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Integration of HBV-DNA Into Liver and Hepatocellular Carcinoma Cells During Persistent HBV Infection

David A. Shafritz

Departments of Medicine and Cell Biology, Liver Research Center, Albert Einstein College of Medicine, Bronx, New York 10461

The hepatitis B virus carrier state (persistent HBV infection) is characterized by the presence of viral surface antigen (HBsAg) and virion particles (Dane particles) in the blood. From 1% to 10% of carriers develop chronic liver disease and/or hepatocellular carcinoma. Recent studies have demonstrated integrated HBV-DNA in hepatocellular carcinomas and in several human hepatoma cell lines. In hepatoma patients, integrated HBV-DNA has been found in all HBsAg carriers. Nontumorous liver also revealed integrated HBV-DNA with the same or a different hybridization pattern from that observed in the tumor. To explore when integration occurs, carriers of short-term (< 2 years) or long-term (> 8–10 years) were evaluated. DNA extracts from percutaneous (needle) liver biopsies showed free viral DNA with no specific integration bands in short-term carriers. In long-term carriers, HBV-DNA was integrated into the host genome with either a diffuse or a unique hybridization pattern. HBV-DNA integration correlated with the duration of the carrier state and absence of virions in the serum but did not correlate with histologic evidence of chronic hepatitis. These studies suggest that integration of HBV-DNA occurs during persistent HBV infection irrespective of liver disease and precedes development of hepatocellular carcinoma.

Key words: recombinant HBV-DNA, molecular hybridization, Southern blot analysis, HBV-DNA integration, pathogenesis of liver disease, viral oncogenesis

Since there has been no tissue culture system available to study hepatitis B virus infection, it has been difficult to elucidate the properties of the virus, its replication, and function. However, with cloning of the HBV genome which was first permitted in 1979, major advances have been made in elucidating the structure of HBV-DNA [1–4] and in understanding the relationship between hepatitis B virus (HBV) infection, chronic liver disease, and hepatocellular carcinoma. Within 6 months after the initial cloning, the entire sequence of HBV-DNA was determined [5–7], and shortly thereafter integrated HBV-DNA was identified in a human hepatocellular carcinoma cell line, PLC/PRF/5, [8–10]. Subsequently, integrated HBV DNA was found in gross hepatocellular carcinomas from patients in Africa, Europe, the United States, and Asia [11–16], as well as in several other human hepatocellular carcinoma cell lines

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[17]. The most striking feature of HBV-DNA in hepatocellular carcinoma tissue is that it is integrated into the host genome in every case in which it is present in the tumor cell (providing that nucleic acid extraction procedures are satisfactory). In some cases, in addition to integrated HBV-DNA, free viral DNA has been found [16]. Both free and integrated viral DNA have been found in liver tissue adjacent to the tumor [13–16]. Integrated HBV-DNA in liver may show a hybridization pattern that is identical to or distinct from that observed in the tumor [13–16,18,19]. In patients who have persistent HBV infection (chronic carriers) with or without histologic evidence of liver disease, integrated HBV DNA has also been identified [15,16,18,19]. These results suggest that integration of HBV DNA into the hepatocyte precedes the onset and may be related to development of hepatocellular carcinoma in man.

RESULTS

Integrated HBV-DNA in the Human Hepatocellular Carcinoma Cell Line PLC/PRF/5

Our initial studies of HBV-DNA in human cells were performed in the PLC/PRF/5 cell line derived by Alexander and co-workers [20] from a primary hepatic neoplasm in a 24-year-old Mozambican male who was a hepatitis B virus surface antigen (HBsAg) carrier. The cell line produced HBsAg [20–23] and contained 4–6 genomes of HBV in integrated form [8–10]. However, there was no evidence for extrachromosomal (ie, nonintegrated) HBV-DNA or for virus production or replication.

Figure 1 illustrates the methods used to identify integrated HBV-DNA in extracts from liver or tumor tissue. Total DNA is extracted from cells by gentle homogenization; homogenized material is digested with proteinase K followed by SDS-phenol:chloroform:isoamyl alcohol extraction, RNAse digestion, repeat phenol extraction, and ethanol precipitation. The final DNA is applied to a 0.8% agarose gel before or after digestion with restriction endonucleases Hind III (which recognizes no site in HBV-DNA) or EcoR1 (which recognizes one site in the HBV genome). Electrophoresed material is transferred to a nitrocellulose or DBM-cellulose sheet, hybridized to purified cloned HBV-DNA labeled by nick translation with (^{32}P) dATP and dCTP to a specific activity of 2–4 $\times$ 10^8 cpm/μg, and autoradiographed to identify specific molecules containing HBV-DNA sequences. By comparing the size of molecules containing HBV-DNA sequences before or after digestion with Hind III or EcoR1 with the migration position of cloned, full-length, linear, double-stranded HBV-DNA ($\sim$3.3 kb), the integration status of these molecules can be established. If HBV-DNA sequences are not integrated, undigested and Hind III–digested material will produce the same HBV-DNA banding pattern. If HBV-DNA sequences are integrated, undigested material will produce no discrete bands (only a diffuse smear in the high molecular weight region), whereas Hind III–digested material will produce a specific banding pattern, assuming that HBV-DNA is integrated into specific sites in the recipient cell genome. Since HBV-DNA contains no cleavage site for Hind III, all integrated molecules containing HBV-DNA sequences should be larger than the HBV genome (3,250–3,300 base pairs), except for integrated fragments of HBV-DNA which might still be detected in molecules smaller than 3,300 base pairs (irregular line in Fig. 1 under Hind III). Whenever possible, EcoR1 analysis is performed to confirm integration and reduce ambiguities created by concatomers,

