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-

fected cell can occur leading to the development of a hepatocarcinoma in which integrated viral DNA is always detected. The mechanism by which the viral DNA becomes integrated is not known. Although information on viral gene transcription and DNA replication has been obtained from studies of cells transfected with cloned HBV DNA or animals infected with related viruses (*5*), many aspects of HBV infection remain unknown. The use of transgenic mice (*6–7*) offers new possibilities for the study of HBV since the problem of natural entry of the virus into the host can be circumvented by the injection of DNA into fertilized eggs and implantation of the eggs into foster mothers. Several different genes have been introduced into the genome of mice and some of them have retained their tissue specificity of expression and have had a biological effect in the animal (*8–10*). Using this technology we have addressed three main questions: (i) How is the viral gene expression regulated in the different tissues of the animal? (ii) Is there a modulation of this expression by the host? (iii) In what way is the mechanism of liver damage linked to HBV infection?

First, we analyzed the expression of the surface antigen using the recombinant plasmid pAC_2 (*11*) that contains all the HBV genome except for 445 base pairs of the gene coding for the major polypeptide of the viral capsid (the Bgl II fragment 1980 to 2425) (Fig. 1). This plasmid allows the expression of the hepatitis B surface antigen gene (S gene), although at a low level, when transfected into mouse fibroblasts (*11*). The complete pre-S and S coding regions and the X coding region are present as well as most of the P coding region (*12*). The deletion in the core gene prevents the possible production of complete virions. At position 3122 the promoter PrS has been shown to direct efficient transcription in transfected cells of a 2.1-kb RNA species encoding the major polypeptide of HBsAg (*13*). An enhancer sequence is present at position 1080 to 1234 upstream from the putative core promoter (*14*).

About 1500 copies of the plasmid pAC_2 linearized by Hind III were injected into one of the pronuclei of single-cell eggs obtained from superovulated C57BL6 × SJL/J females mated with C57BL6 × SJL/J males. Injected eggs were reimplanted into the oviduct of pseudopregnant mothers and mice referred to as pAC_2F_0 were obtained. At about 4 weeks, DNA was extracted from a fragment of the tail and analyzed by dot hybridization for the presence of pAC_2 sequences. A total of six mice were found to be positive in two series of injections and the quantification of the plasmid copy number per genome was performed with known amounts of plasmid pAC_2 (Table 1). Southern blot analysis of cellular DNA from the six pAC_2-containing mice was performed after digestion with the restriction enzyme Eco RI (Fig. 2). Digestion of pAC_2 produces two fragments of distinct sizes, one 4.7 kb long containing mostly pBR322 sequences and one 2.4 kb long containing mostly HBV sequences. Hybridization with the probe pFC80, a pBR322 recombinant with four complete HBV genomes, gives a signal of equal intensity with the two fragments. The DNA restriction pattern of the males E11, E25, E33, E36, and E47 shows two bands comigrating with the control pAC_2 DNA hydrolyzed with Eco RI. This and the fact that no free plasmid is present in uncut DNA suggest the presence of at least two sequences integrated in tandem head to tail. The only female, E43, shows only one weak band of high molecular weight suggesting a deletion of the integrated pAC_2 sequences. Additional bands of various sizes are detected and may contain genomic flanking sequences. In mice E33, E36, and E47, pAC_2 is inserted at two sites as evidenced by restriction with enzymes that do not cut or cut once in the plasmid. The analysis of the precise organization of each integrated sequence will only be possible in F_1 mice.

Table 1. Characteristics of F_0 mice positive for plasmid sequences in tail DNA. Plasmid copy number was evaluated by dot hybridization using known amounts of pAC_2 as described (*16*). Serum HBsAg was measured by radioimmunoassay (AUSRIA; Abbott). The percentage of transmission was calculated on 50 F_1 mice for E36 and 55 mice for E25. ND, not done.

