Novel Autoregulatory Function of Hepatitis B Virus M Protein on Surface Gene Expression*

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The hepatitis B virus surface gene consists of a single open reading frame divided into three coding regions: pre-S1, pre-S2, and S. By alternate translation at each of the three initiation codons, L, M, and S proteins can be synthesized. Studies have shown that M protein is not essential for viral replication, virion morphogenesis, or in vitro infectivity. In this study, we show that native M protein can regulate surface gene expression at the transcriptional level. The regulatory effect of M protein is mediated through the CCAAT box within the S promoter. Deletion mapping analysis indicated that the transactivating effect of M protein is mediated through amino acids 1–57 of M protein (the MHBsau domain), although its maximal transactivation activity coincides with that of the pre-S2 domain. This conclusion is supported by the fact that disruption of the putative V8 protease site at the pre-S2 domain junction not only rendered M protein incapable of transactivating the S promoter but also inactivated its nuclear translocation potential. Immunoprecipitation and immunoblot experiments demonstrated that pre-S2 interacts with the three subunits of the CCAAT box-binding factor/nuclear factor Y, the cognate binding protein of the CCAAT box. These results demonstrate and define a novel regulatory role of M protein, which, under natural conditions, may undergo a proteolytic process to generate an MHBsauu species that will be translocated inside the nucleus, where it will interact with the CCAAT box-binding factor to regulate surface gene expression. Because the CCAAT box is located at a fixed position within numerous promoters, these observations might provide a plausible explanation for hepatitis B virus-associated hepatocarcinogenesis.

Hepatitis B virus (HBV)1 infection causes a wide spectrum of liver diseases, including acute hepatitis, chronic active hepatitis, cirrhosis, and hepatocellular carcinoma (1–4). HBV belongs to the hepadnavirus family, and its particle coat consists of a lipid bilayer membrane and envelope proteins. It has a partial duplex DNA genome of ~3.2 kb and contains four overlapping open reading frames: DNA polymerase, core protein, surface antigen, and the X gene (5). The HBV surface (envelope) gene encodes three forms of viral surface proteins (6, 7). It is divided into three parts by two internal initiation codons: the pre-S1, pre-S2, and S regions. This combination of three regions forms the large surface antigen LHBsAg or L protein. In addition, the pre-S2 and S regions form the middle surface antigen MHBsAg or M protein. The small surface antigen SHBsAg or S protein, also called major S protein, constitutes the most abundant protein of HBV envelopes. The production of these three forms of surface protein (L, M, and S) is controlled by two tandem promoters. The upstream pre-S1 promoter controls the transcription of a 2.4-kb transcript that encodes L protein only. The downstream S promoter, ~240 bp away, specifies transcripts with heterogeneous 5’ termini (5), with the largest transcript encoding M protein and the remaining transcripts encoding S protein.

In both infected and transfected hepatocytes, the amount of pre-S1 transcript is much less than the amount of S transcript (7, 8), and L protein constitutes only a small percentage of the total surface protein synthesized in the infected cell. This is an important aspect of the viral life cycle because L protein, unlike the other surface proteins, is incompetent for secretion, and relative overexpression of L protein leads to the intracellular retention of all forms of the surface protein (9–12).

Although the pre-S1 promoter appears to be much weaker than the S promoter in the context of either the entire viral genome or a large subviral genomic fragment, in fact, with the reporter gene, the pre-S1 promoter is slightly stronger than the S promoter when they are assayed separately in transfection studies. Within the S promoter, an element containing a CCAAT box (13) can down-regulate L protein expression, and this CCAAT box is functionally important for S promoter activity (14–16). Mutation of the CCAAT motif not only decreases the amount of S transcript, but also increases the amount of pre-S1 transcript (13), indicating that this element plays a critical role in the balanced synthesis of the three forms of this surface protein, thus facilitating the proper assembling and secretion of the viral particle. An element adjacent to the CCAAT motif was identified by Bock et al. (17), and this ele-

adjacent factor; nt, nucleotides; CMV, cytomegalovirus; HA, hemagglutinin; Ch, chymotrypsin; GFP, green fluorescent protein; PBS, phosphate-buffered saline; CBF, CCAAT box-binding factor; aa, amino acid(s).

Received for publication, February 28, 2005, and in revised form, May 16, 2005
Published, JBC Papers in Press, May 16, 2005, DOI 10.1074/jbc.M502209200

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Printed in U.S.A.
ment (temporarily named CCAAT adjacent factor (CAF)) may act in concert with the CCAAT motif to contribute the full activity of the S promoter.

The biological role of M protein in the viral life cycle has been controversial. In vitro studies suggest that M protein is not essential for in vitro HBV replication (18), virion morphogenesis (19), or infectivity (20). Furthermore, because an M protein-deficient mutant can be found in patients with fulminant hepatitis, it was suggested that M protein is not required for in vivo viral replication (21). In this study, we show that M protein has a previously unidentified novel autoregulatory function in surface gene expression. We have also characterized the molecular/genetic mechanism involved in its regulatory function.

