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# Estrogen suppresses hepatitis B virus expression in male athymic mice transplanted with HBV transfected Hep G-2 cells

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## Summary

Hormones are known to regulate both viral and cellular genes. It has been shown previously that estrogen has an effect on liver gene transcription and mRNA stability. Sex hormones might have a role in the chronic persistence of hepatitis B virus (HBV) infection. In fact, there is a male preponderance in the incidence of chronic HBV infection, and HBsAg expression was reported to be much higher in male transgenic mice than in the females. We investigated the effect of estrogen on HBV gene expression and regulation in athymic mice bearing 2.2.15 cells, a human hepatoblastoma cell line derived from Hep G-2 transfected with HBV sequences. Both male and female mice were treated with estradiol after tumors could be observed. Episomal DNA was extracted from the tumors and hybridized with $^{32}$P-labelled HBV DNA. Southern blot and slot blot analyses demonstrated that male mice had higher expression of HBV DNA. Estrogen treatment suppressed HBV DNA expression in males, but had only a minor effect on females. HBeAg production in male mice was also inhibited by estrogen treatment. HBV RNA extracted from 2.2.15 cells showed 2–3-fold reduction following $\beta$-estradiol treatment. Moreover, inhibition of HBV transcription by estrogen was demonstrated by an RNA pulse-labelling experiment. These data indicate that estrogen inhibits HBV expression in the in vivo model presented in this study. These results might contribute to a better understanding of the effect of sex hormones on the pathogenesis of HBV-induced liver disease.

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Hepatitis B virus; Gene expression; Estrogen

## Introduction

The Hepadna viruses are small DNA viruses that multiply preferentially in the liver, potentially inducing chronic infection. Much is known about their structure and gene products, but the regulation of gene products and the effect of sex hormones on such regulation, although previously studied, is still poorly understood (Tiollais et al., 1985; Babinet et al., 1985; Palmiter and Brinster, 1986).

We have previously shown that corticosteroids stimulate hepatitis B virus DNA, mRNA, and protein production in a stable expression system (Tur-Kaspa and Laub, 1990). Hepatitis B surface antigen and gene expression is further regulated by sex steroids and glucocorticoids in transgenic mice (Farza et al., 1987). It was also shown that HBsAg levels in male transgenic mice (Farza et al., 1987) and in adult males are 5–10-times higher than in females (Shouval et al., 1981). These observations led us to hypothesize that estrogen has a role in the regulation of HBV gene expression.

In order to determine the validity of our hypothesis, we used a quasi in vivo model, utilizing Hep G-2 cells transfected with four tandem copies of HBV (2.2.15 cells) (Sells et al., 1987) transplanted into athymic mice and treated with $\beta$-estradiol. To evaluate whether estrogen had a direct effect on HBV gene expression and regulation, we investigated the effects of estrogen on 2.2.15 cells by measuring the amount of extracted HBV RNA in $\beta$-estradiol-treated and non-treated cells. In this study we were able to demonstrate that serum HBeAg and HBV DNA in tumor tissues were higher in male than female mice. Estrogen suppresses HBV expression in male mice, while no effect was noted in females. HBV RNA expression in 2.2.15 cells was suppressed by estrogen, and, most probably, this inhibition is at the level of transcription.

## Materials and Methods

*Cell line*

HBV-transfected Hep G-2 (2.2.15 cells) (Sells et al., 1987) cells were grown in culture in MEM supplemented with L-glutamine, non-essential amino acids, antibiotics, G418, as well as 10% fetal bovine serum. Cells were harvested at sub-confluence with trypsin/EDTA.

*RNA extraction and pulse labelling*

Nuclear RNA and cytoplasmic RNA were extracted using NP40, as previously described (Laub and Rutter, 1983). For RNA pulse labelling, cells were treated for 30 min with 200 $\mu$Ci/ml of 5′[$^3$H]uridine. Medium was then

removed and cells were extensively washed with phosphate-buffered saline (PBS); nuclear and cytoplasmic RNA were subsequently extracted, as described above. Labelled RNA fractions were hybridized to filters containing HBV DNA. Hybridization was in 6 × SSC at 68°C for 16 h. Filters were then washed, treated with RNAse-free DNAse (pancreatic GSB).

