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Rearrangement of the surface antigen gene of hepatitis B virus integrated in the human hepatoma cell lines

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SUMMARY

The rearrangement of integrated HBV DNA sequences in three different hepatoma cell lines, huH-1, huH-2, KG-55-T from Japanese patients, were studied by blot hybridization using whole HBV genome or a HBsAg or HBcAg DNA as a probe. The characteristic existence of multiple integration sites of HBV DNA sequences in each HindIII-restricted hepatoma cell DNA was revealed by the HBV genome probe. Detection of the isolated HBsAg gene in the HindIII fragment indicates that the integration of HBV DNA was not always related to the maintenance of the whole viral genome, and that movement of the HBsAg gene to another location occurred by rearrangement. On the other hand, the presence of the HBV DNA sequence without the intact HBcAg gene was shown in some of the HindIII fragments, when the HBcAg gene probe was used, but a HindIII fragment, containing only the HBcAg gene, was not detected so far. The absence of the intact HBcAg gene suggests that the viral genome may lose a part of the HBcAg gene in the process of integration. This is consistent with recent findings of Ogston et al. (1982) that in Woodchuck hepatocellular carcinoma viral sequences are extensively rearranged.

INTRODUCTION

Infection with hepatitis B virus (HBV) is wide spread in man. Most infections are followed by an apparent complete recovery with the development of a virus-specific antibody. However, a significant proportion of infections (ca.1-5%) may produce chronic diseases including persistent infection, chronic hepatitis (CH) of various types, liver cirrhosis (LH) and hepatocellular carcinoma (HCC)[1]. Many epidimiological studies show a close relation between persistent HBV infections and primary liver cancer[2,3,4].

Because of the adr subtype of HBV is the predominant form in the Far East, China and Japan[5], it is important to characterize the HBV DNA from the adr subtype and its integrated form. Recently, we cloned the HBV DNA of adr subtype using plasmid pBR322 as a vector, and determined the nucleotide sequences of the genes for HBsAg containing 226 amino acids and HBcAg consisting of 183 amino acids and other open reading frames[6,7]. We were primarily concerned with characterization of the HBV genome and the expression of the HBsAg

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gene in Escherichia coli for diagnostic purposes and vaccine production. Recent approaches to studying the relationship between HBV integration and human liver disease have been to perform blot hybridization experiments using cloned HBV DNA as a specific probe to detect HBV DNA sequences in the liver or hepatoma cell lines[8,9,10,11]. This technique allows determination of the state of viral DNA in hepatocytes, either free or integrated in the host cellular DNA. The presence and integration of HBV DNA sequences in hepatoma cells is documented, but little is known about the intracellular state of viral genes for HBsAg and HBcAg during the different stages of the disease and the nature of their relationship with HCC. Our approach to the study of the structure of these integrated genes has been to digest cellular DNA in hepatoma cell lines into fragments and determine gene arrangement by blot hybridization using the coding region obtained from the cloned HBV DNA of adr subtype virions as a specific probe for finding their roles in the formation of the HCC. Moreover, the detection of HBV gene sequences is a direct indication of HBV infections in the patients without gene products.

MATERIALS AND METHODS

Human hepatoma cell lines

Two hepatoma cell lines, huH-1 and huH-2, were previously described[12]. According to liver histology, both are hepatocellular carcinoma. For huH-1, the serological status indicated the presence of HBsAg, a marker of a current HBV infection, the absence of the e antigen (HBeAg), a marker of HBV multiplication, and the presence of the antibody to HBcAg, which usually reflects a past HBV infection. One of these cell lines, huH-1, has been shown to continuously produce a small amount of HBsAg in the form of 22-nm particles. In contrast to huH-1 cells, the absence of the HBsAg production was noted in the case of huH-2 cells (unpublished data).

The third hepatoma cell line, KG-55-T, was derived from cancer cell clumps from human hepatocellular carcinoma nodules by needle puncture, as described previously[13]. These clumps were obtained from a cirrhotic patient with hepatocellular carcinoma. The serological status indicated the presence of HBsAb and absence of serum HBsAg. Proteins secreted into the cell culture medium were biochemical markers of hepatocytes, but no production of HBsAg was detected.

