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Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA

(recombinant DNA/eukaryotic gene transfer/gene expression in transformed cells)

MARIE-FRANÇOISE DUBOIS*, CHRISTINE POURCEL†, SOLANGE ROUSSET*, CHARLES CHANY*, AND PIERRE TIOLLAIS†

*Unité de Recherche sur les Infections Virales, U. 43, Institut National de la Santé et de la Recherche Médicale, Hôpital Saint-Vincent-de-Paul, 74 avenue Denfert-Rochereau, 75014 Paris, France; and †Unité de Recombinaison et Expression Génétique, U. 163, Institut National de la Santé et de la Recherche Médicale, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris cedex 15, France

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ABSTRACT **A plasmid containing two cloned hepatitis B virus genomes in a tandem head-to-tail arrangement has been introduced into mouse fibroblasts by using cotransformation with the cloned herpes simplex virus thymidine kinase gene. Several copies of the plasmid were integrated into high molecular weight cellular DNA. The original tandem structure of the hepatitis B virus DNA was conserved. Hepatitis B surface antigen was synthesized by all the 15 clones examined. The other viral antigens were not detected. The surface antigen was excreted into the cell culture medium as particles having the same characteristics as those found in human serum. It is estimated that 2–4 × 10^4 particles were produced per mouse cell per 24 hr in two clones. This value corresponds to approximately 2–4 × 10^6 surface antigen polypeptides per cell per 24 hr.**

Hepatitis B virus (HBV) has a limited host range and, in nature, seems to infect only man and perhaps a few additional primates (1). So far, the virus has not been propagated in cell culture, and some viral surface antigen-producing cell lines from human hepatocellular carcinomas constitute the only cell culture systems that synthesize a HBV marker (2, 3). For these reasons, study of the virus multiplication at the molecular level has been greatly hampered. Recently, information on the genetic organization of the virus was obtained from the nucleotide sequence of the genome. Genes coding for surface and capsid antigens and perhaps for DNA polymerase are located on the long strand of the genome (4–7). However, nothing is known concerning virus gene expression and its regulation.

One approach to the study of HBV gene expression is to examine the functional capacity of the cloned viral DNA after its introduction into a mammalian cell culture. A gene that does not code for a selectable marker can be transferred into a cell by cotransformation of mutant mouse L cells deficient in thymidine kinase (TK) with this gene and the herpes simplex virus (HSV) *tk* gene (8). It appears that most of the selected TK^+ colonies contain the nonselectable marker. In the present study, mouse LTK^- cells were cotransformed with HBV cloned DNA and the HSV cloned *tk* gene. All the selected TK^+ clones synthesized hepatitis B surface antigen (HBsAg) particles which were excreted into the culture medium without apparent damage to the cells.

MATERIALS AND METHODS

Construction of the Recombinant and Bacterial Transformation. Plasmid pBR322 (200 ng) was digested with *Eco*RI endonuclease and treated with 2.6 units of alkaline phosphatase (9) in 100 mM Tris·HCl (pH 8.0) at 60°C for 60 min. After two phenol extractions and three ether extractions, DNA was precipitated with ethanol. The pellet was dissolved in water and 100 ng of *Eco*RI-digested HBV DNA was added. The ligation was performed as described (10). *Escherichia coli* DP50 was grown in L broth medium containing 100 μg of diaminopimelic acid (Sigma) and 20 μg of thymidine (Sigma) per ml. Bacteria were transformed as described (10) with the ligation mixture and were selected for resistance to ampicillin (100 μg/ml) and tetracycline (15 μg/ml). Nine hundred colonies were obtained and tested for the presence of HBV DNA by *in situ* hybridization (11, 12). Eight hundred colonies were positive; 16 giving a hybridization signal of higher intensity were selected. Plasmids were extracted and their structures were analyzed by *Eco*RI, *Xho* I, *Hin*dIII, and *Xba* I (BioLabs) digestion. Two *Eco*RI HBV DNA fragments inserted in a tandem head-to-tail arrangement were found in both possible orientations in 14 clones.

