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State of Hepatitis B Viral DNA in a Human Hepatoma Cell Line

PATRICIA L. MARION,[1] FELIX H. SALAZAR,[1] JENNIFER J. ALEXANDER,[2] AND WILLIAM S. ROBINSON[1]*

Department of Medicine, Stanford University School of Medicine, Stanford, California 94305,[1] and National Institute of Virology, Johannesburg, South Africa[2]

PLC/PRF/5, a tissue culture cell line isolated from a human hepatocellular carcinoma and producing hepatitis B surface antigen, was studied for the presence of hepatitis B virus (HBV)-specific DNA and RNA. PLC/PRF/5 cell DNA accelerated the rate of reassociation of HBV [^{32}P]DNA, and quantitative experiments indicated that the cells contained approximately four copies of viral DNA per haploid, mammalian cell DNA equivalent. PLC/PRF/5 DNA accelerated the rate of reassociation of all individual restriction endonuclease *Hin*cII and *Hae*III fragments of HBV [^{32}P]DNA, indicating that DNA from all regions of the viral genome is present in the cells. This suggests that these cells contain at least most, and possibly all, of the viral genome. Digestion of PLC/PRF/5 cell DNA with restriction endonuclease *Hin*dIII (an enzyme found not to cleave the DNA of any HBV isolate so far examined) yielded only three fragments, all larger than virion DNA, which contained HBV DNA base sequences, suggesting that HBV DNA is integrated in high-molecular-weight DNA at three different sites in these cells and that there is no viral DNA in an episomal form. PLC/PRF/5 cell [^{32}P]RNA was found to hybridize with all restriction fragments of HBV DNA adequately tested, indicating that at least most, and possibly all, of the viral DNA in these cells is transcribed.

Hepatitis B virus (HBV) is a DNA-containing, spherical virus (diameter, 42 nm) infecting humans and a few other primates (14). It is a nearly unique type of animal virus, having been joined only recently by a similar hepatitis virus found to infect woodchucks (20). HBV has not been shown to infect cells in culture. Three antigens have been associated with HBV: (i) hepatitis B surface antigen (HB_sAg), located in the lipid-containing outer coat of the virion (4); (ii) hepatitis B core antigen, which is exposed when the virion outer coat is removed (1); and (iii) hepatitis B e antigen, which appears to be in a cryptic form in the interior of the virion (22). HB_sAg is present in patient serum, not only in the form of virions, but in even greater quantities as spherical, lipid-containing particles (diameter, 22 nm) (2) which lack nucleic acid and hepatitis B core antigen. These smaller particles appear to be incomplete forms of the virus. HB_sAg is a complex antigen consisting of at least one group-specific determinant *a* and more than one type-specific determinant, such as d/y and w/r, which can be used to distinguish different viruses (3).

The size of hepatitis B virion DNA is approximately 1.6×10^6 daltons (15), and the circular molecule is single stranded for 15 to 45% of its length (6, 8, 19). The virion, also known as the Dane particle, contains a DNA polymerase activity (7) which converts HBV DNA into a fully double-stranded form with a size of approximately 2.1×10^6 daltons, or 3,200 base pairs (bp) (6, 8, 19). A map of restriction endonuclease cleavage sites in HBV DNA has been determined, and it has been found that systematic differences in restriction sites occur in the DNAs of viruses of different HB_sAg subtypes and that differences sometimes also occur among viruses of the same subtype (16).

Numerous studies have shown an association between persistent HBV infections and primary liver cancer in some parts of the world (21). Although it is not known whether the relationship is a causal one, the fact that woodchuck hepatitis virus is also associated with liver carcinoma (20) lends credibility to the idea that HBV may induce hepatomas in some cases.

A tissue culture cell line, PLC/PRF/5, isolated in 1975 from a primary liver carcinoma of an African man with persistent HBV infection, has been shown to continually produce small amounts of HB_sAg (subtype ad) in the form of 22-nm particles (10). This paper describes studies to determine (i) whether HBV DNA base sequences are present in these cells; (ii) if present, whether the sequences are in an integrated

or episomal form; (iii) whether the entire HBV genome is present; (iv) how much HBV DNA is present per cell; and (v) what virus-specific RNA is produced by these cells.

MATERIALS AND METHODS

Cell culture. The PLC/PRF/5 cells were grown in Dulbecco-modified Eagle medium supplemented with 10% fetal bovine serum.

Another cell line, HS703T, isolated in 1977 from hepatocellular carcinoma tissue of a patient from the United States, was obtained from Helene Smith of the Naval Biosciences Laboratory in Oakland, Calif., and grown under the same culture conditions. We have been unable to detect any HBV antigens in these cells.

Cellular DNA extraction and purification. Cells in a confluent monolayer in 10-cm dishes were lysed by a brief incubation in 1 to 2 ml of 1% sodium dodecyl sulfate-containing buffer A (0.02 M Tris, pH 8.5; 0.01 M EDTA; and 0.1 M NaCl) at room temperature. Autodigested pronase was added to 1 mg/ml, and the lysate was incubated at 37°C for 2 h. The solution was then extracted two to three times with chloroform-isoamyl alcohol (19:1) and once with buffer-saturated phenol, and the nucleic acids were precipitated with 2 volumes of ethanol. The precipitate was dissolved in buffer B (0.01 M Tris, pH 7.5; 0.001 M EDTA; and 0.15 M NaCl); solid CsCl was added (5 g/4 ml), and the solution was centrifuged in a Spinco type 35 rotor at 29,000 rpm for 48 h at 18°C. The band of DNA in the middle of the tube was collected, diluted sixfold in buffer B, precipitated with ethanol as before, and resuspended in a minimum amount of buffer B.

Liver tissue was homogenized in a Waring blender in 25 volumes of buffer A; sodium dodecyl sulfate and pronase were added as to the cultured cell lysate, and the DNA was extracted and purified in the same manner.

Viral DNA extraction and purification. HBV DNA was prepared both from virions recovered from patient serum, as previously described (8, 15), and from a recombinant plasmid. The entire DNA of hepatitis B virions from patient 1083 (HB_sAg subtype adw_2) was introduced at the unique *Eco*RI cleavage site into the pACYC184 and pBR322 plasmid vectors and cloned in *Escherichia coli* K-12 by Sninsky et al. (17). Viral DNA recovered after endonuclease *Eco*RI cleavage of hybrid plasmid and gel electrophoresis is designated HBV (plasmid) DNA, and that from virus in serum is designated HBV (virion) DNA. No difference was observed in results of hybridization with radiolabeled DNA from either source. All viral DNA used in these experiments exhibited the typical adw_2 restriction endonuclease pattern previously described (16).

Quantitation of DNA was done by adding ethidium bromide and comparing the intensity of fluorescence under UV illumination with that of various known concentrations of phage PM-2 DNA with ethidium bromide.

Phage PM-2 [^{32}P]DNA and unlabeled DNA were prepared as previously described (6, 9).

