

## In Vivo Regulation of Hepatitis B Virus Replication by Peroxisome Proliferators†

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**The role of the peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) in regulating hepatitis B virus (HBV) transcription and replication in vivo was investigated in an HBV transgenic mouse model. Treatment of HBV transgenic mice with the peroxisome proliferators Wy-14,643 and clofibric acid resulted in a less than twofold increase in HBV transcription rates and steady-state levels of HBV RNAs in the livers of these mice. In male mice, this increase in transcription was associated with a 2- to 3-fold increase in replication intermediates, whereas in female mice it was associated with a 7- to 14-fold increase in replication intermediates. The observed increases in transcription and replication were dependent on PPAR $\alpha$ . HBV transgenic mice lacking this nuclear hormone receptor showed similar levels of HBV transcripts and replication intermediates as untreated HBV transgenic mice expressing PPAR $\alpha$  but failed to demonstrate alterations in either RNA or DNA synthesis in response to peroxisome proliferators. Therefore, it appears that very modest alterations in transcription can, under certain circumstances, result in relatively large increases in HBV replication in HBV transgenic mice.**

As the host range of hepatitis B virus (HBV) is limited to humans and primates (26), the majority of studies analyzing the mechanisms of HBV transcriptional regulation and viral biosynthesis have been performed in cell culture systems. With cell culture systems, it has been demonstrated that the 3.2-kb viral genome can encode four viral transcripts (3, 33, 35, 38, 41). The levels of the 3.5-, 2.4-, 2.1-, and 0.7-kb transcripts are regulated by the nucleocapsid, large surface antigen, major surface antigen, and X-gene promoters, respectively (29, 43). The 3.5-kb pregenomic transcript is translated into the HBV polymerase and nucleocapsid or core antigen (HBcAg) (24). The HBV polymerase binds to the  $\epsilon$  stem-loop structure at the 5' end of the pregenomic RNA, and the RNA plus polymerase complex is encapsidated by dimers of the core polypeptide, generating the immature capsid (17). The HBV polymerase reverse transcribes the pregenomic RNA within the capsid to produce minus-strand HBV DNA and then synthesizes a partial plus-strand HBV DNA, using the minus strand as the template (39). Mature intracellular capsids containing the 3.2-kb partially double-stranded HBV DNA bind to the envelope antigen (HBsAg) in the membrane of the endoplasmic reticulum and subsequently bud into the lumen of the endoplasmic reticulum (10, 18, 42). Mature virus is secreted from the hepatocyte after passing through the endoplasmic reticulum and Golgi apparatus, where the envelope polypeptides are glycosylated (1, 18, 34).

As the 3.5-kb pregenomic HBV RNA is the substrate for the synthesis of virion DNA, it is apparent that regulating the level of transcription of this RNA is likely to have a major influence on viral biogenesis. Consequently, it has been of interest to determine the mechanisms regulating the level of transcription from the nucleocapsid promoter. A variety of studies has iden-

tified the *cis*-acting sequences and *trans*-acting factors regulating the nucleocapsid promoter activity (4, 5, 14, 22, 40, 44, 47, 48). Several liver-enriched transcription factors, including C/EBP, hepatocyte nuclear factors 3 and 4, retinoid X receptor  $\alpha$  (RXR $\alpha$ ), and peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), and ubiquitous transcription factors including Sp1 and RFX1 have been shown to modulate nucleocapsid promoter activity in cell culture (2, 13, 16, 23, 25, 45, 46, 49). The observation that members of the nuclear hormone receptor family of transcription factors can modulate the activity of the nucleocapsid promoter suggested that transcription of the 3.5-kb pregenomic RNA and therefore replication might be influenced by the availability of the ligands for these nuclear hormone receptors. Evidence supporting this contention has been obtained in cell culture where the nuclear hormone receptor ligands 9-*cis* retinoic acid and clofibric acid, respectively, have been shown to increase the level of transcription from the nucleocapsid promoter in an RXR $\alpha$ - and PPAR $\alpha$ -dependent manner. RXR $\alpha$  and PPAR $\alpha$  mediate their effects through the peroxisome proliferator response elements (PPREs) located at nucleotide positions -28 to -16 in the nucleocapsid promoter and within the enhancer 1 region of the HBV genome (25).

In this study, these observations have been extended to an in vivo model system of HBV viral replication. By using an HBV transgenic mouse model system of viral replication (12) and the PPAR $\alpha$ -null mouse (21), it has been possible to examine the effects of two ligands for PPAR $\alpha$  on viral transcription and replication in the presence or absence of the nuclear hormone receptor PPAR $\alpha$ . This analysis demonstrated that these ligands activated transcription from responsive cellular promoters in a PPAR $\alpha$ -dependent manner, whereas they produced concomitantly limited alterations in the levels of HBV RNAs. Despite these limited effects on HBV transcription, female HBV transgenic mice demonstrated PPAR $\alpha$ -dependent 7- to 14-fold increases in viral replication, indicating that modest changes in the level of HBV transcription can be associated

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TABLE 1. Effect of PPAR $\alpha$  on HBeAg synthesis in HBV transgenic mice

Gender	PPAR $\alpha$ genotype	No. of mice	Mean (SD) HBeAg $A_{492}$ <sup>a</sup>
Male	+/-	9	0.56 (0.22)
	-/-	10	0.62 (0.18)
Female	+/-	8	0.37 (0.19)
	-/-	15	0.44 (0.19)

<sup>a</sup> Twenty-microliter volumes of serum were assayed for HBeAg by using the Abbott Laboratories HBe enzyme immunoassay.

with dramatic effects on viral replication intermediates under certain circumstances.

#### MATERIALS AND METHODS

**Transgenic and knockout mice.** The production and characterization of the HBV transgenic mouse lineage 1.3.32 have been described elsewhere (12). These HBV transgenic mice contain a single copy of the terminally redundant, 1.3-genome-length copy of the HBV genome integrated into the mouse chromosomal DNA. High levels of HBV replication occur in the livers of these mice. The mice used in the breeding experiments were homozygous for the HBV transgene and were maintained on the C57BL/6 genetic background.

The production and characterization of the PPAR $\alpha$ -null mice have been described elsewhere (21). These mice do not express PPAR $\alpha$  and are refractory to the pleiotropic effects of peroxisome proliferators. These mice do not display any gross phenotypic defects and are viable and fertile. The mice used in the breeding experiments were maintained on the Sv/129 genetic background.

