

Promyelocytic Leukemia Nuclear Bodies Link the DNA Damage Repair Pathway with Hepatitis B Virus Replication: Implications for Hepatitis B Virus Exacerbation during Chemotherapy and Radiotherapy

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Abstract

The mechanism responsible for hepatitis B virus (HBV) exacerbation during chemotherapy and radiotherapy remains unknown. We investigated whether the activation of DNA repair pathways influences HBV replication. The upregulation of the promyelocytic leukemia (PML) protein and its associated PML nuclear body (PML-NB) by chemotherapy and irradiation-induced DNA repair signaling correlated with the upregulation of HBV pregenomic transcription, HBV-core expression, and HBV DNA replication. The HBV-core protein and HBV DNA localized to PML-NBs, where they associated with PML and histone deacetylase 1 (HDAC1). Chemotherapy and radiotherapy affected the interactions between PML, HBV-core, and HDAC1. The enhanced protein-protein interaction between PML and HBV-core inhibited PML-mediated apoptosis and decreased PML-associated HDAC activity. The reversal of HDAC-mediated repression on the HBV covalently closed circular DNA basal core promoter resulted in the amplification of HBV-core and pregenomic expression. These results suggest that PML in PML-NBs links the DNA damage response with HBV replication and may cooperate with HBV-core and HDAC1 on the HBV covalently closed circular DNA basal core promoter to form a positive feedback loop for HBV exacerbation during chemotherapy and radiotherapy. (Mol Cancer Res 2009;7(10):1672–85)

Introduction

Hepatitis B virus (HBV) infection causes acute and chronic hepatitis and increases the risk for developing hepatocellular carcinoma (HCC). Most of the patients with HBV-related HCC are not candidates for surgery at the time of diagnosis because of liver cirrhosis, vascular invasion, multifocality, and

large tumor size (1). Although the induction of DNA damage is one approach for controlling unresectable tumors, radiotherapy has been unsatisfactory for treating HCC primarily because the cirrhotic liver has a low tolerance to irradiation and radiation-induced HBV reactivation or exacerbation is a major complication in 12% to 43% of patients (2). Chemotherapy is also associated with a risk of HBV reactivation or exacerbation in 38% to 78% of HBV carriers (3, 4). HBV reactivation is defined as the reemergence of HBsAg in HBsAg-negative/anti-HBc-positive patients (reverse seroconversion). HBV exacerbation means more increase in HBV DNA levels compared with baseline levels or an absolute increase in the HBV DNA levels. The mechanism by which HBV is reactivated or exacerbated by DNA-damaging therapies remains unknown.

Previous studies have identified radiotherapy- and chemotherapy-induced DNA damage-dependent focal accumulations of many proteins that form DNA repair foci in the nucleus (5, 6). These proteins have been used to study the signal transduction pathways triggered by DNA damage and aided in the identification of factors involved in DNA repair. DNA damage repair foci are found juxtaposed to the promyelocytic leukemia (PML) nuclear body (NB; ref. 7). PML-NBs are dynamic, intranuclear substructures about 0.2 to 1 μm in diameter that are defined by the presence of the PML protein. Their frequency varies from 2 to 30 per cell, depending on the cell type and status. Many specific regulatory proteins or transcriptional factors have been detected within PML-NBs, either transiently [such as histone deacetylase 1 (HDAC1), CBP, Daxx, p53, and TRADD] or constitutively (such as PML and Sp100; ref. 8). The composition of PML-NBs changes during the cell cycle and depends on cellular responses to interferon treatment, genotoxic stress, chromatin integrity, oncogenic transformation, premature cellular senescence, and viral infection. The PML protein, which is the PML-NB organizer, was first identified as the product of the *PML* gene that fuses with the retinoic acid receptor- α gene (*RARA*) in the t(15;17) translocation of acute PML (9). PML-NBs do not exist in PML knockout cells (10). Posttranslational SUMOylation of PML is essential for PML-NB formation and provides a matrix for recruiting specific proteins to these structures (8, 11). The PML transcript undergoes alternative splicing to generate both nuclear and cytoplasmic isoforms (PML I to VII) that all encode a common RBCC (RING finger, B boxes, and coiled-coil) domain [also termed TRIM (TRiPartite motif)] at the 5' end (12). Most PML proteins accumulate in the PML-NB, but cytoplasmic PML isoforms have been implicated in

Received 3/18/09; revised 7/19/09; accepted 7/21/09; published OnlineFirst 10/6/09.
Grant support: Taiwan National Science Council grants NSC 96-2320-B-368-001 and NSC 97-2320-B-368-001.

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 doi:10.1158/1541-7786.MCR-09-0112

TGF- β signaling (13). The nuclear PML isoforms can associate with transcriptional coactivators, corepressors, or other regulatory proteins in PML-NBs to regulate chromatin dynamics and gene transcription, cell growth and apoptosis, protein degradation and modification, p53 function and DNA repair, and MHC class I expression and immune responses (8-16). Receipt of the DNA damage stimulus initiates a rapid increase in the number and alterations in the size and content of PML-NBs by fission and, subsequently, by upregulation and recruitment of the PML protein and other factors from the nucleoplasm, as has been observed following irradiation and chemotherapy (17, 18). This phenomenon is inhibited by the disruption of DNA damage signal transduction pathways (19), suggesting that PML-NBs are involved in relaying DNA damage signals and promoting DNA repair.

PML-NBs are not only docking sites on damaged DNA but they are also sites for viral genome deposition, transcription, and replication (20-23). It seems to be a general feature of nuclear-replicating DNA viruses that their parental genomes preferentially become associated with PML-NBs and that their initial sites of transcription and DNA replication center development are frequently juxtaposed to PML-NBs. In the early phase of some DNA viral infections, the viral genome adjacent to a PML-NB forms replication compartments that contain DNA repair and host recombination proteins, such as ATM, MRN (MRE11-RAD50-NBS1) complexes, RPA, Rad51, BRCA1, and CHK2, which correlate with the induction of the DNA damage response and participate in cell response to DNA repair. The number of PML-NBs per nucleus increases in the presence of viral genomes. In the late or lytic phase of viral replication, a number of viruses, such as Ebola virus, rabies virus, herpesviruses, adenoviruses, papovaviruses, papillomaviruses, and arenaviruses, have developed genes whose products target PML or PML-NB-associated proteins and subsequently modify or disrupt the structure and biological function of PML-NBs to benefit the viral replicative program and/or to inhibit host defenses (21, 24-26).

Although PML and PML-NBs are involved in DNA damage repair and viral replication, little is known about whether the PML protein in PML-NBs link DNA damage repair to HBV replication during chemotherapy and radiotherapy. This study was designed to determine the interactions between DNA damage/repair, PML/PML-NBs, and HBV reactivation/exacerbation by examining DNA damage repair signal transduction pathways and the HBV replication life cycle. We found that the PML protein and its associated coregulators in PML-NBs relayed the DNA repair signal to inadvertently activate the HBV basal core promoter to enhance HBV pregenomic transcription, HBV-core protein expression, and replication. The results further suggested that a positive feedback interaction between PML and HBV-core on the HBV basal core promoter in PML-NBs might aberrantly amplify the HBV replication response in HBV-infected hepatoma cells during chemotherapy and radiotherapy.

Results

The PML Protein and PML-NBs Link DNA Repair and HBV Replication in Response to the Stimuli of DNA Damage

Chemotherapy and radiotherapy generate different levels of single- and double-strand DNA breaks. HBV replicates through

a cycle involving a partially defective double-strand relaxed circular DNA, a covalently closed circular DNA (cccDNA), and a RNA pregenomic intermediate (27, 28). To address whether chemotherapy- and radiotherapy-mediated activation of DNA repair pathways might inadvertently trigger conversion of relaxed circular DNA to cccDNA to stimulate HBV replication, we first characterized the response of DNA repair signal transduction pathways and HBV replication to double-strand DNA damage in a HBV-expressing human hepatoma cell line, 1.3ES2, carrying a chromosomally integrated 1.3-fold HBV genome and chromatinized long-lived HBV cccDNA episomes (29).

In agreement with previous studies (13, 14), we found that the number of PML-NBs increased rapidly within 30 minutes of irradiation (5 Gy) and reached a peak at 3 hours after etoposide (20 μ mol/L) or doxorubicin (1.5 μ mol/L) treatment, and then declined gradually over 3 days (Fig. 1A). The increase in the number of PML-NBs was followed by an increase in the HBV DNA copy number in the culture medium (Fig. 1B). At these doses of chemotherapy and irradiation, we did not observe cell lysis and apoptotic appearance but cell proliferation was somewhat inhibited (Fig. 1C). The PML-NBs rapidly colocalized with γ -H2AX and BRCA1 (Fig. 2A and B). γ -H2AX is a marker of chromatin containing double-strand DNA damage and BRCA1 signals protein recruitment for homologous DNA repair.

In addition to DNA damage, PML-NBs also responded to viral infection. HBV infection increased the size of PML-NBs and altered the morphology of PML-NBs from small dot-like spots to larger donut-shaped spots (Fig. 2C). Immuno-fluorescence *in situ* hybridization (immuno-FISH) analysis using an anti-PML antibody and a HBV DNA probe showed that most of the PML proteins were deposited around the HBV foci, and some HBV DNA and replicative intermediates were located to the inner periphery of PML-NBs (Fig. 2D). The alterations in the number, location, size, and morphology of PML-NBs in response to HBV infection paralleled the alterations to PML-NBs in response to DNA damage (Fig. 2E). Two days after exposure of 1.3ES2 cells to etoposide or irradiation, the number of various-sized PML-NBs and the amount of HBV DNA/replicative intermediates in the nucleus were markedly increased. The levels of PML/HBV colocalized foci relative to control (no DNA damage induction) were about 2-fold increased when cells were etoposide-treated or irradiated. Measuring fluorescence recovery after photobleaching (FRAP), we also observed faster motility of the PML protein depositing around the HBV foci at PML-NBs and a 10% increase in the maximum PML fluorescence recovery in etoposide-treated or irradiated cells compared with control (Fig. 2F). Thus, it is possible that the recruitment of DNA repair proteins to the HBV replication compartment in and around PML-NBs during chemotherapy and radiotherapy is a mechanism by which the DNA damage signal triggers HBV replication.

