We have previously shown that IFN-β inhibits hepatitis B virus (HBV) replication by noncytolytic mechanisms that either destabilize pregenomic (pg)RNA-containing capsids or prevent their assembly. Using immortalized murine hepatocyte cell lines stably transfected with a doxycycline (dox)-inducible HBV replication system, we now show that replication-competent pgRNA-containing capsids are not produced when the cells are pretreated with IFN-β before HBV expression is induced with dox. Furthermore, the turnover rate of preformed HBV RNA-containing capsids is not changed in the presence of IFN-β or IFN-γ under conditions in which further pgRNA synthesis is inhibited by dox removal. In summary, these results demonstrate that types 1 and 2 IFN activate hepatocellular mechanism(s) that prevent the formation of replication-competent HBV capsids and, thereby, inhibit HBV replication.

Materials and Methods

Plasmids. The reverse tetracycline transactivator (rTA) sequences were excised by EcoRI/BamHI digestion of pTet-On (BD Biosciences, San Jose, CA), blunt-end treated, and inserted into the Klenow-treated Sall site in pARV2 (17) to yield pARV2-rTA expressing rTA from an unspliced mRNA and neomycin from a spliced mRNA under the control of the Moloney murine leukemia virusLTR (17). The tetracycline inducible promoter (TRE)HBV plasmid was obtained by cloning a terminally redundant HBV (ayw) genome extending from nucleotides 1821 (transcriptional start site of the pgRNA) to 1986 (BglII) into pTRE (BD Biosciences), fusing the 5′-end of the pgRNA to the transcriptional start site of the CMV minimal promoter in pTRE. Briefly, pTRE was modified by insertion of a linker sequence into the unique XhoI site, creating pTREpac with a unique PacI site. Next, an overlength genome of HBV ayw EcoRI (position 1) to BglIII (position 1986) was inserted into pTREpac opened with EcoRI and HindIII by using a linker to fuse the HBV BglIII site with the vector HindIII site. The sequences from PacI to BspEI (ayw position 2331) in this plasmid were replaced by two PCR products spanning the sequences from PacI to the transcriptional start site in pgTRE (using primers CMVPAC1 5′-cctcaacaacag-3′ and TRESAP 5′-tgaggctcttcaagtcggttcactaaacgagctctg-3′) and from the viral transcriptional start site to BspE I (using primers HBVSAP 5′-gcatgcacttcaacattcttaacgtctgctaatc-3′ and HBVBSPE 5′-gctggctgcgtctaatcag-3′), respectively, creating pTREHBV. Both PCR products contained a SalI site, which after digestion and religation fused the viral transcriptional start site to the CMV minimal promoter. The plasmid pTREHBV was obtained by digestion of pTREHBV with PvuII and insertion of a puromycin resistance gene (pac) expression cassette, excised from pPUR (BD Biosciences) by PvuI and BamHI digestion. Finally, the KpnI/SalI fragment of pTRE- HBV was cloned into the KpnI/SalI-digested pBluescriptII plasmid (BD Biosciences), yielding pPGFPTREHBV containing a bidirectional tetracycline-inducible promoter driving the expression of the complete HBV pgRNA and GFP in opposite directions and, in addition, constitutively expressing the pac gene conferring puromycin resistance (Fig. 1). Plasmid pPGFPTREHBV-V encoding a viral polymerase with a Met to Val substitution in the catalytically active domain YMDD, which strongly reduces reverse transcriptional activity (18) and thereby allows HBV RNA-containing capsids to accumulate in the cell, was constructed by using the QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. All plasmids were verified by sequencing.

Cell Culture. Parental MMHD3 (19) and stably transfected cell lines were maintained in collagen I-coated plasticware (BD Biosciences) in complete RPMI medium as described (14). MMHD-On cell lines were established by transfecting the pARV2-reverse tetracycline transactivator into MMHD3 cells followed by selection

Abbreviations: HBV, hepatitis B virus; pg, pregenomic; TRE, tetracycline inducible promoter; dox, doxycycline; miIFN-β, murine IFN-β; OAS, 2′,5′-oligoadenylate synthetase.

