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Integration Pattern of Hepatitis B Virus DNA Sequences in Human Hepatoma Cell Lines

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Four human hepatoma cell lines established from primary hepatocellular carcinomas were examined for the presence of hepatitis B virus DNA sequences. Reassociation kinetic analysis indicated that the cell lines HEp-3B 217, HEp-3B 14, HEp-3B F1, and PLC/PRF/5 contained two, one, one, and four genome equivalents per cell, respectively. Southern blot hybridization analysis demonstrated that hepatitis B virus DNA was integrated into the cellular DNAs of these cell lines. Further liquid hybridization studies with ^{32}P-labeled *Hin*cII restriction fragments of hepatitis B virus DNA established that DNA sequences from all regions of the HBV genome were represented in the integrated viral sequences. Although the three HEp-3B cell lines were derived from the same tumor, they differed significantly in their patterns of integration of hepatitis B virus DNA, the number of copies of viral DNA per cell, and their ability to produce the virus-coded surface antigen.

The virion of hepatitis B virus (HBV) is known to be a 47-nm particle (the "Dane particle") composed of two protein coats surrounding a double-stranded DNA genome. The structure of the genome of HBV virions found in human serum was shown by Summers et al. (10) to be unique when compared with the circular DNA genomes of other DNA viruses in that it is a nicked circular DNA with a single-stranded region of variable length. The outer protein coat or surface antigen (HBsAg) of the virion is apparently overproduced during the replicative cycle and assembled into 22-nm particles lacking DNA. These represent the major virus-coded gene product (HBsAg) found in the sera of acute and chronically infected patients (6). HBsAg has also been shown to be the major immunogenic polypeptide of the virion (3).

Because all attempts to propagate HBV in cell culture have been unsuccessful, studies of the replicative mechanisms of this virus and the production of a tissue culture vaccine have not been possible (6). However, two cell lines (HEp-3B and PLC/PRF/5) that actively produce HBsAg have been established from patients with hepatocellular carcinoma (1, 4). These cell lines have an epithelial morphology and secrete proteins characteristic of differentiated liver cells, but do not produce HBV virions or any detectable virion-coded protein other than HBsAg (1, 4, 5).

Because HBV has been implicated in liver oncogenesis in humans (12), and in view of the fact that these cell lines were derived from human liver tumors, we were interested in determining the physical state of the HBV DNA in these cells which codes for HBsAg.

MATERIALS AND METHODS

Cells. Three of the hepatoma cell lines used in this study were established from a single hepatocellular carcinoma (1) and are designated HEp-3B 217, HEp-3B 14, and HEp-3B F1. The cell lines were individual cell colonies isolated from a single culture of tumor cells cultivated in a 75-cm^2 flask containing an irradiated layer of fibroblastic mouse cells (feeder cells) as follows: HEp-3B F1, isolated at 5 weeks after initial cell plating and maintained as feeder cell dependent; HEp-3B 14, isolated at 9 months and maintained without feeder cells; and HEp-3B 217, separated at 10.5 months and also grown without feeder cells. Each HEp-3B subline contains a distinctive rearrangement of chromosome 1; each has an epithelial morphology and produces a number of serum proteins characteristic of differentiated liver cells. The PLC/PRF/5 hepatoma cell line, derived from a hepatocellular carcinoma by Macnab et al. (4), was also used in this study.

Purification and cloning of HBV DNA. Virions were pelleted from the plasma of chronically infected patients by centrifugation at 200,000 × *g* for 5 h in a Beckman SW41 rotor. The virus pellet was suspended in CsCl (1.37 g/ml) and centrifuged for 40 h at 180,000 × *g* in a Beckman SW50.1 rotor. Fractions were collected from the bottom of the tube, and 1 μl of each fraction was placed in 2 μl of an endogenous reaction mixture containing 10 μM dATP, 10 μM dGTP, 10 μM dTTP, 5 μM [^{32}P]dCTP, 10 mM $MgCl_2$, and 20 mM Tris-hydrochloride (pH 8.0)-0.1% Nonidet P-40-0.1 M NaCl. Fractions containing DNA polymerase (DNA nucleotidyltransferase) activity were pooled, and the virions were pelleted by centrifugation at 200,000 × *g* for 4 h in a Beckman SW50.1 rotor.