Next, we tested whether blocking the DNA repair signal transduction pathways would affect how PML-NBs and HBV replication respond to DNA damage. DNA-dependent protein kinase (DNA-PK), ATR, and ATM are immediate DNA-damage signal sensors; CHK2 kinase is a midpathway DNA-damage signal transducer; and PML is a downstream DNA damage signal effector that regulates p53 (30). We used caffeine to inhibit both ATR and ATM kinases, wortmannin to inhibit DNA-PK

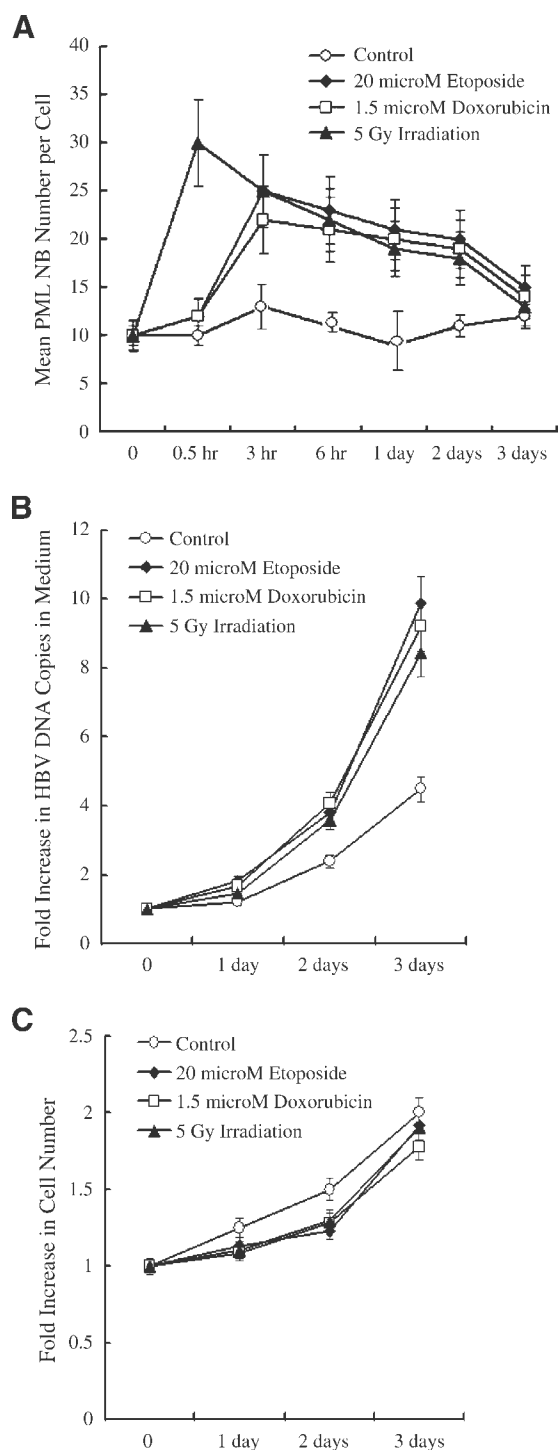


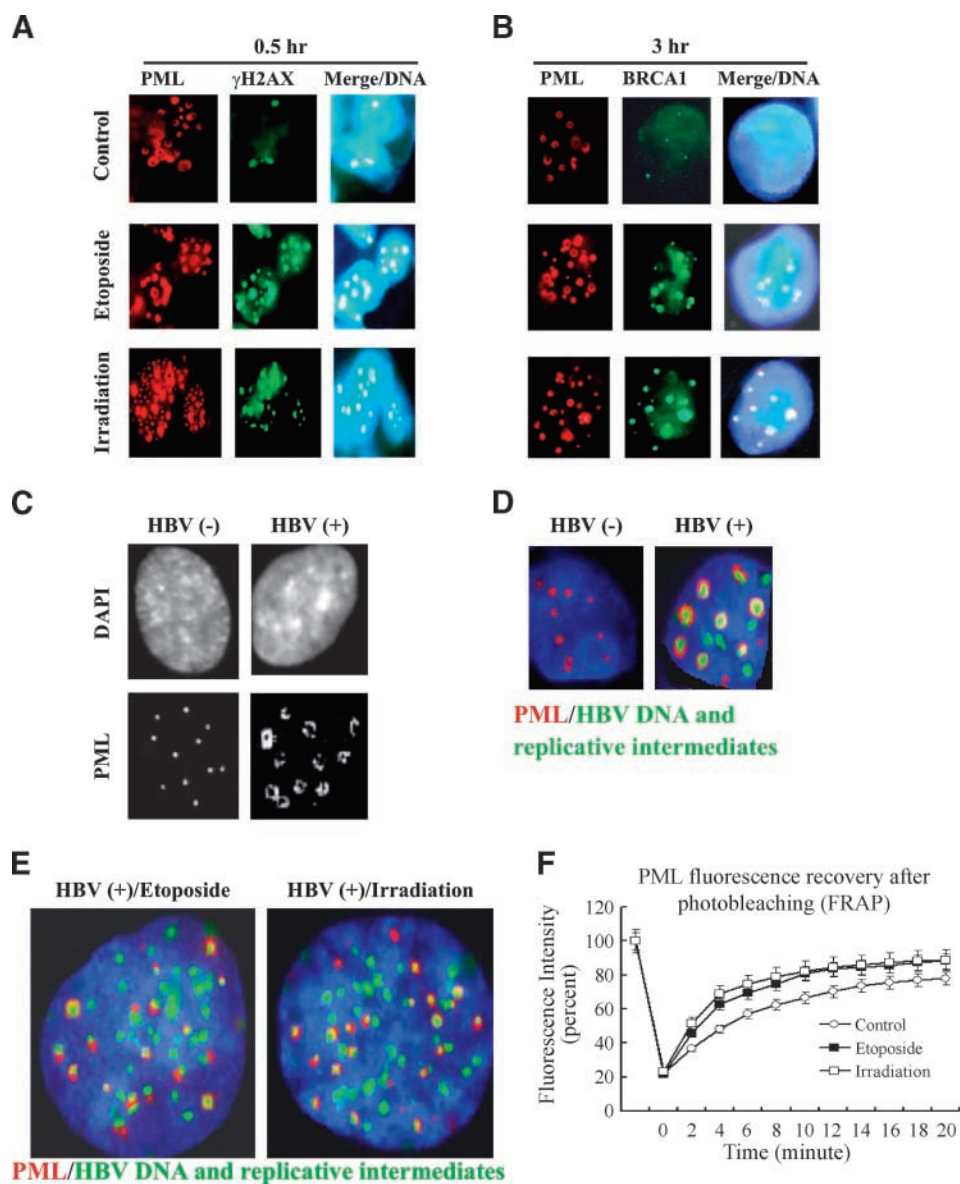
FIGURE 1. Correlation between the increased number of PML-NBs and the increased HBV DNA copy number in response to chemotherapy and irradiation-induced DNA damage. Human hepatoma 1.3ES2 cells were treated with etoposide (20 $\mu\text{mol/L}$) or doxorubicin (1.5 $\mu\text{mol/L}$) for 30 min or irradiation (5 Gy) in one fraction to induce DNA damage. **A.** Increases in the number of PML-NBs in individual cells over time after DNA damage. To reflect the dynamic change of the number of PML-NBs with cell cycle, PML-NBs were counted in at least 50 cells in the same area per time point regardless of cell cycle distribution. **B.** Increases in the number of HBV DNA molecules secreted into the medium over time after DNA damage. **C.** Cell growth curves following DNA damage. Cell number, viability, and lysis were determined by the trypan blue exclusion method.

and ATM kinase, and CHK2 kinase inhibitor II to inhibit CHK2 kinase. To avoid any pharmacologic interference on the activity of nontarget kinases, we treated cells with small interfering RNAs (siRNA) of ATR, ATM, CHK2, PML, or negative control (a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA). Cells were pretreated with the various kinase inhibitors 30 minutes before or were transfected with the siRNAs 12 hours before induction of DNA damage by irradiation (5 Gy) or etoposide (20 $\mu\text{mol/L}$ for 30 minutes). The effects of DNA repair inhibition with drugs or siRNAs on the persistence of irradiation- or etoposide-induced double-strand breaks were assessed in cells by microscopy of $\gamma\text{-H2AX}$ focus formation (a marker of DNA double-strand break; Fig. 3A). By 24 hours after irradiation or etoposide treatment, the number of $\gamma\text{-H2AX}$ foci was the same as for control samples (no etoposide and irradiation), consistent with complete repair of double-strand breaks. In contrast, the number of prolonged $\gamma\text{-H2AX}$ focus formation relative to the etoposide-treated control (no drug or siRNA added) were markedly increased when cells were etoposide-treated after exposure to caffeine; wortmannin; CHK2 inhibitor II; or siRNAs of ATR, ATM, CHK2, and PML. With the exception of the siRNA of PML, the number of PML-NBs per cell and the number of HBV DNA molecules in the medium did not change when cells were pretreated with the kinase inhibitors or transfected with the siRNAs (Fig. 3B and C). During pharmacologic inhibition of the DNA repair signaling pathways, the kinase inhibitors were removed 18 hours after induction of DNA damage to prevent inhibition of cell proliferation from prolonged drug treatment. However, during gene-targeted inhibition, the siRNAs, which suppressed their targeted gene expression efficiently but had no effects on cell proliferation and apoptosis when compared with the controls, were not removed to maintain optimal blockage of the signaling pathway (Fig. 3C and D). Transient pharmacologic inhibition of ATR, ATM, or CHK2 kinases had short-term inhibitory effects on the etoposide-induced increases in the number of PML-NBs and HBV DNA molecules in the medium. In the presence of targeted siRNAs, we observed sustained suppression of the PML-NB response and HBV replication. Among the various kinase inhibitor and siRNA treatments, PML suppression seemed to have the greatest inhibitory effect on HBV replication.

