Journal of Cellular Biochemistry 20:293–301 (1982)
Tumor Viruses and Differentiation 173–181

Organization and Expression of Hepatitis B Sequences Cloned From Hepatocellular Carcinoma Tissue DNA

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We have constructed a phage λ library of liver DNA fragments from West African patient who died of liver failure due to advanced hepatocellular carcinoma. Four hepatitis B virus (HBV) DNA-carrying recombinants have been isolated, one clone (λIA22) being analyzed in greatest detail. It contains approximately 3.8 kb of HBV DNA without detectable deletions or rearrangements. One site of integration lies close to the nick in free viral DNA. The restriction map of the HBV sequences is close to those published for the ay subtype. Coconvection of mouse Ltk$^-$ cells with λIA22 and cloned thymidine kinase gene results in the expression of gene S and the excretion of hepatitis B surface antigen (HBsAg) particles into the culture supernatant.

Key words: hepatocellular carcinoma, hepatitis B virus, integrated DNA, gene cloning, tk cotransformation, HBsAg expression

The relationship of prior hepatitis B viral (HBV) infection to the subsequent development of hepatocellular carcinoma (HCC) in humans remains unknown. However, many studies have indicated a correlation between the geographical distribution of HBsAg, the viral surface antigen, and HCC [1,2]. A prospective study among Chinese chronic carriers of HBsAg have shown that the relative risk of HCC is about 200 times greater than among noncarriers [3,4], a phenomenon believed to be unrelated to dietary factors or lifestyle [5]. Furthermore, there is an analogous virus and attendant hepatocellular carcinoma found in the woodchuck [6,7].

More recently, using molecular hybridization techniques, HBV DNA has been found to be integrated into the host cellular DNA of the neoplastic hepatocyte [8–10]. It was also found that HBV DNA was integrated into the cellular DNA of chronically infected liver [9,11] and in a few cases of acute hepatitis [9]. Interestingly, Bréchot et al [9] found that in cases where the cellular DNA was extracted from both the

Received June 29, 1982; accepted July 22, 1982.

tumoural and nontumoural parts of the same liver the HBV hybridizing Southern blot patterns were different.

Searching for HBV antigens within the neoplastic tissue has shown that most often one does not find HBsAg or HBcAg (hepatitis B core antigen) in the tumourous cells, whereas the nontumourous hepatocytes are positive for HBsAg (or HBcAg) by direct immunofluorescence [12,13]. The generally low levels of serum HBsAg in patients with HCC [14] might reflect the absence of HBsAg expression in the neoplastic cells, the HBsAg present being the product of the few nonneoplastic hepatocytes remaining.

To investigate both the organization of integrated HBV sequences and the control of HBsAg expression in the neoplastic hepatocyte we have constructed a phage bank of the total liver DNA from a patient who died of liver failure due to HCC [8]. We report here some preliminary studies.

MATERIALS AND METHODS

DNA

Patient 53 (a West African black) from whom the liver DNA was extracted was serum positive for both HBsAg and anti-HBc (antibody to HBcAg) [9]. By direct immunofluorescence the liver was negative for both HBsAg and HBcAg. Five bands were found in a Southern blot of a HindIII digest using cloned HBV as a probe. Phage λL47.1 was kindly given by Dr. W. Loenen [15].

Bacteria

Strains LA101 [16] and later JC5495 [17] were used for phage infections. For in vitro packaging the strains BHB2688 and BHB2690 [18] were provided by Dr. John Collins.

Cloning

In brief cellular DNA was partially digested with MboI fractionated on a sucrose gradient, the 10–20 kb fractions being pooled and ligated into BamHI cleaved phage arms. In vitro packaging was made according to the method of Ish-Horowicz [20] or Hohn [18].

Transformation of Ltk$^-$ Cells

About 5 μg of cloned phage DNA were coprecipitated with 5 μg of sonicated herring DNA and 0.2 μg of the plasmid pAGO (containing the cloned herpes simplex virus-1 thymidine kinase gene [21]) by the calcium phosphate technique [35]. The molar excess of cloned phage DNA to pAGO is thus 3.5:1. Mouse Ltk$^-$ cells were transformed with the precipitate and grown on HAT selective medium as described previously [22]. Several transformants, both HBsAg producers and nonproducers, were selected and cloned.

