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Expression of cloned hepatitis B virus DNA in human cell cultures

(Dane particle/hepatitis B surface and core antigens/immunoelectron microscopy/immunofluorescence/12-*O*-tetradecanoylphorbol 13-acetate)

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ABSTRACT **DNA was isolated from the ayw subtype of hepatitis B virus (HBV) that had been incubated *in vitro* with all four deoxynucleoside triphosphates in order to complete the circular viral genome by means of the endogenous DNA polymerase. The purified viral DNA was cleaved with *Eco*RI restriction endonuclease, inserted into the *Eco*RI site of plasmid pBR322, and cloned in *Escherichia coli* χ1776. DNA from a clone, pHBV-1, that contained a 3200-base-pair insert of HBV DNA was cleaved with *Eco*RI and incubated with phage T4 ligase under conditions favoring intramolecular ligation. HeLa cell cultures exposed to this DNA showed marked cytopathic changes, accompanied by production of hepatitis B core and surface antigens, 11–14 days after subculture. Electron microscopic examination of anti-hepatitis B surface antigen immunoprecipitates from culture media of these cells revealed both 42-nm particles with central cores and 20-nm round particles. Although neither intact circular nor *Eco*RI-cleaved linear pHBV-1 DNAs evoked these effects in HeLa cells, both cytopathic changes and intranuclear hepatitis B core antigen were detected in HeLa cells infected with Dane particles.**

The Dane particle (1), which is believed to be the hepatitis B virion, is a 42-nm particle, with an outer shell and an inner 27-nm core. These particles possess at least three of the viral antigens: hepatitis B surface antigen (HBsAg), which can be divided into antigenic subtypes (2), and the hepatitis B core antigen (HBcAg) (3) and "e" antigen (HBeAg) (4, 5), antigens associated with the virus core. The core also contains DNA polymerase and a double-stranded, 3200-nucleotide-long DNA with a single-stranded gap of variable length (for reviews see refs. 6 and 7); the shorter DNA strand serves as primer for the viral DNA polymerase *in vitro* (8). Hepatitis B virus (HBV) DNA has been cloned (9–12), the nucleotide sequence of the entire genome has been determined (13), and the cloned DNA has been shown to direct synthesis of HBcAg in *Escherichia coli* (9, 14). However, the study of the biology of HBV has been hampered by the lack of a suitable cell culture system for the virus.

We have isolated a hybrid plasmid of pBR322 with a 3200-base pair insert of DNA from HBV of subtype ayw. We now report that HeLa cells exposed to cloned HBV DNA, excised from the plasmid and recircularized, synthesize both HBsAg and HBcAg. In addition, particles morphologically similar to Dane particles can be detected in the culture media of these cells.

MATERIALS AND METHODS

Preparation of HBV DNA. DNA from HBV of HBsAg subtype ayw was cloned by ligation into the *Eco*RI restriction endonuclease site of DNA from plasmid pBR322. The recombinant DNA was manipulated under P2 + EK2 containment under an approved memorandum of understanding and agreement as outlined in the National Institutes of Health recombinant DNA research guidelines, part VII (see *Federal Register*, December 22, 1978). HBV was isolated from serum by ultracentrifugation and labeled with [^{32}P]dTTP (New England Nuclear, 470 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) by using the endogenous DNA polymerase, and the labeled DNA was isolated from viral cores as described (15). Viral DNA cleaved by *Eco*RI (New England BioLabs) was inserted into pBR322, reisolated, cloned in *E. coli* χ1776, identified, and purified, using the methods described by Curtis *et al.* (16). The only modification of these methods was the treatment of *Eco*RI-digested pBR322 DNA with alkaline phosphatase (17) prior to ligation.

