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Tumor Necrosis Factor Alpha Negatively Regulates Hepatitis B Virus Gene Expression in Transgenic Mice†

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It is well known that several inflammatory cytokines can modulate hepatocellular gene expression in a complex physiological process known as the hepatic acute-phase response. Since hepatitis B virus (HBV) characteristically induces a vigorous lymphomononuclear inflammatory response in the liver during acute and chronic hepatitis, it is possible that hepatocellular HBV gene expression may also be modulated by one or more of the cytokines produced by these cells. Using bacterial lipopolysaccharide (LPS) as a surrogate inducer of inflammatory cytokines in vivo, we have tested this hypothesis in a transgenic mouse model system. In experiments with two independent transgenic mouse lineages that express the HBV envelope region under the control of either HBV or cellular promoters, we observed a 50 to 80% reduction in the hepatic steady-state content of a 2.1-kb HBV mRNA following administration of a single intraperitoneal dose of LPS. The regulatory influence of several inflammatory cytokines known to be induced by LPS was also examined in this system. The negative regulatory effect of LPS was consistently reproduced by the administration of a single nontoxic dose of tumor necrosis factor alpha, and it was occasionally observed following the administration of high doses of alpha interferon and interleukin-6, while no effect was detectable in response to high-dose interleukin-1 alpha or to gamma interferon. These observations suggest that tumor necrosis factor alpha and perhaps other cytokines may activate a heretofore unsuspected intracellular pathway that negatively regulates HBV gene expression. The intracellular mechanism(s) responsible for this effect and its pathophysiologic relevance remain to be elucidated.

Hepatitis B virus (HBV) causes a necroinflammatory liver disease characterized by an intrahepatic lymphomononuclear and polymorphonuclear cell infiltrate and evidence of hepatic macrophage activation (Kupffer cell hyperplasia) (26, 39). These classes of inflammatory cells produce an assortment of cytokines, such as tumor necrosis factor alpha (TNF-α), alpha interferon (IFN-α), gamma interferon (IFN-γ), interleukin-1 alpha (IL-1α), and interleukin-6 (IL-6), which mediate many of their inflammatory functions and also modulate the expression of several liver-specific genes in a coordinated reaction known as the hepatic acute-phase response (2, 5). The regulatory influence of these cytokines may be positive or negative; for example, the expression of metallothionein and serum amyloid A may be increased by 10- to 100-fold, whereas albumin gene expression is decreased by more than 50% in response to selected inflammatory cytokines (13, 21, 27, 42), and IL-6 has been shown to downregulate alpha-1-inhibitor III gene expression by 10- to 20-fold during the hepatic acute-phase response (1).

Since the HBV genome resides within the hepatocyte, either as an episome or integrated within cellular chromosomal DNA (38), it is possible that HBV expression might also be modulated by one or more of these inflammatory mediators. Though it is difficult to determine whether TNF-α and other inflammatory cytokines exert a direct antiviral effect, it is possible to examine the potential modulatory effect that they may have on HBV gene expression by stimulating the intrahepatic inflammatory response in HBV-transgenic mice. To pursue this line of investigation further, HBV gene expression was monitored following the administration of bacterial lipopolysaccharide (LPS) (a potent stimulant of cytokine generation in vivo [14]) and the recombinant cytokines TNF-α, IFN-α, IFN-γ, IL-1α, and IL-6 to several lineages of HBV-transgenic mice.

Transgenic mice. Two generically different categories of HBV-transgenic mice were used in this study. The first category consists of a lineage that contains the HBV *Bgl*IIa fragment (ayw subtype) under the control of the mouse metallothionein promoter (official designation Tg[MT-1, HBV]Bri28; designated lineage 23-3 herein). The construction and characteristics of this lineage have been described previously (9, 10). The second category consists of the independently derived lineage 80-219, which was produced by microinjection of a 13-kb *Hin*dIII-*Pst*I fragment of plasmid pFC80, which contains a tandem head-to-tail tetramer of the entire HBV genome (ayw subtype), originally derived from plasmid pCP10 (8), which was generously provided by P. Tiollais. Restriction endonuclease analysis (not shown) reveals that this lineage of transgenic mice carried at least one full-length copy of the microinjected construct integrated into a single site in the mouse genome. Total RNA was extracted from frozen tissue that was mechanically pulverized and solubilized by the acid-guanidinium-phenol-chloroform method (11). Hepatic RNA (40 μg) was electrophoretically fractionated in 0.8% formaldehyde–agarose gels, blotted by capillary transfer, and fixed onto nylon membranes (Hybond-N; Amersham, Arlington Heights, Ill.). Membranes were prehybridized at 42°C for 4 to 24 h in 50% formamide–5× SSPE–2× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–200 μg of denatured salmon sperm DNA per ml (40) and hybridized in the same solution plus 10% dextran sulfate for 18 to 24 h at 42°C. The nylon

