Glucocorticoid-induced S-Adenosylmethionine Enhances the Interferon Signaling Pathway by Restoring STAT1 Protein Methylation in Hepatitis B Virus-infected Cells*

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Background: It is necessary to improve the antiviral response of IFN-α for chronic hepatitis B (CHB) patients. An alternative approach to increase the response rate of IFN-α might be to immunologically stimulate the host with glucocorticoids (GCs) before treatment with IFN-α, but the underlying mechanism remains unclear. We hypothesized that the GCs enhance IFN signaling by inducing S-adenosylmethionine (AdoMet) when hepatitis B virus (HBV) replication was effectively suppressed by IFN-α. Here, we investigated the effect of GCs and IFN-α on AdoMet production and methionine adenosyltransferase 1A (MAT1A) expression by hypermethylation within the MAT1A promoter.

Results: Hepatitis B virus (HBV) disrupted glucocorticoid-induced S-adenosylmethionine and methionine adenosyltransferase 1A (MAT1A) expression by hypermethylation within the MAT1A promoter.

Conclusion: Glucocorticoid-induced S-adenosylmethionine enhances the response of IFN-α by restoring STAT1 methylation in HBV-infected cells.

Significance: The combination therapy of glucocorticoids, S-adenosylmethionine, and IFN-α is possibly useful for CHB patients.

Patients with chronic hepatitis B usually exhibit a low response to treatment with interferon α (IFN-α). An alternative approach to increase the response rate of IFN-α might be to immunologically stimulate the host with glucocorticoids (GCs) before treatment with IFN-α, but the underlying mechanism remains unclear. We hypothesized that the GCs enhance IFN signaling by inducing S-adenosylmethionine (AdoMet) when hepatitis B virus (HBV) replication was effectively suppressed by IFN-α. Here, we investigated the effect of GCs and IFN-α on AdoMet production and methionine adenosyltransferase 1A (MAT1A) expression by hypermethylation within the MAT1A promoter. HBV reduced AdoMet production by increasing methylation at GRE sites within the MAT1A promoter. The X protein of hepatitis B virus led to hypermethylation in the MAT1A promoter by recruiting DNA methyltransferase 1, and it inhibited GR binding to the GRE in the MAT1A promoter. Dex could increase an antiviral effect by inducing AdoMet production via a positive feedback loop when HBV is effectively suppressed by IFN-α, and the mechanism that involves Dex-induced AdoMet could increase STAT1 methylation rather than STAT1 phosphorylation. These findings provide a possible mechanism by which GC-induced AdoMet enhances the antiviral activity of IFN-α by restoring STAT1 methylation in HBV-infected cells.

The hepatitis B virus (HBV) is the most common hepatitis virus, and it causes chronic infections in the human liver (1). Complete eradication of HBV is rarely achieved due to the persistence of its covalently closed circular DNA in host hepatocytes (2). One key component of the host antiviral responses is the interferon (IFN) system. The immunomodulatory agent interferon α (IFN-α) is known to reduce the quantity of covalently closed circular DNA, presumably by inducing T-cell cytotoxicity and lysis of infected hepatocytes, along with the production of cytokines for control of viral replication (3). However, patients with chronic hepatitis B (CHB) usually respond poorly to IFN-α treatment, and the underlying mechanism remains unclear (4). It is noteworthy that the HBV genome contains a specific DNA-binding site for the GR, and this HBV GR domain can be categorized as a functional glucocorticoid-response element (GRE). Treatment of CHB would benefit from an improved antiviral response to IFN-α.

An alternative approach to increase the efficacy and response rate observed with IFN might be to immunologically stimulate the host by withdrawing glucocorticoids (GCs) before treatment with IFN. In CHB infection, pulse GC treatment followed by abrupt withdrawal has been associated with an enhanced cellular immune response to hepatitis B, as indicated by a rise in alamine transaminase values and a transient reduction in markers of viral replication upon withdrawal of GCs (5). Pretreatment with GCs (“immunologic priming”) is believed to be synergistic when followed by treatment with IFN-α in a subgroup.

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The abbreviations used are: HBV, hepatitis B virus; CHB, chronic hepatitis B; Dex, dexamethasone; DNMT, DNA methyltransferase; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid-response element; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBx, the X protein of hepatitis B virus; HCC, hepatocellular carcinoma; ISG, interferon-stimulated genes; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; nt, nucleotide.
of patients (with low initial alanine transaminase values) (5, 6). Although there are different opinions concerning the rationale for a combination regimen of GCs and IFN-α, most studies suggest that sequential treatment with GCs and IFN-α for HBeAg-positive chronic hepatitis B may be more effective than IFN-α monotherapy in promoting the loss of hepatitis B "e" antigen and hepatitis B virus DNA (7). However, the antiviral mechanism of the combination regimen is unknown.

S-Adenosylmethionine (AdoMet), a principal biological methyl donor, is synthesized from methionine and ATP in a reaction catalyzed by methionine adenosyltransferase (8, 9). In mammals, there are two genes (MATIA and MAT2A) that encode two homologous methionine adenosyltransferase catalytic subunits (10, 11). AdoMet biosynthesis is depressed in chronic liver disease (12). Preclinical studies indicate that this depression could exacerbate liver injury, and therefore, supplementation may be a useful therapy. AdoMet has been widely adopted worldwide as a therapy for chronic intrahepatic cholestasis liver disease (13). However, the effectiveness of AdoMet therapy in CHB has not been adequately addressed. It was previously reported that hepatitis AdoMet synthetase activity is altered in adrenalectomized animals, suggesting a role for GCs in its regulation (14). This finding was further supported by a study indicating that GCs strongly up-regulate AdoMet synthetase, both in vivo and in hepatic cultured cells, and that they have a direct effect on enzyme gene expression (15). However, the mechanism by which AdoMet synthetase activity was up-regulated by GCs was not investigated in these studies. Interestingly, AdoMet synthetase activity has been reported to be significantly reduced in various liver disorders (16). Thus, it is tempting to speculate that at least some of the beneficial effects of GCs in chronic HBV-related liver diseases could be due to the direct stimulation of AdoMet synthetase, which in turn would increase the availability of AdoMet. In addition, AdoMet may improve IFN signaling and antiviral effects through increased methylation of STAT1, leading to enhanced STAT1-DNA binding and greater expression of interferon-stimulated genes (ISGs) (17). AdoMet represents the first IFN-sensitizing agent with enhanced STAT1-DNA binding and greater expression of interferon-stimulated genes (ISGs) (17). AdoMet appears to be a useful adjunct to IFN-based therapy, a factor that may be particularly important in the era of direct antivirals for HBV infection.

