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State of hepatitis B virus DNA in hepatocytes of patients with hepatitis B surface antigen-positive and -negative liver diseases

(recombinant DNA/hepatocellular carcinoma/chronic and acute hepatitis/Southern blot technique/immunofluorescence)

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ABSTRACT Using the Southern blot technique and cloned hepatitis B virus (HBV) DNA as a probe, we studied the state of HBV DNA in the liver of 13 patients with hepatocellular carcinoma, 17 patients with chronic hepatitis, and 2 patients with acute hepatitis. The hybridization results were compared with the serological and immunohistological data. Integration of HBV DNA in cellular DNA of the liver from patients with hepatocellular carcinoma was demonstrated. In two patients from which tumorous and nontumorous liver tissue samples were available the integration patterns were different. In one patient with hepatitis B e antigen (HBeAg)-positive early hepatocellular carcinoma, free viral DNA was present in the liver. In some patients with HBeAg-negative chronic hepatitis, without tumor, integration of HBV DNA in cellular DNA was also demonstrated. This suggests that HBV is not the only factor involved in the development of a tumor. In patients with HBeAg-positive chronic hepatitis, free viral DNA was detected in the liver. In the two acute hepatitis patients analyzed, the restriction endonuclease patterns strongly suggested HBV DNA integration. Therefore, viral DNA integration seems to occur early in infection. Whatever the form of the disease, discrete bands were observed, suggesting the existence of limited and specific integration sites in host cellular DNA. The presence of integrated or free DNA sequences has implications for antiviral therapy. In addition, detection of HBV DNA in the liver is another sensitive viral marker that could be useful for diagnostic purposes.

Exposure to hepatitis B virus (HBV) can be followed by acute and chronic hepatitis (1). In addition, human HBV chronic carriers have a significantly increased risk of developing hepatocellular carcinoma (2). The Southern blot technique (3) using cloned HBV DNA as a probe (4) provides another approach to investigating the relationship between HBV and these pathological conditions. Presence and integration of HBV DNA sequences in the cellular DNA of hepatocellular carcinomas and of a hepatoma cell line (5–8) have been demonstrated. This observation is an additional argument for HBV as a factor in this human cancer. Thus, it is important to investigate the state of the viral DNA in chronic hepatitis and acute hepatitis. Additionally, the results of the hybridization technique should be compared to the serological and histological tests of HBV infection to evaluate its diagnostic usefulness. In this report, we present evidence for integration of HBV DNA in tumorous and nontumorous parts of the liver of patients with hepatocellular carcinoma and in the liver of patients with chronic and acute hepatitis B.

MATERIALS AND METHODS

Patients and Liver Tissue Samples. The 42 patients investigated were categorized into four groups. Group I included patients with hepatocellular carcinomas. Liver tissue samples from patients 1–9 from the Ivory Coast were obtained at autopsy. Liver tissue samples from patients 10 and 11 were obtained by a surgical biopsy, and the liver tissue sample (≈20 mg) from patient 12 was obtained by a needle biopsy. For patients 1 and 5, a nontumorous cirrhotic part of liver tissue was also available. Patient 13 had e antigen (HBeAg)-positive chronic hepatitis and underwent a portocaval shunt to prevent recurrent bleeding. During surgery, a small tumorous nodule of early hepatocellular carcinoma was discovered and resected. An open biopsy of nontumorous cirrhotic tissue was also performed. There was no evidence of other tumorous nodules on scanning, angiographic, and surgical examination. Group II included patients with chronic hepatitis, without apparent tumor. Liver tissue samples from patients 14, 15, 17, 18, 19, 20, 21, 22, 23, 25, and 26 were obtained by a surgical biopsy and liver tissue samples from patients 16, 24, 27, 28, 29, and 30 by a needle biopsy (≈15 mg). Group III included two patients with acute hepatitis. Liver tissue sample from patient 31 was obtained by a needle biopsy and liver tissue sample from patient 32 with fulminant hepatitis was obtained at autopsy in San Francisco. Group IV included liver tissue samples obtained at autopsy from ten patients (nos. 33–42) without any histological evidence of liver disease.