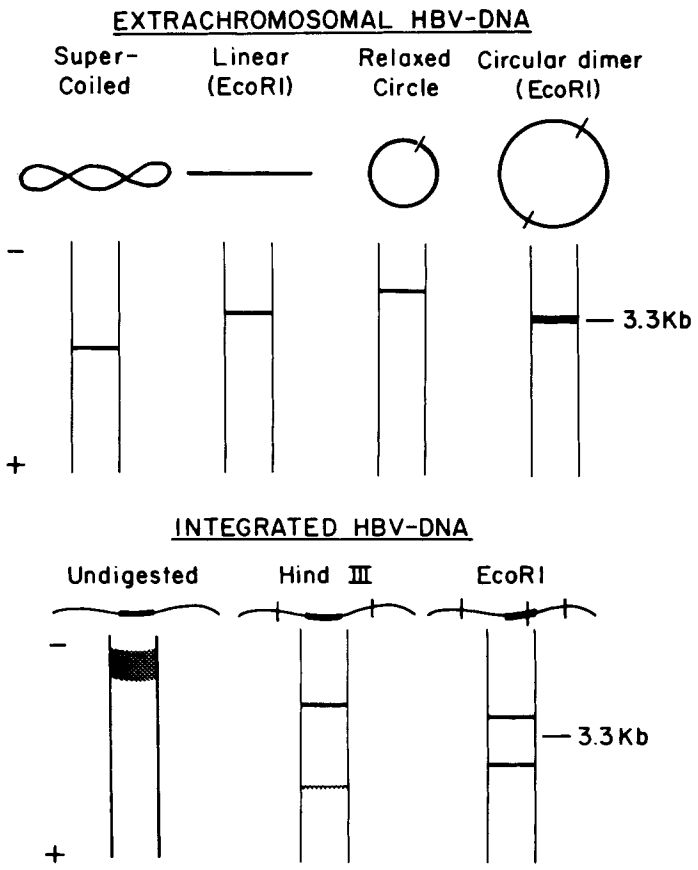

Fig. 1. Strategy for restriction enzyme analysis of HBV-DNA (see text for further details).

tandem repeats, and various circular forms of HBV-DNA (Fig. 1, upper portion). After EcoR1 digestion, all extrachromosomal HBV-DNA molecules should migrate as a single linear species of 3,250–3,300 base pair length. Thus, all HBV-DNA-containing molecules of sequence length greater than 3,250–3,300 base pairs after EcoR1 digestion must contain covalently linked DNA sequences derived from the host cell (Fig. 1, lower portion). The only exception would be concatomers of HBV-DNA in which the EcoR1 site is deleted, or genomes containing partial duplications, inversions, or rearrangements and to date no such molecules have been identified in human HBV infection.

Figure 2 shows the size distribution pattern of molecules containing HBV-DNA sequences in the PLC/PRF/5 cell line. With undigested DNA, hybridization occurred within the high MW region corresponding to cellular DNA, as shown in Figure 2, lane A. Free or nonintegrated HBV-DNA should appear as a sharp band at 3,200–3,300 base pairs, but no hybridization was present in this region (Fig. 2, lane A). With restriction enzyme Hind III (Fig. 2, lane B), 5–6 specific bands were present. One band below 3,300 base pairs represents a fragment of HBV-DNA integrated into PLC/PRF/5 cellular DNA. The other five bands are of molecular weight greater than 3,300 base pairs. It should be mentioned that this technique does not determine whether individual high MW bands with HBV-DNA sequences contain the complete

or only a partial copy of the HBV genome. Other studies, however, have reported that the entire sequence of the HBV genome is present in PLC/PRF/5 cells [10]. Figure 2, lane C, shows the digestion of PLC/PRF/5 cell line DNA with restriction enzyme EcoR1 which can produce one or two bands from each integrated HBV-DNA molecule depending on how close or far the EcoR1 site is located from the integration site. On the average, the EcoR1 bands containing HBV DNA sequences are smaller than the Hind III bands (Fig. 2, lane B vs lane C).