In this study, we propose that a combination regimen of GCs and IFN-α is associated with the GC induction of AdoMet production. Here, we investigated whether HBV, alone or in combination with Dex, could alter the alternative expression of MATIA and AdoMet production, which was potentially associated with DNA methylation in the putative GRE of the MATIA gene promoter in the examined hepatoma cells. We then analyzed the possible epigenetic mechanisms involved. Recent evidence suggests that HBV has evolved strategies to block the nuclear translocation of signal transducers and activators of transcription (STAT1) to limit IFN-α-induced cellular antiviral responses (18). Furthermore, we explored the effect of the GC-induced increase of AdoMet production on STAT1 methylation and phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Cell Culture—** A 1474-bp promoter construct of the MATIA gene, corresponding to the sequence from nt −1474 to 0 (relative to the transcriptional start site) of the 5′-flanking region of the human MATIA gene, was generated from human genomic DNA by PCR using pMATIA1.4Luc-F and pMATIA1.4Luc-R as forward and reverse primers carrying the MluI and Xhol sites at the 5′ and 3′ ends, respectively. The PCR product was cloned into the MluI and Xhol sites of the pGL3-Basic vector. The resulting construct was confirmed by DNA sequencing. The 5′-flanking deletion constructs of the MATIA promoters, pMATIA1.2Luc, pMATIA0.9Luc, and pMATIA0.8Luc, were similarly generated by PCR, using the pMATIA1.4Luc construct as a template. The forward primers were pMATIA1.2Luc-F1, pMATIA0.9Luc-F2, and pMATIA0.8Luc-F3. Site-directed deletion of two GRE sites was performed by 30 cycles of PCR using the pMATIA1.4Luc or pMATIA0.9Luc construct as a template and the appropriate primers. In the pMATIA1.4Luc plasmid, MATIA-GRE1 (nt −876 to −862) was deleted. In the pMATIA1.4Luc2 plasmid, MATIA-GRE2 (nt −1022 to −1008) was deleted. In the pMATIA1.4Luc3 plasmid, both MATIA-GRE1 (nt −876 to −862) and MATIA-GRE2 (nt −1022 to −1008) were deleted. In the pMATIA0.9Luc plasmid, MATIA-GRE1 (nt −876 to −862) was deleted. Four site-directed mutations were constructed by PCR using pMATIA-1.4Luc as a template. Four CpG sites were mutated separately from C to A. Ligation was verified by sequence analysis. The PCR primer sequences are shown in Table 1.

Cell lines, including the human normal liver cell L02 and the hepatoma cell lines Huh7, Hep3B, and HepG2, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), where they were characterized by mycoplasma detection, DNA fingerprinting, isozyme detection, and determination of cell viability. The HepG2.2.15 cell line was derived from HepG2 cells and stably expresses HBV (Genotype D, Serotype ayw, U95551), which was used as an HBV replication model (19–21). The stable cell lines were maintained in DMEM containing 400 μg/ml G418. The plasmid pCMV-HBV-1.3, which expresses HBV (genotype C, serotype adr, FJ899793), was a gift from Dr. Ying Zhu (State Key Laboratory of Virology, College of Life Sciences, Wuhan University, China). All cells were cultured in the recommended media supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and streptomycin at 37 °C in an incubator with 5% CO₂.

**Quantitative qRT-PCR Analysis—** For the analysis of mRNA levels, total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Quantification of total RNA was performed with a Nanodrop™ spectrophotometer (Thermo Scientific) at 260 and 280 nm. cDNA was synthesized using a cDNA synthesis kit (Toyobo, Japan). For the analysis of production levels in ChIP assays, the enriched DNA fragments in ChIPs were quantified with quantitative RT-PCR. Amplification was performed with the iQ5 quantitative PCR system (Bio-Rad) and SYBR® Green Master Mix (Toyobo, Japan). GAPDH was used for normalization of the relative expression. Relative mRNA levels were determined using the 2−ΔΔCt method. The gene-specific primers are listed in Table 1.
Transfection and Luciferase Reporter Assays—All transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. For the luciferase assay, the cells were transiently co-transfected with the pRL-TK plasmid (Promega) containing the Renilla luciferase gene, which was used for internal normalization, and various constructs containing different lengths of the MAT1A 5’-flanking region. After 48 h post-transfection, cell lysates were prepared, and the luciferase activity was measured using a luciferase assay system (Promega). The cell lysates (10 μl) were mixed, and the luciferase assay substrates (50 μl) (Promega) were added before tumor excision. Both the tumors and corresponding peritumoral noncancerous tissues were selected for each case. All patients tested positive for HBV surface antigen in their serum. Matched normal human liver tissues were obtained from patients undergoing partial hepatectomy. Written informed consent was obtained from each patient. Written informed consent was obtained from each patient.

Immunohistochemistry—Fifty cases (25 pairs) of HBV-associated hepatocellular carcinoma (HCC) were collected from Zhongnan Hospital, Wuhan University, between January 2011 and October 2012. Chemotherapy and radiation therapy were not administered before tumor excision. Both the tumors and corresponding peritumoral noncancerous tissues were selected for each case. All patients tested positive for HBV surface antigen in their serum. Matched normal human liver tissues were obtained from liver trauma patients undergoing partial hepatectomy. Written informed consent was obtained from each patient.

Both HCC tissues and adjacent nontumor liver tissues were sectioned to produce 4-μm thick slices. The tissue sections were deparaffinized in xylene and rehydrated with ethanol, and the endogenous peroxidase was inactivated with 0.3% hydrogen peroxide for 10 min. The fixed cells were incubated in PBS without the antibody under the same experimental conditions. The total immunostaining settings were used within each dataset.

Fluorescent Immunocytochemistry—Hepatoma cells were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.5% Triton X-100 for 10 min. The fixed cells were blocked with 3% fetal calf serum for 30 min. For specific detection of GR protein, the cells were incubated with polyclonal rabbit anti-rat GR antibody (1:100, Cell Signaling Technology) overnight at 4 °C. The samples were incubated with Cy3-labeled goat anti-rabbit IgG (1:100, eBioscience) in PBS for 1 h at 37 °C. Then the cells were washed twice with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The images were analyzed using fluorescence microscopy (Nikon Eclipse 80i, Japan). Identical illumination and camera settings were used within each dataset.
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score was calculated as the sum of the positive percentage and
the staining intensity of the stained cells, which ranged from 0 to
6. The percent positivity was scored as 0 (0–25%), 1 (26–
50%), 2 (51–75%), and 3 (∞75%). The staining intensity was
scored as 0 (no staining), 1 (weakly stained), 2 (moderately
stained), and 3 (strongly stained). The results of the immuno-
histochemical staining were scored by two experienced pathol-
ogists, who were blinded to the clinical data. A negative expres-
sion of protein was defined as a total score ≤3, and a positive
expression was defined as a total score ≥4.