Serological Investigations. Serum samples were collected at the same time as the liver tissue and tested for hepatitis B surface antigen (HBsAg), antibody to hepatitis B core antigen (anti-HBc), and antibody to hepatitis B surface antigen (anti-HBs), using radioimmunoassay (Ausria II, Corab, and Ausab, Abbot). Rheophoresis was performed in the reference laboratory of J. Pillot for detection of HBeAg.

Immunohistochemical Methods. Sections of liver tissue were examined for HBsAg and hepatitis B core antigen (HBcAg) by means of fluorescein isothiocyanate-labeled anti-HBs and anti-HBc. The details of the reagents and the procedures used have been reported (9).

DNA Preparation and Hybridization. Immediately upon collection, the liver tissue was frozen in liquid nitrogen and stored at −80°C until it was used for DNA extraction. The fro-

Abbreviations: HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; anti-HBs, antibody to HBsAg; anti-HBc, antibody to HBcAg; anti-HBe, antibody to HBeAg; kb, kilobase.

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zen tissue was pulverized in liquid nitrogen, quickly transferred into lysis buffer, and digested overnight at 37°C with proteinase K (Boehringer) at 50 μg/ml as described (5). The cellular DNA, extracted with phenol/chloroform and dialyzed, was again digested with RNase A (Boehringer) at 100 μg/ml and RNase T1 (Boehringer) at 2 μg/ml for 2 hr at 37°C. After another extraction with phenol/chloroform and dialysis, the DNA was precipitated with ethanol. For one patient (group II, no. 18), the Hirt procedure was used for DNA extraction (10).

For surgical and autopsy samples, cellular DNA was digested with *Hin*dIII or *Eco*RI endonucleases (New England BioLabs) overnight at 37°C, using 2 units of the enzyme for 1 μg of DNA. The yield of DNA extracted by needle biopsy ranged between 7 and 20 μg (average 10 μg) and only one restriction enzyme digestion could be performed. DNA fragments were then electrophoresed in 0.8% agarose gel and transferred to a nitrocellulose filter by using a modification of the Southern technique described by Wahl *et al.* (11). Instead of soaking the gel in 0.25 M HCl twice for 15 min, we used it once for 10 min. The DNA immobilized on nitrocellulose paper was hybridized with cloned HBV DNA as a probe labeled with ^{32}P by the nick-translation procedure (5). The specific activity of HBV probe ranged between 3 and 4 $\times 10^8$ cpm/μg of DNA. For hybridization, 10^7 cpm was added to each filter paper. After extensive washing, the nitrocellulose was autoradiographed with preflashed Kodak-X-Omat film in the presence of a Du Pont Lightning Plus screen at −70°C with an average exposure of 5 days, except for patient 13, for whom the exposure was both overnight and 5 days.

RESULTS

Specimens of serum and liver tissue from each of the patients were analyzed for the serologic markers of HBV infection and immunohistologic localization of HBsAg and HBcAg. The presence and the physical state of HBV DNA in liver cells were studied by using the Southern blot technique with cloned HBV DNA as a probe. We chose *Hin*dIII and *Eco*RI endonucleases to digest the liver DNA because *Hin*dIII does not cleave HBV DNA and *Eco*RI cleaves once in a majority of the viral DNAs that have been examined so far (4, 8, 12–14). Uncut cellular DNA was analyzed when enough DNA was available.

Hepatocellular Carcinoma. Thirteen patients were analyzed (10 Blacks and 3 Caucasians). Their serological status is reported in Table 1. Eight of nine patients from the Ivory Coast had HBsAg and none had HBeAg in their serum. Patient 9 had only anti-HBc in the serum. Patients 10, 11, and 12 were of Caucasian origin; one had HBsAg in the serum, one had only anti-HBs, and another had no serological markers of HBV infection. Patient 13 of Black origin with hepatocarcinoma had HBsAg and HBeAg in the serum. Immunofluorescence revealed HBsAg in four of the ten patients examined; tissue from patients 1, 2, 3, and 13 showed only a few scattered tumorous cells with HBsAg, while HBcAg was not demonstrable at all. In contrast, the nontumorous tissue of patients 1, 5, and 13 showed both HBsAg and HBcAg.