*RNA extraction and analysis*

Transfected Hep G-2 cells (2.2.15 cells) (Sells et al., 1987) were treated with $10^{-6}$ M β-estradiol for 24 h. RNA was extracted from treated and non-treated cells and was hybridized using a $^{32}$P-labelled HBV DNA probe.

*Generation of tumors in athymic mice*

BALB/c athymic mice 4–6 weeks old, were further immunosuppressed by cyclophosphamide, 200 mg/kg, i.p. After 24 h, mice were injected in the flank region with $5 \cdot 10^6$ cells in 0.2 ml of serum-free medium. Mice were examined every 48–72 h for tumor appearance. At the end of experiments, mice were exsanguinated under chloral hydrate anesthesia.

*Estrogen treatment*

Tumors could be palpated about 14 days post-Hep G-2 cell injection. Both male and female mice were then injected (s.c.) three times, every other day, with 0.5 mg/kg β-estradiol (Sigma) in 0.2 ml arachis oil. Control mice were injected similarly with arachis oil.

*HBV markers*

HBeAg was measured in serum by RIA (Abbott, Chicago, IL).

*Detection of HBV DNA in tumor tissue*

Episomal DNA was extracted from tumor tissue using the Hirt method (Hirt, 1967). HBV DNA was analyzed using Southern blot transfer and hybridization and by slot blot hybridization, using a $^{32}$P-labelled probe (Tur-Kaspa and Laub, 1990).

## Results

*Effect of estrogen treatment on HBV RNA in 2.2.15 cells*

To evaluate the effect of estrogen on the levels of HBV RNA in HBV-producing 2.2.15 cells, total RNA was analyzed after 24 h of continuous treatment with $10^{-6}$ M β-estradiol. Fig. 1 shows that estrogen caused reduction in the amount of HBV RNA. In order to establish whether estrogen affects mRNA stability or the rate of transcription, pulse-labelled RNA was studied. Estrogen treatment reduced the level of pulse-labelled HBV RNA, that hybridized to HBV DNA-containing filters, by 2.2-fold (27 000 cpm in non-treated cells as compared to 12 000 cpm in treated cells).

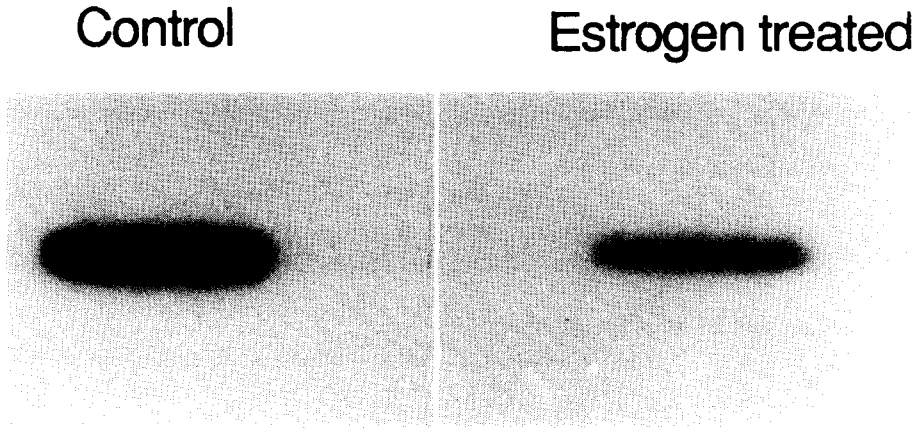


Fig. 1. Slot blot analysis of total RNA extracted from 2.2.15 cells. RNA was extracted from cells treated with $10^{-6}$ M $\beta$-estradiol (estrogen-treated) and non-treated cells (control). For hybridization, a $^{32}$P-labelled HBV DNA probe was used. Lanes contain 3.00 $\mu$g of RNA.