DNA from a hybrid plasmid containing a 3200-base-pair HBV DNA insert (pHBV-1) was linearized by *Eco*RI cleavage and recircularized in dilute solution [DNA at 12 μg/ml in 0.05 M Tris·HCl at pH 8, 0.05 M dithiothreitol, 0.02 M $MgCl_2$, containing 1 mM ATP and phage T4 ligase at 0.05 unit/ml (New England BioLabs)] to favor intramolecular religation. After both the linearization and religation reactions, the DNAs were extracted with phenol and precipitated with ethanol. After drying under reduced pressure the DNAs were dissolved in Hepes-buffered saline for calcium precipitation (18).

Infection of Cell Cultures with pHBV-1 DNA. HeLa cells grown in Eagle's minimal essential medium containing 10% fetal calf serum and 50 μg of gentamicin (Schering) per ml at a density of 2.5×10^5 cells per 35-mm culture dish were exposed to 15 μg of pHBV-1 DNA (uncleaved, linear, or religated), following the method of Graham and van der Eb (18) with the exception that the cells were exposed directly to $CaCl_2$-precipitated DNAs for 25 min at 25°C before culture medium was added. After 4 hr at 37°C the cultures were washed and fresh culture medium was added.

In some experiments the cultures were grown in the presence of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) at 10 ng/ml for 48 hr prior to the addition of the DNAs. Eight to eleven days after transfection, the cells (2.5×10^6) from each 35-mm culture dish were transferred to a 25-cm^2 culture bottle.

Infection of Cell Cultures with HBV. HBV was isolated from human serum as described (15). HeLa cell cultures at a density of 2.5×10^5 cells per 35-mm dish were incubated with 0.2 ml of HBV suspension (corresponding to 20 ng of HBV DNA) for 1 hr at 37°C in 8% CO_2/92% air. Two milliliters of culture medium was added, and after incubation for 24 hr the cells were washed and fresh culture medium was added. Cells were passaged as described above.

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; P_i/NaCl, phosphate-buffered saline.