In the tumorous part of all the HBeAg-negative hepatocellular carcinoma (patients 1–12) and in the nontumorous cirrhotic parts from patients 1 and 5, the *Hin*dIII cellular DNA pattern showed the presence of bands corresponding to hybridized DNA fragments of high molecular weight (representative cases are shown in Fig. 1 *Upper*). When uncut cellular DNA was analyzed, hybridization took place only in the very high molecular weight DNA fragments. In both the tumorous part and the nontumorous cirrhotic part of the liver from the HBeAg-positive early hepatocellular carcinoma (patient 13), the *Hin*dIII pattern

Table 1. Serological, immunohistochemical, and hybridization results

			Serologic tests				Liver fluorescence		HBV DNA in liver	
Patients	Histologic diagnosis	Race*	HBsAg	HBeAg	Anti-HBc	Anti-HBs	HBsAg	HBcAg	Integrated	Free
Group I										
1, 2, 3		B	+	−	+	−	+	−	+	−
4, 5, 6, 7, 8	Hepatocellular carcinoma	B	+	−	+	−	−	−	+	−
9		B	−	−	+	−	−	−	+	−
10		C	+	−	+	−	−	−	+	−
11		C	−	−	−	−	−	−	+	−
12		C	−	−	−	+	−	−	+	−
13	Early hepatocellular carcinoma with chronic active hepatitis and cirrhosis	B	+	+	+	−	+	−	+?	+
Group II										
14, 15, 16	Inactive cirrhosis	C	+	−	+	−	+	+	+	−
17, 18, 19, 20	Inactive cirrhosis	C	−	NT	+	−	+	+	+	−
21, 22, 23	Chronic active hepatitis without cirrhosis (21) or with inactive cirrhosis (22, 23)	C	−	NT	+	+	+	+	+	−
24	Inactive cirrhosis	C	−	NT	−	+	+	+	+	−
25, 26, 27	Inactive cirrhosis	C	−	NT	−	−	+	+	+	−
28, 29, 30	Chronic persistent hepatitis (28, 29) or chronic active hepatitis without cirrhosis (30)	C	+	+	+	−	+	+	?	+
Group III										
31	Acute hepatitis	B	−	NT	+	+	NT	NT	+	−
32	Fulminant hepatitis	C	+	−	+	−	NT	NT	+	−
Group IV										
33–42	Normal liver (control)	C	−	NT	−	−	NT	NT	−	−

NT, not tested.
* Race: B, Black; C, Caucasian.

showed an intense broad band at the 3.2-kilobase (kb) position and a smear in a lower position (Fig. 1 *Upper*). After a prolonged exposure, intense hybridization appeared in the entire track without any discernible bands. The same pattern of 3.2 kb and a smear in a lower position was obtained with uncut cellular DNA (data not shown). The *Eco*RI restriction pattern for patients 1–7 showed bands corresponding to DNA fragments of variable lengths, some of them located at a high molecular weight DNA position (Fig. 1 *Lower*). In four cases, a band at the 3.2-kb position was observed. In patients 10 and 11, an additional but faint band at a higher molecular weight was observed. In patient 13, one intense band was observed at the 3.2-kb position and two other bands appeared at the 1.5-kb position with a smear downstream. No hybridization in the high molecular weight DNA fragments was seen after a 5-day exposure (Fig. 1 *Lower*).

For patients 1 and 5, the tumorous and nontumorous samples had different restriction patterns. For patient 13, with early hepatocellular carcinoma, the hybridization patterns of tumorous and nontumorous parts were identical after both *Hin*dIII and *Eco*RI digestion (Fig. 1).