HBV-DNA Studies in Percutaneous Liver Biopsies From Chimpanzees

By adapting DNA extraction methods to small amounts of tissue, hybridization studies for HBV-DNA sequences were performed with DNA isolated from percutaneous liver biopsies from chimpanzee and human HBV carriers. In chimpanzee liver, only free viral DNA could be identified; there was no evidence for integration [24], even though four of the five animals studied showed histologic evidence of chronic hepatitis. Two molecular forms of HBV-DNA were identified in chimpanzee carrier

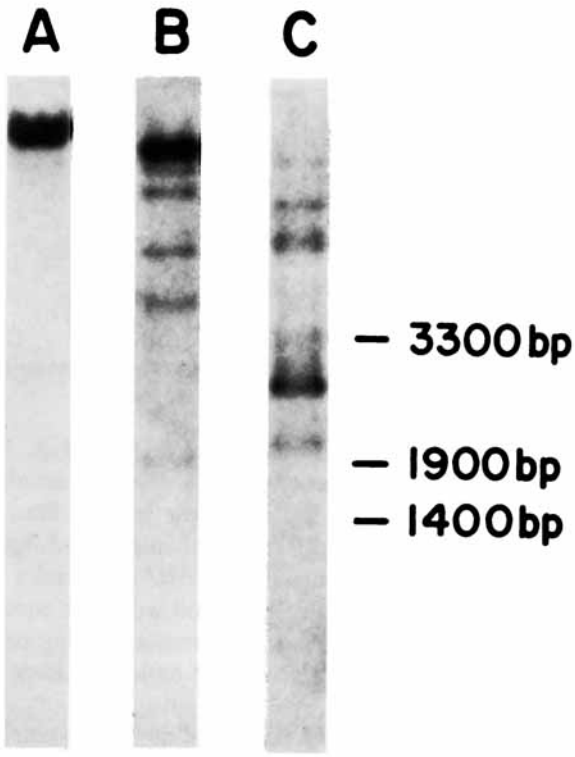

Fig. 2. Hybridization pattern of HBV-DNA sequences in the human hepatocellular carcinoma cell line, PLC/PRF/5. Total DNA was extracted from subconfluent cells in tissue culture as previously reported [9]. DNA (20 μg each lane) was applied to a 0.8% agarose slab, gel, electrophoresed, transferred to a DBM-cellulose filter sheet, and hybridized to ^{32}P-labeled clone purified HBV-DNA (specific activity 2–4 $\times$ 10^8 cpm/μg DNA). Autoradiography was used to identify bands containing HBV-DNA sequences. [For further details, see references 9, 12, and 15.] A) Undigested DNA. B) Hind III–digested DNA. C) EcoR1-digested DNA. The migration position of purified, full-length, linear HBV-DNA ($\sim$ 3,300 base pairs) and Bam H1 restriction fragments of HBV-DNA, 1,900 base pairs (containing HBcAg gene sequences), and 1,400 base pairs (containing HBsAg gene sequences) are shown on the right.

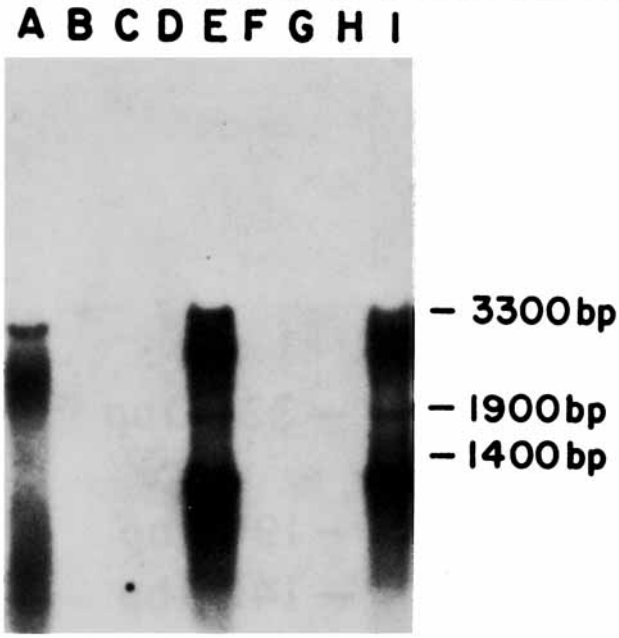

Fig. 3. Hybridization pattern of HBV-DNA sequences in the liver of patients with chronic persistent or chronic active hepatitis. DNA extracts from a portion of a percutaneous liver biopsy of patients undergoing histologic evaluation for the first time for suspected chronic liver disease were studied. Patients A, E, and I were serologically positive for HBsAg, and patients B, C, D, F, G, and H were negative for HBsAg. In some of these cases, anti-HBs and/or anti-HBc was present in the serum. In all patients, liver disease had become apparent within the recent past, and in those patients who were HBsAg-positive, the carrier state was known to be present for less than 2 years. Each DNA extract was digested with Hind III prior to electrophoresis.

