Activation of Hepatitis B Virus S Promoter by the Viral Large Surface Protein via Induction of Stress in the Endoplasmic Reticulum

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Hepatitis B virus (HBV) codes for three forms of surface protein. The minor, large form is translated from transcripts specified by the preS1 promoter, while the middle and small forms are translated from transcripts specified by the downstream S promoter. When the large surface protein is overexpressed, the secretion of both subviral and virion particles is blocked within the secretory pathway. We show here that overexpression of the large surface protein leads to up to a 10-fold activation of the S promoter but not of an unrelated promoter. Neither the middle nor the small surface protein, nor a secretable form of the large surface protein, activates the S promoter, but agents that induce endoplasmic reticulum (ER) stress have an effect similar to that of the large surface protein. The large surface protein also activates the S promoter in the context of the entire viral genome. Therefore, it appears that HBV has evolved a feedback mechanism, such that ER stress induced by accumulation of the large surface protein increases the synthesis of the middle and small surface proteins, which in combination with the large surface protein can form mixed, secretable particles. In addition, like other agents that induce ER stress, the large surface protein can activate the cellular *grp78* and *grp94* promoters, raising the possibility that it may alter the physiology of the host cell.

Hepatitis B virus (HBV) is an enveloped hepatotropic DNA virus that continues to infect a large number of people worldwide (for a review, see reference 11). Three forms of the HBV surface (envelope) protein are found in the virion envelope (for reviews, see references 5 and 6). The large surface protein (LS) is translated from the first ATG codon of the surface open reading frame, while the middle and small forms are translated from in-frame ATG codons further downstream (Fig. 1). All three forms are cotranslationally inserted into the endoplasmic reticulum (ER) as transmembrane proteins and, together with envelope cytoplasmic core (nucleocapsid) particles, form mature virions that are secreted via the constitutive secretory pathway (for a review, see reference 6). In addition, the middle and/or small forms, in the absence of other viral proteins, can bud into the lumen and be secreted in the form of spherical and filamentous subviral particles. LS, in contrast, cannot be secreted when expressed alone but instead is retained within the cell in the form of intraluminal particles (3, 4, 17, 20, 22, 33). If LS is coexpressed with the other forms of surface protein, they form heteromultimers whose phenotype depends on the relative amounts of the various surface proteins: a small relative amount of LS results in secretion, while a large amount results in retention. This retention affects the secretion not only of noninfectious subviral particles but also of the infectious virion particles and, therefore, is deleterious to the viral life cycle (1). Not surprisingly, in the infected cell, LS is usually synthesized in much smaller amounts than the middle and small surface proteins. This differential regulation is achieved by the presence of two independent promoters (Fig. 1) (for a review, see reference 35). The upstream preS1 promoter gives rise to transcripts that are translated mainly

into LS, while the downstream S promoter gives rise to transcripts capable of translation into only the middle and small surface proteins. Since the amount of preS1 mRNA is normally much smaller than the amount of S mRNA, there is insufficient LS to prevent secretion.

Because the relative ratio of LS to the middle and small surface proteins synthesized by HBV is crucial to its replication, we wondered if there is a feedback mechanism to ensure a balanced synthesis of these proteins. In this study, we demonstrated that indeed such a mechanism exists, in that LS significantly activates the S promoter. This activation is correlated with the intracellular retention of LS and is mimicked by agents that induce ER stress. Conversely, LS also activates cellular promoters known to respond to ER stress, but not irrelevant promoters. Therefore, overexpression of LS and subsequent intracellular particle retention appear to activate the S promoter by an intracellular signaling pathway induced by ER stress. This activation would in turn lead to increased synthesis of the middle and small surface proteins and, hence, allow secretion of both subviral and virion particles. Our findings not only reveal a new layer of complexity in the regulation of HBV gene expression but also suggest a mechanism by which LS can affect host cell physiology during chronic HBV infection.