Chronic Hepatitis Without Apparent Carcinoma. Seventeen patients were analyzed. The histological and immunohistochemical results are reported in Table 1. Patients 14–16 had HBsAg-positive inactive cirrhosis without HBeAg in the serum. Patients 17–24 had HBsAg-negative cirrhosis with anti-HBc, anti-HBs, or both in the serum. Patients 25–27 had chronic hepatitis without serological markers of HBV infection. Patients 28 and 29 had HBeAg-positive chronic persistent hepatitis and patient 30 had HBeAg-positive chronic active hepatitis. Im-

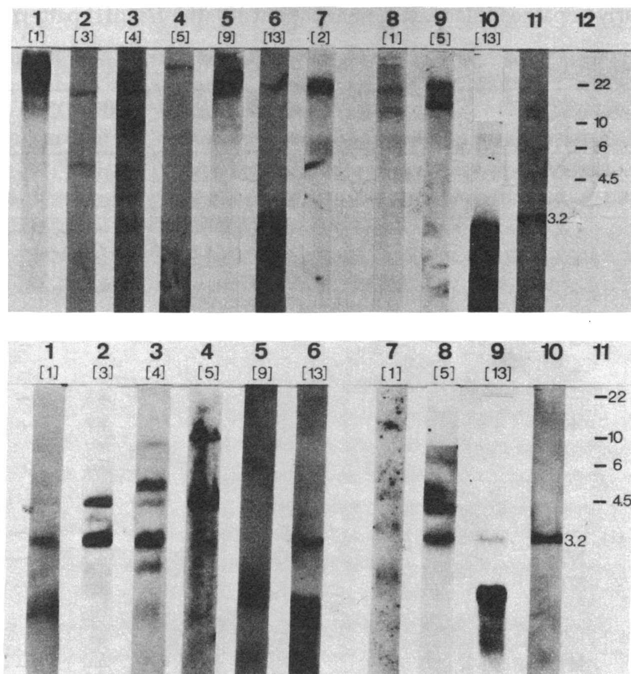

FIG. 1. Southern blot of hepatocarcinoma liver tissue samples (group I) (the number in the brackets corresponds to the number of the patient in Table 1). (*Upper*) *Hin*dIII restriction patterns. Lanes 1–7, tumorous parts of the samples; lanes 8–10, nontumorous cirrhotic part of the samples; lane 11, cloned HBV DNA; lane 12, reference sizes, expressed in kb, of DNA fragments obtained from *Hin*dIII digestion of λ*plac*5cI857S7. (*Lower*) *Eco*RI restriction patterns. Lanes 1–6: tumorous parts of the samples; lanes 7–9, nontumorous cirrhotic parts of the samples; lane 10, cloned HBV DNA; lane 11, reference sizes, expressed in kb, of DNA fragments obtained from *Hin*dIII digestion of λ*plac*5cI857S7. For all the patients 80 μg of cellular DNA was analyzed.