For the huH-1 and huH-2 cells, the culture medium was DM-160 (Kyokuto Pharmaceutical industrial Co. Tokyo) supplemented with 10 % fetal bovine serum and 50 µg/ml kanamycin. Cells of each passage were washed with phosphate

buffer and stored at -80°C (10% DMSO) until use for DNA extraction. KG-55-T cells were cultured using Ham's F-12 medium supplemented with 10% fetal bovine serum[13]. Cells of each passage were washed with phosphate buffer and also stored at -80°C for DNA extraction.

Extraction of human hepatoma cell DNA

Whole DNA was extracted from three different hepatoma cells as follows. The packed cells were suspended in 10 vol. of 10 mM Tris-HCl (pH 7.4), 10 mM NaCl and 2 mM EDTA-3Na and treated with 2 % SDS and 500 μg/ml proteinase K at 37°C over-night. The resultant lysate was extracted twice with an equal volume of saturated phenol for 15 min and then extracted with an equal volume of chloroform/isoamylalcohol (50/1) for 15 min. Nucleic acids in the aqueous phase were precipitated with 2 vol. of ice-cold ethanol, centrifuged at 15,000 rpm for 10 min, and dissolved in a half volume of the same buffer. Nucleic acids were then subjected to digestion with 40 μg/ml RNase and 10 μg/ml RNaseT1 at 37°C for 1 h, followed by a further round of extraction with saturated phenol and chloroform/isoamylalcohol. The DNA was precipitated with 2 vol. of cold ethanol, redissolved in 0.3 M sodium acetate and precipitated again with cold ethanol. Finally the purified DNA was dissolved in water to final concentration of 250 μg/ml.

Gel blotting and hybridization

After electrophoresis, blotting of the DNA fragments from agarose gels to nitrocellulose filters (Schleicher & Shull) was performed essentially by the method of Southern (1975)[14]. Filters were pretreated by the method of Denhardt (1966)[15]. Detection of HBV DNA sequences in the hepatoma DNA fragments on the filters was performed by hybridization with a nick-translated ^{32}P-labeled HBV DNA probe (a specific activity of at least 10^8 cpm/μg of DNA) at 65°C over-night in a solution of 6 x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.02 % bovine serum albumin, 0.02 % polyvinylpyrrolidone, 0.02 % Ficoll, 0.5 % SDS, 100 μg/ml sonicated and heat-denatured salmon sperm DNA. Filters were blotted and autoradiographed with Kodak X-Omat XRP-1 and Du Pont Cronex Lightning Plus screens at -80°C for 1-10 days.

Preparation of nick-translated probes

Probe HBV DNA was prepared from a recombinant plasmid pHBV1-1, in which the entire DNA of HBV virions from patients (HBsAg subtype adr) was introduced at the BamHI cleavage site of pBR322, as previously described[6]. Since the HBV surface antigen gene and the core antigen gene were located within the cloned viral genome[6,7], these genes can be isolated as a TaqI fragment (0.82 kb) for HBsAg and a BglII fragment (0.44 kb) for HBcAg gene, respectively, from

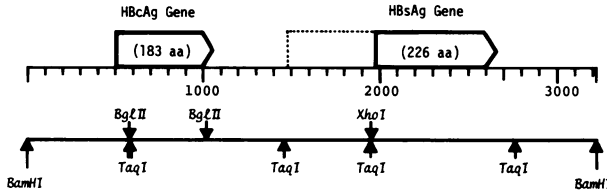

Figure 1. Restriction map and gene organization of cloned HBV genome linearized by BamHI digestion.
Cloned HBV DNA (subtype adr) was digested with XhoI, TaqI and BglII. Relevant fragments to the hybridization probes for the whole HBV genome and HBsAg and HBcAg genes were purified by gel electrophoresis and individually nick translated. Solid boxes represent the coding regions of HBsAg and HBcAg genes. The dotted line represents the putative precursor region of the HBsAg protein.

