Integration of Region X of Hepatitis B Virus Genome in Human Primary Hepatocellular Carcinomas Propagated in Nude Mice

By MICHIKO MIYAKI, 1• CHIEKO SATO, 1 TOHRU GOTANDA, 2 TAKANE MATSUI, 3 SHUNJI MISHIRO, 4 MITSUNOBU IMAI 4 AND MAKOTO MAYUMI 4

1 Biochemistry and 2 Hepatitis Division, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyoku, Tokyo 113, 3 Department of Veterinary Pathology, Obihiro University of Agriculture and Veterinary Medicine, Inadacho, Obihiro 080 and 4 Immunology Division, Jichi Medical School, Minamikawachi, Tochigi-Ken, 329-04, Japan

(Accepted 20 March 1986)

SUMMARY

Tissues of human primary hepatocellular carcinoma (PHC) from six patients infected with hepatitis B virus (HBV) were propagated in nude mice, as well as a strain of hepatitis B surface antigen-positive PHC (PLC/PRF/5). Integration of viral DNA into chromosomal DNA of tumour cells was evaluated by the capacity to hybridize with radiolabelled DNA probes, each representing fundamental parts of the HBV genome, that is S and C genes and regions pre-S and X. All PHC cells possessed region X integrated in their chromosomes. However, integration of the S gene, C gene and region pre-S was found in only six of the seven PHCs. Based on these findings, the integration of region X seems to be most closely associated with carcinogenesis in HBV infection.

Epidemiological surveys (Szmuness, 1978; Beasley et al., 1981) indicate a close correlation between infection with hepatitis B virus (HBV) and the incidence of primary hepatocellular carcinoma (PHC). Moreover, DNA sequences of HBV (HBV-DNA) are integrated into genomic DNA of most patients with PHC seropositive for hepatitis B surface antigen (HBsAg), and also in an HBsAg-positive hepatoma cell line (Brechot et al., 1980; Edman et al., 1980; Shafritz et al., 1981). An exact causal relationship between HBV infection and PHC has yet to be established, however. Attention has been focused on the S gene of HBV-DNA (coding for the major polypeptide of HBV, P22) which is found integrated in DNA of tumour cells without any evidence of being a viral oncogene (Ziemer et al., 1985).

In HBV-DNA there is a region called X (Tiollais et al., 1981) downstream from the C gene which codes for nucleoprotein P19 (Takahashi et al., 1979). As its designation implies, neither the role of region X nor its product is known. We prepared an HBV-DNA region X probe, as well as probes recognizing three other principal sequences of nucleotides in the viral genome, and tested them for binding with chromosomal DNA extracted from PHC tissues from six patients infected with HBV and a PHC strain propagated in nude mice.

Tissues of PHC from six HBsAg-positive patients were inoculated subcutaneously into athymic nude (BALB/c nu/nu) mice, and maintained through two to 30 passages. The identification of tumours and the passage in nude mice at the time of study are as follows: PLC/342 (10th passage), KT495 (17th to 20th), KT589 (8th, 12th, 13th and 26th to 30th), KT669 (9th), KT740 (2nd to 4th) and KT778 (2nd). In addition, PLC/PRF/5 strain, originally propagated from HBsAg-positive human PHC (Alexander et al., 1976) and made tumourigenic in nude mice, as well as two PHCs propagated from HBsAg-negative patients (KT800, 6th to 7th passage; KT842, 4th passage) were studied.
Tissues were homogenized with guanidinium thiocyanate–sodium dodecyl sarcosinate, and the homogenates were centrifuged on a cushion of CsCl solution as described by Chirgwin et al. (1979); DNAs were banded on the CsCl solution as viscous layers, while RNAs were precipitated at the bottom of the tubes. Also, DNAs were extracted from tissues, lysed with SDS and proteinase K, using phenol (Perucho et al., 1981). Poly(A) RNAs were prepared from the RNAs obtained by chromatography on oligo(dT)–cellulose.

