Chromosomal Sites for Hepatitis B Virus Integration in Human Hepatocellular Carcinoma

TAKASHI TOKINOTO and KENICHI MATSUBARA*
Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565, Japan

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The discovery that hepatitis B virus (HBV) integrates into host chromosomes raises the question of whether such viral DNA integration correlates directly with the activation of specific oncogenes or the inactivation of anti-oncogenes. To obtain insight into this problem, we randomly collected HBV integrant samples from different human hepatocellular carcinomas and identified the site of chromosomal integration by using in situ hybridization and/or linkage analysis with the flanking cellular DNAs as probes. Our findings did not specifically identify particular HBV DNA integration sites in chromosomes, although chromosomes 11 and 17 seemed to have more than the average number of integrants.

Hepatitis B virus (HBV) integrants have been found frequently in human hepatocellular carcinoma (HCC). Although the possible precursor form of the viral DNA for integration has been inferred (for example, see reference 16), we still do not know whether the integrated HBV DNA prefers a specific chromosomal site(s) or whether it integrates randomly. Studies with woodchucks, an animal model of hepatitis B virus-induced primary HCC, have shown that in some tumors the viral DNA integrates at or close to the cellular proto-oncogene c-myc and disturbs its expression (12, 15). In humans, Dejean et al. (6) and Wang et al. (29) have reported integration of HBV into the retinoic acid receptor and cyclin A genes, respectively, and proposed that such integration may play a crucial role in hepatocarcinogenesis (7). On the other hand, Rogler et al. (22), Hino et al. (11), and Tokino et al. (25) have presented evidence showing that some of the integrated HBV DNA causes rearrangement of cellular DNA, an effect that may directly or indirectly relate to integration and development of HCC.

The problem in evaluating these findings is the paucity of information as to whether they can be generalized, i.e., whether hepatocarcinogenesis can be correlated reproducibly with any one of these events or whether they merely represent isolated and poorly reproducible events. To obtain an insight into this problem, we investigated chromosomal integration sites by using many randomly collected integrant samples and in situ hybridization and/or linkage analysis. In addition, we reviewed the published data from our laboratory and others.

MATERIALS AND METHODS

Clones carrying the integrant HBV DNA. Clones DA2-2, DA2-6, and C29 were obtained from EcoRI-digested HCC DNA, with DA2-2 and DA2-6 being derived from the same tumor. Clone pY was obtained from the HindIII-digested DNA of another HCC. These clones carried 8- to 13-kb inserts in λ vectors. For details of these clones, see reference 16.

In situ hybridization. The DNA of the integrant clones was cleaved by several restriction enzymes and subjected to Southern analysis. Fragments that did not have repetitive sequences were selected and recloned into plasmid vectors. These unique cellular sequence DNAs were a 3-kb HindIII fragment from PY, a 1.7-kb BglII-EcoRI fragment from DA2-2, a 1.5-kb PstI fragment from DA2-6, and a 1.2-kb BglII fragment from C29. After amplification of the recombinant plasmids, the fragments were cut out, radiolabeled with [3H]dTTP, and used for in situ hybridization.

Metaphase chromosomal spreads were obtained from phytohemagglutinin-stimulated peripheral blood lymphocytes from a normal male. Before hybridization, the chromosome preparations were pretreated with RNase A and denatured at 70°C in 70% formamide-0.3 M sodium chloride-0.03 M sodium citrate. The probe was [3H]labeled by nick translation to a specific activity of 2 × 10^7 dpm/μg and used for hybridization at a concentration of 3 μg/ml. Hybridization, washing, and autoradiography were carried out essentially as described by Harper and Saunders (8), and the slides were subsequently Q banded by double staining with quinacrine mustard and Hoechst 333258 (30). Silver grains on the chromosomes were scored and plotted on an ideogram established by the International System for Human Cytogenetic Nomenclature (13).

