

Analysis of the molecular state of HBV-DNA in the liver and serum of patients with chronic hepatitis or primary liver cell carcinoma and the effect of therapy with adenine arabinoside

M J F FOWLER, J MONJARDINO, I V D WELLER, A S F LOK,
AND H C THOMAS

From the Departments of Physiology and Medicine, Royal Free Hospital School of Medicine, London

SUMMARY The pattern of replicative intermediates seen in the liver of HBe antigen and antibody positive patients was determined. During the phase of HBe antigenaemia the 3.2 Kb species of HBV-DNA (complete HBV genome) is present in the liver but not in the serum. When HBe antigen to antibody seroconversion occurs, either spontaneously or during antiviral therapy, the 3.2 Kb and lower molecular weight intermediates disappear from the liver and the 3.2 Kb band appears transiently in the serum. Integrated HBV-DNA was found in one of 15 patients during the period of HBe antigenaemia and in three of seven patients in the HBe antibody positive phase of the chronic infection before detection of primary liver cell carcinoma. Integrated sequences were found in tumour tissue of two patients with primary liver cell carcinoma who were anti-HBc positive but were absent from the tissues of two patients developing primary liver cell carcinoma at a late stage of autoimmune liver disease. These studies suggest that integration of the HBV genome occurs rarely or in only a small proportion of hepatocytes during the early (HBe antigen positive) phase of infection in Caucasians. They also show that not all primary liver cell carcinomas necessarily contain HBV-DNA.

The epidemiological evidence suggesting that hepatitis B virus (HBV) is an aetiological agent for primary liver cell carcinoma is strong.^{1 2} There is a positive correlation between the geographic prevalence of chronic HBV infection and that of primary liver cell carcinoma,^{3 4} multiple studies show an excess incidence of HBs antigen positivity in primary liver cell carcinoma cases compared with normal individuals and patients with other tumours,⁵⁻⁷ and a prospective study in Taiwan has shown that HBs antigen positive individuals are 390 times more likely to develop primary liver cell carcinoma than HBs antigen negative subjects.⁸

Following the cloning of HBV-DNA, molecular hybridisation techniques were used to study the relationship of the viral DNA to the host genome. Initially several groups examined the Alexander primary liver cell carcinoma cell line,⁹⁻¹¹ and subsequently samples of primary liver cell

carcinoma from patients in Africa, Europe, USA, and Asia.^{12 13} The most striking feature of all these subjects was the presence of HBV-DNA integrated into the host genome. In rare cases episomal HBV-DNA was present.¹⁴ These studies were initially interpreted as evidence of direct oncogenic potential of the virus. Subsequently, tissues from chronically infected patients without evidence of malignant disease were studied and a variable prevalence of integration was found.¹⁵ Thus it became apparent that integration of HBV-DNA into the hepatocyte genome preceded development of primary liver cell carcinoma.

In the present study we have examined serum and liver tissue from patients with HBe antigen and antibody positive chronic HBV infection for the presence of, and molecular state of, HBV-DNA sequences. These data provide insight into the sequence of molecular events occurring during conversion from HBe antigenaemia to the phase of anti-HBe production when it occurs spontaneously and during therapy with adenine arabinoside mono-

Address for correspondence: Dr H. C. Thomas, Department of Medicine, Royal Free Hospital, London NW3.

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phosphate, and also on the stage when integrated sequences are detected.

Methods

PATIENTS

Serum samples and percutaneous liver biopsy cores were obtained from 22 patients with chronic HBV related liver disease and four with primary liver cell carcinoma. Clinical and histological details are shown in the Table. Serial serum specimens were collected from three additional patients undergoing therapy with adenine arabinoside monophosphate 10 mg/kg/day for five days, followed by 5 mg/kg/day for a further 23 days.¹⁶

BIOCHEMICAL TESTS AND HBV SEROLOGICAL MARKERS

Biochemical tests were carried out by routine laboratory methods. HBs and HBe antigens and alpha-fetoprotein were detected and quantified by radioimmunoassay (Abbott and Hoerst Laboratories) and DNA polymerase was measured by the method described by Marion *et al.*¹⁷

ANALYSIS OF HBV-DNA SEQUENCES IN LIVER AND SERUM

Extraction of liver DNA and restriction endonuclease digestion

DNA was extracted from liver biopsy specimens and digested with either restriction endonuclease Hind III or EcoRI.¹⁸ Large molecular weight DNA was checked for size by agarose electrophoresis followed by staining with ethidium bromide. Restriction endonuclease digestion was similarly monitored by analysing approximately 1 µg of DNA from the incubation mixture.