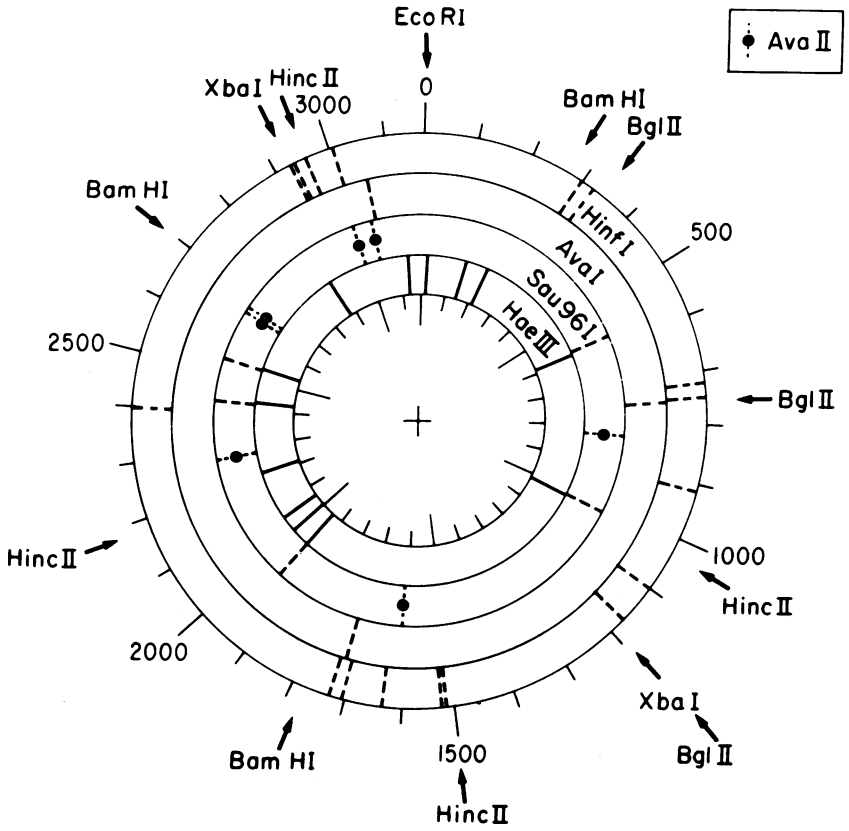

FIG. 1. Restriction endonuclease map of HBV (subtype ayw) DNA derived from pHBV-1. The sites marked with solid bars occur at the same position as those reported by Charnay *et al.* (10); the sites marked with interrupted bars have not been previously reported but occur where predicted by the sequence of HBV DNA subtype ayw (13). The cleavage site for *Eco*RI was chosen as the zero position for the map.

Assay of HBsAg and HBcAg. HBsAg was assayed in culture media by the Ausria II-125 radioimmunoassay (Abbott), using the criteria of Ling and Overby (19) for determining positive results. To detect HBcAg by indirect immunofluorescence, HeLa cells subcultured on LabTek slides were washed with phosphate-buffered saline (P_i/NaCl), fixed in acetone for 2 min at −20°C, washed again with P_i/NaCl, and incubated with 25 μl of a 1:10 dilution of human serum containing anti-HBcAg (20). The slides then were washed with P_i/NaCl, incubated with 25 μl of a 1:10 dilution of fluorescein-conjugated rabbit anti-human globulin at room temperature, washed with P_i/NaCl and covered with Elvanol (Du Pont). Serum containing anti-HBcAg, absorbed with a preparation of HBcAg as described (20), and a normal human serum negative for HBsAg, HBcAg, anti-HBsAg, and anti-HBcAg were used at the same dilution as the serum containing anti-HBcAg as controls.

Immunoelectron Microscopy. Cell culture media were clarified by centrifugation at 6000 rpm for 7 min at 5°C in a

