Cloning and Analysis of Integrated Hepatitis B Virus DNA of the \textit{adr} Subtype Derived from a Human Primary Liver Cell Carcinoma

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\textbf{SUMMARY}

A 10 kb genomic DNA fragment derived from a human primary liver cell carcinoma (PLC) and containing integrated hepatitis B virus (HBV) DNA was cloned and analysed. Physical mapping showed the viral DNA to comprise a linear sequence of at least 2.8 kb (87\%) of the HBV genome and to be of the \textit{adr} subtype. Integration appeared to have occurred in the region of the viral genome spanning the cohesive ends. The cellular flanking DNA sequences to one side of the integrated viral DNA contained repeats of the \textit{Alu} family. The finding of no apparent rearrangements of the integrated HBV DNA sequences in this clone is in contrast to the situation in the huSP and PLC/PRF/5 PLC cell lines in which the integrated viral DNA sequences are greatly rearranged and suggests that such rearrangements may be atypical of solid PLCs.

The epidemiological evidence linking hepatitis B virus (HBV) with the development of primary liver cell carcinoma (PLC) is strong (Beasley, 1982). This association has recently been further strengthened by the finding of HBV DNA sequences integrated into the genomes of many PLCs (Brechot \textit{et al.}, 1981; Shafritz \& Kew, 1981; Shafritz \textit{et al.}, 1981; Chen \textit{et al.}, 1982; Hino \textit{et al.}, 1984; Fowler \textit{et al.}, 1984) and has led to the hypothesis that integration of the viral DNA may be directly involved in the development of PLC (Shafritz, 1982). In order to investigate the possible molecular mechanisms by which integration may lead to the development of the transformed phenotype, several groups have cloned integrated HBV DNA sequences derived from two HBV surface antigen (HBsAg)-positive PLC cell lines (PLC/PRF/5 and huSP) (Dejean \textit{et al.}, 1983; Koshy \textit{et al.}, 1983; Shaul \textit{et al.}, 1984; Mizusawa \textit{et al.}, 1985) and from three solid PLCs (Dejean \textit{et al.}, 1983, 1984; Yaginuma \textit{et al.}, 1984). In the cases of the two cell lines the integrated viral DNAs are greatly rearranged, whereas in the cases of two of the three solid tumours rearrangements of the viral DNA are of a far lesser extent and in the third case no rearrangements are apparent. The aim of the present study was to investigate whether the major rearrangements of integrated HBV DNA as observed in the two cell lines are typical of solid PLCs and in this short communication we report the cloning and physical mapping of an integrated HBV DNA derived from an HBsAg-positive PLC of Japanese origin.

High molecular weight DNA was extracted from the PLC specimen (designated L1) as described by Monjardino \textit{et al.} (1982). Initial analysis for the presence of integrated HBV DNA sequences was by digestion with the restriction endonuclease HindIII followed by Southern blotting and hybridization with a nick-translated \textsuperscript{32}P-labelled HBV DNA probe (Monjardino \textit{et al.}, 1982). Autoradiography gave a hybridization pattern comprising two distinct bands of DNA corresponding to sizes of approximately 10 and 20 kb (Fig. 1a). Since the restriction endonuclease HindIII does not cleave any of the six cloned HBV DNAs that have been

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sequenced (Wain-Hobson & Tiollais, 1984), the presence of hybridizing bands of DNA of sizes considerably greater than the 3.2 kb size of fully double-stranded HBV DNA demonstrated that the viral sequences were integrated into the PLC genome.

In order to characterize further the nature of the integrated viral DNA sequences, a genomic library of L1 PLC DNA was prepared (Maniatis et al., 1982) using the bacteriophage lambda vector L47.1 (Loenen & Brammar, 1980). L1 PLC DNA was digested to completion with HindIII, fractionated on a 10 to 40% sucrose gradient and the 10 to 20 kb fractions were pooled. Vector L47.1 arms were prepared by digestion of the phage DNA with both HindIII and XhoI, followed by purification on a 10 to 40% sucrose gradient. Ligation of a twofold weight excess of vector arms to L1 PLC DNA at a total concentration of 200 μg/ml was for 18 h at 14 °C using 1 unit of bacteriophage T4 DNA ligase (Amersham) and was followed by in vitro packaging using commercially available packaging mixes (Amersham).