a genome of recombinant plasmid pHBV1-1. Figure 1 shows the restriction maps and the positions of two respective genes within the viral genome from a plasmid pHBV1-1. The complete nucleotide sequence of this cloned HBV DNA will be published elsewhere. HBV DNA from the plasmid pHBV1-1 was digested with a combination of restriction enzymes, BamHI and XhoI to yield two fragments roughly 1.8 kb and 1.4 kb. These fragments were separated by gel electrophoresis, and the 1.4 kb fragment containing the HBsAg gene was further digested with TaqI to isolate a whole HBsAg gene. HBcAg DNA was recovered after BglII cleavage of the hybrid plasmid DNA, since no cleavage site for BglII is present in the pBR322. Two fragments were generated by the digestion, one of which contains about 80% of the entire HBcAg gene. Both gene DNAs were separated by polyacrylamide gel electrophoresis and subjected to nick translation reaction.

Nick translation was performed essentially as described by Rigby et al. (1977)[16]. To 1-2 µg of HBV DNA, HBsAg DNA or HBcAg DNA purified as described above in 50 µl of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, bovine serum albumin (50 µg/ml), 1 mM dGTP, dATP and dTTP were added 100 µci of [α-^{32}P]dCTP (Amersham; 5000-7000 Ci/mM), 2 x 10^{-4} Kunitz units of DNase I (Worthington), and 2 units of DNA polymerase I (Boehringer Mannheim). The reaction mixture was incubated at 15°C for 2 h, and then 100 µl of 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 % SDS, containing 50 µg of sonicated salmon sperm DNA were added. The solution was passed through a Sephadex G-50 column, and the peak fraction of DNA were precipitated by ethanol. Specific activities of 3-7 x 10^8 cpm/µg were achieved with 30-50 % incorporation of label.

Restriction endonuclease digestion and gel electrophoresis

Restriction endonuclease digestion and gel electrophoresis were essen-

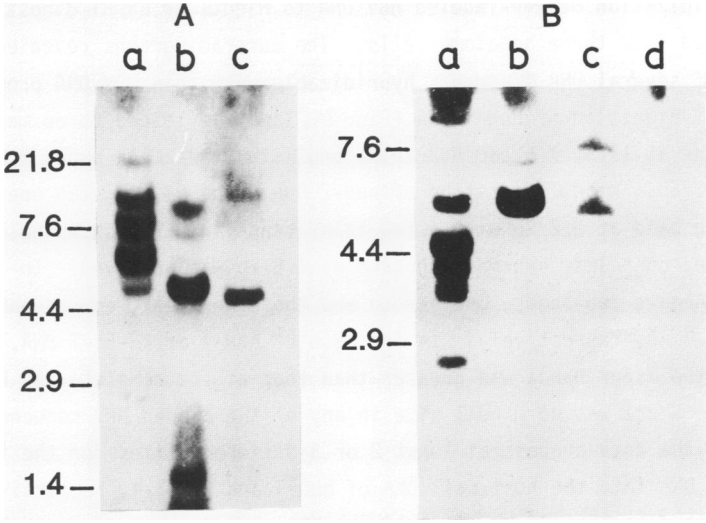

Figure 2. Hybridization of HBV DNA to filter-blotted DNA from human hepatoma cell lines.
DNAs from huH-1, huH-2 and KG-55-T cells were cleaved with either HindIII or BamHI restriction endonuclease. Blotted DNAs were hybridized with nick-translated ^{32}P-labeled HBV DNA. A, HindIII-cleaved cellular DNAs probed with ^{32}P HBV DNA; b, BamHI-cleaved DNAs probed with ^{32}P HBV DNA. a, b, c, d, DNA from huH-1, huH-2, KG-55-T and human placenta, respectively. Lambda phage and pBR322 DNAs were used as molecular weight standards (in kilobase pairs).