Immunoblotting—For the detection of protein, cytoplasm
and nuclear protein extracts were prepared from cells treated
with vehicle or dexamethasone. The protein concentration of
nuclear protein extracts were prepared from cells treated
expression was defined as a total score
sion of protein was defined as a total score
histochemical staining were scored by two experienced pathol-
for the assay, 1
China).

was equilibrated with 80% A and 20% B. The flow rate was 0.5
Sigma. The wavelength for detection was 254 nm. The column
was sheared to reduce the DNA length to between 200 and 1000
bp by sonication 9 times for 10 s each time, using an Ultrasonic
Processor VCX 600 (Sonic and Materials, Newtown, CT) at
30% power. The recovered supernatants were incubated with
an antibody directed against GR (5 µg, Cell Signaling Technol-
y) or an isotype control IgG for 4 h in the presence of ChIP
Dilution Buffer (900 µl) and ChIP blocked protein G-agarose
(60 µl). The immunoprecipitated DNA was retrieved from the
beads with a 1% SDS and 1.1 M NaHCO3 solution maintained at
65 °C overnight. The DNA was then purified using a PCR puri-
fication kit (Axygen), and PCR was performed on the extracted
DNA using specific primers (Table 1).

Electrophoresis Mobility Shift Assay (EMSA)—Nuclear
extracts were prepared from the cells treated as described
above. EMSA was performed using a nonradioactive EMSA kit
following the manufacturer’s instructions (Pierce). The sequence
of the GRE1 probe was P1, 5′-CACACAACACACACATTGTT-
TCTGTA-3′. The GRE2 probe was P2, 5′-GAGTTATGGAA-
CAGATGTTATTACATG-3′, and the HBV-GRE probe
was P3, 5′-CCACCTCTCTGTCCCCAAATTGCTTCTGT-
3′. The 5′ end of the oligonucleotides was biotin-labeled. Ten
micrograms of crude nuclear protein were incubated for 20 min
at room temperature in a 15-µl binding reaction system,
including 1.5 µl of 10× binding buffer, 1.5 µl of poly(dl-dC) (1.0
µg/µl), and double-distilled H2O to a final volume of 15 µl.
Then 0.6 µl of Bio-GRE1 probe or Bio-GRE2 probe or Bio-
HBV-GRE probe (500 fm) was added, and the reaction was
incubated for 20 min at room temperature. Where indicated, 2 µl of
specific unlabeled competitor oligonucleotide was added before
the labeled probe to the ×100× competing system and
incubated for 20 min. Protein-DNA complexes were
resolved by electrophoresis at 4°C on a 6.5% polyacrylamide gel
and subjected to autoradiography. Electrophoresis was
conducted on a 6.5% nondenaturing polyacrylamide gel at 175 V in
0.25× TBE (1× TBE is 89 mM Tris-HCl, 89 mM boric acid, and
5 mM EDTA, pH 8.0) at 4°C for 1 h. For the supershift experi-
ments, purified polyclonal antibody directed against GR (4 µg,
Cell Signaling Technology) or IgG was incubated with protein-
DNA complexes on ice for 20 min. The gels were placed on
the binding membrane, and the proteins were transferred at 394
mA in 0.5× TBE at room temperature for 40 min. Then the
membrane was cross-linked in a UV cross-linking apparatus for
10 min (immobilization), blocked, streptavidin-HRP labeled,
washed, and then equilibrated. Images were obtained using an
Imager apparatus (Alpha Innotech, San Leandro, CA).
Quantitative Methylation Changes of MAT1A Promoter—To perform bisulfite conversion of the genomic DNA, an Epitect bisulfite kit (Qiagen AG, Basel, Switzerland) was used according to the manufacturer’s protocol. We designed primers for the MAT1A promoter to cover the regions with the CpG sites (Table 1). The selected amplicon was located in the core regulatory regions of the promoter, which covered the two GREs. The primers were designed using MethPrimer. For PCR amplification, a T7 promoter tag was added to the reverse primer, and a 10-mer tag sequence was added to the forward primer to balance the PCR primer length. The following PCR conditions were used for the amplification of the bisulfite-treated genomic DNA: one cycle, 94 °C for 4 min; 45 cycles, 94 °C for 20 s; 56 °C for 30 s; 72 °C for 1 min; and one cycle, 74 °C for 3 min. Unincorporated dinucleotide triphosphates were removed by shrimp alkaline phosphatase (Sequenom, San Diego) treatment. Typically, 2 μl of the PCR product was then directly used as a template for the transcription reaction. Twenty units of T7 R&DNA™ polymerase (Epicenter, Madison, WI) were used to incorporate dTTP in the transcripts. Ribonucleotides were used at 1 mmol/liter and the dNTP substrate at 2.5 mmol/liter. After dilution with double distilled H2O to a final volume of 7 μl, the samples were incubated at 37 °C for 3 h. Next, 20 μl of double distilled H2O were added to each sample. Phosphate backbone conditioning was achieved by adding 6 mg of Clean Resin (Sequenom, San Diego) before performing MALDI-TOF MS analysis. A total of 12 nl of the RNase A-treated product was robotically dispensed onto a silicon matrix of preloaded chips (SpectroCHIP; Sequenom, San Diego), and the mass spectra were collected using a MassARRAY Compact MALDI-TOF (Sequenom, San Diego). The methylation ratios of the spectra were generated using EpiTYPER software version 1.0 (Sequenom, San Diego).

**Statistical Analysis—**Measurement data are presented as the mean ± S.D. or the mean ± S.E., and count data were analyzed using a χ² or Fisher’s exact test. Data that satisfied the normal distribution criterion were used in the group design of the t test for the statistical analysis. The differences among the groups were tested by a one-way analysis of variance followed by a post hoc test (LSD). A value of p < 0.05 was considered significant. All statistical analyses were performed using professional statistical software (SPSS 15.0 for Windows, SPSS Inc., Chicago).