Radiolabeling HBV DNA by nick translation. Viral DNA, after conversion of the single-stranded region to double-stranded DNA by an extensive virion DNA polymerase reaction at high nucleotide concentrations (8, 16), was uniformly labeled by nick translation (13) with one or more [^{32}P]deoxynucleoside triphosphates with specific activities of 250 to 3,000 Ci/mmol (Amersham Corp., Arlington Heights, Ill., or New England Nuclear Corp., Boston, Mass.). DNA specific activities of 1×10^8 to 3×10^8 cpm/μg of DNA were usually obtained.

Radiolabeling, extraction, and purification of cellular RNA. To label the RNA of PLC/PRF/5 cells, confluent cell monolayers in 10-cm culture dishes were incubated with 250 μCi of $^{32}P_i$ per ml for 1 to 3 days in 5 ml of phosphate-free Eagle minimal essential medium supplemented with 10% fetal bovine serum, 1% Dulbecco-modified Eagle medium, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 2 mM glutamine. The supernatants were removed from the cells, and each monolayer was washed twice with 25 mM Tris, pH 7.3. The cells were then lysed as previously described and extracted with phenol four times, and the nucleic acids were precipitated with 2 volumes of ethanol. Most of the DNA was spooled on a glass rod and removed. After remaining at −20°C overnight, the RNA was pelleted by centrifugation and resuspended in 2 ml of buffer B. Four milliliters of saturated Cs_2SO_4 (in 0.01 M Tris-hydrochloride [pH 7.5]–0.001 M EDTA) was added to the solution for centrifugation in a Spinco 50 Ti rotor at 39,000 rpm for 48 h at 18°C. The band of RNA in the middle of the tube was collected and dialyzed against buffer B for 24 h at 4°C. The RNA was then precipitated with ethanol and resuspended in 100 μl of buffer B for further use.

DNA reassociation in solution. Radioactive HBV DNA and unlabeled cellular DNA were brought to a concentration of 1 to 1.8 mg/ml in 0.1 M Tris (pH 7.5)–0.5 M NaCl. The mixture was sonicated to give DNA fragments of approximately 400 bp, and 50-μl samples were sealed in micropipettes. The DNA was denatured by incubating for 15 min at 100°C. The samples were quick-chilled in an ice bath; four samples were removed and frozen at zero time, and the remaining samples were placed at 68°C for incubation. When all time points had been frozen, the samples were thawed, and two samples of each time point were incubated at 37°C for 1 h with nuclease S1 to digest single-stranded DNA (9), while the other two were incubated in the same buffer without the enzyme. The acid-precipitable radioactivity was then determined as previously described (9).

Restriction endonuclease digestion and gel electrophoresis. Restriction endonucleases were purchased from New England Biolabs and used as recommended by them.

DNA fragments were fractionated by electrophoresis in horizontal gels of 0.7 to 2% agarose, depending upon the size distribution of the fragments. Further details of the electrophoresis have been previously described (16).

Viral DNA fragments were also fractionated by polyacrylamide gel electrophoresis (16), and DNA fragments were recovered from the polyacrylamide gel by electroelution in a Studier box for at least 4 h at 150 V in electrophoresis buffer diluted 10-fold. The

fragments were then extracted with phenol and precipitated with 2 volumes of ethanol before further use.

Detection of DNA fragments on nitrocellulose filters by hybridization. A modification (12) of the method described by Southern (18) was used to transfer DNA from agarose gels to nitrocellulose filters. Detection of HBV DNA base sequences in PLC/PRF/5 DNA (PLC DNA) on nitrocellulose filters by hybridization was best if the DNA probe had a specific activity of at least 10^8 cpm/μg of DNA and was present at 100 to 200 ng/ml in the hybridization mixture.

RESULTS

Detection of HBV DNA sequences in PLC/PRF/5 hepatoma cells. To determine whether PLC DNA contained HBV-specific base sequences, we measured the ability of PLC DNA to accelerate reassociation of nick-translated HBV [^{32}P]DNA and compared it with the effect of (i) DNA from the liver of a patient with chronic hepatitis B who was chronically immunosuppressed to maintain an organ transplant (productively infected liver DNA), (ii) DNA from uninfected human liver, (iii) DNA from the HS703T hepatoma cell line (HS703T DNA), (iv) calf thymus DNA, (v) herring sperm DNA, and (vi) *E. coli* DNA.

Figure 1 shows reassociation in percentage of trichloroacetic acid-precipitable, nuclease S1-resistant ^{32}P as a function of days incubated at 68°C. HS703T DNA not producing HB_sAg, calf thymus, herring sperm, and *E. coli* DNAs did not accelerate probe reassociation and formed parallel curves. The uninfected liver DNA also did not appear to accelerate reassociation. The repeatedly high zero point obtained with this DNA appeared to be due to impurities in the preparation. Since reassociation with DNA from human liver was no more rapid than with DNA from distantly related species, the serum-derived probe appeared to have little contaminating cellular DNA. DNA from productively infected liver which contained much HBV DNA caused a rapid reassociation of the ^{32}P probe. With the PLC DNA, reassociation was clearly and repeatedly accelerated above that with uninfected liver, calf thymus, herring sperm, or *E. coli* DNAs, although the rate was less than that with DNA from productively infected liver. HBV DNA sequences in PLC/PRF/5 cells were,

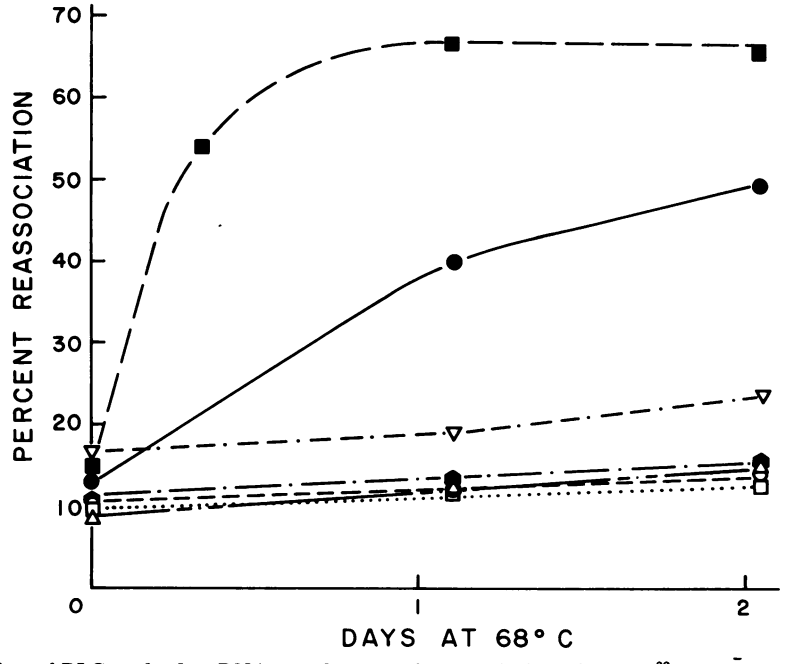

FIG. 1. *Effect of PLC and other DNAs on the rate of reassociation of HBV [^{32}P]DNA in solution. Seven reaction mixtures each contained nick-translated HBV (virion) [^{32}P]DNA (12,000 cpm/ml, 10^7 cpm/μg) and a 1.8-mg/ml amount of either (i) DNA from productively infected human liver (■), (ii) PLC DNA (●), (iii) uninfected human liver DNA (▽), (iv) HS703T cell DNA (◆), (v) calf thymus DNA (○), (vi) herring sperm DNA (□), or (vii) E. coli DNA (△) in 0.1 M Tris-hydrochloride (pH 7.5)–0.5 M NaCl. Ten 50-μl samples of each were sealed in micropipettes, heat denatured, incubated at 68°C for various lengths of time, and stored at −20°C, and nuclease S1-resistant ^{32}P was determined as described in the text.*

therefore, clearly demonstrated in these experiments.