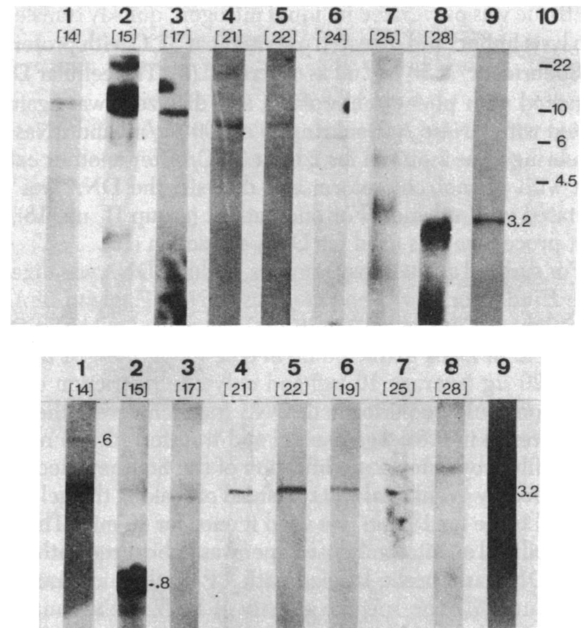

FIG. 2. Southern blot of chronic hepatitis liver tissue samples (group II) (the number in the brackets corresponds to the number of the patient in Table 1). (*Upper*) *Hin*dIII restriction patterns. Lanes 1–8, patient DNAs as indicated. Lane 9, cloned HBV DNA; lane 10, reference sizes, expressed in kb, of DNA fragments obtained from *Hin*dIII digestion of λ*plac*5cI857S7. (*Lower*) *Eco*RI restriction patterns. Lanes 1 to 8, patient DNAs as indicated. Lane 9, cloned HBV DNA. For patients 14, 15, and 25, 80 μg of cellular DNA was analyzed. For patients 17, 21, and 22, 30 μg of cellular DNA was analyzed. For patients 24, 26, and 28, 15 μg of cellular DNA was analyzed.

munofluorescence revealed HBsAg and HBcAg in all cases. In all the patients with HBeAg-negative chronic hepatitis (14–27), the *Hin*dIII restriction pattern showed bands corresponding to high molecular weight DNA fragments (representative cases are shown in Fig. 2 *Upper*). In five cases (patients 14, 18, 21, 23, and 25) the uncut DNA pattern showed hybridization to a very high molecular weight DNA without any discrete band (data not shown). For the three patients with HBeAg-positive chronic hepatitis (28–30), the *Hin*dIII restriction pattern showed an intense band at the 3.2-kb position with a smear downstream. The

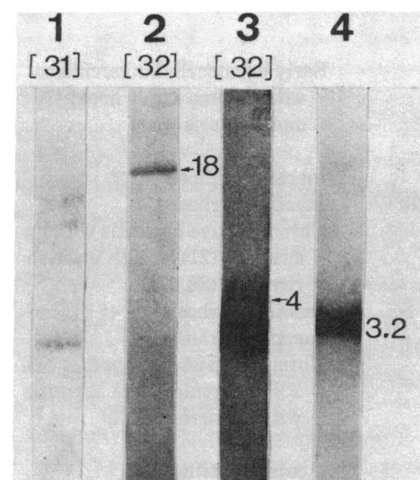

FIG. 3. Southern blot of acute hepatitis liver tissue samples (group III) (the number in the brackets corresponds to the number of the patient in Table 1). Lanes 1 and 2, *Hin*dIII restriction patterns; lane 3, *Eco*RI restriction pattern; lane 4, cloned HBV DNA. For patients 31 and 32, 15 μg and 60 μg of cellular DNA were analyzed, respectively.

*Eco*RI restriction pattern showed that in seven patients (14, 17, 18, 19, 21, 22, and 25) out of ten, an intense band was located at the HBV DNA position. In one case (patient 17), this band was observed in cellular DNA only after a Hirt extraction procedure was used. Representative cases are shown in Fig. 2 *Lower*. In addition, faint bands at higher molecular weight DNA position were also observed, but for some cases these bands were visible only on the original autoradiographs. In patient 15 (Fig. 2 *Lower*, lane 2), an intense band at the 0.8-kb position and a faint band at the 3.2-kb position were observed. In patient 28, a single band at the 4-kb position was observed (Fig. 2 *Lower*, lane 8).

Acute and Fulminant Hepatitis. Two patients were analyzed. Patient 31 had an HBsAg-negative acute hepatitis with anti-HBs and anti-HBc present in his serum (Table 1). Patient 32 had an HBsAg-positive fulminant hepatitis. For these patients, the *Hin*dIII patterns showed bands corresponding to the high molecular weight DNA fragments (Fig. 3). Additionally, in patient 31, a band was also observed at the 3.2-kb position. In patient 32, hybridization of uncut cellular DNA occurred only in the very high molecular weight DNA. For patient 32, the *Eco*RI restriction pattern showed an intense band at the 3.2-kb position with a weak band at the 4.0-kb position (Fig. 3, lane 3).