For the purpose of detecting integrated nucleotide sequences of HBV-DNA, the entire HBV genome cloned from extrachromosomal DNA in the PLC/342 tumour and its restriction endonuclease fragments were used as probes. The nucleotide sequence of PLC/342 extrachromosomal HBV-DNA [3158 base pairs (bp)] is identical with the reported sequence of HBV-DNA (Ono et al., 1983) except for a minor deletion and some point mutations in the region pre-S (N. Okamoto et al., personal communication). The BglII–BglII fragment (439 bp, 1985 to 2423) represented the probe for detecting the C gene, the XbaI–SnaI fragment (450 bp, 376 to 825) for the S gene, the BstEII–AvaI fragment (470 bp, 2816 to 825) for the pre-S region, and the BamHI–Sau3A fragment (362 bp, 1401 to 1762) for region X. Although no sequences of plasmid vector pBR322 were found in any probes, it was included in the battery of probes in order to ascertain the absence of such sequences in DNA and RNA bands with the capacity to bind with HBV-DNA probes. Probes were labelled with [%-32P]dCTP by nick translation (Rigby et al., 1977).

DNA preparations (10 μg each) were digested with BamHI, EcoRI, HindIII, SacI, PstI or TaqI, and the digests were subjected to electrophoresis in agarose gels. DNA fragments were transferred to nitrocellulose membrane filters, and hybridized with 32P-labelled probes (3 × 10^6 c.p.m./ml) at 42 °C for 24 h in a mixture of Denhardt’s solution, 5 × SSC, 50% formamide, 100 mM-PIPES pH 6.8, 2 mM-EDTA and 100 μg/ml salmon sperm DNA as described by Southern (1975). Filters were washed three times with 2 × SSC containing 50% formamide and then with 2 × SSC containing 0.2% SDS at 25 °C, and autoradiographed on X-ray film. The sizes of DNA fragments were determined with reference to HindIII fragments of phage lambda DNA run in parallel.

When the preparations of DNA from seven PHCs were digested with SacI and hybridized with the entire HBV-DNA probe, fragments with the sequence complementary to the probe were visualized in all of them corresponding to the positions from 1.7 to 22 kilobase pairs (kbp) (Fig. 1). They were further tested for the capacity to hybridize with probes representing the S gene, C gene, region pre-S and region X and the results are summarized in Table 1. All samples revealed distinct DNA bands after digestion with restriction endonucleases, indicating that the integration of HBV-DNA into host DNA did not occur at random; random integration would have given rise to smears, rather than distinct bands (Kam et al., 1982). The region X was integrated in all HBsAg-positive PHCs tested, although the orientation of the HBV sequence was not uniform. The S gene was integrated in all except one PHC (KT589). This tumour failed to show the integration of gene S, gene C or region pre-S (Fig. 1). Also, the restriction fragments of this tumour formed by EcoRI, PstI and TaqI hybridized only to the probe for region X. The restriction patterns of this tumour did not change from the 8th to the 30th passage through nude mice. DNA preparations of two HBsAg-negative PHCs (KT800 and KT842) gave no positive staining with HBV-DNA probes. Sequences of pBR322 were not detected in any HBV-related DNA fragments (data not shown).

The expression of integrated HBV-DNA was examined by Northern blot hybridization of poly(A) RNA to the radiolabelled HBV-DNA probes. The poly(A) RNAs (2 to 10 μg each) extracted from carcinoma tissues were subjected to electrophoresis in agarose gels containing formaldehyde. Thereafter, RNAs were transferred to nitrocellulose membrane filters and tested for the capacity to hybridize with 32P-labelled HBV-DNA probes as described by Southern (1975). The size of RNA was determined in comparison with the migration positions of 18S and 28S ribosomal RNAs (1.8 and 4.7 kb respectively).

