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Genetics

Expression and replication of hepatitis B virus genome in transgenic mice

(core particle/reverse transcription/extrachromosomal DNA/animal model)

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ABSTRACT **We produced transgenic mice by microinjecting a partial tandem duplication of the complete hepatitis B virus (HBV) genome into fertilized eggs of C57BL/6 mice. One of eight transgenic mice was a high producer for HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) in the serum. The HBV genomes were transmitted to the next generation and these F_1 mice also produced HBsAg and HBeAg. mRNAs of 3.5, 2.1, and 0.8 kilobases were detected in the livers and the kidneys of these mice. In addition, a 0.8-kilobase RNA was detected in the testis. Single-stranded and partially double-stranded HBV DNAs were shown to be produced in the cytoplasm of the liver and kidneys. These HBV DNAs were associated with the core particles, indistinguishable from nucleocapsid produced in an infected human liver. Viral genome DNA was detected in the serum. These results demonstrate that the HBV genome integrated into the mouse chromosome acted as a template for viral gene expression, allowing viral replication. Thus, these transgenic mice should be useful for detailed studies of the replication and expression of HBV and for pathological studies of hepatitis, including the development of hepatocellular carcinoma.**

Hepatitis B virus (HBV) is a causative agent of hepatitis. This viral genome DNA is a partially double-stranded circular molecule. After infection, it is converted into a covalently closed circular molecule (1), which is transcribed into two main species of mRNA, 2.1 and 3.5 kilobases (kb) in size (2). These molecules are then translated to produce viral proteins, HBV surface antigen (HBsAg) and HBV core antigen (HBcAg), and presumably other proteins called Pre-S, X, and Pol as inferred from the open reading frames. The 3.5-kb RNA, which is called pregenome RNA, is reverse transcribed (3) presumably by the viral polymerase (4), and the product, single-stranded minus DNA, then serves as a template for the synthesis of a plus strand. This reaction often terminates before completion, resulting in the formation of partially double-stranded DNA.

HBV infection is linked to later development of cirrhosis and hepatocellular carcinoma (HCC). Beasley *et al.* (5) showed from prospective epidemiological studies that the relative risk to HBsAg carriers of developing HCC was 217 times as compared with noncarriers. Despite the crucial role of HBV in human health problems, there is only limited knowledge of its mode of replication, integration, and tumor induction because the virus multiplies only in human and chimpanzee livers.

Recently, cell culture systems have been established that allow expression and replication of the HBV genome following transfection with cloned HBV DNA (6–9). These systems have allowed detailed molecular and genetic studies of HBV replication and protein synthesis, but they are not suitable for studies on the outbreak of hepatitis and the induction of HCC. One approach to overcoming these problems is to make a transgenic animal carrying HBV DNA. In this approach the introduced DNAs are located on the same chromosomal site in all cell types of the animal, allowing analyses of tissue-specific expression and of pathophysiological consequences of the expression of the HBV genome. Three groups of investigators have demonstrated successful expression of HBsAg in transgenic mice by introducing a partial or a whole fragment of the HBV genome (10–12).

To express all viral proteins, we employed partially duplicated copies of the HBV genome for injection into fertilized mouse eggs. This structure was chosen because it is sufficient for production of the complete 3.5-kb pregenome RNA. Here we report a line of transgenic mice that produces mRNAs of defined size, viral antigens, and viral DNAs.

MATERIALS AND METHODS

DNA. The HBV genome used in our studies was derived from plasmid pBRHBadr4 (13). Plasmid 1.2HB-BS carries one full length of the HBV genome (*Bam*HI fragment) plus a 619-base-pair (bp) overlapping region (*Bam*HI/*Stu* I fragment) (Fig. 1). This HBV fragment contains the minimum region necessary for transcription of the 2.1-kb and 3.5-kb RNAs (Fig. 1) (see ref. 2). Prior to injection into fertilized mouse eggs, plasmid 1.2HB-BS was digested with *Hin*dIII and *Nde* I, and the resulting 4.4-kb fragment was isolated and used for microinjection.

