Reports

A Transgenic Mouse Model of the Chronic Hepatitis B Surface Antigen Carrier State

Abstract. *In an attempt to establish a model of the healthy carrier state in hepatitis B virus (HBV) infections, transgenic mice expressing HBV genes were produced. Fertilized one-cell eggs were microinjected with subgenomic fragments of HBV DNA containing the coding regions for the HBV surface antigen (HBsAg) and pre-S and X antigens. Either the normal (HBV) or metallothionein promoters were used to obtain expression of the HBV genes. There was no evidence of viral replication or tissue pathology. The integrated HBV DNA sequences were inherited in a normal Mendelian fashion. Three of 16 transgenic mice expressed HBV-encoded gene products to which they were immunologically tolerant. Expression was not tissue specific and may be influenced by the genomic integration site and cellular factors. Both HBsAg and pre-S antigen were detectable within the cytoplasm of hepatocytes and renal tubular epithelial cells. High serum concentrations of HBsAg were detectable and the secreted product appeared authentic as judged by mean density, morphology, mean particle diameter, polypeptide composition, and antigenicity. The absence of tissue pathology in these immunologically tolerant animals supports the hypothesis that cellular injury under these conditions is not a direct consequence of expression of the pre-S or HBs regions of the HBV genome.*

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The human hepatitis B virus (HBV) causes a spectrum of acute diseases ranging from inapparent infection to fatal hepatocellular necrosis. Approximately 10 percent of acutely infected patients continue to carry the virus, and although many of them display variable degrees of liver cell injury, nearly one-half are clinically normal (healthy carriers). Worldwide, there are more than 200 million chronic HBV carriers. Those with severe liver disease often progress to cirrhosis and, after three to four decades, to primary hepatocellular carcinoma (PHC) at a rate more than 200 times greater than the general population (*1*).

Much is known about the structure, organization, and replication of HBV (*2*, *3*). The virus is a circular, partially double-stranded DNA, 3.2 kb in length, which probably replicates via an RNA intermediate and has the capacity to integrate randomly into the host genome. Acute hepatocellular injury generally occurs in the context of free, episomal, viral replication and is usually resolved with the elimination of free virus and virus-infected cells. In the absence of viral clearance, viral sequences may integrate into the human genome. The combined effects of prolonged presence of integrated viral DNA with associated chronic hepatocellular injury and regeneration appear to predispose the infected hepatocyte to neoplastic transformation (*4*).

The mechanisms responsible for acute and chronic hepatocellular injury, and the events leading to the development of PHC, are unknown, in part because it has not been possible to propagate HBV in tissue culture and no suitable inbred animal model has been available for experimentation. In view of the healthy carrier state, it is thought that HBV is not directly hepatocytopathic, but that chronic hepatocellular injury is mediated by a cellular immune response to one or more viral antigens expressed at the hepatocyte membrane (*5*). This hypothesis is untested because of the absence of an appropriate model system. In a similar manner, the relation between viral genome integration and PHC is not well understood. Clearly, chronic HBV infection leads to the development of PHC and most HVB-associated PHC have been shown to contain integrated viral sequences (*6*, *7*). However, since HBV is not an acutely transforming virus, additional cofactors must be necessary for malignant transformation to occur. With the development of mouse embryo microinjection technology, it became apparent that many of those questions might be addressed by creating a model in which integrated copies of the HBV genome are present, expressed, and transmitted in strains of mice that have been previously characterized in terms of their immunological responsiveness to HBV-encoded antigens (*8*). This report describes a prototype of such a model.

Several hundred molecules of DNA fragments containing the coding regions for the HBV surface (*5*) antigen (HBsAg), pre-S, and X antigens downstream of either viral (plasmid pAC_2) or mouse metallothionein I (pMT-PSX) promoters (Fig. 1) were microinjected into the male pronucleus of fertilized one-cell ova of C57BL/6 × SJL hybrids. The offspring were analyzed for the presence of microinjected DNA by tail blot analysis as described (*9*). Ten and six transgenic animals containing pAC_2 and pMT-PSX sequences, respectively, were produced. Southern blot analysis of restricted genomic DNA was consistent with integration of the microinjected DNA in tandem arrays of various copy numbers at one or two presumably random sites within the mouse genome as described in other transgenic systems (*10–12*). Breeding experiments illustrated transmission of the transgene in a normal Mendelian fashion.