liver [25]. These molecules were distinct from the incomplete, double-stranded genome of the Dane particle—one representing a completely double-stranded, supercoiled HBV-DNA molecule migrating at 2.3 kb (form I), and the other a "nicked" or relaxed circular form of the complete HBV genome migrating at 4.0 kb (form II).

Lack of HBV-DNA integration may be the reason that an increased incidence of hepatocellular carcinoma has not been found in chimpanzee carriers. In one chimpanzee with active chronic hepatitis, integrated HBV-DNA was still not identified even when 50 or 100 μg of DNA was applied to the gel (unpublished observations). Our present feeling is either that HBV-DNA is not integrated in these animals or that integration is present in a diffuse manner throughout the host genome or in such a low percentage of cells that these molecules cannot be visualized by the techniques employed.

HBV-DNA Sequences in Human Hepatitis B Virus Carriers

In patients who were hepatitis B carriers for less than 2 years, we did not find integrated HBV-DNA in percutaneous liver biopsies, regardless of the form or state of liver disease [15]. Hybridization analysis of Hind III–digested DNA from the liver of three of these patients, together with specimens from six other patients who were non-HBsAg carriers with comparable liver disease, showed HBV-DNA of 3,300 base pair length or more rapid migration (other "vegatative" forms of extrachromosomal HBV DNA) only in the HBsAg carriers (Fig. 3). Undigested, Hind III–digested, and

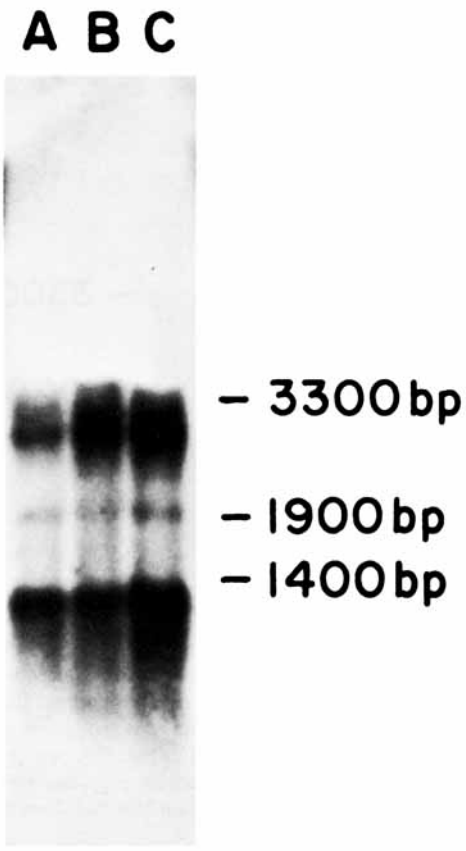

Fig. 4. HBV-DNA hybridization pattern in a 31-year-old male with chronic active hepatitis, known to be an HBV carrier for less than 1 year. A) Undigested DNA; B) Hind III-digested DNA; C) EcoR1-digested DNA. In this experiment ~ 10 μg DNA was used for each lane.

EcoR1-digested liver DNA from one short-term carrier ($HBeAg^+$) analyzed side by side in the same gel showed no evidence for specific HBV-DNA integration (Fig. 4). However, as mentioned above, these results do not rule out the possibility of random or multiple site integration in these cells at relatively low frequency, or even unique site integration in a very small subpopulation of hepatocytes. This could be excluded only by cloning high MW DNA from the liver of such carriers and screening for recombinants containing HBV-DNA sequences in molecules larger than genome length. These would then, of course, need to be characterized for both viral and human cellular sequences.