DNA was extracted from the virions after complet-

ing the minus strand of the DNA genome by using the endogenous DNA polymerase activity. The purified HBV DNA was cleaved with the restriction endonuclease *Eco*RI and ligated into plasmid pBR322 which had been digested with *Eco*RI and treated with alkaline phosphatase. *Escherichia coli* strain χ1776 made competent with $CaCl_2$ was transfected with the HBV-pBR322 ligation mixture. Colonies were screened for the presence of HBV-pBR322 recombinants containing full-length HBV DNAs by using Southern blot analysis. Full-length HBV DNA was cut from one such clone (HBV-T1) with *Eco*RI and purified by electrophoresis on a 1% agarose gel.

Preparation of HBV [^{32}P]DNA probes: HBV DNA was labeled in vitro by the method of Summers et al. (10). Briefly, 30 ng of base-denatured HBV DNA was replicated in a 10-μl reaction containing 10 μM each dCTP, dGPT, and dTTP, 5 μM [^{32}P]dATP (400 Ci/mmol), 10 mM $MgCl_2$, 0.2 μg of calf thymus DNA primers, and 1 U of *E. coli* DNA polymerase I (Boehringer Mannheim). The reaction, which ceased incorporation after 40 min, was made up to 20 mM EDTA-sodium (pH 7.2)–0.5% dodecyl sodium sulfate, layered onto a column of Sephadex G-50 (40 × 0.6 cm), and eluted with buffer containing 10 mM Tris-hydrochloride (pH 7.0), 10 mM EDTA, 0.1 M NaCl, and 0.1% sodium dodecyl sulfate.

Isolation of hepatoma cell DNA and hybridization to a HBV [^{32}P]DNA probe. Monolayers of cells were harvested and washed twice with a solution containing 10 mM Tris-hydrochloride (pH 7.5), 10 mM EDTA, and 100 mM NaCl. The cells were suspended in a buffer containing 10 mM Tris-hydrochloride (pH 8.0), 10 mM EDTA, 0.5% sodium dodecyl sulfate, and 500 μg of *Tritirachium* alkaline proteinase (proteinase k, Boehringer Mannheim) per ml and incubated for 2 h at 37°C. After incubation, the DNA was extracted twice with an equal volume of phenol-chloroform (1:1) and precipitated with 2 volumes of ethanol. Aliquots (4 mg) of PLC/PRF/5 and human control DNAs as determined by measurement of optical density at 260 nm were heated in a boiling water bath in the presences of 0.3 N NaOH for 20 min. The samples were chilled, and the DNA was precipitated by adding HCl to a 0.1 N excess. The precipitated DNA was sedimented at 2000 × g; the pellet was suspended in 1.0 M Tris-hydrochloride (pH 9.0). Samples (500 μg) of cell DNA were mixed with HBV [^{32}P]DNA and cell [^{3}H]DNA probes in a 100-μl reaction mix containing 10 mM Tris-hydrochloride (pH 7.5), 10 mM EDTA, and 1 M NaCl. The final concentrations of the cell DNA, HBV [^{32}P]DNA probe, and normal human cell control [^{3}H]DNA per reaction mixture were 5 mg/ml, 0.1 ng/ml, and 6 μg/ml, respectively. The annealing was carried out at 68°C; 20-μl aliquots were removed at various times to assay for the annealing of the probes by digestion to completion with S1 nuclease.

Restriction endonuclease digestions. *Hin*dIII, *Eco*RI, and *Hin*cII restriction endonucleases were purchased from Bethesda Research Laboratories. Enzyme digestions were carried out at DNA concentrations of 50 μg/ml for 5 h at 37°C in digestion buffers recommended by the supplier. Completeness of digestion was monitored by the addition of bacteriophage lambda DNA to each sample.