PPAR $\alpha$ -null HBV transgenic mice were generated by mating the HBV transgenic mice with the PPAR $\alpha$ -null mice. The resulting F<sub>1</sub> mice were subsequently mated with the PPAR $\alpha$ -null mice, and the F<sub>2</sub> mice were screened for the HBV transgene and PPAR $\alpha$  null allele by PCR analysis of tail DNA. Tail DNA was prepared by incubating 1 cm of tail in 500  $\mu$ l of 100 mM Tris hydrochloride (pH 8.0)–200 mM NaCl–5 mM EDTA–0.2% (wt/vol) sodium dodecyl sulfate containing 100  $\mu$ g of proteinase K per ml for 16 to 20 h at 55°C. Samples were centrifuged at 14,000 rpm in a microcentrifuge for 5 min, and the supernatant was precipitated with 500  $\mu$ l of isopropanol. DNA was pelleted by centrifugation at 14,000 rpm in a microcentrifuge for 5 min and subsequently dissolved in 100  $\mu$ l of 5 mM Tris hydrochloride (pH 8.0)–1 mM EDTA. The HBV transgene was identified by PCR analysis using oligonucleotides XpHNF4-1 (TCGA TACCTGAACCTTTACCCCGTTGCCCG; HBV coordinates 1133 to 1159) and CpHNF4-2 (TCGAATTGCTGAGAGTCCAAGAGTCTCTT; HBV coordinates 1683 to 1658) plus 1  $\mu$ l of tail DNA. The samples were subjected to 32 amplification cycles involving denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension from the primers at 72°C for 2 min. A PCR product of 551 bp indicated the presence of the HBV transgene. The PPAR $\alpha$  null allele was identified by PCR analysis using the oligonucleotides mPPAR1 (CCTGGCCCTTCTAAACATAGG; 5' sequence of exon 8) and mPPAR2 (TCCCTGCTCTCC TGTATGGG; 3' sequence of exon 8) plus 1  $\mu$ l of tail DNA. The samples were subjected to 35 amplification cycles involving denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension from the primers at 72°C for 2 min. A PCR product of 319 bp indicated the wild-type PPAR $\alpha$  genotype, whereas a PCR product of 1.4 kbp indicated the mutated PPAR $\alpha$  genotype. The 20- $\mu$ l

reaction conditions used were as described by the manufacturer (Boehringer Mannheim) and contained 2.5 U of *Taq* DNA polymerase.

**HBV DNA and RNA analysis.** Total DNA and RNA were isolated from livers of HBV transgenic mice as described elsewhere (6, 28). DNA (Southern) and RNA (Northern) filter hybridization analyses were performed with 20  $\mu$ g of *Hind*III-digested DNA and 10  $\mu$ g of total cellular RNA, respectively, as described previously (28). Filters were probed with <sup>32</sup>P-labeled HBV genome DNA (9) to detect HBV sequences, the human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) cDNA (36) to detect the *GAPDH* mRNA, and the rat cytochrome P450 4A1 cDNA (21) to detect the *CYP4A* mRNA.

RNAse protection assays were performed with a PharMingen Riboquant kit, and riboprobes were synthesized by using an Ambion Maxiscript kit as described by the manufacturers. Transcription initiation sites for the 3.5-kb HBV transcripts were examined by using 20  $\mu$ g of total cellular RNA and a 333 (HBV coordinates 1990 to 1658)-nucleotide-long <sup>32</sup>P-labeled HBV riboprobe.

Nuclei were prepared from mouse livers by a modification of a previously described procedure (30). Briefly, liver samples were homogenized in a Potter-Elvehjem tissue grinder in 5 ml of 10 mM HEPES (pH 7.9)–25 mM KCl–1.0 mM EGTA–1.0 mM EDTA–0.32 M sucrose–0.15 mM spermine–0.5 mM spermidine–1.0 mM dithiothreitol (DTT)–0.5 mM phenylmethylsulfonyl fluoride (PMSF) containing leupeptin (0.5  $\mu$ g/ml), aprotinin (1.0  $\mu$ g/ml), and pepstatin (1.0  $\mu$ g/ml) (buffer A). Samples were diluted with 10 ml of 10 mM HEPES (pH 7.9)–25 mM KCl–1.0 mM EGTA–1.0 mM EDTA–2.0 M sucrose–0.15 mM spermine–0.5 mM spermidine–1.0 mM DTT–0.5 mM PMSF containing leupeptin (0.5  $\mu$ g/ml), aprotinin (1.0  $\mu$ g/ml), and pepstatin (1.0  $\mu$ g/ml) (buffer B) and centrifuged over two 3.5-ml sucrose cushions of buffer B for 30 min at 24,000 rpm in a Beckman SW41 rotor at 4°C. The two nuclear pellets from each sample were resuspended together in 2.5 ml of buffer A. After the addition of 5 ml of buffer B, the samples were centrifuged over a 3.5-ml sucrose cushion of buffer B for 30 min at 24,000 rpm in a Beckman SW41 rotor at 4°C. The nuclei were resuspended in 1 ml of 20 mM Tris hydrochloride (pH 7.9)–75 mM NaCl–0.5 mM EDTA–50% glycerol–0.15 mM spermine–0.5 mM spermidine–0.85 mM DTT–0.125 mM PMSF containing leupeptin (0.5  $\mu$ g/ml), aprotinin (1.0  $\mu$ g/ml), and pepstatin (1.0  $\mu$ g/ml). Purified nuclei were stored in liquid nitrogen at approximately 10<sup>7</sup> nuclei per vial until used for nuclear run-on analysis.

Nuclear run-on analysis was performed as previously described (37). The labeled transcripts were hybridized to Hybond N (Amersham) filter strips containing 1 to 2  $\mu$ g of plasmid DNAs. The plasmids used contained the human *GADPH* cDNA insert (36), the rat acyl coenzyme A oxidase (*ACO*) cDNA (21), the rat cytochrome P450 4A1 cDNA (21) and the complete HBV genome (9). pBluescript SK(-) plasmid DNA (Stratagene) was used as a negative control.

Results of filter hybridization, RNase protection assays, and nuclear run-on analyses were quantitated by phosphorimaging using a Packard Cyclone storage phosphor system.

**HBV antigen analysis.** HBeAg analysis was performed with 20  $\mu$ l of mouse serum and the HBe enzyme immunoassay as described by the manufacturer (Abbott Laboratories). The level of antigen was determined in the linear range of the assay. Immunohistochemical detection of HBeAg in paraffin-embedded mouse liver sections was performed as previously described (12).

