

# Hepatitis B viral DNA in liver and serum of asymptomatic carriers

(hepatitis B surface antigen carriers/hepatitis B viral DNA hybridization/hepatitis B virus serum markers/liver histopathology)

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**ABSTRACT** Cloned DNA probes were used to test for hepatitis B virus (HBV) DNA in the liver and serum of 14 asymptomatic hepatitis B surface antigen (HBsAg) carriers and two former carriers. The results were compared with serological markers of HBV infection and liver histopathology. Three groups of carriers were distinguished. In group I, HBV DNA was present in both liver and serum. Hepatitis B e antigen (HBeAg), a marker of viral replication, was uniformly positive in serum. In one individual in this group, viral DNA was also integrated into liver genomic DNA. In group II, lower levels of nonintegrated HBV DNA were detected in the liver, but no HBV DNA was found in the serum. HBeAg was negative and with one exception there were antibodies to HBeAg. Integrated viral DNA was present in each case. In group III, there was no detectable nonintegrated viral DNA in serum or liver, but one individual had integrated sequences. All carriers lacked antibodies to HBsAg and had antibodies to hepatitis B core antigen and demonstrated nonspecific histological abnormalities in the liver. These findings indicate significant quantitative and qualitative differences among asymptomatic HBsAg carriers and suggest that their infectivity may be highly variable.

Infection with hepatitis B virus (HBV) is endemic throughout much of the world (1). HBV infection may either result in an acute and self-limited form of hepatitis or lead to a more protracted, chronic illness of variable severity and prognosis. On the basis of histological criteria (2), chronic hepatitis B is divided into chronic persistent hepatitis, which usually has a benign course of unpredictable duration, and chronic active hepatitis, which commonly is progressive and in the majority of cases eventually leads to cirrhosis (3). In addition, infection with HBV may result in a chronic carrier state of prolonged duration. Such HBV carriers usually are asymptomatic and the presence of hepatitis B surface antigen (HBsAg) in the serum is detected incidental to routine screening of blood donors or during epidemiological surveys (4). The world population includes about 200 million carriers, who are thought to represent a major reservoir of HBV infection (5).

We have used cloned HBV DNA (6, 7) to probe for viral DNA in the liver and serum of 14 asymptomatic carriers and have correlated these findings with conventional serological markers of HBV infection and with structural changes in the liver.

## MATERIALS AND METHODS

**Selection of Subjects.** Sixteen HBsAg-positive blood donors identified by the Irwin Memorial Blood Bank in San Francisco gave informed consent to this study, which was approved by the Committee on Human Research, University of California, San Francisco. None of the subjects reported previous or current liver disease, had a history of jaundice, or was receiving medication at the time of the study. One (F) had received blood transfusions at the age of 6 months, one (P) had a history of pre-

vious intravenous drug abuse, and seven (C, D, F, G, I, L, and P) admitted to occasional or modest alcohol intake. Three subjects (C, D, and O) were homosexuals and seven (E, F, J, K, L, O, and P) acknowledged previous occupational or intimate contact with individuals known or suspected to have viral hepatitis.

After admission to the General Clinical Research Center, University of California, San Francisco, all subjects had a complete physical examination. No overt evidence of liver disease was observed and the results of blood tests were within the normal range, except for four individuals (B, C, D, and P) who had a slight elevation of glutamic-oxaloacetic transaminase or glutamic-pyruvic transaminase activity. A percutaneous liver biopsy was performed on each individual and the tissue was divided into three sections. One was fixed in 1% osmium tetroxide/s-collidine buffer for electron microscopy (8), another was fixed in 10% (vol/vol) formaldehyde for light microscopy (9), and the third was stored in liquid nitrogen for DNA analysis.

**HBV Serological Markers.** HBV serological markers were detected by radioimmunoassay (AUSRIA 11-125, AUSAB, CORAB, and ABBOTT-HBe, all from Abbott). In addition, we determined the HBsAg serum titer by assaying serially diluted samples in 50 mM sodium phosphate (pH 7) containing 1% bovine serum albumin. The results are reported as the dilution equivalent defined in the Abbott procedure.