Cell Culture. Mutant mouse LM cells, clone 1D, deficient in TK (LTK^-, kindly provided by F. Colbère-Garapin) were grown in minimum essential medium (GIBCO) supplemented with 10% calf serum. After transformation, cell cultures were maintained in this medium containing hypoxanthine (15 μg/ml), aminopterin (1 μg/ml), and thymidine (5 μg/ml) (HAT selective medium), according to Littlefield (13).

Transformation of LTK^- Cells. Confluent monolayers (2 × 10^6 cells per 25-cm^2 Falcon flask) were inoculated with DNA according to the method of Graham and Van Der Eb (14), modified by Stow and Wilkie (15). Twenty-four hours after transformation, HAT solution (100-fold concentrated) was added to the medium, which was changed 1 week later and then every 3 days. After 20 days, HAT-resistant TK^+ colonies were picked with a pasteur pipette and transferred to tissue culture microplates. The colonies were passaged every 5 days and maintained under continued selective pressure in HAT culture medium.

Analysis of DNA from Transformed Cells. Cells were washed twice with phosphate-buffered saline and lysed directly in the flasks by the addition of 10 mM Tris·HCl, pH 7.8/10 mM NaCl/10 mM EDTA/0.5% $NaDodSO_4$. After digestion with proteinase K (Boehringer Mannheim; 100 μg/ml, at 37°C for 2 hr), DNA was extracted with phenol and chloroform/isoamyl alcohol (24:1, vol/vol), dialyzed, treated with RNase A

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Abbreviations: HBV, hepatitis B virus; HSV, herpes simplex virus; TK, thymidine kinase (*tk* for TK gene); HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HAT, hypoxanthine/aminopterin/thymidine mixture; kb, kilobase(s).

