The metabolic sensors FXRα, PGC-1α, and SIRT1 cooperatively regulate hepatitis B virus transcription

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ABSTRACT **Hepatitis B virus (HBV) genome transcription is highly dependent on liver-enriched, metabolic nuclear receptors (NRs). Among others, NR farnesoid X receptor α (FXRα) enhances HBV core promoter activity and pregenomic RNA synthesis. Interestingly, two food-withdrawal-induced FXRα modulators, peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) and deacetylase SIRT1, have been found to be associated with HBV genomes *ex vivo*. Whereas PGC-1α induction was shown to increase HBV replication, the effect of SIRT1 on HBV transcription remains unknown. Here, we showed that, in hepatocarcinoma-derived Huh-7 cells, combined activation of FXRα by GW4064 and SIRT1 by activator 3 increased HBV core promoter-controlled luciferase expression by 25-fold, compared with a 10-fold increase with GW4064 alone. Using cell lines differentially expressing FXRα in overexpression and silencing experiments, we demonstrated that SIRT1 activated the core promoter in an FXRα- and PGC-1α-dependent manner. Maximal activation (>150-fold) was observed in FXRα- and PGC-1α-overexpressing Huh-7 cells treated with FXRα and SIRT1 activators. Similarly, in cells transfected with full-length HBV genomes, maximal induction (3.5-fold) of core promoter-controlled synthesis of 3.5-kb RNA was observed in the same conditions of transfection and treatments. Thus, we identified a subnetwork of metabolic factors regulating HBV replication, strengthening the hypothesis that transcription of HBV and metabolic genes is similarly controlled.—Curtil, C., Enache, L. S., Radreau, P., Dron, A.-G., Scholtès, C., Deloire, A., Roche, D., Lotteau, V., André, P., and Ramière, C. The metabolic sensors FXRα, PGC-1α, and SIRT1 cooperatively regulate hepatitis B virus transcription. *FASEB J.* 28, 1454–1463 (2014). www.fasebj.org**

Key Words: HBV · epigenetic · metabolism · nuclear receptors

Chronic hepatitis B is a major worldwide health problem, with >350 million infected individuals at risk of developing cirrhosis and hepatocellular carcinoma (1). Hepatitis B virus (HBV) replicates in the liver, a central organ for metabolism. There is increasing evidence to suggest that metabolic regulation pathways play a crucial role in the regulation of HBV transcription and replication (2).

HBV is an enveloped virus with an extremely compact, partially double-stranded DNA genome of 3.2 kb. Following cell entry, the viral genome is transported to the nucleus and repaired to constitute the covalently closed circular DNA (cccDNA). The cccDNA is structured as a minichromosome by associating with cellular histones and X and core viral proteins (HBx and HBc) and serves as a template for viral RNA transcription (3). Viral gene expression is mainly controlled at the transcriptional level by core, pre-S1, pre-S2/S, and X promoters and 2 enhancer regions (EnhI and EnhII). The core promoter activity, which is modulated by EnhII, plays a major role in HBV life cycle since it governs transcription of both precore and pregenomic RNAs. The precore RNA encodes the precore protein, while

Abbreviations: Act3, activator 3; cccDNA: covalently closed circular DNA; EnhI/II enhancer region I/II; FOXO1, forkhead box protein O1; FXRα, farnesoid X receptor α; HBV, hepatitis B virus; HDAC, histone deacetylase; HIV-1, human immunodeficiency virus 1; HNF-4α, hepatocyte nuclear factor 4α; LRH-1, liver receptor homolog 1; NR, nuclear receptor; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PPARα, peroxisome proliferator-activated receptor α; RXRα, retinoid X receptor α; SHP, small heterodimer partner

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doi: 10.1096/fj.13-236372

This article includes supplemental data. Please visit *http://www.fasebj.org* to obtain this information.

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the pregenomic RNA has a dual function: it encodes both core and polymerase proteins and is reverse transcribed following its encapsidation. Resulting nucleocapsids are then enveloped and released as new virions or return to the nucleus and increase the cccDNA pool (4).

Transcription of HBV genes is intensively regulated by nuclear receptors (NRs), which are transcription factors activated by ligands (hormones or metabolic by-products). HBV enhancer and promoter regions are targeted by liver-enriched NRs. Indeed, ectopic expression of either hepatocyte nuclear factor 4α (HNF-4α), peroxisome proliferator-activated receptor α (PPARα), liver receptor homologue 1 (LRH-1), or farnesoid X receptor α (FXRα) is sufficient to initiate HBV transcription and replication in nonhepatoma cell lines (5–8). On a physiological level, those NRs are major regulators in metabolism processes occurring in the liver, such as gluconeogenesis, lipid metabolism, and bile acid homeostasis. Those factors are now considered to be involved in the hepatic tropism of HBV (8).

The activity of those metabolic regulators is modulated by coactivators, corepressors, and epigenetic factors. Among the numerous modulators of NR activity, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and SIRT1 appear of particular interest regarding HBV infection. PGC-1α is a major NR coactivator, which is activated by food withdrawal, cold, and exercise (9). PGC-1α enhances HBV transcription *in vitro* through HNF-4α, FXRα, LRH-1, and PPARα (5, 6). Moreover, in a murine model, short-term food withdrawal has been shown to increase HBV replication in a PGC-1α-dependent way, and the protein has been detected on viral genomes in the livers of infected mice following chromatin immunoprecipitation (10).

SIRT1, a NAD^+-dependent deacetylase, is another major sensor of energy metabolism activated by nutrient deprivation that has been identified on HBV minichromosome *ex vivo*. SIRT1 has been detected in low replicative viral genomes, suggesting that the histone deacetylase activity of SIRT1 may decrease transcription of HBV genes (11). However, SIRT1 has also been shown to target proteins implicated in HBV core promoter transcriptional regulation, especially PGC-1α and FXRα. SIRT1 deacetylates and activates PGC-1α in response to food withdrawal (12), and SIRT1-induced deacetylation of FXRα increases binding of FXRα and its partner retinoid X receptor α (RXRα) onto the promoter of small heterodimer partner (SHP), an FXRα target gene (13). Using primary mouse hepatocytes, Wei *et al.* (14) showed that in a context of FXRα overexpression, the cooverexpression of SIRT1 led to an up-regulation of SHP transcription. This result indicates that SIRT1 is able to increase FXRα activity on an FXRα target gene (14).

The aim of the present study was to clarify the effect of SIRT1 on HBV transcription *in vitro*. We first used a reporter construct, allowing the specific study of the core promoter transcriptional activity. Next, we sought to determine the global effect of SIRT1 on the transcription of different viral RNAs, using transfection of a full-length HBV genome. Moreover, the involvement of PGC-1α and FXRα on SIRT1 influence was determined using FXRα specific ligands as well as silencing and overexpression experiments.