Restriction fragment length polymorphism (RFLP) test, genotyping, and linkage analyses. Cosmid clones covering the integration sites were selected from a human genomic DNA library by using the unique DNA fragments in the flanking cellular sequences obtained from the integrant clones obtained as probes. The cosmids thus obtained were HB118, HB140, and HB159, which covered the respective integration sites of PY, C1, and P10-1. These cosmid clones were tested for the presence of RFLPs with six enzymes (MspI, TaqI, RsaI, BglII, PstI, and PvuII), and polymorphic fragments were isolated (see Table 1) and subcloned into vector plasmid pUC18. These polymorphic fragments were employed for genotyping and linkage analysis in 59 three-generation families (including 40 families in the reference panel maintained by the Centre d'Etude du Polymorphisme Humain, Paris, France) by using the LINKAGE program package (14). The methods and conditions used for hybridization were described elsewhere (19).

* Corresponding author.
† Present address: Cancer Institute, Division of Biochemistry, Kamiebekuuro, Toshima-ku, Tokyo 170, Japan.
RESULTS

In situ hybridization tests with integrants PY, C29, DA2-2, and DA2-6. In our previous studies, we assigned integrants PY and C29 to chromosomes 11 and 5, respectively (16). We also showed that integrants DA2-2 and DA2-6 are the products of chromosomal rearrangements, whose left and right flanking cellular DNAs are from chromosomes X and 17 in the case of DA2-2 and from chromosomes 5 and 9 in the case of DA2-6 (25). In this study, we determined the regional locations of these integrants by in situ hybridization to metaphase chromosomes by using the radiolabeled unique cellular sequence DNAs derived from the flanking cellular DNAs as probes. For each probe, approximately 50 metaphase chromosomes were counted and the chromosomal distribution of 50 silver grains was scored. The results are shown in Fig. 1. The main peaks of silver grains were seen on bands 11q23 ($\chi^2 = 120, P < 0.001$), 5q14 ($\chi^2 = 100, P < 0.001$), 17q24 ($\chi^2 = 80, P < 0.001$), and 5q23 ($\chi^2 = 120, P < 0.001$) for the probes from PY, C29, DA2-2, and DA2-6, respectively. No secondary peaks of hybridization were found on other chromosomes (data not shown). The other two integration sites for the other arms of DA2-2 and DA2-6 have already been located on chromosomes Xp and 9p, respectively (25).

RFLP tests with cosmids clones. We prepared three cosmid clones (HBI18, HBI40, and HBI59), each of which covered the integration site along the cellular DNA as found in clones PY, C1, and p10-1. Each of the cosmids was tested for polymorphism. These cosmids clones revealed RFLPs with more than one enzyme, and the RFLP markers were employed for genotyping and linkage analysis (14). The results are shown in Table 1. Chromosomal sites of 11q, 1q, and 11q were assigned for cosmids HBI18, HBI40, and HBI59, respectively (covering the respective integration sites of PY, C1, and p10-1). The result for PY was in complete concordance with that obtained by in situ hybridization. All six of the chromosomal sites thus assigned are shown in Fig. 2.

To obtain information on the possible preference for a certain chromosomal site(s), it is desirable to collect as much data as possible. Therefore, we have assembled all of the available data from our own and other laboratories in Fig. 2. Integrants assigned only to a chromosome and not to a region are represented under the corresponding chromosome. The results seem to show that there is no preferential site for HBV integration.

![Diagram showing chromosomal localization of HBV integrants](image)

**FIG. 1.** Chromosomal localization of HBV integrants in PY, C29, DA2-2, and DA2-6 as determined by in situ hybridization with metaphase human chromosomes. The names of the integrant clones subjected to analysis are displayed at the lower right of each chromosome. The hybridization probes used were the unique cellular sequence DNAs obtained from flanking cellular DNA (see Materials and Methods).