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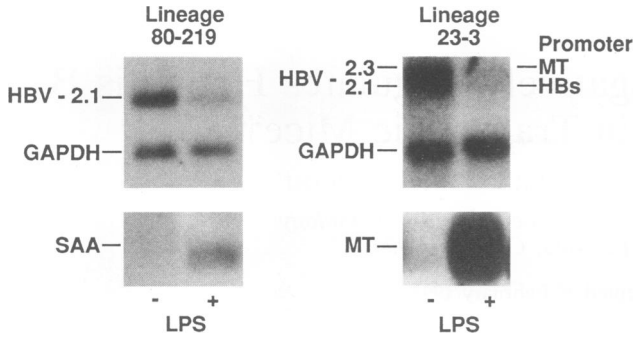

FIG. 1. LPS negatively regulates hepatic HBV 2.1-kb steady-state mRNA level in transgenic mice. HBV-positive transgenic offspring were routinely identified by detection of circulating HBsAg as described previously (9, 10). Endotoxin-treated transgenic mice (+) received a single intraperitoneal injection of LPS (*Escherichia coli*; serotype O127:B8; lot 764035; 1.6×10^7 endotoxin units [EU] per mg in the *Limulus* amebocyte lysate assay [LAL]; Sigma Chemical Co., St. Louis, Mo.) diluted in Dulbecco's phosphate-buffered saline (0.9 EU/ml, lot119F-4617; Sigma); 200 μg of LPS was administered to lineage 80-219 and 50 μg of LPS was administered to lineage 23-3. Heterozygous age-, sex-, and serum HBsAg-matched transgenic littermate controls were treated with diluent only (−). Sixteen hours following LPS treatment, mice were anesthetized (Metofane; Pitman-Moore, Mundelein, Ill.) and killed by cervical dislocation. The left hepatic lobe was immediately removed, snap frozen in liquid nitrogen and stored at −70°C. Shown are Northern blots of two pairs of mice (LPS treated and diluent treated) representative of the effect observed in 10 of 14 animals of lineage 80-219 and 4 of 4 mice of lineage 23-3 treated with LPS. Filters were hybridized with HBV-, metallothionein I (MT)-, serum amyloid A (SAA)-, and GAPDH-specific probes as described in the text.

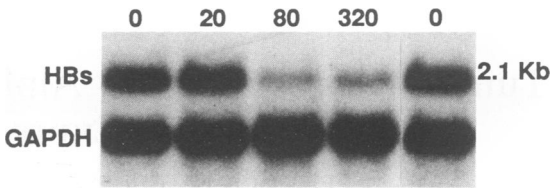

FIG. 2. TNF-α negatively regulates hepatic HBV 2.1-kb steady-state mRNA level in a dose-dependent manner in HBV-transgenic lineage 80-219. Transgenic mice (lineage 80-219) received a single intraperitoneal injection of 20,000, 80,000, or 320,000 U of recombinant murine TNF-α (lot 4296-17; specific activity, 1.2×10^6 U/mg [L929 assay]; 0.98 mg/ml, 0.511 EU/ml [LAL]) diluted in 10% autologous mouse serum in sterile nonpyrogenic 0.9% sodium chloride injection (USP; lot 51-023-DK; <0.25 EU/ml [LAL assay]; Abbott Laboratories); liver tissue was obtained 20 h after treatment. Shown is a Northern blot containing (far left) hepatic steady-state total RNA pooled from 10 saline-treated female transgenic mice (first lane 0), RNA pooled from two females treated with 20,000 U of TNF-α, RNA pooled from two females treated with 80,000 U of TNF-α, representative of the effect observed in 8 of 8 mice, RNA from a female treated with 320,000 U of TNF-α, and RNA pooled from two female transgenic littermates administered saline simultaneously with TNF-α-treated animals (second lane 0). Filters were hybridized with HBV- and GAPDH-specific probes as described in the text.