MATERIALS AND METHODS

Plasmids. All manipulations, enzyme treatments, and growth of plasmids were performed by standard techniques (27). All HBV genomic DNA was derived from strain adw2 (29). The plasmid pSVLM-S- (2) contains the simian virus 40 (SV40) enhancer-early promoter upstream of a fragment of HBV DNA containing the S and X genes and the polyadenylation signal (Fig. 1). The initiating ATG codons for the middle and small surface proteins have been mutated; thus, cells containing this plasmid express the LS and X proteins but not the middle or small surface protein. The plasmid pSVsignalLM-S- (2) is similar to pSVLM-S-, except that a DNA sequence coding for the β-lactamase signal sequence has been fused in frame to the 5′ end of the LS open reading frame. This change results in the synthesis of a secretable form of LS (2). The plasmid pCMVLM-S- is similar to pSVLM-S-, except that the SV40 promoter has been replaced by the cytomeg-

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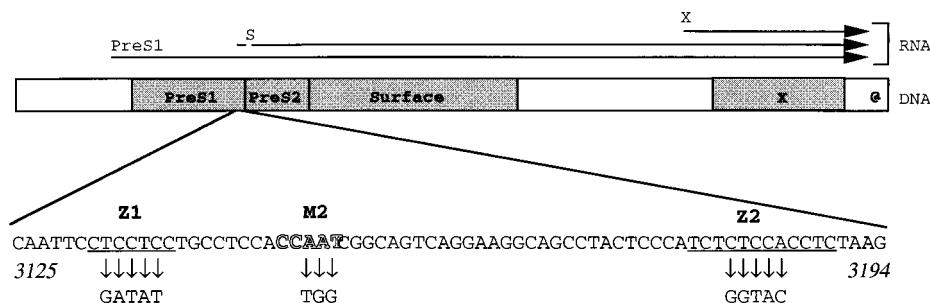† Present address: Fort Dodge Laboratories, Fort Dodge, IA 50501.

FIG. 1. (Top) Map of the region of the HBV genome coding for surface proteins. Open reading frames are shown as shaded boxes. The preS1 promoter gives rise to 2.4-kb transcripts that code for LS (comprising preS1, preS2, and surface domains). The S promoter gives rise to 2.1-kb transcripts that straddle an internal ATG codon and, hence, gives rise to both the middle and small surface proteins (respectively comprising preS2 and surface domains or the surface domain only). In the plasmid pCMVLM-S-, the endogenous preS1 promoter has been replaced by the CMV immediate-early promoter and the ATG initiating codons for the middle and small surface proteins have been mutated to ACG codons. @, polyadenylation signal. (Bottom) The sequence of a portion of the S promoter is shown. The CCAAT element is in outline type, while two sites that bind in vitro to Sp1 are underlined. The sequences of the clustered point mutations in each site are shown below the main sequence.

alovirus (CMV) immediate-early promoter by substituting a fragment of pCMVL (33), PCR amplified using primers 5′GGCGTCGACCGAGCTTGCAT GCCTGCAGG and 5′GTGGAATTCCACTGCGTGGCC (note that the latter contains a nucleotide change [underlined] so that the ATG initiating codon for the middle surface protein is changed to ACG, just as in pSVLM-S-), for the SalI-to-EcoRI fragment of pSVLM-S-. The sequence of the amplified fragment was confirmed to be free of other mutations. The plasmid pCMVL-M-S- contains a frameshift mutation within the LS gene, so that full-length LS cannot be synthesized. It was derived from pCMVL-M-S- by treatment with the restriction endonuclease EcoRI, repair of the staggered ends by the Klenow fragment of DNA polymerase I, and religation. The plasmid pSVLM-S-X- contains two lesions within the X gene: a frameshift mutation as a result of an extra G residue between residues 1682 and 1683, and a premature termination codon as a result of a C-to-A mutation at residue 1685. These mutations were originally generated by overlap PCR amplification with mutagenic primers in a full-length HBV construct (pHBV1.2) (31) and were introduced into pSVLM-S- by replacing the SpeI-to-KpnI fragment of the latter with the SpeI-to-ApaI fragment of the former plasmid (both the KpnI- and ApaI-digested ends were repaired by the Klenow fragment of DNA polymerase I). The plasmid pSAgΔHin (38) contains the native S promoter driving the expression of the middle and small surface proteins, while the plasmid pCMVS contains the CMV promoter driving the expression of the small surface protein. The plasmid pRVL- (1) contains a greater-than-full-length fragment of the entire HBV genome without any exogenous promoters, and it also contains a point mutation that ablates LS expression without affecting any of the other HBV gene products. The plasmid mutant 1 (32) contains a greater-than-full-length fragment of the entire HBV genome, with a 129-bp in-frame deletion of the S promoter comprising the CCAAT element. This deletion leads to decreased synthesis of the middle and small surface proteins and increased synthesis of LS in transfected cells, such that surface protein particles accumulate within vesicles (32).