<table>
<thead>
<tr>
<th>HBV integrant</th>
<th>Probe name</th>
<th>Vector/size (kb/site)</th>
<th>Enzyme</th>
<th>Alleles sizes (kb), frequencies</th>
<th>Locus symbol</th>
<th>Chromosomal location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PY</td>
<td>HBI18P2</td>
<td>pUC18/4.0/PstI</td>
<td>PstI</td>
<td>A1,5.0,0.25;A2,4.4,0.75</td>
<td>D11S147</td>
<td>11q, 7 cM proximal to APOA1 (11q23-11q24)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>HBI40</td>
<td>pUC18/4.4/Acc1</td>
<td>MspI</td>
<td>A1,8.0,0.73;A2,4.4,0.27</td>
<td>D1S66</td>
<td>1q, 21 cM distal to Duffy (FY) (1q21-1q25)</td>
<td>20</td>
</tr>
<tr>
<td>p10-1</td>
<td>HBI159</td>
<td>pUC18/1.6/Acc1</td>
<td>MspI</td>
<td>A1,4.3,0.37;A2,3.8,0.63</td>
<td>D11S146</td>
<td>11q, 0.5 cM proximal to INT2 (11q13.3)</td>
<td>18</td>
</tr>
<tr>
<td>p10-1</td>
<td>HBI159</td>
<td>pUC18/1.6/Acc1</td>
<td>TaqI</td>
<td>B1,1.2,0.55;B2,0.8,0.45</td>
<td>D11S146</td>
<td>11q, 0.5 cM proximal to INT2 (11q13.3)</td>
<td>18</td>
</tr>
</tbody>
</table>

*Polymorphic fragments were isolated from the cosmid clones covering the integration sites and subcloned into vector plasmid pUC18. For example, a 4.4-kb PstI fragment from cosmid clone HBI40 revealed RFLP with PstI; the sizes and frequencies of the polymorphic alleles are presented here.

*Official human gene map symbols for DNA segments (reference 2).

*cM, centimorgans.
**DISCUSSION**

Because HBV integrants can cause secondary rearrangement of chromosomes (11, 25), some of the assigned sites in Fig. 2 may not be the actual sites of integration but may instead be sites of secondary DNA recombination. From our own experience, 8 of 19 integrants analyzed were of the rearranged type and included chromosomal translocations, inversions, or deletions (25, 25a). However, since we have no way of telling whether a particular viral-cellular DNA junction was made in the process of primary integration or secondary rearrangement, we have ignored the distinction and simply assumed that the sites in Fig. 2 are more or less related to the integration event. Accepting this limitation, the available data (Fig. 2) show that HBV integration events do not seem to select a specific site(s), although chromosomes 11 and 17 are involved to a more that average extent.

Interestingly, these chromosomes are known to carry several sites for oncogenes or tumor suppressor genes. Chromosomal region 11q13, where oncogenes int-2 and hst-1 are located (5, 31), is often found amplified in HCC (9), as well as in other cancers (1, 26–28). We have found that integrant p4 at site 11q13 was associated with regional amplification (7). Integrant p10-1 at site 11q13, on the other hand, is associated with a 25-kb chromosomal deletion (25a), and pY at 11q23 is associated with a microdeletion (17). Whether or not these changes are associated directly or indirectly with hepatocarcinogenesis is a topic for further studies. No information is available for the other integrants.

Although the integration events of HBV are not limited to a few sites, all of these integration events could be mutagenic. We are still far from determining what type of integration (and thus mutation) plays a role in the development of HCC. Of course, some of the integrations may have played no role in the etiology of HCC, but one should encounter a higher incidence of a genetic change(s) that plays a role in the etiology or pathogenesis of HCC when surveying a collection of mutated sites than when surveying nonmutated regions. Therefore, we suggest that studies with HBV integrants from HCCs should promise a way to detect more genes and sites related to hepatocarcinogenesis.

**REFERENCES**