PML Affects HBV Transcription, Expression, and Replication at the HBV-Specific Basal Core Promoter

Based on the implicated involvement of PML-NBs in both DNA repair and viral DNA replication, we tested whether the level of PML expression essential for PML-NB formation influenced HBV transcription, expression, and replication. RNA interference analysis revealed that repeated dosing of the PML siRNA every 24 hours was necessary to maintain suppression of PML in 1.3ES2 cells. The persistent suppression of PML had no effect on cell proliferation but reduced both HBV precore/pregenomic mRNA and replicative intermediates (Fig. 4A and B). After withdrawal of PML siRNA, the amount of precore/pregenomic and PML RNA transcripts and HBV replicative intermediates increased in parallel. PML suppression also decreased the amount of HBV DNA secreted into the medium; after withdrawal of the PML siRNA,

FIGURE 2. The structure and dynamics of PML-NBs are coupled to DNA damage, DNA repair, and HBV replication. **A.** In 1.3ES2 cells, the PML protein accumulates in ~10 PML-NBs per cell. Immunofluorescence analysis shows the increase in the number of PML-NBs (*red*), the increase in γ -H2AX (*green*), and their colocalization (*yellow*) 30 min after etoposide or irradiation treatment. **B.** Immunofluorescence analysis shows the increase in the number of PML-NBs (*red*), the increase in BRCA1 (*green*), and their colocalization (*yellow*) 3 h after etoposide or irradiation treatment. **C.** PML-NB morphology in HBV-negative HepG2 (parental cells of 1.3ES2 cells) and HBV-positive 1.3ES2 cells. During HBV infection the dot-like PML-NBs become donut-shaped. **D.** Immuno-FISH shows that HBV DNA and replicative intermediates (*green*) are juxtaposed to the donut-shaped PML-NBs (*red*) at the inner periphery of the nucleus. The cells shown are representative of the majority of the cells in the population. **E.** Immuno-FISH shows that the number of PML (*red*)/HBV (*green*) colocalizing foci (*yellow*) and the amount of HBV DNA/replicative intermediates (*green*) were increased in the nucleus, and various-sized PML-NBs were induced in a representative 1.3ES2 cell 2 d after exposure to etoposide or irradiation. **F.** Quantification by FRAP for dynamics of PML deposit around the HBV foci at PML-NBs in 1.3ES2 cells 2 d after exposure to etoposide or irradiation. Data are presented as the mean values (\pm SD; from at least 20 FRAP experiments each) of fluorescence recovery plotted as percent of initial fluorescence intensity of the PML-NBs over 20 min.



the amount of HBV DNA in the medium increased (Fig. 4C). However, unlike the significant decline in the number of HBV DNA molecules in the medium, the amount of secreted proteins HBsAg and HBeAg in the culture medium was not affected by PML suppression, as determined by ELISA. In contrast, Western blot analysis showed that PML suppression decreased expression of the HBV-core protein efficiently (Fig. 4D). These results indicate that PML suppression inhibited HBV precore/pregenomic transcription and genome replication and differentially regulated HBV-core, HBeAg, and HBsAg viral protein expression. The precore transcript encoding HBeAg is almost identical to the pregenomic transcript encoding HBV-core and polymerase and is only 31 bases longer than the pregenomic RNA at the 5' end (31, 32). However, only the pregenomic RNA can be used as a template for packaging and reverse transcription to form new viral DNA capsids and to generate new DNA genomes. Because expression of HBV-core protein, but

not HBeAg, was dramatically decreased by suppression of PML, it was likely that HBV pregenomic RNA was inhibited more profoundly than was precore RNA. Thus, PML suppression might decrease HBV-core expression and HBV DNA replication through selective reduction of HBV pregenomic transcription while sparing precore transcription and HBeAg and HBsAg expression.

Next, we examined how overexpression of PML affected HBV transcription, expression, and replication. The initiation of pregenomic RNA transcription is naturally controlled by the HBV basal core promoter in infected cells (28, 31, 32). Either of the two full-length HBV-related plasmids in which the transcription of pregenomic RNA was driven by the cytomegalovirus (CMV) promoter or the HBV basal core promoter were cotransfected with a CMV promoter-driven *PML* plasmid into HBV-negative HepG2 cells (parental cells of 1.3ES2 cells; Fig. 4E). Although the basal activity of the CMV promoter was

stronger than the basal activity of HBV basal core promoter in driving HBV transcription after a 48-hour transfection, over-expression of exogenous PML enhanced HBV pregenomic transcription, HBV-core expression, and cccDNA replication in cells transfected with the HBV basal core promoter-driven HBV plasmid but not in the cells transfected with the CMV promoter-driven HBV plasmid. These results indicate that the influence of PML on HBV transcription, expression, and

replication is mediated specifically through the HBV basal core promoter.

HBV-Core Targets PML in PML-NBs: Interactions among HBV, PML, and the DNA Damage Response

The close association between HBV genomes and PML-NBs raises several questions: What viral factors other than the genomes themselves are involved in this association? Are