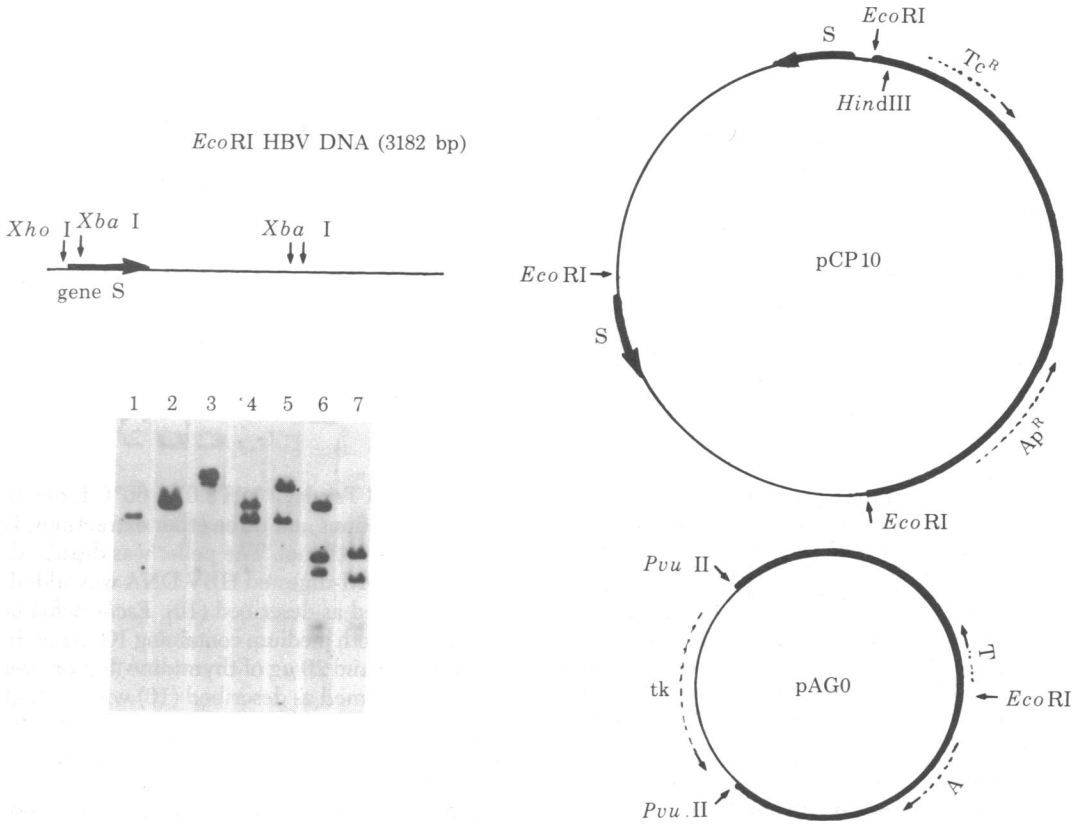

FIG. 1. Restriction endonuclease map of the two plasmids used for cell cotransformation. *Eco*RI HBV DNA is an *Eco*RI restriction fragment that corresponds to the whole HBV genome cloned in *E. coli* (24). Plasmid pAG0 is a derivative of plasmid pBR322, carrying the HSV *tk* gene (26, 27). Plasmid pCP10 is a derivative of plasmid pBR322 carrying two *Eco*RI HBV DNA fragments in a tandem head-to-tail arrangement. (*Inset*) The physical structure of pCP10 was deduced from restriction endonuclease digestions. Lanes: 1, *Eco*RI HBV DNA; 2, pBR322 digested with *Hin*dIII; 3, pCP10 digested with *Hin*dIII; 4, pCP10 digested with *Eco*RI; 5, pCP10 digested with *Xho* I; 6, pCP10 digested with *Xba* I and *Hin*dIII; 7, *Eco*RI HBV DNA digested with *Xba* I.

(Boehringer Mannheim; 100 μg/ml), extracted again with phenol and chloroform, and finally dialyzed. Cellular DNA was analyzed by the Southern blot technique as modified by Wahl *et al.* (16, 17). The *Eco*RI-digested HBV DNA probe was prepared as described by Weinstock *et al.* (18).

Detection of HBV Markers. The presence of HBsAg was detected with an AUSRIA II radioimmunoassay kit (Abbott). A direct passive hemagglutination test (HA screening kit, Wellcome, Beckenham, England) was used to determine the quantity of HBsAg produced. Hepatitis B core antigen (HBcAg) was assayed by neutralization of ^{125}I-labeled antibody to HbcAg with concentrated cell supernatants or with cell lysates before incubation with HBc-coated beads (CORAB test; Abbott). The presence of hepatitis B e antigen (HBeAg) was detected by using the HBeAg radioimmunoassay kit (kindly provided by L. O. Overby, Abbott).

Immunofluorescence Staining. Indirect immunofluorescence testing for HBsAg was performed with rabbit anti-HBsAg antiserum (Behring Institute) and fluorescent anti-rabbit IgG (Institut Pasteur). HBcAg and HBeAg were detected by direct immunofluorescence with fluorescent human anti-HBc and anti-HBe/1,2,3 antibodies (19).

DNA Polymerase Assay. DNA polymerase activity was measured by the method of Kaplan *et al.* (20) in the pellet obtained after cell supernatant ultracentrifugation.

Electron Microscopy. Samples were adsorbed on carbon/Formvar-coated grids by using the negative staining technique (21). For immunoelectron microscopy, grids were coated with anti-HBsAg antiserum as described by Milne and Luisoni (22). The grids were examined with a Siemens–Elmiskop 101 transmission electron microscope.

Biohazards. Biohazards associated with the experiments described in this article have been examined previously by the French National Control Committee, and the experiments were carried out according to the rules established by this Committee.

Table 1. Cotransformation of LTK$^-$ mouse cells with plasmids pCP10 and pAG0

Flask	DNA per flask, μg: Linearized* pAG0	Linearized* pCP10	Circular pCP10	Salmon sperm DNA	No. of TK$^+$ colonies	*P/N*†
b	0.010	10	—	—	140	24
c	0.005	—	5	15	200	16
f	0.010	—	—	15	26	1

* Plasmids pAG0 and pCP10 were linearized with *Hin*dIII.