Mice were screened for expression of HBsAg. One of ten mice (TM 7-2) in the pAC_2 group and two of six mice (TM 18-11, TM 23-3) in the pMT-PSX group were positive for expression (Fig. 2). The frequency of expression with these constructions was lower than in several previous experiments (*9*, *12–14*), suggesting that sequences within the injected construct may, in some manner, interfere with expression.

Mouse 7-2 (five to ten copies of pAC_2

per cell) produced trace quantities of serum HBsAg (25 ng/ml). Analysis of organ homogenates (Fig. 2) from this mouse revealed highest HBsAg concentration (as percent of total protein) in the stomach, followed by brain, small intestine, and liver. The low level of expression HBsAg in TM 7-2 suggests that HBV may not carry strong regulatory sequences that are recognized by the mouse. The variable tissue distribution of HBsAg in this mouse, and in others, suggests that expression is strongly influenced by the integration site and by poorly defined cellular factors in these animals. Although TM 7-2 transmitted his transgene to 50 percent (46 out of 92) of his progeny only two of these were expressors at the serum and tissue levels. This is unusual but has been described before (*12*).

For these reasons we placed the HBV Bgl II A fragment under the control of the strong, inducible, mouse metallothionein (MT) promoter (*12, 13*) and repeated the microinjection experiment. Two of six transgenic mice (TM 18-11 and TM 23-3) expressed HBsAg in the serum, at 0.05 and 5.6 μg/ml, respectively (Fig. 2). The basis for this variability could not be investigated since TM 18-11 died after a liver biopsy had been obtained. Tissue obtained postmortem from TM 18-11 was suitable for analysis of HBsAg but not RNA. TM 23-3 thus far has produced 40 progeny, 20 of which are transgenic and express HBsAg at serum levels between 0.5 and 10 μg/ml.

The distribution of HBsAg in tissue homogenates was not identical in TM 18-11 and 23-3 (Fig. 2). Thus, even in the presence of the MT promoter, gene expression appears to be influenced by host factors. This hypothesis is supported by the different patterns of antigen expression detectable by immunofluorescence analysis of frozen liver sections from TM 18-11 and 23-3 (Fig. 3). Both pre-S antigen and HBsAg were detectable in each animal as coarse granular deposits in the cytoplasm of some hepatocytes. In TM 18-11, positive cells were distributed randomly throughout the liver lobule. In TM 23-3, in contrast, antigen positive cells were restricted to the periphery of the hepatic lobule, and in this site most of the hepatocytes were positive. Although it is possible that TM 18-11 might have been mosaic with regard to the transgene, TM 23-3 clearly is not mosaic since 50 percent of his progeny are transgenic and they display the same peripheral hepatic lobular distribution of HBsAg by immunofluorescence. The important influence of cellular factors on expression is apparent since many hepatocytes and all of the Kupffer cells, endothelial cells, and bile duct epithelium in the tissue are antigen negative. It is notable that all the tissues of these transgenic mice and their progeny studied thus far have been entirely normal histopathologically.

Expression of the integrated genes was analyzed at several levels. First, HBV-specific transcripts examined by Northern blotting of total liver RNA from TM 23-3 revealed a predominant

