Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 1187–1191, March 1987
Biochemistry

Hepatitis B surface antigen gene expression is regulated by sex steroids and glucocorticoids in transgenic mice

(hepatitis B virus/hormonal regulation/animal model/chronic carrier)

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Communicated by Hans Popper, November 3, 1986 (received for review August 14, 1986)

ABSTRACT **We have investigated the basis for liver-specific and sex-linked expression of hepatitis B surface antigen (HBsAg) gene in transgenic mice by monitoring the level of liver HBsAg mRNA and serum HBsAg at different stages of development and in response to sex-hormone regulation. Transcription of the HBsAg gene starts at day 15 of development, together with that of the albumin gene, and reaches a comparable level at birth. HBsAg mRNA level and HBsAg production are parallel in males and females during prenatal development and until the first month of life, but HBsAg gene expression increases 5–10 times in males at puberty. After castration, the level of expression decreases dramatically in both males and females and is subsequently increased by injection of testosterone or estradiol. Glucocorticoids also regulated positively expression of the HBsAg gene. Our results suggest that sex hormones play a role in hepatitis B virus gene expression during natural infection and could explain the difference in incidence of chronic carriers between men and women.**

The hepadnaviruses are small DNA viruses that multiply preferentially in the liver and establish chronic infection in their respective hosts. Much is known about the structure of the viral DNA, RNA, and gene products, but the regulation of hepadnavirus gene expression is still poorly understood, as are the mechanisms of chronicity and pathogenicity (1). To obtain an animal model for the chronic carrier state of hepatitis B virus (HBV), we produced in previous experiments two transgenic mouse strains containing the HBV DNA (except for the core gene) integrated into their genome (2). Both had high amounts of hepatitis B surface antigen (HBsAg) particles in the serum, and the liver was the main place of HBsAg mRNA synthesis. We also showed that in adults HBsAg levels in males were 5–10 times higher than in females. These observations led us to hypothesize that HBV gene expression is regulated by both liver-specific factors and sex steroids. The validity of our hypothesis was assessed by the fact that several genes maintained their normal regulation when injected into transgenic mice (3). Tissue specificity and hormonal control are conserved in transgenic mice containing, for example, genes fused to mouse mammary tumor virus regulatory sequences (4) or the avian transferrin gene (5). In experiments with the chloramphenicol acetyltransferase assay, HBV sequences were responsible for tissue specificity and glucocorticoid control (6, 7). However, the physiological regulation of gene expression can better be explored in a living animal. Therefore, we studied the time of appearance of HBsAg synthesis during development of transgenic mice and the differences between males and females. We also analyzed the effect of steroid hormones by castrating mice and then injecting different hormones. In this report we show that HBsAg gene expression is detected at day 15 of prenatal development, reaching a maximum at birth in both males and females. After birth, HBsAg gene expression decreases and increases again after sexual maturation. Injection of testosterone, estradiol, and dexamethasone increases the level of HBsAg mRNA and its protein.

MATERIALS AND METHODS

Serum HBsAg levels were measured using the AUSRIA radioimmunoassay (Abbott), and the steady-state amount of liver HBsAg mRNA was estimated by using the RNA blot hybridization technique. The only viral RNA species detected was the 2.1-kilobase (kb) mRNA also present in L cells containing the HBV genome (G7), which was used as the control (2). As an internal reference for a specific liver function, we determined albumin mRNA.

RNA Preparation. Total embryos at day 9 were collected and frozen for RNA isolation. Sex was determined by hybridization of placental DNA with a chromosome Y-specific probe. From day 12 until birth, livers were removed in phosphate-buffered saline (Dulbecco's) containing 50 μM aurintricarboxylic acid and then frozen. Total RNA was extracted by the hot-phenol procedure as described (8). Frozen samples were pulverized and homogenized in 10 mM NaCl/10 mM NaOAc/0.5% $NaDodSO_4$/50 μM aurintricarboxylic acid, pH 5.0. Proteins were extracted with 1 vol of phenol at 65°C, 1 vol of phenol/chloroform, 1:1 (vol/vol), and 1 vol of chloroform. The RNA was then precipitated with 2 vol of 100% ethanol/0.2 M NaCl.

