Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1815–1819, March 1982
Biochemistry

Amplification of expression of hepatitis B surface antigen in 3T3 cells cotransfected with a dominant-acting gene and cloned viral DNA

(methotrexate resistance/gene amplification/gene transfer/restriction endonuclease analysis)

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Communicated by Hans Popper, December 17, 1981

ABSTRACT **3T3 cells containing hepatitis B virus DNA sequences can be efficiently selected by exposure to methotrexate after cotransfection with cloned viral DNA and DNA coding for a methotrexate-resistant dihydrofolate reductase. More than 75% of methotrexate-resistant cells isolated after cotransfection with a head-to-tail tandem of the hepatitis B virus genome synthesized viral surface antigen. The antigen was released into the culture medium in the form of 22-nm particles with buoyant density of 1.20 g/ml. No other virally coded proteins were detected in the cells or the culture medium. Application of selective pressure by increasing the concentration of methotrexate resulted in an amplification of viral DNA sequences and a concomitant increase in the rate of synthesis and release of hepatitis B surface antigen. The ability to produce large amounts of surface antigen appears to be a stable trait and has been maintained in these cultures through more than 30 passages.**

The clinical manifestations, epidemiology, and pathology of hepatitis B virus (HBV) infection are well established (1). However, due to the lack of an *in vitro* culture system in which the virus can be efficiently propagated, little is known about the replicative cycle of the virus or about its direct effect on the metabolism of infected cells. Although these difficulties have not yet been overcome, it is possible to study the expression of viral genes in mammalian cells transfected with cloned HBV DNA sequences (2–4).

As we have reported (2), it is possible to obtain expression of viral proteins in HeLa cells after simple transfection with recircularized cloned HBV genomes. Further studies of the expression of HBV genes in transfected HeLa cells were severely limited by the absence of an efficient selection system for isolating the small number of cells producing viral proteins. Here, we describe the selection of 3T3 cells containing HBV DNA by use of a method developed by Wigler *et al.* (5) for introducing and amplifying nonselectable genetic elements in methotrexate-sensitive mouse cells. Although this method could not be applied to HeLa cells because of the high frequency with which they developed methotrexate resistance, it allowed the selection of HBV-transfected 3T3 clones that efficiently synthesized and secreted HBV surface antigen (HBsAg). We further report that conditions that lead to amplification of methotrexate resistance result not only in amplification of HBV DNA sequences but also in increased HBsAg production.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. NIH 3T3 cells (mouse fibroblasts) were maintained in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% fetal bovine serum, penicillin at 250 units/ml, and streptomycin at 0.2 μg/ml. A derivative of the hamster line A29 (6), containing at least 40 copies of a mutated gene for dihydrofolate reductase (generously provided by R. Axel) was grown in DME medium with 3× nonessential amino acids supplemented with methotrexate at 40 μg/ml, 10% calf serum, and antibiotics as above. All cultures were maintained at 37°C in a moist atmosphere containing 5% CO_2.

Construction and Cloning of a Plasmid Containing Tandem Copies of the HBV Genome in a Head-to-Tail Arrangement. HBV DNA sequences were excised from pHBV-1, a recombinant plasmid constructed by inserting *Eco*RI-cleaved DNA from Dane particles into the *Eco*RI site of plasmid pBR322 (2). These sequences were purified by electrophoresis on 1% agarose, collected by electroelution, and ligated (7) to pHBV-1 that had been partially digested with *Eco*RI. The ligated DNA was used to transfect *Escherichia coli* (strain 294). DNA was extracted from tetracycline- and ampicillin-resistant colonies after chloramphenicol amplification (8).

Colonies containing plasmids with tandem head-to-tail HBV insertions were identified by restriction analysis of the isolated plasmid DNA. Fig. 1 shows the restriction analysis of one such plasmid, pTHBV-1, which was used for the transfection experiments described below. All experiments were performed in a P2 facility in the Department of Biochemistry of Mount Sinai School of Medicine, following the National Institutes of Health recombinant DNA research guidelines.

