Detection of Hepatitis B Virus-specific DNA in the Genomes of Human Hepatocellular Carcinoma and Liver Cirrhosis Tissues

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SUMMARY

Hepatitis B virus-related DNA was detected in the chromosomal DNA of three out of seven hepatocellular carcinomas and two out of five cirrhosis samples examined, by means of the blot-hybridization technique, described by Southern (1975). The integration patterns were not identical but some similarities raise the question of whether there are some preferred sites of viral integration.

INTRODUCTION

The association between hepatitis B virus (HBV) and human hepatocellular carcinoma (HCC) has been well studied and a causal role for HBV has been strongly suggested (Szmuness et al., 1978). Epidemiological studies have shown a distinct correlation between the incidence and frequency of HCC and the prevalence of HBV; moreover, a significantly higher proportion of these HCC cases contain one or more viral antigenic markers or the corresponding antibodies in their serum, than do normal matched controls (Maupas et al., 1980; Goudeau et al., 1979). It is therefore reasonable to suspect that HBV either by itself or, more likely, in concert with other factors might be involved in malignant transformation of the hepatocyte. We have analysed cellular DNA of several HCC-derived cell lines as well as liver tissue samples in order to study the frequency with which HBV sequences can be detected in HCC, and furthermore, whether they are integrated into the host genome or present in a free state. Two of the six cell lines studied had HBV sequences integrated into their DNA as shown by hybridization with radioactively labelled cloned HBV DNA, using the method of Southern (1975). Three out of seven HCC tissue samples contained HBV sequences in their DNA. Two out of five cirrhosis tissues also contained integrated HBV sequences.

METHODS

Liver tissues were obtained following surgical ablations or at autopsy, and frozen immediately and stored at −70 °C until used. Most of the samples were received blind and pathology reports were available only after the experimental results had been obtained. In all, samples from 13 patients were obtained. Serologically, 11 of them were HBV-infected, and 8 were HBsAg-positive. One patient was serologically negative and for another one, G3, no data were available. Seven of the samples were HCC, five were cirrhosis and one was an angiosarcoma (details in Table 1). In addition, six cell lines derived from HCC tissues were studied. They were PLC/PRF/5, Mahlavu (Macnab et al., 1976), Hep 3B (Aden et al., 1979), BEL 7402, BEL 7404 and BEL 7405 (Ruiming et al., 1980) (Table 2). All the lines have been well-characterized as HCC-derived by demonstration in them of alpha-feto...
Table 1. **Summary of liver tissues used in hybridization experiments and the results**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Origin</th>
<th>Diagnosis</th>
<th>Serum HBsAg</th>
<th>Unintegrated HBV sequences</th>
<th>Integrated HBV sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2</td>
<td>Senegal</td>
<td>HCC</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D9</td>
<td>Senegal</td>
<td>HCC</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D10</td>
<td>Senegal</td>
<td>HCC</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D12</td>
<td>Senegal</td>
<td>HCC</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D21</td>
<td>Senegal</td>
<td>HCC</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G3</td>
<td>F.R.G.</td>
<td>HCC</td>
<td>NT*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G5</td>
<td>F.R.G.</td>
<td>HCC</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F1</td>
<td>France</td>
<td>Cirrhosis</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F2</td>
<td>France</td>
<td>Cirrhosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G1</td>
<td>F.R.G.</td>
<td>Cirrhosis</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>G2</td>
<td>F.R.G.</td>
<td>Cirrhosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G4</td>
<td>F.R.G.</td>
<td>Cirrhosis</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D18</td>
<td>Senegal</td>
<td>Angiosarcoma/HCC?</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*NT, Not tested.

Table 2. **Summary of HCC-derived cell lines used in hybridization experiments and the results obtained**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>HBsAg</th>
<th>Integrated HBV sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC/PRF/5</td>
<td>S. Africa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mahlavu</td>
<td>S. Africa</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hep 3B</td>
<td>U.S.A.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BEL 7402</td>
<td>China</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BEL 7404</td>
<td>China</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BEL 7405</td>
<td>China</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