Blotting and Hybridization. For the blot-hybridization analysis, 15 μg of total RNA was separated on 1.5% formaldehyde gels and transferred to nitrocellulose (Schleicher & Schuell BA 85). Ribosomal RNA from the ethidium bromide-stained gel served as a size marker. After transfer of RNA to nitrocellulose, the filters were prehybridized and hybridized with ^{32}P-labeled nick-translated probes as described (9). The following probes were used: (*i*) an *Eco*RI HBV fragment purified from pCP10 (8), (*ii*) an albumin cDNA clone (gift of S. Alonso and M. Buckingham), and (*iii*) a mouse chromosome Y-specific probe (gift of C. Bishop).

Hormone Treatment. In a basic experiment, mice were bled and, a few days later, were anesthesized with Avertin prior to a liver biopsy. Some animals were castrated at the same time. One month later the mice were bled and injected with the hormones. One milligram of testosterone or estradiol diluted in oil was injected subcutaneously. Aqueous solutions of dexamethasone were used; two injections were given, one subcutaneously (2 mg) and an additional one intraperitoneally (1 mg) (17). The animals were bled 2, 4, 6, and 9 days after

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.

hormone injection. On day 9 a second hormone injection of 1 mg was given, and 4 hr later the animals were sacrificed and the livers were recovered. The hormone solutions were testosterone enanthate (Schering), 17-β-estradiol valerianate (Schering), Soludecadron (Merck), and Dectancyl (Roussel, France).

RESULTS

We previously showed that males of one of the transgenic mouse strains, E36, had 5–10 times more serum HBsAg than did females. This was observed in the different generations, although HBsAg titers were generally lower in the F_2 than in the F_1 animals. We now report the same observation upon analysis of F_1 animals (five females and four males) of the second strain, E11. The mean titer of males was 3.5 μg/ml and of females was 0.5 μg/ml. This confirmed that the difference in HBsAg levels between sexes was not due to the insertion of the viral DNA in a particular region of the mouse DNA.

In the following experiments, we only used progenies of backcrosses between F_1 E36 males and C57/B16 females. E11 mice were not included in the study because of their defective growth and fertility.

Relation of HBsAg Gene Expression to Development, Sex, and Age. To determine the start of the HBsAg gene expression in the developing embryo, we analyzed RNA from whole embryos at day 9.5 and from liver at day 15 of gestation until birth (day 20). The 2.1-kb HBsAg mRNA was detectable at day 15, and the amount was increased gradually to reach a maximum at birth (Fig. 1*A*). The increase was particularly steep during the last 2 days before birth.

To compare the kinetics of HBsAg synthesis and that of a liver-specific protein, the same blot was rehybridized with a rat albumin probe. Albumin mRNA also appeared around day 15, but the level was higher at that time than the HBsAg mRNA level and then increased gradually until birth (Fig. 1*A*).

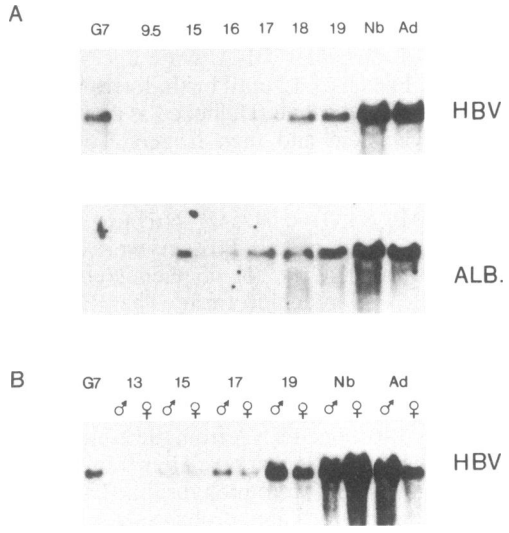

FIG. 1. Developmental regulation of HBsAg mRNA synthesis in embryonic and fetal liver of males only (*A*) and males and females (*B*). Total mRNA was extracted from a pool of four whole embryos at day 9.5 (lane 9.5) and from pools of three livers from day 13 until birth (lane Nb). Adult liver mRNA (lane Ad) was also extracted. About 15 μg was fractionated by electrophoresis on 1.5% denaturing agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled nick-translated probe as described. mRNA of cells transfected with HBV (lane G7) was used as a control, showing the 2.1-kb major hepatic HBsAg mRNA species. (*A*) Samples extracted from males and RNA blot-hybridized first with the HBV DNA probe and then with the albumin (ALB.) cDNA probe. (*B*) Comparison between HBsAg mRNA synthesis in male and female embryos and livers at days 13, 15, 17, and 19 of gestation, at birth (lane Nb), and in adults (lane Ad). The blot was hybridized with a HBV DNA probe. A slightly larger amount of RNA from males at day 17 and 19 was loaded as shown by staining with ethidium bromide.