**Isolation of DNA from Serum or Liver.** Two hundred microliters of serum was added to 250  $\mu$ l of 150 mM NaCl/10 mM EDTA/10 mM Tris-HCl, pH 8.0 (Tris/NaCl/EDTA), containing 2% (wt/vol) NaDodSO<sub>4</sub>, salmon sperm DNA at 40  $\mu$ g/ml, and proteinase K at 2 mg/ml. After incubation for 4 hr at 37°C, the solution was extracted once with an equal volume of buffer-saturated phenol followed by an equal volume of chloroform/isoamyl alcohol, 24:1 (vol/vol). The DNA was precipitated by adding 1/10 vol of 3 M NaOAc and 2 vol. of 100% EtOH. The DNA pellet was washed with 70% (vol/vol) EtOH, dried, and dissolved in 20  $\mu$ l of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (Tris/EDTA).

For isolation of liver DNA, the tissue samples (5-20 mg per biopsy) were homogenized in 450  $\mu$ l of Tris/NaCl/EDTA. After addition of NaDodSO<sub>4</sub> and proteinase K to a final concentration of 1% (wt/vol) and 1 mg/ml, respectively, the homogenates were incubated for 12-16 hr at 37°C and the DNA was purified as described above. No carrier DNA was used and the average yield was about 20  $\mu$ g of DNA per mg of tissue.

**HBV DNA Spot Test.** DNA was partially purified from 200  $\mu$ l of serum as described above. After extraction with phenol, the aqueous phase was dried under reduced pressure, the DNA was denatured in 10  $\mu$ l of 0.35 M NaOH for 10 min at room temperature, and the solution was neutralized by the addition

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBs, -HBe, and -HBe, antibodies to HBsAg, HBeAg, and hepatitis B core antigen; bp, base pair(s).

of 10  $\mu$ l of 2 M  $\text{NH}_4\text{OAc}$  (10). In six cases (G, I, J, K, M, and N) viral particles were concentrated from 10 ml of serum by centrifugation at  $274,000 \times g$  for 20 hr at  $4^\circ\text{C}$ . The pellet was suspended in 7 ml of Tris/NaCl/EDTA containing 2% Na-DodSO<sub>4</sub> and proteinase K at 1 mg/ml and the suspension was incubated for 14 hr at  $37^\circ\text{C}$ . The solution was then extracted with phenol and the aqueous phase was dried under reduced pressure. Ten micrograms of salmon sperm DNA was added to the residue and all the DNA was suspended in 1 ml of  $\text{H}_2\text{O}$ , redried, and treated as above. In all cases, the total volume of 20  $\mu$ l was spotted onto a nitrocellulose filter (Schleicher & Schuell) that had been washed first in water and then in 1 M  $\text{NH}_4\text{OAc}$ . One hundred microliters of 1 M  $\text{NH}_4\text{OAc}$  was added to each air-dried spot and the filter was then rinsed in 0.6 M NaCl/0.06 M sodium citrate, dried in a vacuum oven at  $80^\circ\text{C}$  for 2 hr, and hybridized to  $^{32}\text{P}$ -labeled HBV DNA as described (11).

**HBV DNA Restriction Enzyme Analysis.** Unless otherwise specified, 25  $\mu$ g of purified liver DNA was digested by 5 units of *Hind*III (New England BioLabs) for 12–16 hr at  $37^\circ\text{C}$ . The digested DNA was precipitated by adding 1/10 vol of 3 M NaOAc and two volumes of 100% EtOH. The DNA pellet was washed with 70% EtOH, dried, and dissolved in 20  $\mu$ l of Tris/EDTA. The sample was electrophoresed on 1% agarose gels and the pattern was analyzed by the Southern technique (12) as described (11).