In five long-term HBV carriers, integrated HBV-DNA was found. Hybridization analysis showed diffusely integrated HBV-DNA in the liver from two of these patients (Fig. 5). Three other patients showed integrated HBV-DNA in unique bands, even though histologic analysis of the liver showed no evidence for chronic liver disease (Fig. 6). It should be remembered, however, that only limited material was available for analysis from these individuals, and direct evidence for integration of

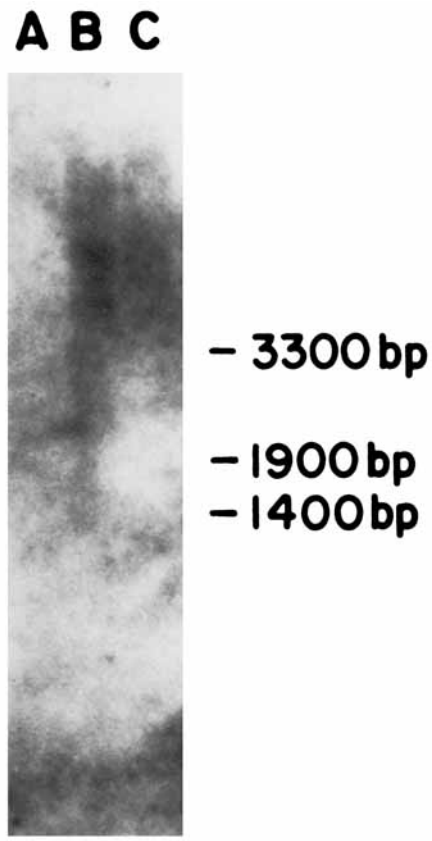

Fig. 5. HBV-DNA hybridization pattern in two long-term HBsAg carriers and in one control specimen. Total DNA was extracted from a portion of the liver biopsy of two young adult males known to be HBsAg-positive for at least 8 years (lanes B and C) and one other patient with primary biliary cirrhosis serving as a control (lane A). One of the surface antigen carriers was $HBsAg^+$, $HBeAg^+$, and anti-HBc-positive with chronic active hepatitis (lane B), and another patient was $HBsAg^+$, anti-HBe^+, and anti-HBc^+ with "ground-glass" cells in the liver but no evidence of chronic active hepatitis (lane C).

HBV-DNA has not been obtained by cloning these molecules and demonstrating covalently linked host and viral sequences. Such analysis could also address the question of HBV-DNA "superforms"—ie, higher MW forms of HBV-DNA containing only viral sequences but with duplications, inversions, deletions, and other rearrangements, as found recently in woodchuck and ground squirrel chronic carriers of viruses related to hepatitis B virus [26,27].

There was a "ground-glass" appearance to the cytoplasm in hepatocytes from most long-term HBV carriers, and this finding has been correlated with high production and/or intracellular accumulation of HBsAg [28]. The finding of integrated HBV DNA in liver tissue from some patients with "ground-glass" hepatocytes suggests that HBsAg production may be directed from an integrated HBV-DNA template rather than free virion DNA. These "ground-glass" cells containing HBsAg may therefore represent a subpopulation of hepatocytes that are nonpermissive for viral replication and which may be or may ultimately become dysplastic or premalignant.

