Enhancement of Hepatitis B Virus Replication by Androgen and Its Receptor in Mice

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Hepatitis B virus (HBV) is an important pathogen that chronically infects more men than women. To understand the molecular mechanism of this gender disparity, we analyzed HBV replication in transgenic mice that carried the HBV genome with or without the ability to express the HBV X protein (HBx). We found that gender had no effect on HBV surface antigen (HBsAg), DNA, and RNA levels in mice before puberty, but its effect on HBV after puberty was apparent, with HBV replicating approximately twice more efficiently in male mice than in female mice whether or not HBx was expressed. The castration of male mice resulted in a reduction of HBV HBsAg, DNA, and RNA levels, which could be partially restored by the injection of the androgen agonist R1881, indicating a positive role of androgen in HBV replication. The introduction of HBV genomic DNA and androgen receptor (AR) short hairpin RNA (shRNA) into the liver of naïve mice by hydrodynamic injection revealed that the effect of androgen on HBV was dependent on its receptor, which apparently could also stimulate HBV replication via an androgen-independent pathway. Further studies indicated that the two previously identified androgen response elements (AREs) in the HBV genome could indeed mediate the effect of androgen on HBV RNA transcription and DNA replication in vivo. These effects of androgen and its receptor on HBV thus provide an explanation for why men have a higher risk of HBV infection than women.
MATERIALS AND METHODS

DNA plasmids and cell cultures. Plasmid p1.3xHBV, which contains the 1.3-mer overlength HBV genome, was described previously (22). Plasmid p1.3xHBV-ARE-mt is identical to p1.3xHBV, with the exception that it contains four nucleotide mutations (nucleotide [nt]: 923G→C, nt 924 T→A, nt 959 G→C, and nt 960 T→A). These mutations abolished the two ARE sites in the HBV genome and were created by using the Stratagene QuikChange site-directed mutagenesis kit. Plasmid p1.3xHBV-X-mt was also described previously (15). It is identical to p1.3xHBV, with the exception that the expression of the HBx protein was abolished by an A-to-C mutation at nt 1377, which removed the initiation codon of the X protein, and by a C-to-T mutation at nt 1398, which created a premature termination codon in the X coding sequence. The expression plasmids for mouse AR short hairpin RNA (shRNA) (GenBank accession number NM_013476) and control shRNA were purchased from Sigma-Aldrich. The AML12 mouse liver cell line (a gift of Shelly Lu) was cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 medium containing 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, and 10% fetal bovine serum. The Huh7 human hepatoma cell line was cultured in DMEM with 10% charcoal-stripped fetal bovine serum. The transfection of the shRNA expression plasmids into Huh7 cells was performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

HBV transgenic mice, castration, and hydrodynamic injection. The HBV transgenic mouse lines Tg05, Tg31, and Tg38 were described previously (15, 20). The Tg05 mouse line carries the 1.3-mer, overlength, wild-type HBV genome. The Tg31 and Tg38 mouse lines also carry the 1.3-mer HBV genome, with the exception that the expression of HBx was abolished by an A-to-C mutation at nt 1377 and by a C-to-T mutation at nt 1398, as mentioned above. All of the experiments were conducted using age-matched mice with the B6 genetic background. For castration, 6-week-old male mice were used, and both testes were removed. For hydrodynamic injection, 8-week-old male mice were used, and both testes were removed. For hydrodynamic injection, 8-week-old male mice were injected via the tail vein with saline in a volume that was equivalent to 8% of the body weight. The injection was completed in 5 to 8 s. The saline contained plasmid p1.3xHBV with or without mutations in the AREs or the X gene. Depending on the experiments, the control shRNA expression plasmid or the AR shRNA expression plasmid may also have been included in the injection mixture. In all of the experiments, the total amount of DNA used for the injection was the same among different samples. The reporter plasmid pRL-SV40 (Promega), which expresses Renilla luciferase, was also included in the injection mixture to serve as an internal control for monitoring the transfection efficiency in vivo (12). For injection with the androgen agonist R1881 (Sigma-Aldrich), R1881 was first dissolved in 30% ethanol and injected into mice at a dose of 10 mg/kg body weight/day. Our mouse studies were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (8a). Our animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Southern California.

Southern and Northern blot analyses. Total liver DNA was isolated by use of our previously reported protocol (12). For RNA isolation, liver tissues were homogenized in TRIzol (Invitrogen), and total RNA was isolated according to the manufacturer’s protocol. Both Southern and Northern blot analyses were conducted by using a 32P-labeled HBV DNA probe.

