a review, see reference 7). The study of the biology of HBV is limited by its restricted host range and by failure to propagate the virus in tissue culture. Information on the genetic organization of HBV has been provided by cloning and sequencing of the HBV genome (for a review, see reference 21). These studies have revealed that the virus DNA contains four open reading frames (ORF) clustered in the 3.2-kilobase viral DNA minus strand. In addition to the genes that code for the major structural proteins, the surface (HBsAg) and core (HBcAg) antigens, and a gene coding for the putative viral polymerase, the genome contains an ORF which could code for a protein termed X (20, 22). The viral promoters located upstream from the core and surface genes have been detected and mapped by in vitro transcription experiments (16), by expression of HBV in chimpanzee liver cells (2, 3), by expression in alternative mammalian cells by a viral expression vector system (13), and by DNA-mediated gene transfer studies (19).

Kay et al. (12), Elfassi et al. (6), and Meyers et al. (15) have recently detected, in samples from some HBV-infected patients, antibodies that recognized the X gene product synthesized as a fusion protein in a bacterial expression system. These results suggest that the X gene of HBV is expressed during HBV infection. However, the mechanism by which this gene is expressed was still unclear.

We have recently identified an HBV enhancer element located between the end of the HBsAg gene and the beginning of the X gene ORF. This enhancer appears to act relatively independently of position and orientation and is essential for activity of the HBcAg gene promoter located 450 base pairs downstream (18). The location of the HBV enhancer upstream from the X gene ORF suggests that this enhancer may also control the expression of the X gene.

The assayable chloramphenicol acetyltransferase (CAT) system (8) was used to survey for the X gene promoter. Figure 1 describes the structure of recombinant plasmids in which the HBV sequences preceding the X gene ORF were inserted upstream of the bacterial CAT gene. The *Hpa*I-*Tha*I HBV fragment (nucleotides 964 to 1355), bearing the HBV enhancer element and sequences located upstream from the X gene, was ligated to synthetic *Bam*HI linkers (14). This fragment was then inserted in the two possible orientations upstream of the CAT gene in the pSV0CAT plasmid, which does not contain a promoter (8). In the resulting pEXp$^+$cat plasmid, the putative X gene promoter was positioned in the sense orientation relative to the CAT gene, while in pEXp$^-$cat, this fragment was inserted in the opposite orientation. DNAs from these plasmids and from control plasmid pSV2CAT (8) were transfected (10) into Hep SK cells, and CAT activity was monitored as described previously (8). The X gene promoter (pEXp$^+$cat) efficiently directs CAT expression, strongly suggesting that this HBV region contains an active promoter (Fig. 2). The inactivity of pEXp$^-$cat excludes the possibility that the CAT activity expressed by pEXp$^+$ cat results from an aberrant promoter within pSV0CAT which is induced by the presence of the HBV enhancer element.

The primer extension procedure was used to map the 5′ ends of the RNA initiating from the putative X gene promoter. Poly(A)$^+$ RNA from cells transfected with the pEXp$^+$cat plasmid was annealed with a labeled 19-mer CAT primer (23) and extended with reverse transcriptase. The extended products were analyzed on a urea-acrylamide sequencing gel. The 5′ ends of these transcripts are heterogenous, with multiple start sites (three major sites and two to four minor sites) spanning some 100 nucleotides between nucleotides 1250 and 1350 on the HBV genome map (Fig. 3). The three major initiation sites (Fig. 3, bands a, b, and c) on the HBV genomic map were mapped to nucleotides 1310 ± 5, 1332 ± 5 and 1340 ± 5. Similar results obtained by the riboprobe technique confirm the primer extesion mapping (data not shown).

To test the effect of the HBV enhancer element on the adjacent X gene promoter, we deleted sequences within the enhancer region and assayed the effect of these deletions on CAT expression driven by the X gene promoter. The pEXΔStu (Fig. 1) deletion removes sequences between nucleotides 970 and 1116. When assayed in Alexander cells (Fig. 4), CAT expression was reduced 12-fold. Deletion of the entire enhancer region, from nucleotides 964 to 1217 (pEXΔSphI), abolishes CAT expression. This result suggests that the X gene promoter requires the enhancer for its function. However, because of the close proximity of the