In a second set of experiments, we compared the amount of hepatic HBsAg mRNA of developing males and females (Fig. 1*B*). There was no significant difference between males and females between day 15 and birth. The level of HBsAg mRNA in the liver of a newborn female was 5–10 times higher than that of an adult female.

We also measured the level of serum HBsAg in mice in the last 2 days of fetal development, at birth, and regularly thereafter until high age (Fig. 2). Because the basic serum HBsAg levels vary from one animal to the other, we calculated the mean titer of five siblings. The concentration of HBsAg in the serum of both males and females was about 0.8 μg/ml 2 days before birth and up to 5 μg/ml at birth, which in some females corresponds to 10 times more than the usual adult titer. It then decreased in both males and females during week 1 of life. At that time, however, the amount of liver HBsAg mRNA was still comparable to that of newborns (data not shown). After puberty, HBsAg increased again and reached in some males 10–15 μg/ml. In adult females, titers never exceeded 2 μg/ml in the F_1 generation and 0.5 μg/ml in the F_2 generation.

HBsAg gene expression was tested several times in the same animal during its life (data not shown). The titers remained stable for several months after puberty, but 18-month-old females had titers less than 1 ng/ml. Males also had low levels of HBsAg, although still 100 times higher than in females.

HBsAg Gene Expression After Modulation of Endogenous and Exogenous Hormone Levels. Serum HBsAg was titrated and liver HBsAg mRNA was analyzed by blot hybridization of total RNA in three adult males and three adult females before and after castration (Fig. 3). In every case, the HBsAg titer decreased 80%. Males still had higher levels than females (mean, 0.5 μg/ml for males and 0.1 μg/ml for females). Hepatic HBsAg mRNA also declined to the same extent (Fig. 3 *Insets*, lane 2). These experiments suggest a

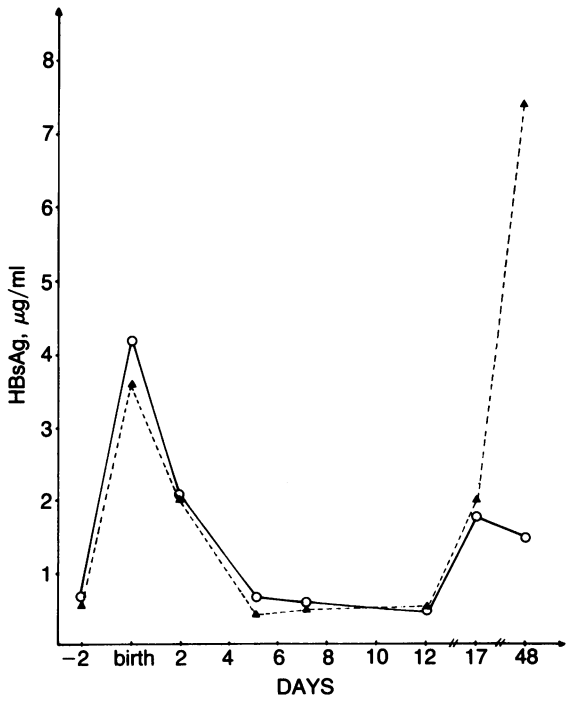

FIG. 2. Age-related concentration of serum HBsAg in males (▲) and females (○). Each value corresponds to the mean titer of five siblings. Animals under 12 days of age were sacrificed for bleeding.