None of the 10 patients without liver disease (normal controls) had serological evidence for HBV infection and no hybridization was observed in the *Eco*RI restriction patterns.

DISCUSSION

DNA extracted from liver tissue can be employed to determine the state of HBV DNA by hybridization using radioactive cloned HBV DNA as a probe. Free monomeric viral DNA would appear at the HBV DNA position in either uncut DNA or *Hin*dIII restriction fragment patterns. In contrast, HBV DNA sequences covalently integrated in the host genome or oligomerized viral genomes would be revealed by the presence of bands corresponding to DNA fragments larger than 3.2 kb. Distinction between these two possibilities can be achieved by the analysis of uncut cellular DNA. For free oligomers, the uncut cellular DNA pattern and the *Hin*dIII restriction pattern would be the same, whereas for integrated fragments the uncut cellular DNA pattern would show hybridization to a very high molecular weight DNA without bands. In addition, the use of *Eco*RI restriction endonuclease can also provide some information on the arrangement of HBV DNA sequences.

In patients with hepatocellular carcinoma, the viral DNA is integrated into the cellular DNA, in both the tumorous and the nontumorous tissue. This is demonstrated by hybridization of high molecular weight DNA after *Hin*dIII and *Eco*RI digestion. In patients with HBeAg-negative chronic hepatitis, when enough cellular DNA was available, the presence of integrated HBV DNA was demonstrated. In certain patients, the presence of free oligomers was considered unlikely because uncut cellular DNA showed hybridization only in very high molecular weight DNA. This was confirmed by the Hirt technique in one patient. In other patients, when these controls could not be made, the hybridization of the high molecular weight DNA after *Hin*dIII digestion suggested HBV DNA integration, but the presence of free oligomers could not be excluded. Similar findings were also observed in the two patients with acute hepatitis. The presence of a band at the 3.2-kb position after digestion with *Eco*RI in a majority of patients from the three histological groups suggested the existence of several complete HBV sequences in tandem with a head-to-tail orientation.

In four patients with HBeAg in the serum, an intense smear with an upper limit at the 3.2-kb position was observed after digestion with *Hin*dIII. This demonstrated the existence of a large quantity of free HBV DNA in the liver. The smear may be explained by viral multiplication and molecular heterogeneity due to the single-stranded region of variable length. In patient 13, with HBeAg in the serum and with early hepatocellular carcinoma, hybridization was also observed in a high molecular weight DNA. However, free viral DNA in very large amounts may get trapped in the host DNA during electrophoresis, causing a dark smear throughout the track. In this case, the presence of a small amount of integrated DNA may be difficult to discern. In the three patients with HBeAg-positive chronic hepatitis, no hybridization occurred in high molecular weight DNA, but the amount of cellular DNA obtained by needle biopsy was low ($\approx 15\ \mu g$).

Concerning hepatocellular carcinomas, our findings with these 12 patients confirm previous observations that a hepatoma cell line (PLC/PRF/5) and tumorous tissues contain integrated HBV DNA sequences (5–8). This is additional evidence in support of HBV as a causative agent of hepatocellular carcinoma. We had in three cases the opportunity to compare the restriction patterns in the tumorous and nontumorous cirrhotic parts of the liver. In the two patients (nos. 1 and 5) with advanced hepatocellular carcinomas, the integration was observed in tumorous and nontumorous tissues and the two restriction patterns were different. This could be, for instance, explained either by additional factors other than HBV involved in tumorogenesis or by a relationship between the position of integration sites and malignant cell transformation. In the HBeAg-positive patient (13) similar patterns in tumorous and nontumorous tissue, with the presence of free HBV DNA, suggest that viral multiplication has occurred at the beginning of the tumor growth. In 10 patients studied by immunofluorescence, integration of HBV DNA was never associated with detection of HBcAg in tumor cells and was associated in four patients with synthesis of HBsAg in only a few scattered cells. In contrast, in the three nontumorous parts of the liver studied, both HBsAg and HBcAg were detected.