DNA Injection. C57BL/6 mice were used for production of transgenic mice. Several hundred molecules of the 1.2HB-BS fragment were microinjected into the pronucleus of fertilized eggs according to the method described (14, 15).

Isolation and Analyses of Nucleic Acids. Samples were lysed with $NaDodSO_4$/Pronase E (Kaken-Kagaku, Tokyo) and RNase A (Sigma). They were treated twice with phenol/chloroform, 1:1 (vol/vol), precipitated with ethanol, and dissolved in TE (10 mM Tris·HCl, pH 7.5/1 mM EDTA). Total RNA was prepared as described (16). DNAs were subjected to electrophoresis in a 1.2% agarose gel and transferred to nylon membranes (GeneScreen*Plus*) according to the manufacturer's recommendations. RNAs were subjected to electrophoresis in a 1% agarose gel containing 6.6% formaldehyde and then transferred to nylon membranes (GeneScreen*Plus*). Hybridizations were done under stringent conditions with a random-primed ^{32}P-labeled whole or regional HBV DNA probe (17) prepared from pBRHBadr4 (13) or with a ^{32}P-labeled strand-specific probe prepared by the M13 system (18). S1 nuclease

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Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBsAg, HBV surface antigen; HBcAg, HBV core antigen; HBeAg, HBV e antigen.

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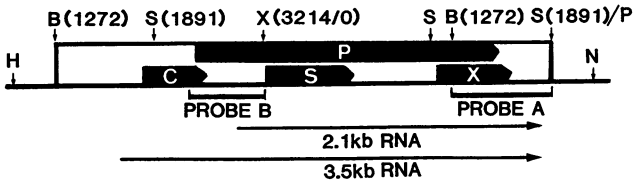

FIG. 1. Structure of plasmid 1.2HB-BS. The coding regions of HBcAg, HBsAg, HBx, and polymerase are displayed with blackened arrows marked C, S, X, and P, respectively. The expected transcripts from this HBV fragment are represented by thin arrows marked 3.5-kb RNA and 2.1-kb RNA. Probe A and probe B represent the fragments that were used in RNA blot analyses. Short vertical arrows with B, H, N, P, S, and X represent the sites of restriction endonucleases *Bam*HI, *Hin*dIII, *Nde* I, *Pvu* II, *Stu* I, and *Xho* I, respectively. The nucleotide numbers marked along the HBV genome start at the unique *Xho* I site.

mapping was carried out according to the method described by Burke (19).

Isolation of Core Particles of HBV from Liver. Liver tissue was homogenized in TE, and cellular debris was removed by centrifugation. PEG 6000 was added to the supernatant to a final concentration of 3% (wt/vol), and high molecular weight components were precipitated by centrifugation. The precipitate was sonicated in 1% Triton X-100 in TE for 5 min and was sedimented through a 7.5–60% (wt/wt) sucrose gradient made up in TE at 240,000 × *g* for 2 hr. Fractions were collected from the bottom. DNA was extracted from each fraction. Part of each fraction was also assayed for HBV e antigen (HBeAg)/HBcAg titer by a commercial enzyme immunoassay kit (Abbott).

Isolation of Cytoplasmic DNA. Liver and kidney tissues were disrupted with eight full strokes in a Dounce homogenizer in extraction buffer [20 mM Tris·HCl, pH 7.4/7 mM $MgSO_4$/50 mM NaCl/0.1% 2-mercaptoethanol/0.25 M sucrose (EB)] (3). After removal of cellular debris and nuclei by centrifugation, the supernatant was layered on 15%, 20%, and 30% stepwise sucrose gradients made up in EB (wt/vol) and centrifuged at 240,000 × *g* for 4 hr at 4°C. DNA was prepared from the pellet as described above.