Mouse	DNA copy number per cell	Serum HBsAg (μg/ml)	Percentage of transmission to progeny
E11*	>1	0.5	Sterile†
E25*	4	0	30
E33*	2	ND	ND
E36*	4	1.9	59
E43‡	<1	0	ND
E47*	2	0	ND

*Male. †One progeny was obtained after in vitro fertilization. ‡Female.

All the F_0 mice were tested for the presence of HBsAg in their serum by means of a radioimmunoassay (AUSRIA; Abbott). Two of them, E11 and E36, were positive at a level of 0.5 μg/ml and 1.9 μg/ml, respectively (Table 1). E25, E33, and E47 were not producing HBsAg, although they contain intact plasmid sequences. Such a situation was already observed in different systems previously studied and points to the relevance of the site of integration (*15–16*).

Breeding was performed between males E11, E36, and E25 and normal C57BL/6 females. E11 was sterile, apparently because of sexual indifference,

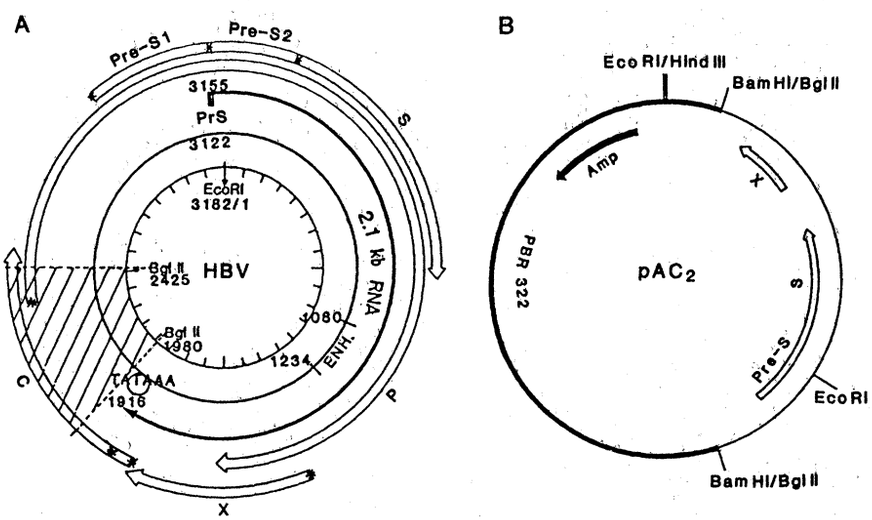


Fig. 1. Genetic map of (A) the HBV genome and (B) the plasmid pAC_2 used for injection. (A) The thin closed circle represents the viral DNA. The open arrows represent open reading frames on HBV DNA: Pre-S1, Pre-S2, S, X, C, and P (*12*). Dark points are AUG codons. PrS represents the promoter for gene S transcription and Enh represents an enhancer element located between position 1080 and 1234. The 2.1-kb messenger RNA (dark arrow) encoding the major S polypeptide extends from position 3155 to the polyadenylation site at position 1916. The hatched region corresponds to the Bgl II fragment absent in recombinant pAC_2. (B) The HBV Bgl II fragment 2425 to 1980 was inserted in the Bam HI site of plasmid pBR322 (*11*). pAC_2 was restricted with Hind III prior to injection. Eco RI cut the plasmid at two sites producing a 4.7-kb and a 2.4-kb fragment.