**RESULTS**

**HBV Serological Markers.** The relevant characteristics of the 16 asymptomatic, HBsAg-positive blood donors studied are listed in Table 1. On retesting (intervals of 6 and 8 months, respectively), two (O and P) were found to be HBsAg-negative and one (O) of these was anti-HBs-positive. The remaining 14 individuals were positive for HBsAg at the time of the study and for preceding periods ranging between 4 and 18 months and in

one case (L) 6 years. In addition, all 14 carriers were tested for HBsAg 12 months after the liver biopsy and were found to be positive.

These 14 asymptomatic carriers can be divided into three groups based on several criteria, including serological markers (Table 1). Individuals in group I had the highest titers of HBsAg (corresponding to 200–500  $\mu\text{g/ml}$ ) and were also positive for HBeAg and negative for anti-HBe. Group II individuals had somewhat lower HBsAg levels, were negative for HBeAg, and, with one exception (E), were positive for anti-HBe. Individuals in group III had very low levels of HBsAg, were also negative for HBeAg, and uniformly positive for anti-HBe. All 14 HBsAg carriers were positive for anti-HBc but negative for anti-HBs.

**Liver Histology.** Although subtle structural abnormalities were observed in all of the tissue samples, only one (O) had portal lymphocytic infiltration of sufficient extent to be classified as chronic persistent hepatitis. In two instances (B and J), tissue samples were too small to permit satisfactory evaluation. In the remaining biopsy samples, cells lining the vascular endothelium were unusually prominent, frequently forming distinct cell “knots.” Large cytoplasmic fat globules were present in the hepatocytes of three (M, N, and P) and in one (H) the portal tracts were wide and filled with collagen. In the remaining samples (A, C, D, E, F, G, I, K, and L), various degrees of portal inflammation were present, ranging from scattered to large aggregates of lymphocytes. Although the terminal plates separating the portal zones from the liver parenchyma were intact, they were irregular in form. Furthermore, examples of isolated cell necrosis and binucleate cells in the parenchyma were common. Ground-glass cytoplasm characteristic of cells producing HBsAg (13) was noted in A, B, H, I, and L.

**HBV DNA in Serum.** Viral DNA isolated from serum-derived HBV particles is circular but only partially double stranded. The long strand is about 3,200 nucleotides and is nicked (7, 14–16). The short strand overlaps the nick by about 300 nucleotides and extends to variable degrees in the 3’ di-

Table 1. Results of HBV antigen, antibody, and DNA tests

Subject	Age, yr	Sex	Race	Serological markers					HBV DNA		
				HBsAg*	HBeAg	Anti-HBe	Anti-HBs	Anti-HBc	Serum	Liver	
										Non-integrated	Integrated
<b>Group I</b>											
A	18	M	Asian	400	+	–	–	+	+	++	+
B	21	M	Asian	400	+	–	–	+	+	++	–
C	19	M	Caucasian	400	+	–	–	+	+	++	–
D	36	M	Caucasian	400	+	–	–	+	+	++	–
<b>Group II</b>											
E	33	F	Asian	100	–	–	–	+	–	+	+
F	28	M	Black	100	–	+	–	+	–	+	+
G	36	M	Asian	100	–	+	–	+	–	+	+
H	35	F	Asian	70	–	+	–	+	–	+	+
I	30	M	Black	100	–	+	–	+	–	–	+
<b>Group III</b>											
J	27	M	Asian	15	–	+	–	+	–	–	–
K	37	M	Asian	4	–	+	–	+	–	–	–
L	31	F	Asian	3	–	+	–	+	–	–	+
M	31	M	Asian	3	–	+	–	+	–	–	–
N	33	M	Asian	2	–	+	–	+	–	–	–
<b>Former carriers</b>											
O	21	M	Black	–	–	–	+	–	–	–	–
P	22	M	Black	–	–	–	–	–	–	–	–

HBeAg, hepatitis B e antigen; anti-HBe, -HBs, and -HBc, antibodies to HBeAg, HBsAg, and hepatitis B core antigen, respectively.  
\* HBsAg titers  $\times 10^{-3}$ .