and lactic dehydrogenase isoenzyme. PLC/PRF/5 and Hep 3B are lines producing HBsAg whereas the others are not. BEL 7405 was originally reported as being HBsAg-positive but it is no longer so, as tested by radioimmunoassay using the Ausria Kit (Abbott Laboratories). PLC/PRF/5 (Marion et al., 1980; Chakraborty et al., 1980; Brechot et al., 1980; Edman et al., 1980) served as a positive control. High mol. wt. DNA was extracted from tissues and cultured cells as follows. Tissues were finely minced with a pair of scissors and homogenized in a Sorvall Omnimix homogenizer with 3 vol. 10 mM-EDTA and 50 mM-tris pH 9. Cultured cells were harvested by trypsin treatment and collected by low-speed centrifugation. Cells or tissue homogenates were lysed with 1% SDS for 20 min at 60 °C. The lysates were repeatedly extracted with PCI-9 (phenol, chloroform and isoamyl alcohol, 5:5:1, pH 9). High mol. wt. DNA was recovered by precipitation with 2 vol. ethanol. The DNA was dried, dissolved in 50 mM-tris pH 9 and 0.5% SDS and treated with 300 μg/ml proteinase K at 37 °C for 4 h, followed by a further round of extraction with PCI-9 and precipitation in ethanol. The purified DNA was dissolved in water and stored at 4 °C. Digestion of DNA with restriction endonucleases was done using 2-5 units of enzyme/μg DNA under conditions provided by the manufacturer (HindIII, Boehringer, Mannheim; HhaI, Biolabs). A 30 to 40 μg amount of DNA was used in each case. Electrophoresis was carried out in 0.8% horizontal agarose slab gels at 100 V and 80 mA. The electrophoresis buffer contained 40 mM-tris pH 8, 18 mM-NaCl, 20 mM-sodium acetate and 2 mM-EDTA. DNA was transferred from the gels to nitrocellulose filters by a modification (Koshy et al., 1980) of the Southern technique. Filters were hybridized with cloned HBV DNA (pAO1-HBV) (Cummings et al., 1980), labelled with α-[32P]dATP (New England Nuclear, 400 Ci/mmol) to a specific activity
of $10^8$ ct/min/µg, by the nick translation procedure of Rigby et al. (1977). Reactions contained 50 mM-tris–HCl pH 7.5, 5 mM-MgCl$_2$, 10 mM-2-mercaptoethanol, 50 µg/ml bovine serum albumin, 1.5 mM each of three non-labelled dNTPs and 5 µM of the fourth dNTP which was α-$^{32}$P-labelled (400 to 600 Ci/mmol), 1 µg DNA and 100 units DNA polymerase I. Hybridization reactions were done in the presence of 10% dextran sulphate as described by Wahl et al. (1979) and also contained 50% formamide, 5 × SSC, 5 × Denhardt’s reagent, 20 mM-sodium phosphate pH 6.5, 100 µg/ml sonicated, denatured salmon sperm DNA and 100 ng labelled probe per filter. Incubations were at 37 °C for 24 h. Filters were extensively washed in 1 × SSC and 0.5 % SDS at 60 °C until no significant radioactivity was detectable in the wash. The filters were then dried and exposed to Kodak X-Omat films in the presence of DuPont Quanta III intensifying screens. Fragment sizes are given in kilobase pairs (kb), and were calculated using HindIII-digested bacteriophage lambda DNA as molecular size markers.

The purified cellular DNA samples were undegraded as seen by ethidium bromide staining of gels following electrophoresis of undigested DNA; the majority of the DNA in each sample remained very close to the origin of the gel lanes. Completion of restriction enzyme digestions was ensured by using two- to threefold excesses of enzyme and prolonged incubations. Ethidium bromide staining of gels after electrophoresis showed homogeneous smears characteristic of complete digestion. Furthermore, completeness of digestions is evident from the relative intensities of the bands after hybridization. The sensitivity of the experiments was high enough to detect at least one HBV copy/cell, assuming the same site of integration in each cell; this was determined by reconstitution experiments using a series of HBV genome equivalences mixed with normal cellular DNA. Each figure represents a composite of different experiments. Each gel had its own mol. wt. markers from which the sizes of hybridized fragments were calculated. Autoradiograms were aligned according to calculated sizes of the bands. The specific activities of the probes varied from $5 \times 10^7$ to $2 \times 10^8$ ct/min/µg and autoradiograms were sometimes over-exposed in order to see minor bands; therefore, direct comparisons of band intensities cannot be made for estimating copy numbers. For the same reason, the background was unproportionately high as compared to the heavy bands.