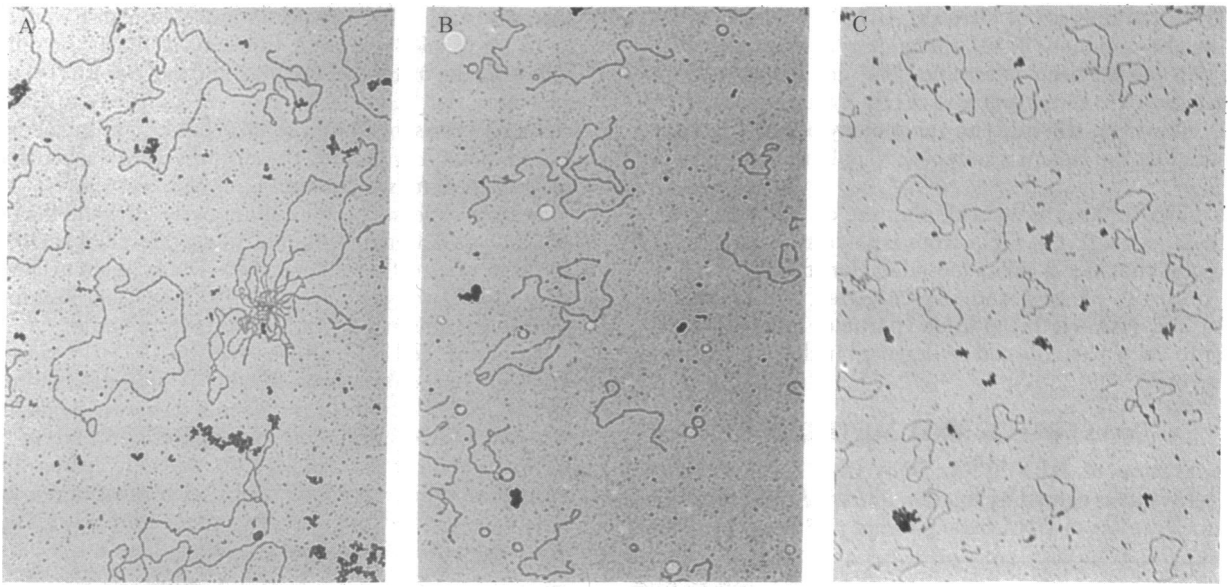

FIG. 2. Electron micrographs of DNA preparations. (×18,900.) (*A*) pHBV-1 DNA, showing supercoils and open circles. (*B*) *Eco*RI-cleaved pHBV-1 DNA, showing linear molecules. This preparation gave two bands (approximately 4300 and 3200 base pairs) on agarose gel electrophoresis. (*C*) Religated linear DNA, demonstrating predominantly open circles (note size difference from circles shown in *A*).