Isolation and Restriction Analysis of DNAs. Plasmid DNAs were isolated from *E. coli* as described by Curtis *et al.* (8). Cellular DNAs were extracted and purified by the method of Jeffreys and Flavell (9). DNAs were digested with *Eco*RI, *Bgl* II, or *Hin*dIII under conditions suggested by the supplier (New England BioLabs). Aliquots of genomic DNA (approximately 20 μg) were electrophoresed in 1% agarose and analyzed for HBV sequences by the method of Southern (10). HBV DNA purified from pHBV-1 or pBR322, radiolabeled to a specific activity of 10^8 cpm/μg by nick-translation with [α-^{32}P]dCTP (11) was used as probe.

Transfection of NIH 3T3 Cells. Cells were seeded at 5×10^5 per 100-mm dish 24 hr prior to transfection. Each culture was transfected with 20 μg of undigested genomic DNA from the A29 derivative and 5 μg of pTHBV-1 DNA in a calcium phosphate precipitate (12), following the protocol described by Wigler *et al.* (5). Initial selection for methotrexate resistance at 0.2 μg/ml was begun 1 day later when the cultures were split into three flasks. After 5–7 days the methotrexate concentration was

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Abbreviations: HBV, hepatitis B virus; HBsAg, surface antigen of HBV; DME medium, Dulbecco's modified Eagle's medium; bp, base pair(s).

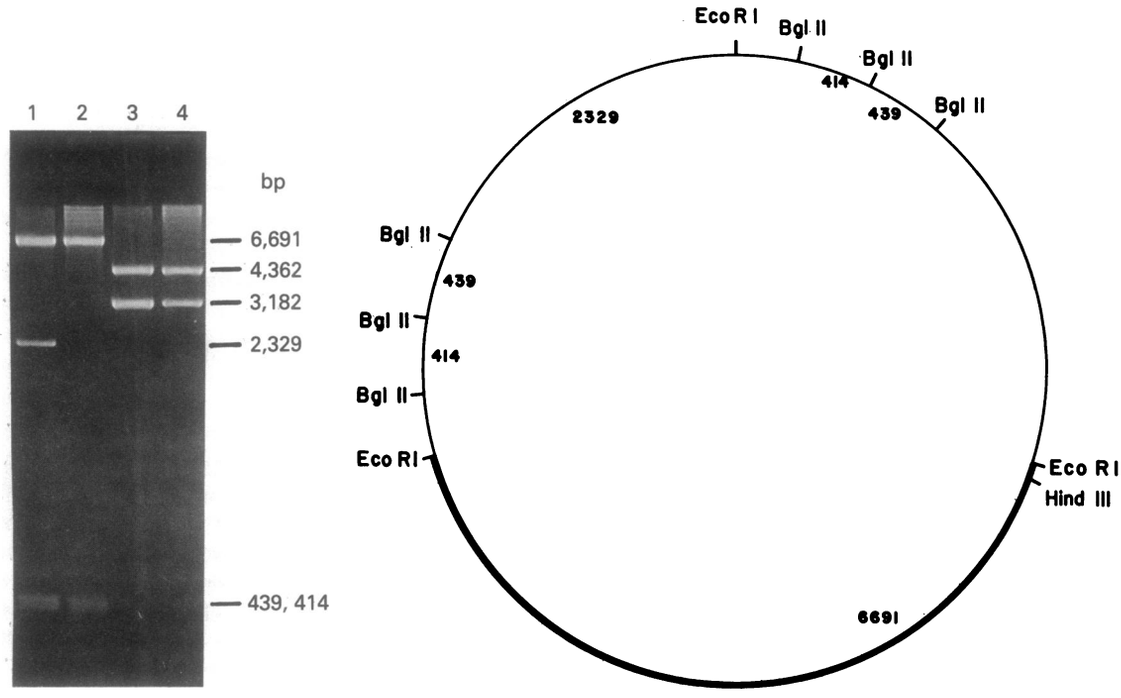

FIG. 1. (*Left*) Restriction enzyme analysis of recombinant plasmid pTHBV-1. Lanes 1 and 3 contain pTHBV-1 DNA (1 μg) digested with *Bgl* II and *Eco*RI, respectively. Lanes 2 and 4 contain DNA (1 μg) from pHBV-1 (pBR322 with one copy of HBV in the *Eco*RI site) digested with the same enzymes. The presence of a double insertion of HBV at the *Eco*RI site is indicated by the intensity of staining of the 3,182-base pair (bp) HBV genome (compare lanes 3 and 4). The head-to-tail orientation of the tandem is indicated by the presence of a 2,329-bp fragment (lane 1). The head-to-tail orientation was confirmed by *Bam*HI and *Hae* III digestion (not shown). (*Right*) Restriction map of pTHBV-1. Thick line, pBR322; thin line, HBV. Fragment lengths in bp are given on the inside.

increased to 0.4 μg/ml. Methotrexate-resistant colonies were visible to the naked eye (20–30 per plate) within 2–3 weeks after transfection. At this time clones were isolated by cloning rings and released by treatment with trypsin.