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of stable cell lines in the presence of 300 μg/ml neomycin (Invitrogen). MMHD-On subclones were tested for robust tetracycline-regulated gene expression by transient transfection of pBl-EGFP (BD Biosciences) expressing GFP under the control of a bidirectional TRE, and a MMHD-On subclone was selected with the puromycin resistance gene (pac) for selection of stable transfectants in mammalian cells. The bidirectional TREminCMV promoter also drives expression of the EGFP to monitor tetracycline induction in cell cultures. Plasmid amplification is supported by the bacterial origin of replication (ori) and the β-lactamase gene (Amp') providing ampicillin resistance. Numbers indicate nucleotide map positions in HBV.

**Results**

**Conditional HBV Gene Expression and Accumulation of HBV pgRNA-Containing Capsids.** To investigate the antiviral effect of IFN-β and -γ on the formation and/or stability of replication-competent HBV pgRNA-containing capsids, an immortalized mouse hepatocyte cell line TREHBV-V that allows for tetracycline-inducible HBV gene expression and accumulation of pgRNA-containing capsids was established as described in Materials and Methods. In the absence of the tetracycline derivative dox, TREHBV-V cells did not express pgRNA, whereas the viral envelope transcripts were expressed from the endogenous HBV promoters as shown by Northern blot analysis of total cellular RNA (Fig. 2A Upper, lane 2). Consequently, TREHBV-V cells did not contain any encapsidated HBV RNA, as demonstrated by HBV-specific Northern blot analysis of nuclease-resistant cytoplasmic RNA (Fig. 2A Lower, lane 2). Two days after the addition of dox (1 μg/ml) to the culture medium, pgRNA was strongly induced while expression of the envelope transcripts from the HBV endogenous promoters remained constant (Fig. 2A Upper, lane 3). The additional RNA species migrating between pgRNA and envelope RNAs (Fig. 2A Upper, lane 3) represented spliced pgRNA transcripts (data not shown). Importantly, dox-induced pgRNA expression resulted in the accumulation of pgRNA-containing capsids, as shown by Northern blot analysis of nuclease-resistant cytoplasmic RNA (Fig. 2A Lower, lane 3). Steady-state levels of total and encapsidated pgRNA plateaued between days 2 and 4 after dox addition (Fig. 2A, lanes 3 and 4). These results show that mechanisms that regulate the de novo formation of HBV RNA-containing capsids can be studied in the TREHBV-V system.

When dox was removed at day 4, the intracellular pgRNA decreased over time and was virtually undetectable 3.5 days after dox removal (Fig. 2A Upper, lanes 5–8). Similarly, encapsidated HBV RNA also decreased and was virtually undetectable 5 days after dox removal (Fig. 2A Lower, lanes 5–8). However, encapsidated HBV RNA decreased with delayed kinetics compared with total pgRNA, because there was still a pool of HBV RNA-containing capsids present 3.5 days after dox removal when total pgRNA was almost undetectable (Fig. 2A, lane 7). Fig. 2B shows that the mutation of methionine 539 to valine in the HBV polymerase impaired reverse transcription in the TREHBV-V cells compared with TREHBV cells that express the wild-type HBV polymerase. Although the pattern of dox-induced HBV gene expression at 1.5 and 4 days after dox addition is similar between the TREHBV-V and TREHBV cells (Fig. 2B Top), the TREHBV-V cells accumulate higher levels of cytoplasmic HBV RNA-containing capsids, as shown by Northern blot analysis of nuclease-resistant cytoplasmic RNA (Fig. 2B Middle). Despite the higher levels of pgRNA-containing capsids in the TREHBV-V cells (Fig. 2B Middle), they contained fewer HBV DNA-replicative intermediates as measured by Southern blot analysis of total cellular DNA (Fig. 2B Bottom), further demonstrating the reverse transcription deficiency of the mutant polymerase (18). Together, these results demonstrate that the

**HBV DNA Analysis.** Cells were lysed in the culture dish by adding 500 μl of DNA lysis buffer (50 mM Tris-HCl, pH 8.0/20 mM EDTA/1% SDS). Samples were then digested overnight at 37°C with proteinase K (1 mg/ml), and total DNA was extracted as described (20). Twenty micrograms of total DNA was analyzed by Southern blotting with a 32P-labeled full length HBV DNA probe after EcoRV digestion (20). All quantifications were done with a Cyclone storage phosphor system (Packard).