**Experimental Procedures**

**Construction of Expression Plasmids**—All constructs used in this study were made using standard recombinant DNA techniques (22). Plasmid p3A3Ag (subtype adw2), which contains the full-length S gene in two forms of small proteins from the surface pre-S1 and S promoters, has been described previously (23). An M protein initiation codon mutation (pHBV1.2Mint, ATG → ATA) was constructed by site-specific mutagenesis using two-step PCR and plasmid pHBV1.2 (24). The first round of PCR was carried out using primers 5'-CATATTCTGGGCTGATCACA-3' (5'-primer, also named D1A; with the A of the unique EcoRI site numbered 1 and the BstEII site underlined) and 5'-CTCTCCATTTGATGATGAGTG-3' (3'-primer; with the initiation codon point mutation underlined). The second round of PCR was carried out using primers 5'-3205TCATGCCGCAATGCTGGA3' (5'-primer; with the initiation codon point mutation underlined) and 5'-GTTCATACACCCAGCTGTA-3' (3'-primer, also named D5; with the XbaI underlined). Finally, these two rounds of PCR products were mixed in equal molar parts and amplified with the 5'-primer (492 bp) and 3'-primer (427 bp) PCR primers. The amplified 919-bp PCR product was digested with BstEII and XbaI and then substituted for the BstEII-XbaI fragment of the surface gene carried on pHBV1.2. To introduce the protein initiation codon mutation into p3A3Ag, “cassette exchange” experiments were carried out as described previously (23). Briefly, pHBV1.2Mint was digested with BstEII and EcoRV, and the resulting 1.5-kb BstEII-EcoRV fragment was exchanged for the BstEII-EcoRV fragment of p3A3Ag to create the p3A3AgMint mutant. p3A3AgMint, which will make M and S proteins from the S promoter, was constructed using PstI-digested p3A3Ag (25) and replaced with a 130-bp fragment containing the S promoter amplified from p3Ag (ARG45); with the NotI site (underlined) is preceded by six random nucleotides and followed by a Kozak sequence (ACCAGT) and an HBV sequence (X1p), corresponding to the bracketed amino acids.

The fragment was amplified with the forward primer and reverse primer (5'-GCGGCGCCGCTTAXAATTX1p) using p3A3Ag as the template, digested with NotI, and ligated to the NotI-restricted and dephosphorylated pCMV-Basic vector. For construction of pCMV-pre-S-(1-55) (nt 4–156), pCMV-pre-S-(1-156) (nt 37–156), and pCMV-pre-S-(1-195) (nt 46–150), the forward primer used was 5'-GGGATCCATCGGCGCATCGGCGC-3' and the reverse primer was 5'-CCTAGGATCCGTTTATTATACCTACG-3'.

**Deletion of the S Promoter**

In the second method, truncated M proteins were tagged at the N terminus of the pCruz HA vector (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which tags the hemagglutinin (HA) epitope at the 5'-end of the expressed proteins. The primers were designed to bracket the truncated proteins exactly; they spanned amino acids 1-281, 1-152, 1-76, 1-71, 1-57, 1-55, 1-52, 1-48, 1-47, 5-55, 1-55, and 19-55 of M protein. The forward primer used was 5'-GCGGCGCCGCTTAXAATTX1p, where the site (underlined) was preceded by six random nucleotides and followed by a Kozak sequence (ACCAGT) and an HBV sequence (X1p), corresponding to the bracketed amino acids. The fragment was amplified with the forward primer and reverse primer (5'-GCGGCGCCGCTTAXAATTX1p) using p3A3Ag as the template, digested with NotI, and ligated to the NotI-restricted and dephosphorylated pCMV-Basic vector.

**Construction of Putative V8 Protease and Chymotrypsin Site-specific Mutants of the S Gene**—The PCR-based site-specific mutagenesis method described above was used to replace either nt 160–165 or 202–207, the putative proteolytic sites for V8 protease and chymotrypsin, respectively. The V8 protease mutant construct pCMV-MHBs/V8 has two amino acid replacements (NMENITS → NHMNIITS). Primers MHBs-1 and MHBs-2 (the same primer pair used to construct pCMV-MHBs) were each combined with reverse primer 5'-CATATGCATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACCGCCGCGTATAAGTGTCATGTCGATTACCATGGGTTAGACGTCCAGAGGACCTAACAGCATGATCACGACG
Autoregulatory Function of HBV M Protein

Preparation of HuH-7 Nuclear Proteins—The HuH-7 cell nuclear extracts were prepared as described previously (26, 27). For immunoprecipitation, nuclear proteins (200 μg) were incubated with the appropriate antibody (1 μg) in a total volume of 300 μl in radioimmune precipitation assay buffer at 4 °C for 18 h with agitation, incubated in protein A-agarose beads (Santa Cruz Biotechnology, Inc.), and washed three times with radioimmune precipitation assay buffer. Finally, the precipitates were resuspended in loading sample buffer, boiled, and subjected to SDS-PAGE with 12.5% acrylamide as described previously (28).

RNA Preparation and Primer Extension Analysis—Total RNA was isolated from transfected cells using a RNeasy kit (Promega). cDNA was synthesized by reverse transcription with the acid guanidine/thiocyanate/phenol/chloroform method according to the manufacturer’s instructions. The levels of pre-S1 and S RNAs were quantified by primer extension analysis with 32P-end-labeled oligonucleotides by T4 polynucleotide kinase as described previously (13, 14, 29, 30). To control for variations in transcription efficiency, cells were cotransfected with pCMVβ, which contains the CMV immediate-early promoter driving the β-galactosidase gene. The oligonucleotide used for primer extension analysis was 5’-TTGGACACCCACCGATTCGGAGAC-3’ (corresponding to nt 702–726 of pCMVβ); GenBankTM/EBI accession number U02451). The intensities of the extended bands were determined using a fluorimeter (FLA1000 system, Fuji Photo Film, Tokyo, Japan). As an internal control, in the same membranes, the antibodies were stripped and reprobed with anti-α-tubulin antibody (IgG1, T-9026, Sigma) or anti-proliferating cell nuclear antigen antibody (PC10, mouse monoclonal IgG2a, Santa Cruz Biotechnology, Inc.) or anti-CCAT box-binding factor (CBF) A (FL-207, rabbit polyclonal IgG), anti-CBF-B (H-209, rabbit polyclonal IgG), or anti-CBF-C (H-120, rabbit polyclonal IgG) (all from Santa Cruz Biotechnology, Inc.) antibody. After being washed with PBS for 15 min, the bound primary antibody was detected with horse-radish peroxidase-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG antibody, which was visualized by enhanced chemiluminescence (Amerham Biosciences). The intensities of the protein bands were determined using a densitometry image analyzer (FLA1000 system, Fuji Photo Film, Tokyo, Japan). As an internal control, in the same membranes, the antibodies were stripped and reprobed with anti-α-tubulin antibody (IgG1, T-9026, Sigma) or anti-proliferating cell nuclear antigen antibody (PC10, mouse monoclonal IgG2a, Santa Cruz Biotechnology, Inc.).