Extraction of DNA from serum and dot hybridisation

DNA was extracted from 0.25 ml serum¹⁹ and the final preparation dissolved in 20 µl water. Dot hybridisation of one µl of these preparations was then undertaken.¹⁹

Agarose gel electrophoresis and Southern blotting

Electrophoresis of DNA samples (20–50 µg of liver DNA, 2–8 µl of serum DNA) was through 0.8% agarose gels.¹⁸ Each gel contained a sample of

Table Clinical, histological and serological details and molecular state of HBV-DNA in liver

Patient (no)	HBV serology						Liver HBV-DNA					
	HBsAg	HBeAg	HBeAb	DNAP	HBV-DNA	HBeAb	Free	Integrated	Age	Sex	Origin	Histology
1	+	+	-	+	+	+	+	-	42	M	British	CAH
2	+	+	-	+	+	+	+	-	23	M	British	CPH
3	+	+	-	+	+	+	+	-	30	M	Italian	CAH
4	+	+	-	+	+	+	+	-	37	M	British	CAH
5	+	+	-	+	+	+	+	-	36	M	British	CAH
6	+	+	-	-	+	+	+	+	36	M	Turkish	CPH
7	+	+	-	+	+	+	+	-	32	M	British	CPH
8	+	+	-	+	+	+	+	-	30	M	Dutch	CPH
9	+	+	-	+	+	+	+	-	32	M	Greek	CAH
10	+	+	-	+	+	+	+	-	30	M	British	CAH
11	+	+	-	+	+	+	+	-	29	F	Saudi	CAH
12	+	+	-	+	+	+	+	-	43	M	Malaysian	CAH
13	+	+	-	+	+	+	+	-	35	M	British	CAH
14	+	+	-	+	+	+	+	-	30	M	Indian	CAH/CPH
15	+	+	-	+	+	+	+	-	42	M	Indian	CAH
16	+	-	+	+	+	+	+	-	30	M	British	CAH
17	+	-	+	-	-	+	-	+	23	M	Spain	CAH
18	+	-	+	-	-	+	-	+	55	M	Kuwait	Cirrhosis
19	+	-	+	-	-	+	-	+	50	M	Iranian	Cirrhosis
20	+	-	+	-	-	+	-	-	61	M	British	CPH
21	+	-	+	-	-	+	-	-	27	M	Iranian	CAH
22	+	-	+	-	-	+	-	-	33	M	Australian	CAH
23*	+	+→-	-→+	+→-	+→-	+	NT	NT	33	M	Japanese	Cirrhosis active
24*	+	+→-	-→+	+→-	+→-	+	NT	NT	25	M	British	CAH
25*	+	+→-	-→+	+→-	+→-	+	NT	NT	28	M	British	CAH
26	-	-	+	-	-	+	-	+	63	M	Nigerian	PLC+CALD
27	-	-	+	-	-	+	-	+	58	M	British	PLC+CALD
28	-	-	-	-	-	-	-	-	72	F	British	PLC+PBC
29	-	-	-	-	-	-	-	-	36	F	British	PLC + lupoid CALD

* Treated with ARA-AMP

NT = not tested, DNAP = HBV-DNA polymerase.

bacteriophage DNA digested with Hind III, 20 µg of the human hepatoma cell line (PLC/PRF/5) DNA digested with either Hind III or EcoRI and approximately 2–5 pg of plasmid pBH20-HBV DNA digested with EcoRI.