Isolation of restriction fragments. Five micrograms of HBV DNA digested with *Hin*cII was fractionated by polyacrylamide gel electrophoresis (10). The individual restriction fragments were cut out of the gel and crushed by passage through a 23-gauge needle, and the DNA was eluted out of the gel by storage overnight at 4°C in buffer containing 10 mM Tris-hydrochloride (pH 7.0), 10 mM EDTA, and 0.1 M NaCl. The eluted DNA fragments were precipitated with 2 volumes of ethanol, dried, and suspended in 20 μl of water each for preparation of probes.

Southern blot hybridization analysis of DNA extracted from hepatoma cells. DNA extracted from hepatoma cells was digested with the restriction endonuclease *Hin*dIII, *Eco*RI, or *Hin*cII (Boehringer Mannheim) and electrophoresed on a 1.4% agarose gel, using a continuous buffer system containing 20 mM Tris-acetate (pH 7.0), 20 mM sodium acetate, and 1 mM EDTA. The DNA was denatured in the gel, neutralized, transferred to a nitrocellulose membrane as described by Southern (9), and baked onto the membrane by heating at 80°C for 2 h. Before hybridization, the membrane was sealed in a plastic bag containing 6× SSC (2× SSC: 0.15 M NaCl–0.015 M sodium citrate), 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone and incubated at 68°C for 1 h. The pre-hybridization solution was removed and replaced with 4 ml of the same solution containing in addition 0.5% sodium dodecyl sulfate and 2 ng of denatured in vitro-labeled HBV [^{32}P]DNA per ml. Incubation was continued for an additional 16 h, after which the blot was washed three times with 2× SSC–0.05% sodium dodecyl sulfate at 1-h intervals. The blot was dried at 37°C for 30 min, covered with polyvinyl chloride wrap, and prepared for autoradiography with Du Pont Cronex 4 X-ray film and a Du Pont Cronex Lightning Plus intensifying screen at −70°C.

RESULTS

Detection of HBV DNA sequences in hepatoma cells. The abilities of the cellular DNAs from the hepatoma cell lines HEp-3B 217, HEp-3B 14, and PLC/PRF/5 to accelerate the annealing of a HBV [^{32}P]DNA probe are shown in Fig. 1, bottom. The rate of reassociation of the viral probe in the presence of known concentrations of HBV DNA mixed with calf thymus DNA (Fig. 1, top) was used to estimate the number of copies of HBV DNA per cell present in the three hepatoma cell lines. Analysis of the reassociation kinetics indicated that cells of hepatoma cell lines HEp-3B 217, HEp-3B 14, and PLC/PRF/5 contained approximately two, one, and four copies, respectively, of the HBV genome per diploid equivalent of cell DNA. Because of the slow rate of growth of the HEp-3B F1 cell line, we were unable to obtain sufficient purified cellular DNA for liquid hybridization analysis.

Southern blot hybridization analysis. The three restriction endonucleases used for Southern blotting analysis of the four hepatoma cell lines were *Hin*dIII, which does not cleave HBV