filters were hybridized to radiolabeled [^{32}P]DNA probes of specific activities approaching 10^9 dpm/μg that were synthesized from random oligonucleotide-primed plasmids containing the cloned sequences of the entire HBV genome (pFC80), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (20), and metallothionein I (42). Membranes were washed three times for 30 min each in 0.1× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C according to the standard protocol and exposed onto X-ray film (Eastman Kodak, Rochester, N.Y.). Radioactive probes were removed from nylon membranes by washing several times in distilled water at 95°C prior to reprobing. Northern (RNA blot) analysis (see below) reveals that the only HBV-derived mRNA in lineage 80-219 is a 2.1-kb species corresponding to the expected size of the transcript originating from the pre-S2 promoter (HBs). This transcript codes for the major envelope antigen (HBV surface antigen [HBsAg]), which was detectable at 4 to 8 μg/ml in the serum of these animals (Ausria II; Abbott Lab., North Chicago, Ill.). In addition to the 2.1-kb transcript, lineage 23-3 also produces a 2.3-kb transcript encoding additional in-frame upstream codons containing the pre-S1 antigen and shown to be controlled by the mouse metallothionein I promoter (9). No significant variation in baseline expression of hepatic HBV steady-state mRNA was detected among heterozygous age-, sex-, and serum HBsAg-matched transgenic offspring.

LPS negatively regulates hepatic HBV 2.1-kb steady-state mRNA level. As estimated by Northern blot hybridization, hepatic HBV steady-state 2.1-kb mRNA (HBsAg) expression was significantly reduced in both transgenic lineages 16 h following LPS administration compared with age-, sex-, and serum HBsAg-matched control littermates treated with diluent only (Fig. 1). Densitometric scanning of Northern autoradiographs and counting of Cerenkov radiation (40) on dry hybridization membranes indicated that hepatic HBV 2.1-kb steady-state mRNA expression was reduced by more than 50% in both lineages 16 h following LPS treatment. The reduction in HBV gene expression coincided with the induction of the hepatic acute-phase response, as assessed by the simultaneous increase in the steady-state content of serum amyloid A and metallothionein mRNA (Fig. 1). The housekeeping gene GAPDH was used to normalize each lane for the amount of RNA bound to the membrane. Similar to serum amyloid A and albumin, as reported previously for many acute-phase genes, HBV gene expression was transiently modulated following LPS injection, with an initial lag period of approximately 12 h, followed by maximal reduction between 16 and 24 h and a return to baseline by 48 h (not shown). Accordingly, all studies were performed between 16 and 20 h postinjection.

Although some mouse-to-mouse variation was observed, most (10 of 14) of the animals in lineage 80-219 and all of the animals (4 of 4) in lineage 23-3 responded to LPS with a reduction in expression of these HBV transcripts. Importantly, the decrease in HBV mRNA content was observed in the absence of hepatocellular injury, which was assessed by measurement of serum glutamic pyruvic transaminase (SGPT) activity, a sensitive indicator of liver cell injury (49). The average SGPT activity of the 14 mice in lineage 80-219 prior to LPS administration was 33 ± 1 (standard error of the mean) U/liter, whereas at 16 h post-LPS injection, the average SGPT activity was 46 ± 2 U/liter. Similarly, SGPT activity was unchanged (n = 2; pre-LPS = 16 ± 4 U/liter; post-LPS = 22 ± 2 U/liter) in lineage 23-3 following LPS challenge. It is important to note the increase in the steady-state content of the endogenous mouse metallothionein mRNA following LPS injection in lineage 23-3 even though the 2.3-kb HBV mRNA level, regulated by the mouse metallothionein promoter in the transgene, was simultaneously decreased in these animals (Fig. 1). Furthermore,