After electrophoresis, the gels were stained with ethidium bromide, photographed and blotted according to Wahl's modification of the method of Southern.²⁰

Preparation of ³²p-HBV-DNA probes and hybridisation

The ³²p-HBV-DNA probes were prepared by nick translation²¹ of HBV-DNA that had been purified from plasmid pBH20-HBV-DNA after digestion with EcoRI.¹⁸ The probes had specific activities of 2–3 × 10⁸ cpm/µg HBV-DNA.

Hybridisation to Southern blots was carried out in the presence of dextran sulphate²⁰ and autoradiography was with preflashed Fuji Rx film and Fuji Mach II intensifying screen at –70°C²² for varying lengths of time.

Results

HBV RELATED CHRONIC LIVER DISEASE

(a) HBe antigen positive patients (Fig. 1).

All had chronic hepatitis (Table) without evidence of primary hepatocellular carcinoma. A smear of HBV-DNA ranging from 2.8 to 2.0 Kb in size was seen in the sera of all these patients (Fig. 1B). There was no 3.2 Kb band but a discrete band at 2.1 Kb was visible. The mobility of this band was unaltered by digestion with EcoRI.

Free HBV-DNA was found in the liver tissue of all of the HBe antigen positive patients (Fig. 1A). The pattern in these patients was characterised by a prominent 3.2 Kb band and two smears varying from 2.8 to 2.0 Kb and 1.6 to 0.4 Kb. Superimposed on the upper smear was a single discrete band at 2.1 Kb and on the lower smear two less well defined bands at 1.8 and 1.6 Kb. The 2.1 Kb band was also present after EcoRI digestion.

In one HBe antigen positive Turkish patient, HBV-DNA sequences were also shown in the higher