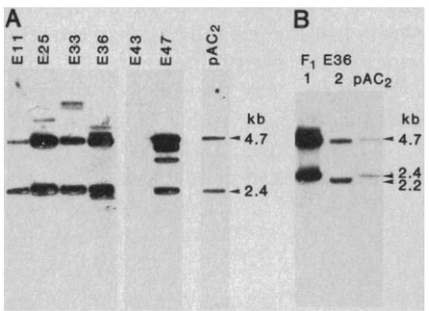
and the animal was killed after sperm from the vas deferens were used for in vitro fertilization. One F_1 male, containing HBV sequences integrated with the same pattern as the parent E11 and expressing HBsAg, was obtained. Among 55 F_1 of E25, 30 percent contained the same sequences as the parent. None were producing HBsAg or antibodies to HBsAg in their serum. These animals were kept for further analysis of the transmission of the genes to the F_2 and for use as controls in RNA analysis. Fifty F_1 from E36 (28 males and 22 females) were analyzed for plasmid sequences and HBsAg. Thirty animals were positive for HBV DNA and could be separated into two groups on the basis of the intensity of the hybridization signal on the dot test and the production of HBsAg. Those in group 1 (20 mice) expressed HBsAg and had a signal corresponding to one copy of the plasmid per diploid genome. Those in group 2 (10 mice) did not express HBsAg but had a signal corresponding to four copies of the plasmid. The ratio of males to females was 1:1 in the two groups. The DNA of the 30 F_1 mice was analyzed by digestion with Eco RI and the Southern blot technique (Fig. 2). In group 1, two fragments of 4.7 and 2.2 kb were detected, suggesting that the integrated plasmid had a short deletion in the smaller Eco RI fragment containing the HBV insert. In group 2, the two reference fragments of 4.7 and 2.4 kb were present showing the presence of tandem integration of four pAC_2 copies. Two additional bands were detected and probably represent flanking sequences. All the Eco RI fragments from group 1 and group 2 were present in the parent E36 pattern. However, no F_1 having the pattern of E36 was found among the 30 mice tested. This suggests the presence of two integration sites in the E36 genome, segregating separately. Analysis of the plasmid sequences in group 1 mice with other restriction enzymes showed that the integration occurred toward the Hind III site used to linearize the plasmid.

Table 2. Serum HBsAg level in E36 F_1 mice of group 1. The animals from the F_1 progeny of E36 are numbered from 1/1 to 1/50. Serum HBsAg titers from 11 males and 9 females belonging to group 1 were measured by radioimmunoassay (AUSRIA; Abbott).

Male mice	Serum HBsAg (μg/ml)	Female mice	Serum HBsAg (μg/ml)
1/2	6	1/14	0.7
1/3	4.6	1/15	1.6
1/4	13.6	1/18	1.5
1/6	1.5	1/19	1.8
1/7	9	1/21	1.3
1/24	10	1/31	2
1/26	9	1/32	2
1/29	9.3	1/35	2
1/39	2	1/44	2
1/49	3.3		
1/50	7		

The expression of HBsAg in the group 1 progeny of E36 was investigated by measurement of serum HBsAg levels when the mice were 4 weeks of age (Table 2). Eleven had HBsAg levels in the same range as the parent E36 and nine had two to ten times more than E36. All the females were in the low producer group and six males had levels of at least 7 μg/ml comparable with that of some human chronic HBV carriers.

To analyze the tissue specificity of the viral transcription in the HBsAg positive mice, we extracted total RNA from different organs of E11 and three F_1E36 (males 1/2 and 1/6 and female 1/14 in Table 2) and analyzed by dot and northern blot hybridization (Fig. 3). A strong signal was found by dot blot analysis in the liver of the four mice tested with a pBR-HBV probe. In E36 1/2, which produces a serum concentration of 6 μg of HBsAg per milliliter, the signal was much higher than in the three other animals that produce less HBsAg. In the testis of E11 and E36 1/6, a signal was detected that was about two times weaker than in E36 1/2 and was sensitive to ribonuclease digestion and NaOH treatment. Spleen and intestine gave a very faint signal and kidney, thymus, brain, heart, lung, pancreas, uterus, ovary, and muscle were negative. Northern blots revealed the presence of one major 2.1-kb RNA species in the liver, comigrating with the HBsAg messenger RNA present in infected humans and in the cell line used as control. In the testis, a smear starting at about 6 kb with two or three superimposed bands was seen in both E11 and E36 1/6. No hybridization was detected in any other organ although presence of intact RNA could be shown after rehybridization with a β_2-microglobulin probe (*17*). However, a more sensitive technique for detecting RNA will be necessary to obtain conclusive data on the viral gene expression in nonliver tissue. The presence of large transcripts in the testis may be due to read-