rection (7, 14–16). The serum from each individual in this study was tested for the presence of the HBV genome by hybridization with a cloned probe. Positive signals were obtained only in those samples (group I) with the highest titer of HBsAg and positive HBeAg (Table 1). As shown in Fig. 1 (lane 2), the pattern of hybridization of  $^{32}\text{P}$ -labeled HBV DNA to viral DNA isolated from serum and electrophoresed on agarose gels is heterogeneous, no doubt reflecting the length dispersion of the short strand. In addition, small amounts of a more rapidly migrating species were detected. None of the other 12 individuals had measurable levels of HBV DNA in the 200- $\mu\text{l}$  serum samples or in the viral fractions obtained by centrifuging 10 ml of serum (G, I, J, K, M, and N).

To estimate the concentration of HBV in serum, spot tests were performed on serially diluted samples. Cloned HBV DNA was used as a standard. From a comparison of the densitometer tracings (Fig. 2), it was estimated that about  $10^8$  viral particles were present per ml of serum. The limit of HBV detection in 10-ml samples is about 15,000 viral particles per ml of serum (that is, 0.5 pg of DNA or 150,000 copies per spot).

**HBV DNA in Liver.** The results of the DNA analysis performed on the 16 liver biopsy samples are summarized in Table 1. The four carriers in group I that had high levels of HBV DNA in the serum also had nonintegrated viral DNA in the liver tissue sampled. As shown in Fig. 1 (lane 3), the electrophoretic pattern of undigested liver DNA shows two low molecular weight bands that hybridized to the HBV DNA probe. They are present in approximately equal amounts and the broad width of the bands indicates molecular heterogeneity. This profile was not changed when the DNA sample was digested by the restriction enzyme *Hind*III (data not shown), an enzyme that does not cut cloned isolates of the HBV genome (7, 15). The slower-migrating species corresponded in average mobility (slightly faster than the 3,200-bp marker) to that found in serum (Fig. 1, lane 2). A faster-migrating form is also sometimes present in very low concentrations in serum (Fig. 1, compare lanes 2 and 3). In some cases the liver DNA pattern was more complex. For example, in carrier B at least five species of differing mobility were resolved

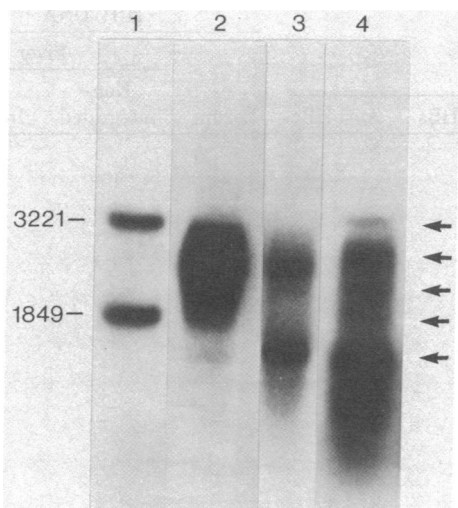


FIG. 1. Hybridization of  $^{32}\text{P}$ -labeled cloned HBV DNA to the viral DNA isolated from the liver and serum of group I asymptomatic carriers. HBV DNA was isolated from the serum (lane 2) or liver (lane 3) of HBsAg carrier A and fractionated on a 1% agarose gel. The electrophoretic pattern of the HBV-specific DNA isolated from the liver of carrier B is shown in lane 4. (The arrows indicate five distinct bands of hybridization visible in the original autoradiogram.) HBV standards [3,221 and 1,849 base pairs (bp)] are included in lane 1. The Kodak X-Omat AR film was exposed for 24 hr at  $-80^\circ\text{C}$ .