RESULTS

In a first series of experiments unrestricted cellular DNA samples were tested as described for hybridization with an HBV-specific DNA probe. By this procedure the bulk of cellular DNA is clearly separated from free virus DNA. The results are listed in Table 1. Three HCC samples (D2, D9, G5), two cirrhosis samples (F1, G1) and one sample, an HBsAg-positive angiosarcoma from liver (D18), gave positive signals within the region of the bulk DNA, i.e. very close to the origin of the gel lanes, thus strongly indicating integration of HBV DNA into cellular DNA. Samples G1 and G5 gave, in addition, positive signals in a region corresponding to approx. 3 to 4 kb, showing the presence of free virus DNA (3-2 kb). No signals were seen in positions corresponding to multiples of 3-2 kb, thus ruling out the possibility of low multimeric HBV DNA. For further and more detailed evidence for integration, the samples were reexamined following digestion with restriction endonucleases HindIII and HhaI respectively. For comparison, DNA from some hepatoma cell lines were included in this study. In a second set of experiments, samples were studied after digestion with HindIII which does not cleave any HBV genome studied so far (Sninsky et al., 1979: Marion et al., 1980; Chakraborty et al., 1980; Brechot et al., 1980; Edman et al., 1980). The DNA of PLC/PRF/5 generated six HBV-specific bands of 25-1, 22-1, 17-4, 12-4, 6-3 and 4-4 kb (Fig. 1 a). A number of previous reports (Marion et al., 1980; Chakraborty et al., 1980; Brechot et al., 1980; Edman et al., 1980) of integrated HBV sequences in this cell line
Fig. 1. HindIII restriction endonuclease analyses of DNA from hepatocellular carcinoma-derived cell lines and tumour tissues. DNA extractions, enzyme digestions and hybridization reactions were performed as described in Methods. Fragment sizes are given in kilobase pairs (kb). (a) PLC-PRF/5 DNA; (b) Hep 3B DNA; (c) D9 HCC tumour DNA; (d) D2 HCC tumour DNA.

are at variance with each other with respect to number and position of the bands. Our measurements are in concurrence with those of Brechot et al. (1980). However, the differences reported may mean that there are clonal differences in each case. In agreement with the other studies, no band of 3.2 kb, indicative of free monomeric HBV DNA, was seen. The DNA of the cell line Hep 3B revealed two bands of 24.2 and 12.1 kb (Fig. 1b). The latter band corresponds to a similar band in the PLC/PRF/5 cell DNA. Again, there was no band corresponding to free monomeric virus DNA. The remaining cell lines tested, namely Mahlavu, BEL 7402, BEL 7404 and BEL 7405, were all negative for HBV sequences. DNA from HCC sample D2 produced two bands of 12.3 and 5.9 kb (Fig. 1d). The 12.3 kb band corresponds to similar bands in both PLC/PRF/5 and Hep 3B DNAs. When DNA from both tumour and normal (peritumoural) tissues of HCC case D9 were studied, two bands were seen with tumour DNA of 8.5 and 6.5 kb (Fig. 1c). In this case also, the 6.5 kb band corresponds to a similar band in PLC/PRF/5 DNA. No bands could be observed in the case of the normal (peritumoural) tissue DNA. DNA from liver tissue G1 (cirrhosis), and G5 (HCC), produced smears which were concentrated at 3.2 kb (not shown), indicating the presence of free virus DNA. Any other band that might have been present in the vicinity of the smear would have been obscured by the background. The remaining two positive samples (Table 1) did not show any bands after HindIII cleavage of their DNA. Failure to detect hybridization in this assay does not necessarily mean absence of specific sequences. It could mean that there is heterogeneity in the cell sample studied with respect to integration sites. In such cases, digestion with an enzyme that cuts the virus genome more than once would concentrate pure virus fragments from different integration sites into one or more bands depending on the number of cuts. For this reason, in a third series of experiments, the restriction enzyme HhaI, which recognizes three cleavage sites in the DNA of HBV with the complex serotype adyw (Pasek et al., 1979), was used to test the DNA samples which were
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Fig. 2. Restriction enzyme HhaI analyses of DNA from HCC-derived cell lines and liver tissues. DNA extractions, enzyme digestions, gel electrophoresis, hybridization and autoradiography were done as described in Methods. The sizes of the fragments are given in kb. (a) PLC/PRF/5 DNA; (b) D18 peritumour DNA; (c) F1 cirrhosis liver DNA; (d) G1 cirrhotic liver DNA; (e) G5 HCC DNA.