RESULTS

M Protein Regulates Surface Protein Expression at the Transcriptional Level—To determine the effect of M protein on surface expression, we constructed a pre-S2-defective mutant (Fig. 1A) frequently seen in hepatitis patients (20, 21, 31). As expected, after the mutant was transfected into HuH-7 cells, we detected no M protein by Western blot analysis. Surprisingly, however, L protein expression more than doubled, and S protein expression decreased to 23% of that of the wild type (Fig. 1, B and C). Similar results were obtained when the amounts of L, M, and S protein-specific RNAs were assayed by
These data indicate that the change in the levels of L and S protein expression, which was caused by the loss of M protein expression, was regulated at the transcriptional level. These findings are somewhat unexpected and surprising.

**M Protein Regulates Surface Gene Expression through the Major Surface Gene Promoter—**

Expression of HBV surface proteins is regulated by several elements within the HBV genome (Refs. 7 and 32–34; reviewed in Refs. 7 and 34). However, expression of the L, M, and S proteins is regulated primarily by the pre-S1 and S promoters at the transcriptional and post-transcriptional levels (13–16, 35–38). Therefore, to further characterize the effect of M protein on surface gene expression, the pre-S1 and major S promoter regions of surface genes were cloned into the luciferase reporter pGL3-Basic vector (Fig. 2A).

### Fig. 1. Effect of M protein initiation codon mutation on HBV surface gene expression.

**A**, schematic diagram of M protein translation initiation codon mutant p(3A)SAg/Mint. The M protein initiation codon mutation (ATG → ATA) is indicated (*). Nucleotides 2856, 3213, and 157 are the first nucleotides of the translation initiation codons of L, M, and S proteins, respectively. **B**, effect of M protein initiation codon mutation on HBV surface protein expression. HuH-7 cells were transiently transfected with wild-type (WT) p(3A)SAg or the mutant plasmid p(3A)SAg/Mint (see "Experimental Procedures"). After 48 h, proteins (25 μg) of total cell lysates were subjected to SDS-PAGE and Western blot analysis with L, M, and S protein-specific antibodies and α-tubulin (loading control) (left panel). The results from quantitative analysis of the mutant (Mint) relative to the wild type (WT) are shown in arbitrary units after normalizing to α-tubulin levels (right panel). X = times.

**C**, quantitative RNA analysis by primer extension. HuH-7 cells were cotransfected with pCMVβ and the wild-type or mutant plasmid. After 48 h, 5 μg of total RNA was annealed to pre-S1 or S transcript-specific-labeled primers and subjected to primer extension analysis (see "Experimental Procedures"). To control for transfection efficiency, pCMVβ, coding β-galactosidase (β-gal), was included. cDNA products corresponding to the CMVβ, L, M, and S proteins are indicated (left panel). The results from quantitative analysis of specific RNA levels normalized to the internal control CMVβ are shown in arbitrary units (right panel). One representative experiment is shown. Similar results were obtained in two independent experiments.

**Transient cotransfection experiments with a luciferase reporter gene plasmid (p(pre-S1-Luc) and an M protein-expressing plasmid (p(3A)5ΔPre/Sint, in which the major surface gene translation initiation codon is mutated from ATG → AGG) indicated that M protein had no effect on pre-S1 promoter activity (Fig. 2B). In contrast, cotransfecting the major S promoter-reporter gene plasmid (pLuc) with the M protein-expressing plasmid resulted in as much as a 5-fold dose-dependent increase in major S promoter activity (Fig. 2C). M protein may thus regulate surface gene expression through the major S promoter.

To exclude the possibility that some cryptic coding sequence is hidden within the native MHBs gene, plasmid pCMV-MHBs was constructed. In pCMV-MHBs, the coding region of MHBs (pre-S2 and S domains) was cloned into the CMV immediate-
early promoter-driven expression plasmid. We found that specific expression of the M protein coding region by a heterologous promoter had the same regulatory effect on major S promoter (Fig. 2D). This supports the notion that M protein regulates surface gene expression through the major S promoter.

The Maximal Transactivation Region Coincides with the Pre-S2 Domain—We wanted to use deletion mapping experiments to investigate the specific region within M protein involved in regulating surface gene expression. We hypothesized that the N terminus of M protein was the most likely candidate region because the main difference between M and S proteins is
there. Using pS(3A)(ΔPst0)/Sint as the backbone template for PCR amplification, we conducted a series of deletions from the C terminus of M protein, starting at amino acids 281 through 47. When cotransfecting a full-length M protein-expressing plasmid (pCMV-MHBs/Sint) and the S promoter-luciferase reporter plasmid (pSLuc) (Fig. 3B, bar 3), as expected, the S promoter was activated, and its level of activity rose to 2.5 times that of the activity detected in the cotransfection experiment with pSLuc and the empty pCMV-Basic vector (bar 2). Serial C-terminal truncations through amino acids 152, 76, and 71 yielded levels similar to that of full-length M protein (Fig. 3B, bars 4–6). Our initial idea was to determine the minimal transactivation domain of M protein. To our surprise, however, further deletion of the C terminus of M protein to amino acids 57 and 55 caused the activity levels to increase rather than to remain the same (Fig. 3B, bars 7 and 8). It is noteworthy that the highest level of activity coincided with the pre-S2 domain of M protein (amino acids 1–55). Attempts to further narrow down the region critically involved in transactivating the S promoter by deleting the N or C terminus of the pre-S2 domain failed to demonstrate any further increase (Fig. 3B, bars 9–14). These results demonstrate that the maximal transactivation region of M protein for S promoter activation is between amino acids 1 and 55, the pre-S2 domain.