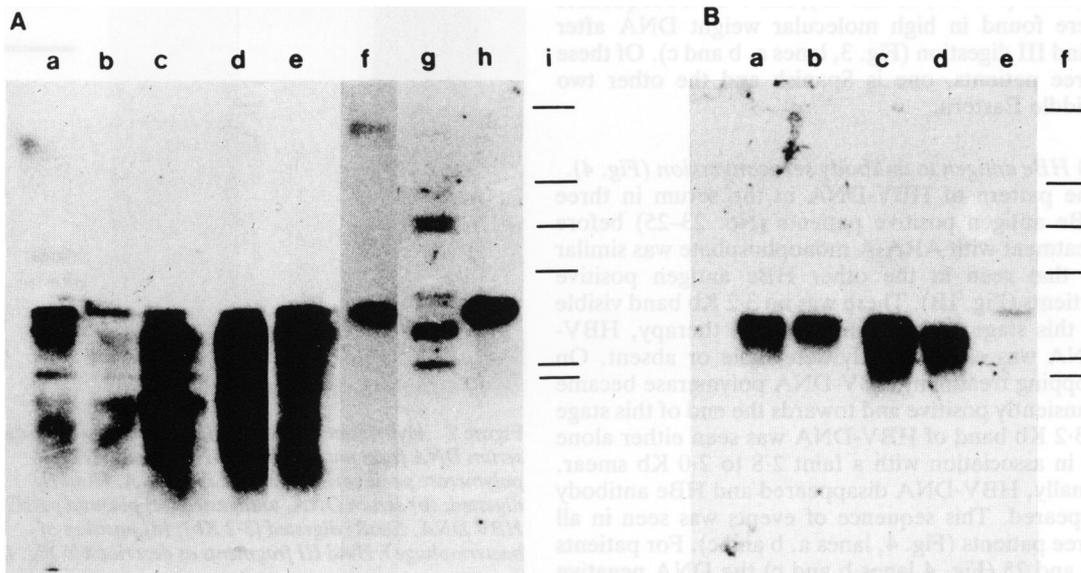


Figure 1 Examples of hybridisation pattern of HBV DNA probes to liver and serum DNA from HBe antigen positive patients with chronic HBV infection.
 (A) Liver DNA: lanes (a) patient 1 EcoRI digested; (b) patient 2, EcoRI digested; (c) patient 3, EcoRI digested; (d) patient 4, Hind III digested; (e) patient 5, Hind III digested; (f) patient 6, Hind III digested; (g) human hepatoma cell line PLC/PRF/5 DNA, EcoRI digested; (h) plasmid pBH20-HBV DNA, EcoRI digested (3.2 Kb); (i) position of bacteriophage λ Hind III fragments (from top: 23.5, 9.7, 6.6, 4.3, 2.2, 2.1 Kb). Liver DNA extracted from patients 7–15 showed similar patterns to those shown above for patients 2–5.
 (B) Serum DNA: lanes (a) patient 2; (b) patient 3; (c) patient 7; (d) patient 5; (e) plasmid pBH20-HBV DNA EcoRI digested (3.2 Kb); (f) positions of bacteriophage λ Hind III fragments as described in Part A of this figure. Serum DNA from patients 1, 4, 6, and 8–15 gave hybridisation patterns similar to those shown above.

molecular weight regions of the gel (>3.2 Kb) (Fig. 1A, lane f).

In this patient (No. 6 in Table) the two low molecular weight smears which are characteristically found in HBe antigen positive patients, were absent in spite of the presence of the 3.2 Kb band. Two faint bands visible in lane a of Figure 1A were artefacts which were not present on further filters prepared from this patient's liver DNA.

(b) HBe antibody positive patients (Figs. 2 and 3)

Seven patients with chronic liver disease, without liver tumours were examined. Six were negative for HBV-DNA in serum. In the remaining patient (No. 16 in Table) who was HBV-DNA polymerase positive, HBV-DNA was present in the serum in the form of a 2.8 to 2.0 Kb smear (Fig. 2, lane b).

Free HBV-DNA was not detected in liver tissue from all six patients who were also HBV-DNA and HBV-DNA polymerase negative in serum. The results for five of these patients (No. 17-21) are shown in Fig. 3 lanes a-e. In patient 16, who was positive for HBV-DNA in serum, free HBV-DNA was also present in the liver (Fig. 2, lane a). In three patients (No. 17, 18 and 19), HBV-DNA sequences were found in high molecular weight DNA after Hind III digestion (Fig. 3, lanes a, b and c). Of these three patients, one is Spanish and the other two Middle Eastern.

(c) HBe antigen to antibody seroconversion (Fig. 4).

The pattern of HBV-DNA in the serum in three HBe antigen positive patients (No. 23-25) before treatment with ARA-A monophosphate was similar to that seen in the other HBe antigen positive patients (Fig. 1B). There was no 3.2 Kb band visible at this stage. After four weeks of therapy, HBV-DNA was either weakly detectable or absent. On stopping treatment HBV-DNA polymerase became transiently positive and towards the end of this stage a 3.2 Kb band of HBV-DNA was seen either alone or in association with a faint 2.8 to 2.0 Kb smear. Finally, HBV-DNA disappeared and HBe antibody appeared. This sequence of events was seen in all three patients (Fig. 4, lanes a, b and c). For patients 24 and 25 (Fig. 4 lanes b and c) the DNA negative tracks post treatment were omitted. In every case HBV-DNA in serum has remained negative in subsequent follow-up.

PRIMARY LIVER CELL CARCINOMA

Two of the four liver cell carcinomas contained HBV-DNA sequences in the high molecular weight DNA (Fig. 5, lanes a and b). In the two that were initially negative, the DNA load on the gel was