*Sex differences in HBeAg expression in athymic mice bearing 2.2.15 cell tumors*

Fig. 2 demonstrates the difference in HBeAg expression between male and female athymic mice bearing subcutaneous 2.2.15 cells. HBeAg production in males was more than twice that in the females.

*Effect of estrogen on serum HBeAg in male athymic mice bearing 2.2.15 cell tumors*

Male and female athymic mice, bearing subcutaneous 2.2.15 cell tumors,

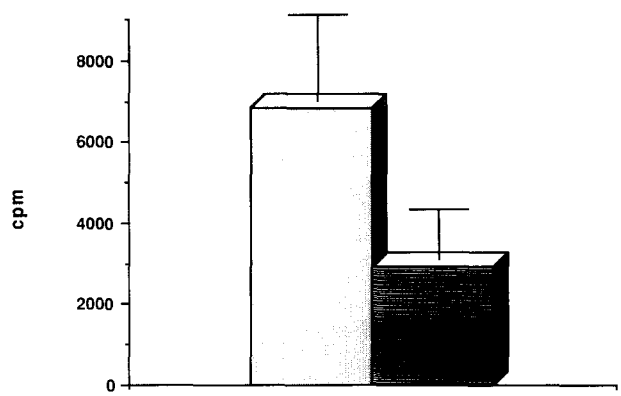


Fig. 2. Serum HBeAg in 15 male and female athymic mice bearing s.c. HBV transfected Hep G-2 cells (2.2.15 cells). HBeAg was measured 21 days post-2.2.15 cell injection. Lightly shaded: male; darkly shaded: female.

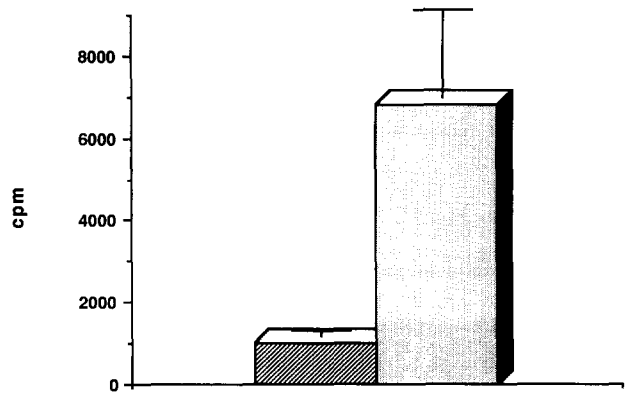


Fig. 3. Serum HBeAg in 14 athymic male mice transplanted with 2.2.15 cells. HBeAg vas measured in mice treated with 0.5 mg/kg $10^{-6}$ M $\beta$-estradiol three times s.c. every other day post-appearance of the 2.2.15 cell tumor. Estrogen treatment caused significant reduction in HBeAg in the mice sera ($P < 0.02$). Darkly shaded: estrogen-treated; lightly shaded: non-treated.

were treated s.c. with $\beta$-estradiol at 0.5 mg/kg. Fig. 3 presents the significant reduction in HBeAg expression in estrogen-treated male mice. No effect of estrogen on HBeAg production could be shown in female mice (data not shown).

*The effect of estrogen treatment on tumor tissue HBV DNA of athymic mice bearing subcutaneous 2.2.15 cell tumors*

DNA extracted from tumors of estrogen-treated and non-treated mice was hybridized using a $^{32}$P-labelled HBV DNA probe. The results of Southern blot analysis and slot blot analysis are shown in Fig. 4a and b, which demonstrate that HBV DNA expression is clearly much higher in the male mice than in female mice. The expression of replicative HBV DNA was remarkably suppressed by estrogen treatment in male mice, while no effect was noted in the estrogen-treated females.