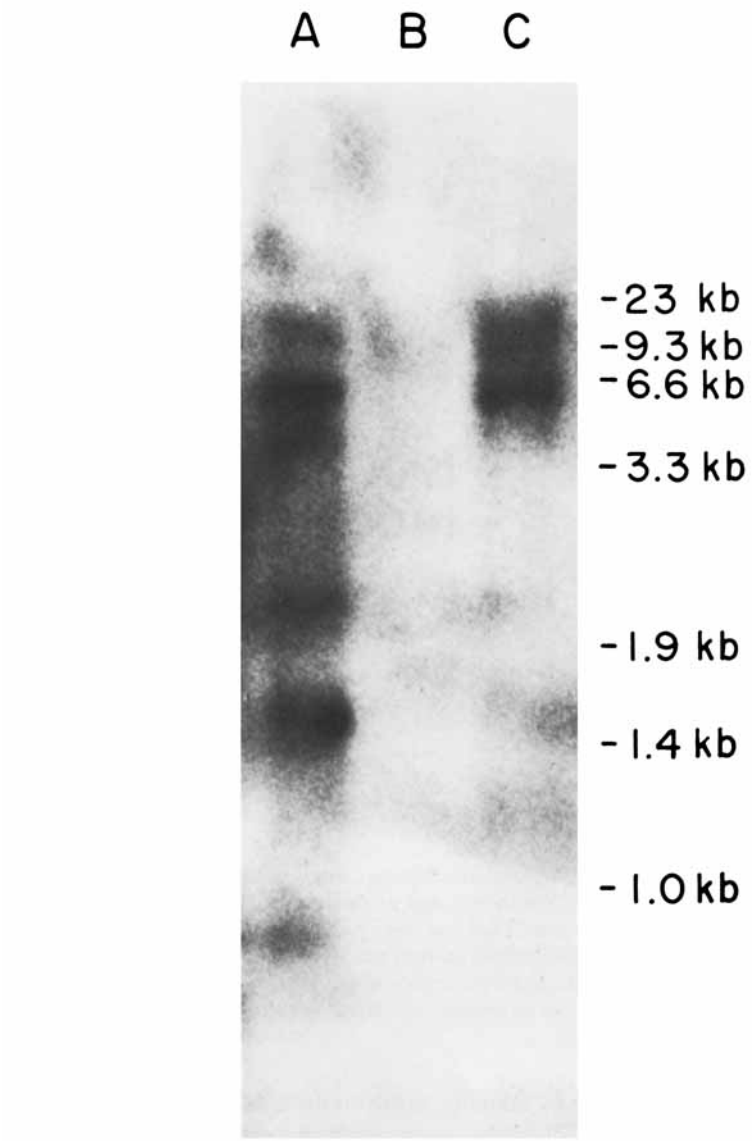

Fig. 6. HBV-DNA integration pattern of long-term HBsAg carriers from Greece. The patients illustrated in lanes A and C were long-term HBsAg carriers who were anti-HBe^+ and anti-HBc^+. Both patients revealed "ground-glass" hepatocytes and intracellular HBsAg on immunohistochemical staining of liver, but there was no histologic evidence of significant chronic liver disease. The markers on the right indicate the migration positions of restriction fragments of bacteriophage λ of known sequence length. In patient A, there is a small amount of free viral DNA (~ 4.0 and 2.3 kb) as well as two bands of integrated HBV-DNA, whereas in patient C all of the HBV-DNA appears to be integrated. DNAs were digested with Hind III prior to electrophoresis. Lane B contained the liver DNA extract from a patient with chronic liver disease who was not an HBV carrier as determined by analysis of serologic markers.

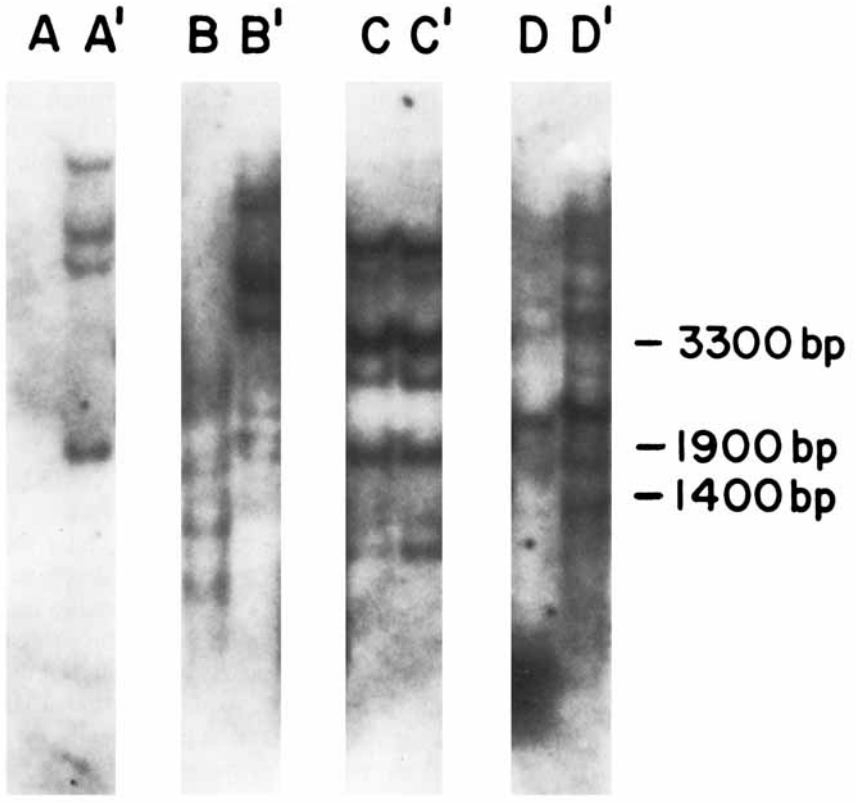

Fig. 7. Hybridization pattern of liver and tumor from four South African patients with hepatocellular carcinoma. In each pair of specimens (AA', BB', CC', and DD'), the left-hand member is liver and the right-hand member is adjacent tumor. Cross contamination of the specimens of tumor versus liver was not apparent as judged by histologic analysis of companion specimens. [Details concerning these specimens and their analysis are given in reference 15.]

Side-by-side analysis of hepatocellular carcinoma tissue and nontumorous liver regions from the same patient revealed variable results. We have found [15] either the same or a different pattern in liver vs tumor in individual cases of paired liver and tumor specimens (see Fig. 7). Histologic analysis was performed to rule out cross contamination of liver and tumor tissue. These studies were performed with specimens from South African blacks, where hepatocellular carcinoma develops in young patients often without evidence of chronic liver disease and progresses to a fatal outcome within months. Brechot et al [16] and Gerin and co-workers [13] reported different integration patterns in liver and tumor from the same patient, although some bands may be in common [13,19]. These patients were of French or Oriental extraction, in whom tumors usually develop on a background of chronic active hepatitis and/or cirrhosis, and tumor progression occurs more slowly.