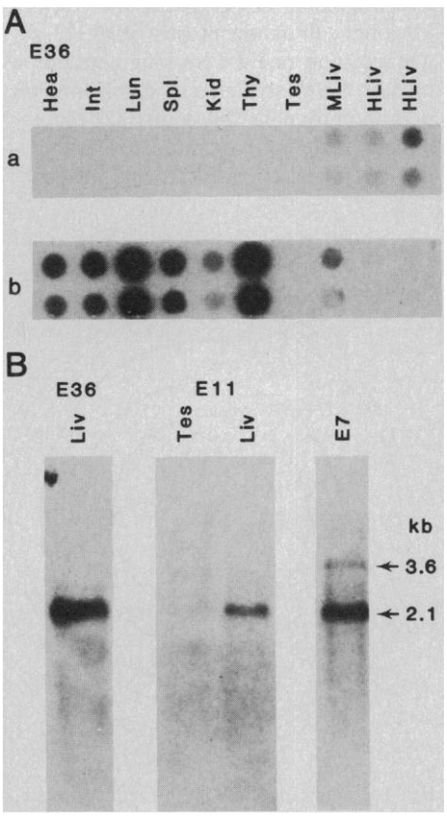


Fig. 2 (left). (A) Southern blot analysis of the integrated plasmid sequences in tail DNA of F_0 mice. DNA was extracted from a fragment of tail, and 15 μg, restricted with Eco RI was subjected to electrophoresis in a 1.5 percent agarose gel and transferred to nitrocellulose as described (*4*). As a control, 20 pg of pAC_2 digested by Eco RI was used. A recombinant plasmid, pFC80, containing four HBV genomes was used as probe. Prehybridization, hybridization, and washing were as described (*4*). (B) Southern blot analysis of Eco RI restricted DNA of F_1 E36 mice. Lane 1: mouse from group 2. Lane 2: mouse from group 1. Lane pAC_2: Eco RI restricted pAC_2 DNA. Fig. 3 (right). (A) Dot blot and (B) Northern blot analysis of E11 and E36 1/2 total RNA. RNA was extracted from different organs with phenol at 65°C, *p*H5, as described (*11*). (A) Total RNA (5 μg) from mouse E36 1/2 was denatured in 20× standard saline citrate (SSC) for 15 minutes at 65°C and filtrated onto a nitrocellulose filter soaked in 20× SSC. The filter was then treated as in the Southern blot procedure. The probe used was pFC80 (a) or β_2-microglobulin (b). Abbreviations; H, human; M, mouse; L, liver; Tes, testis; Thy, thymus; Kid, kidney; Spl, spleen; Lun, lung; Int, intestine; Hea, heart. (B) Total RNA (20 μg) was run on a 1.5 percent formaldehyde agarose gel and transferred to nitrocellulose as described (*20*). The blot was hybridized to the pFC80 probe. Liver (Liv) from mice E36 1/2 and E11 and testis (Tes) from mouse E11 were analysed. E7 is an RNA sample extracted from mouse L cells transfected with cloned HBV DNA and containing a 3.6-kb and a 2.1-kb RNA species.

through of the 2.1-kb RNA or initiation in the cellular flanking sequences. The different tissues of E36 1/6 were also analyzed by an immunoperoxidase assay for the presence of HBsAg. A positive but faint staining was seen in all the hepatocytes of E36 1/6 and in a few spleen lymphocytes. This implies an efficient secretion of HBsAg in the serum of the mice. In the liver of a human chronic carrier used as control, about 10 to 20 percent of the cells were detected as strongly positive. Histological examinations of the livers showed a normal liver for E36 1/6 and a mild inflammation for E11.