**RESULTS**

**AdoMet Homeostasis Was Disrupted by Pharmacologic Concentrations of Glucocorticoids through Inducing MAT1A Expression—**To determine the effects of GCs on AdoMet and AdoHcy, we treated different liver cells with Dex. Dex was chosen in our studies because it is similar to GCs and has been used extensively in humans. We observed that the levels of AdoMet and the ratio of AdoMet/AdoHcy were markedly increased in Dex-treated cells, including normal hepatic L02 cells and HepG2 cells. Next, we determined the specificity of Dex in the activation of AdoMet production. We treated these cells with RU486 (an antagonist of GR) before supplying Dex. The results indicated that RU486 can counteract the stimulatory effect of Dex on AdoMet production, which indicates that GCs induced AdoMet production by binding to a specific receptor, GR (Table 2). Because AdoMet production was dependent on MAT1A expression, to elucidate the mechanism of the GC-induced increase of AdoMet production, we analyzed the effect of Dex on MAT1A expression. The results showed that the half-life of MAT1A mRNA was identical, whereas the absolute level of MAT1A mRNA was higher in Dex-treated L02 cells compared with vehicle-treated cells (Fig. 1, A and B), which suggested that Dex did not affect the stability of the MAT1A mRNA. Furthermore, we found that MAT1A protein was significantly induced when Dex was supplied at doses of 100 and 1000 nM (Fig. 1C). We analyzed the possible mechanisms involved by investigating the effects of Dex on the MAT1A promoter activity. The luciferase activity of MAT1A was significantly increased in a dose-dependent manner in the Dex-treated cells (Fig. 1D). These results were confirmed in other hepatoma cell lines, including Huh7, Hep3B, and HepG2. However, MAT1A expression was blocked dramatically with RU486 treatment in the aforementioned cells (Fig. 1E). The results showed that GCs induced MAT1A expression by binding to the GR. Next, we analyzed the GR localization in hepatoma cells. We observed an increased amount of GR importation to the nucleus in response to ligand binding in different hepatoma cells. The level of GR increased in the nucleus and decreased in the cytoplasm of the Dex-treated cells compared with the vehicle-treated cells (Fig. 1F). These results demonstrated that the GR participated in Dex-induced MAT1A expression via translocation to the nucleus.

**Role of the GRE in the Stimulatory Effect of GCs on the MAT1A Alternative Promoter Activity—**To further explore the mechanism of the effect of GCs on MAT1A expression, we investigated the role of the cis-regulatory elements of the MAT1A promoter in response to Dex regulation. When a series of truncated promoter mutants was generated, we found that the Dex-induced increase of MAT1A promoter activity was inhibited by a deletion from nt −1474 to −874 (Fig. 2A), which suggested that the sequence between nt −1474 and −874 is critical for the activation of MAT1A by Dex. Analyses of the cis-regulatory elements of the MAT1A promoter revealed two GR-binding sites in this region, including MAT1A-GRE1 (nt −876 to −862) and MAT1A-GRE2 (nt −1022 to −1008). To evaluate the roles of these GREs in the activation of the MAT1A promoter by Dex, experiments involving deletion and site-directed mutagenesis at positions GRE1 and GRE2 were conducted (Fig. 2, B and C). The results showed that the luciferase activity in cells transfected with pMAT1A1.4Luc or pMAT1A0.9Luc was significantly induced by Dex, but the actual luciferase activity units of pMAT1A0.9Luc was less than 50% compared with that of pMAT1A1.4Luc. However, the induction of Dex on pMAT1A1.4Luc or pMAT1A0.9Luc was disrupted when the GRE1 or GRE2 site was deleted or mutated. These results suggested that GREs were required for the activation of MAT1A expression mediated by Dex.

To explore the interactions between the GRE sites and the GR, ChIP assays were performed. The results showed that PCR products were only produced from DNA isolated from the Dex-treated cells (Fig. 2D, Chip1). Then we deleted the two GRE
sites separately and found that only PCR products of GRE1 were produced when GRE2 was deleted, and only PCR products of GRE2 were produced when GRE1 was deleted after being treated with Dex (Fig. 2D, Chip2 and Chip3). The results showed that the production of Chip-GRE1 (254 bp) was higher than that of Chip-GRE2 (154 bp), which suggested that there was more binding of GR protein to the GRE1 site than to the GRE2 site. These findings indicated that the two complete GRE sequences are functional in the context of the MAT1A promoter by binding with GR.

To determine the specific binding of the GR to the GRE sites in the MAT1A promoter, EMSAs were performed. We observed one faint band in the absence of Dex, indicating the presence of a protein-DNA complex (binding shift band) (Fig. 2E, 2nd lane); however, the binding shift band was enhanced in the presence of Dex (Fig. 2E, 3rd lane). Moreover, the binding shift band was eliminated in the presence of a cold probe (Fig. 2E, 4th lane). In addition, a specific protein-DNA complex (supershift band) was detected in the presence of an anti-GR antibody (Fig. 2E, 5th lane). Similar results were observed when the GRE2 probe (P2) was used (Fig. 2E, 6th to 10th lanes).

HBV Down-regulated MAT1A Expression by Up-regulating DNMT1 but Not DNMT3A—Although AdoMet production and MAT1A expression were induced by Dex, we found that the levels of AdoMet and the ratio of AdoMet/AdoHcy were not...
The effect of Dex on the binding of the GR to GRE1 (P1) and GRE2 (P2) in the MAT1A promoter was determined by ChIP assays. The productions of Chip-GRE1 and Chip-GRE2 were quantified by qPCR. The effect of Dex on the binding of the GR to the GREs of the MAT1A promoter was determined by ChIP assays. The productions of Chip-GRE1 and Chip-GRE2 were quantified by qPCR. Shown is a representative result from three independent experiments. **, p < 0.01 and ***, p < 0.001.

**TABLE 2**

Changes of AdoMet, AdoHcy, and the AdoMet/AdoHcy ratio in L02, HepG2, and HepG2.2.15 per 10⁵ cells treated with different concentrations of Dex and RU486

| Cell lines | Treatment | Concentration | AdoMet | AdoHcy | AdoMet/AdoHcy |
|------------|-----------|---------------|--------|--------|---------------|
| L02        | Dex (nm)  | 0             | 4.13 ± 0.18 | 2.18 ± 0.14 | 1.89 ± 0.13 |
|            |           | 1             | 5.51 ± 0.11 ** | 2.40 ± 0.12 | 2.40 ± 0.15 ** |
|            |           | 10            | 8.03 ± 0.19 ** | 2.48 ± 0.15 | 3.24 ± 0.14 ** |
|            |           | 100           | 9.37 ± 0.17 ** | 2.60 ± 0.17 | 3.60 ± 0.11 ** |
| HepG2      | RU486 (nm)| 100           | 3.78 ± 0.13 | 2.12 ± 0.03 | 1.79 ± 0.13 |
|            | Dex (nm)  | 0             | 3.57 ± 0.15 | 1.93 ± 0.11 | 1.85 ± 0.13 |
|            |           | 1             | 5.30 ± 0.17 ** | 2.10 ± 0.16 | 2.53 ± 0.16 ** |
|            |           | 10            | 7.24 ± 0.11 ** | 2.21 ± 0.19 | 3.28 ± 0.17 ** |
|            |           | 100           | 8.87 ± 0.14 ** | 2.43 ± 0.37 | 3.66 ± 0.21 ** |
| HepG2.2.15 | RU486 (nm)| 100           | 3.47 ± 0.12 | 1.99 ± 0.09 | 1.75 ± 0.11 |
|            | Dex (nm)  | 0             | 3.17 ± 0.07 | 1.74 ± 0.06 | 1.82 ± 0.07 |
|            |           | 10            | 3.09 ± 0.04 | 1.77 ± 0.12 | 1.75 ± 0.08 |
|            |           | 100           | 3.17 ± 0.08 | 1.75 ± 0.05 | 1.81 ± 0.06 |
|            | RU486 (nm)| 100           | 3.19 ± 0.02 | 1.69 ± 0.04 | 1.89 ± 0.03 |
|            |           | 100           | 2.97 ± 0.11 | 1.65 ± 0.09 | 1.80 ± 0.10 |

*a* p < 0.05 versus Dex 0 nm by unpaired Student’s t test.