As can be seen in Fig. 2 and Table 1, mRNA capable of hybridizing with the probe for region X was detected in six out of seven HBsAg-positive PHCs tested. mRNA complementary to the S gene as well as to the pre-S region was found in five PHCs and that to the C gene in four PHCs.
Fig. 1. Southern analysis of integrated HBV sequences in DNAs from human PHCs propagated in nude mice. DNAs from PHCs were digested by SacI and hybridized with the specific probes of the HBV genome as described in the text. Lane 1, KT495; lane 2, KT589; lane 3, KT669; lane 4, KT740; lane 5, KT778; lane 6, PLC/342; lane 6S, short exposure of lane 6; lane 7, PLC/PRF/5. Marker DNAs are HindIII fragments of phage lambda DNA.
Fig. 2. Northern analysis of HBV sequences in mRNAs from human PHCs. Poly(A) RNAs from PHCs were hybridized with specific probes of the HBV genome as described in the text. Lane 1, KT495; lane 2, KT589; lane 3, KT669; lane 4, KT740; lane 5, KT778; lane 6, PLC/342; lane 7, PLC/PRF/5; lanes 3L, 4L and 5L, long exposures of lanes 3, 4, and 5 respectively; lane 6S, short exposure of lane 6. Markers are 28S (4.7 kb) and 18S (1.8 kb) ribosomal RNA. After washing out the HBV probes, the nitrocellulose filters were re-hybridized with v-myc or v-Ba-ras (related to c-Ha-ras-1) probe. The positions of 2.5 kb c-myc mRNA (left arrow) and 1.1 kb c-Ha-ras-1 mRNA (right arrow) are shown.
Table 1. Integration of parts of hepatitis B virus DNA and their expression in human primary hepatocellular carcinomas propagated in nude mice

<table>
<thead>
<tr>
<th>DNA from PHC</th>
<th>RNA from PHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region</td>
<td>Gene</td>
</tr>
<tr>
<td>PHC</td>
<td>pre-S</td>
</tr>
<tr>
<td>KT495</td>
<td>+</td>
</tr>
<tr>
<td>KT589</td>
<td>+</td>
</tr>
<tr>
<td>KT589</td>
<td>+</td>
</tr>
<tr>
<td>KT740</td>
<td>+</td>
</tr>
<tr>
<td>KT778</td>
<td>+</td>
</tr>
<tr>
<td>PLC/342</td>
<td>+</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>+</td>
</tr>
</tbody>
</table>

In the case of KT589, HBV-positive mRNA could not be detected under the present conditions, although other species, such as mRNAs of cellular oncogene c-myc and c-Ha-ras-1, were strongly expressed (Fig. 2). Control poly(A) RNAs prepared from HBsAg-negative PHCs did not show any binding with HBV-DNA probes. No mRNA bands from PHCs bound to 32P-labelled pBR322 probe.

The high frequency of the integration of region X in PHCs (seven out of seven) is consistent with the recent report by Moriarty et al. (1985) who found that as many as eight out of 11 cases of PHC produced the protein, capable of binding to antibodies raised against synthetic oligopeptides representing amino acid sequences of region X. Region X may be important for HBV integration into chromosomes of PHCs, and it has been suggested that this integration occurs by specific recombination between host cell DNA and HBV-DNA through the direct repeat of region X (Dejean et al., 1984). Integration of region X may also be important for the induction of HBsAg-associated PHCs. The occurrence of one PHC with integrated region X but without mRNA for its transcription favours the assumption that integration of the X gene, rather than its product, is essential for putative carcinogenesis by HBV. It would be worth establishing whether the PHCs of Moriarty et al. (1985), lacking X-related protein, contain integrated region X.

Recently, the presence of an enhancer sequence upstream from the X region has been suggested (Ziemer et al., 1985). Further analysis of DNA from KT589 revealed that the restriction fragments that hybridized with the X probe also hybridized to the two probes containing the region between X and S, the 302 bp immediately upstream from X and the 245 bp further upstream, respectively.

We thank Dr K. Nishioka, Vice-Director of The Tokyo Metropolitan Institute of Medical Science, for valuable help throughout this work, and are grateful to Dr N. Tsuchida, Tokyo Medical and Dental University, for providing v-myc and v-Ba-ras probes.

REFERENCES


Short communication


(Received 20 June 1985)