† *P/N* = cpm in supernatant/cpm in negative control, estimated by radioimmunoassay for HBsAg 20 days after cell cotransformation. *P/N* > 2.1 is considered to be positive.

RESULTS

Construction of the HBV DNA Recombinant Plasmid (pCP10). The 3.2-kilobase (kb) *Eco*RI DNA fragment corresponding to the entire HBV genome (23) was obtained by *Eco*RI digestion of a bacteriophage λ derivative DNA carrying the cloned HBV genome (24). Because the initiation codon of the HBsAg gene (gene *S*) is located at 150 base pairs from one extremity of this fragment (5), we decided to construct a plasmid containing two *Eco*RI HBV DNA fragments in a tandem head-to-tail arrangement. This should enable gene *S* transcription from the HBV genome in transformed cells, whatever the location of the transcription initiation site in the viral DNA might be. The plasmid used to construct the recombinant DNA was pBR322 (25). Integration of two *Eco*RI HBV DNA fragments was shown by *Xho* I digestion which excises from the hybrid plasmid a DNA fragment of the same size as the *Eco*RI HBV DNA (Fig. 1). The direction of *Eco*RI HBV DNA insertion was tested by double digestion with *Hin*dIII and *Xba* I. The recombinant plasmid (10.6 kb) that contained two HBV genomes in a tandem head-to-tail arrangement, oriented as described in Fig. 1, will be referred to as pCP10.

Transformation of Mouse L Cells and Detection of HBsAg. Mouse LTK$^-$ cells were cotransformed with HBV DNA recombinant plasmid pCP10 and plasmid pAG0 (26) carrying the cloned HSV *tk* gene (Fig. 1). Plasmid pAG0 was linearized with *Hin*dIII, and plasmid pCP10 was used as circular form or linearized with the same enzyme. In all experiments, the molar ratio of pAG0 to pCP10 was about 1:1000. Salmon sperm DNA was added as a carrier to adjust the total DNA concentration to at least 10 μg/ml. After growth for 2 weeks in HAT selective medium, the colonies were examined. Twenty days after cotransformation, detectable amounts of HBsAg were found in the cell culture medium by radioimmunoassay (Table 1). Cultures corresponding to cells transformed with plasmid pAG0 alone did not release HBsAg. It is noteworthy that the number of surviving colonies was higher after cotransformation than after transformation with pAG0 alone. Five colonies from flask b (linearized pCP10) and 10 from flask c (circular pCP10) were picked and grown in HAT medium. All 15 cultures released HBsAg into the supernatant, and the amount synthesized, varying over a 30-fold range according to the clones, was stable during subsequent passages. When HBsAg released into the supernatant was mixed in a 10:1 ratio with anti-HBsAg antiserum and incubated at 37°C for 1 hr, the antigen was completely neutralized as shown by radioimmunoassay. With anti-HBsAg antiserum, indirect immunofluorescence revealed discrete cytoplasmic granules in the majority of cotransformed cells (Fig. 2). The highest yielding clones, b3 (transformed with linearized pCP10) and c4 (transformed with circular pCP10), were selected for further studies.

Neither HBcAg nor HBeAg could be detected by radioimmunoassay in supernatants concentrated 25-fold or in cell lysates. No significant fluorescence could be seen in the cells of clone b3 after incubation with anti-HBc or anti-HBe conjugates.

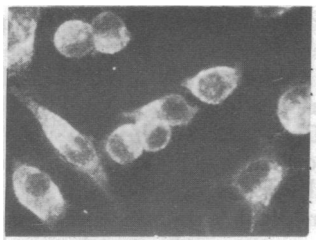

FIG. 2. Indirect immunofluorescence staining of cloned b3 cells showing HBsAg cytoplasmic granular fluorescence.

