Tissue-Specific Expression and Replication of Hepatitis B Virus DNA in Transgenic Mice

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Chapter I

Production of Transgenic Mice and Tissue-Specific Expression of Hepatitis B Virus DNA

SUMMARY

We produced transgenic mice using two construts, HB-GII and 1.2HB-BS, of hepatitis B virus (HBV) DNA. The former has been designed to express mRNAs for HBV surface antigen (HBsAg), and the latter to express all mRNAs of HBV. Several lines of the transgenic mice carrying each construct were examined for the tissue-specificity and level of HBV DNA expression, and for the expression and methylation of the transgenes. Only one out of ten for HB-GII and one out of eight for 1.2HB-BS were high producer for viral antigens. In high producers, transgenes were expressed in the livers and the kidneys. But in low producers, transgenes were usually expressed only in the kidneys. There is a reciprocal relationship between the level of expression and the degree of methylation, that is, the higher the level of expression, the lower the degree of methylation. We also observed that the expression of the integrated HBV-DNA was irreversibly repressed by methylation following its passage through the female germline in one strain. Thus, the expression of integrated HBV DNA reversely related with the degree of methylation, and this methylation may be involved in the regulation of HBV genes.

During the past decade, a number of techniques for manipulating the mammalian embryo have been developed that allow the stable introduction of new genetic material into the germ line. These techniques have revolutionized mouse genetics by making it possible to transfer any cloned gene into the germ line and subsequently to study the expression of the introduced gene. Most cloned genes that have been introduced into the mouse germ line have shown appropriate tissue-specific and stage-specific patterns of expression (1-5; for review 6), despite their integration into apparently random sites in the host genome. Therefore, gene transfer into the mouse embryo can serve as an experimental assay for the *cis*-acting DNA sequences or *trans*-acting cellular factor(s) that dictate specific patterns of expression. In addition, novel genes not normally expressed in the mouse (7, 8), or mutant forms of normal mouse genes, can be introduced. These approaches provide new experimental strategies for answering basic questions in many areas of mammalian biology, and many also allow the production of animal models of human diseases.

Hepatitis B virus (HBV) is a causative agent of hepatitis. Its infection is linked to later development of cirrhosis and hepatocellular carcinoma (HCC). Beasley *et al.* (9) 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.

The hepatitis B virion consists of an electron-dense internal core structure (the nucleocapsid) and an envelope. The envelope is made up of the HBV surface antigen (HBsAg) which share antigenic determinants with the small HBsAg particles. The nucleocapsid contains the HBV c antigen (HBcAg), a DNA

polymerase/reverse transcriptase and the viral DNA which is a partially double-stranded circular molecule (see review 10).

Recently, cell culture systems have been established that allow expression and replication of the HBV genome following transfection with cloned HBV DNA (11-14). 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 (15-17).

We produced several transgenic mouse lines using two constructs of HBV DNA. In this chapter, we analyzed for the tissue-specificity of the expression and the relationship between the level of expression and the degree of methylation of HBV transgene in seven lines of HBV transgenic mice. We here reported that the tissue specificity and level of HBV expression was under the influence of the degree of methylation, and that in one line HBsAg gene was irreversibly repressed following its passage through the female germ line.

MATERIALS AND METHODS

DNA The HBV genome used in our studies was derived from the plasmid pBRHBadr4 (18). The plasmid HB-GII was constructed by inserting a 2,6-kb *BglII* fragment from 3HB-neo (13) into *Bam*HI site of pBR322. This fragment retains the putative promoter region for the 2.1-kb RNA, thus allowing the translation of HBsAg (see Fig. 1). The plasmid 1.2HB-BS (19) carries one full length of HBV (*Bam*HI fragment) plus a 619 bp overlapping region (*Bam*HI/*Stu*I fragment) (Fig. 1). This HBV fragment contains the minimum region necessary for transcription of all kinds of mRNA including 2.1-kb and 3.5-kb RNA (10, 19) (Fig. I-1). Prior to injection into fertilized mouse eggs, the plasmid HB-GII was digested with *Hin*dIII and *Sal*I, the plasmid 1.2HB-BS was digested with *Hin*dIII and *Nde*I, and resulting 3.4-kb and 4.4-kb fragment, respectively, were isolated and used for microinjection.

DNA Injection C57BL/6 mice were used for production of transgenic mice. Several hundred molecules of each construct were microinjected into the pronucleus of fertilized eggs according to the method as described (20, 21).

Isolation of DNA and RNA Tissues 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 isopropylalcohol and dissolved in TE (10mM Tris-HCl, pH7.5/1mM EDTA). Total RNA was prepared as described (22).

Hybridization Studies DNAs digested with appropriate restriction enzymes were subjected to electrophoresis in 1% agarose gel and transferred to nylon membranes (GeneScreen *Plus*) according to the manufacturer's recommendations. RNAs were subjected to electorophoresis in a 1% agarose gel containing 6.6% formaldehyde, then transferred to nylon membranes (GeneScreen *Plus*). Hybridizations were done under stringent conditions with a random-primed ^{32}P-labeled whole HBV DNA probe (23) prepared from pBRHBadr4.

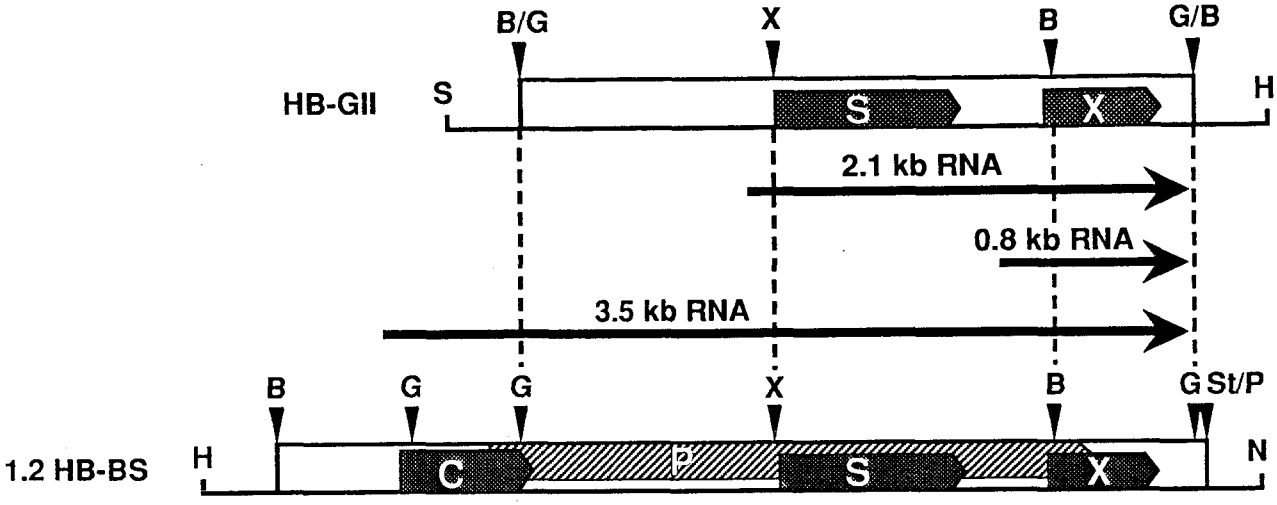


Fig.I-1 The structure of HB-GII and 1.2HB-BS. The coding region of HBcAg, HBsAg, HBx and polymerase are displayed with hatched arrows marked C, S and X, and striped arrows marked P, respectively. The expected transcripts from these HBV fragments are represented by thin arrows marked 2.1-kb RNA, 0.8-kb RNA and 3.5-kb RNA. B, G. H, N, S, St, P and X represent the site of restriction endonucleases *Bam*HI, *Bgl*II, *Hin*dIII, *Nde*I, *Sal*I, *Stu*I, *Pvu*II and *Xho*I, respectively.