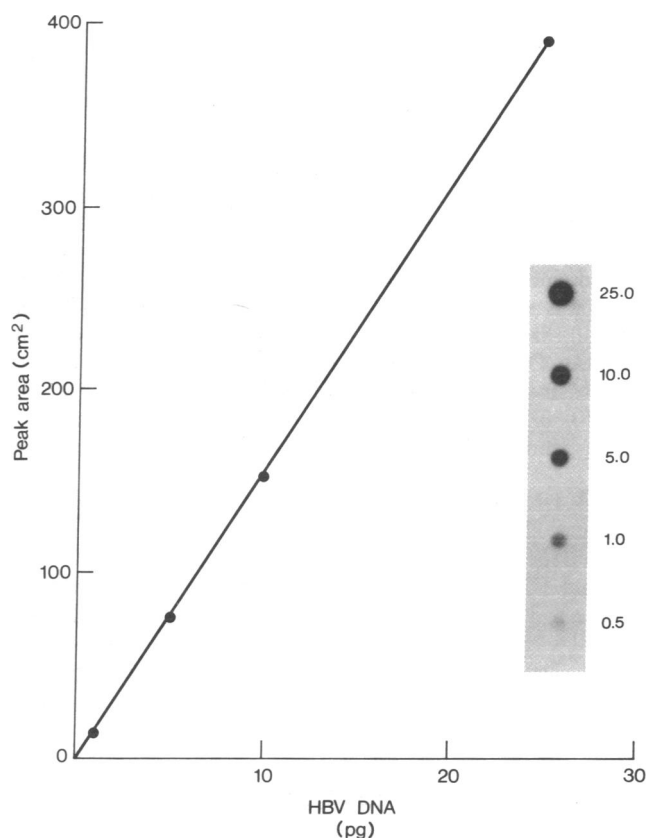


FIG. 2. Spot test of HBV DNA. Increasing amounts of HBV DNA were spotted onto nitrocellulose filters. The intensity of each  $^{32}\text{P}$ -labeled spot (Inset) was estimated by calculating the area of the densitometer tracing. The Kodak X-Omat AR film was preflashed and exposed for 7 days at  $-80^\circ\text{C}$ .

that hybridized to the HBV probes (Fig. 1, lane 4). Of the five carriers in group II, who had no detectable HBV DNA in serum, four had two bands of HBV-specific DNA in the liver (Fig. 3, lane 2). The pattern, however, differed from that observed in the group I carriers. One band migrated as a species of about 3,200 bp and the other was integrated into high molecular weight DNA. The latter may reflect random or specific integration of viral DNA into the host genome; alternatively, HBV DNA may have been trapped in chromosomal DNA during

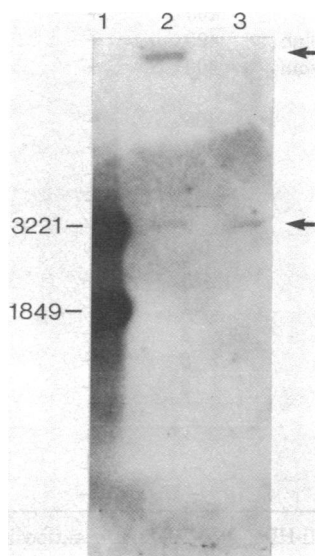


FIG. 3. Hybridization of  $^{32}\text{P}$ -labeled cloned HBV DNA to the viral DNA isolated from the liver of a group II carrier, E. The electrophoretic pattern of the HBV-specific DNA is shown in lanes 2 (undigested) and 3 (plus *Hind*III endonuclease digestion). HBV DNA standards (3,221 and 1,849 bp) are included in lane 1. The Kodak X-Omat AR film was exposed for 14 days at  $-80^\circ\text{C}$ .

electrophoresis. This latter possibility seems unlikely because integrated viral DNA was detected in only one (A) of the four liver samples in group I that contained very high levels of nonintegrated DNA (data not shown). Further, this high molecular weight band was sensitive to digestion with *Hind*III endonuclease (Fig. 3, lane 3). However, no distinct additional bands were evident, suggesting that the viral DNA is not integrated into a unique site(s) in cellular DNA. *Hind*III digestion did not alter the mobility or the intensity of the low molecular weight form. This is consistent with the view that these molecules represent forms of nonintegrated viral DNA.