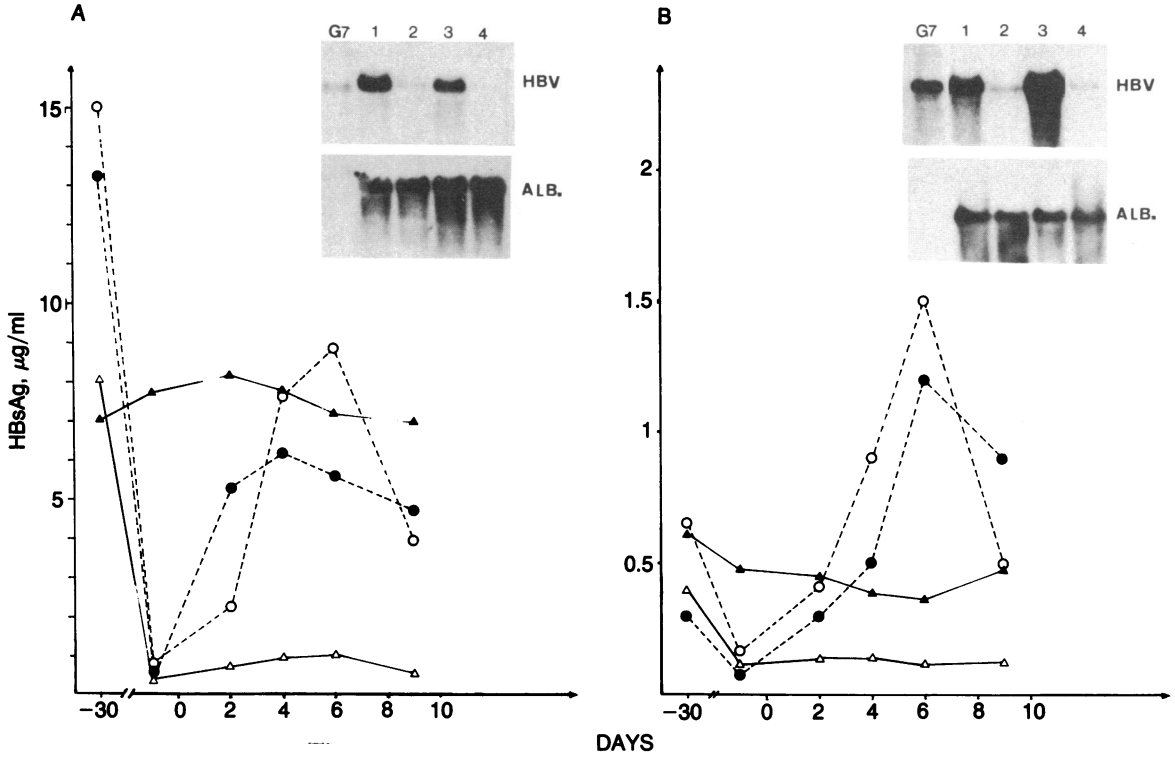

FIG. 3. Effects of testosterone on serum HBsAg and on liver HBsAg mRNA in males (*A*) and females (*B*). One month before hormone injection, animals were simultaneously subjected to partial hepatectomy and bleeding, and some of them were castrated. They were bled again 1 day before the first hormone injection (day 0) and several times thereafter. As a control, noninjected animals [normal (▲) and castrated (△)] were analyzed. At day 0, two castrated mice (○ and ●) were injected with hormone. (*Insets*) Total RNA was extracted from liver samples taken before and after treatment and hybridized first with a HBV probe and then with the albumin (ALB.) cDNA probe. mRNA in lanes: G7, from HBV-transfected cells; 1, from a normal mouse; 2, from a castrated mouse injected with oil; 3, from a castrated mouse injected with testosterone; 4, from a castrated mouse. A longer exposure was used for RNA blots in experiments with females.

positive control by both androgens and estrogens, although natural stimulation by testosterone in males was more efficient.

We then analyzed serum HBsAg and liver HBsAg mRNA levels after injection of testosterone to normal and castrated animals (Fig. 3). To obtain a base value, a liver sample was taken for RNA analysis before the castration and 4 hr after the second injection at day 9. About 60% of expressed serum HBsAg was restored in males 4–6 days after the first injection of testosterone (Fig. 3*A*). The amount of HBsAg mRNA at day 9 was also about 60% that of a normal male (Fig. 3 *Insets*, lane 3). A large dose of testosterone into a normal male led to a small increase (data not shown).

In females injected with testosterone, serum HBsAg and liver HBsAg mRNA levels were 10 times higher, in some cases leading to levels 2 times higher than in normal females (Fig. 3*B*), although they were still low compared to males. In noncastrated females the increase was 2- to 3-fold (data not shown).

Rehybridization with an albumin probe showed that castration or sex hormone injection had no effect on the amount of albumin mRNA.

Similar experiments were performed with estradiol in castrated males and females (Fig. 4). Four days after estrogen injection, serum HBsAg increased 10-fold in castrated males and 20-fold in castrated females. Hepatic HBsAg mRNA levels paralleled the amount of serum HBsAg. Thus, estrogens had a positive effect on expression of the HBsAg gene. However, the maximum expression in the female was still less than in the male.