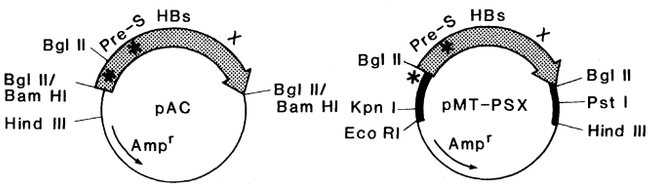


Fig. 1 (left). Plasmid constructions used for microinjection. The pre-S, S, and X coding regions of HBV were introduced into two plasmids. The first, designated pAC_2 contains a 2743-bp DNA fragment of HBV extending between Bgl II sites at nucleotides 2425 and 1986 [see(*3*) for details], cloned into the Bam HI site of pBR322. This construction contains putative sequences necessary for pre-S and HBsAg expression: that is, a TATATAA sequence (asterisk 5′ to the pre-S region) beginning at nucleotide 2776, and transcriptional start sites in the vicinity of nucleotide 3122 (asterisk within the pre-S region), an mRNA polyadenylation signal starting at nucleotide 1916, three translation initiation codons at nucleotides 2848, 40, and 155; and a stop codon at nucleotide 833. It also contains the initiation and termination codons that define the X region open-reading frame at nucleotides 1374 and 1836, respectively, and a putative enhancer element recently described (*19*) as being located in the region between the S and X genes. This 7143-bp plasmid was linearized at the unique Hind III site located within the vector prior to microinjection. The second construction, designated pMT-PSX, contains a 2329-bp HBV fragment which spans Bgl II sites at nucleotides 2839 and 1986 and therefore contains all of the coding information of the first construction but lacks the viral TATATAA sequence. This fragment was cloned into plasmid pMT-I (*20*) at its Bgl II site which is located 65 bp downsteam from the MT cap site (asterisk 5′ to Bgl II site within the MT-1 sequence). Similar constructions with other genes cloned in MT-1 have led to high level expression in transgenic mice (*12, 13*). Plasmid pMT-PSX was digested with Kpn I and Pst I and the resulting 3469-bp fragment containing only HBV and MT sequences was used for microinjection. Broad dotted arrow, HBV sequences; heavy black line, MT sequences; thin black line, pBR-322.

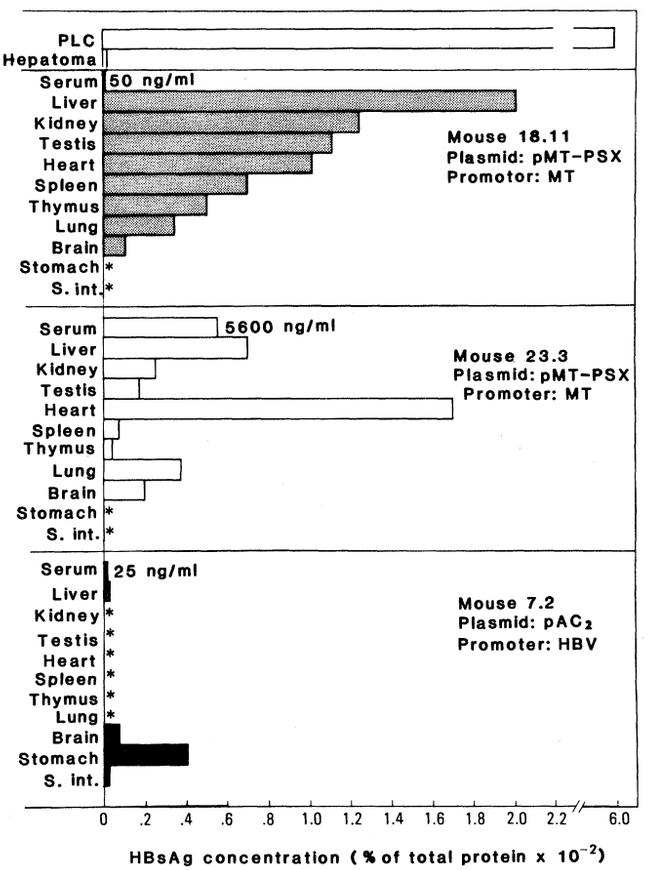
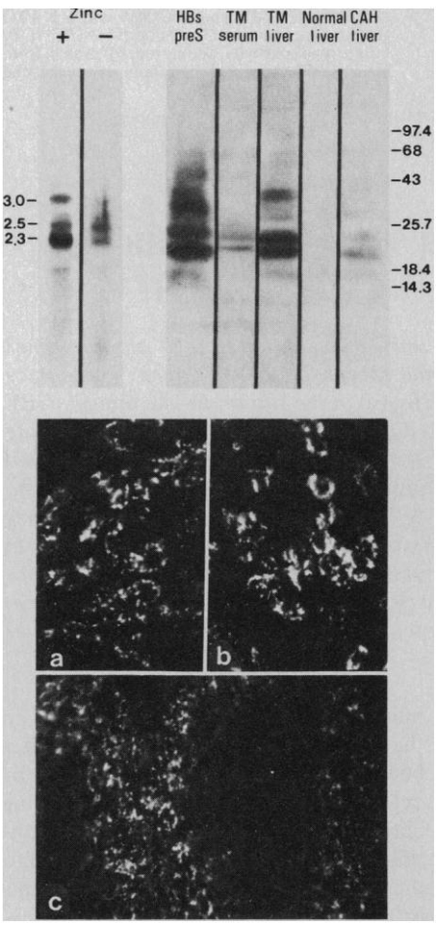