The plasmid pS1CAT contains the 130-bp S promoter fragment, generated by PCR and confirmed by DNA sequencing, inserted between the HindIII and XbaI restriction sites of the promoterless chloramphenicol acetyltransferase (CAT) reporter gene pCAT-basic (Promega). The primers used were 5′CACA AGCTTAACAATTCCTCCTCCTGCCTCCAC and 5′CAGTTCTAGACTG CAGAGGTTGGTGCAAGGCAG. Similar reporter plasmids with mutations in the CCAAT elements and Sp1 binding sites of the S promoter were generated by using as template HBV derivatives containing these mutations, previously generated by overlap PCR (16). The plasmid pSBCAT contains the HBV preS1 promoter driving the CAT gene (38). The plasmids pgrp78(−457)CAT (13) and pgrp94(−357)CAT (23) contain the grp78 and grp94 promoters, respectively, driving the CAT gene and were kind gifts of A. S. Lee (University of Southern California). The plasmid pSp1CAT(3) (12) contains a minimal human immunodeficiency virus type 1 promoter with three tandem Sp1 sites driving the CAT gene and was a kind gift of K. T. Jiang (National Institutes of Health) via S. Tong-Starksen (University of California, San Francisco). The plasmid pSp1CAT(3)CCAAT contains the CCAAT element of the HBV S promoter inserted between the PstI and SalI sites of pSp1CAT. The insert was generated by annealing two oligodeoxynucleotides with the sequences 5′GCCACCAATCG GCAGTCAGGAAGGCAGA and 5′TCGATCTGCCTTCCTGACTGCCGAT TGGTGGCTGCA.

Cell culture and transfection. HuH-7 human hepatoma cells (18) were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, under an atmosphere of 7% CO_2. Cells were transfected with plasmid DNA by the calcium phosphate method (10) and harvested on the second day after transfection. Where indicated, cells were treated for the last 16 h before harvesting with 2-deoxyglucose (10 mM, diluted from a 1 M stock solution), tunicamycin (50 μg/ml, diluted from a stock solution of 5 mg/ml in dimethyl sulfoxide [DMSO]), EGTA (5 mM, diluted from a 0.25 M stock solution), thapsigargin (4 μg/ml, diluted from a stock solution of 5 mg/ml in DMSO), A23187 (200 μg/ml, diluted from a stock solution of 500 mg/ml in DMSO), or DMSO (0.8 μl/ml).

CAT assay and RNA analysis. CAT activity in transfected cells was quantitated by the thin-layer chromatography method (10). Cell extracts were diluted when necessary so that the activity would remain within the linear range of the assay (<50% acetylation of chloramphenicol). RNA was purified from transfected cells with RNAzolB (Biotecx), and primer extension was used to quantitate the amounts of preS1 and S transcripts, as described previously (37).