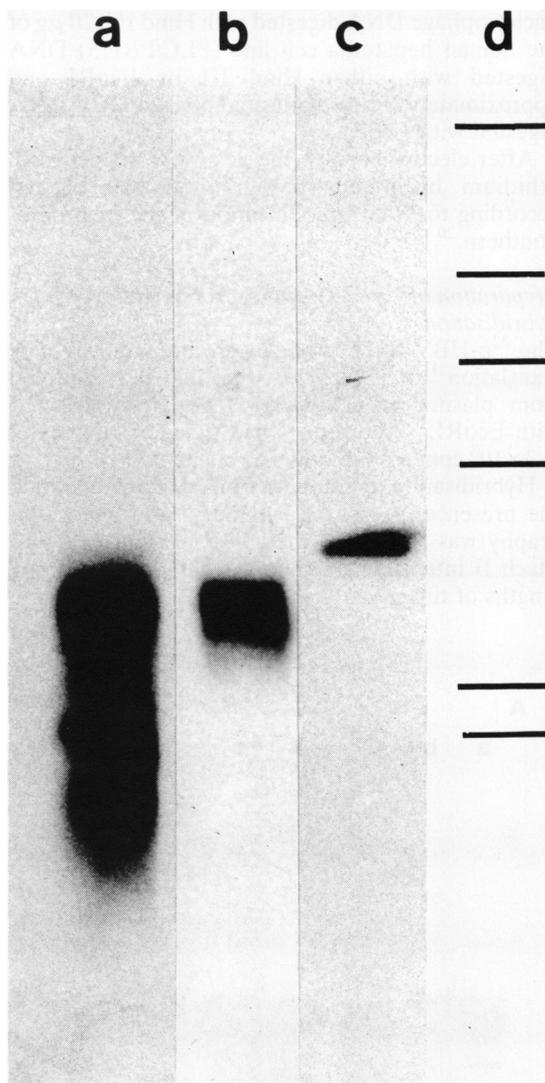


Figure 2 Hybridisation of HBV DNA probe to liver and serum DNA from patient 16 (anti-HBe positive, DNA polymerase positive). Lanes (a) Liver DNA, Hind III digested; (b) serum DNA, undigested; (c) plasmid pBH20-HBV DNA, EcoRI digested (3.2 Kb); (d) position of bacteriophage λ Hind III fragments as described in Fig. 1.

increased from 20 to 50 μ g, but these remained negative for HBV-DNA sequences.

Discussion

In the patients with HBe antigen positive chronic HBV infection, the pattern of viral DNA in the liver

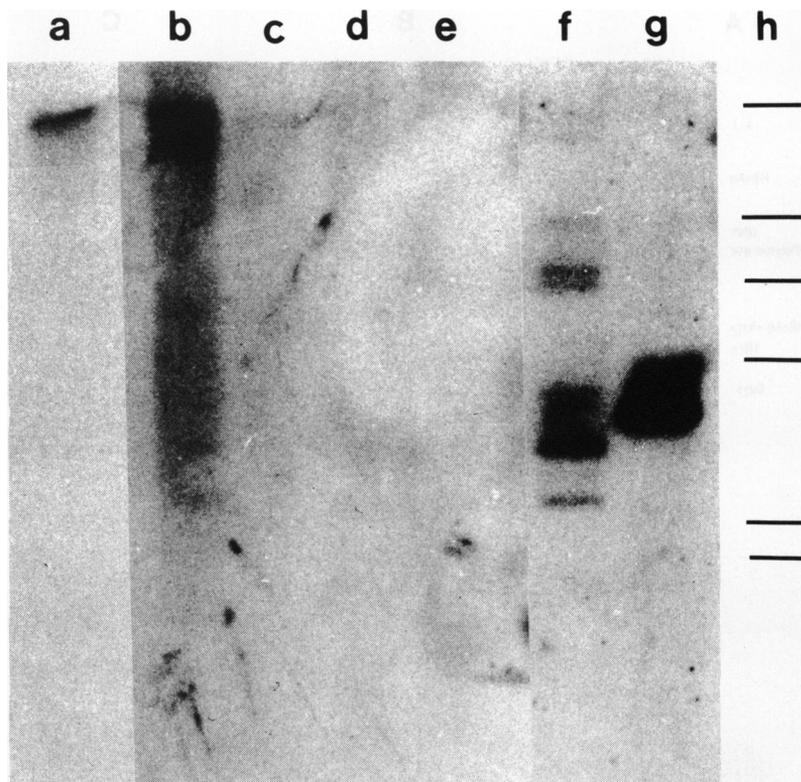


Figure 3 Hybridisation of HBV-DNA probe to liver DNA from HBe antibody positive patients. (a) Patient 17, Hind III digested; (b) patient 18, Hind III digested; (c) patient 19, Hind III digested; (d) patient 20, Hind III digested; (e) patient 21, Hind III digested; (f) PLC/PRF/5 cell line DNA, EcoRI digested; (g) plasmid pBH20-HBV-DNA EcoRI digested (3.2 Kb); (h) position of bacteriophage λ Hind III fragments as described in Fig. 1. Liver DNA from patient 22 showed an HBV hybridisation signal (not shown). Sera from patients 17 to 22 were negative for HBV-DNA when analysed by dot hybridisation (not shown) and were therefore not analysed by gel electrophoresis.