#### RESULTS

Previous *in vitro* and cell culture studies have demonstrated that the nuclear hormone receptor PPAR $\alpha$  can bind as a heterodimer with RXR $\alpha$  to PPREs in the enhancer 1 region and the nucleocapsid promoter of the HBV genome. As a result of the binding of these factors to their recognition sequences, the

TABLE 2. Effect of Wy-14,643 on HBeAg synthesis in HBV transgenic mice

Gender	PPAR $\alpha$ genotype	Treatment <sup>a</sup>	Mean (SD) liver size (% of total body wt)	Mean (SD) HBeAg $A_{492}$ <sup>b</sup>		Mean HBeAg fold induction (day 7 $A_{492}$ /day 0 $A_{492}$ )
				Day 0	Day 7	
Male	+/-	Control	5.73 (0.38)	0.78 (0.07)	0.71 (0.06)	0.91
	+/-	Wy	10.90 (0.59)	0.70 (0.08)	0.89 (0.07)	1.27 <sup>c</sup>
	-/-	Control	5.68 (0.45)	0.76 (0.08)	0.69 (0.00)	0.91
	-/-	Wy	6.17 (0.39)	0.77 (0.09)	0.66 (0.06)	0.86
Female	+/-	Control	4.08 (0.44)	0.48 (0.20)	0.37 (0.16)	0.77
	+/-	Wy	10.00 (0.13)	0.65 (0.05)	1.05 (0.19)	1.62 <sup>c</sup>
	-/-	Control	4.40 (0.51)	0.54 (0.02)	0.50 (0.14)	0.93
	-/-	Wy	5.02 (0.36)	0.55 (0.16)	0.43 (0.10)	0.78

<sup>a</sup> HBV transgenic mice (three mice per group) were fed normal rodent chow (control) or rodent chow containing 0.1% (wt/wt) Wy-14,643 (Wy).

<sup>b</sup> Twenty-microliter volumes of serum were assayed for HBeAg by using the Abbott Laboratories HBe enzyme immunoassay immediately before treatment (day 0) of the mice and on day 7 of treatment.

<sup>c</sup> Statistically significant by paired Student's *t* test (*P* < 0.05).

TABLE 3. Effect of clofibric acid on HBeAg synthesis in HBV transgenic mice

Gender	PPAR $\alpha$ genotype	Treatment <sup>a</sup>	Mean (SD) liver size (% of total body wt)	Mean (SD) HBeAg A <sub>492</sub> <sup>b</sup>		Mean HBeAg fold induction (day 14 A <sub>492</sub> /day 0 A <sub>492</sub> )
				Day 0	Day 14	
Male	+/-	Control	4.55 (0.63)	0.61 (0.04)	0.54 (0.06)	0.89
	+/-	CA	6.90 (0.36)	0.58 (0.04)	0.69 (0.08)	1.19 <sup>c</sup>
	-/-	Control	4.99 (0.58)	0.65 (0.03)	0.58 (0.04)	0.89
	-/-	CA	5.47 (0.07)	0.66 (0.05)	0.54 (0.08)	0.82
Female	+/-	Control	4.22 (0.39)	0.52 (0.05)	0.41 (0.03)	0.79
	+/-	CA	6.67 (0.27)	0.57 (0.14)	0.70 (0.09)	1.23
	-/-	Control	4.16 (0.16)	0.48 (0.04)	0.38 (0.07)	0.79
	-/-	CA	4.78 (0.36)	0.49 (0.06)	0.40 (0.12)	0.82

<sup>a</sup> HBV transgenic mice (three mice per group) were fed normal rodent chow (control) or rodent chow containing 0.5% (wt/wt) clofibric acid (CA).

<sup>b</sup> Twenty-microliter volumes of serum were assayed for HBeAg by using the Abbott Laboratories HBe enzyme immunoassay immediately before (day 0) treatment of the mice and on day 14 of treatment.

<sup>c</sup> Statistically significant by paired Student's *t* test (*P* < 0.05).

level of transcription from the nucleocapsid promoter can be modulated (15, 25). Alteration in the level of transcription from the nucleocapsid promoter would be expected to change the abundance of the pregenomic HBV RNA and consequently the level of viral replication. In an attempt to determine the role of PPAR $\alpha$  in modulating viral replication *in vivo*, we examined the effects of exogenous ligands for PPAR $\alpha$  on HBV transcription and replication in the liver of wild-type and PPAR $\alpha$ -null HBV transgenic mice.

**Effect of PPAR $\alpha$  on HBeAg synthesis in HBV transgenic mice.** The nucleocapsid promoter directs the expression of the

precore and pregenomic RNAs that are translated to produce HBeAg and the nucleocapsid polypeptide, respectively (26). Consequently, HBeAg expression is an indirect measure of nucleocapsid promoter activity. Therefore, analysis of HBeAg in the sera of HBV transgenic mice was initially used to examine the role of the nuclear hormone receptor PPAR $\alpha$  in regulation of HBV transcription from the nucleocapsid promoter.

HBV transgenic mice were bred with PPAR $\alpha$ -null mice, and HBV transgenic mice heterozygous (+/-) or homozygous (-/-) for the PPAR $\alpha$  null allele were identified in the F<sub>2</sub> generation. For these studies, HBV transgenic mice that were