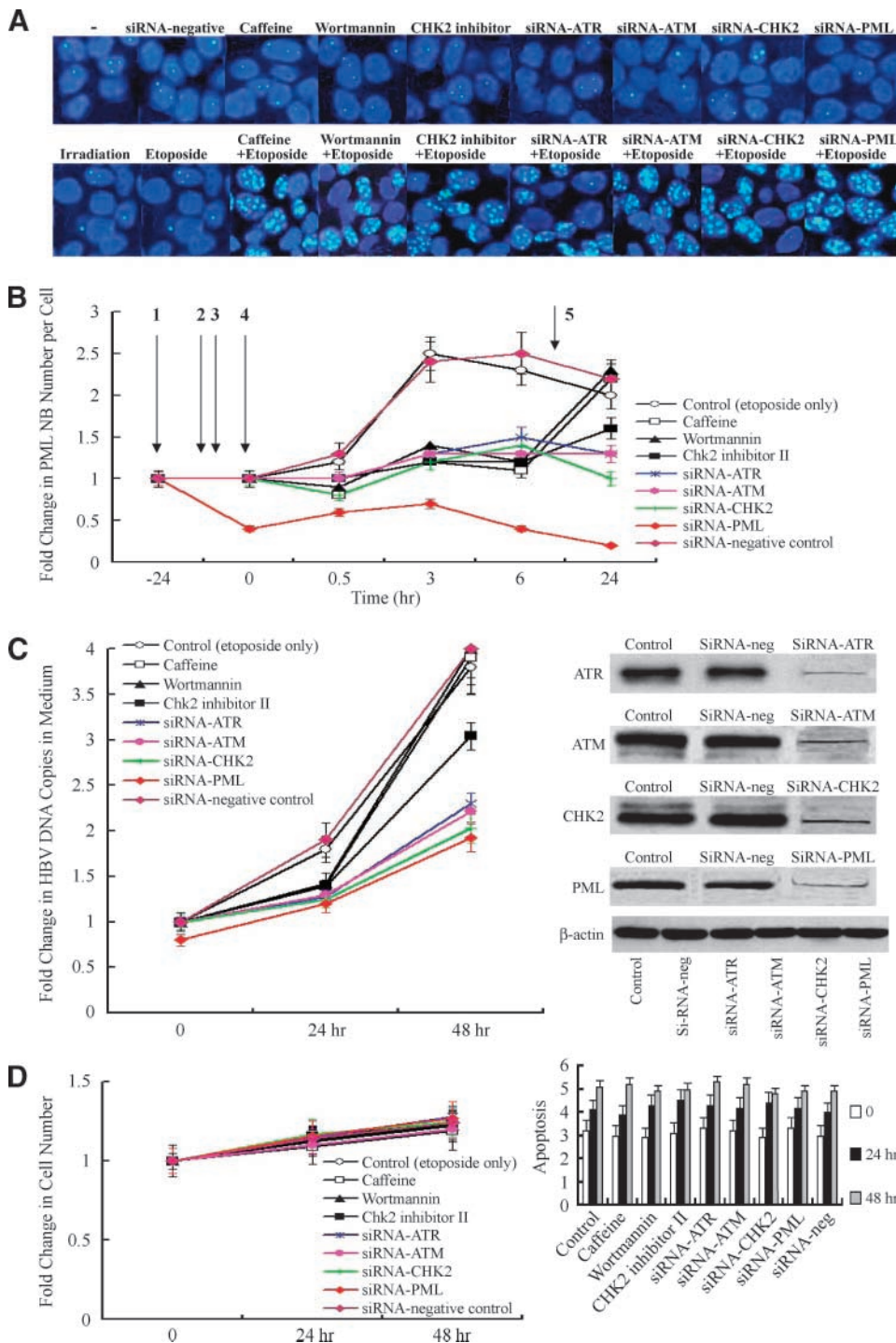
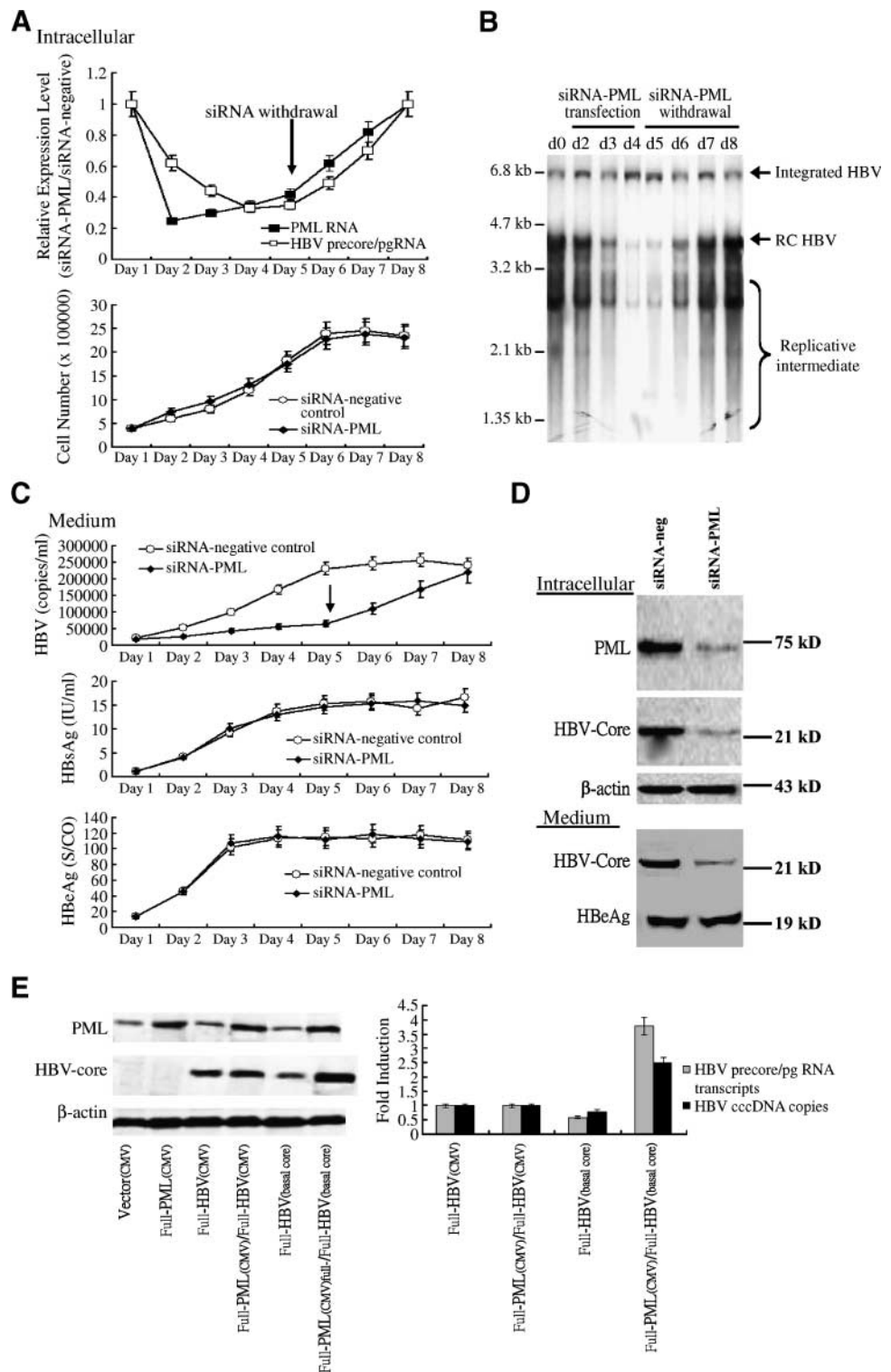


FIGURE 3. The DNA damage-induced increases in the number of PML-NBs and the number of HBV DNA molecules are inhibited by disruptions in the DNA damage repair signal transduction pathways. 1.3ES2 cells were pretreated with various kinase inhibitors (5 mmol/L caffeine, 20 μ mol/L wortmannin, or 10 μ mol/L CHK2 inhibitor II) or transfected with siRNAs of ATR, ATM, CHK2, PML, or siRNA-negative control to interfere with the DNA repair signal transduction pathways before treatment with irradiation (5 Gy) or etoposide (20 μ mol/L) for 30 min. **A.** Confocal microscopy of γ -H2AX focus formation in 1.3ES2 cells at 24 h after different treatments. Inhibition of the DNA damage repair signal transduction pathway maintains the double-strand DNA breaks induced by etoposide. **B.** The numbers 1, 2, 3, 4, and 5 indicate the times for cell seeding, siRNA transfection, addition of kinase inhibitors, etoposide treatment, and removal of kinase inhibitors, respectively. Inhibiting DNA repair signaling pathways with kinase inhibitors and siRNAs affects the DNA damage-induced increase in the number of PML-NBs. **C.** Inhibiting DNA repair signaling pathways with kinase inhibitors and siRNAs affects the DNA damage-induced increase in the amount of HBV DNA secreted into the medium. One day after transfection, cell lysates were harvested and analyzed by Western blot analysis using antibodies specific for ATR, ATM, CHK2, PML, and β -actin. **D.** Cell growth and apoptosis following treatment with etoposide only, etoposide plus various kinase inhibitors, and etoposide plus different siRNAs. Cell number, viability, and lysis were determined by the trypan blue exclusion method. Apoptosis was quantified by measuring the percentage of cells with subdiploid DNA content. Data (mean \pm SD) from three independent experiments are shown.

FIGURE 4. PML influences HBV transcription, expression, and replication through the HBV basal core promoter. **A.** PML siRNA was transfected into 1.3ES2 cells every 24 h after plating (day 0) to maintain suppression of PML expression; PML siRNA was withdrawn after day 5. Real-time reverse transcriptase-PCR was used to determine the correlation between PML expression and HBV transcription. Suppression of PML by repeated siRNA dosing did not affect cell growth. **B.** Total DNA was extracted from 1.3ES2 cells repeatedly transfected with PML siRNA or with PML siRNA withdrawn at the indicated times. After *Hind*III digestion, the integrated HBV genome, the partial double-strand relaxed circular (RC) HBV DNA, and the replicative intermediates were detected by Southern analysis using a HBV-specific probe. **C.** After PML siRNA was transfected into or removed from 1.3ES2 cells, the amounts of HBV DNA, HBsAg, and HBeAg secreted into the culture medium were measured at the indicated times. Black arrow, the siRNA withdrawal time on day 5. **D.** On day 4, the culture medium and cell lysates from 1.3ES2 cells with or without PML siRNA were harvested. Equal amounts of the cell lysates were subjected to Western blot analysis using antibodies specific for PML, HBV-core, and β -actin. Equal amounts of the culture medium were treated with immunoprecipitating antibodies specific for HBV-core; the immunoprecipitates were subjected to Western blot analysis to compare the levels of HBV-core and HBeAg simultaneously. **E.** HBV-negative HepG2 cells were cotransfected with the CMV promoter-driven full-length HBV genome or the HBV basal core promoter-driven full-length HBV genome with or without the CMV promoter-driven full-length *PML*. Two days after transfection, cell lysates were harvested for Western blot analysis. Total RNA and HBV cccDNA were extracted for quantification by real-time PCR.



PML isoforms and/or other PML-NB-associated proteins involved in the interaction? What are the functional consequences of this interaction? PML has been shown to regulate gene expression and induce apoptosis by associating with transcriptional coregulators and apoptosis-related proteins in PML-NBs (8,

12, 14). Among the PML-NB-associated regulatory proteins, the cellular acetyltransferase CBP and the class I HDAC1 are the best known coregulators of gene transcription. Histone acetylation by CBP and deacetylation by HDAC1 remodel chromatin to modulate the access of transcriptional factors to