MATERIALS AND METHODS

Chemicals, antibodies, and siRNA

GW4064 was obtained from Sigma-Aldrich (St. Louis, MO, USA). SIRT1 activator 3 (Act3; ref. 15) was obtained from Cayman Chemical (Ann Arbor, MI, USA). FXRα inhibitor CAS936123-05–6 (described in patent WO 2007052843; Takeda Pharmaceutical Co. Ltd., Osaka, Japan) was synthesized by D.R. Antibodies against FXRα (sc-13063) and PGC-1α (sc-13067) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TBP (TATAbox binding protein; ab6141), and SIRT1 (ab32441) antibodies were obtained from Abcam (Paris, France). Actin antibody was purchased from Sigma-Aldrich. Stealth RNAi duplexes for low-GC negative control (si-ctrl), FXRα (GGAGUAUGCUCUGCUUACAGCAAUU), PGC-1α (GGGCAGAUUUGUUCUUCCACAGAUU), and SIRT1 (UACAAAUCAGGCAAGAUGCUGUUGC) were purchased from Invitrogen Life Technologies (Saint-Aubin, France).

Cell culture

Human hepatoma cell lines Huh-7 and HepG2 (HB-8065; American Type Culture Collection, Manassas, VA, USA) and human embryonic kidney cell line HEK293T were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium, containing 100 U/ml of penicillin and 100 μg/ml of streptomycin, supplemented with 1% HEPES, 1% NEAA, and 10% of a selected batch of fetal bovine serum (PAN-Biotech, Aidenbach, Germany).

Plasmids

The plasmid pGL2-EN2/CP, which contains a firefly luciferase reporter gene under the control of the HBV EnhII/core promoter region (nt 1400 to 1902, HBV genome, ayw subtype; GenBank accession no. U95551), has been previously described (16). Expression plasmids pSG5-FXRα and pSG5-RXRα are gifts from B. Staels [Pasteur Institute, Institut National de la Santé et de la Recherche Médicale (INSERM) U545, Lille, France]. pcDNA4-myc-PGC-1α (Addgene plasmid 10974; ref. 17) was obtained from T. Finkel [U.S. National Heart, Lung, and Blood Institute, National Institutes of Health (NIH), Bethesda, MD, USA], and expression vector for mutant SIRT1 pECE-SIRT1 H363Y (Addgene plasmid 1792; ref. 18) was obtained from M. E. Greenberg (Division of Neuroscience, Children's Hospital, Institute for Biomedical Research, Harvard Medical School, Boston, MA, USA). The expression vector for wild-type SIRT1, pCI-neo3xFlag-SIRT1, was obtained using Gateway cloning technologies, according to the manufacturer's instructions (Invitrogen Life Technologies).

Circular full-length HBV genomes were obtained as described previously (19), with some modifications. A complete HBV genome from ayw subtype (GenBank accession no. U95551) was amplified and cloned into the pCMV-βgal-vector. Following plasmid amplification and purification, the HBV genome was released by *Eco*RI digestion; 3.2-kb fragments corresponding to the viral genome were recovered by gel purification and then ligated using T4 DNA ligase. The

resulting ligation product was precipitated with ethanol and cleaned on a QIAQuick column (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions.

Quantitative RT-PCR analysis of FXRα expression

Endogenous expression of FXRα was quantified by quantitative RT-PCR analysis. Total cellular RNA was isolated using the RNeasy kit (Qiagen), according to the manufacturer's instructions. Following DNA digestion with Ambion Turbo DNase (Invitrogen Life Technologies), 500 ng of RNA was reverse transcribed using the high-capacity RNA-to-cDNA Master Mix (Invitrogen Life Technologies). cDNAs were then subjected to 30 PCR cycles with FastStart Universal SYBR green PCR master mix with ROX (Roche Applied Science, Penzberg, Germany) using StepOnePlus real-Time PCR systems (Applied Biosystems; Life Technologies, Foster City, CA, USA). The following primers were used for FXRα: forward, 5′-TGTGAGGGGTGTAAAGGTTTCT-3′, and reverse, 5′-GCCTGTATACATACATTCAGCCA-3′. TBP quantification was used for normalization using the following primers: forward, 5′-CCACGAACCACGGCACTGATTT-3′, and reverse, 5′-CAGTCTGGACTGTTCTTCACTCTT-3′.

Luciferase assays

Transfections for luciferase assays were performed in 24-well plates containing 1×10^5 cells using jetPEI (Polyplus Transfection, Illkirch, France), according to the manufacturer's instructions. Transfection mixtures for each well comprised 200 ng of plasmid pGL2-EN2/CP, 20 ng of plasmid pRL-SV40 (Promega, Charbonnières, France), serving as an internal control for transfection efficiency, and 100 ng of expression vectors for FXRα, RXRα, PGC-1α, SIRT1, and H363Y-SIRT1 when indicated. At 24 h post-transfection, FXRα and SIRT1 modulators were added as indicated. At 48 h after transfection, firefly and *Renilla* luciferase activities were measured using the dual-luciferase reporter assay system (Promega).

For silencing experiments, 10 pmol of stealth RNAi for control (ctrl), FXRα, PGC-1α, and SIRT1 was added to transfection mixtures for each well. Lipofectamine 2000 (Invitrogen; Life Technologies) was used as a transfection agent, according to the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed according to standard techniques. For FXRα expression analysis, enrichment in nuclear proteins was performed as follows. Pelleted cells were washed with PBS and disrupted for 15 min on ice in a hypotonic buffer (100 mM HEPES, pH 7.9; 15 mM $MgCl_2$; 100 mMKCl; 1 mM DTT; and 1% Nonidet P-40). Cell lysates were then centrifuged at 10,000 *g* for 15 min at 4°C. Pelleted nuclei were resuspended in extraction buffer (20 mM HEPES, pH 7.9, with 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM DTT, and 1% Nonidet P-40) and centrifuged at 12,000 *g* for 15 min at 4°C. Nuclear protein-containing supernatants were recovered and used for further analysis.