Preparation of Extract of Tissues Samples were homogenized in extraction buffer (0.2% Triton X-100, 1mM phenylmethylsulphonyl fluoride in phosphate buffered saline) and centrifuged. The supernatants were used for enzyme immuno assay (Abbot).

RESULTS

Establishment of the Transgenic Mouse Lines Ten and eight founder mice were obtained by the introduction of HB-GII and 1.2HB-BS, respectively (data not shown). Table I-1 shows the titers of HBsAg and HBV e antigen (HBeAg) in their sera. In HB-GII lines, no. 26 was a high producer of HBsAg, nos 5, 27 and 30 were low producers, and the rest did not have any detectable amount of HBsAg. In 1.2HB-BS lines, no. 10 was a high producer for both HBsAg and HBeAg, and the others had no or very low titers of these antigens. We chose 7 lines, HB-GII 25, 26 and 27, and 1.2HB-BS 1, 2, 10 and 15, for the subsequent studies. From the result of Southern blot analysis it was found that 1.2HB-BS 10 carried 1 copy of the transgene, HB-GII 26 and 27 carried 2-3 copies, 1.2HB-BS 1 carried 3-5 copies, HB-GII 25, 1.2HB-BS 2 and 15 carried about 10 copies (Table I-1).

Transmission of HBV DNA in the HB-GII 26 Line The integrated HBV DNAs of these transgenic mice were transmitted to their offspring. We observed that HBsAg expression was irreversibly repressed following its passage through the female germ line (Fig. I-2). The same observation was reported by Hadchouel et al.(24). Since it was expected that this repression was due to methylation of HBV DNA in this line, these HB-GII 26 mice, HB-GII 26 (-), were also used for further analyses.

Expression of HBV RNA in These Transgenic Mice Total RNAs prepared from liver, kidney, testis and brain of these transgenic mice were subjected to RNA blot analysis (Fig. I-3). In case of HB-GII construct, three kinds of mRNA, that is, 2.4-kb, 2.1-kb and 0.8-kb mRNAs can be transcribed from HB-GII fragment (see Fig. I-1). In the HB-GII 26 mouse, which was a high producer of HBsAg, the 2.1-kb RNA was detected in the liver and the kidneys, and the 0.8-kb RNA that may be the transcript for X protein (19) (see Chapter II) was detected in the testis. In the HB-GII 27 mouse, which was a low HBsAg producer, no HBV RNA was detected in the

Tablel I-I. Titers for HBsAg and HBeAg in the sera of HB-GII and 1.2HB-BS founder mice and the copy number of HBV transgene of these mice.

HB-GII										
No.	5	6	25	26	27	30	37	40	42	45
sAg titer	28	<1	<1	754	17	15	<1	<1	<1	<1
Copy Number	2-3	3-5	>10	2-3	2-3	3-5	>10	>20	>10	3-5

1.2 HB-BS								
No.	1	2	6	10	13	15	17	19
sAg titer	<1	<1	<1	45	<1	<1	4.4	<1
eAg titer	<1	<1	<1	20	<1	<1	1.8	<1
Copy Number	3-5	>10	7-8	1	>10	>10	3-5	5-7

Titers were assayed by enzyme immuno assay kit (Abbot) and are expressed as the ratios against cut-off values (=A492 of negative control + 0.050 for HBsAg and + 0.06 for HBeAg). Values under 1.0 were considered negative, and represented by <1. One ng of HB sAg perml in a particle form is roughly equivalent to the value of 6. Quantitation between the titer and concentration of HBeAg has not been done. Values above 2.1 were considered positive. Copy number of HBV transgene was estimated from the result of Southern blot analysis.

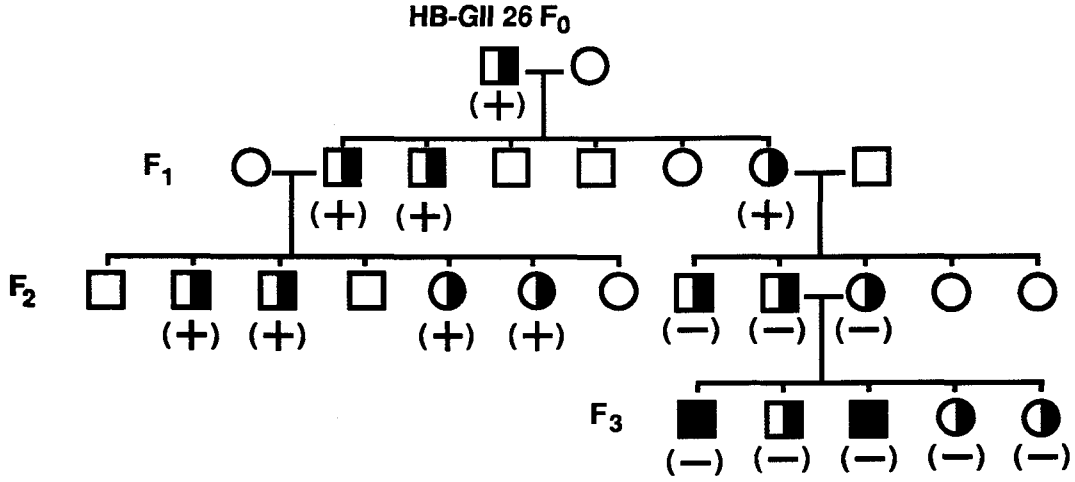


Fig. I-2 Pedigree chart of strain HB-GII 26. The founder male was mated with C57BL/6 female to produce offspring. The mice were tested for HBV DNA by Southern blot hybridization. Serum HBsAg was measured with the AUSZYME II (Abbot), and the mice which had high titer for HBsAg were represented as (+).

liver, instead in the kidneys and the brain two RNAs of about 3.5 kb and 2.1 kb in size, were detected. The latter is the transcript for HBsAg, but the former is not clear. The transcriptional initiation may start at flanking cellular DNA or abnormal site of HBV DNA. In the testis, there was the additional unknown RNA of about 3 kb in size beside the 0.8-kb RNA for X protein. The low titer of HBsAg in HB-GII 27 mice may be due to the expression of the 2.1-kb RNA only in the kidneys. In the case of HB-GII 25 , only smear RNAs were observed, suggesting the most of these RNAs are read-through. This expression pattern is quite similar to p3HB transgenic mice, which Yamamura *et al.* reported previously (25). No RNAs were detected in any tissues in the HB-GII 26 (-). The 2.4-kb RNA was not clear in this analysis.

The expression pattern in 1.2HB-BS mice was similar to that of HB-GII mice. In 1.2HB-BS 10, the expected RNAs, 3.5-kb and 2.1 kb in size (see Fig.I-1), were expressed in the liver and the kidneys, and the 0.8-kb RNA was detected in these tissues as well as in the testis. To our surprise, HBV RNAs were detected in 1.2HB-BS 1 mice which produced no HBV related antigens in their sera. Interestingly, HBV RNAs were observed in the kidneys, the brain and the testis like as HB-GII 27. There are three bands in the kidneys. However, these all RNAs except for the 0.8-kb RNA in the testis were not translatable because any HBV-related proteins could not be detected in these tissues (see below). The 1.2HB-BS 2 mouse expressed only 0.8-kb RNA in the testis, and in the 1.2HB-BS15 mouse, no RNA was detected in these tissues. We could not detect any HBV RNAs in the other tissues including spleen, heart, lung, intestine, thymus and muscle in the all mice (data not shown).