DISCUSSION

From various studies it appears that DNA extracts from liver biopsies of HBsAg carriers show integrated HBV-DNA [15,16,18]. Precisely when integration occurs, however, has not been elucidated, and if only small amounts of HBV-DNA are integrated, or if early in the course of disease integration sites are spread throughout the host genome, this will be difficult to determine. During progression of the carrier state, it is possible that hepatocytes expressing all viral components are continuously being removed or selectively destroyed by viral cytopathic effects or host immunological responses. As suggested by Eddleston [29], the latter may involve HBcAg-anti-HBc complexes and cytotoxic T lymphocytes or recognition of components other than HBsAg during virion production. Therefore, hepatocytes containing integrated HBV-DNA, but which are not expressing full virions, may be preferentially retained. This could lead to an increased proportion of integrated HBV-DNA in longer-term carriers, and would correlate with an increased ability to detect integrated HBV-DNA in HBsAg carriers who are anti-HBe rather than HBeAg-positive. This could lead to two stages in the integration process—early nonspecific integration in which HBV-DNA is diffusely distributed throughout the host genome (stage I), and later specific integration in which cells containing integrated HBV-DNA in unique sites have been selected for survival and/or multiplication by host or other factors related to immune resistance or increased cell division rates (stage II). A similar selection mechanism for hepatocytes containing integrated HBV-DNA could occur during antiviral therapy of carriers with Ara-A and/or interferon [30]. This could explain continued HBsAg expression in most patients in whom viral replication has been permanently inhibited by these agents [31], and could render these patients at increased risk to develop hepatocellular carcinoma later [30].

Integration of HBV-DNA in Relation to Potential Oncogenicity

All published studies are consistent with the interpretation that integration of HBV-DNA into the hepatocyte genome precedes the development of hepatocellular carcinoma by months or years. The exact time at which integration occurs, the relationship between integration and HBV serum markers (as well as their progression), and the frequency with which integration leads to development of hepatocellular carcinoma require further study. From epidemiologic evidence [32,33], it would appear that 10 years of the HBV carrier state, and more often 20–30 years, is usually required to develop hepatocellular carcinoma. Therefore, individuals developing the carrier state in the first few years of life may be at greatest risk to develop hepatocellular carcinoma.

The finding of integrated HBV-DNA in many hepatocellular carcinomas and in all hepatocellular carcinomas in which viral sequences are present suggests strongly that HBV is oncogenic or stimulates oncogenesis. In various animal models and tissue culture systems infected with "tumor" viruses, the presence of integrated viral DNA (proviral DNA in the case of RNA tumor viruses) often correlates with the ability of these viruses to transform cells [34,35]. Cells that are transformed and contain integrated viral genomes are generally "nonpermissive" for viral replication, as found for the PLC/PRF/5 cell line. In contrast, when cells that are permissive for replication of the same virus are infected, the virus does not become integrated and the cells are not transformed. Therefore, whether HBV persistence and integration lead to cellular

transformation needs to be explored. Clearly, the presence of a unique or specific HBV-DNA integration pattern in gross liver and tumor specimens (regardless of whether these patterns are the same or different) suggests that the bulk of cells in these specimens are derived from single progenitor cells that contain integrated HBV-DNA and which have been stimulated to divide. In some instances, the histologic appearance of these abnormal cells may be difficult to distinguish from normal hepatocytes or cells within regenerating liver nodules.

Proposed Events During Integration of HBV-DNA Transformation of Hepatocytes and Development of Hepatocellular Carcinoma

A schematic model of events leading to transformation of hepatocytes during HBV infection is presented in Figure 8. Whether integration of HBV-DNA occurs during acute HBV infection or follows establishment of persistent HBV infection has not been fully clarified. Populations of hepatocytes which contain integrated HBV-DNA but have not been stimulated to divide (ie, are not transformed) would be expected to contain HBV-DNA in many different sites randomly distributed in the host genome (initial multisite integration step or stage I). This would produce a diffuse or nonspecific hybridization pattern (as found in some short-term HBV carriers), and may lead to cellular transformation with activation of cell division. Further selection of hepatocytes containing integrated HBV-DNA in specific sites in which cell division or resistance to removal by host surveillance mechanisms is favored could lead to enrichment of cells containing integrated HBV-DNA (unique-site integration step, or stage II). Once a unique configuration of integrated HBV-