Real-time PCR analysis of HBV core-associated replicative intermediate (RI) DNA. The HBV core particle–associate DNA was isolated by use of our previously described protocol (22). Briefly, 50 mg mouse liver tissues was homogenized in 500 μl lysis buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 50 mM NaCl, and 0.5% Nonidet P-40) containing 100 μg DNase I and micrococcal nuclease for 30 min at 37°C to remove free DNA. The reaction was stopped by the addition of 50 μl 0.5 M EDTA and 300 μg proteinase K to the mixture. After incubation at 65°C overnight, core particle–associated HBV DNA was isolated by phenol-chloroform extraction and ethanol precipitation. The DNA pellet was rinsed with 70% ethanol and resuspended in 50 μl TE (10 mM Tris-HCl [pH 7.0], 1 mM EDTA). HBV DNA was quantified by real-time PCR as previously described (8).

Semi-quantitative RT-PCR analysis for AR RNA. Semi-quantitative reverse transcription (RT)-PCR was conducted as previously described (12). The primers used for AR RNA were forward primer 1081-TACGAGATTCTGCGAC-1103 and reverse primer 1667-TTCATCTGCCATAC-1103 and reverse primer 650-TCATGAGATTCTGCGAC-631. The primers used for α-actin RNA were forward primer 321-ACATGGAGAAGATTCTGGCAC-340 and reverse primer 650-TCATGAGATTCTGCGAC-631.

RESULTS

Effect of gender on HBV replication is age dependent and HBx independent. Our laboratory previously produced transgenic mouse lines that carried either the wild-type or the HBx-negative 1.3-mer overlength HBV genome (12). These mouse lines contained replicating HBV DNA in the liver and produced mature viral particles in the blood. The HBx-negative genome contained a missense mutation in the translation initiation codon and a premature termination codon in the HBx coding sequence. These mutations abolished the expression of HBx without affecting the overlapping polymerase-coding sequence (15). We examined the possible effect of gender on HBV replication in these mouse lines before and after puberty. As shown in Fig. 1A, for 4-week-old prepuberty Tg05 mice, which carried the wild-type HBV genome, gender had no effect on serum HBsAg levels. However, for 10-week-old adult Tg05 mice, the serum HBsAg level was approximately 2-fold higher in male mice than in female mice. Similar results were observed with Tg38 and Tg31 mice, which were two independent mouse lines that carried the HBx-negative HBV genome. The serum HBsAg levels were similar between prepuberty male and female mice, but they were higher in male mice after puberty (Fig. 1A). We also examined HBV replicative intermediate (RI) DNA and RNA levels in the mouse liver. As shown in Fig. 1B, there was no difference in HBV RI DNA and RNA levels in the livers of 4-week-old male and female mice, irrespective of the mouse lines. However, in 10-week-old mice, the replicating HBV RI DNA levels were approximately 70% higher in male mice than in female mice of the Tg05 and Tg38 mouse lines and more than 2-fold higher for male Tg31 mice. This increase of the HBV RI DNA level in the male mouse liver was likely due to an increase of viral gene expression, as the HBV RNA levels were also correspondingly higher in male mice than in female mice of these mouse lines.

Thus, the results shown in Fig. 1 indicated that gender had no effect on HBV replication prior to puberty but that it affected HBV replication in adult mice. These results also indicated that HBx did not affect the effect of gender on HBV replication.

Androgen enhances HBV replication in mice. The results shown in Fig. 1 suggested a role of sex hormones in HBV replication. To test the possible role of androgen in HBV replication, we castrated male mice at 6 weeks of age and analyzed serum HBsAg levels for 4 weeks afterwards. As shown in Fig. 2A, the castration of male Tg05 mice (i.e., wild-type HBV mice) resulted in a reduction of the HBsAg levels in the serum. In contrast, the HBsAg levels in the serum of Tg05 mice were not reduced by a sham operation. These results were consistent with data from a previous report (13) and suggested a role of androgen in enhancing HBV replication. To test this possibility, we injected mice with R1881, a synthetic androgen, 2 weeks after castration. As shown in Fig. 1, this injection partially restored the HBsAg level in the serum. To fur-
ther investigate how R1881 might have affected serum HBsAg levels, we also analyzed HBV DNA and RNA levels in mouse liver. As shown in Fig. 2B, the castration of Tg05 wild-type HBV mice reduced the HBV RI DNA level to about 40%, which was nearly totally restored by the injection of R1881. Similar results were observed for the HBV RNA level, which was reduced by castration and restored by the injection of R1881. These results confirmed a role of androgen in stimulating HBV RNA expression and DNA replication in the mouse liver.

The same experiments were also conducted on male Tg38 and Tg31 HBx-negative HBV mice. Similarly, castration reduced the HBsAg levels in mouse serum, which could be partially restored by the injection of R1881 (Fig. 3A). Castration also reduced HBV DNA and RNA levels in the liver, which again could be restored by R1881 (Fig. 3B). These results again confirmed that the effect of androgen on HBV was independent of HBx.