In numerous experiments in which DNA incubation was continued until a reassociation plateau was reached, a maximum of 60 to 70% of the probe became nuclease S1 resistant in the presence of either PLC DNA, productively infected liver DNA (as shown in Fig. 1), or HBV DNA extracted from virions purified from serum. The failure of the probe to become more than 70% nuclease S1 resistant even during reassociation in the presence of high concentrations of added HBV DNA extracted from virions or infected liver was probably due to the small fragment size of a fraction of the probe DNA, which resulted from labeling the DNA to very high specific activity by the nick translation procedure used. Reassociation of as much as 60 to 70% of the probe was not observed in the presence of control DNAs even after prolonged incubation times because of the very low concentrations of HBV-specific sequences present when the high-specific-activity probe was the only source of these sequences in the reaction. It can be concluded from these experiments that at least 70% of HBV DNA base sequences are present in PLC DNA. Since it was not possible with this probe to demonstrate by the extent of hybridization a difference between the fraction of HBV DNA base sequences in PLC DNA and that fraction in the DNA of productively infected liver or in DNA extracted from virions, it is possible that PLC/PRF/5 cells contain DNA base sequences from more than 70% of the HBV genome.

Detection of specific parts of the HBV genome in PLC/PRF/5 cells. To identify specific parts of the genome of HBV in PLC DNA, nick-translated HBV [^{32}P]DNA was cleaved with *Hin*cII, and the resulting five DNA fragments (8, 16) were separated into four bands by gel electrophoresis. The [^{32}P]DNA of each band was recovered from the gel, and the effect of PLC DNA on reassociation of each was determined. Figure 2 shows that reassociation of all [^{32}P]DNA fragments, as well as the whole HBV [^{32}P]DNA probe, was accelerated by PLC DNA compared with HS703T control DNA.

A similar experiment with 11 HBV [^{32}P]DNA fragments obtained by endonuclease *Hae*III cleavage (8) again showed that PLC DNA accelerated the reassociation of each individual fragment (Fig. 3). The smallest difference in reassociation rates between PLC DNA and control DNA occurred with fragments L and M, the smallest *Hae*III fragments. These experiments indicate that regions of the HBV genome contained in all of the restriction fragments investigated are present in PLC DNA, and no evidence was obtained that any part of HBV DNA is missing. The experiments did not establish, however, that the entire sequence of each fragment is present.

Estimation of number of copies of HBV DNA per PLC/PRF/5 cell by hybridization kinetics. To estimate the number of copies of HBV DNA per cell, we compared the rate of reassociation of HBV [^{32}P]DNA in the presence of known concentrations of PLC DNA with rates in the presence of known concentrations of unlabeled HBV DNA. Herring sperm carrier DNA was added to make the total DNA concentration the same in each tube. The results are shown in Fig. 4. The curves for [^{32}P]DNA reassociation in the presence of PLC DNA (0.9 mg/ml) and of unlabeled HBV DNA (3.8 ng/ml) were nearly superimposed, permitting an estimate of four HBV DNA copies per PLC/PRF/5 cell, assuming a DNA content of a haploid, mammalian cell for the purposes of this calculation.

HBV DNA base sequences in high- and low-molecular-weight cellular DNA. To determine whether the HBV DNA in PLC/PRF/5 cells was in a low- or high-molecular-weight form, we carried out a Hirt precipitation (5) of high-molecular-weight PLC DNA, using DNA from approximately 5×10^8 cells. After precipitation and centrifugation, the DNA in the pellet and that in the supernatant were purified as described above. The final DNA preparations were made up to the same volume, and equal samples of the two were tested for the effect of HBV [^{32}P]DNA on reassociation. Figure 5 shows that DNA from the Hirt precipitate significantly accelerated reassociation of the probe and that Hirt supernatant DNA from an equivalent number of cells had little effect. Clearly, a majority of the HBV DNA sequences in PLC/PRF/5 cells was in the high-molecular-weight DNA fraction.

Restriction fragments of PLC DNA which contain viral DNA base sequences. To identify specific restriction fragments of PLC DNA which contain HBV DNA base sequences, PLC DNA was digested with various restriction endonucleases. The digests were fractionated by agarose gel electrophoresis, and the DNA was transferred to nitrocellulose and incubated with HBV [^{32}P]DNA as described above. In all experiments, the completeness of restriction endonuclease digestion of DNA was monitored by including parallel samples of HBV [^{32}P]DNA and PM-2 [^{32}P]DNA plus 10 μg of carrier DNA. Electrophoresis of these samples containing radioactive DNA fragments of known size in the same gel with PLC DNA provided useful size markers. We carried out multiple experiments with each restriction enzyme to establish the