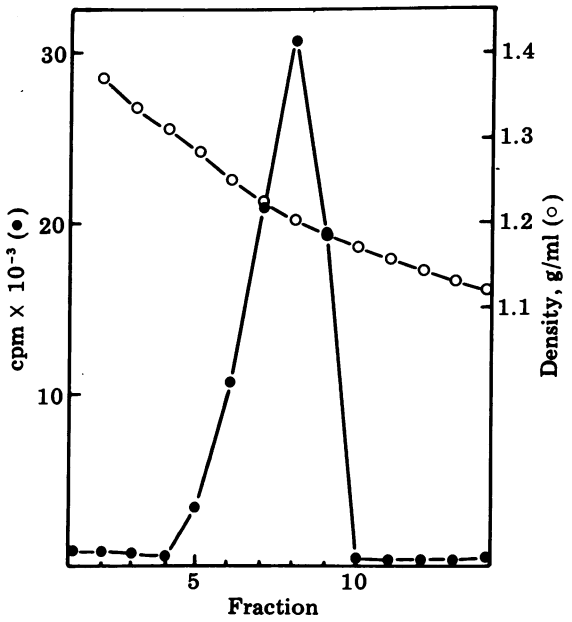

FIG. 3. Isopycnic centrifugation, in a CsCl gradient, of HBsAg particles present in the clone b3 supernatant. After low-speed centrifugation, 29 ml of b3 culture medium was ultracentrifuged at 73,000 × *g* (4°C, 23 hr). The pellet was suspended in 4.5 ml of 10 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM EDTA (TNE buffer). CsCl (1.38 g) was added to give a density of 1.2 g/ml. Centrifugation was at 190,000 × *g* and 4°C for 64 hr. Fourteen fractions were collected, aliquots were diluted 1:30 in TNE buffer, and HBsAg was measured by radioimmunoassay.

Moreover, no DNA polymerase activity was detected in the pellet obtained by ultracentrifugation of the cell culture medium.

Excretion of HBsAg as Particles in the Cell Culture Medium. The question of whether HBsAg is released outside the cell in a soluble or in a particulate form was examined. The supernatant of a b3 clone culture was centrifuged and the pellet was analyzed in a CsCl buoyant density gradient. HBsAg was present in a fraction corresponding to a buoyant density of 1.20 g/ml (Fig. 3), which is also that of the particles found in human serum (28). By electron microscopy, spherical particles were observed, ranging in diameter from 18 to 25 nm with an average of 22 nm. Their shape was similar to that of the spherical 22-nm surface antigen particles present in human serum. Filamentous structures were not observed. Anti-HBsAg antiserum clearly agglutinated and coated the particles (Fig. 4). No Dane particles could be detected.

The kinetics of intracellular and extracellular HBsAg pro-

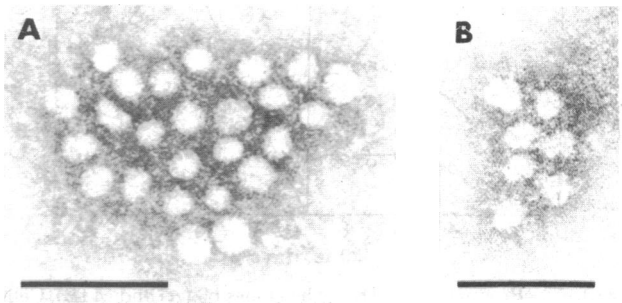

FIG. 4. Electron micrographs of spherical HBsAg particles were trapped in aggregates on antibody-coated grids. Some of them were surrounded with a halo of specific antibodies. Bars denote 100 nm. (*A*) Produced by clone b3 cells. (*B*) From a reference human serum.

duction were further studied. At time 0, cells were plated at 250,000 per petri dish. Supernatants and cell lysates were collected from series of replicate cell cultures every 24 hr for 9 days and the amount of surface antigen was estimated by radioimmunoassay. Results corresponding to clones b3 and c4 are shown in Fig. 5. HBsAg synthesis reached a plateau after 7 days. The amount of intracellular antigen per flask was about one-third the amount of extracellular antigen. In parallel experiments, at cell saturation density, the medium was removed every 24 hr. Under these conditions, the quantity of intracellular and extracellular HBsAg per cell remained approximately constant each day. We attempted to measure the amount of antigen synthesized by using direct passive hemagglutination, with a positive human serum as reference. Clone b3 produced about 150 ng of HBsAg per ml per day at saturation density. In comparison, the hepatoma cell line PLC/PRF/5 (2) produced 250 ng/ml under similar conditions.