We finally investigated the effect of dexamethasone on HBsAg gene expression to assess the positive stimulation described in HBsAg-producing cell cultures (10). We used normal females with basic serum HBsAg levels ranging from 0.2 to 0.6 μg/ml and parallel liver HBsAg mRNA amounts (Fig. 5*B*). In the three females injected with hormone, serum HBsAg was increased significantly—in one to >10 μg/ml 6 days after injection. HBsAg mRNA levels also increased conspicuously (Fig. 5). The effective response of females to glucocorticoids excludes a blockage at the DNA level.

Albumin mRNA was not affected by the hormones as shown by rehybridization with an albumin probe.

DISCUSSION

We investigated the developmental and hormonal regulation of HBsAg expression in transgenic mice to better understand which host factors influence the viral cycle. Synthesis of the 2.1-kb HBsAg mRNA and of albumin mRNA starts at day 15 of development. However, at that time, albumin mRNA is more efficiently expressed, and it is only around birth that both reach the same levels. During fetal life and until puberty, expression of HBsAg is equal in males and females probably because of the low levels of sex steroids and/or of receptors during this period. Perinatally both hepatic HBsAg mRNA and serum HBsAg are present in males and females at levels similar to those in adult males. They decrease during the first 4 weeks of life and increase again in males after sexual maturation. The peak at birth could be due to the large amounts of glucocorticoids present at that time (11). Indeed, injection of dexamethasone leads to a very rapid and conspicuous increase in HBsAg production.

HBsAg production in castrated mice was reduced at least by a factor of 10 compared to young mature animals. Injection of testosterone and estradiol into normal or castrated mice increased the production of serum HBsAg and

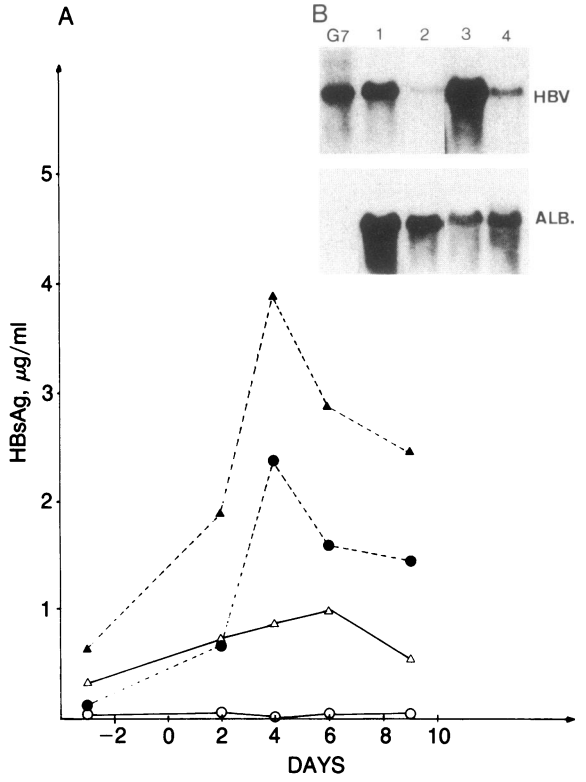

FIG. 4. Effects of 17-β-estradiol on serum HBsAg (*A*) and liver HBsAg mRNA (*B*) in males and females. Castrated males (▲) and females (●) were given a hormone injection at days 0 and 9. They were bled 3 days before the first injection and several days thereafter. A castrated male (△) and female (○) not injected served as controls. (*Inset*) Blot-hybridization analysis was performed on the liver samples as described in Fig. 3. mRNA in lanes: 1, from a castrated female treated with 17-β-estradiol; 2, from a castrated female; 3, from a castrated male treated with 17-β-estradiol; 4, from a castrated male; G7, from HBV-transfected cells. ALB., albumin mRNA.