Northern blot analysis

Huh-7 or HEK293T cells (3×10^5) seeded in 12-well plates were transfected using jetPEI (Polyplus Transfection) with 150 ng of circular full-length HBV genomes, 200 ng of expression vectors for FXRα, RXRα, and PGC-1α when indicated, and 150 ng of a yellow fluorescent protein (YFP) expression vector used to monitor transfection efficiency. At 24 h after transfection, FXRα and SIRT1 modulators were added as indicated. At 24 h after treatment, cells were harvested for viral RNA analysis. Total RNA was extracted using the RNeasy kit (Qiagen). Northern blot analysis was performed as described previously (20). Briefly, 1 μg of RNA per sample was separated on a 1% agarose-formaldehyde gel and transferred onto a positively charged nylon membrane (Roche Applied Sciences) in 10× SCC buffer. Probe synthesis, labeling, and revelation were performed using the DIG System (Roche Applied Sciences), according to the manufacturer's instructions. Primers used for probe synthesis were forward, 5′-CTGAATCCTGCGGACGACCCTTCTC-3′, and reverse, 5′-GGGCAACATTCGGTGGGCGTTCA-3′. Quantification of HBV RNA was performed using ImageJ software (NIH, Bethesda, MD, USA).

RESULTS

SIRT1 enhances FXRα-induced activation of the HBV core promoter

The effect of SIRT1 activation on HBV transcription was first studied in human hepatoma cell lines Huh-7 and HepG2 and in human embryonic kidney cell line HEK293T by measuring the activity of a luciferase reporter gene under the control of the HBV EnhII/core promoter region (pGL2-EN2/PC plasmid; ref. 16). This region harbors multiple response elements for NRs, in particular, HNF-4α, PPARα, LRH-1, and FXRα (**Fig. 1**). As it has been recently shown that SIRT1 targets FXRα (13), we decided to investigate whether

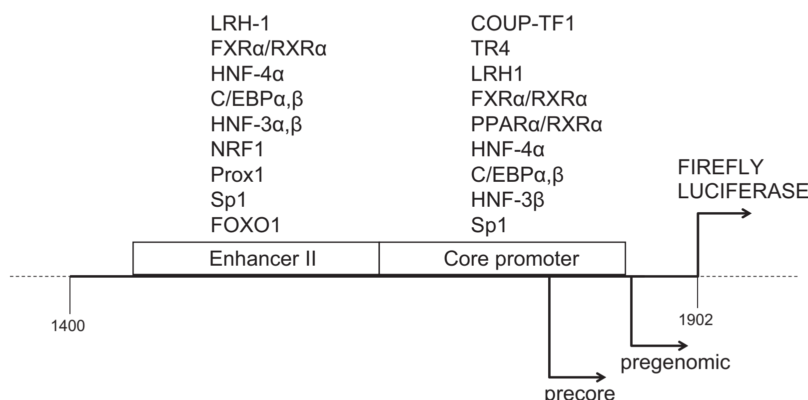

Figure 1. Schematic representation of reporter plasmid pGL2-EN2/CP. HBV sequence comprising nt 1400–1902, which was cloned into plasmid pGL2, is depicted in full line. Bacterial sequences are represented by a dotted line. HBV EnhII and core promoter regions are schematized as boxes. Angled arrows indicate HBV RNA start sites. Transcription factors and NR binding on either EnhII or core promoter are listed above targeted regions.

FXRα and SIRT1 cooperate to control the activity of the HBV core promoter. Cells were incubated with GW4064, a synthetic FXRα ligand, and increasing doses of Act3, a specific activator of SIRT1 (15). Treatment with Act3 alone did not significantly modify the core promoter activity in any of the 3 cell lines (**Fig. 2*A***). As expected, treatment of Huh-7 cells with GW4064 increased the core promoter activity by ~10-fold (16). Combined treatment with Act3 and GW4064 in Huh-7 cells further increased the luciferase activity in a dose-dependent manner, up to 25-fold with 20 μM of Act3 (Fig. 2*A*). However, treatment with GW4064 alone or in combination with Act3 did not modify the core promoter activity in HepG2 and HEK293T cell lines. Notably, we observed that the magnitude of Act3-induced activation varied significantly with batches of FCS (data not shown). The serum batch that gave the most reliable results was selected to be used for subsequent experiments.

To understand the differences between the 3 cell lines in response to the same treatments, we used RT-PCR to compare the expression of SIRT1, FXRα, and its coreceptor RXRα, as well as PGC-1α, an FXRα coactivator, in Huh-7, HepG2, and HEK293T cells. All cell lines expressed SIRT1, RXRα, and PGC-1α mRNAs, while FXRα mRNA was detected in Huh-7 cells but not in HEK293T cells, and hardly in HepG2 cells (data not shown). To confirm this result, we performed FXRα mRNA quantification in the 3 cell lines by RT-qPCR (Fig. 2*B*). As expected, HEK293T did not express FXRα