tially as described previously[17]. DNA samples were digested with HindIII, BamHI, BglII or TaqI (Takara) separately or in combination. The complete digestions were monitored by adding excess restriction enzyme to the reaction mixtures. The resultant DNA fragments were electrophoresed on an agarose or polyacrylamide gel. After the run, the DNA bands were stained with 2 μg/ml of ethidium bromide. DNA was subjected to gel blotting or extracted from the gel by homogenization and incubation with 0.5 M ammonium acetate, 0.1 % SDS, 0.1 mM EDTA for 2-16 h at room temperature. Agarose gels of 0.7-2 % (w/v) and polyacrylamide gels of 5-10 % (w/v) were prepared.

RESULTS

Detection of HBV sequences in hepatoma cells using the whole HBV DNA probe

We examined the physical integration of the HBV sequences in huH-1, huH-2 and KG-55-T cells using the blot hybridization technique and compared the integration patterns with the data from uninfected human placenta DNA. In this case, the nick-translated whole HBV DNA was used as the probe. Figure 2

shows hybridization of ^{32}P-labeled HBV DNA to HindIII or BamHI digests of whole DNA prepared from three hepatoma cells. The autoradiographs revealed the presence of several DNA fragments hybridizable with this HBV DNA probe.

HindIII digestion of huH-1 DNA (Fig. 2A, lane a) yielded three major hybridizing bands at 11.8, 7.6 and 6.2 kb, along with other weak bands ranging from 5.5 to 21.8 kb. HindIII digestion of huH-2 DNA (labe b) produced one major hybridizing band at 5.2 kb with a few faint bands in a region up to 10.8 kb. In addition, one clear hybridizing band at 1.5 kb was observed. In lane c, KG-55-T DNA gave two bands, one strong and the other weak, corresponding to 4.9 and 12.7 kb, respectively. In the case of huH-1 or KG-55-T DNA, the length of the major bands was greater than that of the complete HBV DNA (3.2 kb). Since there was no HindIII site in any of the cloned HBV sequences reported[6,7], the data suggest at least 2 or 3 different sites for the integration of HBV DNA into the host cell DNA of huH-1 and KG-55-T, respectively. There was no detectable free viral linear DNA at 3.2 kb or free viral closed circular DNA in the 2.3 kb region. It is important to note that there was a significantly hybridizing band at a lower-molecular weight region than the entire HBV DNA of 3.2 kb. In particular, huH-2 DNA gave one hybridizing HindIII fragment at 1.5 kb. This finding clearly indicates that HindIII digests of the DNA, extracted from huH-2 cells, contain a part of HBV DNA molecule independently integrated into different sites of the host genome. If the low-molecular weight band detected represents a different HBV DNA molecule containing a HindIII-restriction site, such a molecule could be cleaved into small DNA fragments with HindIII. However, no cleavage of HBV DNA into small fragments has been reported so far[19,20,21]. In addition, the multiplicity of bands observed does not reflect cell heterogeneity, since these patterns of hybridization are always observed in hepatoma cells after many passages in the tissue culture.

BamHI digestion (Fig. 2B, lane a) of huH-1 DNA produced a series of 7 bands ranging from 2.7 to 9.2 kb. Since HBV DNA contains at least a single BamHI site, it was therefore expected that BamHI digestion would give more complex hybridizing bands than those of HindIII-restricted DNA. Figure 2B also shows the results from the same analysis of huH-2 DNA (lane b) and KG-55-T DNA (lane c). One major hybridizing band was obtained at 4.9 kb with a faint band in the region around 7 kb (lane b). On the other hand, two bands were observed at 7.4 and 5.0 kb in the KG-55-T genome. The number and size of hybridizing restriction fragments suggest that the BamHI cleavage site of this integrated viral DNA may be modified in the huH-2 DNA. Final proof will re-

quire investigation of these integrated HBV DNA sequences. No hybridization signal was detected in the DNA from normal placenta (lane d). Thus, there is no sequence closely related to HBV DNA in the normal human genome.

