Transcriptional control elements of hepatitis B surface antigen gene

(in vivo transcription/S1 nuclease/primer extension/chloramphenicol acetyltransferase assay)

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ABSTRACT A series of recombinant plasmid vectors containing hepatitis B virus (HBV) DNA sequences was constructed to study the biosynthesis of the hepatitis B virus surface antigen (HBsAg) RNA and to locate transcriptional control elements involved in the regulation of the S and pre-S DNA sequences. We examined the transcription of the HBsAg gene in permanent cell lines that were developed by transfecting with recombinant vectors containing HBV sequences and the neomycin gene followed by G418 selection. We further defined the promoter activities upstream of and within the pre-S sequences using the assayable chloramphenicol acetyltransferase gene. Results obtained from S1 nuclease digestion and primer extension suggest that HBsAg transcripts are initiated at multiple sites in the pre-S region and from a site upstream of the pre-S region. Chloramphenicol acetyltransferase assays indicate that DNA sequences within and upstream of the pre-S region contain promoter activities and that the "TATA" sequence-containing promoter and the internal promoter show similar levels of activities in CV-1 cells and several other cell lines tested.

The study of human hepatitis B virus (HBV) has been severely limited because it only infects humans and because a tissue culture system in which this virus can be propagated is not available. During infection of humans, the virus (Dane particle) and the two prominent subviral particles (filaments and 22-nm particles) are observed in the sera of infected individuals. The virion consists of an icosahedral core surrounded by a lipid envelope that contains the surface antigen (HBsAg) (1, 2). Filaments and 22-nm particles consist only of the HBsAg and cellular lipid. Careful examination of the surface antigen (3, 4) has indicated that it is composed of at least three pairs of proteins: p24 and gp27; gp33 and gp36; p39 and gp42 (p, protein; gp, glycoprotein), where the second protein in each pair is a glycosylated form of the first. It is apparent from the work of a number of investigators (2, 5—9) that these proteins are derived from a large open reading frame (ORF) and originate from the first three strongly conserved ATGs in that region. This particular ORF consists of an "S" region that is preceded by an in-phase reading frame, which has been designated as "pre-surface" or pre-S. The pre-S region may be further subdivided into pre-S(1) and pre-S(2) (2, 10, 11).

By using eukaryotic vectors, expression of HBsAg has been demonstrated in various cultured cells (5—8). Synthesis of HBsAg in mammalian cells has uniformly resulted in production of this gene product and its secretion in the form of 22-nm particles into the culture medium, which facilitates detection and purification. Previously, we demonstrated that a 2.8-kilobase (kb) Bgl II fragment of HBV DNA contained all of the signals necessary to express the HBsAg gene and that the expression resulted in secretion of the 22-nm particles (7).

Our primary interest in the design of vectors (Fig. 1) was to utilize native HBV sequences in order to define the transcriptional control elements involved in the regulation of HBsAg biosynthesis. Promoter activity has been attributed to a sequence before the pre-S region that contains the canonical "TATA" box at 2790 nucleotides (nt) (7, 12—14) and to a less clearly defined site within the pre-S region itself (5, 8). However, the RNAs produced by this region have not been carefully mapped in transfected cells in which the HBsAg synthesis is under the control of the native promoter(s).

In this study, we describe the HBsAg transcripts produced in vivo by permanent cell lines that were developed from transfection with plasmid vectors containing HBV DNA sequences. We also define the HBsAg promoters by using the assayable chloramphenicol acetyltransferase (CAT) system (15).

MATERIALS AND METHODS

DNA Constructions. The HBV genome used in our recombinant vectors was an adw subtype and its cloning has been described (16). Plasmids pSV10 and pML-neo were from R. Tjian and M. Botchan (University of California, Berkeley), and pSV2CAT was from Bruce Howard (National Institutes of Health). For construction of pNEP and pNET, the genome-length HBV DNA was cleaved at its unique Apa I (2612 nt) and Taq I sites (2020 nt), respectively, and was inserted at the Cla I site of pML-neo. The Apa I ends were processed to add synthetic Cla I linkers for insertion in pML-neo. Construction of pSH18 has been described (7). Plasmid pSH4 was constructed by cleaving pSH18 at HindIII and BstEII sites, which removes the putative HBsAg promoter containing the TATA sequences. The termini were blunted with T4 DNA polymerase followed by self-ligation.