In one of the five carriers of group III (L), as well as one of group II (I), only integrated DNA was found in the liver (data not shown). In the four remaining asymptomatic carriers (J, K, M, and N) and in the two former carriers (O and P) no evidence of HBV DNA, nonintegrated or integrated, was detected. In these cases, 50  $\mu$ g of liver DNA was analyzed by both spot hybridization and electrophoresis. Because the sensitivity of spot hybridization is about 0.5 pg of HBV DNA (Fig. 2), this indicates an average of less than 0.02 copy of the viral genome per hepatocyte (assuming 7 pg of DNA per cell).

### DISCUSSION

None of the 16 individuals had entirely normal liver histology and four had a slight elevation of serum transaminase activity (see also ref. 17). These findings emphasize that, even in asymptomatic carriers, persistent infection with HBV usually results in structural and at times functional evidence of hepatic disease (18). However, the extent of these abnormalities did not correlate with the levels of detectable HBV DNA in serum or liver.

Previous investigations (19, 20) have shown that the HBsAg carrier state is heterogeneous with respect to the level of serum HBsAg and the presence of HBeAg and DNA polymerase activity (both correlated with viremia). Recently, Bonino *et al.* (21) have shown differing levels of HBV DNA in the serum of a mixed group of carriers that included patients with chronic active and chronic persistent hepatitis. Our study focuses on a select group of asymptomatic HBeAg carriers. From the various criteria employed, three subgroups of this state can be tentatively identified. Group I carriers had very high serum levels of HBsAg and were positive for HBeAg. Further, HBV DNA was present in high concentrations in both serum and liver. The liver samples contained nonintegrated viral DNA and, in one instance, HBV DNA was found integrated into cellular DNA. Group II carriers had somewhat lower concentrations of serum HBsAg, but HBeAg was undetectable. However, four of the five carriers had anti-HBe in the serum. No HBV DNA was detected in the serum, but nonintegrated viral DNA was found in the liver of four of them. Significantly, all five carriers in this group had HBV DNA integrated into cellular DNA. In group III carriers, the level of serum HBsAg was very low and HBeAg was undetectable, but anti-HBe was present. Viral DNA was not found in the serum nor was nonintegrated viral DNA found in the liver. However, in one individual, HBV DNA was integrated into hepatocyte DNA.

The absence of HBV DNA from the serum or liver of some carriers is subject to at least two qualifications. First, in the concentrated serum samples the limit of detection of viral DNA is about 15,000 copies per ml. The presence of nonintegrated viral DNA in the liver of group II carriers may be regarded, however, as presumptive evidence that low, undetectable concentrations of hepatitis B virions were also present in the serum. Second, liver tissue was obtained by needle biopsy and, consequently, HBV DNA present in cells in localized regions may have been missed. Thus, in group III carriers, in which HBV DNA was not found in liver or serum, the continued presence

of HBsAg in the serum suggested that HBV DNA did exist somewhere in the liver.

The HBsAg present in the serum of carriers may result from nonintegrated or integrated HBV DNA. For example, some lines of cultured hepatocellular carcinoma cells (Alexander) actively synthesize HBsAg but contain only integrated viral DNA (11, 22, 23). Thus in carriers in whom there was no detectable viremia or nonintegrated HBV DNA in the liver (group III) it seems reasonable to propose that serum HBsAg is produced from integrated copies of the viral genome. In fact, such individuals may fail to produce infectious viral particles. It is apparent from the data that the level of serum HBsAg is not a reliable indicator of infectivity. Even when there was a high titer of serum HBsAg, as in groups I and II, the levels of viremia were dramatically different. Group I carriers had an estimated  $10^8$  HBV particles per ml of serum, whereas in group II the HBV titer numbered less than 10,000 per ml, a difference of about  $10^4$ -fold. The infectivity of carriers in group II consequently is likely to be lower than that of group I. The absence of HBeAg from groups II and III and the appearance of anti-HBe are consistent with epidemiological findings relating this pattern of serological markers to low infectivity (24). However, HBV DNA has been found in the serum of some HBeAg-negative patients (21, 25), and we find nonintegrated viral DNA in the liver of some HBeAg-negative carriers. Although the HBeAg/anti-HBe status may be a useful indicator of the degree of infectivity, we propose that quantitation of serum HBV DNA is the most direct and reliable test of infectivity.