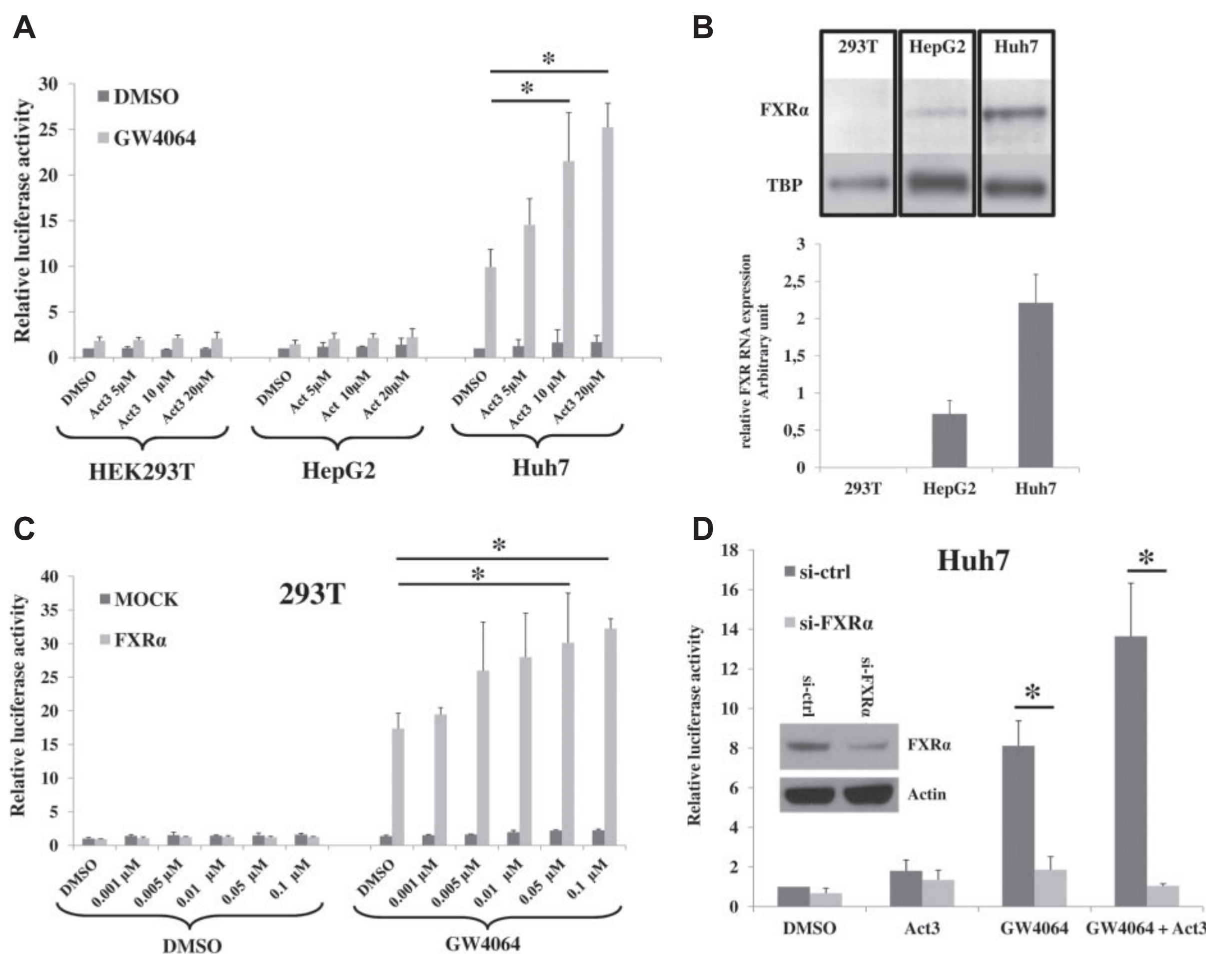

Figure 2. SIRT1 activation enhances FXRα-induced activation of the HBV core promoter. *A*) HEK293T, HepG2, and Huh-7 cells were cotransfected with plasmids pGL2-EN2/PC and pRL-SV40. At 24 h after transfection, cells were treated with DMSO or 1 μM of GW4064 and increasing concentrations of Act3. At 48 h after transfection, cells were harvested, and firefly and *Renilla* luciferase activities were measured. Results were normalized for transfection efficiency with *Renilla* luciferase activity and expressed as fold of the DMSO control condition for each cell line. Results are shown as means ± SD of 3 independent experiments. *B*) FXRα protein expression was assessed by Western blot analysis in protein extracts of HEK293T, HepG2, and Huh-7 cells. TBP antibody was used as control. Data are shown as one representative experiment of 3 independent experiments. RNA extracted from HEK293T, HepG2, and Huh-7 cells were also subjected to quantitative RT-PCR assays for FXRα. TBP gene expression was used for normalization. *C*) HEK293T cells were cotransfected with pGL2-EN2/PC, pRL-SV40, and pSG5-FXRα. *D*) Huh-7 cells were cotransfected with pGL2-EN2/PC, pRL-SV40, and si-ctrl or FXRα si-RNA. At 24 h after transfection, cells were treated with DMSO, GW4064, and Act3. At 48 h after transfection, cells were harvested, and firefly and *Renilla* luciferase activities were measured. Results were normalized for transfection efficiency with *Renilla* luciferase activity and expressed as fold of the si-ctrl plus DMSO control condition. Results are shown as means ± SD of ≥3 independent experiments (*A*–*C*) or means ± SE of ≥4 independent experiments (*D*). $*P < 0.02$; Student's *t* test.

mRNA, and Huh-7 cells expressed ~3 times more FXRα mRNA compared to HepG2 cells. Those results were further confirmed at the protein level by Western blot analysis (Fig. 2*B*). FXRα, thus, might appear as a limiting factor in the two nonresponsive cell lines to SIRT1 and FXRα activation. To confirm this hypothesis, we performed FXRα complementation in HEK293T (Fig. 2*C*) and in HepG2 cells (data not shown). FXRα expression restored the capacity of GW4064 and Act3 to activate the core promoter in a dose-dependent manner. Conversely, FXRα silencing in Huh-7 cells (assessed by Western blot) led to an ~70% decrease in GW4064-induced activation of the core promoter (Fig. 2*D*). Treatment with Act3 and GW4064 in si-control-transfected cells led to a 13-fold increase of the core promoter activity, whereas FXRα silencing nearly completely abolished the core promoter activation induced by Act3 and GW4064. Thus, SIRT1 activation by Act3 increases the transcriptional activity of the core promoter in FXRα-expressing cell lines. Altogether, these data suggest that SIRT1 does not directly modulate the core promoter activity but rather acts through downstream factors including FXRα.

Increase of FXRα-induced core promoter activity by Act3 is dependent on SIRT1 expression and deacetylase function

There has been controversy regarding the specificity and mechanism of different sirtuin-activating compounds used in experimental studies (21, 22). In particular, resveratrol, a natural phenol, which is widely used to activate SIRT1, is known to target numerous cellular proteins, in particular, metabolic regulators, and may not directly activate SIRT1. Thus, we wanted to confirm in our model whether the effect of Act3 on HBV core promoter activity was, indeed, SIRT1-dependent, performing silencing experiments of SIRT1 in Huh-7 cells (**Fig. 3*A***). Interestingly, SIRT1 silencing totally abrogated the FXRα-dependent Act3 activation of the transcriptional activity, since the core promoter activity was similar in SIRT1-silenced cells treated with either GW4064 or GW4064 and Act3, and also very similar to the basal level induced by FXRα activation alone in control cells. These results indicate that Act3-induced increase of the core promoter activity is dependent on the presence and probably the activation of SIRT1.

To determine whether Act3-induced activation of the core promoter required the NAD+-dependent deacetylase function of SIRT1, a deacetylase-defective mutant of SIRT1 (H363Y-SIRT1) was overexpressed in Huh-7 cells and compared with wild-type SIRT1 (Fig. 3*B*). Overexpression of wild-type SIRT1 did not significantly modify the core promoter activity, whether cells were treated with Act3 or not, indicating that the expression level of SIRT1 may not be a critical factor in Huh-7 cells. Strikingly, overexpression of mutant H363Y-SIRT1 completely abolished FXRα-dependent Act3-induced activation of the core promoter, suggesting