In situ screening (Benton & Davis, 1977) of an unamplified library of some 150000 plaques using a 3.2 kb full-length HBV DNA probe revealed one clone carrying HBV DNA sequences. This clone (L1C1) was plaque-purified a further three times and after digestion with HindIII gave a 10 kb HBV hybridizing insert upon Southern blot analysis (Fig. 1b). The similarity in size of this insert to the smaller of the HBV hybridizing restriction fragments present in HindIII-digested L1 genomic DNA (Fig. 1a) suggests that the cloned insert had not been rearranged upon cloning.
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Physical mapping of the L1C1 clone revealed the insert to contain unique sites for the restriction endonucleases XhoI, XbaI, BamHI, and EcoRI, two sites for BglII, and five sites for AvaI (Fig. 2a). No sites in the insert were found for the enzymes SalI, KpnI, SacI, or HpaI. Southern blot analysis using an HBV DNA probe showed that the HBV sequences present in the insert contained the unique restriction sites for XhoI, XbaI, and BamHI together with two sites for BglII and three sites for AvaI (Fig. 2a). By comparison of the restriction sites present in the HBV DNA sequences of L1C1 with those present in six HBV DNA clones of various HBsAg subtypes for which sequences are available (Wain-Hobson & Tiollais, 1984) the integrated HBV DNA appears to be of the HBsAg adr subtype as characterized by Fujiyama et al. (1983). This subtype is predominant in Japan (Yamashita et al., 1975) and is in agreement with the geographical origin of the L1 PLC specimen.

Comparison of the restriction map of the L1C1 insert with that of the HBV adr subtype genome (Fujiyama et al., 1983; Takeshima et al., 1985) (Fig. 2) suggests that whilst small rearrangements below the resolution of the mapping techniques (approximately 200 bp) cannot be excluded, the integrated HBV DNA present in L1C1 does not appear to be rearranged and comprises a linear sequence of at least 2.8 kb (87%) of the HBV genome. Furthermore, whilst the exact locations of the virus-host junctions are uncertain and may extend up to a few hundred bp beyond the minimum extent shown in Fig. 2(a), integration of the viral DNA appears to have occurred in the region of the viral genome spanning the cohesive ends (Fig. 2b).

Hybridization of a plasmid BLUR8 probe (Jelinek et al., 1980) to Southern blots of L1C1 DNA showed sequences of the 

\[ \text{Alu} \] repeat family to be present in the 3.5 kb AvaI fragment located to the extreme right of the L1C1 insert (data not shown). This fragment corresponds to a portion of the human genomic DNA flanking the integrated HBV DNA sequence. Whilst
integration of HBV DNA in the near vicinity of members of this widely dispersed family of repeats has also been shown in the case of the PLC/PRF/5 cell line (Shaul et al., 1984), the biological significance of these findings is uncertain.

The finding in the present study of an integrated HBV DNA species derived from a solid PLC that does not appear to have been rearranged is in contrast to the findings in the cases of the PLC/PRF/5 and huSP lines in which the integrated HBV DNA species are greatly rearranged by deletions, duplications and inversions (Dejean et al., 1983; Koshy et al., 1983; Shaul et al., 1984; Mizusawa et al., 1985). However, as noted above, in the cases of the three clones containing integrated HBV DNA derived from solid PLCs that have been analysed by other workers, one does not show any apparent rearrangement (Yaginuma et al., 1984), one consists of a 1.35 kb linear subgenomic fragment (Dejean et al., 1983) and one comprises a complete linear HBV genome with an additional contiguous 0.8 kb HBV subgenomic fragment (Dejean et al., 1984). Thus, major rearrangements in the form of inversions, deletions and non-contiguous duplications as observed in the cell lines may be atypical of solid PLCs and whilst the observed integration pattern in the PLC/PRF/5 cell line is stable (Monjardino et al., 1983), genetic rearrangements may have occurred in establishing the cell line, resulting in an abnormal karyotype (Alexander et al., 1976) and highly rearranged integrated HBV DNA sequences.

As regards the sites of integration on the viral genome, in the case of the L1C1 clone both virus–host junctions appear to be approximately located in the region of the cohesive ends although it is possible that one junction may be located near the end of the 'single-stranded' region of the viral genome (Fig. 2). In the case of one of the solid PLCs studied previously both virus–host junctions appear to be restricted to around the viral core antigen (HBcAg) gene near the cohesive ends (Dejean et al., 1983). However, any post-integration rearrangements in the form of inversions, deletions and non-contiguous duplications as observed in the cell lines may be atypical of solid PLCs and whilst the observed integration pattern in the PLC/PRF/5 cell line is stable (Monjardino et al., 1983), genetic rearrangements may have occurred in establishing the cell line, resulting in an abnormal karyotype (Alexander et al., 1976) and highly rearranged integrated HBV DNA sequences.

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REFERENCES


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