**HBV RNA Analysis.** Total cellular RNA was isolated by the guanidine thiocyanate method by using standard protocols (21), and 20 μg of RNA was subjected to Northern blot analysis as described (20). Encapsidated RNA was extracted from cells grown in a 60-mm culture dish. Briefly, cells were scraped from the dish in 1 ml of PBS and pelleted by brief centrifugation in a tabletop microtube centrifuge at room temperature. The pellets were lysed in 0.3 ml of lysis buffer (100 mM NaCl/1 mM EDTA/50 mM Tris-base, pH 8.0/0.5% Nonidet P-40). Nuclei were pelleted by centrifugation for 5 min at 12,000 rpm and 4°C in a microtube centrifuge. Encapsidated RNA in the supernatant was extracted as described (13). The RNA was dissolved in 50 μl of H2O, and 15 μl was used for Northern blot analysis as described (20). Quantitative real-time PCR for apolipoprotein mRNA-editing enzymes from total cellular RNA is described in Table 1 and Supporting Text, which are published as supporting information on the PNAS web site.
subjected to HBV-specific Southern blot analysis (micrograms of total cellular DNA isolated from TREHBV-V and TREHBV cells was extracted from parallel dishes of TREHBV-V and TREHBV cells (Northern blotting). Total cellular RNA isolated from TREHBV-V and TREHBV cells cultured in the Tetracycline-regulated expression and replication in TREHBV-V and TREHBV cells. The presence of dox for the indicated time periods was subjected to HBV-specific Northern blotting. (A) HBV- and GAPDH-specific Northern blot analysis of total cellular RNA. (B) Northern blot analysis of encapsidated HBV RNA. (C) Northern blot analysis of total cellular RNA for the mIFN-β response gene 2′,5′-OAS. For experimental details and symbols, see Materials and Methods and the legend to Fig. 2, respectively.

Fig. 2. Tetracycline-regulated expression and replication of HBV. TREHBV-V and TREHBV cells were cultured as described in Materials and Methods with or without dox, as indicated, and individual dishes were harvested at different days (d) during the experiment. (A) Tetracycline-regulated gene expression and accumulation of HBV pgRNA-containing capsids in TREHBV-V cells. Twenty micrograms of total cellular RNA was used for Northern blot analysis for viral pgRNA and viral envelope-encoding transcripts (envRNA) and the cellular gene for GAPDH as a control for loading differences (Upper). Encapsidated HBV RNA was isolated as described in Materials and Methods and subjected to HBV-specific Northern blotting (Lower). Twenty micrograms of total cellular RNA isolated from HBV transgenic (HBVtg) mice was included as size marker for HBV RNA. (B) Tetracycline-regulated expression and replication in TREHBV-V and TREHBV cells. Total cellular RNA isolated from TREHBV-V and TREHBV cells cultured in the presence of dox for the indicated time periods was subjected to HBV-specific Northern blotting (Top). Northern blot analysis of encapsidated HBV RNA extracted from parallel dishes of TREHBV-V and TREHBV cells (Middle). Twenty micrograms of total cellular DNA isolated from TREHBV-V and TREHBV cells was subjected to HBV-specific Southern blot analysis (Bottom). Tg, HBV transgene; DS, double-stranded HBV DNA replicative intermediates; SS, single-stranded HBV DNA replicative intermediates.

TREHBV-V cells retain a pool of HBV RNA-containing capsids even after suppression of pgRNA expression. Thus, the results demonstrate that the impact of potential antiviral mechanisms even after suppression of pgRNA expression. Thus, the results demonstrate that the impact of potential antiviral mechanisms on the stability of preformed HBV RNA-containing capsids can be studied in the TREHBV-V system.