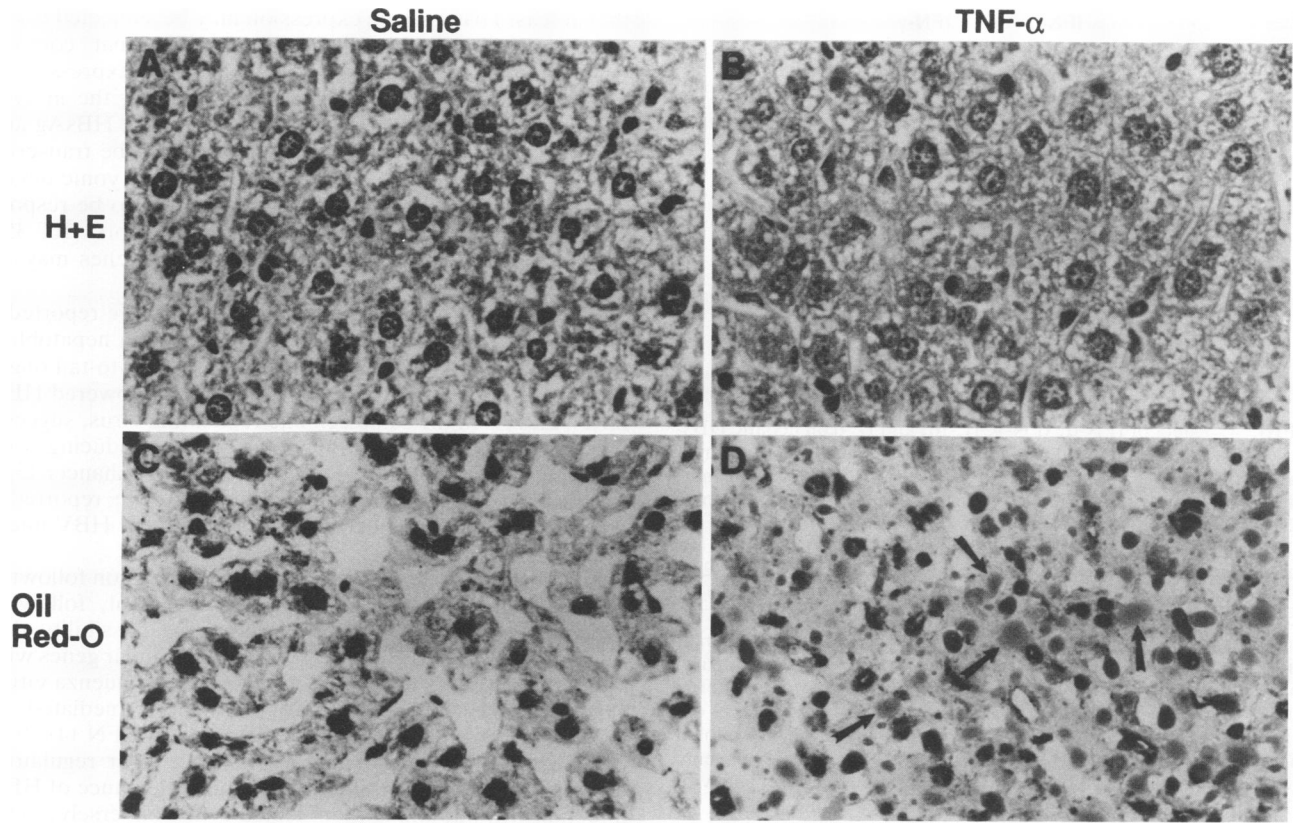

FIG. 3. Histological analysis following TNF-α administration in HBV-transgenic lineage 80-219. (Top) Hematoxylin and eosin (H+E) staining of liver tissue obtained from the same saline- and TNF-α (80,000 U)-treated transgenic liver samples as shown in Fig. 2. (Bottom) Intracellular lipid content (arrows) was revealed by oil red O staining of frozen liver sections. Magnification, ×200.

preliminary examination of several newly derived HBV-transgenic lineages indicated that the levels of the 2.4-kb and 3.5-kb transcripts originating from the viral pre-S1 and core promoters are similarly decreased following LPS administration. These findings will be reported separately.

TNF-α negatively regulates hepatic HBV 2.1-kb steady-state mRNA in a dose-dependent manner. In order to determine whether any of the inflammatory cytokines known to be induced by LPS might be involved as mediators of this effect, hepatic HBV mRNA was monitored in mice of lineage 80-219 at various time intervals following the administration of recombinant TNF-α, IFN-α, IFN-γ, IL-6, and IL-1α. As illustrated in Fig. 2, recombinant murine TNF-α induced a significant (approx. 70%, as determined by densitometric scanning) reduction in the 2.1-kb HBV mRNA level in nine of nine mice 16 to 20 h after they were injected with a single intraperitoneal dose of 80,000 U of TNF-α per mouse. Pilot kinetic analysis indicated lesser reductions at 6 and 12 h postinjection.