Analysis of HBV-Related Materials in Serum. PEG 6000 was added to serum to a final concentration of 12% (wt/vol), incubated at 4°C for 3 hr, and centrifuged. The precipitate was suspended in phosphate-buffered saline, loaded on a discontinuous CsCl density gradient (from 23% to 39%), and spun at 240,000 × *g* for 20 hr. Each fraction was assayed for HBsAg by an enzyme immunoassay kit (Abbott) and DNA was prepared from each fraction.

Pathologic Analysis. Organs (brain, lung, heart, liver, spleen, kidney, testis, and bone marrow of the femur) were obtained, and sections were made for histological, histochemical, immunohistochemical, and electron microscopic examinations according to standard methods (20).

RESULTS

Establishment of the Transgenic Mouse Line Expressing the HBV Genome. The 1.2HB-BS DNA was microinjected into fertilized eggs, and the surviving eggs were then transferred into the oviducts of foster mothers. In total, 23 mice were born. The tail DNAs from these mice were screened for the presence of HBV DNA by Southern blot analysis. Eight mice were shown to carry HBV DNA. Two mice, 1.2HB-BS 10 (male) and 1.2HB-BS 17 (male) were positive for HBsAg and HBeAg in the sera, and their HBsAg concentrations were 15 ng/ml and 2 ng/ml, respectively (quantitation between the titer and concentration of HBeAg has not been done). Since the 1.2HB-BS 10 mouse is a high producer, this mouse was chosen for the subsequent studies. Among 18 offspring from the 1.2HB-BS 10 mouse, 12 mice carried the HBV DNA and were positive for HBsAg and HBeAg in their sera. No sex difference in the titer of these antigens showed in animals at the age of 7 weeks.

Analysis of RNA. Total RNAs prepared from various tissues of the F_1 mice were subjected to RNA blot analysis. When the probe was the whole HBV DNA or probe A (see Fig. 1), two RNAs, 3.5 kb and 2.1 kb in size, were detected in the liver and the kidneys (Fig. 2, probe A). The sizes of these RNAs are in good agreement with those detected in HBV-infected hepatocytes (2). A small amount of 2.4-kb RNA was present in the liver but not in the kidneys (see Fig. 3). Interestingly, the amount of the 3.5-kb RNA was only slightly lower than that of the 2.1-kb RNA in the liver, whereas it was higher than that of the 2.1-kb RNA in the kidneys. However, more 3.5-kb RNA was detected in the liver than in the kidneys (Fig. 2, probe A).

A 0.8-kb RNA was detected in these two tissues and in the testis as well. Siddiqui *et al.* (21) reported the existence of 0.8-kb RNA, which corresponds to the X open reading frame. To examine whether the 0.8-kb RNA is the transcript for the X protein, we employed two kinds of probes (see Fig. 1) for RNA blot analysis: probe A contains the X region, whereas probe B covers a region between C and S. As shown in Fig. 2, the 0.8-kb RNA hybridized only with probe A but not with probe B. Another 0.7-kb RNA that hybridized with probe B but not with probe A was also detected in the liver. However, the nature of this RNA is not clear. No HBV RNA was detected in other organs tested (see Fig. 2), although the presence of intact RNA could be shown by rehybridization with a β-actin probe (data not shown).

The initiation sites for the 3.5-kb and 2.1-kb transcripts were examined by S1 nuclease mapping using two probes that detect the 5′ end in either the C or S promoter regions. The precore region probe revealed three start sites in the liver and the kidneys (Fig. 3*A*, a, b, and c). The major initiation site (a) was located between the ATG of pre-C and C. Two minor initiations (b and c) occurred upstream of the ATG of pre-C and probably represented the initiation sites for the pre-C. These results are consistent with previous studies using a cell culture system (9). We also confirmed that the 2.1-kb and 3.5-kb RNAs terminated at the poly(A) signal in the C region (t). Fig. 3*B* shows that three clusters of initiation sites of the 2.1-kb RNA were mapped around the ATG of the pre-S2 using the pre-S region probe in the liver and the kidneys. Two of them were located downstream of the ATG (d and e), and the other one was upstream of the ATG (f). These results are in good agreement with the previous observation (22). Three additional initiation sites of the 2.4-kb RNA were located around the ATG of the pre-S1 region in the liver but not in the kidneys. Two of these, i and g, correspond to the starts for