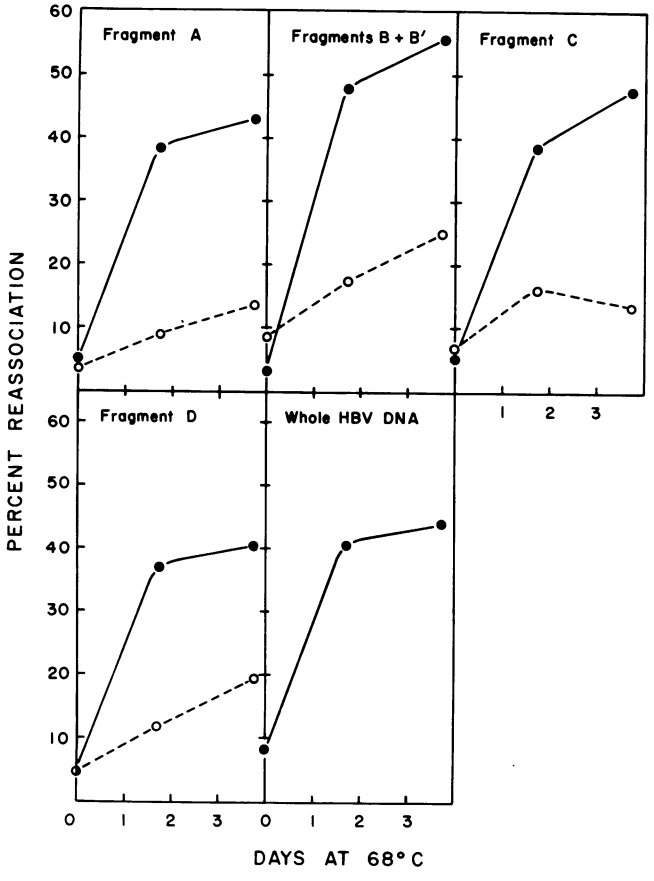

FIG. 2. *Effect of PLC and HS703T DNAs on the rate of reassociation of individual HincII fragments of HBV [^{32}P]DNA. Nick-translated HBV (virion) [^{32}P]DNA (5 × 10^5 cpm, 2.3 × 10^8 cpm/μg) was cleaved with endonuclease HincII, and the fragments were separated by polyacrylamide gel electrophoresis into four bands, which were recovered as described in the text. Reaction mixtures contained either sonicated PLC DNA (●) or HS703T cell DNA (○) at 1.8 mg/ml and 6,000 cpm of [^{32}P]DNA per ml from either HincII fragment A (980 bp), B (766 bp) plus B' (728 bp), C (546 bp), or D (314 bp), or uncleaved (whole) HBV [^{32}P]-DNA. The rate of reassociation of the [^{32}P]DNAs was determined as described in the legend to Fig. 1 and in the text.*

reproducibility of results shown here. In at least one such experiment with each enzyme, PM-2 DNA was mixed with PLC DNA for enzyme digestion, and the PM-2 DNA fragments were detected after gel electrophoresis by staining with ethidium bromide to establish that the hybridization results with the PLC DNA occurred after complete enzyme digestion of the DNA.

An autoradiogram from such an experiment with restriction endonuclease *Hinc*II-digested PLC DNA is shown in Fig. 6. Lanes a, c, and e show the positions of *Hinc*II fragments of phage PM-2 [^{32}P]DNA, which provided size markers and monitored the efficiency of transfer of DNA fragments from the agarose gel to nitrocellulose. Five radioactive DNA fragments in positions corresponding to sizes between 2,170 and 550 bp are clearly visible in these lanes. PM-2 DNA fragments smaller than 550 bp were present in very low amounts in this experiment and were not detected. Lane f shows the position of radioactive fragments of HBV [^{32}P]DNA digested with *Hinc*II after electrophoresis and transfer to nitrocellulose. Three bands consisting of radioactive DNA fragments of 980, 766 and 728 together, and 546 bp, as previously described (8, 16), are clearly present. A fifth *Hinc*II fragment of HBV DNA (314 bp) (16) is not visible in lane f. This fragment contains the single *Eco*RI site at which HBV DNA joins plasmid DNA in the recombinant which was the source of HBV DNA in this experiment. The two parts resulting from *Eco*RI cleavage of the 314-bp fragment were too

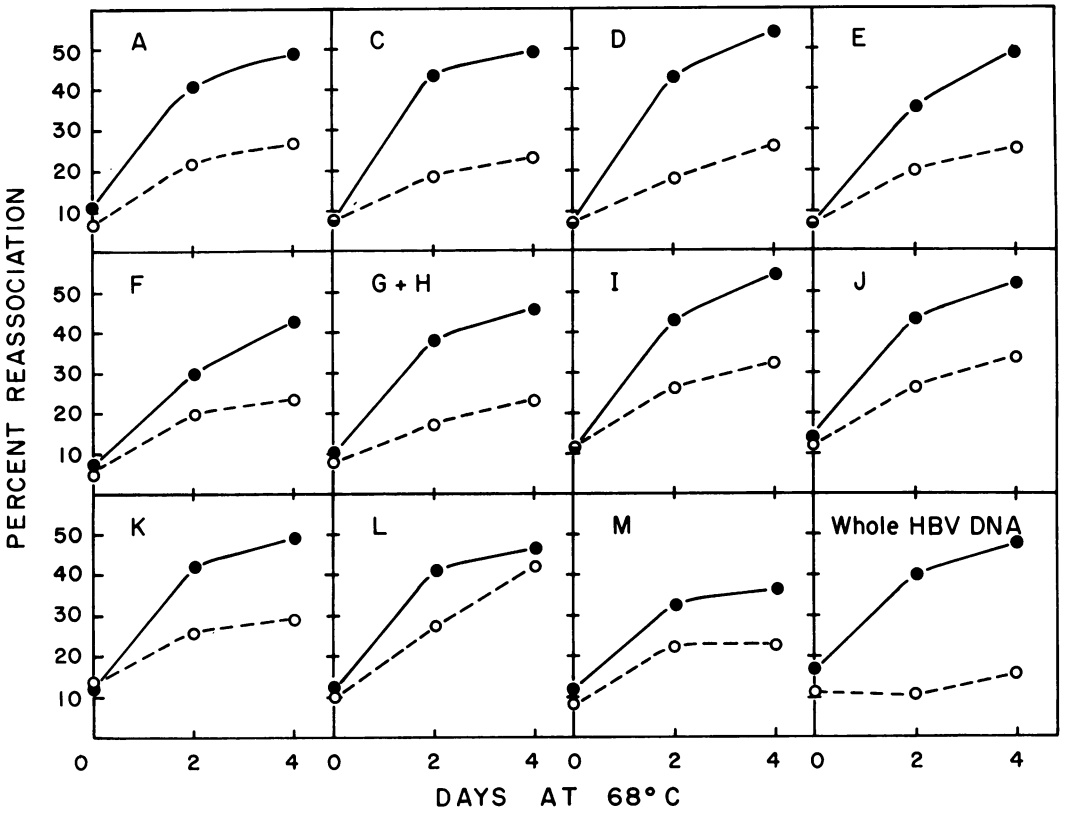

FIG. 3. *Effect of PLC and salmon sperm DNAs on the rate of reassociation of individual HaeIII fragments of HBV [^{32}P]DNA. Nick-translated HBV (virion) [^{32}P]DNA (1.3 × 10^{8} cpm/μg) was digested with endonuclease HaeIII, and individual fragments were isolated as described for Fig. 2. Reaction mixtures contained either sonicated PLC DNA (●) or salmon sperm DNA (○) at 1.0 mg/ml and 7,000 cpm of [^{32}P]DNA per ml from either HaeIII fragment A (970 bp), C (415 bp), D (347 bp), E (310 bp), F (242 bp), G (225 bp) plus H (209 bp), I (159 bp), J (144 bp), K (111 bp), L (100 bp), or M (90 bp), or uncleaved (whole) HBV [^{32}P]DNA. The rate of reassociation of the [^{32}P]DNAs was determined as described in the legend to Fig. 1 and in the text.*

small to transfer from the agarose gel to nitrocellulose. The unlabeled PLC DNA in lane d shows four strongly radioactive bands and one weaker band after hybridization with HBV [^{32}P]DNA. The bands range in size from approximately 850 to 6,000 bp. Only the smallest band was in the range of any of the fragments of the *Hin*cII-digested HBV [^{32}P]DNA shown in lane f, but this band did not actually comigrate with any of the HBV DNA fragments. PM-2 DNA was cleaved completely when added to the *Hin*cII reaction mixture with PLC DNA (data not shown), suggesting that the fragments of PLC DNA containing HBV DNA sequences were not products of partial digestion with *Hin*cII. No specific hybridization of the probe to HS703T DNA processed in the same way was detected (lane b).

