Differential Regulation of the Hepatitis B Virus Surface Gene Promoters by a Second Viral Enhancer*

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The hepatitis B virus surface gene is transcribed from two promoters, and the resulting mRNA species code for three distinct forms of the surface antigen. We show here that the viral transcriptional trans-activator, X protein, has no effect on either promoter. However, a cis-acting element in the downstream half of the X gene, distinct from the previously mapped viral enhancer, selectively activates the major surface gene promoter. Nuclease protection and gel-shift assays reveal that multiple cellular factors bind to two sites within this DNA fragment, both of which are necessary for enhancer activity. Since this region of the viral genome is frequently deleted upon integration into the host chromosome in chronic hepatitis B, loss of this second enhancer can alter the relative amounts of the three forms of the surface antigen in infected hepatocytes and thus possibly contribute to cellular damage.

The hepatitis B virus (HBV) surface gene is transcribed from two tandem promoters (see Fig. 1A). The upstream pre-S1 promoter transcribes an RNA species that mainly encodes the large surface antigen, while the much stronger S promoter transcribes the mRNA for the middle and small (major) surface antigens by virtue of heterogeneous start sites that span the initiating AUG of the middle surface antigen (reviewed in Ref. 1). All three forms of the surface antigens are co-linear in the carboxyl-terminal portion, are viewed in Ref. 1). All three forms of the surface antigens, enhancer located between the surface and X genes up-regulates both promoters (4-6). Since the HBV X protein (pX) trans-activates many promoters (reviewed in Ref. 7, including the HBV core promoter (8), we have performed experiments to determine if pX also regulates the pre-S1 or S promoters. Unexpectedly, pX has no trans-effect on the surface gene promoters. Rather, the downstream half of the X gene is a cis-acting element that selectively enhances transcription from the S promoter.

MATERIALS AND METHODS

Plasmids—The 2.8-kilobase pair BglII fragment of HBV DNA strain adw (9), comprising the surface and X genes (see Fig. 1A), was cloned into the BamHI site of pUC18. A frameshift mutation in the X gene was constructed by digestion with SstII and T4 DNA polymerase, and religating (10). This results in a -2 frameshift known to inactivate the trans-activation function of pX (11). Deletions of the X gene were generated by digestion with NcoI and either Stul or RsrII (see Fig. 1A for restriction sites) and religation following end filling by the Klenow enzyme.

Reporter plasmids were constructed from the plasmid pSVOCAT, which contains a promoterless chloramphenicol acetyltransferase (CAT) gene upstream of the SV40 polyadenylation site (12). The promoter, X, or the S promoter was excised from the HBV genome with SstI and BstEII, or with BstEII and EcoRI, respectively, and inserted into the HindIII site of pSVOCAT with blunt-end ligation. The orientation was checked with suitable restriction enzyme digests. Fragments of the X gene (excised with RsrII and Stul or with ApaLI and Alul) were then inserted downstream of the polyadenylation signal, at the BamHI site, by blunt-end ligation.

The plasmid pECE-X expresses the X gene from the SV40 early promoter, while pECE is the parental plasmid with no inserted gene (13). Cell Transfection, Primer Extension, and CAT Assays—For primer extension analyses, HuH-7 hepatoma cells (which are free of endogenous HBV DNA) were transfected with 10 µg of plasmid DNA for 16 h with the calcium phosphate precipitation method (14). After 2 days, the total cellular RNA was purified by the guanidine/cesium chloride method (15), and 5-µg aliquots were subjected to primer extension with 5 units of avian myeloblastosis virus reverse transcriptase and 1 ng of an oligonucleotide (5'AGAGGCAATATTGGCATCTCGAGACGGGTTC) labeled at the 5' end with polynucleotide kinase and [γ-32P]ATP (10). After 1 h at 42 °C, the mixture was electrophoresed on a denaturing 8% polyacrylamide gel (10). The primer extension products should give a product of 121 bases, while the S transcript should give products ranging from 105 to 136 bases (14, 15) (see also Fig. 1A).

For chloramphenicol acetyltransferase assays, the hepatoma cells were transfected with 5 µg of reporter plasmid and 5 µg of expression plasmid for 4 h. After 2 days, the cells were lysed and analyzed for chloramphenicol acetyltransferase activity with thin-layer chromatography (12).

Nuclease Protection and Gel-shift Assays—For nuclease protection (16), the lower strand of the X gene was labeled at the 3' end of the RsrII site with [α-32P]dGTP and the Klenow fragment of DNA polymerase I (10). One ng of the fragment from this site to the RsrII site was incubated with 30 µg of HuH-7 hepatoma nuclear extract (17) and digested with 40 ng of DNase I (Worthington) for 30 min at room temperature. The partially digested DNA was electrophoresed on a denaturing 8% polyacrylamide gel (10). The lower strand of the X gene was constructed by digestion with SstII and BstEII. or with BstEII and EcoRI, respectively, and inserted into the HindIII site of pSVOCAT with blunt-end ligation.