All restriction enzymes, DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were products of either Boehringer Mannheim, Bethesda Research Laboratories, or New England Biolabs. Synthetic linkers were purchased from P-L Biochemicals.

S1 Nuclease Mapping, Primer Extension, and CAT Assays. For mapping 5' and 3' ends, the procedure of Weaver and Weissman was used (17). Analysis of S1 nuclease-protected DNA fragments was carried out by either alkaline or urea/polyacrylamide gel electrophoresis (18). Primer extensions were according to Ghosh et al. (19). Products of primer extensions were analyzed on 8% urea/polyacrylamide gels (18). Gels were dried and then autoradiographed for 1—3 days at −70°C with an intensifying screen and X-Omat AR film.

Abbreviations: HBV, hepatitis B virus; HBsAg, HBV surface antigen; kb, kilobase(s); nt, nucleotide(s); ORF, open reading frame; bp, base pairs; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.
CAT assays were carried out according to the procedure of Gorman et al. (15) with 0.5 μCi (1 Ci = 37 GBq) of [3H]chloroformenol (New England Nuclear) per assay.

DNA Transformation. Rat fibroblasts (R. Erikson, Harvard University) and COS cells (Y. Gluzman, Cold Spring Harbor Laboratory) were transfected either by the calcium phosphate method (20) or the DEAE-dextran and chloroquine method (21). Transient expression was studied in COS cells. The rat fibroblast cells transfected with pNEP and pNET were subjected to G418 selection to generate permanent cell lines according to the method described previously (22). About 60% of G418 clones were positive for HBsAg expression.

RIAs. The commercial Ausria II RIA kit (Abbott) was used to quantitate the amounts of HBsAg particles in the culture medium. Values of P/N > 2.1 were considered positive.

RNA Extraction. Cytoplasmic RNA from transient or stable cell lines was isolated by a modification of the method of Chirgwin et al. (23). Cells from monolayer plates were harvested by scraping with a rubber policeman and were lysed with 0.5% Nonidet P-40 in 0.05 M Tris-HCl, pH 7.5/0.01 M MgCl₂/0.1 M NaCl. Nuclei were pelleted, and 6 M guanidinium isothiocyanate was added to the cytoplasmic supernatant. This mixture was then layered on 1.2 ml of a 5.7 M CsCl cushion in Beckman centrifuge tubes and centrifuged in an SW 55 rotor at 40,000 rpm for 16 hr at 16°C. RNA pellets were washed with cold water, suspended in large volumes of 0.1 M NaCl/0.5 M Tris-HCl, pH 7.5/1 mM EDTA, and precipitated with ethanol twice. RNA isolated by this method is absolutely free of DNA, obviating the need for DNAse treatment for subsequent RNA studies. Further purification of poly(A)+ RNA was accomplished by oligo(dT)-cellulose chromatography (18). For RNA transfer blot analysis (24) and mapping studies, total cytoplasmic RNA and poly(A)+-purified RNA were used.

RESULTS

The HBV genome was linearized at two unique restriction sites (Taq I, 2020 nt; Apa I, 2612 nt) and the entire genome was inserted into the pML-neo vector (Fig. 1) to produce pNET and pNEP, respectively. These plasmids were used to stably transform rat fibroblasts by G418 selection (22). Two of the G418 clones that were HBsAg-positive, NET-22 and NEP-67, were used for RNA and protein analyses.