Purification of HBsAg From the Culture Medium

The supernatant of an HBsAg-producing clone was first made 1% in sucrose and the proteins pelleted for 5 hr at 34,000 rpm (Beckman SW41). The pellet was taken up in 1X phosphate buffered saline (PBS) and made up to a density of 1.2 gml^{-1} with CsCl. After centrifuging for 22 hr at at 44,000 rpm (Beckman Ti60) the

gradient was fractionated and tested for HBsAg activity. HBsAg positive fractions were pooled, dialyzed against 1× PBS and centrifuged through a 5–20% sucrose/PBS gradient for 2.5 hr at 39,000 rpm (Beckman SW41). HBsAg positive fractions were stored in sucrose at 4°C. HBsAg activity was detected using the AUSRIA^{125}I diagnostic test of Abbot Laboratories.

Electron Microscopy

A Phillips 201 transmission electron microscope was used. An acceleration voltage of 60 kV gave a nominal magnification of 200,000×. Grids (400-mesh) were covered either with colloidon or carbon films. HBsAg particles were absorbed onto the grids for 10 min, drained, stained using 1% uranyl acetate and shadowed. Immunoelectron microscopy was performed by absorbing both undiluted antigen (5λ, in sucrose) with rabbit anti-HBsAg serum (5λ, Behring) for 10 min at room temperature directly onto the prepared grids. After washing in 10 mM Tris Cl pH 7.5 and 10 mM $MgSO_4$, the grids were drained, stained with uranyl acetate, and shadowed.

RESULTS

An unamplified library of some 650,000 plaques was screened for HBV DNA sequences using a modification of the Grunstein and Hogness in situ hybridization technique [34]. As probes we used ^{32}P nick-translated plasmid-cloned HBV (pCP10 [22]) or M13 mp7 cloned HBV [19] between different plaque purifications. This is particularly important when using DNA from autopsy samples. These often contain proliferating bacteria and since many patients have been treated with antibiotics, antibiotic resistance elements are often amplified. Thus if the whole plasmid or phage DNA containing HBV is used as a probe, or there are contaminating plasmid or M13 sequences, the risk of purifying false positive clones is real. Positive plaques were subsequently purified three times.

Four clones carrying HBV sequences have been found. One clone (λIA22) has been studied in the most detail. It carries a 10.5 kb insert of which approximately 3.8–4 kb hybridizes to HBV probes. The restriction map of λIA22 is shown in Figure 1.

The HBV hybridizing sequences contain unique EcoRI, PstI, XhoI, and SacI restriction enzyme sites, two XbaI sites, and three BglII and BamHI sites. There are no SalI, SmaI, ClaI, KpnI or HindIII sites, none of which have so far been shown to

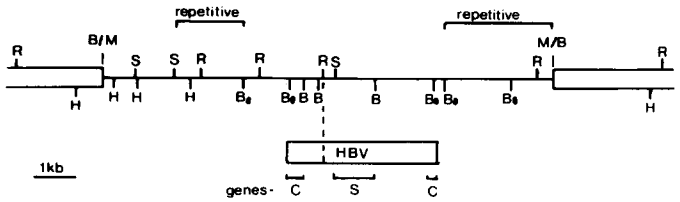

Fig. 1. Restriction map of clone λIA22. Left and right blocks represent phage arms. Restriction enzyme sites are: R, EcoRI; H, HindIII; B, BamHI; B_1, BglII; S, SacI; M, MboI. The HBV hybridizing region is marked HBV and those regions hybridizing to gene C, gene S, and repetitive DNA probes are marked C, S, and repetitive, respectively.

cleave any HBV sequence [23]. By comparison of the restriction maps of six clones sequences [23,24] it is clear that the HBV sequences carried by clone λIA22 are closest to those of the ay subtype. However differences still exist suggesting that there are still further variants witin this subtype.