State of the HBsAg gene integrated in hepatoma cell DNA

To determine what kind of the fragment of HBV genome retained in each of the integrated sites, we hybridized HindIII-digested hepatoma cell DNAs with ^{32}P-labeled HBsAg gene as the probe. In Figure 3A autoradiographs of these hybridization reactions indicate that nearly identical DNA fragments were hybridized compared to those obtained with the whole HBV DNA probe (Fig. 2).

The huH-1 DNA (lane a) yielded three major hybridizing bands along with other weakly hybridizing fragments ranging from 2.9 to 21.8 kb, indicating the HBsAg gene to be present in similar bands following digestion with HindIII, when whole HBV DNA used as a probe. HindIII digestion of huH-2 DNA (lane b) produced one major hybridizing DNA at 5.2 kb with a few faint bands at a higher-molecular-weight region up to 10.8 kb, in addition to one clear band at 1.5 kb. The HBsAg gene was again observed in DNA bands similar to those obtained by the whole HBV DNA probe. KG-55-T DNA (lane c) gave 4.9 and 12.7 kb bands. The results clearly indicate that almost all the HindIII fragments contain the HBsAg gene sequence if not the complete structure. In case of huH-2 DNA, there was one hybridizing band at 1.5 kb smaller than the full-length HBV DNA, indicating that a HBsAg gene-containing fragment alone integrates at a distinct site of the host genome.

To further examine the state of HBsAg gene integration, three hepatoma cell DNAs were digested with TaqI into small fragmnets, and were hybridized to the HBsAg gene probe. As shown in Fig. 3B, TaqI digestion of huH-1 DNA (lane a) produced many hybridizing bands, ranging from 0.7 to 7.6 kb. In the case of huH-2 DNA hybridizing bands were also obtained in the region from 0.7 to 2.0 kb (lane b). A lower band in the 0.8 kb region was weakly detected when whole HBV DNA was used as the probe (data not shown). On the other hand, KG-55-T DNA revealed two bands in the 2.0 and 0.7 kb regions with one faint band at 1.4 kb. There are at least five TaqI sites in any of the cloned HBV DNA sequences reported [19,20,21] and two of these have been found in the regions proximal to the 5' and 3' ends of the HBsAg gene of adr subtype (Fig. 1). Thus, the hybridizing band at 0.8 kb is indicative of the presence of the complete HBsAg gene of the adr subtype. It should be mentioned that one small band detected at 0.5 kb by the whole HBV DNA probe (data not shown) comes from a different TaqI fragment outside the HBsAg gene. It is important to note that in the case of huH-1 DNA, there was a significantly hybridizing band

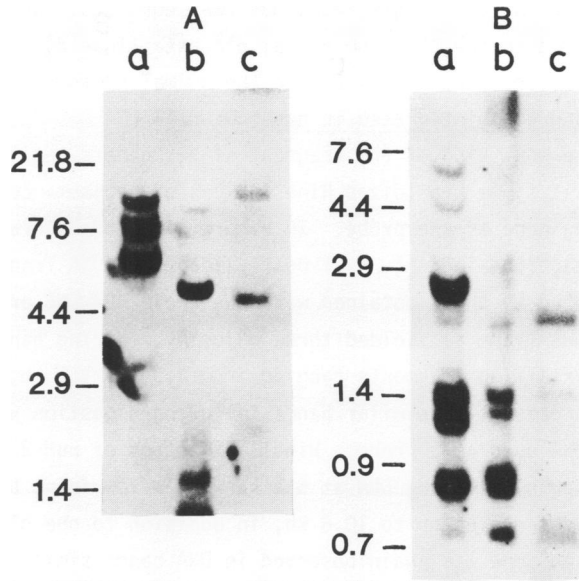

Figure 3. Hybridization of HBV DNA and HBsAg DNA to filter-blotted DNA from human hepatoma cell lines.
DNAs from huH-1, huH-2 and KG-55-T cells were cleaved with either HindIII or TaqI restriction endonuclease. A, HindIII-cleaved cellular DNAs probed with ^{32}P HBsAg DNA; B, TaqI-cleaved DNAs probed with ^{32}P HBsAg DNA. a, b, c, DNA from huH-1, huH-2 and KG-55-T, respectively.

at higher-molecular-weight region than the full-length HBV DNA at 3.2 kb. These findings may possibly be interpreted as either a modification of a TaqI site inside the integrated HBV genome or a rearrangement of a TaqI site in the integrated HBV DNA.