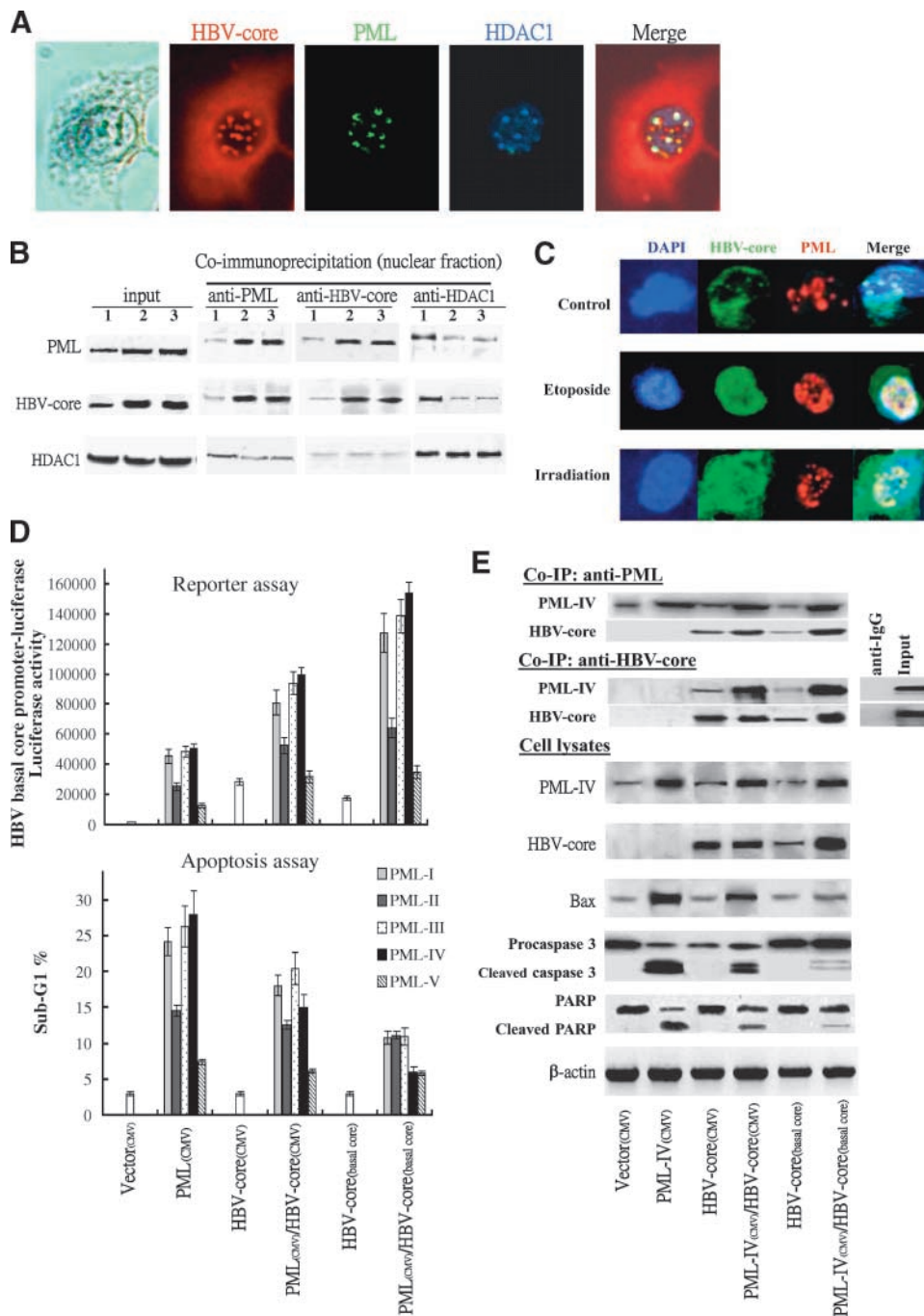


FIGURE 5. The HBV-core protein interacts with PML and HDAC1 in PML-NBs and affects PML activity in gene transcription and induction of apoptosis. **A.** Immunofluorescence analysis shows the colocalization of HBV-core (red), PML (green), and HDAC1 (blue) in PML-NBs in 1.3ES2 cells. **B.** 1.3ES2 cells were treated with etoposide (20 μmol/L) for 30 min or irradiation (5 Gy) in one fraction; 24 h later, the cell lysates were harvested and subjected to immunoprecipitation using antibodies specific for PML, HBV-core, or HDAC1, as indicated. Immunoprecipitates were analyzed for coprecipitation by Western blot. 1, control; 2, etoposide treatment; 3, irradiation. **C.** Immunofluorescence analysis shows the increased colocalization (yellow) of HBV-core (green) and PML (red) after etoposide treatment or irradiation. **D.** HepG2 cells were cotransfected with the indicated CMV promoter-driven PML isoform (I, II, III, IV, or V; 1 μg) and the CMV-promoter-driven or HBV basal core promoter-driven HBV-core (1 μg) expression vectors or empty vector along with a luciferase reporter gene driven by the HBV basal core promoter. Two days after transfection, cell lysates were harvested and analyzed for luciferase activity. Apoptosis was quantified by measuring the percentage of cells with subdiploid DNA content. Data (mean ± SD) from three independent experiments are shown. **E.** Cell lysates with PML-IV transfection were also subjected to coimmunoprecipitation using antibodies specific for PML and HBV-core and Western blot analysis using antibodies specific for PML, HBV-core, Bax, caspase-3, poly(ADP-ribose) polymerase, and β-actin.

promoters for activating and repressing gene transcription, respectively. Some viral factors, such as HBV-core protein, have been shown to bind to the HBV basal core promoter to initiate HBV transcription and replication (27).

We assessed the subcellular localization and the spatial association of PML, viral factors, and PML-NB-associated coregulators by immunofluorescent staining and coimmunoprecipitation. As shown in Fig. 5A, HBV-core protein was detected predominantly in the cytosol, but a small fraction

of HBV-core protein colocalized with PML protein and HDAC1 in the PML-NBs in the nucleus. PML coprecipitated from nuclear extracts with HBV-core protein as well as with HDAC1 (Fig. 5B). Reciprocal immunoprecipitation substantiated the specific coimmunoprecipitation of PML, HBV-core protein, and HDAC1. Chemotherapy and irradiation seemed to affect the interactions between PML, HBV-core, and HDAC1. After chemotherapy or irradiation, we noted that the PML and HBV-core expression was upregulated,

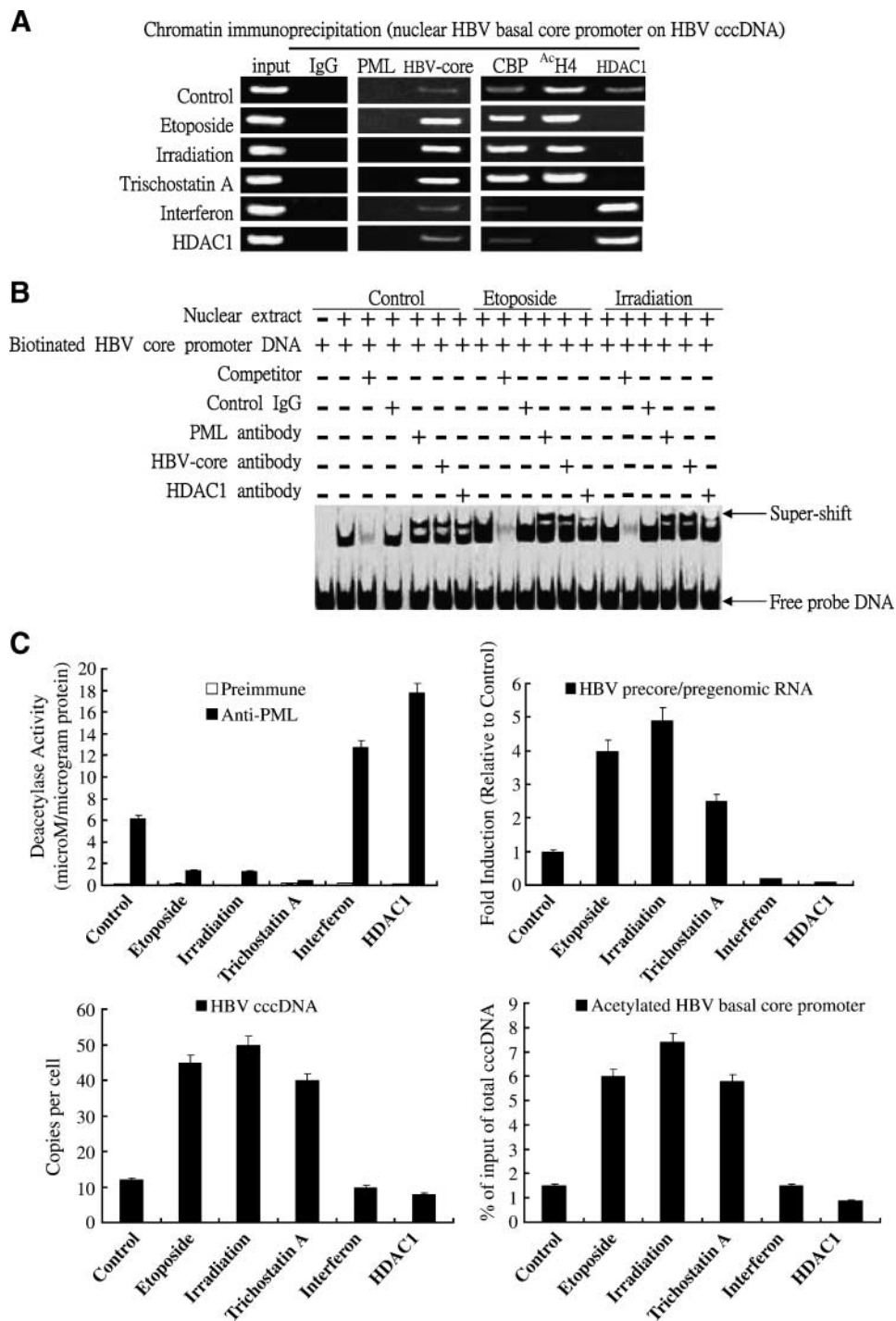


FIGURE 6. The levels of HBV transcription and replication correlate with PML-associated HDAC activity and the acetylation status of HBV cccDNA basal core promoter-bound H4 histones. **A.** Chromatin immunoprecipitation of 1.3ES2 cells treated with etoposide (20 μ M/L) for 30 min, irradiation (5 Gy) in one fraction, trichostatin A (300 nmol/L) for 24 h, interferon- α (1000 u/mL) for 24 h, or transfection with HDAC1 for 2 d was done by using antibodies specific for PML, HBV-core, CBP, acetylated-H4, or control IgG. Specific primers were used to selectively amplify the HBV basal core promoter of HBV cccDNA. **B.** The electrophoretic mobility gel shift assay shows the binding of nuclear proteins obtained from control (untreated), etoposide-treated, or irradiated cells to labeled oligonucleotides corresponding to the HBV basal core promoter sequences, which is abolished in the presence of a 200-fold excess of unlabeled oligonucleotides (competitor). Gel supershift appears in the presence of specific anti-PML, anti-HBV-core, or anti-HDAC1 antibodies but not with control IgG. **C.** The cell lysates from the treated cells were subjected to immunoprecipitation with an anti-PML antibody or preimmune serum. The immunoprecipitates were used for deacetylase assay. Total RNA was extracted for quantitative real-time PCR to determine whether there is a correlation between PML-associated HDAC activity and HBV pregenomic transcription. **D.** The HBV cccDNA accumulation in 1.3ES2 cells subjected to various treatments was assessed by quantitative real-time PCR and is expressed as the number of cccDNA copies per cell (mean \pm SD) from three independent experiments. Quantitative evaluation of the percentage of acetylated-H4-bound HBV basal core promoters in total HBV cccDNA for each experimental condition was done by real-time PCR on the anti-acetylated H4-immunoprecipitated chromatin using primers specific for the HBV basal core promoter. Input refers to the starting amount of cccDNA isolated for each experimental condition.