Fig. 2 (right). Tissue distribution of HBsAg in transgenic mice. HBsAg was measured in serum and cell or organ homogenates by a solid-phase radioimmunoassay (AUSRIA II, Abbott) and quantitative HBsAg levels were determined by end-point serial dilution and comparison with a standard curve with the use of purified HBsAg particles of known concentration (*8*). Total protein was determined by Coomassie blue binding (Bio-Rad). Asterisks reflect the absence of detectable HBsAg. Snap frozen tissue powder was suspended in 0.5 ml of 0.01*M* sodium phosphate buffer, *p*H 7.4 containing 0.15*M* NaCl and 1 m*M* phenylmethylsulfonyl fluoride and subjected to three cycles of freezing and thawing and centrifugation (Microfuge B, Beckman). PLC, human HBV-positive Alexander hepatoma cell line (*21*); hepatoma, human HBV-positive surgically resected hepatoma.

2.3-kb transcript (Fig. 3) consistent with promotion at or near the MT promoter and termination at the HBV polyadenylation signal. Since this transcript was inducible by zinc (Fig. 3), the MT promoter was probably functional. Several larger transcripts, some of which were zinc inducible, were also observable. These inducible transcripts were probably due to termination downstream from the HBV polyadenylation signal. Noninducible transcripts may represent RNA's initiated from other promoters present either in adjacent mouse DNA or in the HBV sequences. HBV-specific transcripts were detectable by Northern blotting only in liver, kidney, testis, heart, and brain, suggesting that tissue HBsAg detectable in other organs may reflect serum contamination. Furthermore, only liver and kidney displayed dominant 2.3-kb transcripts, whereas in other organs either larger transcripts were dominant or all transcripts were equally represented.

The protein was also assessed by Western blotting of HBsAg–positive serum and liver homogenates (Fig. 3). Both serum and liver from TM 23-3 revealed the predicted HBsAg bands at molecular ratios (M_r) of 21,000 and 25,000 representing the unglycosylated and glycosylated forms of the 226 amino acid product of the HBs gene (*15*). Additional, higher molecular weight bands were observed in liver with a dominant band at about 39,000 M_r. This falls between the predicted sizes of the translation products that derive from the two translation initiation codons within the pre-S region (34,000 and 42,000 M_r) and thus may represent an alternative form of pre-S antigen (*16*).

We also examined the secreted serum product. HBsAg activity migrated with an apparent molecular weight greater than 1×10^6 upon gel filtration by high pressure liquid chromatography consistent with the size (2.5×10^6 M_r) of human serum HBsAg particles from HBV-infected patients. This high molecular weight material banded at a mean density of 1.20 g/cm^3 in cesium chloride (Fig. 4), consistent with the density of human HBsAg (*17*). Finally, this highly purified fraction contained filamentous and spherical HBsAg particles with a mean diameter of 22 nm, indistinguishable from human HBsAg particles (*16*), as shown by immunoelectron microscopy