For gel-shift analysis (19), double-stranded oligonucleotides with 5' GATC protruding ends and containing sequences corresponding to the direct or inverted repeats in sites I and II were synthesized. Their sequences for sites I and II, respectively, are 5'TGGGGAGGAGTTCGGGGAGGAGATT and 5'TCTTACATAAGAGGACTCTTGGA. Each fragment was labeled with [γ-32P]ATP and polynucleotide kinase, and 1 ng was incubated with 2 µg of poly(dI-dC) and 5 µg of HuH-7 nuclear extract for 30 min at room temperature. In some experiments, a 50-fold molar excess of unlabeled competitor oligonucleotide was included in the
RESULTS AND DISCUSSION

To determine if pX trans-activated either the pre-S1 or S promoter, we measured the effect of deleting most of the X gene (from the NcoI to Stul sites) on transcription from these promoters. Primer extension analysis of RNA isolated from transiently transfected hepatoma cells showed that this deletion indeed produced a significant (>5-fold) drop in the amount of the S transcripts, without a significant change in the pre-S1 transcript, as judged by autoradiography of three independent transfections (Fig. 1B). However, neither a frameshift mutation in the X gene nor deletion of the upstream portion of the X gene (from NcoI to RsrII sites) had a significant effect on either promoter (Fig. 1B).

These results imply that the effect of deleting the X gene resulted from loss of a cis-acting element in the downstream portion of the gene, rather than from loss of trans-activation by the X protein function. To confirm our inference, we placed a fragment of the X gene (from ApaL1 to AluI sites) downstream of the CAT reporter gene in a plasmid driven by either the S or pre-S1 promoter. As predicted, this DNA fragment in either orientation was able to enhance expression from the S promoter by >5-fold but had no significant effect on the pre-S1 promoter (Fig. 2). In addition, when an expression plasmid containing the X gene was co-transfected with CAT reporter genes driven by either the S promoter or pre-S1

mixture. The samples were then electrophoresed at 140 V in a 8% polyacrylamide gel at room temperature and the bands visualized by autoradiography.
Hepatitis B Virus Enhancer II

FIG. 3. Footprint analysis of the enhancer. A, the lower strand of the X gene was labeled at the 3' end and subjected to DNase I protection assay. The Roman numerals I and II indicate the two protected regions, and the arrows indicate the direct and inverted repeats present in these regions. *, a hypersensitive site; G+A, cleavage reaction at G and A residues; BSA, DNase I digestion in the presence of bovine serum albumin; Hep, digestion in the presence of hepatoma nuclear extracts. B, sequence of protected regions. The Arabic numbers indicate nucleotide position relative to the unique EcoRI site in HBV DNA (9).

promoter, no trans-activation was observed (Fig. 2).

One or two copies of a smaller fragment of the X gene, extending from the RsrII to StuI sites, did not have significant enhancing activity when placed downstream of the S promoter (Fig. 2), even though deletion of this fragment from the HBV genome resulted in a loss of enhancing activity (Fig. 1B). Therefore, it is likely that there are at least two sites within the X gene, one on each side of the StuI site, and that each is
necessary but not sufficient for enhancer activity. To pinpoint these sites, we performed DNase I protection analysis of this fragment of DNA with nuclear extracts from hepatoma cells.

Two major footprints were observed (Fig. 3A), one on each side of the StuI site. The downstream footprint (site I) covers two direct repeats of the sequence GGGAGGAG, while the upstream one (site II) is centered on an overlapping pair of palindromic sequences (Fig. 3B). Additional weaker footprints are possibly present between the two sites and upstream of site I (Fig. 3A).

Gel-shift analysis confirms that both sites I and II bind nuclear factors. Specifically, three shifted bands were observed when an oligonucleotide containing site I was incubated with HuH-7 nuclear extracts, and both were competed by unlabeled excess oligonucleotide (Fig. 4A), while two shifted bands were observed with an oligonucleotide containing site II (Fig. 4B). Additional studies will be done to determine the factors that render the pre-S1 and S promoters differentially responsive to this enhancer, since this may provide a paradigm for selective regulation of closely juxtaposed cellular promoters by enhancers.

Recently, others have shown that the portion of the X gene downstream of the StuI site (site I) enhances transcription from the HBV core and thymidine kinase promoters (20), while the portion of the X gene upstream of the StuI site (site II) enhances transcription from the SV40 early promoter (21, 28). Our results show that these two regions are interdependent in enhancing the S promoter and should be considered a single functional cis-acting unit. Therefore, the downstream half of the X gene comprises HBV enhancer II to be distinguished from the previously mapped enhancer I located between the surface and X genes (4).

Our results show that enhancer II affects the relative strengths of the different surface antigens. The balance in the synthesis of the three forms of the surface antigen is important for the HBV life cycle. On one hand, all three forms are needed for viral morphogenesis. On the other hand, an increased ratio of large to the middle and small forms leads to formation of abnormal surface antigen particles and loss of secretion of all three forms of the surface antigen (2, 3, 22, 23), and the accumulated particles can become inspissated in the endoplasmic reticulum and be toxic to the host cell (2, 3). Therefore, the relative strengths of the pre-S1 promoter (which encodes mostly large surface antigen) and the S promoter (which encodes the middle and small surface antigens) must be stringently controlled. The downstream portion of the X gene, including enhancer II, is frequently deleted when HBV DNA integrates into the host chromosome during chronic infection, while the entire surface gene is usually intact in the integrated state (24-26). According to our results, this deletion would lead to an increased ratio of large to middle and small surface antigens synthesized and thus may contribute to the formation of hepatocytes with inspissated surface antigen particles ("ground glass cells") frequently seen in liver biopsy specimens of patients with chronic hepatitis B (27). Further experiments with transgenic mice will be needed to confirm this scenario for the pathogenesis of these ground glass cells, which may be important for liver injury and even carcinogenesis (3).

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D X Zhou and T S Yen

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