The decrease in HBV gene expression following TNF-α administration was associated with minimal elevation of SGPT activity (n = 7; pre-TNF = 27 ± 6 U/liter; post-TNF = 94 ± 13 U/liter), which was associated with microvesicular fatty change in the hepatocyte cytoplasm (Fig. 3), consistent with the known effects of TNF-α on lipid metabolism (19). Since there was no evidence of hepatocellular necrosis or an inflammatory infiltrate following TNF-α administration and because the degree of reduction in steady-state HBV mRNA content is greatly out of proportion to the minor histopathological and biochemical changes, we conclude that the decrease in HBV mRNA content is due to a change in the transcriptional program in the hepatocyte rather than a nonspecific consequence of liver cell injury.

The mechanism(s) by which LPS and TNF-α modulate HBV gene expression in lineages 80-219 and 23-3 is unknown. However, since the 2.3-kb HBV transcript, which has been shown to be driven by the murine metallothionein promoter in transgenic lineage 23-3 (9), is dramatically reduced even though the endogenous metallothionein transcript is greatly increased in these livers following LPS administration (Fig. 1), it is possible that posttranscriptional events are responsible for the decreased HBV steady-state mRNA level observed in both lineages in this study. It is germane that the 2.1-kb and 2.3-kb HBV transcripts are identical for most of their length and terminate at the same polyadenylation signal, so they are both susceptible to events that might influence transcript elongation, processing, and termination. However, since all transgenes used contain both HBV enhancers and the HBV pre-S2 promoter, the possibility of transcriptional control at least in part cannot be excluded. Further studies will be necessary to examine this important issue.

Although we cannot be sure that TNF-α is principally responsible for the reduction of HBV mRNA levels following LPS administration, TNF-α is known to be the major mediator of LPS activity in many other systems (6). Future studies designed to explore the ability of anti-TNF-α anti-

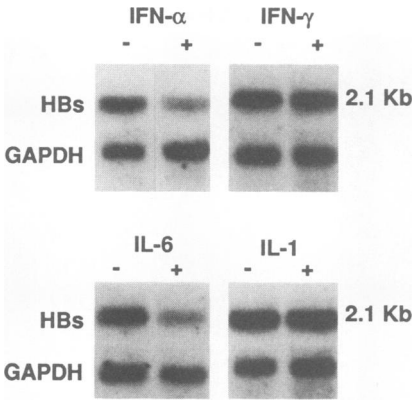

FIG. 4. Effect of IFN-α, IFN-γ, IL-1α, and IL-6 on hepatic HBV 2.1-kb steady-state mRNA level in transgenic lineage 80-219. Shown are Northern blots of total hepatic steady-state mRNA from individual transgenic mice 20 h after a single intraperitoneal injection of either 500,000 U of recombinant human IFN-α A/D (lot 102AA; 6.4 × 107 U/ml; L929 cell-vesicular stomatitis virus assay), 500,000 U of recombinant murine IFN-γ (lot M3-RD48; specific activity, 5.2 × 10^6 U/mg; 1.1 mg/ml, 10 pg of endotoxin per ml), 500,000 U of recombinant human IL-6 (batch PPG9001; specific activity, 52 × 10^6 U/mg; 2.5 mg/ml, 0.4 EU/mg [LAL]), or 500,000 U of recombinant human IL-1α (lot 2/88; specific activity, 3 × 10^8 U/mg; 0.68 mg/ml, 0.5 EU/ml [LAL]) diluted in 10% autologous mouse serum in sterile nonpyrogenic 0.9% sodium chloride; the lanes adjacent to the treated samples contain total hepatic steady-state mRNA pooled from 10 heterozygous age-, sex-, and HBsAg-matched transgenic littermates which received diluent only. Filters were hybridized with HBV- and GAPDH-specific probes as described in the text.

bodies to neutralize the effect of LPS on HBV gene expression will be necessary to examine this possibility.