By using a core antigen gene (gene C [19]) specific probe (BglII fragment C of the ayw subtype [25] cloned in pBR322 [C. Pourcell, unpublished data]) and a surface antigen gene (gene S [19]) specific probe (plasmid pPCXbal [26]) these corresponding sequences were located amongst the HBV hybridizing sequences. The gene C probe strongly hybridized to sequence at the left end of the integrated HBV. There was a weaker hybridization to the right end of the λIA22 HBV sequences. However using the small BqlII fragment at the right end of the λIA22 HBV sequences (Fig. 1) subcloned into pBR322 as a probe, it was shown that the fragment hybridized, albeit weakly, to HBV sequences at the end of gene C (data not shown).

Gene S sequences hybridize to sequences right of the EcoRI site that lies within the HBV sequences (Fig. 1). This would, by analogy with other HBV restriction maps, indicate that the organization and sense of the C and S genes are the same as in free viral DNA [27]. This assumes however that the HBV DNA is present as a continuous unrearranged sequence, an observation confirmed by heteroduplex analysis of phage DNA-cloned HBV DNA. Thus by convention the sense of the HBV genes would be left to right (see Fig. 1) [19] and therefore we denote the left site of integration as the 5′ site, and the right side, the 3′ site of integration.

The nature of the flanking sequences was examined by nick-translating total human liver DNA (extracted from a HBV negative patient, both serologically and by blotting); only repetitive sequences can thus be detected. We found that both 5′ and 3′ flanking sequences contained repetitive sequences as shown by Figure 2 and diagrammed by Figure 1. The 3′ flanking sequences hybridized most strongly but this could be due to a clustering of repetitive sequences in this region.

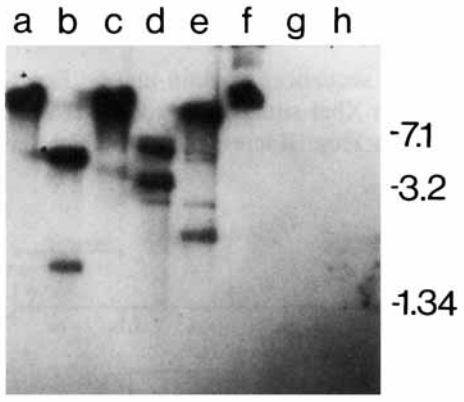

Fig. 2. Southern blot of λIA22 DNA cut with various restriction enzymes and probed with total human DNA. Lanes a–f: λIA22 + BamHI, BglII, SacI, AvaI, Pst I, and uncut, respectively. Lane G: HBV-containing plasmid pTKH9 + AvaI [19] and lane h: λHBV-1 + EcoRI [36]. The faintly hybridizing bands in lanes d and e are due to a small proportion of party digested material. Size markers are in kilobases pairs.

To ascertain whether or not phage λIA22 carried HBV genes capable of being expressed we coconvected mouse Ltk$^-$ cells with λIA22 DNA and the cloned herpes simplex type-1 thymidine kinase gene. After selection on HAT medium for 15 days the culture supernatant was found to be HBsAg positive by radioimmunoassay (AUSRIA II). Six colonies were cloned. One clone, 2a, has expressed continuously HBsAg after passaging six times, whereas clone 1c, originally positive for HBsAg proved unstable with respect to HBsAg expression and ceased after two passages. The four other clones were all negative for HBsAg expression.

The total DNA of all six clones was extracted, digested by HindIII, electrophoresed on 0.8% agarose gel, and Southern blotted to a nitrocellulose filter [28]. When hybridized with an HBV probe, only clone 2a contained HBV sequences (Fig. 3). The HindIII pattern for clone 2a reveals a 11.4 kb HBV hybridizing fragment, identical in size to that of the HindIII cleaved λIA22 DNA. Thus the transfection of λIA22 DNA into clone 2a cellular DNA has occurred without rearrangement of the cloned insert. No free viral DNA, which would be manifested by a band at 3.2 kb, was detected in the cells of clone 2a.