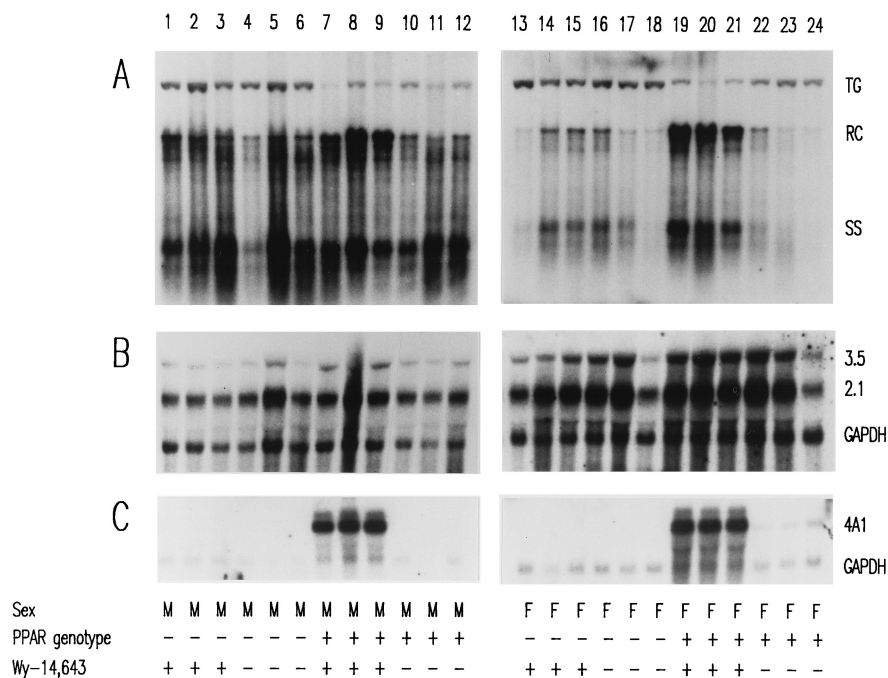


FIG. 1. DNA (Southern) and RNA (Northern) filter hybridization analysis of HBV DNA replication intermediates (A) and transcripts (B) in livers of HBV transgenic mice. Groups of three mice of each gender and genotype were fed rodent chow either with or without Wy-14,643 for 7 days before their livers were analyzed. Induction of the transcript detected by the rat cytochrome P450 4A1 cDNA (4A1) was used as a control to demonstrate the PPAR $\alpha$ -dependent activation of transcription by the peroxisome proliferator from a responsive gene. The *GAPDH* transcript was used as an internal control for quantitation of the HBV 3.5- and 2.1-kb RNAs (3.5 and 2.1). The HBV transgene was used as an internal control for quantitation of the HBV replication intermediates. The probes used were HBV<sub>g/w</sub> genomic DNA (A), HBV<sub>g/w</sub> genomic DNA plus *GAPDH* cDNA (B) and cytochrome P450 4A1 cDNA plus *GAPDH* cDNA (C). TG, HBV transgene; RC, HBV relaxed circular replication intermediates; SS, HBV single-stranded replication intermediates; M, male HBV transgenic mouse; F, female HBV transgenic mouse; PPAR genotype -, PPAR $\alpha$  knockout (-/-) mouse; PPAR genotype +, PPAR $\alpha$  heterozygous (+/-) mouse; Wy-14,643 +, HBV transgenic mouse fed 0.1% (wt/wt) Wy-14,643; Wy-14,643 -, HBV transgenic mouse fed normal rodent chow.





TABLE 5. Effect of clofibric acid<sup>a</sup> on HBV RNA and DNA synthesis in HBV transgenic mice

Gender	PPAR $\alpha$ genotype	Mean (SD) 2.1/ <i>GAPDH</i> ratio		Fold induction of HBV 2.1-kb RNA (+CA/-CA)	Mean (SD) 3.5/ <i>GAPDH</i> ratio		Fold induction of HBV 3.5-kb RNA (+CA/-CA)	Mean (SD) RI/TG ratio		Fold induction of HBV RI (+CA/-CA)
		-CA	+CA		-CA	+CA		-CA	+CA	
Male	+/-	2.24 (0.46)	2.29 (0.60)	1.02	0.48 (0.07)	0.54 (0.22)	1.12	21.30 (12.68)	56.67 (14.39)	2.66 <sup>b</sup>
	-/-	2.29 (0.38)	2.41 (0.36)	1.05	0.37 (0.06)	0.37 (0.05)	1.00	30.80 (17.46)	22.30 (5.16)	0.72
Female	+/-	2.59 (0.75)	4.61 (2.94)	1.78	0.30 (0.11)	0.50 (0.31)	1.67	9.05 (1.08)	69.35 (16.50)	7.66 <sup>b</sup>
	-/-	2.92 (1.16)	1.65 (0.40)	0.57	0.37 (0.14)	0.19 (0.03)	0.51	6.02 (3.30)	12.14 (8.25)	2.02

<sup>a</sup> HBV transgenic mice (three mice per group) were fed normal rodent chow (-CA) or rodent chow containing 0.5% (wt/wt) clofibric acid (+CA) for 14 days before the relative levels of the HBV 3.5- and 2.1-kb RNAs, *GAPDH* RNA, HBV transgene, and replication intermediate DNAs in the liver (Fig. 2) were measured. Other details are as for Table 4, but determined from Fig. 2.

<sup>b</sup> Statistically significant by Student's *t* test (*P* < 0.05).

hepatocytes and hepatocellular hyperplasia (27). Hepatocellular hypertrophy is the primary cause of hepatomegaly and results from a massive increase in peroxisomes within the hepatocytes (27). Treatment of HBV transgenic mice that were heterozygous for a functional PPAR $\alpha$  gene with Wy-14,643 for 7 days resulted in a 1.9- to 2.5-fold increase in the size of the liver (Table 2), whereas a 14-day treatment with clofibric acid resulted in a 1.5- to 1.6-fold increase in liver size (Table 3). Mice lacking a functional PPAR $\alpha$  gene failed to show this large increase in liver size, as expected from earlier results (21). Treatment of HBV transgenic mice with Wy-14,643 or clofibric acid resulted in a small PPAR $\alpha$ -dependent increase in serum HBeAg (Tables 2 and 3). The increase in serum HBeAg with peroxisome proliferators occurred in every PPAR $\alpha$  +/- HBV transgenic mouse examined and was somewhat greater in female HBV transgenic mice than in male HBV transgenic mice. HBV transgenic mice lacking PPAR $\alpha$  failed to show any increase in serum HBeAg in response to either peroxisome proliferator. These observations are consistent with the possibility

that activation of PPAR $\alpha$  in HBV transgenic mice results in increased transcription from the nucleocapsid promoter and therefore increased levels of the precore RNA.

**Effect of PPAR $\alpha$  on viral transcription and replication in HBV transgenic mice.** The HBV transgenic mice described above were also examined for the effects of the peroxisome proliferators on the steady-state levels of HBV transcripts and replication intermediates by analysis of the total liver RNA and DNA (Fig. 1 and 2). Induction of the PPAR $\alpha$ -responsive cytochrome P450 4A1 (*CYP4A*) mRNA by peroxisome proliferators confirmed that these compounds mediated PPAR $\alpha$ -dependent transcriptional activation of responsive genes in the HBV transgenic mice (Fig. 1C and 2C) (21). HBV transgenic mice lacking PPAR $\alpha$  failed to demonstrate increased levels of the *CYP4A* mRNA in response to peroxisome proliferator treatment, indicating that PPAR $\alpha$  is essential for this induction (Fig. 1C and 2C, lanes 1 to 3 and 13 to 15).

Analysis of the levels of the HBV 3.5- and 2.1-kb transcripts in the livers of HBV transgenic mice with and without PPAR $\alpha$