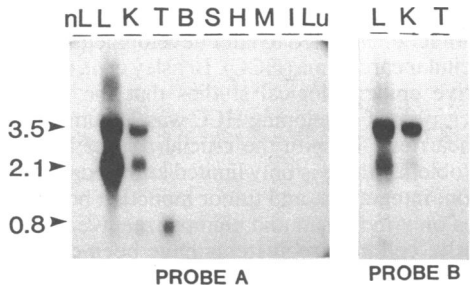

FIG. 2. RNA blot analysis of total RNA (12 μg) prepared from various tissues of the 1.2HB-BS 10 F_1 mouse. Probe A, hybridization with probe A (see Fig. 1) containing the X coding region. Probe B, hybridization with probe B (see Fig. 1) containing a region between C and S. nL, liver from a normal mouse; L, liver; K, kidney; T, testis; B, brain; S, spleen; H, heart; M, muscle; I, intestine; Lu, lung. Sizes are shown in kb.

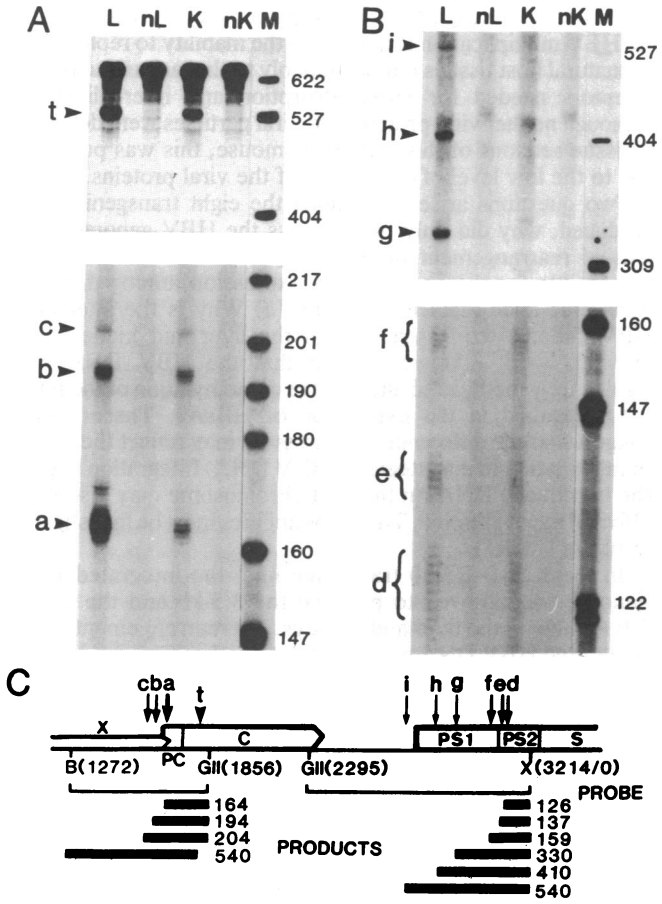

FIG. 3. S1 nuclease mapping. Total RNAs (10 μg each) of the liver and kidneys of the 1.2HB-BS 10 F_1 mouse (lanes L and K, respectively) and liver and kidneys of a normal mouse (lanes nL and nK, respectively) were applied to S1 nuclease mapping. Lanes M, size markers (shown in nucleotides). (*A*) The 5′ initiation in the pre-C region. The *Bam*HI–*Bgl* II single-stranded probe was used (see below). The protected bands are marked a, b, c, and t. (*B*) The 5′ initiation in the pre-S region. The *Bgl* II–*Xho* I single-stranded probe was used (see below). The protected bands are marked d, e, f, g, h, and i. (*C*) Schematic representation of the results of S1 nuclease mapping. The viral open reading frames are shown by open arrows. Thin lines indicate the probes, and thick lines indicate the protected products. Numbers at the right of these lines represent the lengths of the protected fragments in nucleotides. The arrows (a–i) indicate the start positions of transcription, as deduced from the sizes of the corresponding products of S1 nuclease mapping. t, Termination site of the 3.5-kb and 2.1-kb RNAs; B, *Bam*HI; GII, *Bgl* II; PC, precore region; PS1, pre-S1 region; PS2, pre-S2 region.

the pre-S1 and pre-S2 mRNA, respectively, but the other (h) was not determined.