The present findings also provide a possible explanation for the variable response of hepatitis patients to antiviral therapy. A recent study showed that on therapy some patients no longer produced HBsAg, whereas others lost the markers of active viral replication such as DNA polymerase activity and HBeAg but HBsAg production persisted (26). Our findings suggest that aggressive treatment before integration of HBV DNA into cellular DNA has occurred might result in the loss of HBV DNA sequences and hence the capacity for HBsAg production. On the other hand, after integration has taken place, HBsAg expression might persist despite treatment. An effective therapeutic protocol therefore may depend on early detection of HBV infection.

Viral DNA extracted from the liver of group I carriers contained several molecular species, including two dominant but heterogeneous forms. One migrated in the region of the 3,200-bp nicked circular duplex found in serum; the other, more rapidly migrating, forms might represent superhelical structures, largely single-stranded molecules or various replicative forms. A similar pattern has been reported (27, 28) in several patients with chronic active hepatitis. In group II carriers there was only a single nonintegrated species that migrated as the nicked complete HBV DNA molecule. These qualitatively different patterns may represent different stages of HBV infection. Combined with the antigen/antibody data, they suggest that the individuals in group I represent a relatively acute form of the carrier state, whereas groups II and III reflect more protracted stages. Whether these events occur in sequence remains speculative because we do not know how long the carrier state had existed prior to its incidental detection. Long-term studies on individual carriers will help to clarify this issue.

In addition to virion DNA, integrated HBV sequences were found in the cellular DNA in all carriers in group II and in one each of groups I and III. Prior to digestion with restriction enzymes, the HBV-containing DNA migrated with the high molecular weight DNA band. Digestion with *Hind*III, a restriction endonuclease that fails to cleave any of the HBV genomes that have been cloned (7, 15), resulted in the disappearance of the

hybridizing band without the concomitant appearance of lower molecular weight bands. This finding indicates that HBV DNA is integrated into multiple sites within the host genome. We (ref. 29 and unpublished observations) and others (30–33) have demonstrated HBV DNA integration in primary hepatocellular carcinomas, and it recently has been reported that HBV DNA is integrated into hepatocyte DNA in cases of chronic active hepatitis (25, 27, 28) and cirrhosis (30). In each of these instances a distinct banding pattern was observed after *Hind*III digestion. Nonetheless these findings are consistent with the hypothesis that HBV integrates into many genomic sites, because DNA samples may have been derived from clonal populations of regenerating or neoplastic cells. Integration of HBV DNA into genomic DNA may occur stochastically as a function of time and HBV concentration, and therefore the probability of HBV integration may increase with the persistence and intensity of the infection.

The consequences of HBV DNA integration into genomic DNA are not yet fully understood. For example, integrated viral sequences could serve as a source of recurrent viral infection. Findings in cultured hepatocellular carcinoma cells suggest that integrated HBV sequences are stable and are not recruited by bromodeoxyuridine treatment (ref. 34 and unpublished observations). Furthermore, some of the copies represent incomplete or aberrant forms of the virus (11). Therefore, it seems unlikely that integrated viral sequences represent a reservoir of infection. However, integration should not be considered as an inconsequential state. The presence of integrated HBV copies in a high proportion of hepatomas is consistent with a primary or secondary role of HBV integration in oncogenesis. Furthermore, it is possible that integration of viral sequences and persistent surface antigen production contribute to the development of progressive liver disease and cirrhosis observed in clinically asymptomatic carriers (35).

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