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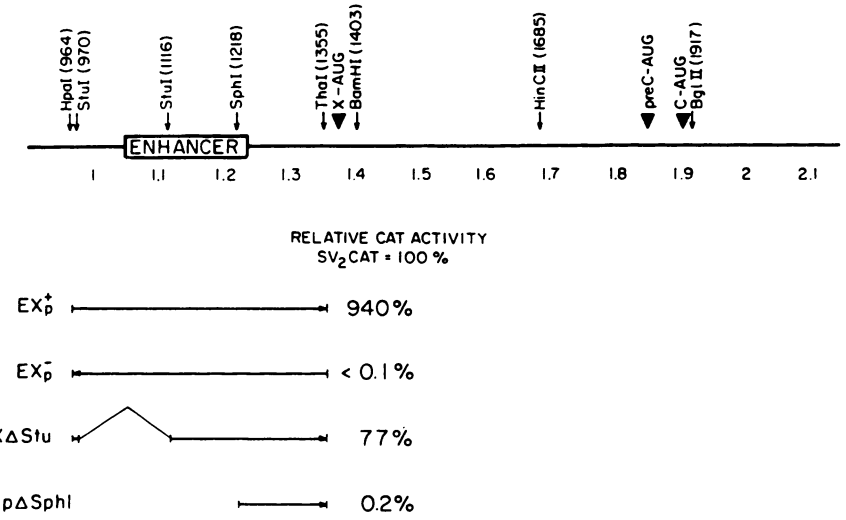

FIG. 1. Structure of the plasmids containing the X gene promoter. The HBV *Hpa*I-*Tha*I (nucleotides 964 to 1355) fragment was ligated with synthetic *Bam*HI linkers and inserted into the unique *Bgl*II site within the promoterless pSV0CAT plasmid (8). In the resulting plasmids, pEXp$^+$cat (EXp$^+$) and pEXp$^-$cat (EXp$^-$), the putative X gene promoter was inserted in the sense and nonsense orientation to the CAT gene, respectively. A deletion between nucleotides 970 and 1116 (EXΔStu) was made by removing part of the enhancer element region by using the two *Stu*I sites within pEXp$^+$cat. The EXpΔSphI deletion, which lacks the entire enhancer element, was produced by subcloning the *Sph*I-*Bam*HI fragment from EXp$^+$cat between the *Sph*I and *Bam*HI sites in pBR322. The map of the HBV genome (22) in this region, including the location of the HBV enhancer, the beginning of the X gene (X-AUG), and the relevant restriction sites, is presented at the top of the figure. The values of CAT activity in transfected Alexander cells are given relative to the activity expressed by pSV2CAT, taken to be 100%.

enhancer and the X gene promoter, the possibility that these two elements overlap rather than affect one another cannot be excluded.

Primer extension analysis demonstrated that the RNA encoded by the X gene promoter initiates at multiple sites spanning nucleotides 1250 to 1350. Gough (9) previously detected, in a rat cell line containing HBV DNA, a transcript which was roughly mapped to the X gene region. Saito et al. (17), used an adenovirus vector to express HBV DNA in human HeLa cells and mapped three major HBV transcripts. One transcript, 0.7 kilobases in length, was mapped to the X region. Nuclease S1 mapping suggested that this 0.7-kilobase transcript has a heterogeneous 5′ end and maps between nucleotides 1250 and 1300. From these studies, however, it was still unclear whether the X-encoding mRNA resulted from transcription initiation, RNA processing, or RNA splicing. Our results demonstrate that the X transcript is initiated from its own promoter. This X gene promoter lacks TATA-box-like sequences, a feature also observed for the simian virus 40 late promoter and the internal HBV surface gene promoter (2), which are likewise characterized by production of RNAs with heterogenous 5′ ends.

We previously described an element in HBV that displays the characteristics of an enhancer element (18). This enhancer maps between nucleotides 1000 and 1250 on the HBV genome (18). The location of this enhancer upstream from the beginning of the X gene ORF (nucleotide 1377) suggests that the enhancer element may also play a role in the regulation of the X gene promoter. Indeed, deletions within the enhancer region dramatically reduced transcription from the downstream X gene promoter. The ΔStuI deletion, which removed 140 nucleotides (nucleotides 970 to 1116), reduced the X gene promoter activity 12-fold. A larger deletion, ΔSphI (nucleotides 964 to 1217), abolished all the X gene promoter activity. These results suggest that the X gene promoter requires the presence of the HBV enhancer for maximal activity. However, the possibility that the X gene promoter and the enhancer element are two independent overlapping elements cannot be excluded.

HBV uses unusual strategies to express its genes and to increase its coding capacity to as many as six or seven functional proteins. The HBV transcript initiates from multiple sites, terminates at a single inefficient poly(A) addition signal, and lacks RNA splicing and processing. The discovery of a new promoter located upstream from the X gene ORF provides evidence that these sequences represent a functional gene which is expressed from its own transcriptional unit. We previously reported that the HBV enhancer element is located 450 base pairs upstream from the HBcAg gene promoter and is required for its efficient expression.