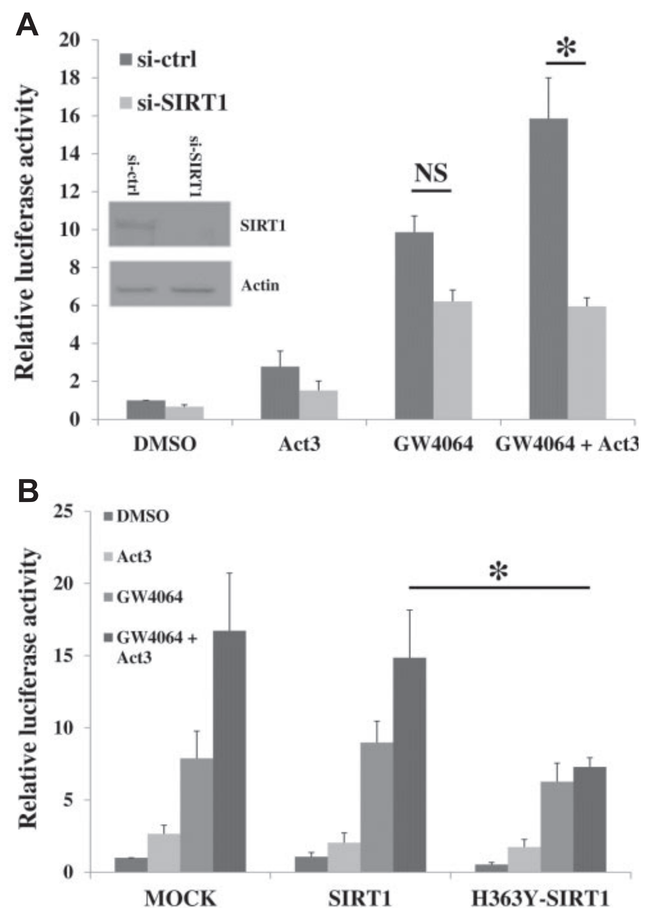

Figure 3. SIRT1 is required for Act3-induced activation of the core promoter. Huh-7 cells were cotransfected with pGL2-EN2/PC, pRL-SV40 and si-ctrl or SIRT1 si-RNA (*A*) or pGL2-EN2/PC, pRL-SV40 and pCI-neo3xFlag-SIRT1 or pECE-H363Y-SIRT1 (*B*). At 24 h after transfection, cells were treated with DMSO, 1 μM of GW4064, and 20 μM of Act3.At 48 h after transfection, cells were harvested, and firefly and *Renilla* luciferase activities were measured. Results were normalized for transfection efficiency with *Renilla* luciferase activity and expressed as fold of the DMSO control condition. Proper expression of both wild-type and mutant SIRT1 was verified in transfected cells by Western blot (not shown). Results are presented as means ± SE of ≥4 independent experiments. NS, not significant. $^*P < 0.03$; Student's *t* test.

that the deacetylase function of SIRT1 is more essential than its expression level in amplifying the FXR-induced HBV core promoter activation.

SIRT1, PGC-1α, and FXRα constitute a regulatory subnetwork controlling the HBV core promoter transcriptional activity

It has been previously shown that PGC-1α, an essential coactivator of FXRα, can be directly deacetylated and activated by SIRT1 during the metabolic response to food withdrawal (12). Moreover, PGC-1α is known to amplify FXRα-dependent activation of the HBV core promoter (5, 6). We, therefore, sought to determine whether PGC-1α was implicated in the core promoter activation induced by FXRα and SIRT1.