**AR enhances HBV replication in mice.** Androgen exerts its effect on cellular gene expression via the AR. To determine the possible role of the AR in HBV replication, we first determined the expression level of the AR in mouse liver by semiquantitative RT-PCR. As shown in Fig. 4A, the expression level of the AR was very low in mouse liver before puberty, indicating that it likely played little role in HBV gene expression and replication at that time. The level of the AR in liver was significantly increased after puberty, with a slightly higher level in female mice than in male mice. To determine whether the AR is important for HBV repli-
cation in the adult mouse liver, we decided to perform a shRNA knockdown experiment to suppress the expression of the AR in mouse livers. We first tested the efficiency of the AR shRNA on the suppression of AR expression using an immortalized mouse liver cell line, AML12. As shown in Fig. 4B, this shRNA could efficiently suppress the expression of the AR in this cell line. We then performed a hydrodynamic injection, which is a rapid and convenient method for gene delivery into mouse liver (16). The 1.3-mer HBV genomic DNA was coinjected with the expression plasmid for control shRNA or AR shRNA into sham-operated or castrated male mice, which were sacrificed 48 h later for the isolation of the liver and analysis of HBV core particle-associated HBV RI DNA by real-time PCR. As shown in Fig. 4C, for mice injected with the control shRNA expression plasmid, castration reduced the level of core particle-associated HBV RI DNA by approximately 50% compared with the sham-operated mice. This result was consistent with the transgenic mouse results shown in Fig. 3. For mice injected with the expression plasmid of the AR shRNA, the HBV RI DNA level was reduced to about 20% whether the mice were sham operated on or castrated. As the suppression of AR expression had a significantly greater effect on HBV replication than did the removal of androgen, these results indicated that some of the ARs might activate HBV replication independently of androgen. Also, since the removal of androgen did not further reduce the

FIG 2 Effect of androgen on wild-type HBV replication in transgenic mice. The Tg05 mouse line, which carried the wild-type HBV genome, was used for these studies. (A) Analysis of serum HBsAg levels. Mouse sera were collected on weeks 0, 2, and 4 from 5 sham-operated mice, 5 castrated mice, and 3 castrated mice with a daily injection of R1881 2 weeks after castration. The serum HBsAg level was measured by ELISA. The results represent the means ± standard deviations (SD) for all of the mice used in each group. (B) Analysis of HBV DNA and RNA. Mouse liver DNA and RNA were isolated from sham-operated mice, castrated mice, and castrated mice treated with R1881. The levels of HBV RI DNA (top) and RNA (middle) were then analyzed by Southern and Northern blotting, respectively, as described in the Fig. 1 legend. The relative HBV DNA and RNA levels are shown under the panels. Quantification was conducted as described in the Fig. 1 legend. The DNA and RNA levels of sham-operated mice were arbitrarily defined as 1.

FIG 3 Effect of androgen on replication of HBx-negative HBV in transgenic mice. Tg38 and Tg31 mice, which are two independent HBx-negative HBV transgenic mouse lines, were used for the studies. (A) Analysis of serum HBsAg levels. (B) Analysis of HBV DNA and RNA. The experiments were conducted as described in the Fig. 2 legend.
HBV RI DNA level in mice with an AR knockdown, these results also indicated that the effect of androgen on HBV was most likely mediated entirely by the AR.

We also conducted the same hydrodynamic injection experiment using the 1.3-mer HBx-negative HBV genome. As shown in Fig. 4D, castration reduced the HBV RI DNA level in mice injected with the control shRNA expression vector by approximately one-half, and the knockdown of AR expression reduced the HBV RI DNA level to slightly above 20%, which was not reduced further by castration. These results were essentially identical to those obtained with the wild-type HBV genome, indicating that the effect of androgen and its receptor on HBV was independent of HBx.