Purification and Analysis of HBsAg

The HBsAg activity in the L-cell supernatant was purified on the assumption that HBsAg particles were excreted. Accordingly the purification procedure of Dubois

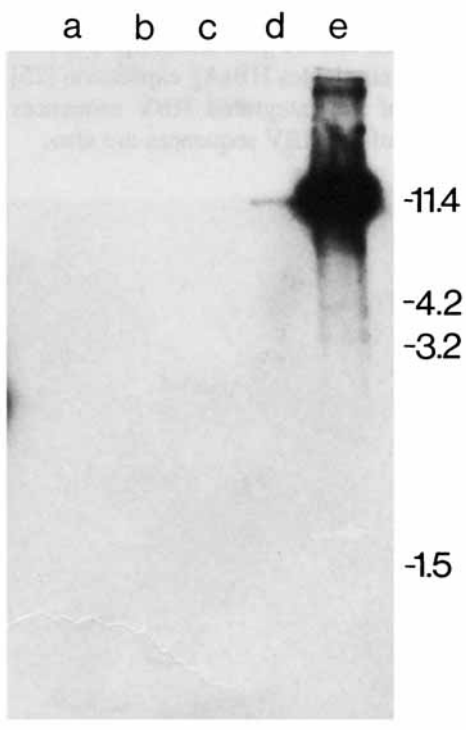

Fig. 3. Southern blot of HindIII digests of cellular DNA extracted from six tk positive clones after cotransformation by pAGO and λIA22 DNAs. Probe plasmid cloned HBV-DNA. Lanes a and b: clones 2b and 1a (HBsAg negative producers); lane c: clone 1c (an unstable HBsAg producer, finally negative); lane d: clone 2a (a stable HBsAg producer); lane e: λIA22 DNA. Size markers are in kilobase pairs.

et al [22] was used with minor variations. HBsAg activity, as determined by radioimmunoassay, was found in CsCl fractions at a density of 1.2 gml^{-1}, typical of the density of human HBsAg 22-nm particles [29]. In an identical manner, the supernatant from clone 1c was analysed. No HBsAg activity was found in the p = 1.2 gml^{-1} fractions nor elsewhere in the CsCl gradient.

Undiluted HBsAg from the sucrose gradient was immunoabsorbed with rabbit anti-HBsAg serum directly onto the grids. We used 1% uranyl acetate as negative stain since it gave better results than phosphotungstic acid in our hands. Figure 4 shows an electron micrograph of the immunoprecipitated HBsAg particles. The particles are clearly seen (arrow). They resemble in morphology human HBsAg 22 nm particles; however their mean diameter is somewhat less (approximately 15 nm). No such particles were found either in the analogously purified supernatant of clone 1c (HBsAg negative) or in the rabbit antiserum alone.

DISCUSSION

We have cloned integrated HBV sequences from a patient who died of liver failure due to HCC. At least one genome equivalent of HBV-DNA has been integrated into the hepatocellular DNA without apparent rearrangement or detection—as in the case of clone λIA22. That this is indeed the case is supported by an electron microscopic analysis and by the finding of gene S expression (coding for the viral surface envelope protein, HBsAg) when λIA22 DNA is convected into mouse Ltk^- cells. It has been shown that the mature gene transcript is 2.3 kb and that any insertion into the corresponding DNA eliminates HBsAg expression [25]. Thus, in the absence of actually sequencing all of the integrated HBV sequences, certainly gene S is complete and probably most of the HBV sequences are also.

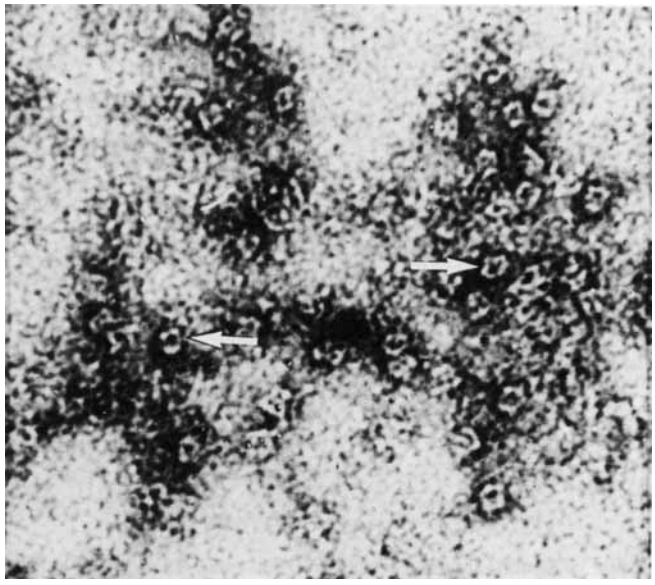

Fig. 4. Immunoelectron micrograph of HBsAg particles purified from the culture supernatant of clone 2a. The arrows denote typical particles.