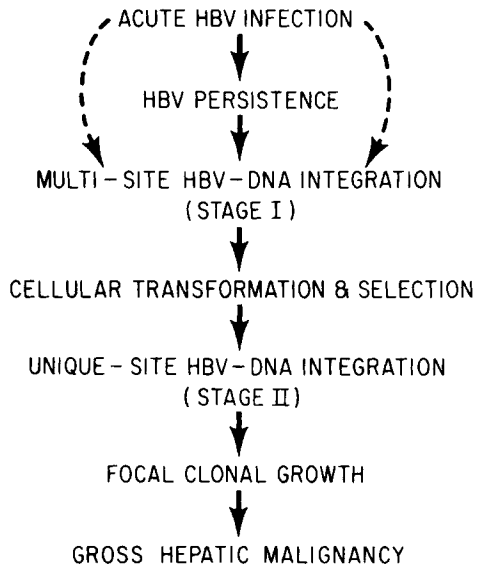

Fig. 8. Schematic model representing a working hypothesis to explain existing patterns of integrated HBV-DNA in liver tissue and hepatocellular carcinoma in HBV carriers. A potential relationship between integration of HBV-DNA, cellular transformation, and development of hepatocellular carcinoma is proposed (see text).

DNA bands emerges, the most likely explanation is that the bulk of cells from which this DNA was derived represent a monoclonal population of transformed hepatocytes (a focal clonal growth). Transformed hepatocytes with different HBV-DNA integration patterns and/or different cellular growth rates (varying oncogenic potential) may develop from individual subclones within a primary focus or from separate foci to form gross hepatic malignancies. Independent growth of such foci may produce tumors with different integration patterns and different histologic features in the same liver. Such a mechanism could explain the apparent multifocal origin of hepatocellular carcinoma in some patients, as well as the finding of different hybridization patterns in liver or tumor cells in a given individual. This mechanism also implies that factors other than integration of HBV-DNA and cellular transformation may be critical in development of gross hepatic malignancy. Other possible explanations for the same integration pattern in liver and tumor in some cases are 1) that DNA was taken up "en bloc" from the extracellular environment from lysed hepatocytes or was transferred from cell to cell by fusion or perhaps as chromosomal fragments or double minutes (which we have observed in the PLC/PRF/5 cell line); 2) that favored sites or more stable sites for HBV-DNA integration exist and vary from individual to individual; or 3) that nests of tumor cells within the liver specimens were missed on histologic evaluation.

Cellular Oncogenes and Other Considerations

The above model represents a working hypothesis to explain existing patterns of HBV-DNA integration in liver and hepatocellular carcinoma; many other interpretations of the data are possible. Although these results provide molecular evidence for a causal relationship between HBV-DNA infection and hepatocellular carcinoma, they do not prove that HBV-DNA is oncogenic, since this hypothesis does not imply that HBV genes themselves are oncogenic. It is possible that the primary influence of HBV-DNA integration is to modify, activate, or rearrange cellular genes or to act in concert with cellular genes or other agents (cellular, hormonal, or chemical) to produce cellular transformation.

In certain animal cell RNA tumor virus (retrovirus) systems, "onc" genes appear to be derived from cellular origin [36–38], perhaps by classical recombination or by integration of proviral DNA adjacent to specific cellular genes followed by reexcision of the viral DNA together with cellular DNA and reorganization of the viral genome. In retrovirus systems, cotranscription of viral and cellular sequences from integrated viral DNA occurs during oncogenic transformation by a "promotor insertion" mechanism, whereby transcription begins at an integrated viral promotor signal and reads through into cellular sequences [39]. How this process relates to oncogenicity has not been clarified.

One possible mechanism might be placement of an active HBV promotor adjacent to a repressed cellular oncogene, resulting in expression of that gene. Another possibility is that during persistent HBV infection, cellular oncogenes become incorporated into replicating HBV mutant genomes similar to those observed in animal virus systems, such as defective substituted variants of SV40 [40], and are subsequently transferred to susceptible hepatocytes. This could lead to enrichment of such sequences, and if these molecules are integrated into the recipient cell genome in the appropriate site, this could lead to enhanced expression of transformation properties. By such a mechanism HBV might represent a convenient passenger for

oncogenic cellular sequences by serving as a mobile DNA element or mediator of gene transfer and/or enrichment. In the PLC/PRF/5 cell line, we have identified a poly A^+ viral transcript (presumably a viral mRNA) which contains HBsAg gene and cellular sequences in covalent linkage (presumably derived from an integrated viral-cellular transcription unit). Currently, in collaboration with Dr Jesse Summers, Fox Chase Cancer Center, Philadelphia, we are analyzing cellular sequences flanking HBV genes in human hepatocellular carcinomas and in the PLC/PRF/5 cell line. We have also begun to study phenotypic transformation of animal cells with various types of cloned HBV DNA including cloned integrated HBV-DNA, as well as these flanking cellular sequences themselves. With tissue culture and recombinant DNA methods currently available, it should be possible to test the various hypotheses presented above and their specific implications in HBV-related hepatic oncogenesis.

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