Integration of the HBV Genome into Cellular DNA. High molecular weight DNA was extracted from clone b3. Undigested DNA and *Eco*RI, *Hin*dIII, and *Xho* I DNA fragments were fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters. DNA was then hybridized with ^{32}P-labeled nick-translated *Eco*RI HBV DNA as a probe according to Southern (16). HBV DNA hybridized to undigested DNA in the region of the gel corresponding to high molecular weight values (Fig. 6). Digestion with *Hin*dIII, which does not cleave HBV DNA (23), revealed about six DNA fragments. These two results prove the integration of several copies of plasmid pCP10 into the cellular DNA and the absence of HBV DNA in an episomic structure. The HBV DNA probe hybridized predominantly with an *Eco*RI and an *Xho* I fragment, both having the same size as the linear HBV genome (3.2 kb). Other DNA fragments of different sizes were also detected in the two patterns. This demonstrates that the HBV genome, in the dimer form, is still present in the cells. The existence of a few weak bands in the *Eco*RI pattern shows that, in some cases, pCP10 could be integrated through the HBV DNA. Similar results were obtained after the analysis of clone c4 DNA.

DISCUSSION

Using cotransformation of LTK$^-$ mouse fibroblasts with two plasmids carrying, respectively, two cloned *Eco*RI HBV DNA fragments in a tandem head-to-tail arrangement and the cloned HSV *tk* gene, we have selected cell lines that synthesize HBsAg. This DNA-mediated gene transfer system gave a highly efficient transformation with HBV DNA because all the 15 LTK$^+$ selected clones synthesized HBsAg. Under our experimental

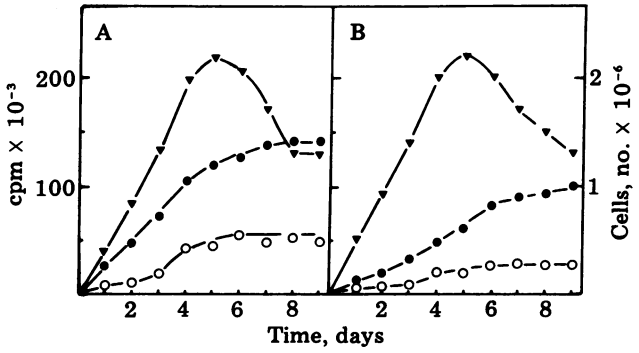

FIG. 5. Production of HBsAg by clones b3 (*A*) and c4 (*B*). Cells were grown in HAT medium in petri dishes and counted each day. Cell lysates were obtained by three cycles of freeze-thawing. HBsAg in cell supernatants and cell lysates was determined by radioimmunoassay. ▼, Cell counts; ●, amount of HBsAg in 2 ml of culture medium; ○, amount of HBsAg in cell lysate from entire petri dish.