Figure 7 shows a similar experiment with restriction endonuclease *Hae*III-digested PLC DNA. PLC DNA fragments which hybridized with HBV [^{32}P]DNA are shown in lane a, and controls consisted of *Hae*III-digested phage PM-2 [^{32}P]DNA (lane b) and HBV [^{32}P]DNA (lane c). The *Hae*III fragments of HBV [^{32}P]DNA are similar to those previously described (8). At least nine bands in the *Hae*III-digested PLC DNA have hybridized with the ^{32}P probe. Some of the less intense bands appear to correspond to the position of the HBV [^{32}P]DNA (e.g., fragments of 970, 415, 347, and possibly 242 bp). However, the two most intense PLC DNA bands do not correspond in position to any of the fragments resulting from *Hae*III cleavage of HBV [^{32}P]-DNA, and there are at least three additional, less intense bands, one of which is larger than the largest (970 bp) *Hae*III fragment of HBV [^{32}P]DNA.

The observations that many of the *Hae*III and *Hin*cII fragments of PLC DNA containing HBV-

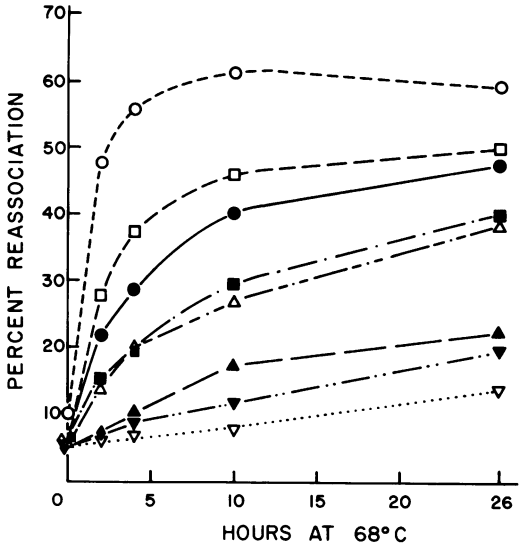

FIG. 4. *Estimation of the amount of HBV DNA in PLC/PRF/5 cells. The rate of reassociation of a nick-translated HBV (plasmid) [^{32}P]DNA (2 × 10^4 cpm/ml, 10^8 cpm/μg) in the presence of (i) 380 ng of HBV DNA per ml and 1.8 mg of herring sperm DNA per ml (○), (ii) 38 ng of HBV DNA per ml and 1.8 mg of herring sperm DNA per ml (□), (iii) 3.8 ng of HBV DNA per ml and 1.8 mg of herring sperm DNA per ml (△), (iv) 1.8 mg of PLC DNA per ml (●), (v) 0.9 mg of PLC DNA per ml and 0.9 mg of herring sperm DNA per ml (■), (vi) 0.18 mg of PLC DNA per ml and 1.62 mg of herring sperm DNA per ml (▲), (vii) 90 μg of PLC DNA per ml and 1.71 mg of herring sperm DNA per ml (▼), or (viii) 1.8 mg of herring sperm DNA per ml (▽) was determined as described in the legend to Fig. 1 and in the text.*

specific sequences were different from the fragments resulting from *Hae*III and *Hin*cII digestion of HBV [^{32}P]DNA suggest either that the HBV DNA is integrated as small pieces in PLC DNA or, more likely, that HBV(ad) DNA in the PLC/PRF/5 cells has different *Hae*III and *Hin*cII sites than the HBV [^{32}P]DNA used as a marker.

PLC DNA was also digested with restriction endonuclease *Hin*dIII, an enzyme found not to cleave any HBV DNA yet studied in our laboratory. Figure 8 shows that *Hin*dIII digestion of PLC DNA (lane b) produced two bands (approximately 3,500 and 5,000 bp) that were made intensely radioactive by hybridization with HBV [^{32}P]DNA and one band (6,500 bp) that was much less intensely labeled. No discrete bands were detected in undigested PLC DNA (data not shown). Controls consisted of *Hin*dIII-digested phage PM-2 [^{32}P]DNA (lane a), unlabeled calf thymus DNA (lane c), and HBV [^{32}P]DNA (lane d). It is apparent that the HBV [^{32}P]DNA was not cleaved by *Hin*dIII. The finding of three discrete DNA bands with HBV-specific base sequences in *Hin*dIII-digested (but not in undigested) PLC DNA which are larger than 3,200 bp (the size of uncleaved HBV DNA) suggests that HBV DNA is probably integrated at three different sites in high-molecular-weight PLC DNA.

Digestion of PLC DNA was also carried out with the restriction endonuclease *Eco*RI, which is known to cleave the typical adw_2 HBV DNA at a single site (16). Figure 9, lane b, shows that *Eco*RI digestion of PLC DNA produced two bands of approximately 2,000 and 2,600 bp which were made strongly radioactive by hybridization with HBV [^{32}P]DNA and two less radioactive bands of approximately 2,200 and 3,000 bp. Other fainter bands may also be present. Lane c shows that no detectable hybridization occurred with calf thymus DNA. Lane a shows the

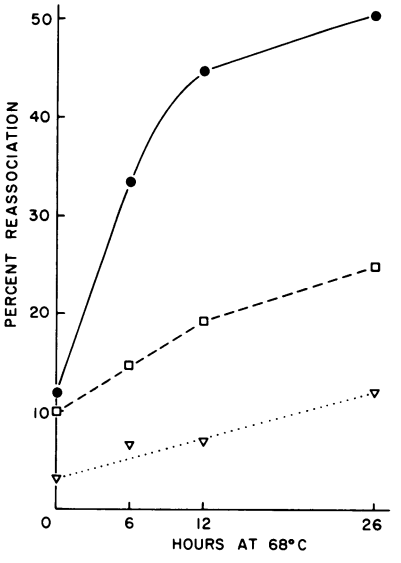

FIG. 5. *Effect of high- and low-molecular-weight fractions of PLC DNA on the rate of reassociation of HBV [^{32}P]DNA. The DNA in a sodium dodecyl sulfate PLC/PRF/5 cell lysate was separated into high- and low-molecular-weight fractions by salt precipitation by the method of Hirt (5), and DNA in each fraction was purified as described in the text. The DNA samples were dissolved in equal volumes of buffer, and equal portions were added to reassociation reactions. Reaction mixtures contained nick-translated HBV (plasmid) [^{32}P]DNA (16,000 cpm/ml, 10^8 cpm/μg) and either (i) 1.0 mg of DNA from the Hirt precipitate (●) per ml, (ii) 0.15 mg of DNA from the Hirt supernatant (□) per ml, or (iii) 1.0 mg of salmon sperm DNA (▽) per ml. The rate of reassociation of the [^{32}P]DNA was determined as described in the legend to Fig. 1 and in the text.*