Detection by HBV Proteins. HBsAg in culture medium and CsCl gradient fractions was detected by using the Ausria II radioimmunoassay (Abbott). Samples with a positive-to-negative ratio greater than that of the positive control were diluted with phosphate-buffered saline until they gave values at or slightly below the value of the positive control. Intracellular HBsAg was detected by indirect immunofluorescence. The method and control reactions have been described (13). Immunoelectron microscopy of HBsAg particles was performed as published (14).

RESULTS

Isolation and Characterization of Methotrexate-Resistant 3T3 Cells Carrying HBV DNA. Methotrexate-resistant cells arising after cotransfection of 3T3 cells with DNA containing genes for a methotrexate-insensitive dihydrofolate reductase and HBV DNA were screened for the presence of HBV sequences and for production of HBsAg. To estimate the efficiency of transfer and expression of HBV sequences, methotrexate-resistant colonies were trypsinized from a single flask (≈30 colonies) and carried as a mixed culture through two passages. The cells were then analyzed by indirect immunofluorescence for HBsAg. Approximately 30% of the cells gave a strong fluorescence and at least another 50% were weakly fluorescent, indicating that cotransfection had occurred with high efficiency and that most cotransfectants synthesized HBsAg. Three colonies were selected at random from a second culture of cotransfected cells and grown to allow a more detailed characterization. All three clones contained HBV sequences that could be detected in 10 μg of genomic DNA by dot hybridization (15). One clone (C1) produced no detectable HBsAg. The other two clones (C2 and C3) produced and released equal amounts of HBsAg into the medium. As shown in Fig. 2, HBsAg could be detected in the culture medium of C2 cells within 24 hr of plating. Over a 4-day period, while the cells were in the logarithmic stage of growth, surface antigen accumulated in the medium at a rate proportional to the increase in cell number. Once the culture reached stationary phase, however, the rate of release of surface antigen increased severalfold. HBsAg could also be detected as fine granules in the cytoplasm of the cells by indirect immunofluorescence (Fig. 3). One day after seeding in fresh medium, 20–30% of the cells gave a positive reaction for HBsAg; by day 2, 40–50% of the cells were strongly positive, a value that did not increase with further time in culture. Similar results were obtained with C3 cells. Cells from the clone that did not release surface antigen were negative for intracellular HBsAg, as were NIH 3T3 cells and NIH 3T3 cells transfected with A29 DNA alone. Core or δ antigen could not be detected by indirect immunofluorescence in cells from any of the cultures.

The surface antigen in the medium of C2 and C3 cells was characterized by isopycnic centrifugation in CsCl (Fig. 4) and by electron microscopy (Fig. 5). All of the HBsAg banded at a density of 1.20 g/ml. This fraction was shown by electron microscopy to contain 22-nm spherical particles similar to those found in the serum of HBV-infected patients (16). No filamentous structures or Dane-particle-like particles were observed. Intracellular HBsAg was found to sediment in CsCl with the same buoyant density as released HBsAg particles.

The HBV sequences in the genome of C2 cells were further analyzed by Southern blotting (Fig. 6). Digestion of genomic DNA with *Hin*dIII gave a single fragment (≥8,000 bp) that hybridized with the ^{32}P-labeled HBV probe (lane 2). Digestion with *Eco*RI yielded two fragments detectable with this probe,