more HBV-core proteins and PML-NBs appeared in the nucleus, and the association of PML with HBV-core protein in the nucleus was increased (Fig. 5B and C); however, the fraction of HDAC1 in the PML–HBV-core protein complex decreased. These findings imply that chemotherapy and radiotherapy increase or enhance the interaction between PML and HBV-core and titrate or diminish the association between PML and HDAC1.

To define the functional relevance of the increased protein-protein interaction between PML and HBV-core, we asked whether the interaction had consequences for PML-associated biological functions, including gene transcription and induction of apoptosis. The CMV promoter–driven *PML-I*, *PML-II*, *PML-III*, *PML-IV*, and *PML-V* isoforms, respectively, were cotransfected with the CMV promoter–driven *HBV-core* or the HBV basal core promoter–driven *HBV-core* plasmid along with a luciferase reporter gene construct controlled by the HBV basal core promoter (Fig. 5D). All five PML isoforms induced HBV basal core promoter activity, which was further enhanced in the copresence of HBV-core. The cooperative effect of PML and HBV-core on the HBV basal core promoter activity was also reflected by higher luciferase activity in the cells transfected with the HBV basal core promoter–driven *HBV-core* than in the cells transfected with the CMV promoter–driven *HBV-core*. On the other hand, all five PML isoforms were functional as apoptosis inducers but they differed in their efficiency to induce apoptosis. Overexpression of exogenous PML-IV in HepG2 cells induced the highest apoptosis rate of 28%, according to subdiploid DNA content. Cotransfection of *PML-IV* and *HBV-core* expression plasmids driven by the same CMV promoter resulted in the production of similar amounts of PML-IV and HBV-core (Fig. 5E); as a result, the rate of PML-induced apoptosis decreased from 28% to 15%. In contrast, if the CMV promoter–driven *PML-IV* was cotransfected with the HBV basal core promoter–driven *HBV-core*, the expression amount of HBV-core was greater than that of PML and the rate of PML-mediated apoptosis was strongly impaired, reaching only 6%. Moreover, upregulation of the proapoptotic Bax protein, activation of procaspase-3, and cleavage of poly(ADP-ribose) polymerase by PML-IV overexpression–induced apoptosis were markedly decreased in the cells transfected with the HBV basal core promoter–driven *HBV-core*, which induced more interactions (coprecipitations) between HBV-core and PML-IV (Fig. 5E). These findings indicate that the interaction of HBV-core with PML inhibits PML-mediated apoptosis and enhances HBV basal core promoter transcriptional activity.

PML-Associated HDAC Activity Regulates HBV Pre-genomic Transcription: The Influence of the Host Transcriptional Machinery on the HBV cccDNA Basal Core Promoter following Chemotherapy and Radiotherapy

We then investigated whether and how the level of HBV pre-genomic transcription from the cccDNA in the nucleus was potentiated by the increase in PML protein and PML-NBs following chemotherapy and irradiation.

Based on the dynamic interaction between PML and HDAC1, we hypothesized that PML could regulate viral chromatin for transcription by association or dissociation with

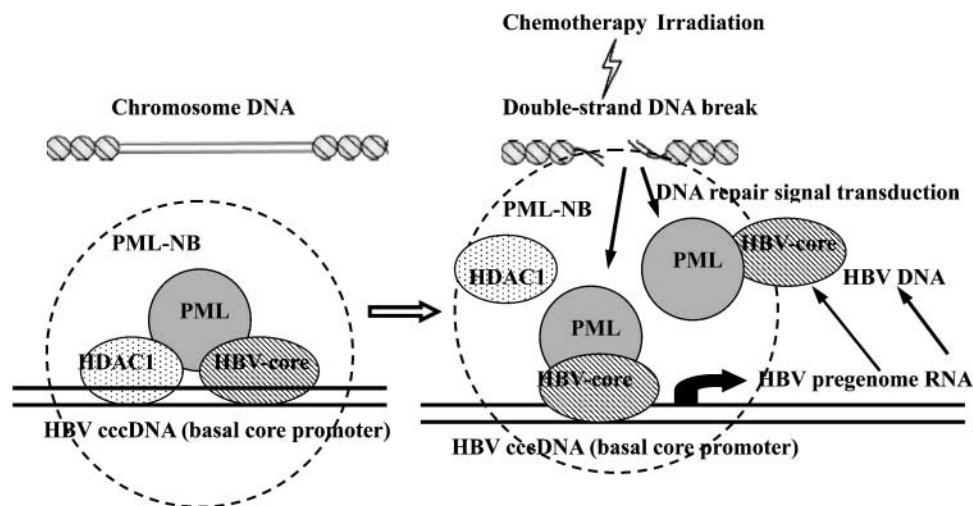
HDAC1. We performed chromatin immunoprecipitation assays in 1.3ES2 cells treated with chemotherapy or irradiation using antibodies specific for PML, HBV-core, CBP (a histone acetyltransferase and transcriptional coactivator), HDAC1, and acetylated H4 to immunoprecipitate formaldehyde cross-linked nuclear chromatin from the HBV cccDNA basal core promoter. The immunoprecipitated cccDNA was treated with nuclease and the HBV basal core promoter region was amplified using PCR primers that could discriminate between nuclear HBV cccDNA, both integrated linear HBV sequences, and HBV relaxed circular DNA (27). We found increased binding of HBV-core protein, CBP, and acetylated H4 but decreased binding of HDAC1 to the basal core promoter of HBV cccDNA following chemotherapy and irradiation, although PML did not bind directly to the basal core promoter (Fig. 6A). However, by the electrophoretic mobility gel shift assay, PML was noted to associate with the HBV basal core promoter in the presence of HBV-core and HDAC1, and even with more other nuclear proteins after etoposide treatment or irradiation (Fig. 6B). Moreover, the supershift assay using anti-PML, anti-HBV-core, and anti-HDAC antibodies showed more binding of PML and HBV-core but less binding of HDAC1 to the HBV basal core promoter upon etoposide treatment or irradiation. Consistently, PML coimmunoprecipitated a significant amount of HDAC activity in 1.3ES2 cells but that the PML-associated HDAC activity was decreased in cells treated with chemotherapy or irradiation (Fig. 6C). The PML-associated HDAC activity was inversely correlated with HBV pre-genomic transcription. Trichostatin A, which inhibits HDAC (27), and interferon- α , which increases HDAC activity (33), were used to verify the effect of HDAC1 on the cccDNA basal core promoter.

Real-time PCR assays were done to quantify the number of HBV cccDNA molecules per cell and the percentage of acetylated HBV basal core promoters in the total cccDNA (Fig. 6D). Both chemotherapy and irradiation resulted in a 4- to 5-fold increase in HBV cccDNA accumulation and in a 6- to 7-fold increase in the cccDNA basal core promoter–bound acetylated histone H4. The effects of chemotherapy and irradiation were similar to those of trichostatin A, a HDAC inhibitor. In contrast, the antiviral effect of interferon was correlated with high PML-associated HDAC activity. These results indicate that chemotherapy and radiotherapy reduce PML-associated HDAC activity and reverse HDAC-mediated transcriptional repression on the HBV basal core promoter of the HBV genome, which may account for the amplified HBV response during chemotherapy and radiotherapy.

Discussion

Previous studies have suggested that systemic chemotherapy might suppress the immune system, leading to enhanced HBV replication and HBV exacerbation. However, conformational delivery of radiation to a discrete part of the liver in an effort to prevent immunosuppression still induces HBV reactivation and exacerbation in a significant portion of patients. Thus, other mechanisms for HBV reactivation or exacerbation by anticancer treatment must be involved. It is possible that chemotherapy and radiotherapy activate HBV

FIGURE 7. Model of chemotherapy and radiation-induced exacerbation of HBV. PML establishes a supramolecular scaffold (PML-NB) in the nucleus for dynamically interacting, associating, or dissociating with viral and host proteins and different gene transcriptional coregulator (such as HDAC1) upon viral infection or DNA damage. PML in PML-NBs links the DNA damage repair signaling pathways with HBV replication cycle. A positive feedback loop for HBV exacerbation may involve PML, HBV-core, HDAC1, and HBV pregenomic RNA.



replication directly in infected hepatocytes and/or indirectly by stimulating cytokine secretion from neighboring nonhepatocytes (34). Because HBV exacerbation is correlated with elevated aspartate aminotransferase/alanine aminotransferase liver enzymes, hepatocytes could be the major target. In this study, we found that direct exposure of HBV-expressing human hepatoma 1.3ES2 cells to doxorubicin, etoposide, or irradiation increased HBV pregenomic transcription, HBV-core expression, and HBV DNA replication.