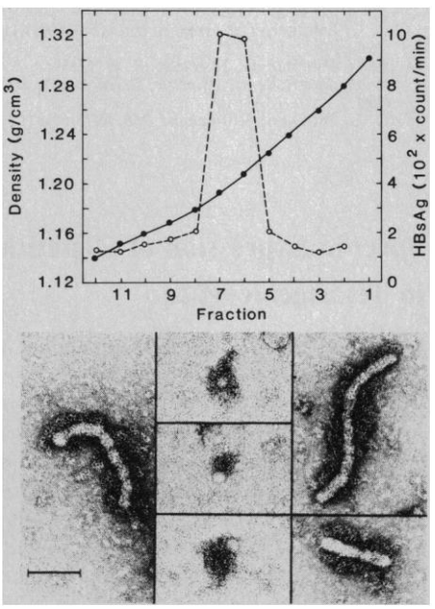


Fig. 3 (left). Expression of the HBV gene in pMT-PSX transgenic mice. (A) Northern blot: Total RNA from snap frozen liver homogenates from TM 23-3 was prepared according to Holmes and Bonner (*22*), subjected to electrophoresis through 1.4 percent agarose gels after glyoxal-dimethyl sulfoxide (DMSO) denaturation and transferred to nitrocellulose. Filters were prehybridized for 4 hours at 65°C in 50 ml of 6× standard saline citrate (SSC), 5 percent Denhardt's solution containing single-stranded salmon sperm DNA (150 μg/ml), and hybridized to a nick-translated, HBV-specific DNA probe labeled with ^{32}P to a specific activity of approximately 2×10^8 cpm/μg. Filters were washed with 2× SSC and 0.1 percent sodium dodecyl sulfate (SDS) in 1 m*M* $NaPO_4$ buffer at room temperature and then with 0.1 × SSC with 0.1 percent SDS at 65°C. Filters were exposed to Kodak XAR film with two enhancing screens at −70°C for 16 to 24 hours. The samples represent 30 μg of total cellular RNA prepared from surgical liver biopsies obtained from TM23-3 before, and 5 days after, his drinking water was supplemented with zinc sulfate (7.5 g/liter). (B) Western blot: Serum and organ homogenates prepared as described (see Fig. 1) were denatured in 2.5 percent SDS and 350 m*M* mercaptoethanol, boiled, and subjected to electrophoresis through 15 percent polyacrylamide gels in tris, glycine, and SDS as described (*23*). Proteins were transferred to nitrocellulose by electroblotting (Bio-Rad) and the filters were blocked with 1 percent bovine serum albumin and 0.5 percent fetal calf serum in 50 m*M* tris and 150 m*M* NaCl, *p*H 7.6, containing Tween 20 (50 μl/liter), and incubated overnight at 4°C as described (*24*). Filters were developed by sequential incubation with a rabbit antiserum to a synthetic peptide fragment (p49a) of HBsAg (*25*), and then an affinity purified goat antiserum to rabbit immunoglobulin (Boehringer Mannheim) and ^{125}I–labeled affinity purified swine antiserum to goat immunoglobulin (2×10^5 cpm/ml; 3 μCi/μg). Washed filters were exposed to Kodak XAR-5 film for 6 to 12 hours at −70°C with one enhancing screen. Samples: HBs/Pre-S is a purified preparation of recombinant HBV particles produced by Chinese hamster ovary cells transfected with an HBV-DHFR construction as described (*26*). Characteristic HBsAg bands are present at 21,000 and 25,000 M_r and a pre-S/HBsAg band is at 39,000 M_r. TM serum and liver are samples from TM 23-3. Normal liver is derived from a nontransgenic littermate of TM 23-3. CAH liver is derived from a patient with chronic active hepatitis B. (C) Immunofluorescence: Cryostat sections (3 μm) of (a and b) TM 18–11 and (c) TM 23-3 were stained with a fluorescein-conjugated human antiserum to HBsAg (b and c) as described (*27*) or with a rabbit antiserum to synthetic pre-S antigen peptide representing the 26 amino terminal residues of the pre-S region (*28*) and then with a fluorescein-conjugated goat antiserum to rabbit immunoglobulin. Specificity of all reactions was assessed by blocking with the relevant antigen. Fig. 4 (right). (Top) Partially purified (by high-pressure liquid chromatography) serum HBsAg from TM 23-3 was ultracentrifuged for 48 hours in a self-generating cesium chloride gradient in a Beckman-type 50 Ti rotor at 40,000 rev/min at 20°C. Fractions collected by bottom puncture were analyzed for the presence of HBsAg as described in the legend to Fig. 2. (Bottom) Fraction 7 above was dialyzed against distilled water, concentrated by evaporation (Speed-Vac), applied to parlodion, carbon-coated grids, and incubated with a rabbit antiserum to HBsAg (Calbiochem-Behring), and then a colloidal gold-labeled affinity purified antibody to rabbit immunoglobulin G. Samples were negatively stained by aqueous uranyl formate. Grids were examined under a Hitachi HV12A electron microscope at ×30,000 magnification. Note the characteristic morphology, diameter, and antigenicity of native HBsAg particles.

with collodial gold-labeled antibodies (Fig. 4). Thus, on the basis of molecular weight, buoyant density, diameter, morphology, and antigenicity, secreted transgenic HBsAg is indistinguishable from authentic human HBsAg particles.