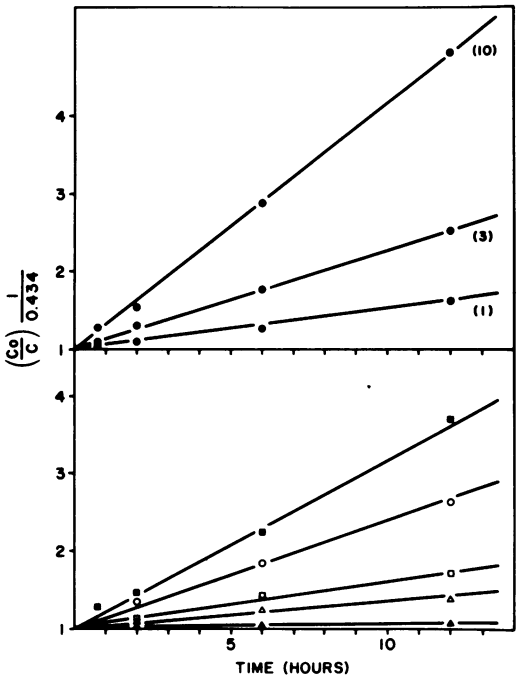

FIG. 1. *Comparison of hybridization of in vitro-labeled HBV [^{32}P]DNA with 1 to 10 copies of HBV DNA with hybridization with hepatoma cell line DNAs. (Top) Rates of annealing of 1.5, 0.45, and 0.15 μg of HBV [^{32}P]HBV DNA (equivalent to 10, 3, and 1 equivalents of HBV DNA per cell, respectively) in the presence of 500 μg of normal human DNA. (Bottom) Curves for the hybridization of the ^{32}P probe to uninfected liver cell DNA (▲), HEp-3B 14 cell DNA (△), HEp-3B 217 cell DNA (□), PLC/PRF/5 cell DNA (○), and normal human cell [^{3}H]DNA (■).*

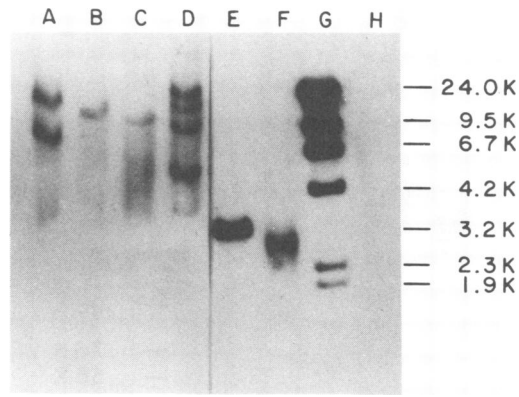

FIG. 2. *Blot hybridization analysis of HindIII digests of hepatoma cell DNAs. Lanes: A through D, 5 μg of HEp-3B 217, HEp-3B 14, HEp-3B F1, and PLC/PRF/5 cell DNA, respectively; E, full-length linear HBV DNA isolated from our recombinant clone HBV-T1; F, HBV DNA extracted from virions purified from human plasma; G, ^{32}P-end-labeled HindIII digest of lambda DNA; H, HindIII digest of uninfected human liver cell DNA. The numbers at the right of the autoradiogram designate the sizes in base pairs of labeled DNA markers.*

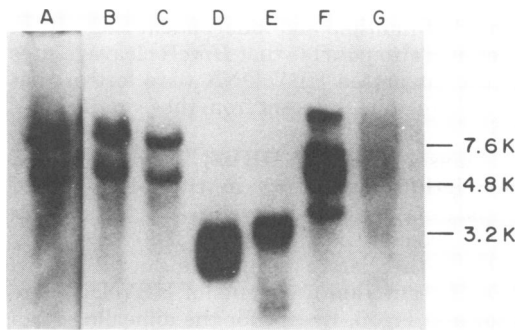

FIG. 3. *Blot hybridization analysis of EcoRI digests of hepatoma cell DNAs. Lanes: A through C, 5 μg each of EcoRI-digested HEp-3B 217, HEp-3B 14, and HEp-3B F1 cell DNAs, respectively; D and E, authentic HBV DNA isolated from virions purified from human sera and digested with EcoRI; F, ^{32}P-end-labeled EcoRI digest of lambda DNA; G, EcoRI-digested uninfected human liver cell DNA. The source of viral DNA probe for this hybridization was virions isolated from infected human plasma. The numbers at the right of the autoradiogram designate the sizes in the base pairs of labeled DNA markers.*

DNA; *Eco*RI, which cleaves HBV DNA once; and *Hinc*II, which cleaves HBV DNA four times (7, 8). *Hin*dIII digestion of the hepatoma DNAs yielded a different pattern of fragments for each of the three cell types (Fig. 2). Each of the bands in these digestions migrated more slowly than either full-length authentic HBV DNA from the bacterial clone or HBV DNA with incomplete minus strands extracted from human plasma HBV. We conclude from this analysis that free HBV genomes are not present in these cells. These results were further corroborated by further Southern blot analysis of the HEp-3B cell DNAs after digestion with *Eco*RI (Fig. 3). This digestion yielded bands of hybridization migrating in the 5- to 10-kilobase region of the gel, and not with full-length viral DNA, as would be expected if episomal HBV DNA were present in these cell lines. The PLC/PRF/5 cell line has previously been shown not to contain episomal viral DNA (5).