Concerning chronic hepatitis, the observation of a few strongly delineated bands suggested the existence of a limited number of integration sites in the host DNA. Because chronic hepatitis is not thought to be a monoclonal disease, presence of a limited number of integration sites suggested the existence of specific sites. This finding would contrast with the random integration pattern generally observed *in vivo* in virus-induced tumors in animals (15) or *in vitro* in cell culture (16). Cloning of the integrated viral sequences would provide further information. The finding of HBV DNA integration in chronic hepatitis is in accord with that of Lutwick *et al.* (17) concerning four patients, but differs from recent observations in chimpanzees with HBeAg-positive chronic hepatitis (18). In contrast with tumorous livers, the integration of HBV DNA in chronic hepatitis was associated with the expression of genes coding for HBsAg and HBcAg as revealed by immunofluorescence.

Integration has implications for antiviral therapy of chronic hepatitis. It is hard to conceive of the elimination of integrated viral DNA by such therapy. However, it may be partially successful in patients with both free and integrated HBV DNA. Demonstration of free HBV DNA in four HBeAg-positive patients is interesting because it supports the association of HBeAg with virus multiplication, as was already suggested by serological studies on infectivity (19). Moreover, interferon and adenine arabinonucleoside reduce the level of HBeAg and DNA polymerase activity in such patients (20). The observation that in some patients adenine arabinonucleoside does not affect the titer of HBsAg in serum could be explained by integrated viral DNA coexisting with free viral DNA (20).

In the four patients with an anti-HBs-positive chronic hepatitis, it is remarkable that the DNA of HBV is present in an integrated form with expression of viral DNA demonstrable by immunofluorescence while HBsAg is undetectable in serum. In one of these patients only anti-HBs is present in the serum. Because a patient with hepatocellular carcinoma also had only anti-HBs in the serum and integrated HBV sequences in the tumor, persistence of integrated sequences and cellular transformation may be unaffected in some patients by humoral immunity against HBsAg. This occurrence of anti-HBs alone in the serum of a few patients with hepatocellular carcinoma has been reported (21). In addition, detection of HBsAg and HBcAg in the liver of patients with chronic hepatitis has been reported, although only anti-HBc (22–24) or even only anti-HBs (9, 25) was detectable in the serum. Additional studies should include, as a control, patients who have recovered from acute hepatitis B. In three patients with chronic hepatitis there were no serological markers of HBV infection, but immunohistological demonstration of HBcAg and HBsAg in the liver was confirmed by the hybridization technique. Antigenic crossreactivity between HBeAg and non-A/non-B antigen has been reported (26). Therefore, the possibility that non-A/non-B agents are genetically related to HBV cannot be excluded. However, the detection of HBsAg and HBcAg in the hepatocyte suggests that HBV is involved.

Although we have studied only two patients with acute hepatitis, the possible integration of HBV DNA suggests that, even in the acute stages of HBV infection, there is integration in the host genome. After *Hin*dIII digestion, in patient 31, a band at the 3.2-kb position was observed without a smear, whereas in patient 32 there was no band at this position. Free viral DNA with the pattern observed in the HBeAg-positive patients was not demonstrated. This could be related to the serological status of these patients, because only anti-HBc and anti-HBs were detectable in patient 31 and because HBsAg titer was decreasing at the time of the sampling in patient 32. Further studies at earlier stages of the viral infection—i.e., HBeAg-positive acute hepatitis—could permit testing the Hirschman hypothesis concerning the role of integration in viral DNA replication (27).

The sensitivity of hybridization is comparable with the immunohistochemical assay in this study. However, hybridization is possibly less variable because differences in the techniques, antibody specificity, and avidity may be a cause of any discrepancies between results from different laboratories. Although more laborious and expensive, for diagnostic purpose this assay has the major merit of uniformity of the cloned probe. Our results demonstrate that the hybridization assay can be performed on a needle biopsy sample. This could be helpful for assessment of patients with chronic hepatitis.

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