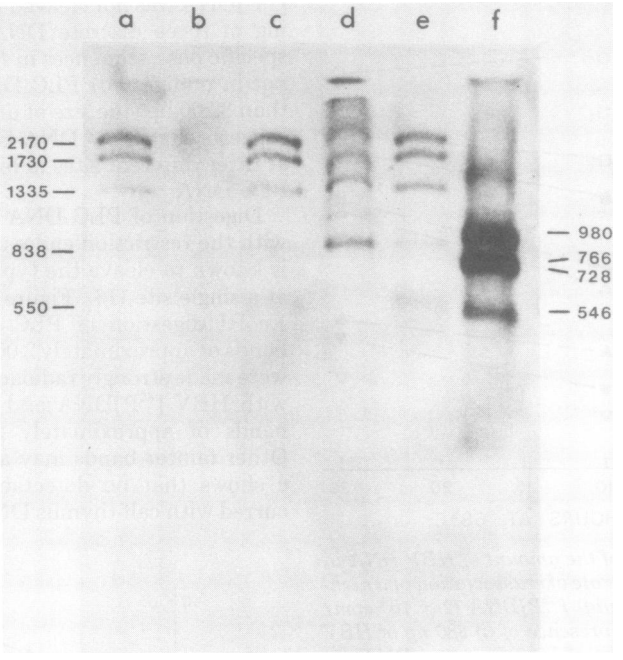

FIG. 6. *Autoradiogram of HBV [^{32}P]DNA hybridized with restriction endonuclease HincII cleavage fragments of PLC and other DNAs on a nitrocellulose filter. Lanes a, c, and e show the results with PM-2 [^{32}P]DNA plus 10 μg of calf thymus DNA. Results are shown with 10 μg of HS703T DNA (lane b), 10 μg of PLC (lane d), and nick-translated HBV (plasmid) [^{32}P]DNA plus 10 μg of calf thymus DNA (lane f) after each had been digested with HincII and after the DNA fragments had been separated by electrophoresis in a 2% agarose gel and transferred to nitrocellulose for incubation in a hybridization reaction with nick-translated HBV (plasmid) [^{32}P]DNA (5 × 10^7 cpm/μg, 0.15 μg/ml) as described in the text. The numbers on the left designate the sizes (bp) of the PM-2 [^{32}P]DNA fragments in lanes a, c, and e, and the numbers on the right are the sizes of the radioactive fragments in lane f resulting from cleavage of HBV [^{32}P]DNA with HincII and EcoRI.*

positions of *Hin*dIII fragments of PM-2 [^{32}P]-DNA.

Virus-specific RNA produced by PLC/PRF/5 cells. To examine the RNA of PLC/PRF/5 cells for virus-specific base sequences, we prepared [^{32}P]RNA as described above. Unlabeled restriction fragments of recombinant HBV-pBR322 DNA were separated by agarose gel electrophoresis and transferred to nitrocellulose, and the nitrocellulose was incubated in a hybridization reaction with [^{32}P]RNA. A subsequent autoradiogram is shown in Fig. 10. To monitor the transfer of DNA fragments to nitrocellulose and to provide molecular weight markers, cloned HBV DNA, which had been recovered from the recombinant after *Eco*RI digestion, was radiolabeled by nick translation, digested with *Ava*II, and run in lane a. Five radioactive fragments with electrophoretic positions corresponding to sizes between 1,130 and 220 bp can be observed in lane a. PM-2 phage [^{32}P]DNA, radiolabeled in vivo and digested with *Hin*dIII, was run in lane d to provide additional markers, and six radioactive fragments with positions corresponding to sizes between 5,000 and 275 bp are apparent.

Lane b shows the hybridization of PLC [^{32}P]RNA with DNA fragments resulting from digestion of HBV-pBR322 recombinant DNA with endonuclease *Ava*II. The four largest DNA fragments (approximately 1,130, 800, 640, and 340 bp), the sum of whose sizes is 2,910 bp, have clearly bound [^{32}P]RNA, and the intensities of the bands indicate that the radioactivity was present in the same proportions as the intensities of the staining with ethidium bromide of unlabeled HBV DNA fragments in the gel before transfer to nitrocellulose (data not shown) and in the same relative proportions as the HBV [^{32}P]DNA fragments generated by *Ava*II (lane a). The 220-bp *Ava*II fragment appears to be underlabeled or not labeled at all in lane b. This could be due to inefficient transfer of the DNA fragment because of its small size or to a low amount or absence of [^{32}P]RNA complementary to this fragment in the PLC RNA preparation.

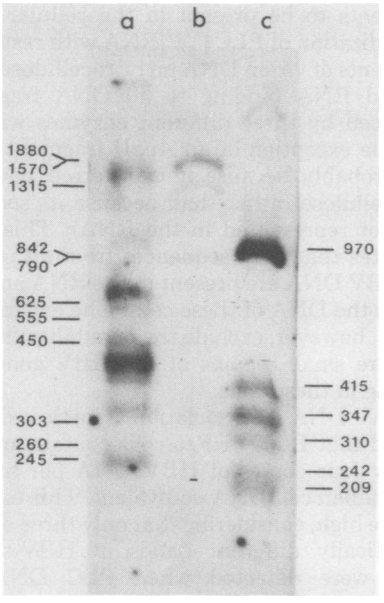

FIG. 7. *Autoradiogram of HBV [^{32}P]DNA hybridized with restriction endonuclease HaeIII cleavage fragments of PLC DNA on a nitrocellulose filter. Lane a shows the results with 10 μg of PLC DNA. Results are shown for PM-2 [^{32}P]DNA plus 10 μg of calf thymus DNA (lane b) and nick-translated (plasmid) HBV [^{32}P]DNA plus 10 μg of calf thymus DNA (lane c) after each had been digested with endonuclease HaeIII and after the resulting DNA fragments had been separated by gel electrophoresis and transferred to nitrocellulose for hybridization with nick-translated HBV (plasmid) [^{32}P]DNA (2.5 × 10^8 cpm/μg, 0.15 μg/ml) as described in the text. The numbers on the left are the sizes (bp) of the HaeIII PM-2 [^{32}P]DNA fragments (lane b) detected 3 weeks before hybridization, and the numbers on the right are the sizes (bp) of radioactive fragments resulting from cleavage of HBV [^{32}P]DNA with HaeIII and EcoRI (lane c).*

Lane c shows the results of hybridization of PLC [^{32}P]RNA with DNA fragments resulting from digestion of HBV-pBR322 DNA with endonucleases *Eco*RI and *Bgl*II. DNA fragments with sizes of approximately 1,900, 800, and 400 bp have hybridized with [^{32}P]RNA. These sizes correspond to those of the only three fragments which have been shown to result from digestion of HBV DNA typical for subtype adw$_2$ with *Eco*RI and *Bgl*II (16).