Digestion with *Hinc*II (Fig. 4) yielded a series of bands, all migrating more slowly than bands found in a *Hinc*II digest of HBV DNA, with the exception of one band in the lane containing DNA from PLC/PRF/5. This band comigrated with the *Hinc*II A fragment (980 base pairs) of HBV DNA. The smallest *Hinc*II fragment (fragment F, 320 base pairs) ran off the gel. Because all but one of the bands of hybridization in the

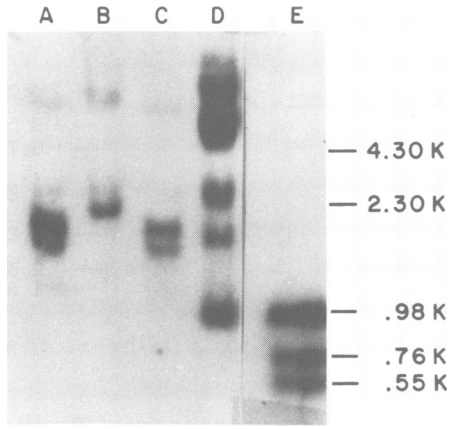

FIG. 4. *Blot hybridization analysis of HincII digests of hepatoma cell DNAs. Lanes: A through D, 5 μg each of HincII-digested cellular DNAs of HEp-3B 217, HEp-3B 14, HEp-3B F1, and PLC/PRF/5 cell lines, respectively; E, HincII digest of HBV DNA. The numbers at the right of the autoradiogram designate the sizes in base pairs of labeled markers.*

*Hinc*II digests migrated more slowly than *Hinc*II HBV DNA fragments, it would appear that fragments of the viral genome were integrated at multiple and independent sites. However, it is also possible that *Hinc*II cleavage sites in the integrated HBV DNA were lost or that they were quite different from those in the HBV DNA used as a marker.

Reassociation of HBV [^{32}P]DNA *Hinc*II restriction fragments in the presence of hepatoma DNA. The abilities of the cellular DNAs from HEp-3B 217, HEp-3B 14, and PLC/PRF/5 to accelerate the annealing of ^{32}P-labeled *Hinc*II restriction fragments of HBV DNA are shown in Fig. 5. Except for the annealing reaction containing the HBV *Hinc*II E fragment and HEp-3B 14 DNA, all the hepatoma DNAs accelerated the rates of reassociation of the individual viral probes. These results demonstrate that DNA sequences from all regions of the genome were integrated. However, because the observed hybridizations could have been due to partial homologies, they do not prove that the entire region represented by each restriction fragment was present in each cell line.

DISCUSSION

Our findings demonstrate that HBV DNA is integrated into the cellular DNA of four different hepatoma cell lines. In addition, evidence of episomal viral DNA was not found in these cell lines, i.e., no 3,200-base-pair bands were observed in the *Hind*III digests of cellular DNA, and none of the bands of hybridization of an