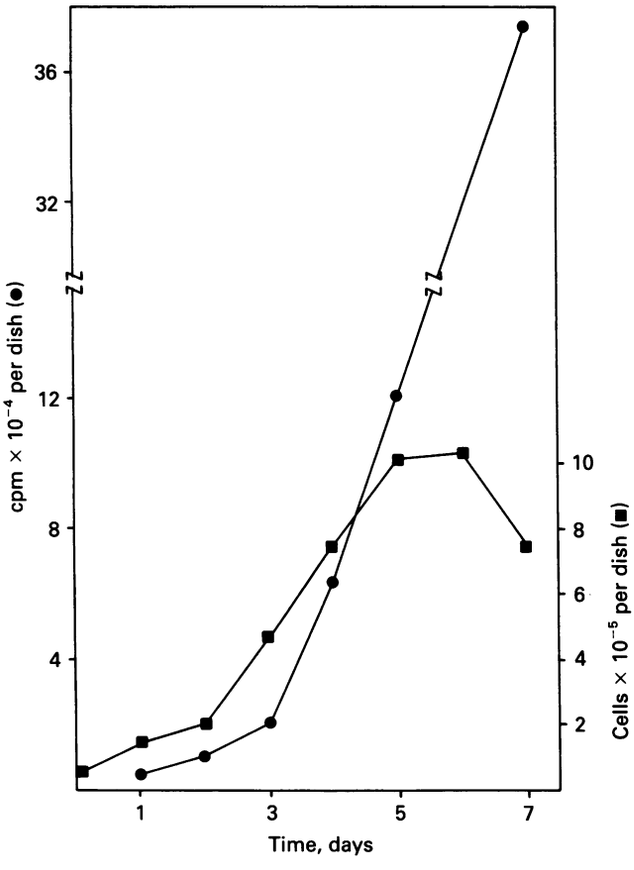

FIG. 2. Release of HBsAg by C2 cells. Cells were seeded at 5 × 10^4 per 35-mm Petri dish in 2 ml of DME medium/10% fetal bovine serum containing methotrexate at 0.4 μg/ml. At the indicated times, medium was removed from the dish and assayed for HBsAg. The cells were removed from the same dish by trypsinization and counted. Values shown are the average of two determinations.

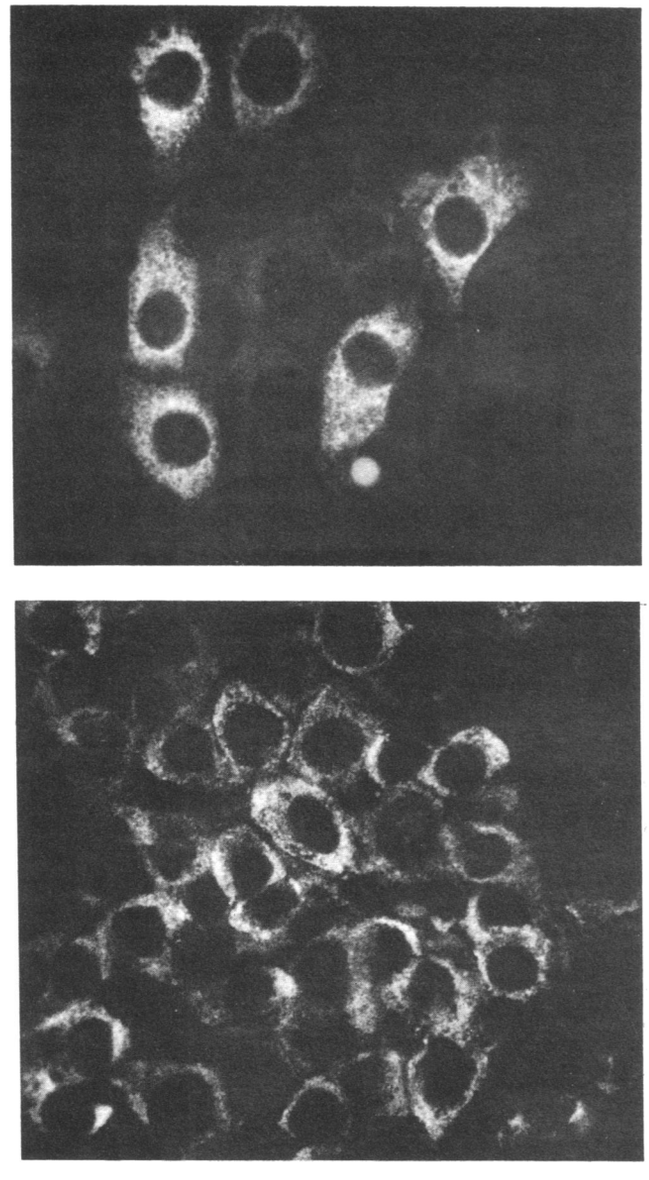

FIG. 3. Indirect immunofluorescent staining of C2 cells showing cytoplasmic HBsAg. (*Upper*) C2 cells in methotrexate at 0.4 μg/ml, 4-day culture. (*Lower*) C2 cells in methotrexate at 40 μg/ml, 4-day culture. These cells had been maintained for 5 weeks in methotrexate at 40 μg/ml. (×400.)