To demonstrate that transactivation was indeed caused by the expression of truncated M protein, we used Western blot analysis. To facilitate detection of expressed protein, the truncated M proteins were HA-tagged at their N termini. Western blot analysis of cytoplasmic fractions with anti-HA antibody revealed that full-length and truncated M protein constructs encoded by HA-MHBs/Sint, HA-MHBs-(1–152), HA-MHBs-(1–76), or HA-MHBs-(1–71) yielded protein bands, as expected (Fig. 3C). Surprisingly, HA-MHBs-(1–57) and HA-pre-S2-(1–55) and those forms further truncated at the N or C terminus of the pre-S2 domain (HA-pre-S2-(1–52), HA-pre-S2-(1–48),HA-pre-S2-(1–47), HA-pre-S2-(4–55), HA-pre-S2-(16–55), and HA-pre-S2-(19–55)) were undetectable within the cytoplasmic fraction (Fig. 3C, lanes 6–12). Even more surprisingly, Western blot analysis of the nuclear proteins of transfected cells indicated that full-length M protein and forms truncated to amino acid 57 at the C terminus expressed a protein band with a molecular mass similar to that of the MHBs-(1–57) domain (Fig. 3C, lanes 2–5). However, N- or C-terminally truncated forms of the pre-S2 domain generated proteins with molecular masses slightly less than that of the pre-S2 domain (Fig. 3C, lanes 7–13). This indicates that, under natural conditions, M protein may undergo some sort of proteolytic process to generate a molecular species with electrophoretic mobility similar to that of MHBs-(1–57). His-tagged full-length and truncated M proteins produced similar results and conclusions (data not shown). Quantitative analysis of the amounts of HA-tagged protein bands within the nuclear fraction demonstrated that HA-pre-S2-(1–55) had the highest level of expression after it had been normalized to the internal control proliferating cell nuclear antigen (Fig. 3D). This was consistent with our deletion mapping results. Similar results were obtained with His-tagged truncated M proteins (data not shown). To further demonstrate that the pre-S2 domain has the highest transactivation activity and that the HA tag would not affect our analysis and conclusions, HA-tagged full-length and truncated M proteins were further cotransfected with the pSLuc reporter gene into HuH-7 cells. Although HA-tagged proteins had less transactivation activity compared with untagged proteins (Fig. 3, B and E), these data further support our previous results and conclusions that the pre-S2-(1–55) domain has the highest level of transactivation activity.

**Nuclear Translocation Potential of the Pre-S2 Domain**—An early study by Cho et al. (39) suggests that the pre-S2 domain may translocate to the nucleus in an energy-independent fashion, although there is no direct evidence to support this. Their observations, in conjunction with our results (Figs. 3 and 5), suggest that, in vivo, M protein may undergo proteolysis to generate a molecular species with a molecular mass close to that of MHBs-(1–57), which then translocates across the nuclear membrane. To further investigate this possibility, the pre-S2 domain was fused to the N or C terminus of GFP (pEGFP-N-pre-S2 or pEGFP-C-pre-S2) and transfected into HuH-7 cells. Regardless of whether the pre-S2 domain was fused to the N or C terminus of GFP, GFP was selectively localized within the nucleus (Fig. 4, A and B). These and previous results indicate that the pre-S2 domain alone may be able to translocate inside the nucleus.

**Disruption of the V8 Protease Cleavage Site Abolishes M Protein Transactivating Function**—Mapping studies indicated that MHBs-(1–57) is detectable only within the nuclear fraction and not the cytoplasmic fraction. This is reminiscent of the V8 protease cleavage site, which is adjacent to the boundary of the pre-S2 and S domains (Fig. 5A) (40) and coincides with MHBs-(1–57) delineated in our experiments (Fig. 3). We hypothesized that the MHBs-(1–57) domain may be released from M protein after undergoing a proteolytic process through the V8 protease cleavage site. To examine this possibility, the V8 protease cleavage site and its adjacent amino acid residue were mutated by site-specific mutagenesis (NMENITS → NHMNITS, with the cleavage residue E in boldface and the mutated amino acids underlined) (Fig. 5A). Mutation of the V8 cleavage residue E rendered the pCMV-MHBs/V8 mutant no longer capable of transactivating the S promoter (Fig. 5B). As a control, one adjacent chymotrypsin cleavage site was also mutated (VLQAG → VHMACG, with the cleavage residue Q in boldface and the mutated amino acids underlined). Mutation of the chymotrypsin cleavage residue Q and its adjacent amino acid did not prevent it from transactivating the S promoter (Fig. 5B). Similar results were obtained when pCMV-MHBs/V8 or pCMV-MHBs/Ch was cotransfected with the pSLuc reporter gene into HepG2 cells (Fig. 5C). As a positive control, an M protein-expressing plasmid (pCMV-MHBs/Sint) was also included (Fig. 5C). These results and those illustrated in Figs. 3 and 4 demonstrate that, in vivo, M protein may undergo a proteolytic cleavage at amino acid 57, through which the MHBs-(1–57) domain may be released and translocated inside the nucleus to activate the S promoter. To further demonstrate that this is indeed the case, the pre-S2 domain, MHBs/Ch, and MHBs/V8 were His-tagged at the N terminus and transfected into HuH-7 cells. Nuclear extracts prepared from transfected cells were immunoblotted with anti-His antibody. The results indicate that His-pre-S2 and His-MHBs/Ch, but not His-MHBs/V8, were detectable within the nuclear fraction (Fig. 5D). These results also indicate that V8 protease-like enzyme may be present within the HuH-7 and HepG2 cells and that it can act on the glutamic acid of M protein to cleave it. Taken together, these results unambiguously demonstrate that, in vivo, M protein expressed within the cytoplasm may be processed at amino acid 57. The released MHBs*domain (amino acids 1–57) may be translocated inside the nucleus to transactivate the S promoter.