All these results suggest that the expression of HBV authentic RNAs are liver and kidney specific. This expression pattern seemed to represent HBV tissue specificity. On the other hand, in the case of HB-GII 27 and 1.2HB-BS 1, HBV

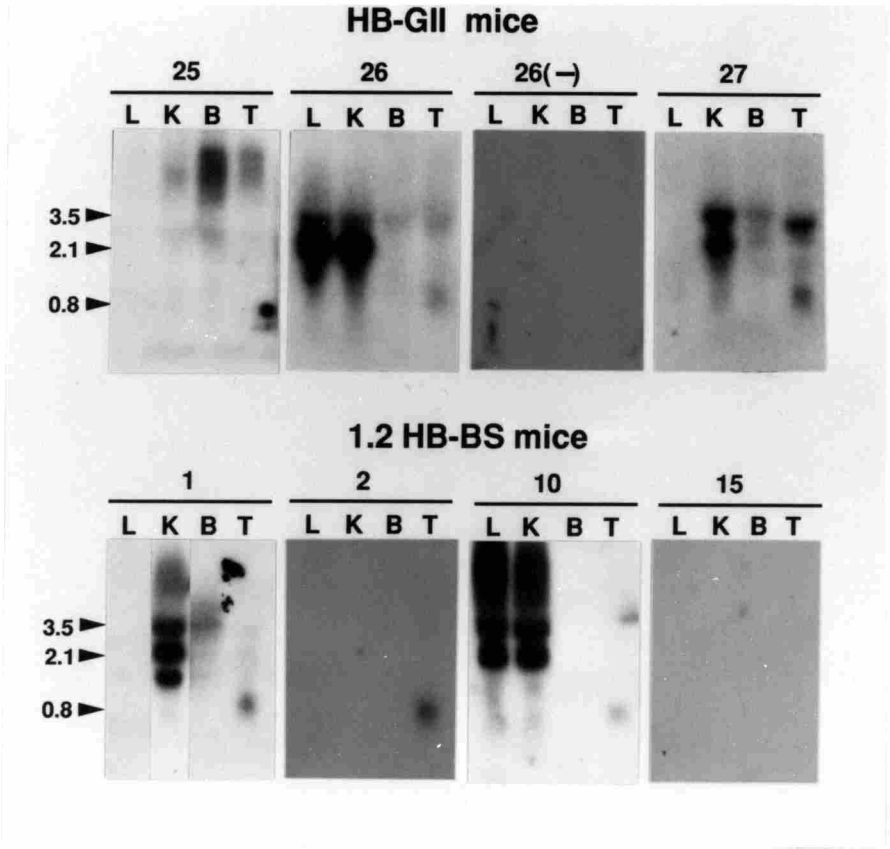


Fig. I-3 RNA blot analysis. Total RNA (10μg) prepared from the mice of HB-GII 25, 26, 26(-), 27, 1.2HB-BS 1, 2, 10 and 15 subjected electrophoresis followed by transfer to nylon membrane and hybridization with ^{32}P-labeled HBV DNA probe. . L, liver; K, kidney; B, brain; T, testis.

RNAs did not express in the livers, but in the kidneys, furthermore some RNAs were unknown. So we expected that these kidney-specific expression was abnormal.

HBV-Related Antigens in Tissues To examine the abnormal RNAs of HB-GII 27 and 1.2HB-BS 1 are translated into HBsAg or HBeAg proteins, we prepared tissue extracts and these antigen titer were assayed (Table I-2). In the HB-GII 27, HBsAg was detected in the kidneys in which 2.1-kb RNA was detected, but not in the other tissues. In 1.2HB-BS 1, neither HBsAg nor HBeAg were detected in any tissue. As expected, in the HB-GII 26 and 1.2HB-BS 10 high titers of both HBsAg and HBeAg were detected in the livers and the kidneys. These results demonstrated that abnormal RNAs produced in HB-GII 27 and 1.2HB-BS 1 were not translated into any HBV-related proteins suggesting that this expression do not reflect the tissue specificity of the expression of HBV DNA.

Methylation of HBV DNA To examine whether the tissue specificity and the level of the expression is correlated with the degree of methylation, we prepared DNAs from liver, kidney, brain and testis. These DNAs were digested with *Hpa*II or *Msp*I (recognizing CCGG sequence) and followed by Southern blot analysis.

As shown in Fig. I-4(A), when HB-GII were digested with *Msp*I, mainly three fragments (1080 bp, 880 bp and 615 bp) were detected in all DNA samples (lane M). Then, the DNAs were digested with methylation-sensitive restriction enzyme, *Hpa*II. Only the DNAs of the liver,kidney and testis from HB-GII 26 were digested to some extent, and these undermethylated tissues corresponded to the tissues in which HBV RNAs are expressed. In HB-GII 27, all the DNAs were methylated suggesting that the expression of abnormal HBV RNA in the kidney, brain and testis is due to the methylation of HBV DNA. In HB-GII 26(-), all the DNAs were heavily methylated. From this results, we could confirm that the maternal inhibition of this line is accompanied by the methylation of the integrated HBV DNA leading to the complete inhibition of HBV DNA expression.

Tble I-2. Titers for HBsAg and HBeAg in the serum and the tissue extracts

	HB-GII								normal			
	26			27								
	S	L	K	S	L	K	B	T	L	K	B	T
sAg titer	621	730	718	25	<1	28	<1	<1	<1	<1	<1	<1

	1.2 HB-BS								normal			
	10			1								
	S	L	K	S	L	K	B	T	L	K	B	T
sAg titer	187	322	318	<1	<1	<1	<1	<1	<1	<1	<1	<1
eAg titer	47	221	243	<1	1.2	1.2	<1	<1	1.3	1.1	<1	<1

Extracts of tissues of normal or transgenic mice were prepared and assayed for HBsAg and HBeAg titer by enzyme immuno assay kit (Abbot). Titers were represented in the same way of Tablel I-1. S, serum; L, liver; K, kidney; B, brain; T, testis.

Fig. I-4(B) shows the results of 1.2HB-BS lines. By digestion of 1.2HB-BS with *Msp*I (lane M), three fragments (1080, 967 and 504 bp) were mainly generated. In 1.2HB-BS 10 DNAs from the liver, the kidneys and the testis were digested with *Hpa*II to considerable degree as judged by the hybridizing intensity of the three main fragments (1080,967 and 504 bp). However, the DNA from the brain was not digested with *Hpa*II. In 1.2HB-BS 1, DNAs were also digested with *Hpa*II but to less extent when compared with 1.2HB-BS 10. In addition, the degree of methylation is lower in kidneys and the testis where the HBV DNA is expressed than in the brain and the liver. In contrast to these two lines, DNAs from 1.2HB-BS 2 and 15 were methylated almost completely. As described before, no expression was observed in all tissues from these two lines except the testis of 1.2HB-BS 2 where the 0.8-kb RNA was detected. All these results suggest that there is a reciprocal relationship between the level of expression and the degree of methylation, that is, the higher the level of expression, the lower the degree of methylation.