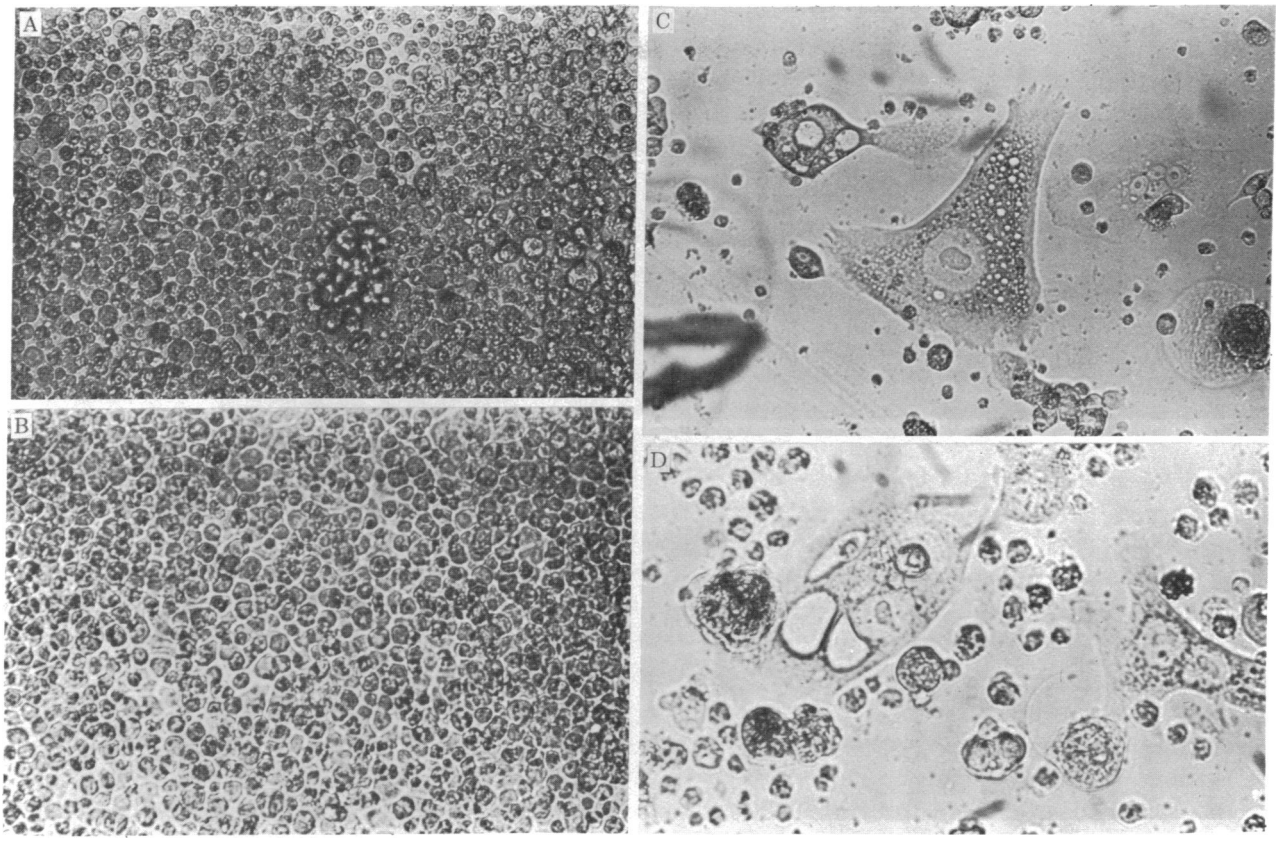

FIG. 3. Photomicrographs of HeLa cell cultures 12 days after passage. (Original magnification for *A*, *B*, and *C* ×250; printer's magnification ×2.65.) (*A*) Untreated cells. (*B*) HeLa cells exposed to *Eco*RI-cleaved pHBV-1 DNA, showing confluent growth. (*C*) HeLa cells exposed to religated HBV DNA. In the center there is a markedly enlarged cell filled with cytoplasmic vacuoles. To the right of this cell is a large cell with multiple nuclei and ground-glass cytoplasm; at the upper left there is a cell with dense nuclear and cytoplasmic bodies. (*D*) HeLa cells exposed to religated HBV DNA. The large cell in the upper left contains three vacuoles with refractile borders; to the right there is a binucleate cell with nuclear inclusions. (Original magnification ×400; printer's magnification ×2.65.)

Sorvall RC2B centrifuge. Five microliters of goat anti-HBsAg (20) were added to 1 ml of supernatant and incubated first for 2 hr at 37°C with gentle shaking and then for 2 hr at 4°C. One milliliter of P_i/NaCl was added and the fluid was centrifuged at 39,000 × *g* for 4 hr at 5°C. The resultant pellet was suspended in 20 μl of P_i/NaCl and stored at −20°C. Samples (10 μl) of the suspension were deposited on carbon-coated copper grids and stained with 2% phosphotungstic acid in 0.4% sucrose.

Electron Microscopy of DNA. DNA at a concentration of 1–2 μg/ml was stained with uranyl acetate by the method of Davis *et al.* (21).

RESULTS

Characterization of the pBR322-HBV Recombinant DNA Clone, pHBV-1. A restriction endonuclease cleavage map of the 3200-base-pair HBV DNA insert of pHBV-1 is shown in Fig. 1. The map indicates the similarity of our cloned HBV DNA to the DNA from the same serotype, cloned in λgt.*WES* by Charnay and coworkers [λHBV-1 (10)].

The infectivity of pHBV-1 was investigated with three different preparations (Fig. 2): (*i*) the intact hybrid plasma DNA (pHBV-1 DNA; Fig. 2*A*); (*ii*) pHBV-1 DNA cleaved by *Eco*RI into two fragments—i.e., the 3200-base-pair HBV DNA and 4300-base-pair pBR322 DNA (linear DNA; Fig. 2*B*): and (*iii*) *Eco*RI-cleaved pHBV-1 DNA recircularized with T4 ligase under conditions favoring intramolecular religation (religated DNA; Fig. 2*C*). It can be appreciated from the electron micrographs that few circular molecules remain after digestion by *Eco*RI and that, after religation, the DNA consists predominantly of circular forms that are approximately half the size of pHBV-1 DNA. We have estimated from electron micrographs that at least 75% of the linear DNA molecules are intramolecularly religated.

Effect of Cloned HBV DNA on HeLa Cell Morphology. HeLa cells exposed to any of the three preparations of pHBV-1 DNA did not differ markedly in appearance from cultures of untreated cells during the primary incubation period of 8–11 days. Similarly, the plating efficiency of DNA-treated cells at first passage was the same as that of untreated cells. However, 11–14 days after passage, cultures treated with religated DNA (HBV circles) exhibited marked cytopathic changes. Although the cells had multiplied in an apparently normal manner during the first week after passage, a large number of cells were lysed or detached at 11–14 days. Many of the remaining cells, some of which were multinucleate, were enlarged and contained dense nuclear inclusions of various sizes, as well as cytoplasmic vacuoles (Fig. 3 *C* and *D*). There were cells with ground-glass cytoplasm (Fig. 3*C*) and cells with long cytoplasmic processes. These cultures have now been maintained through four subsequent passages. During the first week after each subculture only cells with normal morphology were noted. However, after 8–10 days the cells again showed cytopathic changes. In contrast, cultures exposed to uncleaved pHBV-1 DNA or *Eco*RI-cleaved pHBV-1 DNA did not differ in appearance from untreated cells (Fig. 3 *A* and *B*).