M Protein Transactivates the S Promoter through the CCAAT Box—Because we hypothesized that M protein regulates surface gene expression through the S promoter (Figs. 2 and 3), we next defined the target region in which M protein transacti-

\(^{2}\) X. Lu and W. H. Gerlich, unpublished data.
Deletion mapping of M protein reveals that the maximal transactivation region coincides with the pre-S2 domain. A, expression vectors bearing various lengths of M protein sequences were constructed as indicated (not drawn to scale). pCMV-MHBs/Sint contains the entire coding sequence of M protein except for the S protein initiation codon mutation (ATG → AGG, indicated by the asterisk). Solid lines below the map illustrate the regions covered by different truncated forms of M protein. B, the maximal transactivation domain coincides with the pre-S2 domain. Various truncated forms of M protein-expressing plasmids or the empty pCMV-Basic vector was transiently cotransfected with the pS-Luc reporter plasmid into HuH-7 cells. After 48 h, cell lysates were prepared for a luciferase assay (performed as described under “Experimental Procedures”). The relative luciferase activity of each transfection is expressed relative to the value for cells cotransfected with pS-Luc and the empty pCMV-Basic vector (bar 2). Similar results were obtained in three independent experiments. C, nuclear and cytoplasmic fractions of HA-tagged full-length M protein and different truncated forms were subjected to Western blot analysis. The cytoplasmic and nuclear fractions isolated from HuH-7 cells transfected with various truncated forms of M protein were separated by SDS-PAGE and immunoblotted with anti-HA antibody and appropriate secondary antibodies. Internal controls for cytoplasmic and nuclear fractions were detected with anti-α-tubulin and anti-proliferating cell nuclear antigen (PCNA) antibodies, respectively. The bound antibodies were detected as described under “Experimental Procedures.” The vector control was transfected with pCruz HA. The positions of several molecular mass markers are shown on the right. D, the MHBs-(1–55) (pre-S2 domain) construct has the highest level of expression within the nuclear fraction. The nuclear levels of full-length M protein and various truncated forms from C were quantitated using a chemiluminescence image analyzer, normalized to those of proliferating cell nuclear antigen, and expressed as arbitrary units. E, HA-tagged MHBs-(1–55) (pre-S2 domain) has the highest level of transactivation activity. HuH-7 cells were cotransfected with pS-Luc and HA-tagged full-length M protein and various truncated forms as described for C. After 48 h, the cell lysates were harvested for luciferase assay. The results are expressed relative to the value for cells cotransfected with pS-Luc and the empty pCruz HA vector. All transfections were performed in triplicate and normalized to internal control Renilla luciferase activity.
vates the S promoter. Several Sp1-binding sites (14, 36) and one CCAAT box (14–16) have been identified within the S promoter (Fig. 6A). In addition, one unknown transcription factor (adjacent to the 3′-end of the CCAAT box), CAF, is critical for CCAAT box-mediated S promoter activity (17). We hypothesized that transcription factors within the S promoter respond to M protein-mediated transactivation. To examine whether this is the case, mutations were introduced into two Sp1-binding sites separately (pS/SP1-Luc and pS/SP2-Luc) and simultaneously (pS/SP12-Luc) in pS-Luc. As expected (14), mutation of the upstream Sp1 site (nt 3132–3137, pS/SP1-Luc) decreased S promoter activity by ~30% (Fig. 6B, compare bars 2 and 3). However, upon cotransfection into HuH-7 cells, pS/SP1-Luc responded to increasing amounts of pre-S2 domain-expressing plasmid (pCMV-pre-S2) by dose-dependently increasing luciferase activity expression (Fig. 6B, bars 3–6). Mutation of the downstream Sp1 site (nt 3182–3187, pS/SP2-Luc) also decreased S promoter activity (Fig. 6B, bar 7), as expected (14). However, similar to results obtained with the upstream Sp1 site mutation, pS/SP2-Luc reporter gene expression was up-regulated in a dose-dependent manner when increasing amounts of pCMV-pre-S2 expression plasmid were cotransfected (Fig. 6B, bars 7–10). These results indicate that the transactivating effect of the pre-S2 domain on the S gene promoter is not mediated through the Sp1 sites. Similar results were obtained when the two Sp1 sites were simultaneously mutated (pS/SP12-Luc) (Fig. 6B, bars 11–14). Mutation of the two Sp1 sites drastically reduced S promoter activity (Fig. 6B, bars 11), as observed previously (14). In contrast, increasing amounts of transfected pCMV-pre-S2 gradually and dose-dependently increased the amounts of pS/SP12-Luc reporter gene activity (Fig. 6B, bars 12–14). Similar results were obtained upon cotransfection with the M protein-expressing plasmid pCMV-MHBs (data not shown). These results clearly demonstrate that the transactivating function of M protein is mediated through neither Sp1 site of the S promoter.

Next, we examined whether the transactivating effect of M protein is mediated through the CCAAT box. Mutation of the CCAAT box within the S promoter drastically reduced S promoter activity (Fig. 6C, bar 3), as expected (14). However, in contrast to mutation of the Sp1 site, pS/CAT-Luc did not respond to increasing amounts of transfected pCMV-pre-S2 (Fig. 6C, bars 4–6). Similar results were obtained upon cotransfection with pCMV-MHBs (Fig. 6D). These data indicate that the transactivating effect of M protein may be mediated through the CCAAT box. Mutation of CAF only slightly reduced S promoter activity (Fig. 6C, bar 7). Surprisingly, pS/CAF-Luc gradually and dose-dependently decreased luciferase activity in response to increasing amounts of pCMV-pre-S2 (Fig. 6C, bars 8–10). These results suggest that CAF may play a positive regulatory role in mediating the transactivating effect of M protein in conjunction with the CCAAT box, which is consistent with the previous observation that CAF is essential for CCAAT box function. To confirm that CAF is indeed crucial for CCAAT box function, both the CCAAT box and CAF were mutated concomitantly (pS/CAF-Luc). As expected (14), mutation of both the CCAAT box and CAF dramatically reduced S promoter activity (Fig. 6E, bar 3). However, pS/CAF-Luc responded to increasing amounts of pCMV-pre-S2 by dose-dependently decreasing luciferase activity. Similar results were obtained when pCMV-MHBs was cotransfected with pS/CAF-Luc (Fig. 6E, bars 7–10). Taken together, these results demonstrate that transactivation of the S promoter by M protein is mediated through the CCAAT box. Consistent with a previous study (17), CAF is essential for the positive regulatory function of CCAAT box-mediated S promoter activity.