Lane e shows the results of hybridization of PLC [^{32}P]RNA with DNA fragments resulting from digestion of HBV-pBR322 DNA with endonucleases *Eco*RI and *Hpa*I. DNA fragments with sizes of approximately 2,300 and 900 bp have hybridized with [^{32}P]RNA, and these correspond to the sizes of the only fragments previously shown to result from digestion of HBV DNA typical for subtype adw$_2$ with these enzymes (16).

This experiment shows that all or nearly all of the HBV genome in these cells is transcribed, and it is consistent with the above experiments which show that all or nearly all of the HBV genome is present in PLC/PRF/5 cells.

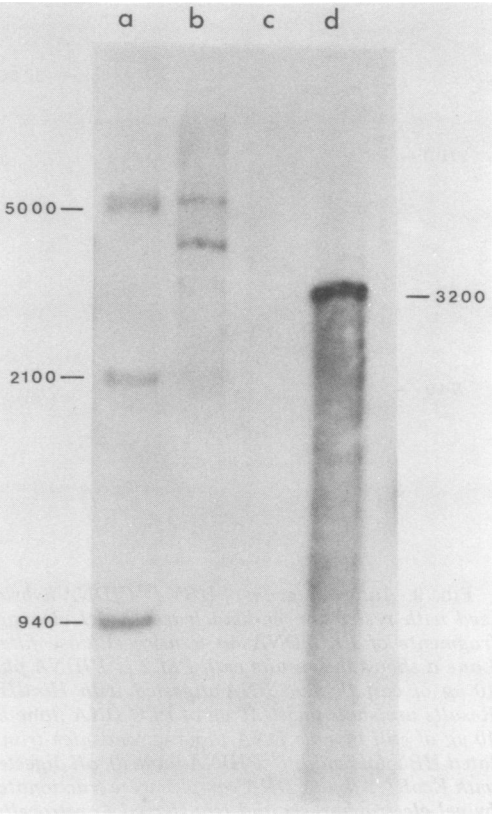

FIG. 8. *Autoradiogram of HBV [^{32}P]DNA hybridized with restriction endonuclease HindIII cleavage fragments of PLC and other DNAs on a nitrocellulose filter. Lane a shows the results with PM-2 [^{32}P]DNA plus 10 μg of calf thymus DNA. Results are shown with 10 μg of PLC DNA (lane b), 10 μg of calf thymus DNA (lane c), and HBV [^{32}P]DNA labeled by nick translation plus 10 μg of calf thymus DNA (lane d) after each had been digested with HindIII and after the resulting DNA fragments had been separated by electrophoresis in a 1% agarose gel and transferred to nitrocellulose for incubation in a hybridization reaction with nick-translated HBV (plasmid) [^{32}P]-DNA (7 × 10^7 cpm/μg, 70 ng/ml) as described in the text. The numbers on the left are the sizes (bp) of the HindIII PM-2 [^{32}P]DNA fragments (lane a), and the number on the right is the size (bp) of HBV [^{32}P]-DNA cleaved with EcoRI (whole linear DNA) shown in lane d.*

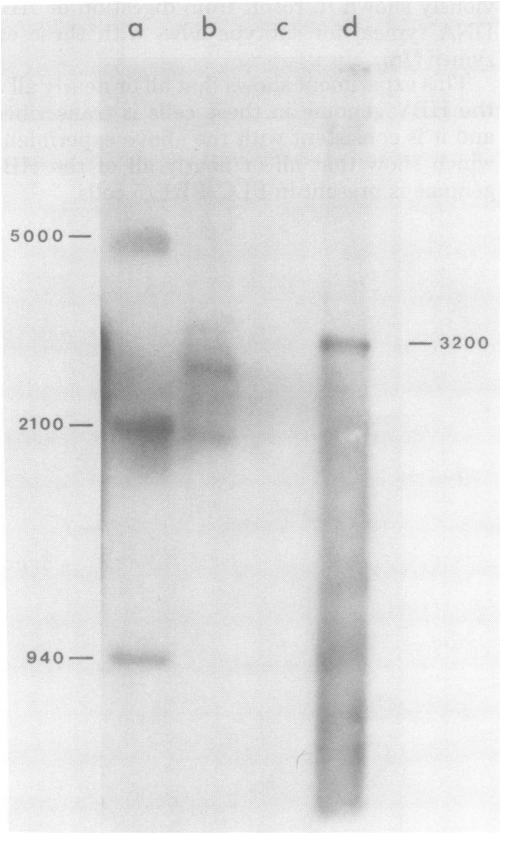

FIG. 9. *Autoradiogram of HBV [^{32}P]DNA hybridized with restriction endonuclease EcoRI cleavage fragments of PLC DNA on a nitrocellulose filter. Lane a shows the results with PM-2 [^{32}P]DNA plus 10 μg of calf thymus DNA digested with HindIII. Results are shown with 10 μg of PLC DNA (lane b), 10 μg of calf thymus DNA (lane c), and nick-translated HBV (plasmid) [^{32}P]DNA (lane d), all digested with EcoRI. All four DNA samples were fractionated by gel electrophoresis and transferred to nitrocellulose for hybridization with nick-translated HBV (plasmid) [^{32}P]DNA (7 × 10^7 cpm/μg, 70 μg/ml), as described in the legend to Fig. 6 and in the text. The numbers on the left are the sizes (bp) of the HindIII fragments of PM-2 [^{32}P]DNA (lane a), and the number on the right is the size (bp) of linear HBV DNA (lane d).*

DISCUSSION

The experiments described here have shown that HBV-specific DNA is present within a primary human liver carcinoma cell line that produces HB_sAg. A high proportion (possibly all) of the HBV DNA appears to be represented in DNA extracted from these cells. Base sequences in all of the virion DNA fragments produced by both restriction endonucleases *Hinc*II and *Hae*III were shown by liquid hybridization experiments to be present in the cellular DNA. Hybridization of PLC [^{32}P]RNA with restriction fragments of virion DNA on nitrocellulose filters showed RNA binding to all DNA fragments produced by three different enzymes with the possible exception of an *Ava*II fragment of 220 bp (probably because it transferred poorly to nitrocellulose rather than because its sequence was not represented in the RNA). This result indicates that base sequences from all parts of the HBV DNA are present in the RNA and thus also in the DNA of these cells. The experiments do not, however, exclude the possibility that one or more small regions of the HBV genome is missing in these cells.