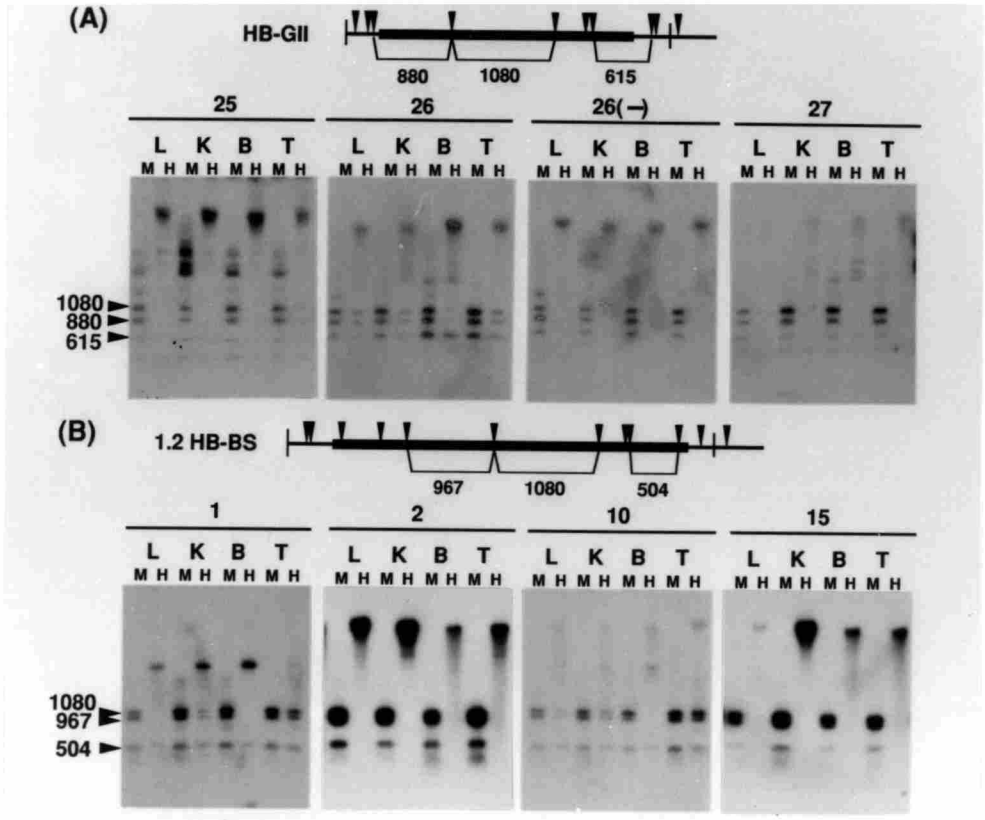


Fig. I-4. Methylation of HBV DNA in the HB-GII mice (A) and the 1.2HB-BS mice (B). The thick line of the maps indicate HBV DNA. The arrows represent the action site of restriction endonucleases *Hpa*II and *Msp*I (CCGG). In HB-GII, the major three expected fragments were 880, 1080 and 615 bp. In 1.2HB-BS, they were 967, 1080 and 504 bp. The DNAs from liver (L), kidney (K), brain (B) and testis (T) were digested with *Msp*I (M) or *Hpa*II (H) followed by Southern blot analysis with ^{32}P-labeled HBV DNA probe.

DISSUCUSSION

We examined seven strains of transgenic mice using two DNA construction, HB-GII and 1.2HB-BS, and found that in high producers of HBV-related antigens HBV DNAs expressed in liver- and kidney-specific manner and were undermethylated in these tissues. This result suggests that methylation can be a means of tissue specific expression.

Several groups have reported transgenic mice carrying HBV DNA (15-17, 19 and 26) derived HBV own promoter. The tissue specificity of HBV expression is not agreement perfectly. But liver- and kidney-specific expression is most frequent, including our results (17, 19 and 26). Therefore, it is expected that liver and kidney have some *trans*-acting factor(s) that HBV regulatory elements can utilize. This hypothesis is supported by other data. An enhancer sequence was identified at about the 450-bp upstream region from the HBcAg gene promoter (27-29) and was shown to be involved in the activation of transcription of the 3.5-kb RNA preferentially in liver cells (28, 29). In addition, Halpern *et al.* (30) reported that the viral DNAs could be synthesized in the kidneys of Pekin ducks infected with duck hepatitis virus.

Two strains (HB-GII 27 and 1.2HB-BS 1) showed kidney specific expression, but some abnormal RNAs were produced and the titers of HBV-related antigens were very low or not detectable. This suggests that the factor(s) of kidney may be different from those of liver and tend to be utilized under suppressed condition and induce uncommon transcription.

The 0.8-kb RNA which we have already reported being the transcript for the X protein (19, see chapter II) was detected in HB-GII 26, 27, 1.2HB-BS 1 and 2 as well as 1.2HB-BS 10. These results suggest independent regulatory mechanisms for the X gene expression. Testis is peculiar tissue in the point of reproduction organ in which meiosis and DNA replication occur frequently. Thus testis may have some

transactivating factor of X gene. Anyway, the implication of the atypical expression of the X gene in these particular organs is not clear at present.

We showed that the HBV-DNA is irreversibly repressed following its passage through the female germ line by methylation in HB-GII 26 line. The same thing was demonstrated by Hadchouel *et al* .(24), however, the other two groups (31,32) found that transgenes are reversibly methylated after passage through the male or female germ line. Because HBV sequences are easily methylated (see below), their methylation may become irreversibly. In any case, the important point is that a gene which has been active is repressed by *de novo* methylation. We have shown that the methylation of HBV DNA is the main cause of gene inactivity by demonstrating that the demethylation by 5-azacytidine induced the expression of HBsAg gene (33). These suggest that methylation play an important role in the regulation of HBV DNA.

We found that the degree of methylation of the integrated HBV DNA related with its copy number (see Table I-1). The mice which carry many copy number of HBV transgene did never express HBsAg or HBeAg in their sera. As reported by some groups (19, 25 and 26), HBV DNA is easy to be methylated in transgenic mice more frequently than mammalian genes of which about 80% usually expressed at high efficiency. . It seems that HBV DNA has some signal sequence of methylation.

Considerable evidence suggests that methylation of cytosine residues in the regulatory region of both viral and eukaryotic genes reduce their transcription (34-36), and that the methylation is a key developmental process, often invoked in models of cell differentiation (37, 38), X-dosage compensation (39, 40), and parental imprinting (24, 31, 32). In addition, DNA methylation influences chromatin structure *in vivo*, suggesting that methylation may regulate transcription by altering protein-DNA interactions (41). So far, however, the molecular mechanisms of the methylation-dependent imprinting and regulation of gene expression are poorly

understood. Transgenic mice using HBV DNA should become useful for the analysis of methylation because it tends to be methylated upon integration into mouse genome.

HB-GII 26 and 1.2HB-BS 10 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. In the next chapter, further analyses of HBV replication in 1.2HB-BS 10 transgenic mice will be demonstrated.

Chapter II

Replication of Hepatitis B Virus in Transgenic mice and Application to Studies of Hepatitis

SUMMARY

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, and virus particles were observed in the sera by immunoelectron microscopic study. These results demonstrate that the HBV genome integrated into the mouse chromosome acted as a template for viral gene expression, allowing viral replication. When these transgenic mice were immunized with HBsAg or HBcAg, they produced anti-HBs or HBc response. In accordance with the appearance of HBsAb, HBsAg became undetectable and. the production of HBsAb resulted in the clearance of viral particles. 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. The pathological consequences of HB virus infection are unpredictable and range from inapparent forms to acute hepatitis and chronic liver disease. Carriers with severe liver disease often progress to cirrhosis and, after three to four decades, to hepatocellular carcinoma (HCC) at a rate more than 200 times greater than the general population (9). Recently, the vaccination to HBV has been developed and hence the new infection will be significantly reduced in near future. However, in Japan, 2% of population, that is, about three million people are HBsAg carriers and we do not have any treatment for these carriers. Thus, HBV infection is still a serious health problem.

HBV genome DNA is a partially double-stranded circular molecule. After infection, it is converted into a covalently closed circular molecule (42), which is transcribed into two main species of mRNA, 2.1 and 3.5 kilobases (kb) in size (10). 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 (43) presumably by the viral polymerase (44), 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.

The mechanisms responsible for acute and chronic hepatocellular injury, and the events leading to the development of HCC, are unknown. But, accumulating data suggest that HB virus is not directly cytopathic and carcinogenetic. Instead, the host immune response to viral antigens play a major role in the development of hepatitis. Concerning the mechanism of the development of HCC, we prefer the following model. Once the hepatitis is induced by the immune response, liver cells may be damaged but will regenerate again. This cycle, that is, degeneration and

regeneration, will repeat for a long time as long as the HB virus exists. During this course. the genetic change such as chromosomal aberration may be caused leading to, for example, the activation of oncogene.

To prove this hypothesis we first attempted to produce a transgenic mouse model for the chronic carrier state of HB virus infection. 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 mature virus.

MATERIALS AND METHODS

DNA. The HBV genome used in our studies was derived from plasmid pBRHBadr4 (18). 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. II-1). This HBV fragment contains the minimum region necessary for transcription of the 2.1-kb and 3.5-kb RNAs (Fig. II-I) (see ref. 10). 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.