M Protein Regulates Surface Gene Expression by Interacting with CBF/Nuclear Factor Y—The above experiments demonstrated that the transactivation ability of M protein is mediated through the CCAAT box of the S promoter. Whether M protein interacts with CBF (also named nuclear factor Y, CP1, and Y- element (CCAAT)-binding protein) (41–44) is unknown. To determine whether M protein interacts with CBF in vivo, we carried out immunoprecipitation and immunoblotting experiments. First, we prepared total cell lysates from HuH-7 cells transfected with the M protein-expressing plasmid pCMV-MHBs and immunoprecipitated them with anti-CBF antibody. Our initial attempts failed to detect M protein in the immunoprecipitates by Western blotting with anti-M protein antibody (data not shown). Because M protein may undergo a proteolytic

FIG. 4. Nuclear translocation potential of the pre-S2 domain. A, dense fluorescence intensity in nuclei of cells transfected with the pre-S2-EGFP fusion protein. HuH-7 cells were transfected with pEGFP-N-pre-S2 (lower right panel) or pEGFP-C-pre-S2 (lower left panel), which express the pre-S2 domain at the N or C terminus of EGFP, respectively, or with pEGFP-N1 (upper right panel) or pEGFP-C1 (upper left panel). After 48 h, transfected cells were examined under a fluorescence microscope. Relatively dense fluorescent intensity was observed in the nuclei of cells transfected with pEGFP-N-pre-S2 and pEGFP-C-pre-S2 compared with the controls, which showed diffuse fluorescent staining. B, preferential expression of the EGFP-pre-S2 fusion protein in the nucleus. To visualize the nuclear location of transfected cells, the cells were stained with propidium iodide (PI). The dense green fluorescence preferentially co-localized with propidium iodide staining.
The cleavage residues at the boundary of the pre-S2 and S domains. The protein coding region and the putative V8 protease and chymotrypsin M protein transactivation potential.

FIG. 5. Mutation of the V8 protease cleavage site abolishes the M protein transactivation potential. A, schematic diagram of the M protein coding region and the putative V8 protease and chymotrypsin cleavage residues at the boundary of the pre-S2 and S domains. Numbers indicate the amino acids covered by various domains and motifs. The relevant residues at the boundary of the pre-S2 and S domains are illustrated. The PEST and fusion sequence motifs are defined as described previously (40). The V8 protease cleavage residue E (aa 57) and the Ch cleavage residue Q (aa 71) are shown in boldface (indicated by vertical arrows). The amino acid substitutions made in the mutants are indicated by horizontal arrow. The minus and plus signs indicate that pCMV-MHBs/V8 and pCMV-MHBs/Ch lost and retained transactivation activity after mutation, respectively. B, mutation of the V8 protease (but not chymotrypsin) cleavage site destroys the M protein transactivation potential in HepH-7 cells. HepH-7 cells were cotransfected with the empty pGL3-Basic vector or pCMV-Basic vector. After 48 h, the cell lysates were prepared for luciferase activity assay (see "Experimental Procedures"). The relative luciferase activity of each transfection is expressed relative to the value for cells cotransfected with pGL3-Basic vector (control). C, mutation of the V8 protease (but not chymotrypsin) cleavage site destroys the M protein transactivation potential in HepG2 cells. HepG2 cells were cotransfected with the empty pGL3-Basic vector or pS-Luc and with increasing doses of pCMV-MHBs/Ch, pCMV-MHBs/V8, or the empty pCMV-Basic vector. After 48 h, the cell lysates were prepared for luciferase activity assay. The relative luciferase activity of each transfection is expressed relative to the value for cells cotransfected with pS-Luc and the empty pCMV-Basic vector. All transfections were performed in triplicate and normalized to internal control Renilla luciferase activity. D, mutation of the V8 protease (but not chymotrypsin) cleavage site destroys the M protein transactivation potential in HepG2 cells. HepG2 cells were cotransfected in the same manner as described for HepH-7 cells in B. The results were treated as described for B and are expressed as the mean ± S.D. Similar results were obtained in three independent transfection experiments. E, V8 protease site mutation abolishes the nuclear translocation ability of M protein. The pCMV-MHBs vector (control), pCMV-His-pre-S2, pCMV-His-MHBs/Ch, or pCMV-His-MHBs/V8 was transfected into HepH-7 cells. Nuclear extracts were prepared from transfected cells and immunoblotted (IB) with anti-His antibody. The positions of several molecular mass markers are shown on the right in kilodaltons. Only the pre-S2 domain or MHBs/Ch (but not MHBs/V8) was detectable in the nuclear fraction.
**FIG. 6.** M protein transactivates the S promoter through the CCAAT box. A, the transcription factor-binding sites, two Sp1 sites, CBF, and CAF (underlined) within the S promoter are illustrated. The mutated sequences are shown below (Sp1 sites, CBF, and CAF) or above (the CCAAT box and CAF) the respective binding sites (mutated nucleotides are shown in lowercase letters). The numbers indicate nucleotide positions of mutated sequences. The transcription initiation sites of the S promoter (horizontal arrows) and the initiation codon of M protein (boldface) are indicated. B, the transactivation ability of M protein is not mediated through the Sp1 site. HuH-7 cells were transiently transfected with pS-Luc, pS/SP1-Luc, pS/SP2-Luc, pS/SP12-Luc, or the empty pGL3-Basic vector and increasing doses of either the pCMV-pre-S2 expression plasmid or the empty pCMV-Basic vector. After 48 h, the cell lysates were prepared for luciferase activity assay (see "Experimental Procedures"). The results are expressed relative to the value for cells cotransfected with pS-Luc and pCMV-Basic. C, the transactivation activity of the pre-S2 domain is mediated through the CCAAT box. Cells were transiently cotransfected with pS-Luc, pS/CAT-Luc, pS/CAF-Luc, or the empty pGL3-Basic vector and increasing doses of either pCMV-pre-S2 or the empty pCMV-Basic vector. The results were treated as described for B. pS/CAT-Luc did not respond to increasing doses of pCMV-pre-S2. In contrast, pS/CAF-Luc responded to increasing doses of pCMV-pre-S2 by gradually decreasing luciferase activity in a dose-dependent manner. D, the transactivation activity of M protein is mediated through the CCAAT box. Cells were transiently cotransfected with pS-Luc, pS/CAT-Luc, or the empty pGL3-Basic vector and increasing amounts of either pCMV-MHBs or the empty pCMV-Basic vector. The results were treated as described for B. E, CAF plays a positive regulatory role in mediating the transactivating effect of M protein on the CCAAT box. Cells were transiently cotransfected with pS-Luc, pS/CAT-CAF-Luc, or the empty pGL3-Basic vector and increasing amounts of pCMV-pre-S2, pCMV-MHBs, or the empty pCMV-Basic vector. The results were treated as described for B. Mutation of CAF caused the mutated CCAAT box to respond negatively to increasing doses of pCMV-pre-S2 or pCMV-MHBs. The data represent means ± S.D. Similar results were obtained in two independent experiments.
domain physically interacts with the three subunits of CBF.