## Discussion

We investigated the effect of estrogen on HBV gene expression and replication in athymic mice bearing 2.2.15 cell tumors, as well as the effect of estrogen on HBV-RNA production in homologous cells. We were able to demonstrate that male mice had a higher expression of HBeAg in their serum, and estrogen treatment significantly reduced HBeAg expression in male mice, while no such effect could be shown in female mice. Moreover, we have shown that HBV DNA expression is clearly much higher in male mice than in female mice, and the expression of replicative HBV DNA was significantly suppressed

by estrogen treatment in male mice, while no such effect was noted in the female mice. We also report an effect of estrogen on HBV RNA production in 2.2.15 cells. A 3–4-fold reduction in HBV RNA production was demonstrated in the β-estradiol-treated cells. In order to clarify whether this effect is on the rate of transcription, or indirectly on mRNA stability and possible mediation of other factors, the level of pulse-labelled HBV RNA was studied. Our results, showing a 2.2-fold reduction in treated cells, imply a direct effect on the

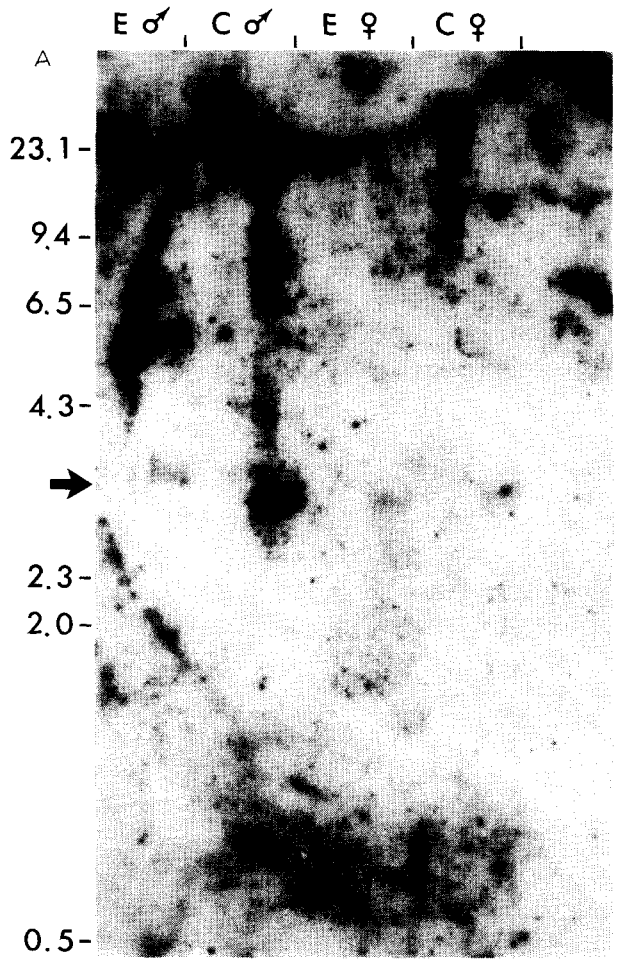


Fig. 4. (a) Southern blot transfer showing DNA extracted from tumors and hybridized with $^{32}$P-labelled HBV DNA probe. Each lane contains DNA extracted from 10 mg of tumor tissue. In each group the left lane contains uncut DNA and the right lane contains DNA digested with *Eco*RI. Groups: E ♂, male mice treated with 0.5 mg/kg β-estradiol; C ♂, untreated male mice; E ♀, female mice treated with 0.5 mg/kg β-estradiol; C ♀, untreated female mice. (b) Slot blot analysis showing the same DNA as in (a). DNA extracted from tumors was hybridized with $^{32}$P-labelled HBV DNA probe.