tissue was similar to that described in HBe antigen positive chimpanzees.^{23 18} The pattern was that of free DNA molecules as indicated by bands at 3.2 Kb, a short smear from 2.8 to 2.0 Kb and a large smear further down the gel (1.6 Kb and below), and also bands of discrete size below 3.2 Kb superimposed on the two smears. All these species, including the discrete bands, were seen in EcoRI digested as well as undigested and Hind III digested samples and therefore cannot be ascribed to the presence of supercoiled HBV-DNA.²⁴ No bands at 3.4 Kb or 4.1 Kb, previously reported by Shouval *et al*,²⁵ were seen in this study. The 3.2 Kb bands which are either linear or relaxed circular forms of HBV-DNA were not seen in the serum and therefore appear to be replicative intermediates which, at this stage, are only found in the liver. Only one patient of Turkish origin had integrated HBV-DNA sequences. These data are concordant with those published^{13 15} in showing a low prevalence of detectable integrated HBV-DNA sequences in patients examined during the HBe antigenaemic phase of the infection. Indeed, if those with primary liver cell carcinoma are excluded, the number of published cases with integrated HBV-DNA at this

stage of the infection, is very small. In addition, negative findings in HBe antigen positive chimpanzees^{23 18} further emphasise the need for more studies in this area before a definitive conclusion can be reached on whether integration of HBV-DNA sequences occurs in these HBe antigen positive patients.

Seven patients were examined during the latter stage of the disease when they had developed HBe antibody. One such patient was still HBV-DNA and HBV-DNA polymerase positive in serum and had free HBV-DNA present in the liver tissue. The remaining patients did not have detectable quantities of free HBV-DNA in their livers, but in three, integrated sequences were found. These patients were of Arabic, Spanish, or Iranian nationality and had probably been HBV infected since childhood. One patient with integrated sequences had been treated with ARA-A monophosphate and had undergone HBe antigen to antibody seroconversion within the last six months.

Of the 18 British patients, both HBe antigen and antibody positive groups, none had detectable integrated HBV sequences. The majority of these patients were homosexuals aged 20–40 years and