**M Protein (Pre-S2 Domain) Transactivates a Multimer of the CCAAT Box, CAF, and a Combination of the CCAAT Box and CAF—To demonstrate that the pre-S2 domain transactivates the CCAAT box, CAF, and a combination of the CCAAT box and CAF, respectively, four copies of each of these elements were cloned into the pLuc-MCS reporter gene (Fig. 8A). Increasing pCMV-pre-S2 expression resulted in increased luciferase activity expression in HuH-7 cells cotransfected with p(CCAAT)₄-Luc. Fig. 8B shows that the pre-S2 domain transactivated the CCAAT element. Similarly, cotransfection experiments with p(CAF)₄-Luc or p(CATCAT)₄-Luc indicated that the pre-S2 domain transactivated CAF and a combination of the CCAAT box and CAF.

**DISCUSSION**

The role of M protein in the HBV life cycle and HBV gene regulation is controversial. Much evidence from in vitro studies shows that M protein is not essential for HBV replication in vitro (20), virion morphogenesis (19, 20), or infectivity (20, 46). Because a pre-S2-defective mutant can be identified in patients with fulminant hepatitis, it has been suggested that M protein is not essential for in vivo infectivity (21). However, results obtained from chronic HBV-infected patients indicate that expression of M protein is a marker of chronicity, implying that it is indicative of active viral replication (47). Moreover, a transcription transactivator (MHBst) function has been ascribed to M protein-expressing plasmic pCMV-MHBs/Sint. After 48 h, cell lysates were harvested for luciferase assay. The results are expressed relative to the value for cells transfected with the reporter gene construct and the empty pCMV-Basic vector.
protein in surface gene expression. The autoregulatory function of the protein in its own promoter has been observed in a number of genes (49, 50). In our study, we found that M protein can transactivate the CCAAT box of the major S promoter to regulate surface expression of L, M, and S proteins. Furthermore, our immunoprecipitation and immunoblotting experiments demonstrated the physical interaction between the three subunits of CBF (CBF-A, CBF-B, and CBF-C) and the pre-S2 domain of M protein. We were unable to determine whether they directly interact with each other, however. The ability of CCAAT-binding protein to regulate L, M, and S protein expression has been shown previously: CBF, the cognate CCAAT box-binding transcription factor, not only augments M and S protein expression, but also serves as a transcription blocker of L protein expression (13). Therefore, the balanced synthesis of these three proteins controlled by CBF is critical to the viral life cycle (13). It is not surprising then that M protein also regulates surface gene expression through interaction with CBF. The question is why HBV needs M protein to augment its surface protein expression. One plausible explanation is that this will ensure that the virus has produced sufficient amounts of M and S proteins for the assembly and release of virions. In vitro, M protein is not required for virus formation, but S protein is (51). We speculate that M protein is required, however, to prevent the overproduction of L protein, which will inhibit surface protein secretion (10, 12, 52) and the subsequent secretion of virions (9). Thus, the extra autoregulatory mechanism of M protein is advantageous for propagating the virus. The CCAAT boxes are present and occupy a fixed location within the promoter region in a substantial number of human genes (53). The transactivation activity of M protein uncovered in this study might provide a plausible explanation for HBV-associated hepatocarcinogenesis, which might be due in part to dysregulated gene expression caused by continuous stimulation of M protein during chronic hepatitis.