IFN Inhibits the Accumulation of Replication-Competent HBV Capsids. To determine whether IFN-β-induced antiviral mechanisms interfere with the formation of replication-competent HBV RNA-containing capsids, we induced HBV gene expression and replication in TREHBV-V cells in which the antiviral mechanism was previously induced by IFN-β. Because we have demonstrated (14, 15) that the IFN-β-mediated antiviral mechanism is effectively induced within 6–12 h after IFN addition to immortalized HBV transgenic hepatocytes, the TREHBV-V cells were pretreated with IFN-β (100 units/ml) for 6 h, and 2′,5′-oligoadenylate synthetase (OAS) mRNA, a marker for IFN-β signaling, was monitored in the cells by Northern blot analysis of total cellular RNA (Fig. 3C, OAS). Six hours after IFN addition, dox (1 μg/ml) was added to the culture medium of the control and the IFN-β-pretreated cultures to induce HBV gene expression and accumulation of pgRNA-containing capsids. As shown in Fig. 3A, dox-induced similar levels of pgRNA synthesis in control and IFN-β-pretreated cells (Fig. 3A, lanes 1 and 2, respectively) as measured by HBV-specific Northern blot analysis of total cellular RNA harvested 36 h after dox addition. As expected, dox-induced pgRNA expression in the control cells resulted in the accumulation of high levels of HBV RNA-containing capsids, as measured by HBV-specific Northern blot analysis of nuclease-resistant cytoplasmic RNA (Fig. 3B, lane 1). In contrast, when TREHBV-V cells were pretreated with IFN-β, accumulation of HBV RNA-containing capsids was inhibited 8-fold in TREHBV-V cells when compared with the control cultures (Fig. 3B, compare lane 2 with 1). Similar pretreatment experiments using IFN-γ were not informative, because IFN-γ interfered with dox-induced expression of HBV pgRNA as well as GFP mRNA from...
the bidirectional TRE-promoter in TREHBV-V cells (data not shown). Nevertheless, the results presented in Fig. 3 demonstrate that the TREHBV-V cells respond to IFN and suggest that IFN-β inhibits HBV replication by preventing the formation of pgRNA-containing capsids. Accordingly, the TREHBV-V cells were used in the next series of experiments to study the effect of IFN on preformed HBV RNA-containing capsids.

**IFN Does Not Shorten the Half-Life of Preformed HBV RNA-Containing Capsids.** To determine whether IFNs reduce the half-life of HBV RNA-containing capsids, we monitored their effect on the cellular content of preformed HBV RNA-containing capsids under conditions that prevent further capsid formation and maturation. HBV pgRNA expression was induced in TREHBV-V cells by addition of dox (1 μg/ml) to the culture medium for 4 days (Fig. 4 A and D, lane 1) to produce a pool of HBV RNA-containing capsids (Fig. 4 B and E, lane 1). At this time (0 h), dox was withdrawn from the culture medium to prevent further pgRNA expression and thus formation of HBV RNA-containing capsids. As shown in Fig. 4 A and D, total HBV pgRNA was reduced at 24 h (Fig. 4 A and D, lane 2) and virtually undetectable at 84 h (Fig. 4 A and D, lane 3) after dox removal, as determined by HBV-specific Northern blot analysis of total cellular RNA. However, at this time, there was still a pool of nuclease-resistant cytoplasmic HBV RNA present in the cells (Fig. 4 B and E, lane 3) reflecting capsids containing full length pgRNA (high molecular-weight species) and those undergoing reverse transcription (lower molecular-weight species). At this time (84 h after dox removal), IFN-β or IFN-γ (200 units/ml) was added to the culture medium of the TREHBV-V cells to determine their impact on the rate at which the preformed HBV RNA-containing capsids disappeared. As shown in Fig. 4 B and E (lanes 4–11), the absolute levels of all species of encapsidated HBV RNA decreased over time, but there was no difference between the IFN-β (Fig. 4 B) or IFN-γ (Fig. 4 E) treated and untreated control cells at any given time point, suggesting that the normal turnover rate of the preformed capsids (including natural decay and maturation through reverse transcription) was not changed. To rule out the possibility that residual HBV DNA replicative intermediates interfered with the analysis of the encapsidated RNA, fractions of the isolated nucleic acids were subjected to RNase digestion before Northern blot analysis. The absence of any HBV-specific signal by Northern blotting after RNase digestion confirmed that the isolated nucleic acids represented encapsidated RNA (data not shown). Because IFN-β and -γ signaling occurred, as shown by the induction of 2′,5′ OAS (Fig. 4 C, lanes 5, 7, 9, and 11) and the small GTPase TGTP (Fig. 4 F, lanes 5, 7, 9, and 11), respectively, these results demonstrate that neither IFN-β nor IFN-γ treatment accelerated the degradation of HBV RNA-containing capsids. Together with the results shown in Fig. 3, these results suggest that IFN-β and -γ induce a cellular mechanism that interferes with the formation of replication-competent HBV RNA-containing capsids, whereas it has no effect on their degradation.

