Defective Hepatitis B Virus DNA Molecules Detected in a Stable Integration Pattern in a Hepatoma Cell Line, and in Induced Tumours and Derived Cell Lines

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SUMMARY

Hepatitis B virus (HBV) DNA was found to be integrated into seven sites in the DNA of the PLC/PRF/5 hepatoma cell line as determined by digestion with the restriction endonuclease HindIII which does not cut through the viral genome. The integration pattern was stable in the cell line, in tumours induced in athymic mice by this line and in cell lines derived from such tumours. Syntheses of hepatitis B surface antigen and alphafoetoprotein were maintained in the induced tumours and derived cell lines. A defective HBV DNA molecule (approx. 2.8 kilobase pairs) appears to be integrated in a head-to-tail tandem arrangement and it is proposed that such defective molecules may be involved in the process of neoplastic transformation by HBV.

The human hepatocellular carcinoma cell line PLC/PRF/5 synthesizes and secretes 20 nm hepatitis B virus (HBV) surface antigen (HBsAg) particles of identical composition to those found in the serum of carriers (Monjardino & Crawford, 1979; Skelly et al., 1979), but not the other major virus gene products (core antigen and DNA polymerase). HBV DNA is present in this cell line integrated into the host cell DNA at a number of sites but free viral genomes have not been detected (Brechot et al., 1980; Chakraborty et al., 1980; Edman et al., 1980; Marion et al., 1980). As well as HBsAg, the hepatocellular carcinoma cell line synthesizes and secretes several serum proteins which can be detected in concentrated cell culture supernatants (Tong & Prince, 1978; Knowles et al., 1980). Alphafoetoprotein (AFP), initially undetectable (Alexander et al., 1976; Tong & Prince, 1978; Knowles et al., 1980), was later found in the cytoplasm of these cells by immunofluorescence staining (Gerber et al., 1981), and in the serum of tumour-bearing nude (athymic) mice after heterotransplantation of the cell line (Bassendine et al., 1980).

The aim of the present study was to evaluate the state and arrangement of HBV DNA sequences in the PLC/PRF/5 cell line, in tumours produced in nude mice inoculated with the cell line, and in cell lines derived from such tumours. Serum from tumour-bearing mice and concentrated cell culture supernatants from both the original cell line and the tumour-derived cell lines were also analysed for the presence of HBsAg and AFP.

The cell line PLC/PRF/5 was grown in minimal essential medium (MEM) (Earle’s salts) and 10% foetal calf serum as previously described (Monjardino & Crawford, 1979). Transplantation of cells into male mice (1 × 10⁷ to 2 × 10⁷ cells/mouse) was carried out as reported by Bassendine et al. (1980). Tumours were detectable after about 2 weeks, and reached 2 to 2.5 cm diam. by 3 to 4 weeks. At this stage, and after decapsulation, the tumour tissue was divided into two portions and used both for DNA extraction and for starting a cell culture. This was achieved by collagenase (0.1%) digestion in phosphate-buffered saline (PBS 'A') at room temperature followed by resuspension of the pelleted cells in fresh MEM.

High molecular weight DNA was extracted from tumour specimens and from tissue culture cells as described previously (Monjardino et al., 1982). DNA (20 μg), either undigested or digested with the restriction endonucleases HindIII or EcoRI, was fractionated through 0.8%
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Fig. 1. Southern blot analysis of DNA from hepatoma NT3, PLC/PRF/5 cell line and NT3-derived cell line, with $^{32}$P-labelled HBV DNA probe. (a) DNA size markers (kb); (b) pBH20-HBV DNA (EcoRI); (c) PLC/PRF/5 (HindIII); (d) PLC/PRF/5 (EcoRI); (e) tumour NT3 (undigested); (f) tumour NT3 (EcoRI); (g) tumour NT3 + PLC/PRF/5 (HindIII); (h) cell line NT3 P2 (EcoRI); (i) NT3 P2 (HindIII); (j) NT3 P2 + PLC/PRF/5 (HindIII); (k) NT3 P4 (HindIII). Variations in intensity of autoradiographic bands reflect differences in DNA loading (10 to 20 μg).

HBV DNA, purified from plasmid pBH20-HBV by EcoRI digestion and agarose gel electrophoresis (Monjardino et al., 1982), was labelled with $^{32}$P to high specific activity (2 × 10⁸ to 3 × 10⁹ ct/min/μg DNA) by nick translation (Weinstock et al., 1978). Hybridization in the presence of dextran sulphate was performed as described by Wahl et al. (1979) and the hybrids were detected by autoradiography using pre-flashed Fuji RX film and intensifying screens at −70 °C (Laskey & Mills, 1977).

HBsAg and AFP in 100-fold concentrated cell supernatants were detected using commercially available radioimmunoassay diagnostic kits (HBsAg-Ausria II, Abbott Laboratories; AFP-RIAgnost AFP, Hoechst).

Indirect immunofluorescence (Vogt, 1969) of AFP was detected using a rabbit anti-AFP first layer (Hoechst) followed by a goat anti-rabbit fluorescein isothiocyanate conjugate second layer (Nordic).