Chemotherapy drugs such as doxorubicin and etoposide, and radiotherapy using high-energy ionizing photon beams, kill proliferating cells efficiently by causing different levels of single- and double-strand DNA damage, which in turn triggers recruitment of the MRN complexes (sensors) that activate ATM, ATR, and DNA-PK, and phosphorylate γ H2AX around the sites of the DNA breaks (5, 6, 30). ATM and ATR phosphorylate CHK1 and CHK2 (signal transducers), which subsequently phosphorylate a number of distinct downstream targets, BRCA1, E2F1, and PML (effectors) in PML-NBs, resulting in DNA repair, cell cycle arrest, senescence, or apoptosis. We uncovered a novel link between the activation of DNA damage repair pathways and initiation of the HBV replication cycle: the PML protein and its associated PML-NBs (Fig. 7). Consistent with results from other studies conducted in different cell systems and with various viral infections (17-26), PML-NBs in hepatoma cells behave as dynamic macromolecular nuclear structures and alter their number, size, and content in response to DNA damage and HBV infection. The association of PML-NB structures with HBV genomes and the recruitment of DNA repair proteins into viral replication compartments have many parallels. Following chemotherapy and radiotherapy, immuno-FISH, indirect immunofluorescence, coimmunoprecipitation, and chromatin immunoprecipitation all showed that the induction of extensive contact between PML, γ -H2AX, BRCA1, HDAC1, HBV-core, and HBV DNA occurs on the newly formed PML-NBs. Further, we showed that PML in PML-NBs relays the DNA damage signals that initiate HBV pregenomic transcription of the HBV-core protein and enhance

HBV replication. In turn, the HBV-core protein targets and interacts with the activated PML protein in PML-NBs not only to inhibit PML-mediated apoptosis but also to decrease the PML-associated HDAC activity, thereby preventing apoptosis and further enhancing the HBV basal core promoter activity and increasing HBV pregenomic production. Thus, PML, HBV-core, and HDAC form a positive feedback loop for HBV pregenomic transcription on the HBV basal core promoter in PML-NBs, thereby enhancing HBV DNA replication.

Although we could not find PML bound directly to the HBV basal core promoter, we show that PML in PML-NBs interacted with the HBV-core and HDAC1 proteins that both bound to the HBV basal core promoter. Because PML has been found to recruit specific proteins through posttranscriptional SUMOylation in PML-NBs (the covalent linkage of the small ubiquitin-related modifier to lysines 65, 160, and 490 of PML; refs. 8, 11, 12), the formation of PML-NBs may provide a scaffold or matrix for cellular protein-viral DNA interaction. Moreover, we found that PML selectively affects HBV-core expression at the transcriptional level through the basal core promoter but has no effect on HBeAg and HBsAg expression. The 3.2-kb HBV genome can be unidirectionally transcribed by four promoters: (a) basal core, to produce precore (encoding HBeAg) and pregenomic (encoding HBV-core and polymerase) transcripts; (b) SPI (encoding large HBsAg); (c) SPII (encoding middle and small HBsAg); and (d) X (encoding X protein; refs. 28, 31, 32). Previous studies have shown that although each of the precore and pregenomic transcripts is produced by the same basal core promoter, they can be regulated coordinately or differentially. Detailed analysis of the basal core promoter and the upstream regulatory sequence has revealed that the promoters driving HBeAg and HBV-core expression overlap physically but can function independently (32).

Given that PML-NBs are associated with the chromatin modifiers BRCA1, HDAC, the Bloom's syndrome DNA helicase BLM, and the Swi/Snf family member ATRX (35, 36), it is not surprising that PML-NBs are involved in both DNA damage repair and the efficiency of cellular and viral gene

transcription. Although our study shows that the induction of PML and PML-NBs and the increased interaction of HBV-core with PML in PML-NBs correlate with enhanced HBV transcription, expression, and replication, PML and PML-NBs have been associated with gene transcription and viral DNA replication in both positive and negative ways (21). The negative effect of PML and PML-NBs on viral gene expression and replication is supported by evidence showing that the immediate-early protein IE1 of the human CMV and the regulatory protein ICP0 of the herpes simplex virus type 1 target PML to disrupt PML-NBs and activate viral gene expression and replication. In contrast, the positive effect of PML and PML-NBs on viral gene expression and replication is supported by evidence showing that PML-NBs allow for efficient human polyomavirus JC propagation by providing scaffolds and that PML-NBs store and release factors required for efficient viral gene expression. Thus, the PML expression level and the number of PML-NBs are not always correlated with viral replication activity. For example, although PML expression and the number of PML-NBs are greatly increased and induced by interferon, chemotherapy, and radiotherapy, HBV replication is inhibited by interferon but promoted by chemotherapy and radiotherapy. Because numerous regulatory proteins have been reported to be transiently associated with PML-NBs under certain circumstances, in different viral replicative phases, or after specific treatments, the function of PML-NBs should be based on the combined effects of the constituent and recruited regulatory proteins. Therefore, although the PML protein is essential for PML-NB formation, PML itself should not be the only factor that determines activation versus repression of viral replication in PML-NBs.

The variability of PML-NB contents might underlie the diverse processes with which PML-NBs have been associated. Among the PML-NB-associated regulatory proteins, CBP and HDAC1 are the best known histone coregulators on promoters for gene transcription. Because HBV cccDNA has a chromatin-like organization and is the template from which cellular polymerases transcribe viral mRNAs and HBV pregenomic RNA, the acetylation status of HBV cccDNA basal core promoter-bound histones is crucial for regulating the transcription, expression, and replication of HBV chromatin. Our results show that the PML-associated HDAC activity affects the acetylation status on the HBV cccDNA basal core promoter and determines the activation or repression of HBV transcription, expression, and replication. Further, this mechanism explains why both interferon and DNA damage signals upregulate PML but have contrasting effects on HBV replication; interferon increases but DNA damage signals decrease the PML-associated HDAC activity. Previous data showed that the deposition of PML-NB-associated proteins at the sites of viral genomes might reflect an intrinsic cellular response aimed at repressing viral gene expression; however, our results show that the DNA damage response induced by chemotherapy and radiotherapy can reverse the HBV repression.

PML in PML-NBs is thought to be important for antiviral defense and induction of apoptosis, but HBV may have evolved a way to counteract the PML-mediated apoptotic pathway to impair cellular antiviral responses. Based on a previous study showing that the core protein of hepatitis C virus (HCV) also

interacts with the PML protein to affect PML-mediated gene transcription and inhibit apoptosis (37), our results are compatible with two potential mechanisms: (a) suppressing HBV expression and replication by HCV-core during superinfection with these two hepatitis viruses through competition for interaction with PML and (b) HBV-associated development of HCC through HBV-core-mediated inactivation of the PML tumor suppressor pathway.

Thus, our data suggest an interactive mechanism by which host response, cellular signaling pathways, viral targets, and environmental factors might cooperatively stimulate HBV replication and pathogenesis. Because the convergence of several proteins in PML-NBs links DNA repair to viral replication, the cellular response to DNA damage might affect viral replication in PML-NBs and vice versa. This study suggests that one of the biological roles of PML-NBs is to act as a DNA damage repair effector whose dynamic responses, including increasing number, altering structure, and recruiting or dissociating chromatin regulators, reflect both the degree of cellular and viral DNA defects and the function of the DNA repair signaling pathways that are implicated in the reactivation of HBV replication. This work also supports prophylactic use of antiretroviral agents in reducing the incidence and severity of HBV reactivation in HBV-carrying cancer patients who undergo systemic chemotherapy or receive liver irradiation (38).

Materials and Methods

Cell Culture and Drug Treatment

HepG2 (parental cells of 1.3ES2 cells) and 1.3ES2 cells obtained from Dr. Chungming Chang (Division of Molecular and Genomic Medicine, National Health Research Institute, Taiwan) were maintained in DMEM (Gibco-BRL) containing 10% fetal calf serum. To generate double-strand DNA damage (17), cells were treated with 20 $\mu\text{mol/L}$ etoposide or 1.5 $\mu\text{mol/L}$ doxorubicin (Sigma-Aldrich) for 30 min or irradiation (5 Gy) using a ^{137}Cs irradiator at 1 Gy/min on ice. For kinase inhibition, cells were treated with 20 $\mu\text{mol/L}$ wortmannin, 5 mmol/L caffeine, or 10 $\mu\text{mol/L}$ Chk2 inhibitor II (Sigma-Aldrich) for 30 min before DNA damage.

Coimmunoprecipitation, Western Blot Analysis, and Histone Deacetylase Activity Assay

Cells were incubated with the lysis buffer [50 mmol/L TrisCl (pH 8.0), 150 mmol/L NaCl, 0.02% sodium azide, 0.1% SDS, 100 $\mu\text{g/mL}$ PMSF, 1 $\mu\text{g/mL}$ aprotinin, 1% NP40, and 0.5% sodium deoxycholate] for 20 min and centrifuged at 12,000 $\times g$ for 2 min at 4°C. The supernatant proteins (400 μg) were incubated with different antibodies (PML, HDAC1, HBV-core, all from Santa Cruz Biotechnology), respectively, in 0.5 mL of NET-gel buffer for 1 h at 4°C. The protein A-Sepharose beads were added for an 1-h incubation at 4°C. The immunoprecipitate Sepharose beads containing protein A-antigen-antibody complexes were collected by centrifugation at 12,000 $\times g$ for 20 s at 4°C, and then washed five times with NET-gel buffer. The immunoprecipitates were subjected to Western blot analysis or HDAC activity assays. Equal amount of proteins were electrophoresed in 15% SDS-polyacrylamide gel and blotted onto polyvinylidenedifluoride membranes.