**Discussion**

This study was triggered by our previous work showing that replication-competent HBV RNA-containing capsids are the target of IFN-β-induced antiviral mechanisms in the liver of HBV transgenic mice (13) and in hepatocytes derived from these mice (14). Those experiments, however, did not elucidate whether inflammatory cytokines prevented the assembly or accelerated the degradation of these RNA-containing capsids. By establishing a dox-inducible HBV gene expression system that permits conditional accumulation of HBV RNA-containing capsids, we were able to demonstrate that IFN-β prevented the accumulation of HBV RNA-containing capsids when administered before and during the induction of HBV gene expression.

Moreover, IFN-β and -γ had no effect on the turnover rate of preformed HBV RNA-containing capsids when further capsid formation was prevented. Together, these results demonstrate that IFN-β and -γ interrupt the HBV life cycle by interfering with the assembly of replication competent (i.e., HBV RNA-containing) capsids.

![Figure 4](https://www.pnas.org/cgi/doi/10.1073/pnas.0504273102)
Nonspecific inhibition of HBV replication in the liver of HBV transgenic mice often occurs under conditions that induce TNF-α together with IFN-γ, and thus TNF-α could also, theoretically, inhibit HBV replication (11). Although studies with immortalized HBV transgenic hepatocytes demonstrated that TNF-α alone did not mediate inhibition of HBV replication (14), it has recently been reported that activation of NF-κB by TNF-α impaired the formation and/or stability of cytoplasmic HBV capsids in the HepG2 human hepatoma cell line (22), suggesting that TNF-α might induce an antiviral activity similar to the one shown for IFN-β and -γ in this report, depending on the cell culture system used.

Formation of replication-competent HBV capsids is a complex process that requires the proper interaction of several viral and possibly cellular functions. For hepadnaviruses, it has been shown that assembly of replication-competent capsids requires the formation of a ribonucleoprotein complex with the viral polymerase in the proper conformation competent for RNA packaging and initiation of reverse transcription (27, 28), and a protein complex is also required for the packaging of the viral core protein, which initiates reverse transcription using a tyrosine residue as a primer (25, 26). Furthermore, the formation of the ribonucleoprotein complex is also required for the packaging of the viral RNA. In general, this results in the formation of replication-competent capsids. In particular, it has been shown that cellular chaperones are important for duck hepatitis B virus capsid formation and for the priming reaction that precedes reverse transcription (29, 38, 39). Therefore, IFN-mediated inhibition of assembly of replication-competent HBV capsids could occur by several nonexclusive mechanisms.

First, it is possible that an IFN-inducible hepatocellular protein sterically prevents core protein dimer and/or multimer association by directly sequestering core protein, as has been suggested for the apolipoprotein mRNA-editing enzyme APOBEC3G or APOBEC3F (30). However, inhibition of HBV by APOBEC3G or APOBEC3F has been observed only after overexpression of the corresponding proteins in human hepatoma cell lines (30, 31), because they express the APOBEC family genes not at all or only at very low levels (9, 30–33). Similarly, mAPOBEC3G, the murine homologue of APOBEC3G (34), is expressed only at extremely low levels in TREHBV-V and HBV-Met cells, and its expression is not induced by IFN-β or IFN-γ treatment (Table 2, which is published as supporting information on the PNAS web site), suggesting that APOBEC genes are not involved in the antiviral mechanism induced by IFN. Consistent with this notion, APOBEC3G and APOBEC3F expression in the liver of chimpanzees is also very low, and it is not induced during clearance of HBV infection as shown in Table 3, which is published as supporting information on the PNAS web site. Second, IFN could induce the activity of kinases and/or phosphatases that modulate the phosphorylation of specific residues in the HBV core protein whose phosphorylation status has been shown to influence the formation of replication-competent (i.e., pgRNA-containing) capsids (35–37). Third, it is possible that IFNs modulate the activity of cellular proteins that are required for formation of replication-competent capsids. In particular, it has been shown that cellular chaperones are important for duck hepatitis B virus capsid formation and for the priming reaction that precedes reverse transcription (29, 38, 39). Therefore, an IFN-mediated change in the activity and/or availability of cellular chaperones could prevent capsid formation. Fourth, it is also possible that the interaction of the viral polymerase with the ε sequence on the pgRNA is prevented by an IFN-inducible cellular factor.

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