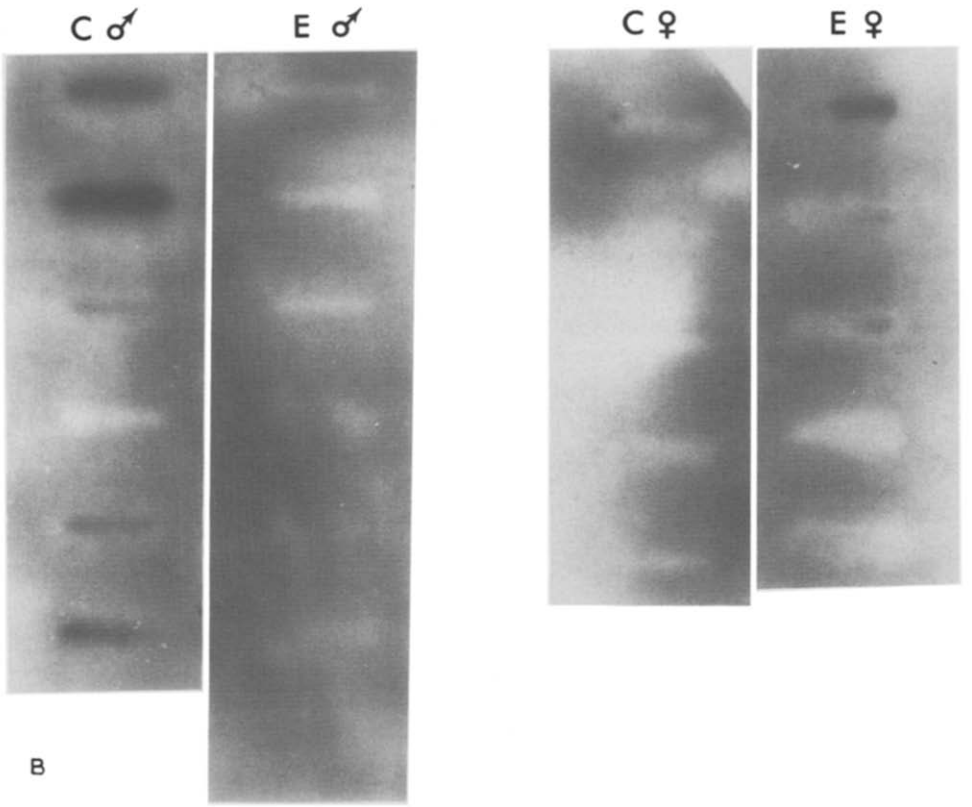


Fig. 4 (continued).

transcription level. This model of nude mice, transplanted with Hep G-2 cells, which contain replicating HBV, can serve as an experimental in vivo model for HBV carriers. This model can be used to study HBV transcription regulation, and replication. This is a better model than the transgenic mice model for studying viral replication or the effect of various drugs and hormones on the virus transcription machinery and replication.

Estrogen was previously shown to regulate cellular gene transcription and mRNA stability by three basic mechanisms (Shapiro et al., 1989): an increase in the absolute rate of gene transcription, an increase in the absolute rate of total nuclear RNA synthesis, and selective stabilization of mRNA against cytoplasmic degradation. Estrogen was also shown to have an effect on viral gene transcription. Specific RNA transcripts of human papillomavirus type 16 in Siha cervical carcinoma cells are stimulated by estrogen (Rosenbaum et al., 1989). This effect is presumably mediated by estrogen-receptor binding to the estrogen-responsive element (ERE) consensus sequence (GGTCANNN-TAACC) (Maurer and Notides, 1987). We were not able to demonstrate any HBV DNA fragment with a high degree of homology to ERE sequences.

Moreover, we are describing a down-regulation effect induced by estrogen. Such an effect, although previously described (Adler et al., 1988), is not well understood, and further studies will be required to clarify its molecular mechanism. Farza et al. have previously reported that transgenic male mice show higher expression of HBV gene products (Farza et al., 1987). In our study, we were able to define the effect of estrogen specifically on HBV replication and HBeAg production. This effect might be the result of down-regulation of the pre-genome RNA (Ganem and Varmus, 1987) production.

The possible clinical significance of such an inhibitory effect of estrogen on HBV is worth mentioning. Reducing the rate of the viral replication by hormonal manipulation might have a positive effect on mitigating the activity of the liver disease, and preventing the long-standing complications of active HBV replication in the liver. However, further studies are needed to evaluate the long-term effects of $\beta$-estradiol treatment.

## Acknowledgements

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