one the same size as the HBV genome (3,182 bp) and one larger (4,000 bp) (lane 1). These results are compatible with insertion of the HBV sequences into a single site with preservation of at least one full copy of the genome. Two major bands could be visualized after *Bgl* II digestion, a doublet containing the 439- and 414-bp fragments of HBV and the 2,329-bp internal fragment characteristic of a head-to-tail tandem orientation of HBV DNA (lane 3). This indicated the preservation of the region where the two copies of the HBV genome were linked. A faint band at 3,500 bp was also detected.

The same blot was washed (15) to remove the HBV probe and reprobed with ^{32}P-labeled pBR322 (Fig. 6, lanes 9–16). This DNA hybridized with the ≥8,000-bp *Hin*dIII fragment (lanes 10 and 14), with a 5,000-bp fragment in the *Eco*RI digest (lanes 9 and 13), and with a 3,500-bp fragment in the *Bgl* II digest (lanes 11 and 15). It did not, however, hybridize to any significant degree with the 3,182- and 4,000-bp *Eco*RI or the 2,329-, 439-, and 414-bp *Bgl* II fragments detected with the HBV probe. This would indicate that one end of the intact HBV genome is still linked to pBR322 and that the partial HBV genome is linked to either hamster or mouse sequences.

Amplification of HBV Sequences and Increased Production of HBsAg. To determine whether HBV sequences would be amplified along with genes conferring methotrexate resistance, C2 cells were seeded into medium containing methotrexate at 40 μg/ml. After an initial lag, most of the cells began to divide at a rate no more than 20% slower than cells maintained in methotrexate at 0.4 μg/ml. Although these cells were not subcloned prior to characterization of integrated HBV sequences, their DNA yielded the same restriction fragments as C2 cells when annealed with a HBV probe. The intensity of the autoradiographed bands was, however, at least 10-fold greater. (By dot hybridization, it could be demonstrated that approximately the same amount of ^{32}P-labeled HBV DNA hybridized to 0.5 μg of DNA from C2 cells maintained in methotrexate at 40 μg/ml and 10 μg of DNA from C2 cells maintained in methotrexate at 0.4 μg/ml.) Thus, it appears that by increasing the concentration of methotrexate in the medium, the HBV sequences in a large proportion of the cells were amplified as a unit without any rearrangement (Fig. 6, compare lanes 1–3 with lanes 5–7).

That this amplification of HBV sequences resulted in increased production of HBsAg was indicated by two findings: (*i*) Almost twice as many cells in 4-day cultures were strongly positive for intracellular HBsAg—i.e., 81–95% in methotrexate at 40 μg/ml versus 44–48% in methotrexate at 0.4 μg/ml (compare Fig. 3 *Upper* with Fig. 3 *Lower*). (*ii*) Both the level and

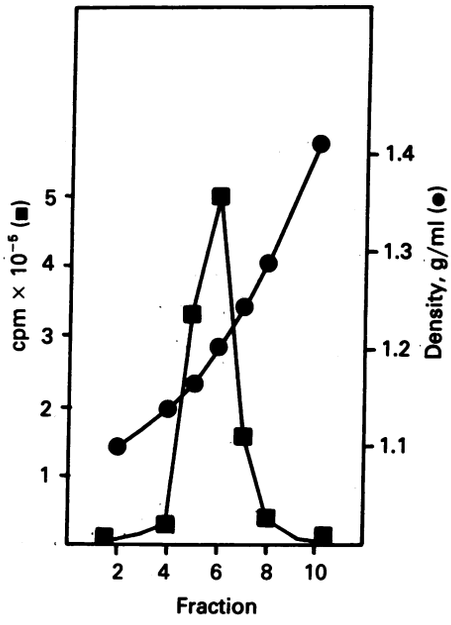

FIG. 4. Isopycnic centrifugation of HBsAg particles present in the medium of C2 cells. After centrifugation at 10,000 × *g* for 10 min, 60 ml of C2 culture medium was centrifuged at 50,000 × *g* (24 hr, 4°C). The pellet was rinsed with 10 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM EDTA (TNE buffer) and suspended in 4.5 ml of TNE buffer. CsCl (1.38 g) was added and the samples were centrifuged at 235,000 × *g* for 60 hr at 4°C. Fractions (500 μl) were collected; aliquots were tested for HBsAg by radioimmunoassay after 1:30 dilution in TNE buffer.

rate of release of HBsAg into the medium were increased 5- to 6-fold (Fig. 7).