The finding of gene C very close to the 5′ site of integration places this site very close to the nick in L strand of free viral DNA [27]. This confirms the suggestion of Edman et al [30] that in the human hepatoma cell line, PLC/PRF/5, some of the HBV sequences are integrated very close to the nick-free viral DNA. A protein covalently bound to the 5′ end of the L strand has been found [31]. It was found not to interfere with the dissociation or association of the cohesive ends of HBV DNA. It is therefore possible that this protein may be involved somehow with viral integration [31].

The mechanism by which HBV DNA integration can result in the presence of tandem or multiple genomes in the cellular DNA is not clear. It was first shown by blotting that there were tandem head-to-tail HBV genomes in hepatocarcinoma DNA [8] and subsequently in the liver DNA of chronic carriers [9]. This has now been directly confirmed by cloning the integrated HBV sequence; clone λIA22 has approximately 1.2 genomes of continuous HBV sequences. It is possible that either HBV-DNA polymerizes prior to integration or else the DNA is amplified after integration. The absence of oligomers in HBV productive liver [11] would seem to support the latter possibility.

That HBsAg particles result from the convection of λIA22 DNA into mouse L cells demonstrates that a functionally intact HBsAg gene is carried by the clone. This result shows that HBV sequences can be transcribed. Bréchot et al [11] have found free viral DNA in both liver and serum, only amongst HBeAg (hepatitis B e antigen) positive patients. A corrolary is therefore that for HBsAg-positive, HBeAg-negative patients (ie, in the absence of viral replication) the source of HBsAg is the transcription of integrated HBV sequences.

Patient 53 was serum positive for HBsAg but negative for HBsAg in the liver as determined by direct immunofluorescence [8]. Such a finding is very common in poorly differentiated HCC tissue [12]. The trivial explanation that gene S is deleted or rearranged or that integration occurred within the HBsAg gene is clearly not the case at least for one sequence in patient 53. There are two possible explanations for the failure to detect HBsAg in poorly differentiated neoplastic hepatocytes: (1) The quantity of HBsAg expressed by the neoplastic cells is below the threshold of detection by immunofluorescence, but once excreted, the HBsAg particles accumulate in the blood; or (2) that neoplastic cells possess a structurally intact but functionally inactive HBsAg gene(s) and that the HBsAg in the serum results from active proviral S genes in the remaining low nontumourous cells.

While the first possibility cannot be ruled out, it is the more unlikely since the total renewal time of HBsAg particles has been estimated at 3.3 days and thus it would be difficult to accumulate sufficient HBsAg particles in the blood [32].

The second hypothesis is supported by immunofluorescence studies of tumourous and nontumourous sections of HBV-infected livers. The nontumourous sections stain positive for HBsAg while the tumourous sections stain negative. In addition, the serum HBsAg titer of patients with HCC is frequently very low such that it can only be detected by solid phase radioimmunoassay [14]. Thus in the limit of there being no nontumourous hepatocytes one would expect the patient to be HBsAg negative. This might suggest that some HBsAg-negative patients with HCC could still contain integrated HBV-DNA sequences—a finding already noted [33]. However not all patients with HBsAg-negative HCC have advanced HCC.

In conclusion, we present a description at the molecular level of integrated HBV sequences. Integration of multiple copies of HBV DNA is possible with the retention of complete HBV gene sequence. The finding of an intact S gene in the DNA from the tumourous part of the liver suggests that there is a certain level of HBV gene control in the development of hepatocellular carcinoma.

ACKNOWLEDGMENTS

We would like to thank Drs. Brigitte Cami, Patrick Charnay, Christine Pourcel, and Jean Weissenbach for advice and discussions. We are also grateful to Eliane Sobczak for kindly making the radioimmunoassays and to Anne Marie Carrasco for typing the manuscript.

This work was supported by INSERM grants ATP 72.79/104 and 124036 and grants from the Ministère de la Recherche et de la Technologie.

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