HBV AREs are required for AR to stimulate HBV replication in vivo. By using HepG2 hepatoblastoma cells (13), it was demonstrated previously that there are two putative AREs located at nucleotides (nt) 913 to 927 (ARE-1) and nt 949 to 962 (ARE-2) in the HBV genome in the upstream region of the enhancer I core sequence (13). However, whether these two AREs are indeed responsible for the effect of the AR on HBV in vivo remained to be determined. To investigate the possible role of these two ARE motifs in HBV replication in vivo, we mutated two critical nucleotides of ARE-1 and ARE-2 in the 1.3-mer HBV genome (Fig. 5A). These mutations were previously shown to abolish the binding of the AR to the two AREs. The 1.3-mer HBV genomes with and without these ARE mutations were first transfected into Huh7 hepatoma cells for an analysis of their response to the androgen agonist R1881. As shown in Fig. 5B, although R1881 was able to increase the wild-type HBV RI DNA and RNA levels approximately 2-fold, it had only a marginal effect on HBV ARE mutant RI DNA and RNA levels, confirming that these ARE mutations were able to abolish most of the enhancing effect of R1881 on HBV replication in cell cultures. The wild-type and ARE mutant HBV genomic DNAs were then hydrodynamically injected into sham-operated and castrated mice. As shown in Fig. 5C, the mutations in the two AREs reduced the HBV DNA level in the liver to approximately 35% in sham-operated mice and to about 18% in castrated mice. As the results shown in Fig. 4 suggested that the activity of androgen was mediated by its receptor, the observation that the removal of androgen further reduced the HBV DNA level of the mutant suggested the possible presence of other minor AREs in the HBV genome. In agreement with the results shown in Fig. 3, the injection of R1881 partially restored the HBV DNA level in castrated mice. However, this injection only marginally increased the HBV DNA level in castrated mice injected with the HBV mutant, confirming the importance of the two putative AREs located at nt 913 to 927 and nt 949 to 962 in HBV replication in vivo.
mediating the effect of androgen on the stimulation of HBV replication.

**DISCUSSION**

Epidemiology studies indicate that men are three to seven times more likely to become HBV carriers than women, and male HBV carriers are more likely to develop HCC than are female HBV carriers (19). By using transgenic mice that carried the entire HBV genome and productively replicated HBV in the liver, we demonstrated that HBV replicated approximately twice more efficiently in male mice than in female mice (Fig. 1). This higher replication efficiency of HBV in males provides an explanation as to why there are more male HBV carriers than female HBV carriers. It also provides an explanation as to the gender discrepancy in the incidence of HCC, as HBV viral load has been shown to be a major risk factor for HCC (2, 4, 11).

The effect of gender on HBV replication was apparently due to androgen, as the castration of male mice to remove androgen led to a reduction in levels of HBV DNA replication, which could be partially restored by the injection of the androgen agonist R1881 (Fig. 2B). The positive effect of androgen on HBV replication was also supported by our observation that gender had little effect on the activation of the AR to enhance HBV replication. The observation that female adult mice expressed a high level of the AR is interesting (Fig. 4A). It is possible that this low level of the AR may be due to the low expression level of the AR in the liver of these female adult mice. It is conceivable that these additional factors may also participate in the activation of the AR to enhance HBV replication. The observation that female adult mice expressed a high level of the AR is interesting (Fig. 4A). It is possible that this low level of the AR may also stimulate HBV gene expression albeit to a much lesser degree due to the low level of androgen in female mice.

The HBx protein can activate the AR either directly via binding to the AR or indirectly via the activation of signaling pathways (17, 21). For that reason, we also investigated the possible effect of androgen and the AR on HBV replication in the absence of HBx. Our results indicated that the absence of HBx did not affect the effects of gender, androgen, and the AR on the replication of HBV (Fig. 1 to 4). This observation that HBx is dispensable for the effect of androgen and the AR on HBV replication is interesting but perhaps not surprising, as the effect of HBx on the AR is dependent on androgen but not vice versa (3, 21). Thus, the removal of HBx would not be expected to abolish the interaction between androgen and its receptor and their effects on HBV.

Previous studies using cell cultures identified two AREs in the upstream region of the HBV enhancer I element (13, 14). By performing mutagenesis to remove these two AREs (Fig. 5A), we confirmed the importance of these two AREs in HBV replication in cell cultures (Fig. 5B) and, in addition, demonstrated their importance in vivo (Fig. 5C). Since we found that the removal of androgen by castration could further reduce the replication of this HBV ARE mutant (Fig. 5C), it is possible that there may be an additional ARE(s) in the HBV genome. Indeed, a minor ARE was
also suggested previously to reside near the 3’ coding sequence of the C gene (14). Whether this ARE is indeed used by androgen and its receptor to stimulate HBV gene expression in vivo, however, will require further studies.

In conclusion, our results indicate that androgen and its receptor can enhance HBV replication and provide an explanation for the gender disparity of HBV carriers. The effect of androgen and its receptor on HBV is independent of HBx and is mediated by the AREs located near the HBV enhancer I core sequence and possibly also other regions of the HBV genome, which together stimulate HBV RNA transcription. It remains to be determined, however, why HBV has evolved this mechanism to enhance its replication in males. It is possible that a higher replication level in men will facilitate the horizontal transmission of this virus, whereas a lower replication level in women may facilitate the establishment of persistent infection after vertical transmission. The latter is supported by our recent finding that low-level HBV infection allows HBV to use the interferon response to enhance its replication and persistence (12). Further research in this area will be required to resolve this question.

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REFERENCES