Production of Intracellular Core Particles and Their Association with Free HBV DNA. Since the 3.5-kb RNA that could serve as a HBV pregenome was detected in the liver and kidneys, the HBV genomes were expected to replicate in these tissues. The intermediate replication product of HBV DNA has been shown to reside in core particles forming a replication complex (3). To examine whether such core particles were produced in the liver, we analyzed the extract of the liver of an F_1 mouse by a sucrose gradient velocity sedimentation. Core particles produced in yeast were used as a reference, which have the same size and sedimentation properties as the particles produced in an infected human liver (23). Fig. 4 shows that the patterns of HBeAg/HBcAg sedimentation in these two samples were indistinguishable, lending strong support to the conclusion that core particles in

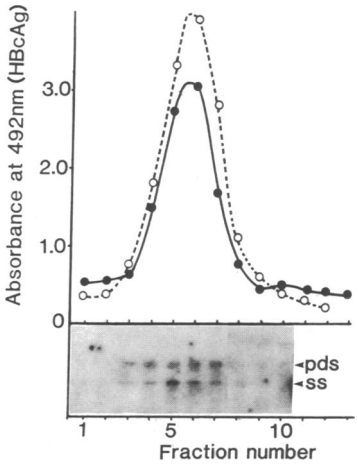

FIG. 4. Sucrose gradient velocity sedimentation of replicative complexes from the liver extract of the 1.2HB-BS 10 F_1 mouse. Total liver extract was sedimented through a sucrose gradient, and 24 fractions were collected from the bottom. Part of each fraction was assayed for HBeAg/HBcAg titer by an enzyme immunoassay kit (Abbott). Core particles produced in yeast (19) were run in parallel. ○, Core particles of yeast; ●, liver sample of the 1.2HB-BS 10 F_1 mouse. The DNAs were recovered from each fraction and were subjected to Southern blot analysis after electrophoresis through a 1.2% agarose gel. The probe used was whole HBV DNA. ss, Single-stranded HBV DNA; pds, partially double-stranded DNA.

the liver of the transgenic mice are very similar to those produced in infected human liver.

To see whether the HBV DNA is produced and assembled in core particles, the DNA from each fraction of the gradient was analyzed by Southern blot hybridization. As shown in Fig. 4, HBV DNA was detected only in the fractions in which core particles were present. These results strongly suggest that this HBV DNA was reverse-transcribed from the 3.5-kb RNA and was associated with the core particles to form a replicative complex.