Detection of HBcAg, HBsAg, and Viral Particles in HeLa Cell Cultures Exposed to Religated HBV DNA. HeLa cells

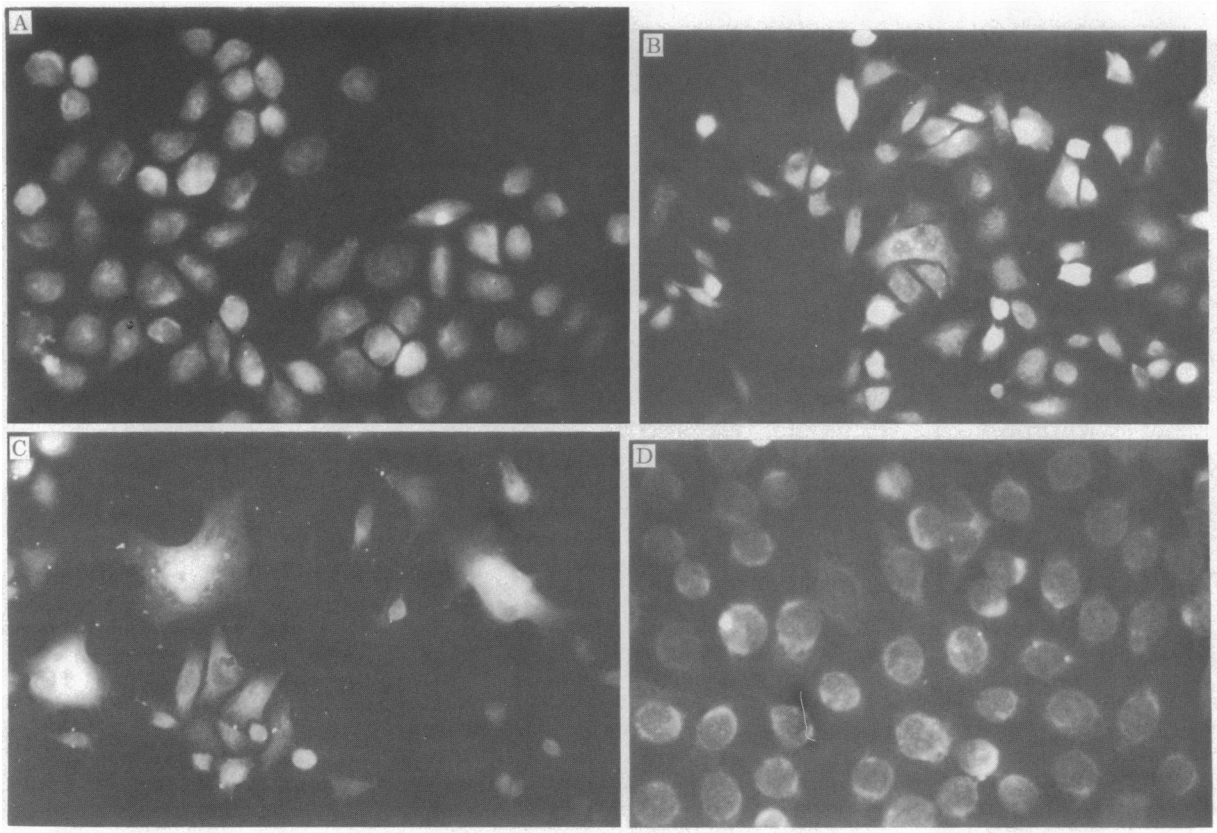

FIG. 4. Indirect immunofluorescent staining of HeLa cells for HBcAg. (Original magnification ×250; printer's magnification ×2.65.) (*A*) Untreated cells (the few bright highlights in the photograph consist of precipitated stain and optical artifacts and do not represent fluorescent cell nuclei). (*B*) HeLa cells exposed to religated HBV DNA, showing cells with nuclear fluorescence. (*C*) HeLa cells pretreated with TPA and exposed to religated HBV DNA, showing large cells with highly fluorescent nuclei. (*D*) HeLa cells exposed to religated HBV DNA. The serum containing anti-HBcAg was absorbed with HBcAg prior to incubation with the cells (20). Cells incubated with a normal human serum negative for anti-HBsAg, anti-HBcAg, HBsAg, and HBcAg also failed to show fluorescent staining.

subcultured onto LabTek slides 16 days after first passage were examined 6 days later for the presence of HBcAg by indirect immunofluorescence. Cells from cultures treated with religated HBV DNA showed nuclear fluorescent staining after exposure to human serum containing anti-HBcAg, while cells treated with linear and uncleaved DNAs were unstained. As shown in Fig. 4, the fluorescence was most pronounced in large cells containing intranuclear and cytoplasmic inclusions and in cells with ground-glass cytoplasm. Because it has been observed that the tumor promoter TPA enhances transformation of human leukocytes by Epstein–Barr virus (22) and induces expression of viral antigens in cells carrying the genome of oncogenic herpes viruses (23, 24), we assessed the effect of pretreatment of HeLa cells with TPA on the infectivity of HBV DNA preparations. TPA did not potentiate infection with uncleaved or linear DNA. After exposure to religated HBV DNA, however, pretreated cells showed cytopathic changes more rapidly, gave