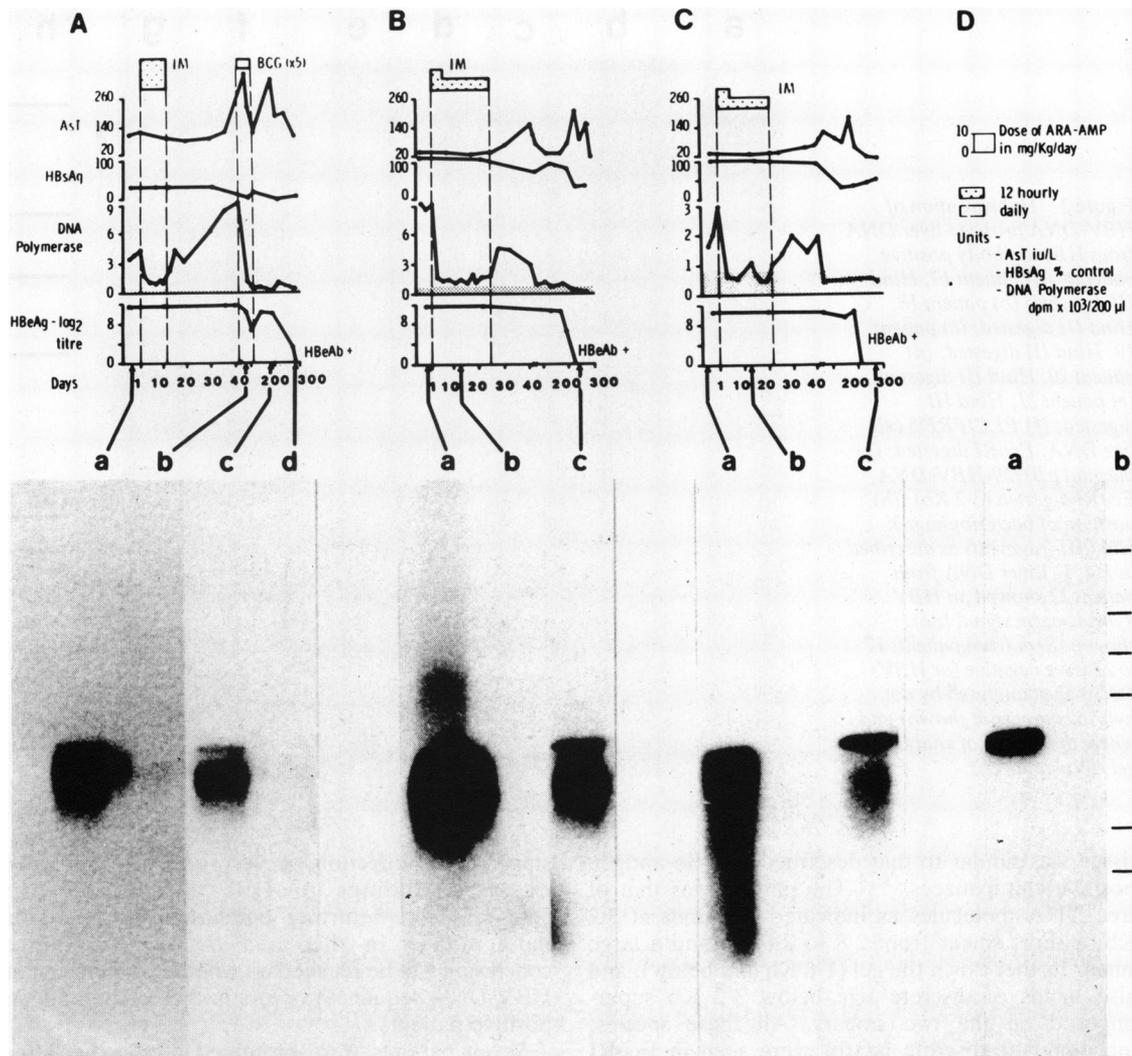


Figure 4 Hybridisation pattern of HBV-DNA probe to serum DNA from patients undergoing HBe antigen to HBe antibody seroconversion. Upper panels: changes in AST, HBsAg, HBV-DNA polymerase and HBe antigen titres during antiviral therapy. Lower panel: hybridisation patterns of HBV-DNA probe to Southern blots of 5 μ l of serum DNA taken at various times and exposed for different lengths of time. (A) Patient 23 treated with ARA-AMP and BCG; (B) patients 24 treated with ARA-AMP; (C) patient 25 treated with ARA-AMP; (D) legends and (a) hybridisation of HBV-DNA probe to plasmid pBH20-HBV-DNA digested with EcoRI (3.2 Kb), (b) positions of bacteriophage λ Hind III digestion products as described in Fig. 1.

had probably been infected as a result of their sexual activities. Thus in this group, in contrast with patients coming from the Mediterranean and Middle East, who were most probably infected in early life, the duration of HBV infection was likely to be less than 10 years. Further studies are indicated to determine whether the apparently higher prevalence of integrated HBV sequences in people of Arab,

Spanish and Iranian origins is related to the duration of the infection or to genetic factors.