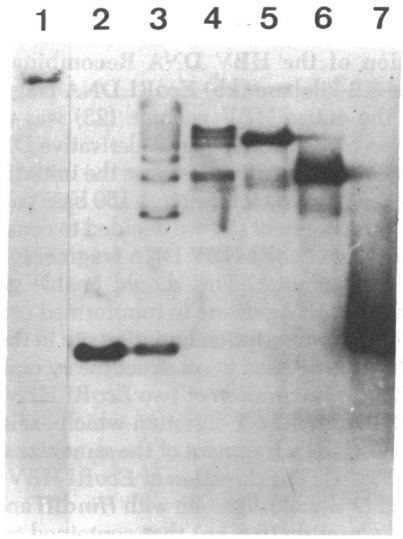

FIG. 6. Autoradiograph of a 0.8% agarose gel containing DNA from clone b3 analyzed by the Southern blot technique. Each cellular DNA sample contained 12 μg of DNA. After electrophoresis, DNA was transferred to a nitrocellulose filter and hybridized with 20 ng of ^{32}P-labeled *Eco*RI HBV DNA (400 × 10^6 cpm/μg). [^{32}P]dCTP and [^{32}P]TTP (Amersham) had a specific activity of 2000–3000 Ci/mmole (1 Ci = 3.7 × 10^{10} becquerels). Lanes: 1, nondigested DNA; 2, *Eco*RI restriction fragments; 3, *Xho* I restriction fragments; 4, *Hin*dIII restriction fragments; 5, 10 pg of undigested pCP10; 6, 10 pg of pCP10 treated with *Hin*dIII (10.6 kb); 7, 10 pg of *Eco*RI HBV DNA (3.2 kb).

conditions, successful cell transformation with HBV DNA, leading to HBsAg synthesis, is only obtained when the recombinant plasmid contains two copies of *Eco*RI HBV DNA. Cells transformed with a recombinant plasmid containing one *Eco*RI HBV DNA fragment do not synthesize HBsAg (data not shown). This may be related to the position of gene *S* compared to the *Eco*RI extremities. On the other hand, the importance of the HSV *tk* marker in our studies is shown by the lack of any detectable HBV expression in cells treated with the HBV recombinant plasmid pCP10 alone. In the two TK$^+$ clones studied, b3 and c4, HBV DNA is found integrated into high molecular weight cellular DNA and the original tandem structure is conserved. No episomic forms were detected.

HBsAg synthesis in mouse L cells clearly demonstrates that these cells possess all the enzymatic equipment necessary for transcription and translation of this human viral genome. This result can be compared with the translation of the chicken ovalbumin gene into ovalbumin polypeptide or with the transcription of the β-globin gene into β-globin mRNA in LTK$^-$ mouse cells, obtained with a similar method (29–31). Whether or not HBsAg gene transcription in transformed cells is initiated at the virus promoter region is not known. The recovery of expression only in cells transformed with two *Eco*RI HBV DNA fragments in a tandem head-to-tail arrangement strongly suggests that HBsAg gene transcription is initiated in the viral genome. Construction of new plasmids containing HBV DNA fragments of different sizes could answer the question. Theoretically, if transcription is really initiated in the viral promoter region, the HBV plasmid pCP10 could be used as a vector for foreign gene expression in eukaryotic cells, in the same way as simian virus 40 DNA (32, 33). The HBV genome has the advantage that it does not lead to the induction of cell lysis.

The other viral markers—i.e., HBc or HBe antigen or DNA polymerase—were not detected in the transformed clones. The reasons for these negative results are unknown, but they cannot be related to the presence of an incomplete genome. Further

investigation is required, especially to determine if the production of HBsAg alone is or is not correlated with a dissociation of HBV gene expression in mouse cells. The preferential production of HBsAg in this artificially constructed cell line may be comparable to selective HBsAg production by some human hepatocellular carcinomas which also contain two HBV genomes integrated into the cellular DNA in a tandem head-to-tail arrangement (34).

The products of HBsAg gene expression in the mouse cell line are assembled in particles which are excreted to outside the cell. These particles are similar to the HBsAg particles present in human serum because their shape, diameter, buoyant density, and antigenicity are the same. In mouse cells, particles are synthesized at a rate of $2–4 \times 10^4$ particles per cell per 24 hr. Assuming that a particle contains about 100 polypeptide molecules, the yield per cell is about $2–4 \times 10^6$ per cell per 24 hr.

In the absence of a cell culture system able to propagate HBV, the experimental model presented here could be used advantageously to study the regulation of HBV gene expression. This model may also be suitable for HBsAg production which, in the absence of virion synthesis, may have potential practical applications.

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