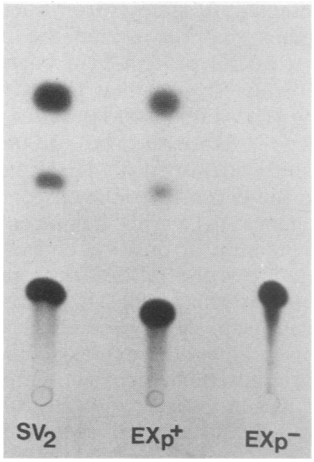

FIG. 2. Expression of CAT activity in Hep SK cells. Hep SK cells were transfected with pSV2CAT (SV2), pEXp$^+$cat (EXp$^+$), and pEXp$^-$cat (EXp$^-$) plasmid. HepSK cells are identical to SK-Hep-1 cells (4). Cell extracts were prepared and assayed for 1 h for CAT activity (8).

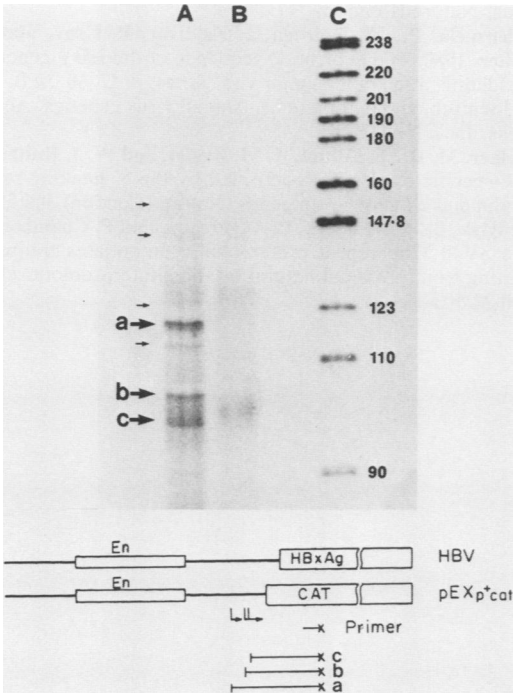

FIG. 3. Mapping of the 5′ end of EXp$^+$cat coded RNA by primer extension. An end-labeled 19-mer CAT primer (23) was annealed with poly(A$^+$) RNA extracted from Hep SK cells transfected with pEXp$^+$cat. The autogram shows the extended products analyzed on a sequencing gel. Lanes: A, primer extension reaction in the presence of pEXp$^+$cat RNA; B, RNA from mock-infected cells; C, size markers. The annealing and extension reaction was carried out as described by Walker et al. (23). The diagram below the figure represents the pEXp$^+$cat template, the corresponding HBV region, and the location of the three major extended transcripts.

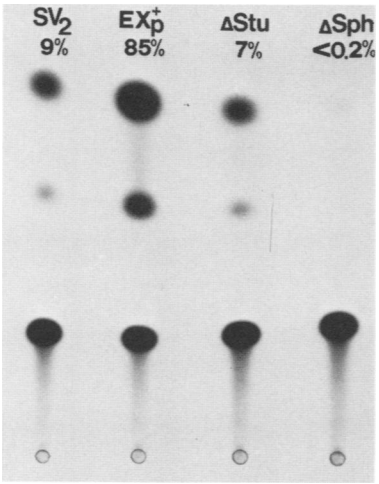

FIG. 4. Effect of the HBV enhancer element of the X gene promoter activity. pSV2CAT (SV2), pEXp$^+$cat (EXp$^+$), pEXpΔStu (ΔStu), and pEXpΔSphI (ΔSphI) DNAs were transfected into Alexander cells. Cell extracts were prepared after 48 h and assayed for CAT activity after 1 h of incubation. The conversion of chloramphenicol into its acetylated forms was determined by counting the region from the chromatogram, and the results are expressed as percent conversion.

The location of the X gene promoter suggests that the HBV enhancer affects the activity of at least two promoters associated with two separate transcriptional units on the same genome. Wasylyk et al. (24) and de Villiers et al. (5) presented data suggesting that enhancer elements preferentially activate promoters adjacent to them rather than those located further downstream. Kadesch and Berg (11) observed similar interference but suggested that promoter occlusion (1) causes reduced transcription at a downstream promoter if transcription is initiated at a nearby upstream promoter. This mechanism may imply that the regulation of the X gene promoter also affects the downstream HBcAg gene promoter.

We thank M. Walker for help with the primer extension analysis, D. Shafritz, J. Traub, and M. Kessler for critically reading the manuscript, and Melvine Stein for preparing the manuscript.

This study was supported by grants from the Israel Cancer Association (O.L 5/85) and the Leo and Julia Forcheimer Center for Molecular Genetics.

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