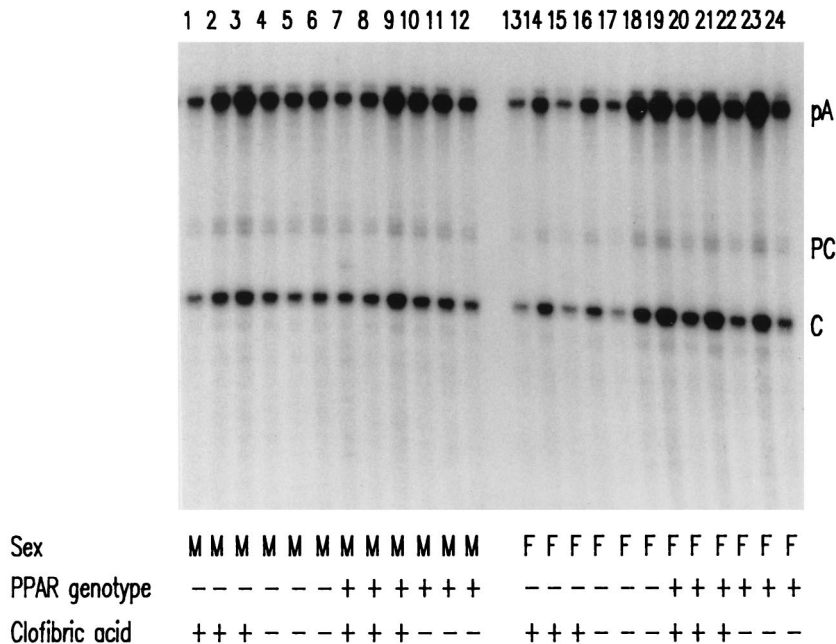


FIG. 3. RNase protection analysis mapping the transcription initiation sites of precore (PC) and pregenomic (C) transcripts from livers of HBV transgenic mice. The 3' ends of all HBV transcripts corresponding to the polyadenylation sites (pA) of these RNAs also generated a protected fragment in this analysis. Groups of three mice of each gender and genotype were fed rodent chow either with or without clofibric acid for 14 days before their livers were analyzed. The riboprobe used included the HBV<sub>ayw</sub> sequence spanning nucleotide coordinates 1990 to 1658. M, male HBV transgenic mouse; F, female HBV transgenic mouse; PPAR genotype -, PPAR $\alpha$  knockout (-/-) mouse; PPAR genotype +, PPAR $\alpha$  heterozygous (+/-) mouse; clofibric acid +, HBV transgenic mouse fed 0.5% (wt/wt) clofibric acid; clofibric acid -, HBV transgenic mouse fed normal rodent chow.

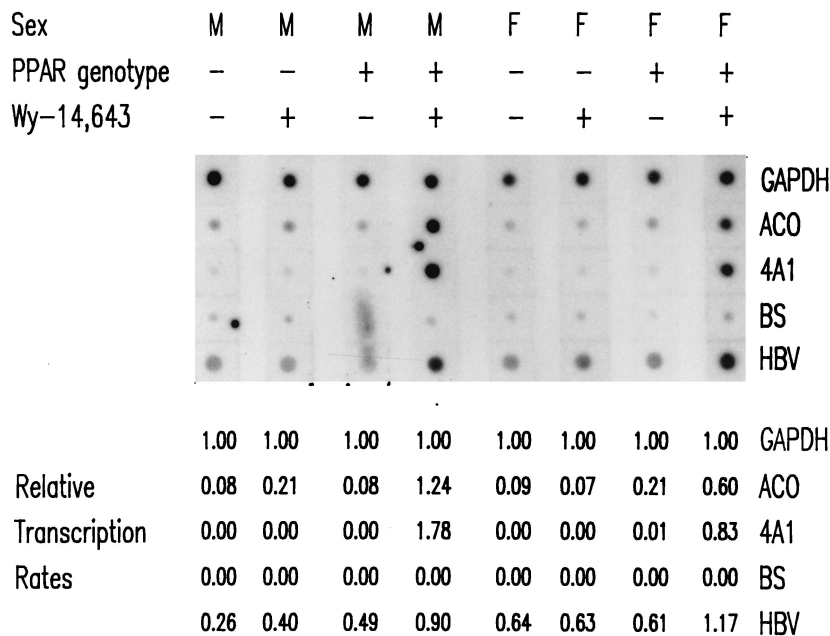


FIG. 4. Nuclear run-on analysis of HBV transcripts in livers of HBV transgenic mice. A mouse of each gender and genotype was fed rodent chow either with or without Wy-14,643 for 7 days before their livers were analyzed. Induction of the transcription rates of the RNAs detected by the rat *ACO* and cytochrome P450 4A1 (4A1) cDNAs were used as controls to demonstrate the PPAR $\alpha$ -dependent increase in transcription rates induced by the peroxisome proliferator from these responsive genes. The rate of transcription from the *GAPDH* gene was used as an internal control for quantitation of the relative transcription rates of the other genes analyzed. The rate of transcription of the *GAPDH* gene was designated 1.00. The nonspecific background rate of transcription detected with the pBluescript SK(-) plasmid (BS) control was designated 0.00. The rates of transcription for the HBV $\gamma$ w (HBV), *ACO*, and 4A1 genes were estimated relative to the *GAPDH* gene after subtracting the nonspecific background rate of transcription. The probes hybridized to each strip of filter were  $^{32}$ P-labeled transcripts isolated from in vitro-labeled mouse liver nuclei. The DNA on each strip of filter is indicated at the right. M, male HBV transgenic mouse; F, female HBV transgenic mouse; PPAR genotype -, PPAR $\alpha$  knockout (-/-) mouse; PPAR genotype +, PPAR $\alpha$  heterozygous (+/-) mouse; Wy-14,643 +, HBV transgenic mouse fed 0.1% (wt/wt) Wy-14,643; Wy-14,643 -, HBV transgenic mouse fed normal rodent chow.

in the presence or absence of peroxisome proliferators was performed. The steady-state levels of the HBV transcripts vary modestly under these different conditions (Fig. 1B and 2B; Tables 4 and 5). Most notably, the only consistent change in the levels of transcripts was a less than twofold induction of the HBV transcripts in the female PPAR $\alpha$  +/- HBV transgenic mice after treatment with peroxisome proliferators (Table 4 and 5). This was somewhat surprising considering the previous cell culture analysis demonstrating that transcription from the nucleocapsid promoter could be increased approximately fourfold by activation of RXR $\alpha$  and PPAR $\alpha$  by 9-*cis* retinoic acid and clofibric acid (25).

In addition to examining the HBV transcripts, the levels of the viral replication intermediates in the livers of these HBV transgenic mice were also determined (Fig. 1A and 2A; Tables 4 and 5). In the absence of peroxisome proliferators, PPAR $\alpha$  does not influence the level of replication intermediates in the livers of the HBV transgenic mice (Tables 4 and 5). In addition, it is apparent that there is an approximately fourfold-higher level of replication intermediates in male HBV transgenic mice than in female HBV transgenic mice. This difference in replication intermediates is qualitatively similar to the levels of HBeAg in the serum of male and female HBV transgenic mice (Table 1). Treatment of PPAR $\alpha$ -null HBV transgenic mice with peroxisome proliferators did not alter the level of replication intermediates observed in livers (Tables 4 and 5). In male HBV transgenic mice expressing PPAR $\alpha$ , a modest, two- to threefold increase in HBV replication intermediates was observed in response to treatment with peroxisome proliferators. However, in female PPAR $\alpha$  +/- HBV transgenic mice, replication intermediates increased approxi-

mately 14-fold with Wy-14,643 treatment and 7-fold with clofibric acid treatment. This effect was not observed in female PPAR $\alpha$ -null HBV transgenic mice treated with these PPAR $\alpha$  activators. Therefore, despite the modest increase in viral transcription observed in female PPAR $\alpha$  +/- HBV transgenic mice in response to peroxisome proliferators, a dramatic PPAR $\alpha$ -dependent increase in viral replication intermediates is observed in the livers of these animals. The reason for the sexual dimorphism in the ability to respond to peroxisome proliferators is unclear, but this observation suggests that under certain circumstances minor alterations in HBV transcription can be associated with dramatic alterations in viral replication. Additionally, it should be noted that although female PPAR $\alpha$  +/- HBV transgenic mice initially have a lower level of replication intermediates in their livers, treatment with peroxisome proliferators results in similar levels of replication intermediates in male and female PPAR $\alpha$  +/- HBV transgenic mice (Fig. 1A and 2A; Tables 4 and 5).