DISCUSSION

There are now a number of reports in the literature describing mammalian cell lines that produce HBsAg. These are lines derived from human hepatocellular carcinomas (17, 18), thymidine kinase-negative mouse cells cotransfected with the herpes simplex virus thymidine kinase gene and a head-to-tail tandem of the HBV genome (3), and monkey kidney cells infected with

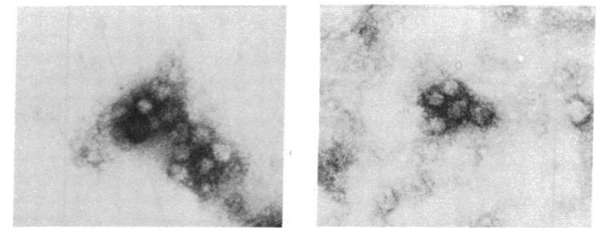

FIG. 5. Electron micrographs of aggregates of 22-nm spherical particles from CsCl density gradient fraction 6. Particles were incubated with antibody to HBsAg prior to negative staining with 2% phosphotungstic acid. (×100,000.)

a simian virus 40 recombinant carrying approximately 40% of the HBV genome (4). All of these lines, even those shown to contain complete copies of the HBV genome, fail to produce detectable amounts of viral proteins other than HBsAg. Both the human and mouse lines contain multiple copies of HBV sequences integrated into different sites in the cellular genome.

C2 cells are similar to these lines in that, even though they contain at least one complete and one partial copy of the HBV genome and even though these copies are amplified many times after growth in the presence of methotrexate at μg/ml, they do not produce detectable amounts of core or δ antigen. They do, however, contain intracellular HBsAg detectable by immunofluorescence that is present in particles of the same density as those released into the medium.

The cell lines we have described here differ from these lines in two respects. First, as indicated by restriction analysis, the HBV sequences appear to be integrated into a single site. Because no new integration sites are generated when the HBV sequences are amplified, it is probable that this integration site is present in a larger amplifiable unit, perhaps the dihydrofolate reductase gene itself. From our data, we cannot completely exclude the possibility that the integration site is present in an episome that is replicated along with genomic DNA. However, we were unable to detect any HBV sequences in a Hirt (19) extract, nor have we observed any double-minute chromosomes among the metaphase chromosomes of C2 cells grown in methotrexate at 40 μg/ml. Furthermore, the level of HBsAg production by C2 cells is undiminished after several passages in