Table 1. Detection of HBsAg in HeLa cell cultures

Treatment of cells	cpm
None	258
48 hr TPA and then religated HBV DNA	890
Religated HBV DNA	668
Linear HBV DNA	270
48 hr TPA and then linear HBV DNA	304
48 hr TPA and then circular pHBV-1 DNA	256

After treatment, cells were maintained for 11 days prior to passage. The culture medium (4 ml) was removed 16 days after passage and radioimmunoassayed for HBsAg. Each value represents an average of triplicate determinations using 0.2 ml of medium per assay; cpm values greater than 519 are positive for HBsAg ($P/N \geq 2.1$ as discussed in ref. 19).

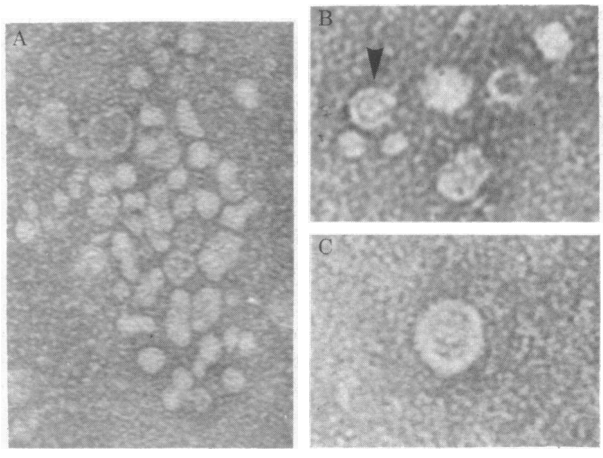

FIG. 5. Detection of virus-like particles in culture medium of HeLa cells exposed to religated HBV DNA. Immunoprecipitates from goat anti-HBsAg-treated medium (removed from cultures 16 days after passage) were collected and processed for electron microscopy. (*A*) Clumped particles containing 20- to 25-nm and 30- to 50-nm spherical forms, and small tubular forms with cross-striations. (×87,600.) (*B*) Arrow shows partially uncoated 40-nm particle with a central core. (×146,000.) (*C*) A 42-nm particle with outer coat and inner core. (×204,400.)

more intense nuclear fluorescence with anti-HBcAg (Fig. 4*C*) and produced more HBsAg. As can be seen in Table 1, the culture medium from cells exposed to recircularized HBV DNA contained small amounts of HBsAg, detectable by radioimmunoassay. HBsAg production occurred only in cultures displaying cytopathic changes; medium collected from cells exposed to linearized or uncleaved pHBV-1 DNA (data not shown) was consistently negative.