The relative abundance of the precore and pregenomic RNAs in HBV transgenic mice was investigated to determine if activation of PPAR $\alpha$  affected the relative utilization of their two start sites (Fig. 3). The presence of PPAR $\alpha$  or its activation by clofibric acid did not alter the relative abundance of the precore and pregenomic RNAs as determined by RNase protection analysis. The pregenomic RNA initiation site is utilized approximately five times more than the precore RNA initiation site in HBV transgenic mice irrespective of the presence of PPAR $\alpha$  or clofibric acid. As observed for the steady-state levels of the 3.5-kb HBV RNA (Fig. 2B), an approximately 60% increase in the level of the pregenomic RNA is observed in



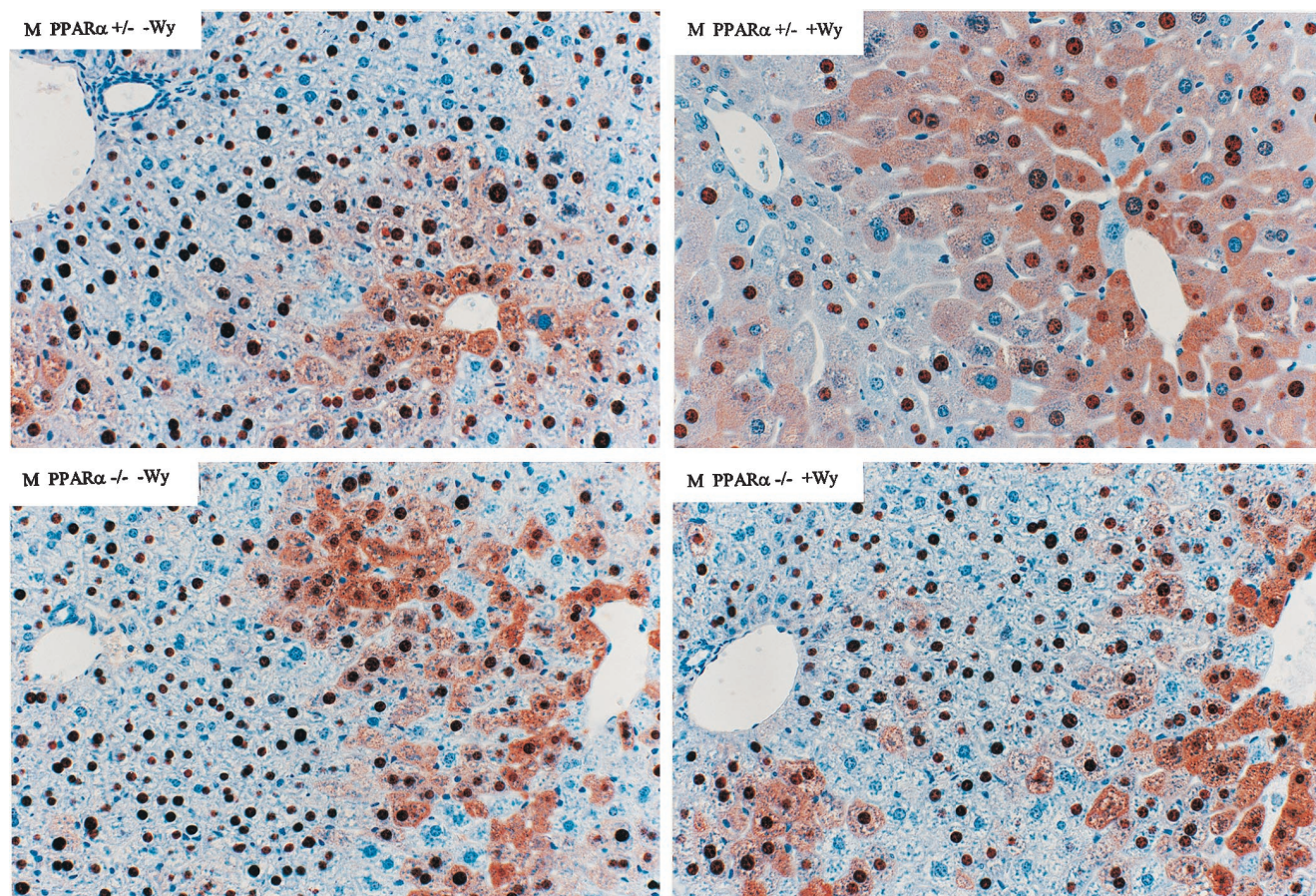


FIG. 5. Immunohistochemical staining of HBcAg in the livers of male HBV transgenic mice (original magnification,  $\times 200$ ). Nuclear staining of HBcAg is observed throughout the liver, whereas cytoplasmic staining is located primarily in the centrolobular hepatocytes in the livers of untreated PPAR $\alpha$  +/- HBV transgenic mice (upper left). Treatment of PPAR $\alpha$  +/- HBV transgenic mice with Wy-14,643 (upper right) results in hepatocyte hypertrophy and HBcAg staining that extends to the periportal hepatocytes. Livers from Wy-14,643-treated (lower right) or untreated (lower left) PPAR $\alpha$  -/- HBV transgenic mice display the same distribution of HBcAg staining as untreated PPAR $\alpha$  +/- HBV transgenic mice.

female PPAR $\alpha$  +/- HBV transgenic mice treated with clofibrate (Fig. 3).