Altered in HepG2.2.15 cells that were stably transfected with HBV after Dex treatment (Table 2). Furthermore, Dex also failed to induce MAT1A expression in HepG2.2.15 (Fig. 1E). These results suggested that the effect of Dex on MAT1A expression may be disrupted by HBV. It has been reported that HBx plays a crucial role in hepatocarcinogenesis by inducing aberrant epigenetic modifications (23). To verify the role of HBV and HBx in the regulation of MAT1A expression, we studied whether post-transcriptional regulation is involved. We observed that the half-life of MAT1A mRNA was identical, whereas the absolute level of MAT1A mRNA was lower in pCMV-HBV1.3-transfected HepG2 cells compared with the mock-transfected cells (Fig. 3, A and B), which suggested that HBV did not affect the stability of MAT1A mRNA. We also found that the levels of the MAT1A protein (Fig. 3C) were lower in HepG2 cells transfected with pCMV-HBV1.3 than with mock-transfected cells. To determine the effects of HBV on luciferase activity, HepG2 cells were transiently transfected with pMAT1A-1.4Luc or pMAT1A-0.8Luc. There was a significant reduction of luciferase activity in pMAT1A-1.4Luc when the cells were transfected with pCMV-HBV1.3 compared with the mock vector (Fig. 3D). This suggests that HBV suppressed MAT1A promoter activity by way of the sequence between nt −1474 and −874, which was critical for the activation of MAT1A by Dex. However, Dex failed to induce MAT1A expression, but DNMT1 and DNMT3A were induced in a dose-dependent manner in HepG2.2.15 cells (Fig. 3F). Furthermore, we found that MAT1A expression was inducible by Dex when DNMT1 was knocked down with siDNMT1 (5′-AGAATTTGTCCTTGGAAGACGG-3′), whereas MAT1A expression was not induced by Dex when DNMT3A was knocked down with siDNMT3A (5′-AGAAGTTGATACACGGACATG-3′) (Fig. 3F). These results suggested that Dex-induced MAT1A expression was disrupted by HBV, perhaps due to HBx recruiting of DNMT1 to increase methylation at the putative GRE of the MAT1A promoter.
HBV Could Suppress the Dex-induced Increase of MAT1A Expression by Promoting DNA Hypermethylation of the MAT1A Promoter—To study HBV suppression of Dex-induced MAT1A expression in vivo, we tested the expressions of HBx and DNMT in HBV-associated HCC tissues, and we searched for a possible linker role for DNA methylation in the Dex-dependent interaction of the GR, the MAT1A promoter, and HBx. As shown in Fig. 4A, HBx had a higher expression in HCC tissue, which was consistent with our previous findings (22); in addition, DNMT1 had a higher level of expression, whereas DNMT3B had a lower level of expression in HCC tissues compared with adjacent nontumor tissues. Interestingly, there is a positive correlation between HBx expression and DNMT1 expression, and a negative correlation between HBx expression and DNMT3B expression in liver tumor tissues (Table 3). As shown in Fig. 4B, the protein level of MAT1A was significantly decreased by 17.82% (0.83 ± 0.06 versus 1.01 ± 0.09, p = 0.015) in the HCC tissues compared with adjacent nontumor tissues. Previous studies have reported that HBx expression increased total DNMT activities by up-regulating DNMT1 and DNMT3A and selectively promoting regional hypermethylation of specific tumor suppressor genes. HBx also induced global hypomethylation by down-regulating DNMT3B (23). As mentioned earlier, we found that HBx could recruit DNMT1 to increase methylation at the putative GRE of the MAT1A promoter (Fig. 3). Therefore, we speculated that HBx might promote regional hypermethylation by up-regulating DNMT1 and lead to repressed MAT1A expression. Next, we investigated the methylation profile of CpG sites in the promoter sequence of MAT1A in four pairs of liver tissues. We found that the rates of methylation of CpG sites of the MAT1A promoter were higher in HBV-associated HCC tissues than in adjacent nontumor tissues (Fig. 4, C and D).

HBV Inhibited MAT1A Expression by Site-specific Hypermethylation within the GRE in the MAT1A Promoter—To clarify the role of HBV in aberrant epigenetic modifications at the putative GRE of the MAT1A promoter, we located two putative GR-binding sites in the GRE1 (nt −876 to −862) and GRE2 (nt −1022 to −1008) in the human MAT1A promoter. Five bases are necessary for maximal GRE function: −3, −2, +2, +3, and +5 (24). Of these five bases, the MAT1A-GRE1 sequence (5′-CACACACATTGTCTT-3′) contains the five optimal bases. However, the MAT1A-GRE2 sequence (5′-TGACACAGTGT-TTAT-3′) has only one different base (+5), where a C is substituted for a T (Fig. 5A). Thus, the MAT1A-GRE2 contains all but one of the nucleotides, which is required for full functional activity. This may be the primary reason for more binding of the GR protein to the GRE1 site than the GRE2 site. To demonstrate HBV-induced aberrant epigenetic modifications at the putative GRE in the MAT1A promoter, CpG methylation was tested by a MALDI-TOF mass array (Fig. 5B). The analysis of the DNA fragments of the MAT1A promoter, containing CpGs between nt −1120 and −620, revealed an increased methylation density in the 2nd and 3rd CpGs with increasing concen-

FIGURE 3. Effect of HBV on MAT1A promoter activity and expression. A, analysis of MAT1A mRNA stability in HepG2 cells transfected with HBV. Each level of Dex-treated and -untreated MAT1A mRNA before actinomycin D treatment was considered as 1, and the relative levels were calculated. B and C, MAT1A mRNA and MAT1A protein were examined after HepG2 cells were transfected with HBV for 24 h. The inset shows the representative immunoblots of MAT1A in different transfections. D, effect of HBV on luciferase activity in HepG2 cells transfected with pMAT1A-1Luc. *, p < 0.05. E, DNMT1, DNMT3A, MAT1A, GR, HBx, and GAPDH protein levels were detected after HepG2.2.15 cell treatment with vehicle or Dex for 24 h. The inset shows the representative immunoblots of DNMT1 and DNMT3A at different concentrations. **, p < 0.01; ##, p < 0.01. F, DNMT1, DNMT3A, MAT1A, and GAPDH protein levels were detected after HepG2.2.15 cells were transfected with siControl, siDNMT1, or siDNMT3A and treated with vehicle or Dex (100 nm) for 24 h. The inset shows the representative immunoblots of MAT1A with different treatments. *, p < 0.05. Shown is a representative result from three independent experiments.
trations of transfected with pCMV-HBV1.3 (Fig. 5C). It was interesting to note that there was no significant reduction of luciferase activity when the CpG2 and CpG3 sites were mutated (Fig. 5D). These CpGs overlap with the GREs, which are important determinants for the induction of MAT1A expression, and the methylation of these CpG sites by HBV significantly reduced the activity of the MAT1A promoter.