State of the HBcAg gene integrated in the hepatoma cell DNA

A series of tests was then performed to demonstrate the correlation between the HBsAg and HBcAg genes in the integrated state. The hybridization probe for the HBcAg gene has been derived in the form of the 0.44 kb BglII fragment from cloned HBV DNA (Fig. 1). We used this HBcAg gene probe to survey the hybridizing DNA fragments of hepatoma cell genomes after digestion with the same restriction enzymes as described above. Figure 4A shows the hybridizing DNA fragments derived from three hepatoma cell genomes after digestion with HindIII. The huH-1 DNA (lane a) yielded only two major hybridizing bands at 6.2 and 7.6 kb, which are quite similar to the DNA bands obtained when using the whole HBV DNA or HBsAg gene probe in position and number. However, these bands differ in intensity. In particular, the 11.8

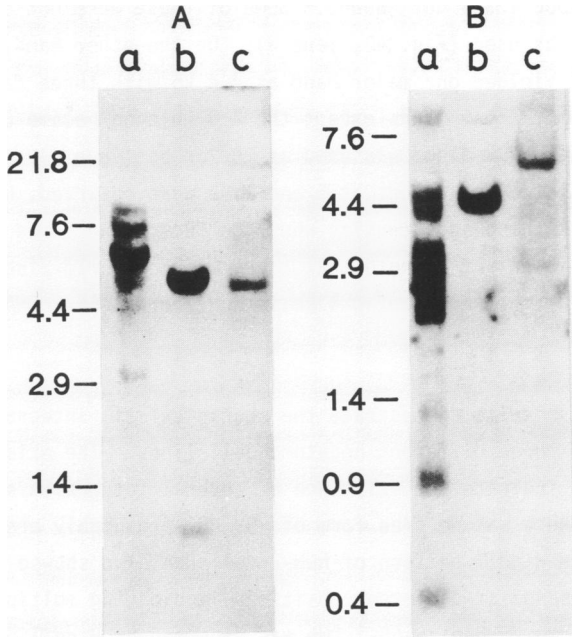

Figure 4. Hybridization of HBcAg DNA to filter-blotted DNA from human hepatoma cell lines.
DNAs from huH-1, huH-2 and KG-55-T cells were cleaved with either HindIII or BglII restriction endonuclease. A, HindIII-cleaved cellular DNAs probed with ^{32}P HBcAg DNA; B, BglII-cleaved DNAs probed with ^{32}P HBcAg DNA. a, b, c DNA from huH-1, huH-2 and KG-55-T, respectively.

kb band was less hybridized than the others. None of the DNA fragment, existing in the HindIII digest was different from those obtained by the HBsAg gene probe. The huH-2 DNA (lane b) produced one intensely hybridizing band at 5.2 kb. There was no extra band which only hybridized to the HBcAg gene probe. In the case of KG-55-T DNA (labe c) one band at 4.9 kb was observed without any extra band. These results clearly indicate that all the hybridizing bands by the whole HBV DNA probe contain the HBsAg gene or a part of it, but only particular bands are hybridizable with the HBcAg gene. A HindIII fragment containing only the HBcAg gene, has not been detected so far.