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 x g for 2 hr. Fractions were collected from the bottom. DNA was extracted from each fraction. Part of each fraction was also assayed for 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)] (43). 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 x 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

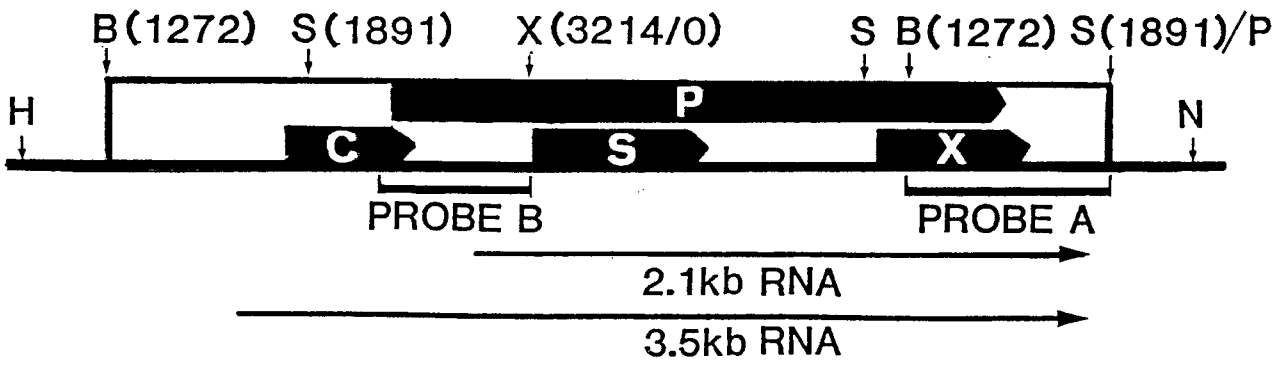


Fig. II-1. Structure of plasmid 1.2HB-BS. The coding regions of HBcAg, HBsAg, HBx, and polymerase are displayed with blackened arrows marked C, S, X, and P, respectively. The expected transcripts from this HBV fragment are represented by thin arrows marked 3 .5-kb RNA and 2. 1-kb RNA . Probe A and probe 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, *Hind*III, *Nde*I, *Pvu*II *and Xho*I respectively. The nucleotide numbers marked along the HBV genome start at the unique *Xho* I site.

on a discontinuous CsCl density gradient (from 23% to 39%), and spun at 240,000 x 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 (45).

Preparation of Serum DNA Samples. One hundred microliters of serum was incubated at 60°C for 3 hr in proteinase K (100μg/ml)/1%NaDodSO$_4$/5mM EDTA/10mM Tris-HCl, pH8. The solution was phenol/ chloroform extracted, and the DNA was precipitated with ethanol in the presence of carrier tRNA (100mg/μl). The precipitate was dissolved in 10μl of TE, and then was digested with *Bam*HI.

Polymerase Chain-Reaction PCR Assay of Serum DNA. Oligonucleotide primers were synthesized on DNA synthesizer (Applied Biosystems). Primer 47, 5'-GGGTACTTTACCGCAAGAA-3', begins at map position 772, and primer 48 (from the complementary DNA strand), 5'-CCGCGTAA-AGAGAGGTGCGC-3', begins at map position 1415 of the HBV genome (18). Amplification using the *Taq* polymerase was performed according to the procedure described by Saiki *et al.* (46). The reaction was performed for 32 cycles in a programmable DNA thermal cycler. Samples were heated to 94°C for 1 min (denaturation of DNA), cooled to 55°C for 1 min (hybridization to primer), and incubated for 2 min at 72°C (polymerase reaction).

RESULT

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) (see Table I-1). Since the 1.2HB-BS 10 mouse is a high producer, this mouse was chosen for the subsequent studies. Fig. II-2 shows the transmission of the 1.2HB-BS. Findings obtained by the analyses of these mice demonstrated that all the mice carrying 1.2HB-BS were positive for HBsAg and HBeAg and that the titers of these antigens in the homozygotes of the 1.2HB-BS were much higher than those of the hemizygotes. No sex difference in the titer of these antigens showed in animals.

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. II-1), two RNAs, 3.5 kb and 2.1 kb in size, were detected in the liver and the kidneys (Fig. II-3, probe A). The sizes of these RNAs are in good agreement with those detected in HBV-infected hepatocytes (10). A small amount of 2.4-kb RNA was present in the liver but not in the kidneys (see Fig. II-4). 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. II-3, probe A).

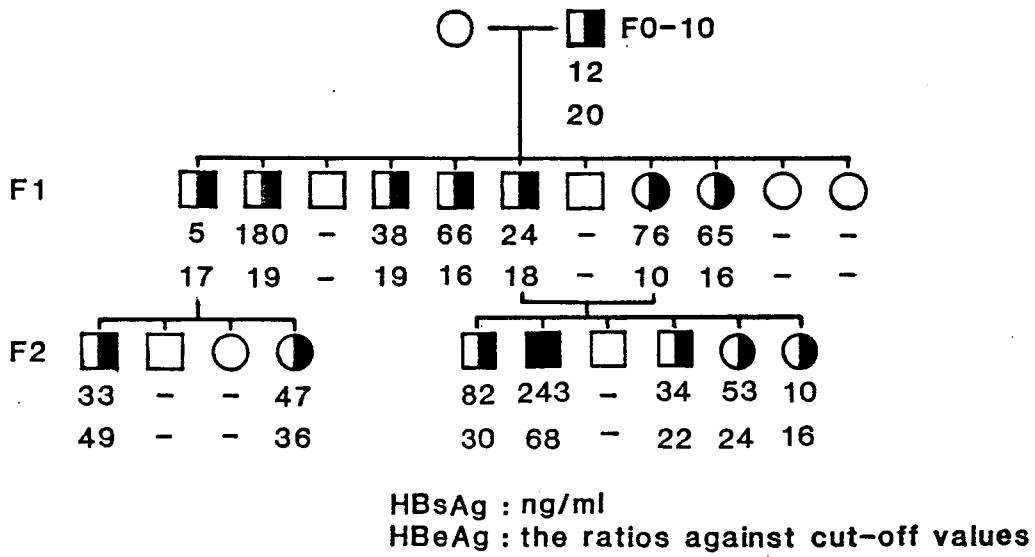


Fig. II-2 Transmission of HBV DNA to offspring in 1.2HB-BS 10 line. The mice were tested for HBV DNA by Southern blot hybridization. The numbers represent titers of HBsAg (ng / ml) (upper) and HBeAg (cut-off values) (lower) assayed by enzyme immuno assay kit (Abbot). For details about enzyme immuno assay, see the legend of Table I-1.

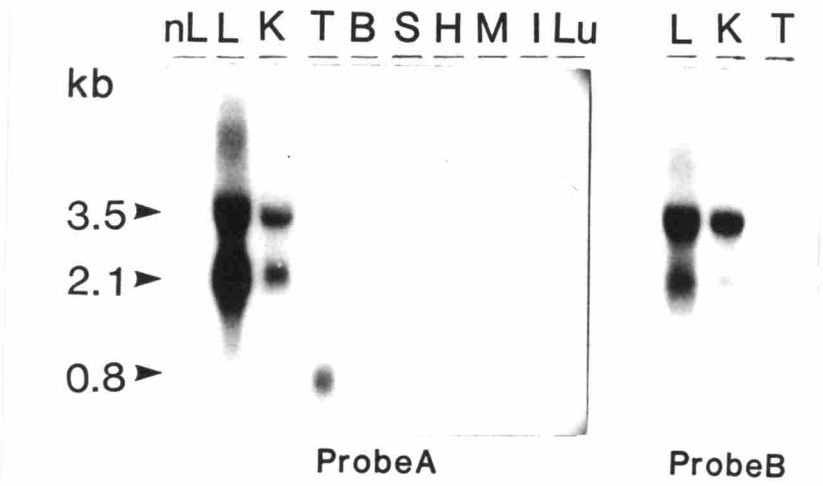


Fig. II-3. RNA blot analysis of total RNA (12 μg) prepared from various tissues of the 1.2HB-BS 10 F_1 mouse. Probe A, hybridization with probe A (see Fig. II-1) containing the X coding region. Probe B, hybridization with probe B (see Fig. II-1) containing a region between C and S . nL, liver from a normal mouse; L, liver; K, kidney; T, testis; B, brain; S, spleen; H, heart; M, muscle; 1, intestine; Lu, lung. Sizes are shown in kb.