Cell media positive for HBsAg were also examined for the presence of viral particles by electron microscopy. Immunoprecipitates with goat anti-HBsAg contained 18- to 25-nm spherical particles, small tubular particles 50 nm in length (Fig. 5 *A* and *B*), and larger circular 38- to 50-nm particles with an outer coat surrounding a 26- to 30-nm core (Fig. 5*C*). Large tubular forms were not observed and there were more 20-nm particles than 42-nm particles. Both types of spherical particles were observed in clumps (Fig. 5*A*), and 42-nm particles with a broken outer coat and partially released core were noted (Fig 5*B*). Thus, particles similar in morphology to both HBsAg and Dane particles could be detected in culture medium of HeLa cells treated with recircularized HBV DNA.

Susceptibility of HeLa Cells to Infection with HBV. Because our results indicated that HeLa cells could support the expression of information encoded in the HBV genome, it was of interest to determine whether HeLa cells could also be infected directly with HBV. For this purpose cells were exposed to HBV particles concentrated from sera of patients with hepatitis B ($\approx 6 \times 10^9$ particles per 2×10^5 cells). Twenty-two to 26 days later some of the cells developed the same cytopathology as did cells in cultures treated with religated HBV DNA. Although altered morphology developed more slowly and involved many fewer cells, the affected cells produced HBcAg detectable by indirect immunofluorescence.

It is likely that the apparent inefficiency of the HBV particles as infectious agents compared to religated HBV DNA is a reflection of DNA concentration. We have calculated that, with 15 μg of religated DNA, the cells were exposed to approximately 4.5 μg of HBV DNA, whereas the inoculum of HBV particles contained no more than 0.02 μg of HBV DNA.

DISCUSSION

All of the evidence presented above indicates that under our experimental conditions HBV DNA can act as an infective agent for HeLa cells, but only when it is in the form of a 3200-base-pair double-stranded circle. It is not yet clear why the HBV genome is not expressed when it is present in a hybrid plasmid or when it has been linearized with *Eco*RI. Although it is likely that the linear form of HBV DNA is more susceptible to destruction by cellular nucleases than the circular form, such an explanation would not account for the inactivity of the HBV genome in the hybrid plasmid. However, it is known that the *Eco*RI cleavage site lies in a region of the HBV genome that contains three open reading frames on the coding (L) strand (13). Thus, it is possible that the cleaved HBV genome, whether present as part of a larger circular DNA molecule or in the linear form, cannot express some function(s) vital to viral replication.

The cytopathic effects observed in the HeLa cells are quite similar to the cytopathic effects previously observed after infection of human hepatocytes in cell culture with suspensions of HBV (25). Such hepatocytes develop nuclear inclusions and cytoplasmic vacuoles. The cultures contain enlarged cells, cells with ground-glass cytoplasm, and cells with long cytoplasmic processes (25). In infected HeLa cells these cytopathic effects as well as HBcAg and HBsAg became evident only after the cells had grown to confluence. This suggests that although infected cells may grow, viral functions are expressed only in growth-inhibited or nondividing cells.

Our data indicate that HeLa cells are a suitable host for expression of HBV viral functions. Because large amounts of infectious HBV genome circles can be easily prepared from cloned DNA, it seems likely that it will now be practical to study the replication of HBV *in vitro*. Furthermore, HeLa cells, so readily available in large amounts, should prove useful for detection of infectious forms of HBV in sera or in purified HBsAg vaccines.

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