The transcription rate of the HBV RNAs was measured directly by nuclear run-on analysis (Fig. 4) and compared with the steady-state levels of these transcripts observed in the livers of the HBV transgenic mice (Fig. 1B; Table 4). The transcription rate of *GAPDH* was used as an internal control and designated a relative transcription rate equal to 1.00. The rates of transcription of the PPAR $\alpha$ -inducible genes, *ACO* and *CYP4A* were low or undetectable in the absence of Wy-14,643 but were similar to the rate for *GAPDH* in the presence of PPAR $\alpha$  and the peroxisome proliferator (Fig. 4). This result is consistent with the observed steady-state levels of these transcripts under these conditions (Fig. 1C) (21). The relative transcription rates of the HBV RNAs were increased approximately twofold in the presence of PPAR $\alpha$  and Wy-14,643 (Fig. 4). In the absence of either PPAR $\alpha$  or Wy-14,643, the rate of HBV transcription was unaffected. These observations are consistent with the effects of Wy-14,643 on the steady-state levels of the HBV transcripts (Fig. 1B; Table 4) and support the suggestion that a small change in HBV transcription can, under certain circumstances, result in a relatively large increase in viral replication.

**Effect of PPAR $\alpha$  on viral HBcAg distribution within the livers of HBV transgenic mice.** Immunohistochemical analysis

of the livers of HBV transgenic mice also demonstrated that peroxisome proliferators influence the distribution of HBcAg within the liver lobule (Fig. 5 and 6). In both male and female PPAR $\alpha$  +/- HBV transgenic mice, treatment with Wy-14,643 results in a higher number of hepatocytes that are positive for cytoplasmic HBcAg staining and consequently cytoplasmic staining extends from the centrolobular region much closer to the periportal region of the liver lobule (Fig. 5 and 6). As cytoplasmic HBcAg staining correlates with viral replication (12), this observation is consistent with the increased level of viral replication intermediates found in the livers of these mice. The hepatocytes of the PPAR $\alpha$  +/- HBV transgenic mice treated with Wy-14,643 display the expected hypertrophy, whereas this effect and the extended distribution of HBcAg staining is not observed in untreated PPAR $\alpha$  +/- HBV transgenic mice and PPAR $\alpha$  -/- HBV transgenic mice (Fig. 5 and 6).

## DISCUSSION

Previously the role of the nuclear hormone receptor PPAR $\alpha$  in modulating transcription from the HBV nucleocapsid promoter had been examined in cell culture (25). In transient transfection analysis, RXR $\alpha$  and PPAR $\alpha$  in the presence of the activating ligands, 9-*cis* retinoic acid and clofibrate, acid,



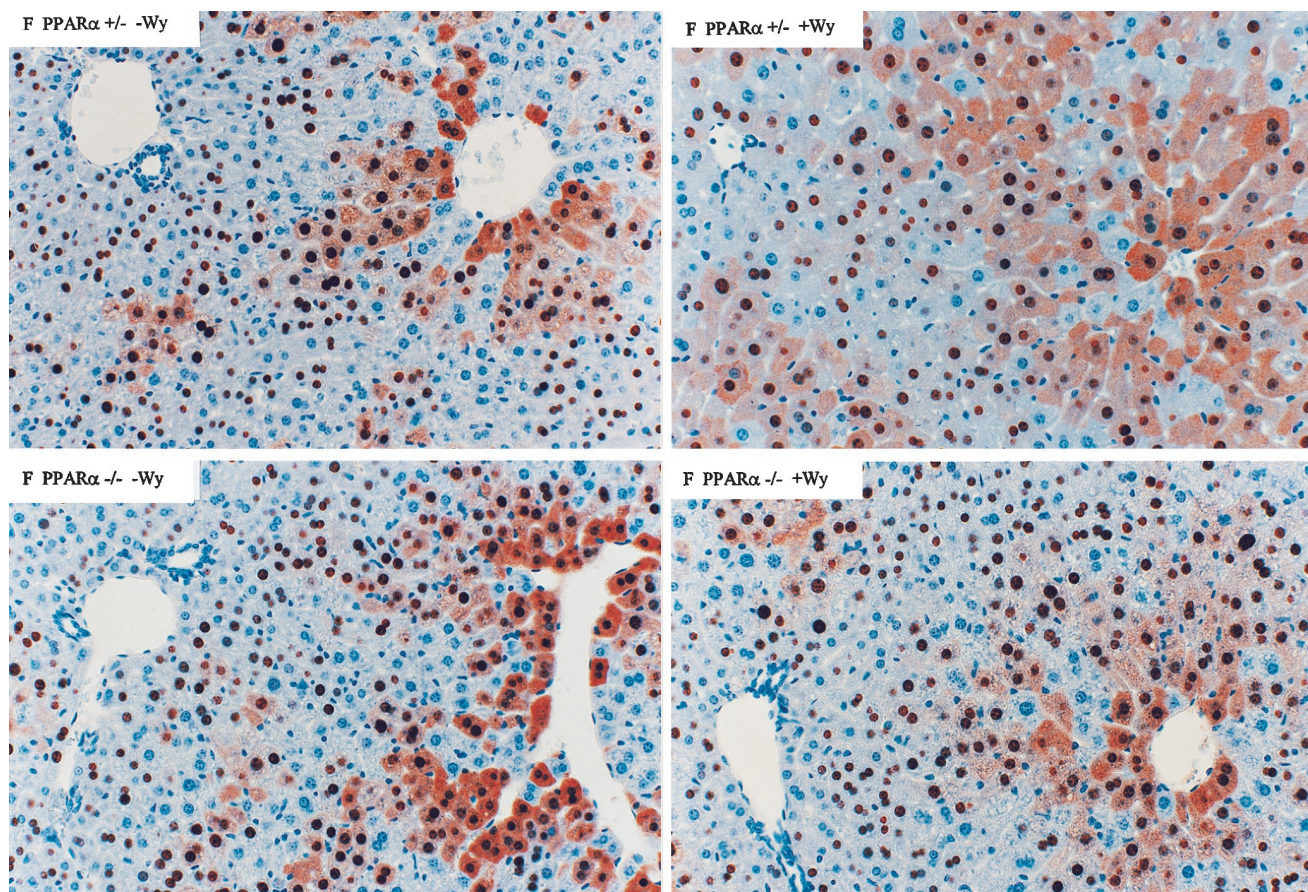


FIG. 6. Immunohistochemical staining of HBcAg in the livers of female HBV transgenic mice (original magnification,  $\times 200$ ). Nuclear staining of HBcAg is observed throughout the liver, whereas cytoplasmic staining is located primarily in the centrolobular hepatocytes in the livers of untreated PPAR $\alpha$  +/- HBV transgenic mice (upper left). Treatment of PPAR $\alpha$  +/- HBV transgenic mice with Wy-14,643 (upper right) results in hepatocyte hypertrophy and HBcAg staining that extends to the periportal hepatocytes. Livers from Wy-14,643 treated (lower right) or untreated (lower left) PPAR $\alpha$  -/- HBV transgenic mice display the same distribution of HBcAg staining as untreated PPAR $\alpha$  +/- HBV transgenic mice.

increased transcription from the nucleocapsid promoter approximately fourfold. RXR $\alpha$  and PPAR $\alpha$  mediated this effect through the PPREs located in the HBV enhancer 1 region and the nucleocapsid promoter (25). In this study, the effect of activating PPAR $\alpha$  with synthetic ligands was investigated in an *in vivo* model of HBV replication. The *in vivo* model system used was the HBV transgenic mouse that replicates virus at high levels in the liver (12). If the abundance of the HBV pregenomic RNA were regulated by PPAR $\alpha$  activation of transcription from the nucleocapsid promoter, one would anticipate that an increase in this transcript would be associated with an increase in HBV replication. This possibility was examined by treating HBV transgenic mice with the peroxisome proliferators Wy-14,643 and clofibrate. To determine if these ligands were mediating their effects through PPAR $\alpha$ , HBV transgenic mice lacking PPAR $\alpha$  were also characterized.