It is noteworthy that the HBV genome contains a specific DNA-binding site for the GR, and this HBV GR domain can be categorized as a functional GRE. Therefore, we further examined GR-binding profiles in HepG2.2.15 cells using ChIP analyses (Fig. 5E). The results indicated that the GR preferred to bind to the DNA sequence of HBV rather than to the promoter of MAT1A. To confirm that HBV was able to compete with

| TABLE 3 |
| --- |
| Correlation of HBx protein expression with DNMT1, DNMT3A, DNMT3B, MAT1A, GR protein expression, and patients’ clinicopathologic characteristics in hepatocellular carcinomas and noncancerous tissues |
| The correlations between the protein expression and tissue types were analyzed using a χ² or Fisher’s exact test. |

| Characteristic | HBx expression HCC tissues | HBx expression noncancerous tissues |
| --- | --- | --- |
| | Negative | Positive | Correlation | p value | Negative | Positive | Correlation | p value |
| DNMT1 expression | Negative | 14 | 3 | 0.557 | 0.010* | 19 | 2 | 0.600 | 0.016* |
| | Positive | 2 | 6 | | | 1 | 3 | | |
| DNMT3A expression | Negative | 5 | 5 | 0.870 | 0.010* | 11 | 4 | 0.615 | 0.016* |
| | Positive | 8 | 7 | | | 9 | 1 | | |
| DNMT3B expression | Negative | 1 | 12 | −0.923 | 0.000* | 17 | 4 | | |
| | Positive | 12 | 0 | | | 3 | 1 | 1.000 | 0.557 |
| MAT1A expression | Negative | 18 | 2 | 0.656 | 0.005* | 7 | 3 | 0.500 | 0.034* |
| | Positive | 1 | 12 | | | 3 | 12 | | |
| GR expression | Negative | 4 | 3 | 1.000 | 0.010* | 8 | 8 | 0.428 | 0.615 |
| | Positive | 8 | 9 | | | 7 | 3 | | |
| Sex | Male | 10 | 10 | 1.000 | 0.010* | 16 | 4 | 1.000 | 0.016* |
| | Female | 3 | 2 | | | 4 | 1 | | |
| Liver cirrhosis | No | 6 | 2 | 0.557 | 0.010* | 5 | 3 | 0.673 | 0.016* |
| | Yes | 3 | 14 | | | 8 | 9 | | |
| AFP (ng/ml) | ≤ 200 | 3 | 0 | 0.538 | 0.024* | 2 | 1 | | |
| | >200 | 5 | 17 | | | 8 | 14 | | |

* p < 0.05 was considered significant.
MAT1A in binding to the GR at the GRE site, EMSAs were performed (Fig. 5F). We observed that the intensity of the band in lane 3 was stronger than that in lane 6 or lane 7 (Fig. 5F). The results indicated that there was more nuclear protein binding to the HBV probe than to the MAT1A promoter probe (GRE1 and GRE2 probes) after treatment with Dex. Taken together, all these results demonstrated that Dex-induced MAT1A gene expression was inhibited by HBV through site-specific hyper-

**A**

-1120 GTGTCAGAC GCCGTGATTTA ATAGAAGAC TACATGTTA ATACCTGTTA CTACCTTTTT
-1060 TGTCGAAAGAC AAAATAAC GCAAGCTTA TGTGACACCG ATGTTTTA CAGTTTAGA
-1000 ACGAATATG CAGTGTGTTA TACAACCCG GAACTGGGAA ACTCAGTTT CATAATTTCC
-940 CTGTAATTGAA TCCTATCTCT AAAAAGCATT TTTCCTAAAA AAAACACACA CACACACACA
-880 CAACACACACATGGTTTT ATGTTACCTC CAGAATGAG ATCTTTTTTT AAGCTTTGCT
-820 GTTAATTGCT CTGGACGTTT TTTAGTAGA GTGGATAGTT AGGTTGCTT CTGTAAGCGA
-760 ACAGCTGGA CTTCAAGCCT TCCCTCTAA ATGAGGAC TGTGCAAGCAT CACACCTGGG
-700 TCAGTAAACA GGTAGGCTCT CTTGTCTTTG CTGAGAAGAT AGATGGGAA GAGACACATC
-660 CAGATGAGAC GCAAGGGAGG

**B**

A 0% Not analyzed 100% B 0% Not analyzed 100%

**C**

| Treatment | Methylation Quantification |
|-----------|-----------------------------|
| Mock      | 0%                          |
| HBV-transient (0.4ug) | 0%                          |
| HBV-transient (0.8ug) | 0%                          |
| HBV-stable | 0%                          |

**D**

| Cond | pCMV-HBV-blue | pCMV-HBV-1.3 |
|------|---------------|---------------|
| MAT1A Luc | * | * |
| Met-CopBlue | * | * |
| Met-CopBlue | * | * |

**E**

| Treatment | Input |
|-----------|-------|
| IgG       | 100%  |
| Dex(-)    | 90%   |
| Dex(+)    | 60%   |

**F**

Lane 1: IgG, Lane 2: HBV, Lane 3: GRE1, Lane 4: GRE2

- Super shift
- Binding shift

Optical Density (%)

Lane 3 (HBV, P3) | Lane 6 (GRE1, P1) | Lane 7 (GRE2, P2)
methylation at the GRE within the MAT1A promoter in hepatoma cells.