negative with HindIII or showed an excess of free virus DNA. DNA of cell line PLC/PRF/5 as a positive control, showed a number of bands from 1.3 to 7.2 kb (Fig. 2a). The greater intensities of the bands of 1.6 kb and 1.7 kb, relative to the others, suggest that they are internal, virus fragments, i.e. without flanking host sequences. DNA from cirrhosis tissue G1, which showed a smear of free virus DNA, after digestion with HhaI produced five bands of 7.0, 2.1, 1.7, 1.1 and 0.7 kb (Fig. 2d); similarly, the DNA of HCC sample G5, which also showed a smear of free virus DNA, produced five bands at 4.3, 2.1, 1.7, 1.1 and 0.7 kb (Fig. 2e). The 2.1 kb band is very faint probably because there is only a small amount of virus DNA in the fragment. These data, in combination with the data on undigested DNA, indicate integration of virus sequences into the cellular DNA. In G1 (Fig. 2d), the four very intense bands (2.0, 1.5, 1.1 and 0.9 kb) which sum up to more than the equivalent of the unit length HBV genome (i.e. 3.2 kb) are probably derived from two different molecular populations, i.e. free HBV DNA and integrated HBV-specific sequences. DNA of cirrhosis case F1 after digestion with HindIII did not produce any band; however, HhaI cleavage resulted in a single band of 1.28 kb (Fig. 2c). As mentioned above, there are three cleavage sites on HBV DNA for HhaI (Pasek et al., 1979). This would mean that unless one or other of these sites is lost, cleavage with HhaI would create two purely virus fragments. Since only a single band is observed in F1, perhaps only a part of the virus sequence is present. Furthermore, integration may be heterogeneous within the cell population. D18 was a case of angiosarcoma of a serologically HBV-positive liver from which DNA of both tumour tissue as well as peritumoural tissues showed no positive band in the HindIII pattern. HhaI digestion of the tumour DNA also failed to reveal any band; however, digestion of the peritumoural tissue
DNA generated two bands of 1.4 and 0.8 kb (Fig. 2b). As in F1, this result also might suggest integration of a part of the virus genome in different sites. The presence of HBV DNA in peripheral tissue but not in the tumour itself is not understood at present. However, as the tumour (angiosarcoma) was of mesenchymal origin, the aetiology may be altogether unrelated to HBV which has a tropism for hepatocytes.

DISCUSSION

The presence of free HBV sequences has previously been reported, in some liver tumours (Summers et al., 1978; London, 1978). Recently Brechot et al. (1980) showed integrated HBV sequences in one liver carcinoma. We have observed HBV sequences in the cellular DNA of three out of seven cases of HCC and two out of five cases of cirrhosis examined. Integrated sequences were also detected in one case of HBV-related liver disease where the diagnosis was not clear on the part of the liver in question, which was somewhat necrotic. An angiosarcoma was present in another area in which HBV sequences were not detected. Two out of six cell lines studies also contained HBV sequences. The complement of virus DNA in the different positive samples will be determined in future experiments. We cannot rule out the possibility in the negative cases of the presence of minor amounts of the virus DNA in a small proportion of the cells. As mentioned earlier, the sensitivity of our experiments was high enough to detect at least one virus genome/cell, providing that the integration is at the same site in each cell. The data suggest copy numbers from less than one/cell to at least six. Some of the integration sites may be preferred (25.2 kb, 12.4 kb and 6.3 kb), although the similarity of the fragment sizes alone does not prove it.

The presence of HBV DNA in cellular DNA of cirrhosis tissue is reported here for the first time. Cirrhosis often progresses to malignancy and indeed if cirrhotic liver is carefully examined microscopically, in many cases, malignant cells can be identified although carcinoma is not clinically evident (R. Müller, unpublished data). The question of when insertion of virus information takes place cannot be answered until pre-HCC events such as acute and chronic hepatitis and cirrhosis are better studied. The distribution of integrated HBV sequences in cirrhotic tissues suggests to us that the event of integration may be systemic, occurring in different cells at different sites.

A further point of interest is the observation (see Table 1) that integrated sequences are only found in HBsAg-positive cases. If such sequences are present, they are expressed at least in part.

The data provide additional evidence for a possible role of HBV in human hepatocellular carcinoma. In known tumour virus systems (Tooze, 1980), proviral integration appears to be a prerequisite step for malignant transformation. The results show that the integration of HBV-specific DNA sequences into the liver cells may not only occur as late and rare events as in the case of primary liver carcinomas, but also rather early and systemically as in another HBV-related liver disease, namely cirrhosis. As has been shown in various other virus–host systems (Gallimore et al., 1974; Svoboda et al., 1977), integration of only a part of the virus genome can take place. Apparently, malignant transformation can result from transfection of specific subgenomic fragments of virus DNA or cDNA (Canaani et al., 1979; Anderson et al., 1979; Copeland et al., 1980; Graham et al., 1974). It is not yet known what the role, if any, of HBV DNA is in the cell. There is no evidence to indicate that HBV DNA codes for any gene product that is related to the initiation and/or maintenance of malignant transformation, such as the src gene product in retroviruses or the T-antigen in SV40 (Tooze, 1980). It may be that HBV DNA has no such function but rather causes alteration of cellular gene expression which may in some cases lead to tumourigenesis. A detailed analysis of integrated HBV sequences and flanking host DNA is in progress.
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The results presented here were reported in a preliminary form at the International Symposium on Hepatitis B vaccine, in Paris, December 1980.

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REFERENCES


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