A 0.8-kb RNA was detected in these two tissues and in the testis as well. Siddiqui et al. (47) 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. II-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. II-3, 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. II-3), 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. II-4A, 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 (14). 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. II-4B 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 (48). 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 the pre-SI and pre-S2 mRNA, respectively, but the other (h) was not determined.

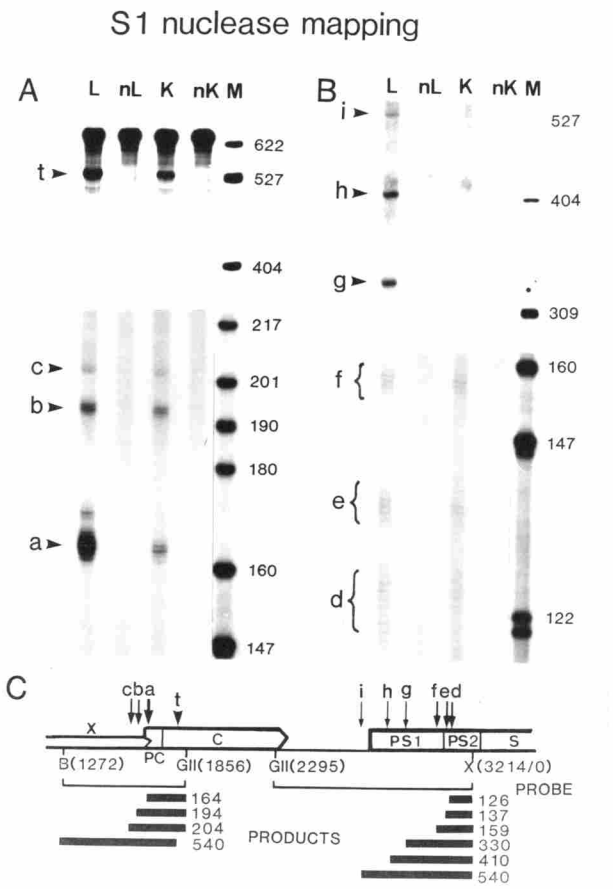


Fig. II-4. S1 nuclease mapping. Total RNAs (10 μg each) of the liver and kidneys of the 1.2HB-BS 10 F_1 mouse (lanes L and K, respectively) and liver and kidneys of a normal mouse (lanes nL and nK, respectively) were applied to S1 nuclease mapping. Lanes M, size markers (shown in nucleotides). (A) The 5' initiation in the pre-C region. The *Bam*HI-*Bgl*I 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*I-*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*I; PC, precore region; PS1, pre-S1 region; PS2, pre-S2 region.

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 (43). 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 (49). Fig. II-5 shows that the patterns of HBeAg/HBcAg sedimentation in these two samples were indistinguishable, lending strong support to the conclusion that core particles in 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. II-5, 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. II-6A). 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 (43). *Bam*HI cleaves the HBV genome at one site in the X region, and *Bgl*II cleaves

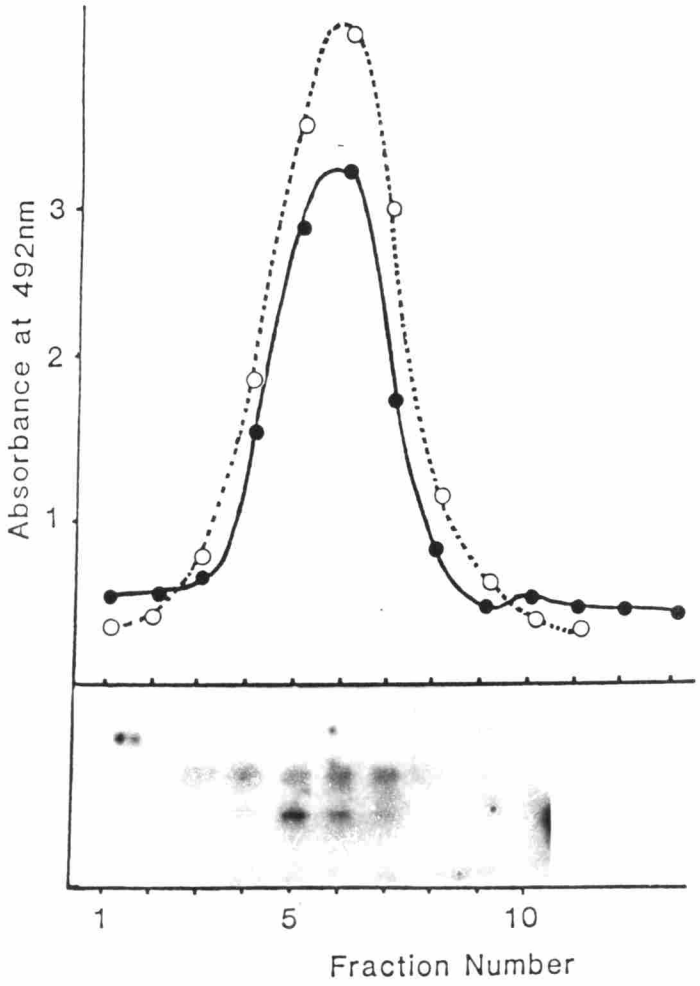


Fig. II-5. Sucrose gradient velocity sedimentation of replicative complexes from the liver extract of the 1.2HB-BS 10 F_1 mouse. Total liver extract was sedimented through a sucrose gradient, and 24 fractions were collected from the bottom. Part of each fraction was assayed for HBeAg/HBcAg titer by an 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.

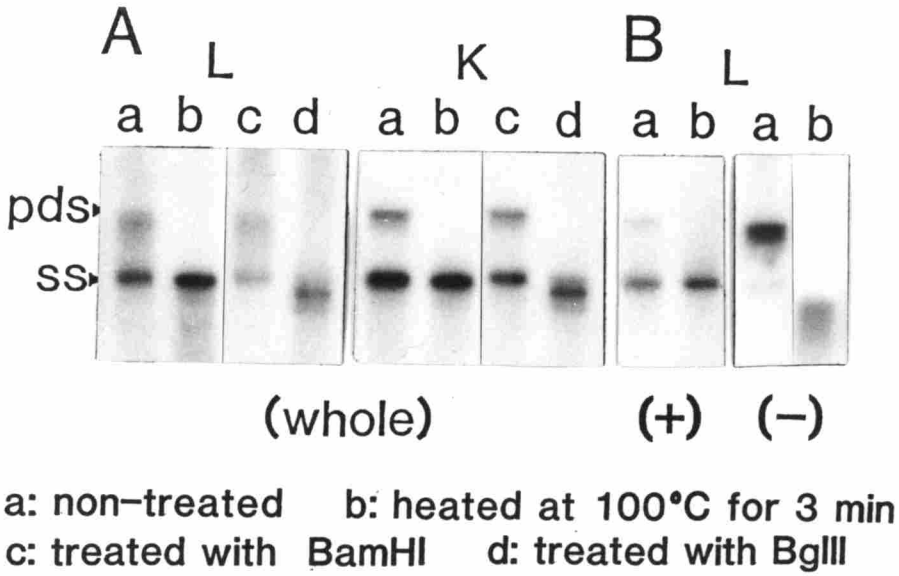


Fig. II-6. Southern blot analyses of DNAs prepared from the cytoplasm of the liver (L) and the kidneys (K). (A) The probe used was a random-primed ^{32}P-labeled whole HBV DNA probe (whole). (B) The probes used were HBV plus strand (+) and minus strand (-). Lanes: a, nontreated DNA; b, DNA treated at 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.

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-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. II-6B). 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. II-7). 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 (50). This HBV DNA migrated to the same position as the partially double-stranded HBV DNA prepared from the liver cytoplasm (Fig. II-7). 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.