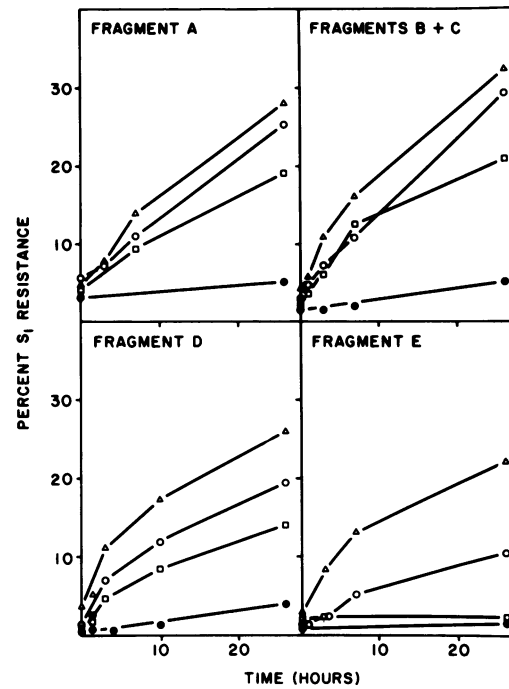

FIG. 5. *Hybridization of in vitro-labeled HincII restriction fragments of HBV [^{32}P]DNA with hepatoma cell line DNAs. The annealing reactions contained 500 μg of hepatoma cell DNA (HEp-3B 14 [□], HEp-3B 217 [○], PLC/PRF/5 [△], or human placental DNA from Calbiochem [●] and 0.15 μg of either HincII fragment A (980 base pairs), fragments B and C (760 730 base pairs), fragment D (550 base pairs), or fragment E (320 base pairs) labeled with ^{32}P per ml.*

*Eco*RI digest of cellular DNA comigrated with linear HBV DNA.

The DNAs from HEp-3B 217, HEp-3B 14, and PLC/PRF/5 cultures accelerated the annealing of individual *Hinc*II restriction fragment probes of HBV DNA, indicating that HBV DNA sequences from all regions of the viral genome are integrated. Experiments using the individual *Hinc*II restriction fragments of HBV DNA for probes in Southern blot analysis of *Hinc*II digests of the hepatoma DNA (data not shown) confirmed the results of the liquid hybridization analysis. However, these experiments do not prove conclusively that all the DNA sequences contained in the *Hinc*II restriction fragments are integrated, since the annealing of only a part of the probe to integrated HBV sequences could account for the observed hybridization.

Because digestion of the hepatoma DNAs with a multiple-cut restriction endonuclease did not yield bands comigrating with viral markers (especially in the case of the HEp-3B cultures),

we could not determine how the HBV DNA sequences were integrated. It is possible that the viral sequences are integrated as fragments of the genome at multiple sites. Alternatively, it is conceivable that the intact viral genomes could be integrated at one or more sites (depending on the cell line) but that the restriction sites have undergone alterations in the integrated DNA sequences. We are cloning restriction fragments containing the integrated viral sequences from HEp-3B 217 and PLC/PRF/5 in *E. coli* in order that they can be sequenced and in order that the segments of integrated HBV genome can be properly identified.

Because the HEp-3B cultures, which were all derived from the same tumor, differed as to ability to produce HBsAg, it was of interest to attempt to correlate the different patterns of HBV DNA integration with HBsAg production. In summary, we found that the HEp-3B 217 culture, which actively produces HBsAg in high concentration, contained two sites of integration of HBV DNA, whereas HEp-3B F1, which produces less HBsAg according to a pattern suggesting some gene regulation (1) contained one site of integrated viral sequence. The HEp-3B 14 cell line contained one site of integration of viral DNA, but did not produce HBsAg.

In regard to production of HBsAg as it relates to the number of copies of integrated HBV DNA per cell, it would appear that the more copies of HBV DNA integrated, the greater the HBsAg production. For example, PLC/PRF/5, which possesses four copies of viral DNA per cell, produced approximately 20-fold more HBsAg than did HEp-3B 217, which has two genome equivalents per cell. HEp-3B F1 and HEp-3B 14 produced little or no detectable HBsAg and contain only one copy of viral DNA. Integration of HBV DNA at multiple sites in cellular DNA may increase the probability that viral DNA sequences will reside in actively transcribed (as opposed to silent) regions of a chromosome in the correct reading frame. Whether or not the integrated HBV DNA sequences we have detected in these hepatoma cultures were responsible for initiating oncogenesis cannot be proven by our analysis. However, our findings certainly evoke favorable comparison with the reports of others who have described the integration of DNA virus genomes in tumors and transformed cells (2, 3, 13). Conclusive proof that hepatitis viruses are oncogenic awaits demonstration of oncogenesis in an animal model, such as the woodchuck (11), or a prospective epidemiological study showing that vaccination against HBV infection will significantly reduce the incidence of hepatocellular carcinomas.

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