RESULTS AND DISCUSSION

Activation of the S promoter by LS and by ER stress. To test the effect of LS on S promoter activity, we cotransfected two plasmids into HuH-7 hepatoma cells. One plasmid, pS1CAT, contains the CAT reporter plasmid driven by the S promoter. The other plasmid, pCMVLM-S-, expresses LS from the CMV immediate-early promoter; to prevent expression of the middle and small surface proteins, the initiation codons for these proteins have been mutated (2). As shown in Fig. 2A, LS expression from pCMVLM-S- caused a >10-fold increase in CAT activity in the transfected cells, compared with cotransfection with pUC18. When the expression of LS was ablated by a frameshift mutation (pCMVL-M-S-), no increase in CAT activity was observed (Fig. 2A). Since these expression plasmids also contain the HBV X gene (Fig. 1), which codes for a transcriptional transactivator (for a review, see reference 36), it was possible that X gene products were also necessary for this activation. However, cotransfection of a plasmid with both frameshift and chain-terminating mutations in the X gene (pCMVLM-S-X-), but an intact surface gene, still resulted in a significant increase in CAT activity (Fig. 2A), indicating that LS was both necessary and sufficient for activation of the S promoter. Similar results were obtained when LS was expressed from the SV40 enhancer-early promoter (pSVLM-S- [Fig. 2A]). This activity of LS was not a nonspecific effect on CAT gene expression, since neither a CAT reporter plasmid driven by the HBV preS1 promoter nor an unrelated CAT reporter plasmid driven by a minimal HIV-1 promoter [pSp1CAT(3)] was significantly activated (Fig. 3). It should be further noted that overexpression of wild-type LS in the presence of the middle and small surface proteins also activated the S promoter (data not shown), indicating that this phenomenon cannot be ascribed to the two methionine-to-threonine mutations introduced into LS as a result of the ablation of the
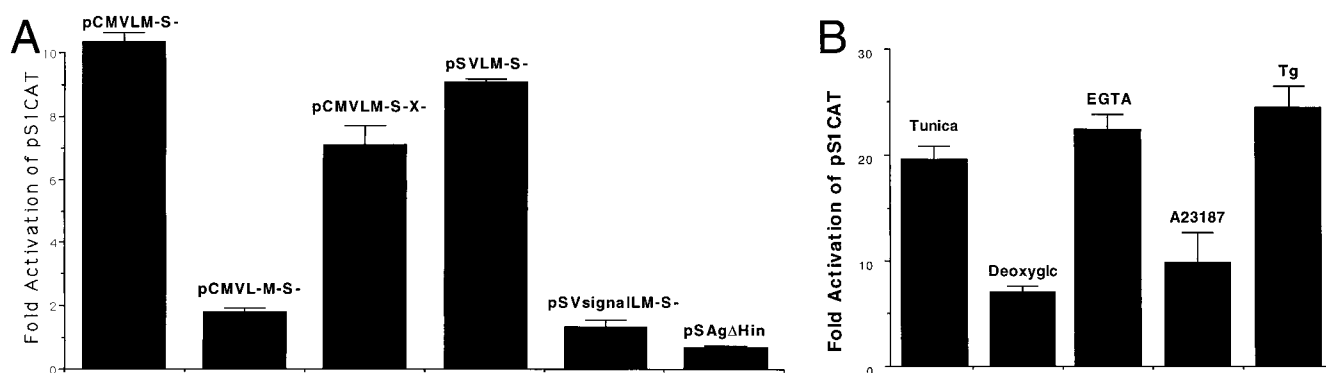
FIG. 2. (A) Effect of various expression plasmids on CAT expression directed by the S promoter in pS1CAT. HuH-7 cells in 60-mm-diameter plates were cotransfected with 2.5 μg of pS1CAT and 2.5 μg of the indicated plasmids, and the CAT activity at 2 days posttransfection was normalized to the activity in cells cotransfected with pS1CAT and pUC18. The results are means ± standard deviations of values for three plates of transfected cells. (B) Effect of various inducers of ER stress on CAT expression from pS1CAT. HuH-7 cells in 60-mm-diameter plates were transfected with 5 μg of pS1CAT and treated for 16 h with the indicated chemicals. The CAT activity was normalized to the activity of cells treated with the same amount of DMSO as used to dissolve the thapsigargin (Tg). The results are means ± standard deviations of values for three plates of transfected cells. Tunica, tunicamycin; Deoxyglc, deoxyglucose.

initiating codons for the middle and small surface proteins in pCMVLM-S- and pSVLM-S-.

Since LS is targeted to the ER as a transmembrane protein and then buds into the lumen to form 20-nm-diameter particles that accumulate in the distal ER-intermediate compartment (33), it seemed unlikely that LS was acting directly in the nucleus to affect transcription. On the other hand, it is well known that misfolded or mutated proteins that are incompetent for secretion can cause stress in the ER, with resultant effects on cellular transcription via intracellular signaling pathways (14, 21, 30). Therefore, we hypothesized that the S promoter was responding to signals generated by ER stress induced by LS retained within the secretory pathway. If so, activation of the S promoter must depend on particle accumulation within the cell. Indeed, a modified LS that is secretable (pSVsignalLM-S-) does not activate the S promoter; nor do the middle and small surface proteins (pSAgΔHin), which behave as normal secretory proteins (Fig. 2A). We then tested the effect of different chemical agents that induce ER stress (for a review, see reference 14): 2-deoxyglucose and tunicamycin, two different inhibitors of glycosylation; EGTA, a calcium chelator; A23187, a calcium ionophore; and thapsigargin, an inhibitor of the ER ATPase. Indeed, all four chemicals caused large increases in CAT expression directed by the S promoter (Fig. 2B). Conversely, LS activated two cellular promoters (for

the *grp78* and *grp94* genes) known to be up-regulated by ER stress (23, 25) (Fig. 3). Thus, ER stress and LS have identical effects in inducing both HBV and cellular promoters.