Cell lines were isolated from six tumours designated NT1 to NT6 but only three, NT3, NT5 and NT6, were the object of this study. DNA samples extracted from the tumour NT3 and from two different passages of the NT3-derived cell line (NT3 P2 and NT3 P4) were first analysed after EcoRI and HindIII digestion. The patterns obtained after Southern blotting and hybridization to $^{32}$P-labelled HBV DNA are shown in Fig. 1. Undigested tumour DNA (lane e) shows a strong band of integrated viral DNA associated with DNA of very high molecular weight and no free DNA [3-2 kilobase pairs (kb)]. All HindIII-digested DNA samples show an identical band pattern clearly seen in lanes (c) (PLC/PRF/5) and (k) (NT3 P4). When the original cell line DNA was digested together with DNA from either tumour NT3 (lane g) or tumour-derived cell line NT3 P2 (lane j), the pattern remains unchanged. The pattern after HindIII digestion
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comprises seven bands and was reproduced for every sample analysed. The sizes of these HBV DNA-containing fragments are 29.5, 24.0, 16.2, 11.0, 6.0, 4.2 and 2.1 kb. Lanes (d), (f) and (h) in Fig. 1 show the patterns observed after EcoRI digestion of PLC/PRF/5, NT3 and NT3 P2, respectively. Here again, an identical pattern is seen in every case consisting of eleven distinct bands (24.0, 10.2, 9.8, 7.1, 6.5, 3.4, 3.2, 2.8, 2.5 and 2.2 kb) which includes a very prominent 2.8 kb band. This pattern was again reproduced and more clearly seen when DNA samples from cell lines derived from two additional tumours (NT5 and NT6) were analysed as shown in Fig. 2. Furthermore, in the case of the cell line derived from tumour NT5, DNA was extracted from cells at six different passages and in every case an identical eleven-band pattern was clearly seen (Fig. 2). Two faint extra bands were found after EcoRI digestion (5.6 and 5.0 kb) both in the original cell line in tumours and tumour-derived cell lines but were not clearly visible in every sample analysed.

Having established a stable pattern of integrated viral DNA during tumour formation, we next analysed the expression of the viral surface antigen as well as of alphafetoprotein both in the serum of tumour-bearing mice and in concentrated medium from tumour-derived cultures. In all cases, HBsAg and AFP were detected by radioimmunoassay (results not shown). AFP was detected by immunofluorescent staining in both PLC/PRF/5 and NT3 P2 cells in the region of the Golgi apparatus as seen in Fig. 3. HBsAg was visualized in the form of 20 nm spheres by immunoelectron microscopy (not shown).

In the present study, we confirm that HBV DNA is integrated into the genome of the PLC/PRF/5 cell and that there is no detectable free HBV DNA. The pattern of integration, which we were able to characterize in greater detail than previous workers (Brecho et al., 1980; Chakraborty et al., 1980; Edman et al., 1980; Marion et al., 1980), remains stable after tumour formation and in tumour-derived cell lines. It is composed of seven distinct integration sites as shown after digestion of the cell DNA with a restriction endonuclease (HindIII) that does not cut through the viral genome. Of the seven fragments, one is 2.3 kb long and thus shorter than
the full genome. *EcoRI* digestion of DNA from the cell lines and tumours studied generates a stable pattern of at least eleven fragments. The most prominent fragment is clearly smaller than the full genome (Fig. 1 and 2) and corresponds to a size of 2.8 kb. The good separation obtained in the gels allowed the resolution of an additional fainter 3.2 kb band. Both these species are likely to be generated from the tandem integration of two or more HBV genomes, since *EcoRI* cuts the viral genome at only one site. This 2.8 kb band corresponds to a defective HBV DNA molecule 12 or 13% shorter than the wild-type viral DNA and containing the *EcoRI* site.

Since the enzyme *EcoRI* cuts the viral genome only once, twice as many *EcoRI* fragments as *HindIII* fragments would have been expected. Additionally, for every head-to-tail tandem arrangement, an extra band corresponding to the full size of the DNA involved in this arrangement should also be found. This apparent discrepancy will only be clarified by restriction enzyme mapping of the individual HBV DNA-carrying fragments.

The finding of retained expression of HBsAg and AFP in the serum of tumour-bearing nude mice had already been reported (Bassendine *et al.*, 1980). In this study, we report the synthesis of both these proteins not only in the original cell line but also in the various tumour-derived cell lines. Similar studies carried out in our laboratory with earlier passages of PLC/PRF/5 (unpublished results) had failed to detect AFP in concentrated supernatants from PLC/PRF/5 cultures. Its presence in the later passages analysed in this study probably reflects a gene dosage effect relating to an increase in chromosome number (96 to 106) compared with the modal number of 56 originally described (Alexander *et al.*, 1976 and our unpublished observations).

Our finding of a stable pattern of integration of HBV genomes in head-to-tail tandem arrangements involving predominantly a defective HBV DNA molecule is similar to what other workers have described in polyoma virus-transformed rat cell lines (Chowdhury *et al.*, 1982). Assuming that integration of viral DNA into the host DNA may be involved during replication of the viral DNA, as suggested by Summers & Mason (1982) in the case of a structurally related virus, one could speculate that the integrated defective viral genome may not be excised from the host genome. Failure of this integrated genome to serve as template for the expression of core antigen would enable the cells carrying it to escape the normal immunological clearance which appears to require membrane display of this antigen (London & Blumberg, 1982). During the subsequent inflammatory process of chronic hepatitis, where cell death and cell renewal are continuously taking place, the cells carrying the integrated viral genome will increase in numbers through active division, leading eventually to amplification of the neoplastic clone.
This proposed model for the oncogenic role of hepatitis B virus would essentially differ from the one suggested by London & Blumberg (1982) in that it would depend on the integration of a defective HBV genome rather than on the existence of two distinct populations of liver cells.

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