HBV DNA reassociation kinetics demonstrated that PLC/PRF/5 cells contain approximately four copies of HBV DNA per haploid, mammalian cell DNA equivalent. This estimate may be high, considering that only three electrophoretically different bands of HBV-specific DNA were detected when PLC DNA was cleaved with *Hin*dIII, a restriction endonuclease that has no sites within the DNA of any of more than 20 different HBV isolates tested in our laboratory to date. One or more of the *Hin*dIII bands, however, could represent more than one copy of viral DNA per cell, or DNA fragments of nearly equal size from more than one integration site. The inefficient transfer of DNA fragments of high molecular weight to nitrocellulose could result in failure to detect any very large *Hin*dIII fragments containing HBV-specific sequences and thus explain the difference between the HBV DNA copy number found per cell and the number of *Hin*dIII bands.

All of the HBV DNA base sequences appear to be integrated in cellular DNA since they are present in high-molecular-weight DNA, shown both by liquid hybridization after a Hirt fractionation and by their exclusive presence in *Hin*dIII cleavage fragments of PLC DNA which are larger than linear virion DNA (3,200 bp). Both the relatively low number of viral genome copies per cell and the absence of detectable viral base sequences in DNA fragments of 3,200 bp or less after cleavage with endonuclease *Eco*RI, which has been found to cleave the HBV genome at only one site, rule out the possibility that HBV sequences are present in a high-molecular-weight form as tandemly arranged genomes (12).

Exactly how HBV DNA is integrated in the DNA of PLC/PRF/5 cells is not clear from our results. Digestion of PLC DNA with restriction endonucleases known to cleave HBV DNA several times (*Hinc*II and *Hae*III) yielded no fragments containing HBV sequences which were clearly identical to those of the typical adw_2

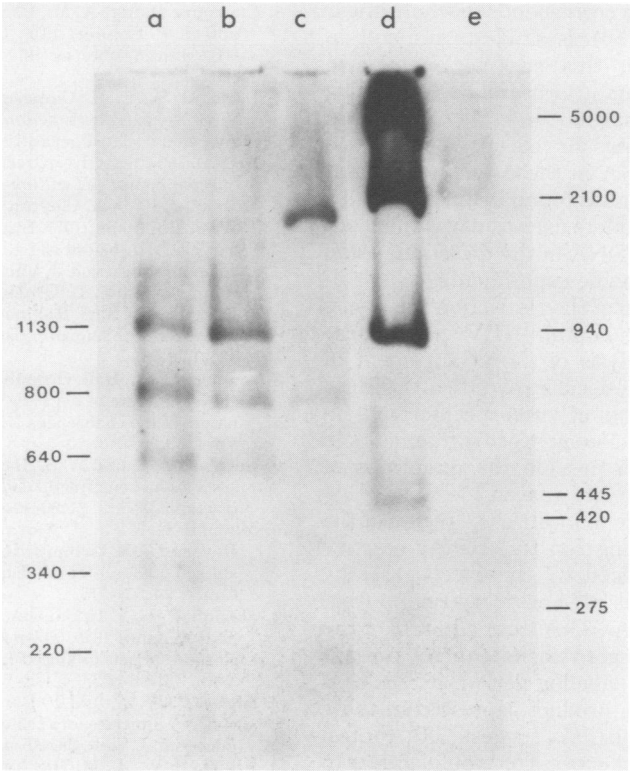

FIG. 10. *Autoradiogram of HBV [^{32}P]DNA hybridized with restriction endonuclease TaqI cleavage fragments of PLC DNA on a nitrocellulose filter. Lane a shows the results with 10 μg of PLC DNA digested with TaqI. Results are shown with PM-2 [^{32}P]DNA plus 10 μg of calf thymus DNA digested with HindIII. Both DNA samples were fractionated by gel electrophoresis and transferred to nitrocellulose for hybridization with nick-translated HBV (plasmid) [^{32}P]DNA (4 × 10^8 cpm/μg, 70 ng/ml) as described in the legend to Fig. 6 and in the text. The numbers designate the sizes (bp) of the HindIII PM-2 [^{32}P]DNA fragments in lane b.*

subtype DNA. The *Hinc*II digest contained only one recognizable fragment containing HBV DNA base sequences in the size range of the largest *Hinc*II cleavage product of typical adw$_2$ virion DNA and at least four larger fragments. The *Hae*III digest of PLC DNA yielded only two or three fragments in small amounts near the sizes of any *Hae*III fragments of typical adw$_2$ virion DNA and at least five prominent fragments that were clearly different. Interpretation of these findings is difficult because of the considerable variation in restriction patterns found among the DNAs isolated from the viruses of different infected patients (16). Although some restriction patterns appear typical for viruses of certain subtypes, variation within subtypes appears to be common. It is likely, therefore, that there are differences in some restriction sites in the DNA of the virus in the PLC/PRF/5 cells compared with the viral DNA used as a marker in these experiments. Virion-containing serum from the patient from whom the PLC/PRF/5 cell line was isolated was not available for restriction analysis of virion DNA. Because of the small amounts of HB$_s$Ag produced by these cells, only partial subtyping has been done (subtype ad), and thus it is not even possible at this time to use DNA from HBV of identical HB$_s$Ag subtype for comparison with PLC DNA. Our results with *Hinc*II and *Hae*III digestion of PLC DNA indicate that either the HBV DNA contained within these cells has *Hinc*II and *Hae*III restriction sites different from those in the HBV DNA used as a marker in these experiments or subgenomic fragments of the DNA, instead of whole genomes, are integrated. The finding that all or almost all of the viral DNA is present in these cells and that it occurs exclusively at three bands after digestion of cell DNA with *Hin*dIII would argue against the latter possibility. Incomplete digestion of the cell DNA was ruled out by appropriate controls.

The finding of multiple major and minor HBV-specific bands in the *Eco*RI digest of PLC

DNA, none of which corresponds to whole linear HBV DNA (3,200 bp), is similarly difficult to interpret. Although this enzyme appears to cleave the DNA of many virus isolates (including typical adw_2) at a single site, it is possible that multiple cleavage sites for this enzyme are present in the viral DNA in these cells to account for the number of bands found in digests of PLC DNA. Again, incomplete digestion was ruled out by including PM-2 DNA in the digestions as an internal control in some experiments.

Further definition of the restriction fragments of PLC DNA which contain HBV-specific base sequences and analysis of the arrangement of the viral DNA in these cells are difficult because of the small amounts of virus-specific DNA in these cells. We are cloning specific fragments in procaryotic cells to provide the quantities of DNA needed for further analysis.

It was not possible to localize the region of the HBV genome coding for the HB_sAg-reactive polypeptides produced by these cells (11) by hybridization of cell RNA with restriction fragments of HBV DNA. RNA from all parts of the HBV genome appeared to be equally present after a 3-day RNA labeling period. If HB_sAg is the only HBV gene product expressed in these cells, as our previous data suggest (11), control of viral gene product expression would appear to be at a step after transcription.

Our finding of HBV DNA integrated in the DNA of this human tumor cell line follows the example provided by the integration of papovavirus, adenovirus, and herpesvirus DNA in the genomes of cells transformed and tumors induced by these viruses. Further evidence that HBV induces cell transformation or hepatocellular carcinomas by its presence or integration still awaits a suitable in vitro culture system for HBV or a study with the related woodchuck virus and its host.

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