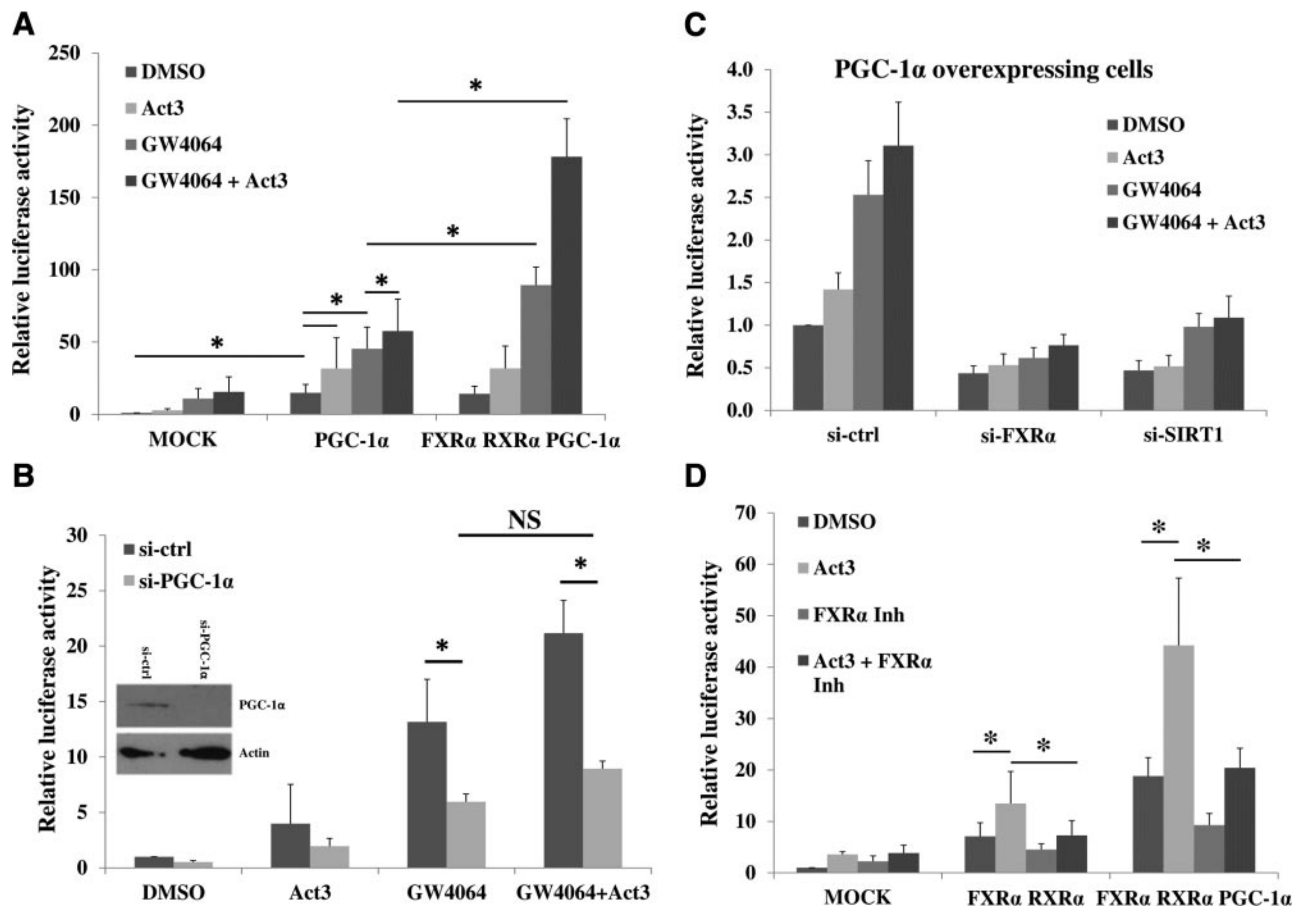

Figure 4. PGC-1α cooperates with FXRα and SIRT1 to activate HBV core promoter. Huh-7 cells were cotransfected with pGL2-EN2/PC, pRL-SV40 and expression vectors for FXRα, RXRα and PGC-1α (*A*) si-ctrl or PGC-1α siRNA (*B*), pcDNA4-PGC-1α plasmid plus si-ctrl or FXRα siRNA or SIRT1 siRNA (*C*), expression vectors for FXRα, RXRα and/or PGC-1α (*D*). siRNA efficiency was assessed by Western blot analysis (see Figs. 2*D* and 3*A*). At 24 h after transfection, cells were treated with DMSO, 1 μM of GW4064, 10 μM of FXRα inhibitor CAS936123-05–6 (named FXR inh in the graph) and 20 μM Act3. At 48 h after transfection, cells were harvested, and firefly and *Renilla* luciferase activities were measured. Results were normalized for transfection efficiency with *Renilla* luciferase activity and expressed as fold of the MOCK or si-ctrl DMSO condition. Results are presented as means ± SD (*A–C*) or means ± SE (*D*) of ≥3 independent experiments. NS, not significant. $*P < 0.03$; Student's *t* test.

We first overexpressed PGC-1α alone or in combination with FXRα and RXRα in Huh-7 cells treated with GW4064 and Act3 (**Fig. 4*A***). In the absence of GW4064 and Act3, PGC-1α overexpression led to an ~15-fold induction of the core promoter activity. This result probably represents the coactivation of endogenous nuclear receptors that control HBV transcription, including FXRα. Treatment of PGC-1α-transfected cells with Act3 increased the core promoter activity by ~30-fold, indicating that PGC-1α may integrate SIRT1 signaling. GW4064 treatment enhanced the core promoter activity by 45-fold, confirming that PGC-1α was able to cooperate with FXRα to enhance the HBV core promoter activity. Moreover, when PGC-1α-overexpressing Huh-7 cells were treated with both Act3 and GW4064, the core promoter activity was significantly increased compared with use of Act3 or GW4064 alone. Interestingly, cooverexpression of FXRα-RXRα did not further modify the core promoter activity in the absence of FXRα activation by GW4064 but very significantly increased this activity in GW4064 and GW4064-Act3 treatment conditions (by more than 150-fold for the latest condition, compared to control and untreated cells). These data confirmed that PGC-1α coactivates FXRα in this model and showed that this effect is potentiated by SIRT1 activation.

We then wondered whether PGC-1α was necessary for FXRα and/or SIRT1 to activate the core promoter. Silencing of endogenous PGC-1α expression decreased by ~50% the activation of the core promoter induced by treatment with GW4064 or with GW4064 and Act3, while no significant variation was observed for untreated or Act3-treated cells (Fig. 4*B*), suggesting that the effect of SIRT1 on FXRα-induced activation of the core promoter requires the presence of PGC-1α.

To further understand the interactions between FXRα, PGC-1α, and SIRT1 in the activation of the HBV core promoter, we next performed the silencing of endogenous FXRα and SIRT1 in Huh-7 cells overexpressing PGC-1α (Fig. 4*C*). FXRα or SIRT1 silencing dramatically decreased PGC-1α-induced activation of the core promoter by between 56 and 74% and between 53 and 66%, respectively, in cells treated with DMSO, Act3, GW4064, or GW4064-Act3. We also per-

formed overexpression of FXRα, RXRα, and/or PGC-1α in Huh-7 cells treated by Act3 and FXRα inhibitor (Fig. 4*D*). Treatment by Act3 led to a significant increase of core promoter activity in both overexpression conditions, confirming that FXRα expression level may influence SIRT1 activation effect. Moreover, inhibition of FXRα activity by the FXRα inhibitor resulted in a decrease of Act3-induced activation of the core promoter in cells overexpressing FXRα, RXRα, and/or PGC-1α, meaning that basal activation of FXRα, probably due to the presence of bile acids in the serum used (data not shown), was responsible for Act3-induced increase of the core promoter activity.

Altogether, these results strongly suggest that PGC-1α is involved in SIRT1 and FXRα-induced activation of the core promoter. Indeed, FXRα, PGC-1α, and SIRT1 seem to constitute a subnetwork of interdependent proteins that modulates the HBV core promoter transcriptional activity.

FXRα/PGC-1α/SIRT1 subnetwork increases synthesis of HBV RNA

In the context of a whole HBV genome, the core promoter and EnhII regions are not autonomous, but dependent on other transcriptional regulatory sequences, in particular, the EnhI region (23). Moreover, viral proteins HBc and HBx associate with HBV minichromosomes, and one of them, HBx, has been shown to play a role in modulating cccDNA transcription (11). We, thus, wanted to examine the effect of FXRα and SIRT1 activation in Huh7-cells and HEK293T cells transfected with circular full-length HBV genomes (designated as HBV-transfected cells), which contain every enhancer and promoter sequence and allow synthesis of all HBV proteins. Synthesis of core promoter-controlled 3.5-kb, preS2/S, and preS1 promoter-controlled 2.1/2.4-kb, and X promoter-controlled 0.7-kb viral RNAs was first assayed by Northern blot in Huh-7 cells (**Fig. 5*A***). Expression of 3.5-kb RNA was significantly induced by GW4064 with or without Act3. Moreover, overexpression of FXRα, RXRα, and PGC-1α increased synthesis of the 3.5-kb RNA by 2.5-fold in cells treated with GW4064 and by 3.5-fold in cells treated with both FXRα and SIRT1 activators, with a statistically significant difference between the two conditions. In the same conditions of treatment, we also observed a parallel increase in synthesis of the 2.1/2.4 kb RNAs. Basal expression level of 0.7-kb X RNA was low but also increased in cells overexpressing FXRα, RXRα, and PGC-1α and treated