Role of NF-Y in responding to ER stress. While the detailed mechanism of transcriptional regulation by ER stress is as yet unknown, it is clear that activation of the cellular *grp78* (26), *grp94* (23), and *ERp72* (28) genes requires the transcription factor NF-Y and the CCAAT elements to which it binds. Since the S promoter contains an important CCAAT element that binds NF-Y (16) (Fig. 1), it seemed likely that this element is also involved in this promoter's response to ER stress. We therefore introduced into the S promoter of the CAT reporter plasmid three clustered point mutations in the CCAAT element that destroy its ability to bind to NF-Y (38) (M2 in Fig. 1). This mutant is entirely unresponsive to induction of ER stress by either LS (Fig. 4) or various chemical inducers (data not shown). Therefore, this CCAAT element is required for ER stress to activate the S promoter, just as the CCAAT elements of cellular genes are required for their activation by ER stress.
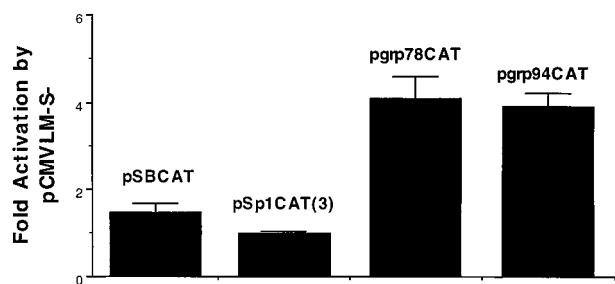

FIG. 3. Effect of LS on CAT expression directed by the HBV preS1 promoter (pSBCAT), the minimal HIV-1 promoter [pSp1CAT(3)], the *grp78* promoter, or the *grp94* promoter. HuH-7 cells in 60-mm-diameter plates were cotransfected with 2.5 μg of pCMVLM-S- and 2.5 μg of the indicated plasmids, and the CAT activity at 2 days posttransfection was normalized to the activity in cells transfected with pCMVL-M-S- instead of pCMVLM-S-. The results are means ± standard deviations of values for three plates of transfected cells.
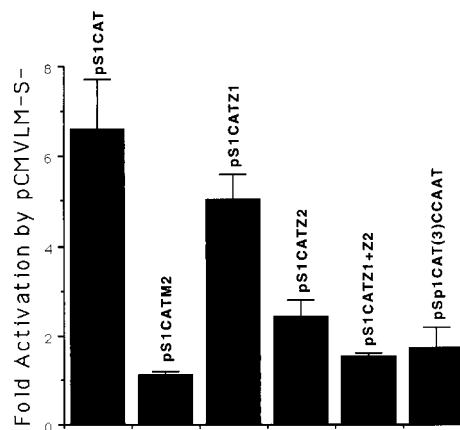

FIG. 4. Effect of LS on CAT expression directed by wild-type or mutated S promoters. HuH-7 cells in 60-mm-diameter plates were cotransfected with 2.5 μg of pCMVLM-S- and 2.5 μg of the indicated plasmids, and the CAT activity at 2 days posttransfection was normalized to the activity in cells transfected with pCMVL-M-S- instead of pCMVLM-S-. The results are means ± standard deviations of values for three plates of transfected cells.
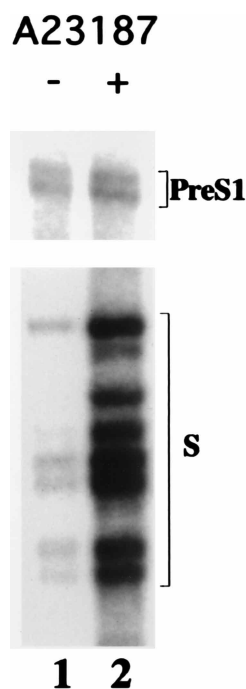
A23187

FIG. 5. Effect of ER stress on preS1 and S transcript levels. HuH-7 cells in 100-mm-diameter plates were transfected with 10 μg of pRVL-, which contains a greater-than-full-length HBV genome that expresses all viral transcripts and proteins except for LS. This plasmid was used to preclude any effects of ER stress on LS translation or secretion that may induce secondary effects on the S promoter. After 16 h of treatment with A23187 or DMSO, RNA was purified from the cells, and the amounts of preS1 and S transcripts were quantitated by primer extension. Note the multiple start sites specified by the S promoter. A repeat experiment produced similar results. Lane 1, DMSO; lane 2, A23187.