**IFN-α Could Restore HBV-suppressed MAT1A Expression through an Antiviral Pathway—**As mentioned above, Dex failed to increase the production of AdoMet in HepG2.2.15, possibly because Dex enhanced the replication of HBV. It was suggested in our previous study that HBV replication can suppress AdoMet production (22). We speculated that the antiviral drug could restore HBV-suppressed MAT1A expression through an antiviral pathway. Therefore, we used IFN-α/H9251 as an antiviral drug to inhibit viral replication in this study, and we investigated the effects of Dex, AdoMet and IFN-α on the expression of MAT1A, HBsAg, and HBeAg in HepG2.2.15 (Fig. 6). The results showed that IFN-α combined with AdoMet could reduce the expression of HBsAg and HBeAg, and induce expression of MAT1A (Fig. 6, A and D). The expression of MAT1A was induced and the expression of HBsAg and HBeAg was repressed when IFN-α was combined with Dex (Fig. 6, B and E). In addition, the expression of MAT1A was significantly induced when Dex and AdoMet were combined with IFN-α (Fig. 6C), and the antiviral effect was enhanced in HepG2.2.15 (Fig. 6F). Interestingly, IFN-α could suppress the expression of HBsAg and HBeAg at a concentration of 2000 IU/ml, and IFN-α could also induce the expression of MAT1A in a concentration-dependent manner (Fig. 7). As shown in Fig. 7A, the protein levels of MAT1A were significantly increased after the

**FIGURE 6. Effect of the combination of IFN-α, AdoMet (SAMe), and Dex on expression of MAT1A, HBsAg, and HBeAg in HepG2.2.15 cells.** A–C, MAT1A protein levels were detected in HepG2.2.15 cells after treatment with AdoMet combined with IFN-α, Dex combined with IFN-α, or AdoMet and Dex combined with IFN-α. The inset shows representative immunoblots of MAT1A with different treatments. D–F, HBsAg and HBeAg were determined by ELISA after treatment with AdoMet combined with IFN-α, Dex combined with IFN-α, or AdoMet and Dex combined with IFN-α in HepG2.2.15 cells. **, p < 0.01, and ***, p < 0.001; #, p < 0.05, and ##, p < 0.01. Shown is a representative result from three independent experiments.

**FIGURE 5. Effect of HBV on the methylation profile of CpGs and competition with the GR for binding to the consensus GRE in the MAT1A promoter.** A, putative GRE-binding sites in the 5′-flanking region of the MAT1A gene are underlined. The human MAT1A-GRE1 and MAT1A-GRE2 were compared with the consensus GRE and the palindromic GRE. B, color of the circles is related to the percent of methylation in each CpG site. C, effect of HBV on the methylation profile of the CpG sites for the MAT1A promoter sequence. D, effect of HBV on the relative luciferase activity of the MAT1A promoter when four CpG sites were mutated in a wild-type pMAT1A-1.4Luc plasmid. *, p < 0.05. E, GR-binding profiles were examined by ChIP assays in HepG2.2.15 cells. Productions of Chip-GRE1, Chip-GRE2, and Chip-HBV were quantified by qPCR. *, p < 0.05. F, analyses of the effect of Dex on the binding of the GR to GRE of HBV (P3), and GRE1 (P1) and GRE2 (P2) of the MAT1A promoter by EMSA. Shown is a representative result from three independent experiments. N.E., nuclear extraction.
treatment with IFN-α/H9251 at 1500 units/ml (1.19 ± 0.03 versus 0.98 ± 0.08, p = 0.014) and 2000 units/ml (1.57 ± 0.23 versus 0.98 ± 0.08, p = 0.013) compared with that after the treatment with IFN-α at 0 units/ml. Interestingly, we observed that IFN-α could not affect the protein expression of MAT1A (Fig. 7), but the combination treatment of IFN-α, AdoMet, and Dex significantly increased the protein expression of MAT1A (Fig. 6) when the concentration of IFN-α was 1000 IU/ml. These findings indicated that the induced expression of MAT1A by IFN-α might be due to the suppression of HBV DNA replication. These results suggested that IFN-α might restore HBV-suppressed MAT1A expression through an antiviral pathway, and Dex-induced increase of AdoMet production might enhance the antiviral effect of IFN-α on HBV.

Dex-induced Increase of AdoMet Production Restored STAT1 Methylation Rather than Phosphorylation—Recent evidence suggests that HBV has evolved strategies to block the nuclear translocation of STAT1 to limit IFN-α-induced cellular antiviral responses (18). Because of the crucial role of STAT1 phosphorylation in IFN-α signaling, we investigated whether Dex and AdoMet could potentially influence the phosphorylation of STAT1 responding to IFN-α in HepG2.2.15 cells. As shown in Fig. 8A, the phosphorylation of STAT1 was decreased by 20.80% (0.40 ± 0.01 versus 0.50 ± 0.02, p = 0.004) after the treatment with IFN-α and Dex compared with that after the treatment with IFN-α alone. The phosphorylation of STAT1 was lower (0.40 ± 0.05 versus 0.50 ± 0.02, p = 0.006) after the treatment with IFN-α, AdoMet, and Dex than that after the treatment with IFN-α alone. However, AdoMet did not enhance the suppression by Dex of the phosphorylation of STAT1 responding to IFN-α (Fig. 8B).

Furthermore, methylation is functionally essential for STAT1, as unmethylated STAT1 can be bound and inactivated by a protein inhibitor of activated STAT1 (PIAS1) (25, 26). We investigated whether AdoMet and Dex could influence the methylation of STAT1 responding to IFN-α in HepG2.2.15. As shown in Fig. 8C, the methylation of STAT1 was effectively induced by AdoMet in a concentration-dependent manner. As shown in Fig. 8D, STAT1 methylation was significantly increased by 1.28-fold (0.55 ± 0.02 versus 0.43 ± 0.02, p <
with IFN-

PRMT1 was knocked down with siPRMT1 (Fig. 9)

methylation was increased by 1.91-fold (0.82

cells. To demonstrate the mechanistic insight into

ratio of pSTAT1/STAT1 with different treatments. STAT1 methylation (immunoprecipitation (IP) with antibody to methyl- and dimethylarginine (MDA), Western blot with anti-STAT1 antibody) was detected by co-IP analysis. STAT1 protein was used as a loading control. C, cells were pretreated with different concentrations (0–1 g/liter) of AdoMet for 16 h, followed by treatment with IFN-α (1000 IU/ml) for 8 h. D, cells were pretreated with or without Dex (100 nM) or/and AdoMet (0.75 g/liter) for 16 h, followed by treatment with IFN-α (1000 IU/ml) for 8 h. The inset shows the ratio of STAT1-met/STAT1 with different treatments. *** p < 0.001; ##, p < 0.01, and ###, p < 0.001. Shown is a representative result from three independent experiments. IB, immunoblot.