Effect of IFN-α, IFN-γ, IL-6, and IL-1α on hepatic HBV 2.1-kb steady-state mRNA. The potential modulatory effect that other inflammatory cytokines might have on HBV gene expression was examined by administration of recombinant human IFN-α (7), murine IFN-γ (31, 46), human IL-1α (32), and human IL-6 (30) at doses which increase hepatic acute-phase gene expression in lineage 80-219 and are known to exert biological activity in other murine models (13, 30, 48, 54). As presented in Fig. 4, single intraperitoneal injections of recombinant human IFN-α (500,000 U) and recombinant human IL-6 (500,000 U) also suppressed hepatic HBV 2.1-kb steady-state mRNA expression 16 to 20 h after administration without biochemical evidence of liver cell injury (post-IFN-α SGPT = 16 U/liter; post-IL-6 SGPT = 26 U/liter). In contrast, recombinant murine IFN-γ (500,000 U) and recombinant human IL-1α (500,000 U) did not suppress HBV gene expression when examined between 12 and 20 h after cytokine administration. However, pilot experiments indicated that exceedingly high doses (10^6 U) of IL-1α and IFN-γ will sporadically reduce hepatic HBV steady-state mRNA levels in occasional animals (not shown), although it is difficult to interpret the significance of these results, since such observations might merely reflect secondary or tertiary events related to IL-1α- and IFN-γ-induced TNF-α production in vivo (17).

The results presented in this report indicate that several soluble mediators of the inflammatory response, but especially TNF-α, negatively regulate HBV gene expression in vivo. The coincident decrease in expression of the 2.1-kb HBV transcript and induction of the hepatic acute-phase response following LPS and TNF administration indicate that at least HBsAg gene expression may be subjected to the same transcriptional and/or posttranscriptional controls which modulate the expression of albumin, the expression of which is decreased by more than 50% during the in vivo hepatic acute-phase response (2, 5). Moreover, HBsAg and albumin gene expression have been shown to be transcriptionally activated at the same time during embryonic development in HBV-transgenic mice (15, 18) and to be responsive to many of the same transcription factors (16, 35), suggesting that the expression of these two genes may be regulated by a common pathway.

In keeping with our observations, others have reported a block in HBV replication by IFN-α in a human hepatoblastoma cell line (HepG2) transfected with a head-to-tail oligomer of the HBV genome and that IFN-α also lowered HBV mRNA levels in the same cell line replicating virus, suggesting that IFN-α inhibits HBV replication by reducing transcription of viral genes driven by the HBV enhancer (52). Furthermore, preliminary clinical studies have reported a possible therapeutic effect of TNF-α in chronic HBV infection (12, 44).

The observed decrease in HBV gene expression following TNF-α administration and, to a lesser extent, following treatment with IL-6 and IFN-α raises the possibility that these cytokines activate the expression of cellular genes with antiviral potential reminiscent of the state of influenza virus-specific "intracellular immunity," which is mediated by induction of cellular *Mx* gene expression by IFN (4). It is conceivable that TNF-α may stimulate cellular regulatory mechanisms that contribute to the natural clearance of HBV infection without killing the infected cell. Conversely, these cytokines may contribute to HBV persistence by causing the decreased expression of viral proteins which otherwise would serve as targets for antigen-specific clearance by cytotoxic T cells. Both of these alternatives are speculative at present.

Although we do not understand the mechanism for the negative regulatory effect of TNF-α on HBV gene expression, this cytokine is known to modulate the expression of many cellular genes. For example, TNF-α transcriptionally activates a number of proto-oncogenes (36) as well as certain cytokines (27, 29), ferritin (51), major histocompatibility complex genes (24, 53), and several hepatic acute-phase genes (36, 45). In contrast, TNF-α is known to inhibit expression of albumin (3), collagen (47), surfactant (54), and genes involved in lipogenesis, such as acetyl coenzyme A carboxylase (34, 50). Although some of these effects appear to be mediated via posttranslational activation of a *trans*-acting factor, NF-κB (33), the mechanism(s) by which TNF-α exerts the majority of its regulatory effects is not known.

The current observations suggest that TNF-α, and perhaps other cytokines, may play a heretofore unsuspected role in HBV biology and pathogenesis. Further studies will be necessary to establish the molecular basis and pathophysiological relevance of this effect.

The MT-HBVenv transgenic mouse lineage 23-3 used in these studies was produced as part of a collaboration between F.V.C. and Ralph Brinster (University of Pennsylvania, Philadelphia, Pa.) and Richard Palmiter (University of Washington, Seattle). HBV-transgenic mouse lineage 80-219 was developed at the Scripps Research Institute transgenic mouse facility.

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