Analysis of the Cytoplasmic HBV DNAs. To characterize the HBV DNA within the core particles, this DNA was analyzed by treatment either with heat at 100°C or with restriction endonuclease *Bam*HI or *Bgl* II, followed by Southern blot analysis (Fig. 5*A*). Two bands were detected before treatment (lane a). The slow-migrating band was eliminated by heating (lane b) and was resistant to digestion with *Bam*HI (lane c) but was digested with *Bgl* II to produce a faster-migrating band (lane d), indicating that it represents partially double-stranded DNA (3). *Bam*HI cleaves the HBV genome at one site in the X region, and *Bgl* II cleaves at two sites in the C region. Therefore, the C region is expected to be in the duplex form. On the other hand, the fast-migrating band was unchanged by any treatment, indicating that it represents single-

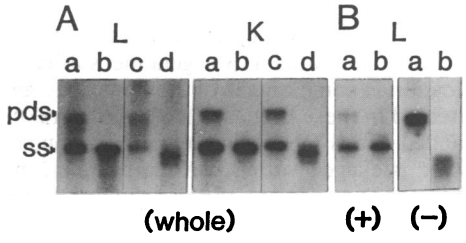

FIG. 5. Southern blot analyses of DNAs prepared from the cytoplasm of the liver (L) and the kidneys (K). (*A*) The probe used was a random-primed ^{32}P-labeled whole HBV DNA probe (whole). (*B*) The probes used were HBV plus strand (+) and minus strand (−). Lanes: a, nontreated DNA; b, DNA treated at 100°C for 3 min; c, DNA treated with *Bam*HI; d, DNA treated with *Bgl* II. ss, Single-stranded HBV DNA; pds, partially double-stranded HBV DNA.

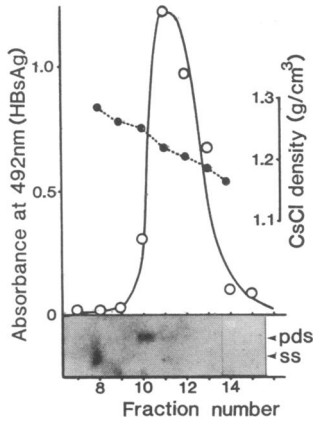

FIG. 6. CsCl density-gradient sedimentation of the serum of the 1.2HB-BS 10 F_1 mouse. A total of 10 ml of serum was concentrated to <0.5 ml and subjected to centrifugation. Fractions collected from the bottom were analyzed for HBsAg titer (○) and for the density of CsCl (●). The DNAs were recovered from each fraction and were subjected to Southern blot analysis using a whole HBV DNA probe. ss, Single-stranded HBV DNA; pds, partially double-stranded HBV DNA.

stranded DNA. In addition, the single-stranded and double-stranded DNAs hybridized with the plus strand probe and with the plus and minus strand probes, respectively (Fig. 5*B*). Therefore, the double-stranded DNA must be the assembly of a single-stranded minus strand and an incomplete plus strand. Thus far, no closed circular form of HBV DNA has been detected.

HBV-Related Materials in Serum. About 10 ml of serum of F_1 mice was concentrated and was subjected to a CsCl density-gradient centrifugation. HBsAg was distributed around the density of 1.2 g/ml (Fig. 6). The DNA recovered from each fraction was then examined by Southern blot analysis. HBV DNA was detected principally in the fraction with a density of 1.25 g/ml, where Dane particles are usually detected (24). This HBV DNA migrated to the same position as the partially double-stranded HBV DNA prepared from the liver cytoplasm (Fig. 6). These results strongly suggest that the partially double-stranded DNAs produced in the liver and kidneys are packaged into Dane particles and secreted into the serum together with small HBsAg particles.

Histological Examination. We performed histological analyses of the F_1 mouse. However, there was no evidence of tissue pathology. No HBV antigens were detected in any of the tissues that were examined. Nor could we find any HBV-related particles, using the electron microscope, in the nuclei or cytoplasm of the liver. Perhaps the HBV antigens were below detectable levels in the tissues.