Another novel aspect of M protein revealed in this study is that it can translocate inside the nucleus after undergoing proteolytic cleavage. More important, the nuclear translocation ability is closely associated with its ability to transactivate the S promoter and is mediated by the MHBs\textsuperscript{au} domain (amino acids (aa) 1–57 of M protein, although MHBs\textsuperscript{au} may be composed of aa 1–56 depending on the cleavage site being located at the N or C terminus of Glu residue). (For simplicity, MHBs\textsuperscript{au} is temporarily assumed to span aa 1–57.) It has been suggested that the pre-S2 domain may have an energy- and mediator-independent nuclear translocation potential (39). In our effort to map the minimal transactivation domain of M protein, we found that, upon transfection, full-length M protein was automatically cleaved by an unknown mechanism to generate a molecular species whose electrophoretic mobility is similar to that of MHBs-(1–57) and which can be translocated inside the nucleus. In other words, although full-length M protein could be detected within the cytoplasm, only the cleaved MHBs\textsuperscript{au} domain (MHBs-(1–57) species) could be detected within the nuclear fraction. The same situation occurred in M proteins C-terminally truncated down to aa 71. All of the M protein mutants that we truncated to aa 57 and those further truncated from the N or C terminus of the pre-S2 domain translocated inside the nucleus and were not detected within the cytoplasm. In mapping experiments in which we used two different approaches, we demonstrated that M protein truncated to aa 55 had the maximal transactivation activity. Furthermore, MHBs-(1–57) and those forms that underwent further truncations had no detectable protein band within the cytoplasm. These findings demonstrate that native M protein may undergo proteolytic cleavage and be translocated inside the nucleus and that the cleavage site is localized at around aa 57. This conclusion is based on two key findings. First, mutation of aa 56 and 57 (NMENITS → NHMNITS) (pCMV-MHBs/V8 in Fig. 5, B and D) not only inhibited its nuclear translocation ability, but also inactivated its transactivation potential. Second, mutation of aa 56 (S protein translation initiation codon, NMENITS → NRENITS) (p(SA)S(APst)/Sint in Fig. 2C and pCMV-MHBs/Sint in Fig. 5C) neither inhibited its nuclear translocation ability nor inactivated its transactivation potential. Interestingly, aa 57 co-localized with the V8 protease cleavage site (Fig. 5A) (40, 54). Virions treated with staphylococcal V8 protease can infect HepG2 cells, which are normally resistant to HBV infection, probably because the cleavage of M and/or L (L/M) protein with V8 protease exposes the fusogenic domain of L/M protein (40). The exposed domain then fuses with either the cellular or endosomal membrane, thus facilitating viral entry into the cell. It has been postulated that HepG2 cells are not susceptible to HBV infection because they lack a fusion-activating protease for cleavage of L/M protein on the cell surface (40). That primary hepatocytes can be infected by HBV in vitro (55) suggests that this "V8 protease-like activity" might be present on the surface of primary hepatocytes and that it is required for the natural course of HBV infection. Our data further indicate that this V8 protease-like activity is present in HuH-7 and HepG2 cells, as evidenced by mutation of aa 57, which rendered M protein unable to transactivate the S promoter and inactivated its nuclear translocation ability. These observations raise a number of interesting questions and implications. First, whether the proteolysis-dependent exposure of the fusogenic domain and the intracellular cleavage of aa 57 at the pre-S2/S junction are mediated by the same V8 protease-like activity is not known. If they are mediated by the same enzyme, then why is it expressed in HepG2 and HuH-7 cells but not on the cell surface of HepG2 and, presumably, HuH-7 cells? Perhaps these two processes are mediated by two different enzymes. Whatever the reason, we believe that this enzymatic activity presents a potential therapeutic target for inhibiting viral entry and replication. Second, it is conceivable that this V8 protease-like enzyme cannot only facilitate HBV infection by exposing the fusogenic domain, but also promote the nuclear translocation of the MHBs\textsuperscript{au} domain. What then is the biological significance of this novel function of M protein? Because of the nuclear membrane permeabilizing effect of the pre-S (pre-S1 + pre-S2) proteins, it is possible that the nuclear translocation ability of MHBs\textsuperscript{au} may target the core particle or viral DNA inside the nucleus (39). Because the nuclear envelope may impede the nuclear transport of the core particle (56), this would require the viral capsid to disassemble so that the nuclear delivery of the HBV genome can be achieved by the nuclear translocation ability of M protein.

Previously, a transcription activation activity was ascribed to truncated forms of M protein (MHBs\textsuperscript{i}) (57). It might be speculated that the autoregulatory function of M protein uncovered in the present study is the same as that of MHBs\textsuperscript{i} described previously. Upon close examination, however, these two are quite different. First, the transcription transactivator function of MHBs\textsuperscript{i} was originally based on its ability to transactivate the SV40 promoter-reporter gene (57). Later, it was found that it may indirectly transactivate reporter genes containing transcription factor-binding sites, e.g. activator protein-1, activator protein-2, nuclear factor κB, etc. (58). The transcription transactivator function of M protein described in this study is autoregulatory and specifically regulates S gene expression through the CCAAT box of the S promoter. Moreover, this regulation involves the physical interaction of the...
MHBsau domain with CBF. Second, full-length M protein itself is autoregulatory once expressed within the cells. However, MHBs acts as a transcription transactivator only after truncation of the C-terminal 70 amino acids; full-length M protein has no transactivating function (48). A trans-activity-on region that requires both the pre-S2 domain and the N-terminal part of the S domain has been defined (48). Furthermore, MHBs-(1–53) is the minimal activation domain of MHBs (59). Our study has revealed that the maximal transactivation domain of M protein coincides with the pre-S2 domain. Frameshift at aa 47 abolishes the MHBs-activator. In contrast, further truncations at either the N terminus down to amino acid 19 (pre-S2-(19–55)) or the C terminus down to amino acid 47 (pre-S2-(1–47)) do not disrupt its transactivation ability. Third, the transcription transactivator function of MHBs requires the cytoplasmic orientation of the pre-S2 domain (59).

In summary, in this study, we have found and defined a novel function of M protein. M protein expressed within the cytoplasm undergoes a proteolytic cleavage through which an autoregulatory domain (MHBsau) is released. MHBsau then translocates inside the nucleus through its nuclear permeabilizing effect to interact with the CCAAT box of the S gene promoter to regulate surface gene expression.

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