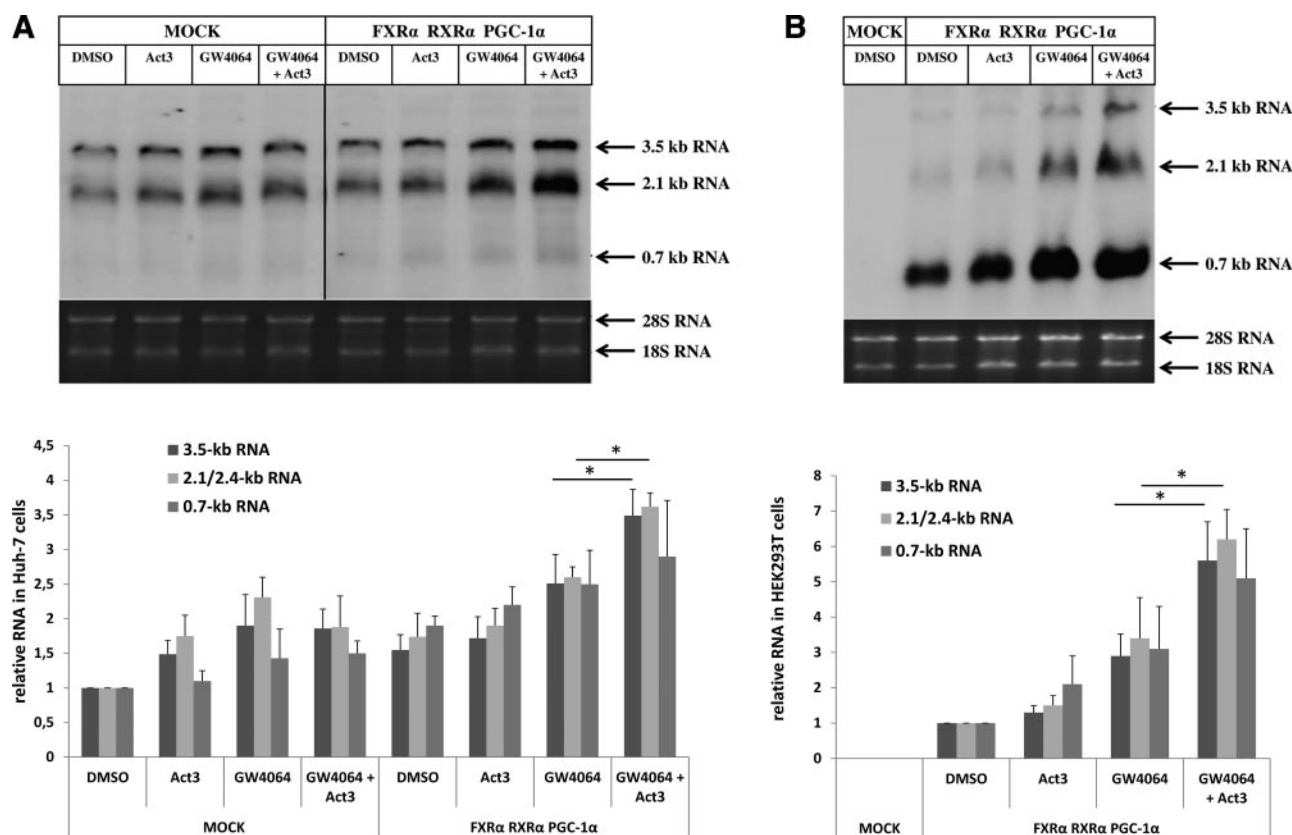

Figure 5. FXRα, PGC-1α, and SIRT1 cooperatively enhance synthesis of HBV RNA. Huh-7 cells (*A*) and HEK293T cells (*B*) were transfected with circular full-length HBV genomes with or without expression vectors for FXRα, RXRα, and PGC-1α. At 24 h after transfection, cells were treated with FXRα and/or SIRT1 activators. At 48 h after transfection, total cellular RNA was extracted, analyzed by Northern blot, and quantified by densitometry. One representative Northern blot for Huh-7 and HEK293T cells is presented. Quantification of 3.5-, 2.1/2.4- and 0.7-kb HBV RNA was performed using ImageJ software. Results are presented as means ± SE of 4 independent experiments. $*P < 0.03$; Student's *t* test.

with GW4064. However, treatment with Act3 and GW4064 did not significantly modify synthesis of 0.7-kb RNA compared to treatment with GW4064 alone. These results indicate that activation of the FXRα/PGC-1α/SIRT1 axis increases not only 3.5-kb HBV RNA transcription from the core promoter, but also synthesis of 2.1/2.4-kb RNA.

We also performed Northern blot analysis in HEK293T cells in order to evaluate the effect of the FXRα/PGC-1α/SIRT1 axis in a cell line that does not express the liver-enriched nuclear receptors that initiate transcription of the HBV 3.5-kb RNAs (Fig. 5*B*). As expected, following transfection of HBV genomes, we did not observe any synthesis of viral RNAs, but overexpression of FXRα, RXRα, and PGC-1α was sufficient to initiate transcription of viral genes. In HBV-transfected HEK293T cells overexpressing FXRα, RXRα, and PGC-1α, synthesis of 3.5-kb RNAs was not significantly modified by treatment with Act3 alone. FXRα activation by GW4064 increased the expression level of the 3.5-kb RNA by ~3-fold, and combined treatment with both Act3 and GW4064 by ~6-fold compared with DMSO-treated cells. As in Huh-7 cells, we observed a parallel increase of 2.1/2.4-kb RNA synthesis in the same conditions of treatment and with nearly the same amplitude. 0.7-kb RNA synthesis was also increased in cells treated with GW4064 and Act3, but to a lower extent. These results, thus, confirm data obtained using the reporter system, even though at a lower level, and indicate that activation of the FXRα/PGC-1α/SIRT1 axis induces transcription of HBV RNA both in hepatoma and nonhepatoma cell lines.

DISCUSSION

In this study, we showed that histone deacetylase SIRT1 participates in the increase of HBV replication dependent on FXRα and PGC-1α *in vitro*. All three proteins appear to constitute a subnetwork that modulates HBV promoter activity and viral transcription. This result may appear surprising as, on the basis of the histone deacetylase activity of SIRT1 combined to identification of SIRT1 on low replicative cccDNA *ex vivo*, it could have been predicted that SIRT1 activation would rather decrease HBV transcription. Indeed, acetylation and deacetylation of histones have been shown to play a major role in the transcriptional regulation of several DNA viruses able to persist in infected cells either as episomes or as integrated genomes, such as members of the *Herpesviridae* family, HBV, or human immunodeficiency virus 1 (HIV-1) (24, 25). In particular, inhibition of HDAC has been shown to induce reactivation of a fraction of latent HIV-1 viruses in different experimental models (26) and is now envisaged as a putative new therapeutic agent (27). The most likely explanation for the SIRT1-induced activation of the HBV core promoter that we observed *in vitro* is that SIRT1 may preferentially target nonhistone proteins (28). In particular, SIRT1 is known to deacetylate several transcription factors and transcriptional modulators involved in metabolism regulation, which have been shown to activate HBV transcription. We focused in this study on FXRα and PGC-1α, which have been shown to activate transcription from the HBV core promoter. We observed that Act3-induced SIRT1 activation did not modify HBV transcription when FXRα was not activated by its ligand, suggesting that SIRT1 only indirectly modulates HBV transcription *via* one or several targets. Indeed, in the three cell lines used in this study, FXRα expression and activation were necessary to observe maximal induction of the core promoter activity by PGC-1α and SIRT1.