DISCUSSION

An important contribution of this work is the finding that the chromosomally integrated HBV genome suffices to allow viral replication in some organs of the transgenic mice. HBV DNAs detected in these tissues consisted of a mixture of the single-stranded minus DNA and the partially double-stranded viral genome packaged within core particles, such as those found in infected human livers. We also showed the presence of a very small amount of partially double-stranded HBV DNA in the serum, probably representing the HBV DNA packaged in Dane particles. Thus it is highly likely that the normal process of HBV replication, including the production of the 3.5-kb pregenome RNA, its packaging into a nucleocapsid, the synthesis of the complete minus strand DNA, and the synthesis of the plus strand, followed by the release of Dane particles into the serum, occur in this transgenic mouse and its descendants. Our results also suggest that the apparent species specificity of the HBV multiplication is not due to the inability to replicate in nonnatural host tissues but presumably to the lack of factors or receptors needed for virus adsorption and internalization. Although neither viral proteins nor viral particles were detected in tissue sections of this transgenic mouse, this was probably due to the low level of expression of the viral proteins.

Two questions arise. (*i*) Among the eight transgenic mice produced, why did only two express the HBV genome? Extensive rearrangement of the integrated HBV DNA is not responsible for the variability in expression among different transgenic mice (data not shown). (*ii*) Why is the integrated HBV genome expressed only in the liver and kidneys? In previous work (25) we showed that the HBV DNA was significantly methylated and that the demethylation of the HBV DNA resulted in the expression of HBsAg. These results suggest that the difference in expression may reflect the [illegible] of methylation in the integrated HBV DNA. Integration sites of the introduced DNA on the host chromosome may also have affected the expression. These possibilities must be investigated in future studies.

In the 1.2HB-BS 10 transgenic line, the integrated HBV genome was allowed to produce the 3.5-kb and the 2.1-kb RNAs only in the liver and kidneys. No rearrangement of the integrated HBV DNA was observed in other tissues (data not shown). Our results are consistent with those of Burk *et al.* (12), who also showed that expression of the HBsAg gene was liver and kidney specific in their transgenic mice. These results may suggest that the HBV regulatory elements can utilize transacting factors produced not only in liver but also in kidneys. This hypothesis is supported by other data. An enhancer sequence was identified at about the 450-bp upstream region from the HBcAg gene promoter (26–28) and was shown to be involved in the activation of transcription of the 3.5-kb RNA preferentially in liver cells (27, 28). In addition, Halpern *et al.* (29) reported that the viral DNAs could be synthesized in the kidneys of Pekin ducks infected with duck hepatitis virus.

It is of interest that the 0.8-kb RNA was produced not only in the liver and the kidneys but also in the testis. Only this type of HBV RNA was produced in the testis. This 0.8-kb RNA was considered to be a transcript for the X protein, as judged by RNA blot analysis using specific probes (see Fig. 2). The same 0.8-kb RNA was detected in the testis of some other lines (data not shown). These results suggest independent regulatory mechanisms for the X gene expression. The implication of the atypical expression of the X gene in these particular organs is not clear at present.

Two groups of investigators reported that HBsAg itself is not harmful in transgenic mice (10, 11), although the overproduction of large HBV envelope polypeptide was toxic to hepatocytes (30). Our animals were clinically normal over 9 months of observation. Thus, the replication and expression of the HBV genome at this level are not toxic to the liver and kidneys.

It is possible to induce immunologically mediated tissue injury by bone marrow transplantation after γ-irradiation of the transgenic mouse because we used an inbred strain of mouse, C57BL/6. Therefore, these transgenic mice should be a powerful tool for pathological studies of hepatitis that may lead to the development of HCC and for molecular studies on the HBV replication and expression.

Finally, although this work was carried out under P2 containment in accordance with the guidelines of Japan, these transgenic mice might possibly be a source of infection to humans. So far as has been examined, transmission of the infection from the transgenic mice to normal littermates or from the transgenic mothers to their offspring has not been observed. In addition, the concentration of Dane particles in the serum is quite low (about 10^4 per ml, as judged by the

Southern blot analysis). Thus, these mice should not pose a serious health danger to humans.

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