To confirm directly the presence of Dane particles in the serum, we carried out electronmicroscopic study. We collected fifty ml of the serum, partially purified and examined by immunoelectron microscopy. As shown in Fig. II-8, about 42 nm particles with double shelled structure and 22nm HBsAg particles were observed suggesting the presence of complete Dane particles in the serum.

Histological Examination. We performed histological analyses of the Fl 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

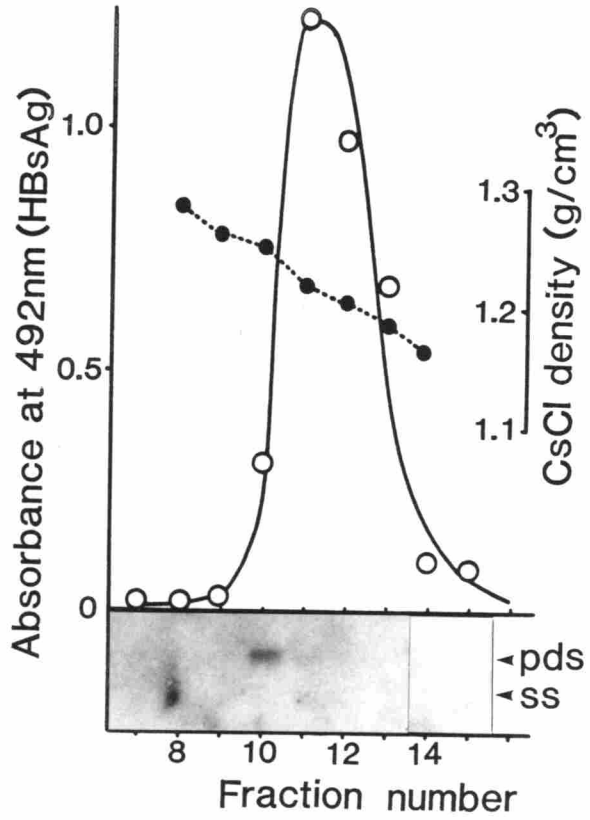


Fig. II-7. CsCl density-gradient sedimentation of the serum of the 1.2HB-BS 10 F_1 mouse. A total of 10 ml of serum was concentrated to <0.5 ml and subjected to centrifugation. Fractions collected from the bottom were analyzed for HBsAg titer (○) and for the density of CsCl (●). The DNAs were recovered 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.

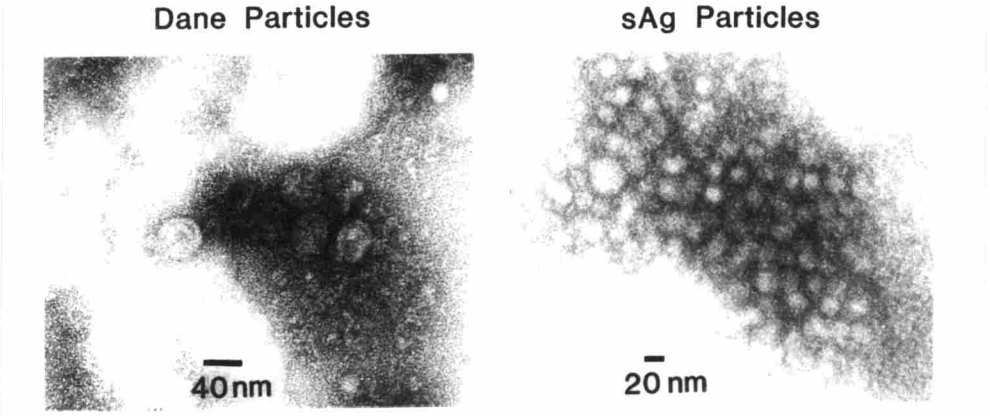


Fig. II-8. Electron micrograph of HBsAg- positive spherical particles in the serum of the transgenic mice. Serum (50ml) were partially purified by centrifugation on sucrose density gradient. The concentrated serum was incubated with anti-HBsAg rabbit IgG. The mixture was then centrifuged and the pellet was stained with uranylacetate.

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.

Immunization with HBsAg or HBcAg. The original founder mouse is now 25 months old, but shows no clinical sign and no pathological change. This suggests that HBV is not directly cytopathic, and so the immunological response to viral antigens play an important role in the induction of hepatitis.

To examine whether these transgenic mice are tolerant to HBsAg, we immunized transgenic and control mice with HBsAg. To our surprise, after immunization the transgenic mice produced anti-HBs response (Fig II-9). In accordance with the appearance of HBsAb, HBsAg became undetectable by radio immuno assay. Interestingly, we observed two fold increase of the HBeAg titer. The normal mice also produced anti-HBs response, but titers of HBsAb were much higher than those of the transgenic mice. These data suggest that the transgenic mice are not fully tolerant to HBsAg, and so can produce anti-HBs response.

We also immunized these mice with HBcAg. As shown in Fig. II-10, the transgenic mice also produced anti-HBc response. The HBcAb titers were also lower than those of normal mice. HBeAg titer showed no drastic change. In about 50% of immunized mice, HBsAg titers were decreased. Whether this is related to anti-HBc response or not is not clear. We tested for serum GPT of these immunized mice, but no elevation was detected.

Now, we are carrying out continuous immunization with HBsAg or HBcAg.

Detection of HBV DNA in the Serum of Immunized Mice. From the clinical stand point of view, it is very important to examine whether Dane particles present in the serum before immunization can be eliminated by the HBsAb or not. To determine the amount of Dane particles in the serum of these immunized mice, we established a detection system of Dane particle using PCR method. DNAs prepared from sera (see MATERIALS AND METHODS) were treated with *Bam*HI

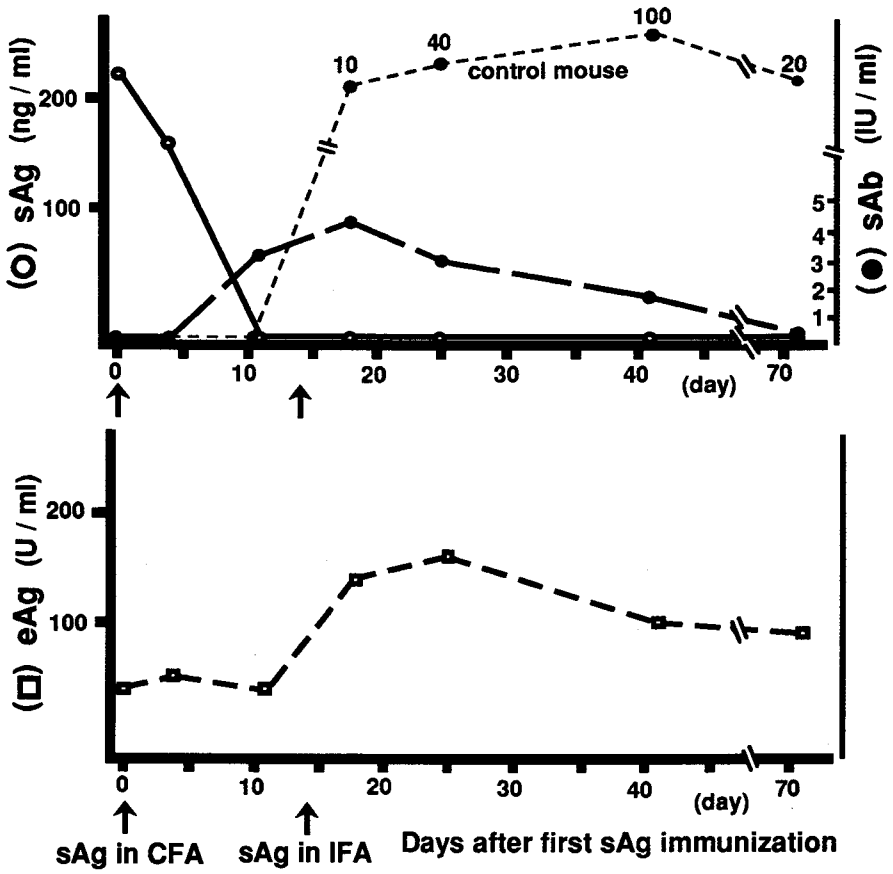


Fig. II-9 Time course the titers of HBsAb(●),HBsAg (o) and HBeAg (□) in the transgenic mice immunized with HBsAg. In first immunization, 100μg of HBsAg in Freund's complete adjuvant was injected into a mouse, then, two weeks later, the same amount of HBsAg in Freund's incomplete adjuvant was injected. We use fifteen transgenic mice. All the mice showed very similar response, and this figure shows the representative case. All the titers were assayed by radio immuno assay (Abbot).