Our results show that FXRα is an important NR modulating the core promoter activity *in vitro*. We confirmed that FXRα, coactivated by PGC-1α, strongly increases HBV gene transcription. On the basis of FXRα-silencing experiments, we also showed that FXRα was involved in PGC-1α-induced activation of the core promoter in Huh-7 cells. Interestingly, Wu *et al.* (29) showed that FXRα was the most up-regulated gene among 84 NRs and related genes studied in HepG2.2.15 cells (which contain an integrated HBV genome) compared to parental HepG2 cells, and it has been shown that miR-1, one of the microRNAs involved in hepatocyte differentiation, modulates HBV replication by increasing FXRα expression and decreasing histone deacetylase 4 activity (HDAC4, a suppressor of HBV biosynthesis; ref. 30). Put together, these observations suggest that, among the NRs that are involved in HBV regulation, FXRα plays an essential role in hepatoma cell lines. Indeed, the level of endogenous FXRα in Huh-7 cells allowed a strong induction of the core promoter activity in response to FXRα and SIRT1 activation, whereas FXRα overexpression was necessary in HepG2 cells to observe the activation of the core promoter in response to SIRT1 stimulation. Whether SIRT1 can modulate HBV transcription in a way that is strictly dependent on the axis composed of FXRα and PGC-1α, or by targeting other factors, still has to be determined. In particular, SIRT1 has also been shown to modulate other transcription factors and NRs involved in HBV transcription, such as PPARα and Forkhead box protein O1 (FOXO1) (31, 32). These findings suggest, nonetheless that, when screening for HBV transcription modulators, levels of FXRα expression and activation (and those of other metabolic NRs and modulators) have to be determined and taken into account.

Experiments using transfections of a full-length HBV genome confirmed that Act3-induced SIRT1 activation, when combined with FXRα activation, increased HBV transcription. However, the level of induction was rather low compared to results obtained using our reporter system. Reasons for this discrepancy are still unclear, but our results suggest that, in the context of a full-length virus, when all viral proteins are expressed, transcription regulation may be regulated by other factors, such as cccDNA-bound viral proteins HBc and/or HBx. Indeed, Belloni *et al.* (11) have previously

identified numerous chromatin-modifying enzymes, such as SIRT1, P300, and histone deacetylase 1 (HDAC1), which are associated with HBV minichromosomes. Among those proteins, recruitment of deacetylases on HBV minichromosomes was enhanced in the absence of HBx. Belloni *et al.* also observed that, compared to wild-type viruses, HBx-deficient viruses had a decreased histone acetylation and a decrease of pregenomic RNA synthesis and HBV replication, but no change in the cccDNA pool (3). Interestingly, a direct interaction between SIRT1 and HBV X protein has been reported (33), but its functional significance remains unclear. Another viral protein, HIV-1 Tat, has been shown to target and inactivate SIRT1, which may contribute to T-cell hyperactivation, thus favoring viral replication (34). Conversely, SIRT1 itself targets Tat and functions as a coactivator of Tat-mediated transactivation by deacetylating K50 in Tat (35). Like Tat, HBV X protein is able to transactivate transcription partly by modulating the acetylation and methylation status of cccDNA-bound histones, thus regulating viral transcription. It is, therefore, tempting to speculate that SIRT1 deacetylates HBx and modifies HBx-mediated transactivation of HBV transcription or, inversely, that HBx modulates SIRT1 deacetylase activity. Overall, this suggests that examining in more details the interplay between SIRT1, HBx, and transcription factors and modulators, such as FXRα and PGC-1α will be of great interest.

Results of Northern blot performed in Huh-7 and HEK293T cells transfected with full-length HBV genomes revealed that Act3-induced SIRT1 activation, when combined with FXRα activation, also increases synthesis of 2.1/2.4-kb S RNA. This result was somewhat unexpected as no FXRα response element has been described to date on either preS2/S or preS1 promoters. Increased synthesis of 2.1/2.4-kb S RNA may be due to either modulation of S promoters by the EnhII/core promoter region, as described previously (36) or to SIRT1-induced activation of PGC-1α, which is known to also activate synthesis of HBV S transcripts (10) or to an effect of SIRT1 on other NR or transcription factors known to modulate synthesis of S RNAs, such as PPARα (31).

The main limitation of our study is that it was performed in hepatoma cell lines. Indeed, epigenetic regulation is known to be deregulated during the carcinogenesis process. Moreover, acetylation and deacetylation of target proteins are dynamic processes that evolve in response to environmental stimuli (37). In particular, SIRT1 expression and activation levels vary according to fed and fasting states, and those natural fluctuations can hardly be mimicked in *in vitro* cell culture conditions. Thus, the relevance of the FXRα, PGC-1α, and SIRT1 axis, both in modulating HBV and metabolic genes, needs to be assessed in more relevant models. *In vivo*, the only data available regarding the effect of food withdrawal on HBV biosynthesis come from two studies performed in mice, showing conflicting results. In a model using HBV genomes injected to mice, Shlomai *et al.* (10) showed that short-term food withdrawal (7 h) significantly increases HBV replication in a PGC-1α-dependent manner. However, using HBV transgenic mice, Li *et al.* (38) found that prolonged food withdrawal (48 h) only leads to a limited increase of HBV replication. These apparently contradictory results might be explained by profound differences between those two models of HBV infection and the protocols followed, in particular, concerning the duration of food withdrawal. Thus, metabolic studies in HBV transgenic mice should be carefully analyzed and confirmed in more relevant animal models, such as mice with chimeric human livers infected with HBV. Moreover, studies in HBV-infected patients would be of great interest to determine whether HBV viral load fluctuates during nychthemeron and fed *vs.* unfed states. FJ

Work was supported by a grant from Agence Nationale de Recherche sur le Sida et les Hépatites Virales (ANRS) and by the Natheb program (French Fond Unique Interministériel). L.S.E. was partly supported by the Sectorial Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian government under contract POSDRU 6/1.5/17. The authors acknowledge the contribution of the AniRA level 3 security laboratory and genetic analysis platforms of SFR Biosciences Gerland-Lyon Sud (AMS3444/US8).

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Received for publication August 9, 2013.
Accepted for publication November 11, 2013.