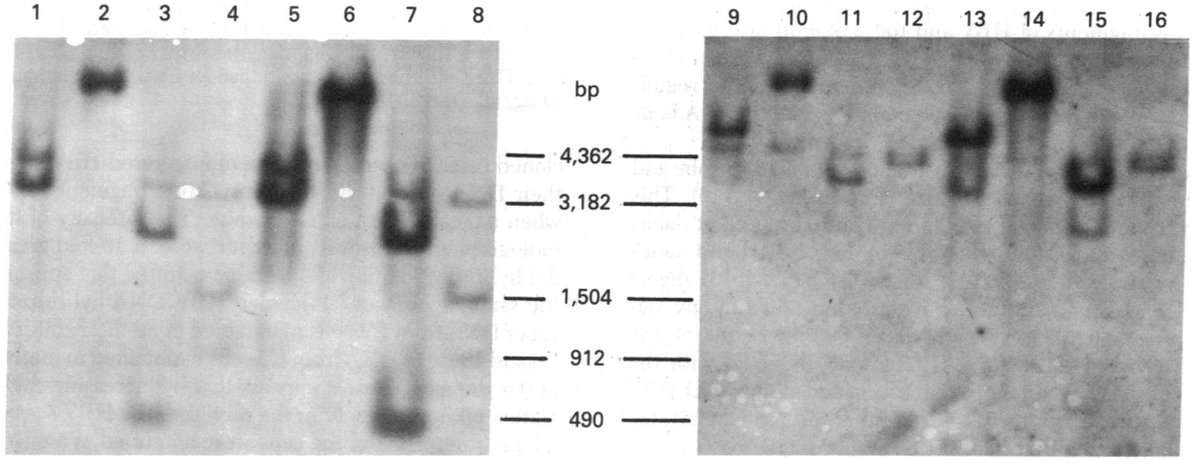

FIG. 6. Restriction analysis of HBV and pBR322 sequences in C2 cell genomic DNA. Autoradiographs of a 1% agarose gel loaded with: DNA from cells maintained in methotrexate (0.4 μg/ml) digested with *Eco*RI (lanes 1 and 9), *Hin*dIII (lanes 2 and 10), or *Bgl* II (lanes 3 and 11) and DNA from cells maintained in methotrexate (40 μg/ml) digested with *Eco*RI (lanes 5 and 13), *Hin*dIII (lanes 6–14), or *Bgl* II (lanes 7 and 15). Each sample contained 20 μg of DNA. Lanes 4, 8, 12, and 16 were loaded with a mixture of *Bam*HI/*Eco*RI and *Eco*RI digests of pHBV-1 DNA (lanes 4 and 12, 21 pg of *Eco*RI digest plus 105 pg of *Eco*RI/*Bam*HI digests; lanes 8 and 16, twice the concentration of lane 4). Fragment size is indicated. Lanes 1–8 show an autoradiograph after annealing with ^{32}P-labeled HBV DNA. Lanes 9–16 show an autoradiograph of the same blot after removal of the HBV probe and annealing with ^{32}P-labeled pBR322 DNA.

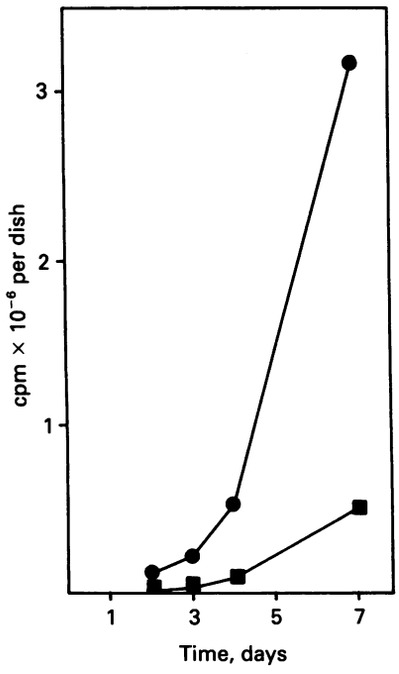

FIG. 7. Release of HBsAg by C2 cells maintained in methotrexate at 0.4 μg/ml (■) and 40 μg/ml (●). All details are as in Fig. 2. Both sets of cultures were seeded with 5×10^4 cells per dish. By day 7, cultures in methotrexate at 0.4 μg/ml contained an average of 9.2×10^5 cells, and cultures in methotrexate at 40 μg/ml, 7.8×10^5 cells.

methotrexate-free medium. All of these findings argue for a stable form of integration of HBV sequences into the mouse genome. Second, both the number of HBV genome copies and the amount of HBsAg synthesized can be readily increased through the selective pressure of increased levels of methotrexate. It can be calculated from the radioimmunoassay data that the maximal release of HBsAg by C2 cells growing in methotrexate at 40 μg/ml is on the order of 8–10 μg per 10^7 cells per day, which approximates that reported for human hepatoma cells at saturation density (17). This level of HBsAg release has been maintained through more than 30 passages.

Although we have not yet attempted further amplification of the HBV sequences, it is clear from our results that it should be possible to increase production of HBsAg by these cells to an even higher level. Because C2 cells do not produce complete hepatitis virus particle and are not transfected with any other human viral sequences, medium exposed to these cells may prove to be a suitable source of HBsAg for vaccine production.

We thank Dr. S. Hirschman for providing the serum Dane particles from which the HBV genome was cloned. We are also grateful to Dr. H. Popper for his helpful discussions. J.K.C. is a Senior Investigator of the New York Heart Association.

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