During successful therapy with ARA-A monophosphate,¹⁶ three patients developed a 3.2 Kb band as the 2.8 to 2.0 Kb smear molecules were disappearing from the serum and as the transaminases became raised. Finally, after development of HBe antibody and return of transaminases to

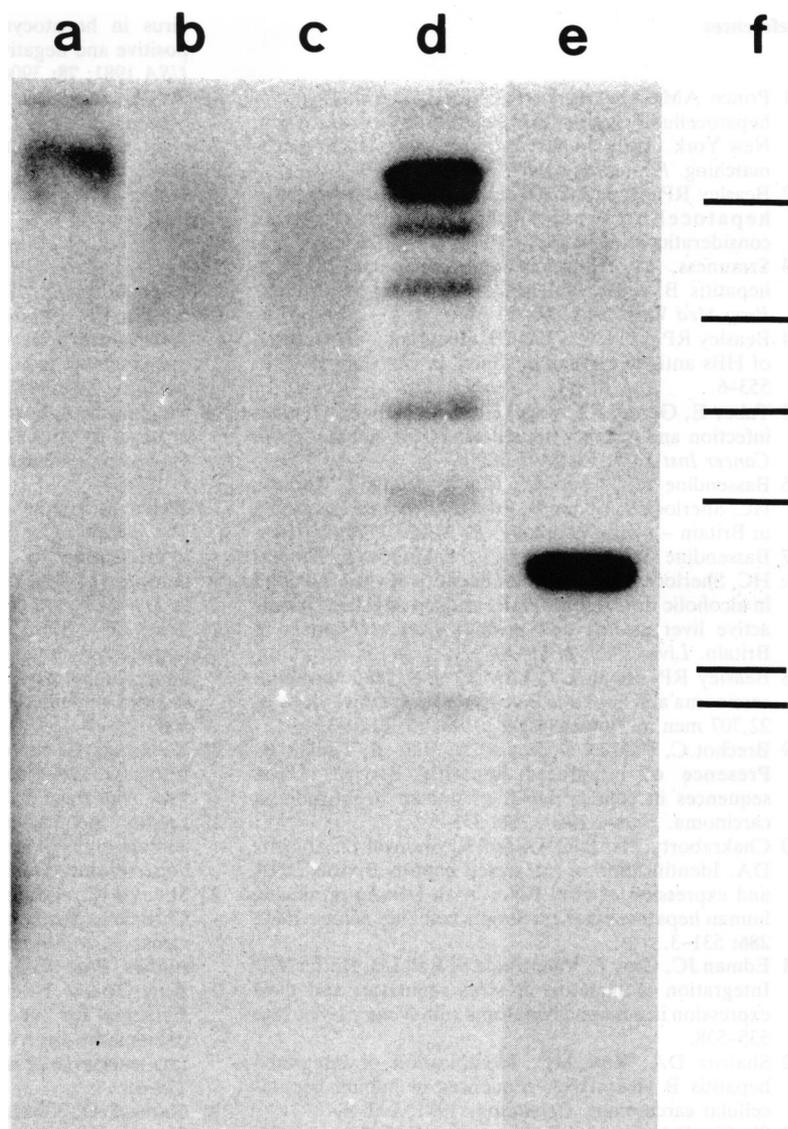


Figure 5 Hybridisation of HBV-DNA probe to tumour DNA from patients with primary liver cell carcinoma. (a) Patient 26, *Hind III* digested; (b) patient 27, *Hind III* digested; (c) patient 27 *EcoRI* digested; (d) PLC/PRF/5 cell line DNA *Hind III* digested; (e) plasmid pBH20-HBV-DNA *EcoRI* digested; (f) positions of bacteriophage λ *Hind III* fragments as described in Fig. 1.

normal, free HBV-DNA disappeared both from the serum and liver. Whether the serum 3.2 Kb band is a replicative intermediate transiently released into the serum during elimination of HBV replicating cells or is a reflection of the liver's ability to produce virus particles with a complete plus strand during low but not high level replication, remains to be determined.

Four patients with primary liver cell carcinoma were studied. All four were HBs antigen negative by radioimmunoassay but two were positive for anti-HBc in the absence of anti-HBs. These two patients had integrated HBV-DNA sequences in their liver

tissue whereas the two without serological markers of HBV infection were negative for HBV-DNA. The latter patients had autoimmune liver diseases (primary biliary cirrhosis and lupoid chronic active hepatitis) and had been treated with prednisolone and azathioprine. In these it seems improbable that HBV had a role and more likely that an immune defect underlay the development of the tumours.²⁶

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