**DISCUSSION**

HBV infection is a serious global health problem, with 2 billion people infected worldwide, and 350 million suffering from chronic HBV infection. Currently, treatment with IFN-α is one of the major therapies that have been approved for CHB patients. Conventional use of IFN-α has produced encouraging results, with HBeAg loss rates of 20–50% (27). However, HBV, as a hepatotropic DNA virus, may have a low sensitivity to IFN-induced ISGs and may counteract IFN actions at different levels, including the IFN signal transduction and antiviral functions of ISG products (28). Although the effect of IFN seems indisputable, response rates are unsatisfactory, from a clinical point of view. Pretreatment with GCs is one of the proposed ways to increase the response to IFN-α treatment. The rationale for GC pretreatment therapy stems from an early clinical observation that patients with chronic HBV infection often cleared markers of viral replication following tapering or discontinued GC treatment (7). The exact mechanism underlying the effectiveness of combination regimen has not been fully elucidated.

As a major methyl donor, the availability of AdoMet potentially has profound effects on liver metabolism, and AdoMet synthesis is depressed in chronic liver disease (12). Thus, there has been considerable interest in the utility of AdoMet to ameliorate disease severity (13). Furthermore, hepatocellular injury in cholestasis is frequently associated with glutathione depletion, and thus, AdoMet may help correct this problem (29, 30). These findings suggest that any drug that can increase the steady-state level of AdoMet could offer substantial clinical effects.
benefits for restoring liver function. Recently, studies have shown that AdoMet may improve IFN signaling and antiviral effects (31, 32). GCs strongly up-regulate AdoMet synthetase both in vivo and in vitro (14, 15). Therefore, we speculated that the GC-induced increase of AdoMet production enhances IFN signaling in HBV-infected cells. To confirm our speculation, we investigated the effect of GCs and IFN-α on AdoMet production and MAT1A expression in HepG2.2.15 cells. We found that AdoMet homeostasis was disrupted by pharmacologic concentrations of GCs. AdoMet and the ratio of AdoMet/Ado-Hcy were markedly increased in Dex-treated cells, including normal hepatic L02 cells and HepG2 cells. However, Dex could not induce MAT1A expression, even at a high dose in HepG2.2.15 cells, which may be due to the induction of the expression of HBSAg and HBeAg by promoting the replication of HBV. The expression of HBSAg and HBeAg was repressed with the use of IFN-α at a dose of 2000 IU/ml, which was consistent with previous studies (18–20), and the expression of MAT1A was induced, and AdoMet production was increased in HepG2.2.15 cells. Interestingly, IFN-α can also induce the expression of MAT1A in a concentration-dependent manner, which may be due to IFN-α suppression of HBV DNA replication. These results indicated that GCs could increase antiviral effects by inducing AdoMet production when HBV was effectively suppressed by IFN-α. Furthermore, we observed that HBV suppressed AdoMet production and MAT1A expression induced by Dex. To investigate the mechanism of the transcriptional regulation of the MAT1A gene by Dex, we evaluated the 5’-flanking sequence of the MAT1A gene within 1474 bp upstream of the transcription start site by a transient transfection assay. We found that the GRE in the promoter was an important cis-regulatory element and that the sequence between nt −1474 and −974 of the MAT1A promoter along with two GRE sites (nt −876 to −862 and nt −1022 to −1008) were required for the functional induction of MAT1A expression by Dex. The GR participates in Dex-induced MAT1A expression by being translocated to the nucleus. We observed that GCs facilitated the binding of the GR to the MAT1A promoter in GRE1 (nt −876 to −862) and GRE2 (nt −1022 to −1008). To further verify the role of HBV and GCs in the regulation of MAT1A expression, we studied whether post-transcriptional regulation is involved in HBV-repressed MAT1A mRNA expression induced by GCs. Our results suggested that Dex-induced MAT1A expression was disrupted by HBV, which may be due to HBx recruiting DNMT1 to increase methylation at the putative GRE of the MAT1A promoter. It has been demonstrated that HBx expression increased total DNMT activities by up-regulation of DNMT1, DNMT3A1, and DNMT3A2 and selectively promoted regional hypermethylation of specific tumor suppressor genes leading to regional hypermethylation and global hypomethylation during the formation of HCC (23). HBV inhibited MAT1A expression through Cpg2 and Cpg3 hypermethylation within the MAT1A promoter. Although Cpg3 is not located within the GRE, HBV may affect the methylation status of Cpg3 in a direct or indirect manner, which is the neighbor dependence mechanism (33).

Previous studies have demonstrated that nucleocapsid proteins of HBV could be involved in a deficient IFN-α response (34, 35). The primary and most important signaling pathway activated by IFNs is the JAK-STAT pathway. By binding to type I IFN receptors, IFN-α triggers the oligomerization and tyrosine phosphorylation of the receptors followed by the activation of receptor-associated Janus tyrosine kinase (JAK) (36). Recently, studies have suggested that type I IFNs are critical GC targets for regulating STAT1 activity and may account for the overall effectiveness of GCs in inflammation suppression in a clinically relevant time (37). However, type I IFN receptors were expressed to a much higher extent in HepG2.2.15 cells.
than in HepG2 cells. Therefore, the potential role of STAT1 methylation remains controversial (18). It is thus necessary to further investigate the effect of the GC-induced increase of AdoMet production on the STAT pathway to obtain a more accurate picture. Recent studies have shown that AdoMet can increase the induction of ISGs and the antiviral effects of IFN-\(\alpha\) by increasing STAT1 methylation, possibly affecting STAT1-DNA binding (31). Inhibition of STAT1 methylation is involved in the resistance of hepatitis B virus to IFN-\(\alpha\). HBV infection leads to hypermethylation in the MAT1A promoter and disturbs GR binding to GRE in the MAT1A promoter. In HBV-infected cells not treated with IFN-\(\alpha\), HBV was able to compete with MAT1A for binding to GR at the GRE site. GCs activate HBV replication, which suppresses the expression of MAT1A and production of AdoMet. GCs induced an increase of AdoMet production through a positive feedback loop, which promoted expression of the ISGs and enhanced the antiviral effect of IFN-\(\alpha\) by improving STAT1 methylation rather than phosphorylation.

Our data demonstrated that GCs directly regulated the MAT1A expression in vitro by enhancing the binding of the GR to GRE in the MAT1A promoter. GCs can also activate HBV replication by enhancing the binding of the GR to GRE in the HBV genome. HBV infection leads to hypermethylation in the MAT1A promoter by recruiting DNMT1 and disturbs GR binding to GRE in the MAT1A promoter. Thus, GC-induced AdoMet production and MAT1A expression were disrupted by
HBV through site-specific hypermethylation at GRE sites within the MAT1A promoter and competitive binding with the GR in vitro. However, when HBV replication was effectively suppressed by IFN-α, GCs induced an increase of AdoMet production through a positive feedback loop, which enhanced the antiviral effect of IFN-α by improving arginine methylation of STAT1, rather than phosphorylation (Fig. 10). These findings suggest that combination therapy of GCs, AdoMet, and IFN-α is possibly useful for patients with CHB.

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