Transcription of HBsAg RNA. Cell lines NEP-67 and NET-22 express HBsAg and secrete 22-nm particles into the culture medium. We further investigated the HBV-related mRNA species produced in the NET-22 cell line. Poly(A)+ RNA was extracted from NET-22 cells (see Materials and Methods) and analyzed by RNA transfer blot hybridization (24) with the nick-translated HBV genome as a probe. Two species of HBsAg-specific RNA were resolved: a predominant mRNA of 2100 nt and a second species of 900 nt (Fig. 2B). These mRNA species have been described by others (5, 6, 8, 13). Similar bands could also be observed with NEP-67 RNA and in COS cells transfected with plasmids pNEP and pNET (data not shown). The 2100-nt band represents the HBsAg RNA transcript; the 900-nt band may correspond to the "X" ORF since it does not hybridize with an S sequence-specific probe (Xba I–Hpa I, 253–967 nt) in an RNA transfer blot hybridization analysis (data not shown). It has been suggested by Simonsen and Levinson (6) that S coding sequences are removed by alternate splicing to produce smaller mRNA transcripts from a large transcript.

To map the initiation sites of the HBsAg transcripts, we first used a Bgl II–Xba I (2436–253 nt) restriction fragment labeled at the 5' ends as a probe for S1 nuclease mapping (Fig. 2). The S1 nuclease-protected band of about 260–270 nt can be seen, which places the point of S1 nuclease cleavage at about 3190 nt, upstream from the EcoRI site (Fig. 2B). Next, we carried out primer extension analysis (19), as shown in Fig. 2C. A 123-base-pair (bp) Tha I–Xba I (130–235 nt) fragment labeled at the 5' end was used as a primer in this analysis. This analysis (Fig. 2C) revealed three bands (267, 248, and 236 nt) that are probably the major initiations of HBsAg mRNA transcripts. These results are in agreement with previous observations by other workers (5, 6, 8, 9) in other cell lines. Extensions of 236 and 248 bp are located downstream of the second ATG and represent transcripts for p24/gp27, whereas the 267-bp extension maps upstream of the second ATG, the ATG for p31/gp35. The 248-nt band appears more intense than the other two, suggesting that it is a stronger initiation site. These results are reproducible and may not represent premature terminations of primer extension.

To determine if the initiation occurred at the pre-S ATG (2859 nt) immediately downstream of the TATA HBsAg promoter, we used a Bgl II–BstEII fragment (2436–2828 nt) as a probe in an S1 nuclease digestion experiment. Results of this experiment, shown in Fig. 2D, indicate that a 25- to 30-nt band was protected, placing the initiation of RNA transcripts upstream from the pre-S ATG. These results suggest that initiation at the pre-S ATG (2860 nt) does occur and the TATA promoter is involved in the synthesis of RNA transcripts from that ATG. The RNA transcripts initiating at this site are infrequent and represent about 1–2% of the HBsAg mRNA population (refs. 8 and 9; unpublished results). It is also possible that most mRNAs that originate from the TATA promoter are spliced to produce X mRNA (6) (Fig. 2A, lane 2).
Assays for HBsAg Promoters. The bacterial CAT gene has been used extensively to assay eukaryotic DNA sequences for promoter activity. Plasmid pSV2CAT was designed by Gorman et al. (15) for expression of the CAT gene in eukaryotic cells. In the present study we assayed two principal areas of the HBV genome: (i) the first fragment contains the putative HBsAg promoter with a canonical TATA sequence located upstream from the pre-S ATG (2860 nt) bound by Bgl II–BstEII (2436–2828 nt) restriction sites; (ii) the second fragment contains DNA sequences bound by BstEII–PstI (2828–34 nt) restriction sites and includes the major portions of the pre-S region. Additionally, the pre-S sequences bound by the Sau3A restriction sites (2938–30 nt) were also tested. These fragments were introduced into pSVOCAT (15) between the Acc I and HindIII sites, as illustrated in Fig. 3 Right. The resulting plasmids—pSVB-gCAT, pSVFTCAT (+/−), and pSVSauCAT—were transiently expressed in CV-1 cells. The results, presented in Fig.
4C, show that plasmids pSVBgCAT, pSVPTCAT (+), and pSVSauCAT are all capable of directing CAT expression. This expression is above the background of pSVOCAT (Fig. 4C, lanes 5 and 6), which does not contain eukaryotic promoter sequences, as well as pSVPTCAT (–) (Fig. 4C, lane 3), which contains the BstEII–Pst I fragment in the antisense transcriptional orientation with respect to the CAT coding region. The low level of CAT activity shown by pSVOCAT is commonly seen in CV-1 cells (Fig. 4C, lanes 5 and 6) (15) and may be due to cryptic promoters present in the adjacent plasmid sequences. It should be noted that insertion of a promoter fragment in an antisense orientation, as in pSVPTCAT (–), totally abolishes this background activity.