Analysis of the HBV transgenic mice without peroxisome proliferators treatment indicated that under normal physiological conditions PPAR $\alpha$  did not influence secretion of HBeAg, HBV transcription, or HBV replication. However, treatment of HBV transgenic mice with the peroxisome proliferators Wy-14,643 and clofibrate demonstrated that these PPAR $\alpha$  ligands increased the levels of secreted HBeAg and HBV transcripts less than twofold (Fig. 1 and 2; Tables 2 to 5). In contrast, the level of viral replication intermediates in male HBV transgenic mice was increased approximately 2.5-fold,

whereas the level of replication intermediates in female HBV transgenic mice was increased 7- to 14-fold, by peroxisome proliferator treatment (Fig. 1 and 2; Tables 4 and 5). The increase in viral replication was dependent on PPAR $\alpha$ , as PPAR $\alpha$ -null HBV transgenic mice failed to show this increase in replication intermediates in response to peroxisome proliferator treatment. Additional evidence that peroxisome proliferators enhanced HBV replication was apparent from immunohistochemical analysis of the livers from HBV transgenic mice (Fig. 5 and 6). HBV transgenic mice displayed cytoplasmic HBcAg in a greater number of hepatocytes after treatment with peroxisome proliferators, suggesting that higher levels of viral replication were occurring in more cells as a result of the activation of PPAR $\alpha$  (12).

This analysis demonstrates that female HBV transgenic mice display a dramatic increase in viral replication that is much larger than the observed increase in viral transcription in response to two different peroxisome proliferators (Fig. 1 and 2; Tables 4 and 5). The explanation for this observation is unclear. However, a model in which small alterations in the level of the HBV pregenomic RNA can result in large alterations in replication intermediates offers a possible explanation for these results. In this model, the level of HBV pregenomic RNA in female mice would be slightly lower than the level in male mice. The observation that the ratio of precore to pregenomic RNA is essentially constant in these mice and that the



level of secreted HBeAg is higher in male mice than in female mice is consistent with this idea (Fig. 3; Table 1). In female mice, the translation of the core polypeptide from the pregenomic RNA is proposed to be insufficient to permit the efficient formation of capsids from core polypeptide dimers (32, 50, 51). Consequently, many of the core polypeptide dimers are transported into the nucleus of the cell, where they accumulate but do not contribute to viral replication. This pattern of HBeAg immunohistochemical staining is observed in many of the cells surrounding the portal vein where nuclear HBeAg is associated with limited viral replication (Fig. 5 and 6) (12). In contrast, male HBV transgenic mice expressing slightly higher levels of pregenomic RNA would synthesize enough core polypeptide to reach a concentration of core polypeptide dimers sufficient to permit more efficient formation of capsids and consequently more efficient conversion of pregenomic RNA into viral DNA replication intermediates. This possibility is consistent with the observation that male and female HBV transgenic mice express very similar levels of pregenomic RNA but male HBV transgenic mice have approximately fourfold-higher levels of replication intermediates in their livers compared with female HBV transgenic mice (Fig. 1 to 3; Tables 4 and 5). After treatment with peroxisome proliferators, the level of pregenomic RNA in both male and female mice is proposed to be increased to a limited degree but sufficiently to permit the efficient conversion of core polypeptide dimers into nucleocapsids. In this situation, both male and female HBV transgenic mice would be expected to synthesize similar levels of viral replication intermediates, as was observed (Fig. 1 and 2; Tables 4 and 5). This explanation for the PPAR $\alpha$ -dependent effects of peroxisome proliferators on viral replication appears the most reasonable. It is consistent with the previous mapping of PPREs to the HBV enhancer 1 region and the nucleocapsid promoter and the observation that transcription from the nucleocapsid promoter can be increased by PPAR $\alpha$  and its ligand in cell culture (25).

Alternative explanations for the effects of peroxisome proliferators on viral replication are possible. There may exist in the HBV biosynthetic process a PPAR $\alpha$ -sensitive step, other than pregenomic RNA synthesis, that is more critical to viral replication in female mice than in male mice. Stimulation of this hypothetical step could be responsible for the dramatic increase in viral replication observed in female HBV transgenic mice treated with peroxisome proliferators. The putative nature of this step is unknown. However, it is reasonable to assume that the initial event in this process would involve the PPAR $\alpha$ -dependent activation of an unknown gene or genes. This gene product would then presumably increase viral replication by activating a posttranscriptional event in the viral life cycle such as pregenomic RNA encapsidation or HBV DNA synthesis.

Two potential alternative explanations for the observed results can probably be excluded. The finding that the level of replication intermediates increases less in male than in female HBV transgenic mice in response to peroxisome proliferators suggests that the simple increase in liver size cannot explain the observed results (Tables 2 and 3). The observation that HBeAg secretion increases in HBV transgenic mice treated with peroxisome proliferators indicates that the increase in intracellular viral replication intermediates is unlikely to be due to retention of virus resulting from a decrease in the ability to secrete the virus from the hepatocytes (Tables 2 and 3).

Regardless of the nature of the PPAR $\alpha$ -mediated increase in viral replication, the *in vivo* demonstration that peroxisome proliferators can increase HBV viral replication intermediates may have important clinical implications. A variety of hypo-

lipidemic drugs mediate their action by activating PPAR $\alpha$  (7, 8, 11, 20, 31). Patients receiving these drugs who are also infected with HBV may activate viral replication, and this could have potentially detrimental effects on the outcome of the viral infection. In addition, a variety of dietary and environmental compounds are ligands for PPAR $\alpha$ , and exposure to these chemicals might also affect HBV replication in infected individuals (7, 8, 19, 31). As the results in the HBV transgenic mouse model suggest that small alterations in the level of pregenomic RNA levels may have a dramatic effect on viral replication, antagonists directed against PPAR $\alpha$  might have therapeutic application in the treatment of HBV infections.

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