Since there are two BglII sites located inside the 5' and 3' ends of the HBcAg gene of the adr subtype (Fig. 1), the hybridizing band at 0.4 kb is therefore indicative of the presence of the HBcAg gene-containing sequence of the adr subtype in the hepatoma cell DNA. BglII digestion of huH-1 DNA produced many hybridizing bands, ranging from 0.4 to 8.5 kb, when whole HBV DNA

was used as a probe (data not shown). Some of these were not observed when the HBcAg probe was used (Fig. 4B, lane a). On the other hand, BglII digestion of huH-2 DNA yielded one major band at 4.4 kb with three faint bands at 8, 1.3 and 0.4 kb, but these bands except the 4.4 kb band became undetectable when the HBcAg gene DNA alone was used as the probe (lane b). In the case of KG-55-T cell DNA, two weak bands at 5 and 9 kb were observed, however, one of these at 9 kb decreased in intensity when the HBcAg gene probe was used (lane c). It is also important to note that there was no hybridizing band in the lower-molecular-weight region of 0.4 kb in huH-2 or KG-55-T DNA.

DISCUSSION

The present results demonstrate the characteristic integration of HBV DNA sequences in three different hepatoma cell lines. The existence of multiple HindIII bands indicate the existence of several integraion sites in the huH-1 genome. There was no free form of HBV DNA presumably present at the 3.2 kb position. The HindIII pattern of huH-2 cell DNA also showed the presence of at least two bands at different positions, indicating multiple integration sites in the genome. When KG-55-T DNA was digested by HindIII, the pattern showed the presence of bands at different positions, compared to the pattern of the other two cell lines. The restriction patterns were relevantly changed to different restriction enzymes, BamHI, TaqI and BglII, studied in three cases of hepatoma cell DNAs.

Attention was directed to the restriction patterns in these three hepatoma cell DNAs using HBsAg or HBcAg gene as the probe, since the restriction pattern would be different according to the probe used. The HindIII patterns of each hepatoma cell DNA by the HBV DNA probe were almost identical to those obtained using the HBsAg gene as the probe. Integration of the HBsAg gene was detected even in the short fragment 1.5 kb, indicating that the integration of HBV DNA was not always related to the maintenance of the whole genome. Present data clearly indicate the existence of the HBsAg gene even if no production of HBsAg is detected in the two hepatoma cell lines huH-2 and KG-55-T. However, due to the lack of DNA sequencing data for each hybridizing band, we are unable to predict the complete coverage of the HBsAg gene in these hybridizing bands.

Multiple integrations of the HBV DNA sequences were again detected in huH-1 DNA with the HBcAg gene probe, but only one intense band was detected in huH-2 or KG-55-T DNA. Thus, the autoradiograms are quite different from each other in the three hepatoma cell DNAs and show the absence of the comp-

lete HBcAg gene in some of the HindIII fragments. These findings may be interpreted as a splitting of the two BglII sites of the HBcAg gene as a result of the integration of the viral genome during persistent infection. Based on the previous data that the HindIII does not cleave the HBV genome[18], the present results suggest that some of the HindIII fragments not containing the HBcAg gene are formed by rearrangement of the integrated HBV genome.

The transfer and integration of genetic material from extra-nuclear compartments into chromosomal DNA and the possible involvement of multi-copy mobile elements have been suggested for several systems[22,23,24]. This pertains to so-called processed genes having genomic pseudogene sequences bearing close resemblance to mRNAs. The presence of a HindIII fragment containing only the HBsAg gene suggests such movement of a HBsAg gene-copy to another locus. It may be speculated that either a RNA intermediate may be involved in the formation of this moving viral gene by reverse transcription of mRNA or a rearrangement may be evoked in the formation of this processed gene by transposition reaction. The absence of a BglII fragment corresponding to the HBcAg gene suggests that the viral genome may lose a part or all the HBcAg gene in the process of rearranged integration into chromosomal DNA. This is consistent with the recent findings of Ogston et al. (1982) that Woodchuck hepatitis virus, being similar to the related hepatitis B virus, is induced in its natural host hepatocellular carcinoma, where viral sequences are extensively rearranged[25]. We attempted to isolate various clones containing the rearranged viral sequences from hepatoma cell DNAs. Among the clones of the HindIII fragments, one clone could be detected by hybridization of the HBsAg gene, but not the HBcAg gene. To locate the HBsAg gene sequence in the HindIII fragment, sequencing of the cloned DNA is now under investigation.

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