We have additionally tested the TATA sequence-containing fragment (Bgl II–BstEII) in plasmid pSVO10 background (25). Plasmid pSVO10 and pSV10-based recombinants replicate with high efficiency in COS cells (7, 26). The scheme of construction of the plasmid is depicted in Fig. 3 Left. Plasmid pPTCAT directs high levels of CAT expression in COS cells over the promoterless plasmid pATCAT (Fig. 4A, lanes 3 and 4). In Fig. 4B, a time course of CAT activity expressed by pPTCAT and pΔTCAT is described. These results clearly show a linear response of the assay as well as establish the presence of a bona fide promoter in the Bgl II–BstEII fragment of HBV. When tested in CV-1 cells, pPTCAT again showed CAT expression, whereas pΔTCAT did not (data not shown).

An independent approach was used to confirm the presence of the internal promoter and its utilization during HBsAg biosynthesis. For this, plasmids pSH18 and pSH4 (Fig. 3 Left) were used. These contain a 2.8-kb Bgl II fragment (2436–1991 nt) and a 2.4-kb BstEII–Bgl II fragment (2828–1991 nt), respectively, cloned into the polylinker of plasmid pSVO10. Although pSH18 contains both, the TATA as well as the internal promoters, pSH4 contains only the internal promoter. When transiently expressed in COS cells, these plasmids directed high levels of HBsAg synthesis, as assayed by RIA of the culture supernatants (data not shown). These levels were not significantly different for the two plasmids. In addition to showing the utilization of the internal promoter, this further suggests that the TATA promoter is dispensable for the synthesis and secretion of HBsAg, in accordance with earlier observations of others (5, 6, 8).

We have also tested CAT expression under the control of both of the HBsAg promoters in rat fibroblasts, rat hepatoma cells, CV-1 cells (monkey kidney), human fibroblasts, and two human hepatoma cell lines (27)—Hep G2 (HBV-negative) and Hep 3B (HBV-positive). Preliminary results (data not shown) suggest that the TATA and the internal promoters of HBsAg gene direct the CAT activity with approximately similar promoter strengths in all of the cell lines tested.

DISCUSSION

We report here in vivo transcription studies of the HBsAg gene in permanent rat fibroblast cell lines, which continually secrete 22-nm HBsAg particles. In agreement with previous reports, we also find that HBsAg mRNAs initiate within the pre-S region and terminate at 1900 bp, beyond a UAAAAA (5, 6, 8, 9) poly(A) signal. Previous studies indicated that the putative HBsAg promoter located upstream of the pre-S ATG containing the TATA sequence (TATA promoter) may regulate the synthesis of HBsAg (7, 12, 13, 28, 29). Subsequently, several reports indicated that pre-S sequences upstream from the EcoRI site (Fig. 2) also contain promoter activity and that a great majority of HBsAg transcripts initiates from this promoter sequence (5, 8, 9). Our studies with CAT plasmids clearly support this second notion.

At the present time it seems reasonable to suggest that two distinct promoter activities are associated with the HBsAg gene. The first is immediately upstream from the second highly conserved ATG codon (3215 nt) and, in our view, controls the production of the p24/gp27 and p31/gp35 HBsAg polypeptides (3–5, 8, 9). The agreement between the S1 nuclease and primer extension data (Fig. 2) suggests that the mRNAs from this promoter are not spliced. This region of the HBV genome is heavily used and evolutionary constraints may have dictated that the S gene products (p24 and p31 and their glycosylated counterparts) are always produced. The internal promoter does not contain the canonical sequences
Preliminary observations on the transient expression of pNEP and pNET in Hep G2 cells [a hepatoma cell line (27)] support this notion.

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