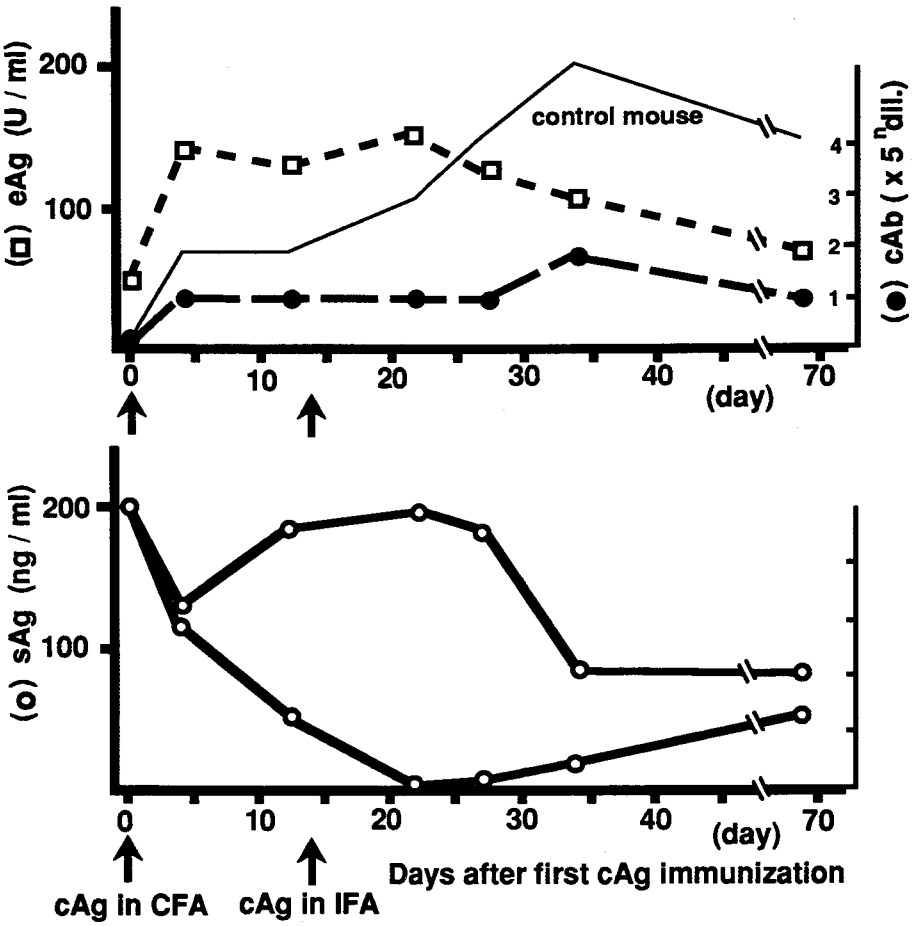
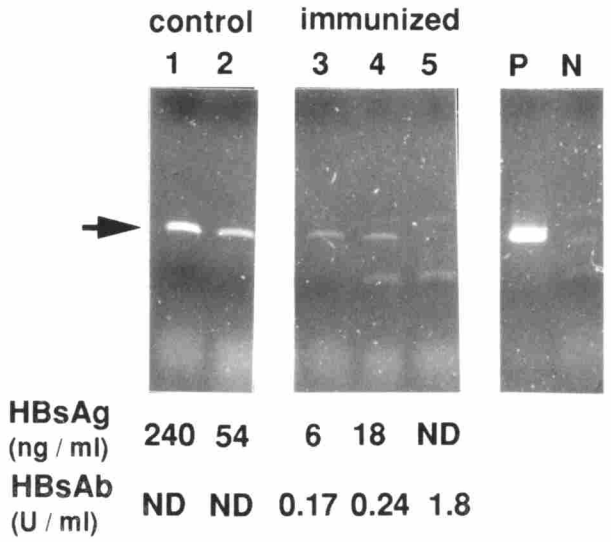


Fig. II-10 Time course the titers of HBcAb(●),HBsAg (o) and HBeAg (□) in the transgenic mice immunized with HBcAg. In first immunization, 25μg of HBcAg in Freund's complete adjuvant was injected into a mouse, then, two weeks later, the same amount of HBcAg in Freund's incomplete adjuvant was injected. We used eight transgenic mice. Four of the mice showed the decrease of HBsAg titer, but not the rest. This figure shows the representative time course of each cases . All the titers were assayed by radio immuno assay (Abbot).

before PCR reaction to avoid contamination of HBV transgene in mouse chromosome from leukocytes. Because *Bam*HI site is located in single-stranded region, free viral DNA is resistant to *Bam*HI and can be amplified. Using this system, we examined the presence or absence of Dane particles in HBsAg immunized mice.

As shown in Fig. II-11, the band of expected size was detected in the sera of control transgenic mice (lane 1 and 2) and positive control sample (lane P), but not in the serum of transgenic mouse in which only HBsAg gene is integrated (lane N). Lane 3-5 showed the result of the DNAs of the sera from transgenic mice after immunization with HBsAg. The band was observed when the HBsAg is still present (lane 3 and 4), but no band was detected when the HBsAb titer is high and the HBsAg is not detected (lane 5), suggesting that the production of HBsAb resulted in the clearance of viral particles.



N; a HBV transgenicmouse without any viral particle.
P; about 1000 molecules of HBV DNA fragment

Fig. II-11. Detection of viral DNA in the serum by PCR. Serum DNAs were prepared and amplified by PCR as described in MATERIALS AND METHODS. A aliquot from the reaction mixture was subjected to agarose- gel electrophoresis, and DNA was visualized under UV light after staining with ethidium bromide. The expected band of about 620 bp was represented by allow. Lane 1; serum DNA from a normal transgenic mouse with high HBsAg titer, lane 2; serum DNA from a normal transgenic mouse with low HBsAg titer, lane 3-5; serum DNA from immunized transgenic mice with HBsAg The titers of HBsAg and HBsAb were assayed by enzyme immuno assay (Abbot).

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 complete viral particle carrying 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.

Although the mechanism of liver damage is not fully understood, the virus itself is thought to be not cytopathic. All transgenic except one case, in which the overproduction of large HBV envelope polypeptide cause liver cell damage (51), are shown to be clinically normal (15-17, 19 and 26). The 1.2HB-BS 10 founder mouse in this experiment is now 25 months of age, but shows no clinical and pathological change. These data suggest that the host immune response to viral antigens present at the liver cell membrane has a major role in the pathogenesis of HBV-related liver disease.

We found that the production of HBsAb by immunization with HBsAg in these transgenic mice resulted in the clearance of viral particles. This result indicates that vaccination to carriers may be a means to prevent the secretion of HBV. In the absence of a small laboratory animal, such transgenic mice should be a powerful tool for the analysis of development of HBV-related diseases.

1.2HB-BS 10 transgenic mice could produce anti-HBs and anti-HBc response. Because we use very large amount of antigens (25-100μg / a mouse), it is expected that B cells produce HBsAb or HBcAg themselves without induction by T cells. It is thought that hepatocellular injury is mediated by a cellular immune response, especially cytotoxic T lymphocyte. We are now cloning cytotoxic T lymphocyte line to HBcAg and going to transfer these cells into these transgenic mice.

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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