The blotted membranes were treated with 5% skim milk in PBS/0.1% Tween 20 and incubated with different primary antibodies for 16 h at 4°C. The membranes were then incubated with a peroxidase-labeled second antibody for 1 h at room temperature. The membranes were rinsed, treated with enhanced chemiluminescent reagent, and exposed to films. The HDAC activities in the immunoprecipitates from the anti-PML antibody were measured with the use of a nonisotopic assay that used a fluorescent derivative of epsilon-acetyl lysine (HDAC Fluorescent Activity Assay Kit, Biomol). The results are expressed as micromolar values of the provided standard per microgram of protein.

Transfection and Luciferase Assay

Cells were grown on 60-mm dishes and transfected with different siRNAs of negative control, PML, ATM, ATR, and CHK2 (all from Santa Cruz Biotechnology) or different plasmids [the CMV or HBV basal core promoter (nt 1636-1851)-driven full-length HBV genome, HBV-core, or luciferase reporter expression vectors as gifts from Dr. Chungming Chang] using Lipofectamine 2000 reagent (Invitrogen). Luciferase assays were done 36 h after transfection. Renilla luciferase vector (Promega) was cotransfected to determine transfection efficiency. The medium was aspirated and 200 μ L of the lysis buffer were added. Fifty microliters of beetle luciferin substrate (0.1 mmol/L total luciferin) were added to 100 μ L of the lysate. Bioluminescence was measured using the LB940 automated analyzer (Berthold Technologies). Duplicate measurements were done for all experiments.

HBV Antigens and Medium HBV DNA Quantification

HBsAg and HBeAg were determined based on the manufacturer's protocol for the ELISA kits (Evernew Biotech, Inc.). Medium HBV DNA was quantified using the AmpliCore HBV monitor kit (Roche Molecular System). Each experiment was done in triplicate and repeated three times independently.

Southern Blot Analysis

DNA (20 μ g) was digested with *Hind*III, separated on a 1.2% agarose gel, and transferred onto nylon membranes. The membranes were hybridized with the ³²P-labeled DNA probe generated by a random-primed labeling kit (Amersham) using full-length HBV DNA.

Measurement of Apoptotic Cell Death

Subdiploid DNA content was measured with a BD Biosciences FACS flow cytometer and CellQuest software, and calculated as 100% \times [experimental apoptosis - spontaneous apoptosis in control (%)] / [100% - spontaneous apoptosis in control (%)].

Immunofluorescence, Immuno-FISH Stainings, and FRAP

For indirect immunofluorescence, cells were grown in 12-well plates on coverslips and fixed for 5 min at -20°C with methanol/acetone (1:1). Cells were blocked for 30 min at room temperature in PBS containing 5% (v/v) goat serum. Cells were then incubated for 60 min with the primary antibodies as indicated (all from Santa Cruz Biotechnology) at room temperature, subsequently washed five times in PBS before incubation for

45 min with the appropriate fluorescein Alexa488 (Molecular Probes)-, Texas red (The Jackson Laboratory)-, or AMCA (Abcys)-conjugated secondary antibodies. Cells were stained with 4',6-diamidino-2-phenylindole, mounted on glass slides, and were examined with a Zeiss Axioplan-2 fluorescence microscope. For immuno-FISH, cells grown on chamber slides were preextracted in CSK [0.1 mol/L NaCl₂, 0.3 mol/L sucrose, 3 mmol/L MgCl₂, 10 mmol/L Pipes (pH 6.8)] containing 0.5% Triton X-100 for 5 min on ice before fixation in 4% formaldehyde for 10 min. Cells were subjected to repeated freeze-thaw in liquid nitrogen and treated with 0.1 mol/L HCl for 10 min. DNA was denatured in 70% deionized formamide, 2 \times SSC at 72°C for 2 min, and washed for 1 min in cold 2 \times SSC before addition of denatured DNA probe. Hybridization was at 37°C overnight, followed by washes at 42°C in 50% formamide, 2 \times SSC for 3 \times 5 min, and in 2 \times SSC for 3 \times 5 min. Digoxigenin-labeled DNA probes were detected using antidigoxigenin FITC (Boehringer Mannheim). Detection of nuclear proteins by immunofluorescence was done simultaneously with the FISH detection steps. Slides were then counterstained with 4',6-diamidino-2-phenylindole and photographed. FRAP experiments were done as previously described (19). Five to 10 images were taken before the bleach pulse and 50 to 100 images after the bleaching of regions of interest that contained one PML-NB each at 0.05% laser transmission to minimize scan bleaching.

Real-time Reverse Transcriptase-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA was reverse transcribed with 50 ng random hexamers in a volume of 20 μ L containing 200 units of Superscript III Reverse Transcriptase (Invitrogen) and 40 units of RNaseOUT Recombinant RNase Inhibitor. Real-time PCR with primers PML forward: 5'-CCGCAAGACCAACAACATCTT-3', PML reverse: 5'-CAGCGGCTTGGAACATCCT-3', HBV precore/pregenomic forward: 5'-GTATTTGGTGTCTTTCCGGAGTG-3', and HBV precore/pregenomic reverse: 5'-GACCTGCCTCGTCTAAC-3' was carried out in a MicroAmp Optical 96-well plate using power SYBR Green PCR Master Mix (Applied Biosystems Inc.), with 2 μ L cDNA in each well. PCR reactions were monitored in real time using the ABI PRISM 7000 real-time PCR system (Applied Biosystems, Inc.). The thermal cycling conditions for real-time PCR were 50°C for 2 min, then 95°C for 10 min, and 40 cycles of denaturation (95°C, 15 s) and annealing/extension (60°C, 60 s). 18S rRNA was used as an endogenous control. Relative quantitation of gene expression was determined using the $\Delta\Delta$ Ct method, according to the manufacturer's directions.

Quantification of HBV cccDNA

Cells were lysed by the addition of 3 mL lysis solution [0.6% SDS, 10 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 7.5)] for 5 min and mixed with 750 μ L of 5 mol/L NaCl on ice overnight. The insoluble components were pelleted by centrifugation at 3,000 \times g for 15 min at 4°C. The supernatant was extracted with phenol/chloroform and precipitated by ethanol. Aliquots of each DNA were treated for 1 h at 37°C with 10 units of plasmid-safe DNase (Epicentre). Real-time PCR experiments were done in a Light-Cycler (Roche) using a 20 μ L

reaction volume containing 20 ng of DNA, 3 mmol/L MgCl₂, 0.5 μmol/L of forward and reverse primers, 0.2 μmol/L of 3' fluorescein (FL)-labeled probe, and 0.4 μmol/L of 5'-Red640 (R640)-labeled probe. Forward and reverse primers were 5'-CTCCCCGTCTGTGCCTTCT-3' (HBV subtype ayw 1547-1565) and 5'-GCCCCAAAGCCACCCAAG-3' (HBV subtype ayw 1902-1885), respectively, whereas the hybridization probes were 5'-GTTACGGTGGTCTCCATGCAACGT-FL-3' and 5'-R640-AGGTGAAGCGAAGTGCACACGGACC-3', respectively. Amplification was done as follows: 95°C for 10 min then 45 cycles of 95°C for 10 s, 58°C for 5 s, 63°C for 10 s, and 72°C for 20 s. The partially defective double-strand relaxed circular (RC) and single-strand (SS) forms of HBV DNAs were eliminated by DNase treatment before PCR. β-Globin amplification was done using the Light-Cycler β-globin control kit (Roche). Serial dilutions of a plasmid containing a monomeric HBV ayw subtype were used as quantification standards.

Chromatin Immunoprecipitation Assay

HBV cccDNA was subjected to immunoprecipitation with specific antibodies as previously described (27). In brief, after the reverse cross-linking step, chromatin immunoprecipitates were treated with Plasmid-safe DNase (Epicentre) for 1 h at 37°C and subjected to PCR amplification (35 cycles) using the HBV ayw subtype forward primer 5'-CTGAATCCTGCG-GACGACCC-3' nt 1443-1462 and reverse primer 5'-CCCAA-GGCACAGCTTGGAGG-3' nt 1890-1871 specific for the HBV basal core promoter region.

Electrophoretic Mobility Gel Shift Assay

The double-stranded oligonucleotides containing the HBV basal core promoter sequence (the HBV ayw subtype 1741-1841) were labeled after synthesis using the Biotin 3' End DNA Labeling Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). Nuclear extracts (5 μg) from untreated, etoposide-treated, or irradiated cells were incubated for 30 min at room temperature with the biotin end-labeled duplex DNA in a total volume of 10 μL in a buffer containing 20% glycerol, 5 mmol/L MgCl₂, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), and 0.25 mg/mL poly (di-dC). After 30 min at room temperature, samples were separated on a nonreducing 4% polyacrylamide gel using 0.5 mol/L TBE buffer. The DNA was then rapidly transferred to a positive nylon membrane, UV cross-linked, probed with streptavidin-horseradish peroxidase conjugate, incubated with the chemiluminescent substrate, and exposed to an X-ray film. Competition assays were done with a 200-fold excess of unlabeled oligonucleotides as a nonspecific competitor. Gel supershift assays were done using anti-PML, anti-HBV-core, anti-HDAC1, and nonspecific IgG antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Mol Cancer Res 2009;7:1672-1685. Published OnlineFirst October 6, 2009.

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