Finally we analyzed the structure of the serum HBsAg. The density measured in a CsCl gradient was about 1.2 g/ml, which corresponds to that of particles found in infected human serum or in culture medium of HBsAg-producing cell lines. Three mice tested contained in their serum the receptor for polymerized albumin, the product of the pre-S2 region (*18*). The expression of the X gene was not investigated.

Our results show that transgenic mice containing HBV sequences can be used as a model to study the regulation of viral gene expression in vivo. Indeed we have shown that synthesis of the major 2.1-kb S mRNA takes place essentially in the liver of two independent mice producing HBsAg. Although this could be due to integration of pAC_2 in host regions specifically transcribed in liver cells we favor the hypothesis of *cis*-acting control sequences brought in with the injected DNA. This last hypothesis is supported by the recent discovery of a liver specific enhancer element in the HBV genome (*14*). We also show that in the progeny of one animal, males produce more HBsAg than females and that the regulation is at the messenger RNA level in the liver. HBsAg production in hepatoma cell lines has been shown to be inducible by steroid hormones (*19*), but no report has yet mentioned a possible effect of sexual hormones on the virus expression in vivo or in vitro. After 6 months of observation, the animals do not show any signs of pathology. This confirms the current thinking that persistent production of HBsAg, itself, by the chronic carrier is not toxic to the liver. In addition, it is likely that transgenic mice tolerate the presence of the viral antigens since they carry the viral DNA as one of their genes, and thus do not produce antibodies against these antigens. However, it should be possible to induce a liver cell necrosis in these mice and study the influence of the viral sequences on the liver regeneration. The use of other recombinant HBV plasmids will allow a more detailed analysis of the viral gene expression and pathology linked to HBV infection.

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Fractal Surfaces of Proteins

Abstract. *Fractal surfaces can be used to characterize the roughness or irregularity of protein surfaces. The degree of irregularity of a surface may be described by the fractal dimension* D. *For protein surfaces defined with probes in the range of 1.0 to 3.5 angstroms in radius,* D *is approximately 2.4 or intermediate between the value for a completely smooth surface* (D = *2) and that for a completely space-filling surface* (D =*3). Individual regions of proteins show considerable variation in* D. *These variations may be related to structural features such as active sites and subunit interfaces, suggesting that surface texture may be a factor influencing molecular interactions.*

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One of the important problems in structural biology is the origin of specificity and recognition in molecular interactions. An essential step in this process is complementary contact between approaching molecular surfaces. Surface representations of proteins have provided a powerful approach for characterizing the structure, folding, interactions, and properties of proteins (*1*). A fundamental feature of surfaces that has not been characterized by these representations, however, is the texture (roughness) of protein surfaces, and its role in molecular interactions has not been defined. The degree of irregularity of a surface may be described by the fractal dimension D (*2, 3*), where $2 \leq D \leq 3$. As a surface becomes more irregular, the fractal dimension increases from the value $D = 2$, for a smooth surface, to $D \leq 3$.

The value of $(2 - D)$ may be obtained from the slope of the plot of log(surface area) against log(probe size) used to define the molecular surface (*2*):

$$2 - D = \frac{d\log(A_s)}{d\log(R)} \quad (1)$$

where A_s and R are the molecular surface area and probe radius, respectively. Such a relationship is illustrated in Fig. 1 for lysozyme, ribonuclease A, and superoxide dismutase. The slopes of these plots approach zero (corresponding to $D = 2$) in the limit of both small and large probe sizes. Small probes predominantly interact with the smooth van der Waals spheres describing the protein atoms, whereas large probes are sensitive only to the overall shape of the molecule. For probes with radii of 1 to 3.5 angstroms (Å), however, the average value of D is approximately 2.4. Since this size range corresponds to the approximate size of water molecules and side chains, such probes should be sensitive to specific interactions between residues.

To examine the variation in D over protein surfaces, we calculated the fractal dimensions of several proteins and displayed them as spherical projections. The spherical grids were sampled at 10° intervals in the polar angles ϕ and ψ. For each probe size, the surface area was calculated for all atoms within an angle θ about a particular (ϕ, ψ) direction. The