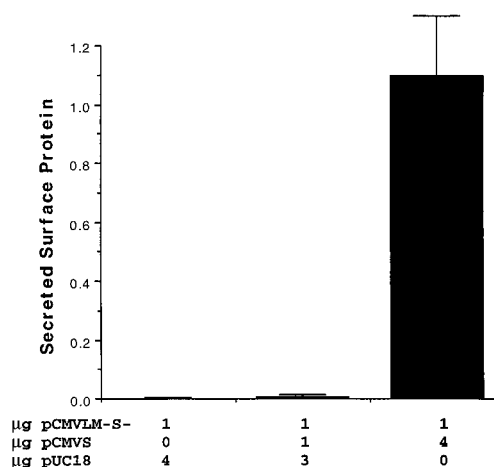|			
μg pCMVLM-S-	1	1	1
μg pCMVS	0	1	4
μg pUC18	4	3	0

FIG. 6. Surface protein secretion depends on the ratio of LS to small surface protein rather than on the absolute amount of LS. HuH-7 cells were transfected with the indicated amounts of plasmids expressing only LS (pCMVLM-S-) or small surface protein (pCMVS) and sufficient pUC18 to make up a total of 5 μg of DNA. After 2 days, the media were harvested and the amount of surface protein was quantitated with the Abbott enzyme immunoassay kit. The data are presented as the means ± range of values from two transfections after subtraction of background.

For the *grp78* (26), *grp94* (23), and *ERp72* (28) genes, other DNA elements that bind a different set of cellular factors, other than the CCAAT elements, are also necessary for responding to ER stress. To determine if this may also be the case for the S promoter, we mutated the other two known upstream *cis* elements in the S promoter, both individually and together (Z1 and Z2 in Fig. 1), and measured the responsiveness of these mutants to ER stress. As seen in Fig. 4, mutation of either of these elements (pS1CATZ1 and pS1CATZ2) depressed but did not eliminate activation of this promoter by LS. When both elements were mutated (pS1CATZ1+Z2 [Fig. 4]), activation was not observed. Therefore, for the S promoter to respond to ER stress, the CCAAT element by itself is also insufficient and at least one Z site is needed. The cellular factor necessary for this function of the Z sites is not known. While Sp1 has been shown to bind Z sites (24), we have not been able to show significant activation by ER stress of a promoter containing the S promoter CCAAT element upstream of authentic Sp1 sites [pSp1CAT(3)CCAAT (Fig. 4)]. Therefore, Sp1 is unlikely to mediate this function of the Z sites.

LS and ER stress in the context of the whole viral genome. The experiments described above were done with expression or reporter plasmids containing portions of the HBV DNA. To determine if the results are valid in the context of the entire genome, we first determined whether the S promoter responded to ER stress when present within a plasmid that contains the entire HBV genome (the plasmid pRVL-). Indeed, when HuH-7 cells were transfected with this plasmid and then treated with A23187, primer extension analysis revealed a large increase in S transcript levels (Fig. 5), commensurate

with the 10-fold increase in CAT activity seen with A23187 treatment of pS1CAT-transfected cells (Fig. 2B). In contrast, the amount of preS1 transcripts changed less than twofold (Fig. 5). Therefore, ER stress also activates the S promoter in the context of the entire HBV genome. Conversely, LS, when expressed from its native promoter in a mutant HBV genome (mutant 1) that overproduces LS and underproduces the middle and small surface proteins, is capable of activating the S promoter in the CAT reporter plasmid 4.8 ± 0.4-fold. Thus, our results are not an artifact of using fragments of the HBV genome.