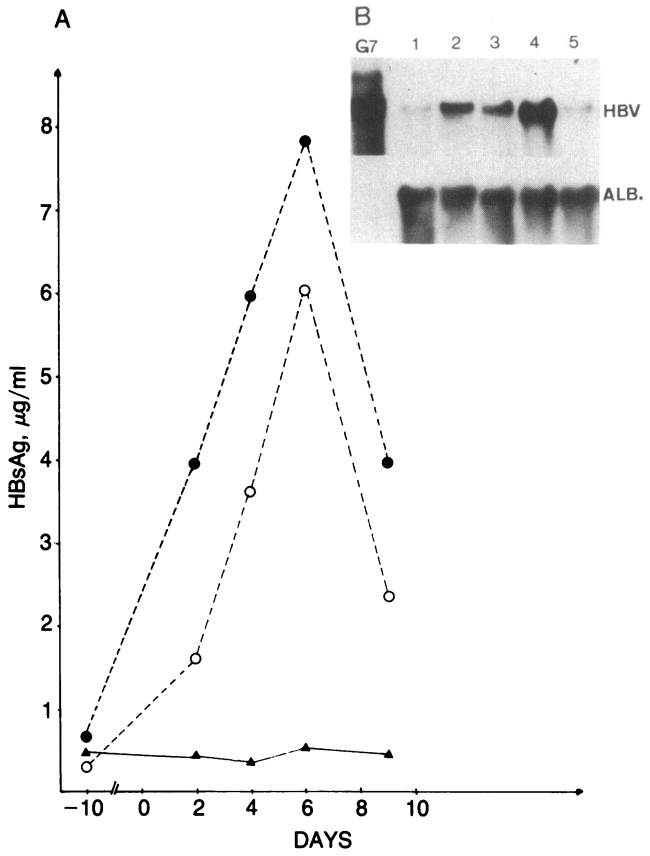

FIG. 5. Effects of dexamethasone on serum HBsAg (*A*) and on liver HBsAg mRNA (*B*). Mature normal females (○ and ●) were injected with dexamethasone at days 0 and 9. They were bled 10 days before the first injection and several days thereafter. mRNA was extracted, and blot-hybridization was performed as described in Fig. 3. Comparison was made with a normal uninjected female (▲). (*Inset*) mRNA in lanes: 1, from a female (○) before treatment; 2, from a female (○) after treatment; 3, from a female (●) before treatment; 4, from a female (●) after treatment; 5, from an uninjected female. G7, HBV-transfected cells mRNA. ALB., albumin mRNA.

hepatic HBsAg mRNA, thus confirming the positive regulation by sex hormones. However, HBsAg levels in females never equaled those in males. This may be due to the different number of liver receptors for sex hormones following the so-called imprinting by testosterone in pre- and neonatal life (12). In aging animals, HBsAg gene expression is also reduced considerably, although no change can be detected at the DNA level (data not shown). Females almost completely lose HBsAg production, possibly because of perturbation of their hormonal status.

In most of our experiments, the amount of hepatic mRNA parallels the serum HBsAg levels. After castration, the amount of mRNA decreases and increases again after injection of hormones. Based on other studies of genes regulated by steroids (13), we postulate that HBsAg gene expression is regulated at the transcriptional level. However, the discrepancy between the large amount of RNA present in the liver 1 week after birth and the low level of serum HBsAg could be due to a posttranscriptional regulation. Run-off experiments should clarify the mechanism of hormonal control.

As the control we determined albumin mRNA and compared it to HBsAg mRNA. In the absence of hormonal stimulation by glucocorticoids and/or sex steroids, HBsAg gene expression is lower than that of the albumin gene, which is constitutionally expressed at a high level and is not affected by hormones. Some liver proteins show sexual dimorphism and are even strictly dependent upon sex-steroid regulation (12). HBV DNA carries in a very condensed form sequences that allow tissue specificity and developmental and hormonal regulation and that could be of cellular origin as suggested (14).

To know how this complex regulation could affect the fate of the natural infection, we compared HBsAg levels of 64 male and 29 female chronic carriers who were HBsAg-positive, HBeAg-negative, and we did not find any significant difference (data not shown). However, such a difference may exist during the incubation period or at the acute stage of infection, before chronicity is established. Infection of infants or young children very often leads to a chronic carrier state without a difference between girls and boys (15). Our results suggest that boys do not have a higher risk of becoming chronic carriers until puberty. However, when adults are infected, 10% become chronic carriers with a male/female ratio of 2 (16). If viral antigens are produced at a higher rate in men, they may allow infected cells bearing these antigens to escape the host immune response and, thus, lead to chronic carriage.

We thank A. M. Courrouce (Centre National de Transfusion Sanguine, Paris) for the data on human chronic carriers, F. Chisari for helpful advice, and K. Paradis for reading the manuscript. This work was supported in part by National Institutes of Health Grant CA37300 and North Atlantic Treaty Organization Grants.

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