All animals were clinically and histologically normal over several months of observation. As expected, HBsAg-positive mice failed to produce specific antibody either spontaneously or after immunization with human HBsAg in complete Freund's adjuvant, whereas HBsAg-negative transgenic littermates produced a normal response to immunization. This suggests that HBsAg expressors are immunologically tolerant to this antigen. These observations are compatible with the hypothesis that liver cell injury in human HBV infection is not a direct consequence of viral envelope antigen expression and may be secondary to a cytotoxic immune response to hepatocyte surface membrane viral antigens. It will now be possible to design studies to circumvent tolerance and monitor immunologically mediated tissue injury specific for each of the viral antigens based on the transgenic mouse model described here.

These transgenic mice thus provide a model analogous to the stage in HBV infection when replication has ceased and the viral DNA has integrated into the host genome as occurs in the chronic carrier state and in hepatocellular carcinoma. Our results suggest that endogenous HBV regulatory signals are weak in this system, and that their expression may be influenced by the integration site and cellular factors. Other data suggest that the pAC_2 construct is able to establish high serum levels of HBsAg in transgenic mice (*18*). Because of the low HBsAg serum titers we achieved with this construct (pAC_2), the MT fusion gene may provide a useful alternative system. The virtue of the MT promoter is its strength and inducibility, but expression also appears to be influenced by host regulatory factors as illustrated by the variable tissue distribution of HBsAg and the nonuniform cellular expression of HBsAg and pre-S Ag among hepatocytes. Nonetheless, the MT promoter leads to predictable expression of high levels of HBV-encoded antigens within the liver and this makes it useful for subsequent studies of immunologically mediated hepatocellular injury in transplantation and adoptive transfer experiments. Although the transgenic mouse model lacks many features of HBV infection, it provides an opportunity to study the consequences of expression of integrated HBV DNA in genetically defined mice of predetermined immune responsiveness to HBV-encoded antigens. Such studies should provide useful information pertaining to the pathogenesis of the diseases associated with HBV in man.

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Specific Expression of Hepatitis B Surface Antigen (HBsAg) in Transgenic Mice

Abstract. *Two transgenic mice were obtained that contain in their chromosomes the complete hepatitis B virus (HBV) genome except for the core gene. These mice secrete particles of HBV surface antigen (HBsAg) in the serum. In one mouse, HBV DNA sequences that had integrated at two different sites were shown to segregate independently in the first filial generation (F_1) and only one of the sequences allowed expression of the surface antigen. Among these animals the males produced five to ten times more HBsAg than the females. A 2.1-kilobase messenger RNA species comigrating with the major surface gene messenger RNA is expressed specifically in the liver in the two original mice. The results suggest that the HBV sequences introduced into the mice are able to confer a tissue-specific expression to the S gene. In addition, the HBV transgenic mice represent a new model for the chronic carrier state of hepatitis B virus infection.*

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Hepatitis B virus (HBV) infects humans and some other primates and may cause acute hepatitis or, in some patients, chronic hepatitis and hepatocellular carcinoma (*1–2*). The role of the immune response to the viral antigens in the appearance of liver cell necrosis has been postulated (*3*), but the target for cytotoxic cells is still unknown. During chronic infection the viral DNA can continuously replicate or it can integrate into the cellular genome where it is apparently maintained passively. When DNA is present only in an integrated form, viral surface antigen (HBsAg) is the only antigen expressed and the patient is asymptomatic and shows mild inflammation of the liver. By contrast, active hepatitis is often associated with viral replication and is accompanied by the synthesis of the core antigen carried by the viral capsid in addition to the HBsAg (*4*). In either situation, transformation of an in-