Significance of secretion of LS. Our results demonstrate that when there is accumulation of LS in the secretory pathway, the synthesis of the small surface protein is activated at the transcriptional level. We suggest that the resultant increased ratio of small surface protein to LS results in the relief of the block to secretion and that therefore this feedback mechanism is helpful in maintaining viral morphogenesis under conditions in which LS is overexpressed. However, the existing data in the literature do not directly address the question of whether it is the absolute level of LS that is important in blocking secretion or the relative level of LS versus the small surface protein. This is an important question, since if the former possibility were true, then increasing the synthesis of the small surface protein would only result in further accumulation of surface proteins in the ER and not in secretion. To answer this question, we cotransfected HuH-7 cells with two plasmids, one that expresses LS alone and the other that expresses the small surface protein alone. When 1 μg of just the LS-expressing plasmid was transfected, no significant secretion of surface protein occurred, as expected (Fig. 6, left bar). Similarly, when 1 μg of the plasmid expressing the small surface protein was transfected in addition to 1 μg of the LS-expressing plasmid, no secretion was observed (Fig. 6, middle bar). However, when the amount of small surface protein-expressing plasmid was increased to 4 μg but the amount of the LS-expressing plasmid was held at 1 μg, a large amount of surface protein was secreted into the medium (Fig. 6, right bar). These results show that it is the relative ratio of LS to small surface protein, and

not the absolute amount of LS, that determines the secretability of surface protein. Therefore, when LS is oversynthesized and accumulates in the secretory pathway, boosting the amount of small surface protein indeed can potentially alleviate the intracellular accumulation of surface proteins.

Summary. It is well known that an appropriate balance of the various HBV surface proteins is important for the viral life cycle, in that even relatively modest increases in the amount of LS result in an intracellular block to secretion of virus-encoded particles (for a review, see reference 5). Our results demonstrate the presence of a previously unsuspected feedback mechanism for controlling the expression of HBV surface proteins. Thus, when there is sufficient LS overexpression to block particle secretion, the subsequent intracellular accumulation of particles initiates a sequence of events that results in the transcriptional activation of the S promoter. The subsequent increased production of the middle and small surface proteins then presumably allows particle secretion to resume by restoring the proper ratio of LS to middle and small surface proteins. In this manner, a disruption of the viral life cycle caused by overexpression of LS can be avoided. Therefore, this appears to be an important regulatory feature of HBV gene expression, although it is unknown at this time how often this regulatory loop actually comes into play during normal infection by wild-type HBV. In transient transfections, wild-type HBV does not appear to synthesize sufficient LS to activate this pathway (data not shown).

Despite the presence of this feedback mechanism to prevent overexpression of LS, the livers of people with chronic HBV infection frequently contain individual hepatocytes with accumulated LS, presumably as a result of LS overexpression (11). These so-called ground-glass cells have large amounts of LS-containing filamentous particles within the hyperplastic ER and/or an intermediate compartment (7). The cause of ground-glass cell formation is unknown. It is possible that changes in host cell *trans*-acting factors lead to a vast overexpression of LS that cannot be compensated for by increased production of the middle and small surface proteins. Alternatively, HBV mutations that accumulate during chronic infection may have lost the ability to respond appropriately to ER stress. Indeed, several groups (8, 19, 34) have independently found many different naturally occurring HBV mutants with deletion and/or point mutations in the S promoter CCAAT element, which not only is important for both the basal S promoter function (16) and its activation by ER stress but also down-regulates the production of preS1 transcripts (15). As a result, these mutants not only would show decreased basal synthesis of the middle and small surface proteins but also would be unable to increase S promoter activity in response to LS accumulation as a result of increased preS1 mRNA levels. Consequently, hepatocytes containing these mutants would overexpress and accumulate LS within the ER. Transfection studies of one such mutant in culture experiments have already shown intracellular accumulation of LS. However, confirmation that the same phenomenon occurs in vivo must await studies in transgenic mice and direct analysis of HBV genomes within ground-glass cells.

Ground-glass cells have also been observed in transgenic mice overexpressing LS (4). Interestingly, these mice show marked fragility of the hepatocytes that results in hepatocellular necrosis and hepatitis, either in response to gamma interferon (9) or spontaneously (4). Our results may explain how LS accumulation can have this dramatic effect on the hepatocyte, since ER stress can activate several cellular transcriptional pathways (14, 21, 30). Future experiments will analyze the signalling pathway from the ER to the nucleus and the resultant changes in cellular physiology that may be relevant to the pathogenesis of HBV-related diseases.

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