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Secretion of the hepatitis B virus surface antigen from mouse cells using an extra-chromosomal eucaryotic vector

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Recombinant DNA molecules which contained a subgenomic fragment of the hepatitis B virus (HBV) genome, the pML2 vector and the bovine papillomavirus type 1 (BPV1) genome were constructed. The HBV fragment includes the entire transcription unit for the hepatitis B surface antigen (HBsAg). After propagation in *Escherichia coli*, the recombinant plasmids were cleaved with endonucleases *Sal*I and *Pvu*I to eliminate most of the bacterial sequences before transfection of mouse C127 cells. Foci were observed 10–14 days after transfection. Cells from selected foci were cloned and the supernatants were assayed for the presence of HBsAg. Most of the clones tested were found to secrete HBsAg particles into the growth medium. These particles appear to be similar to the 22 nm particles present in the serum of HBV chronic carriers. SDS-polyacrylamide gel electrophoresis revealed that the particles contain two polypeptides, probably representing the glycosylated and unglycosylated forms of the HBsAg major polypeptide. An analysis of DNA from the transformed clones revealed that they contain multiple extra-chromosomal copies of the recombinant, which, however, had suffered rearrangement.

Key words: bovine papilloma virus/22 nm particles/antigen secretion/vaccine

Introduction

Numerous systems have been developed for expression of cloned genes in bacteria. With regard to eucaryotic host/vector systems, progress has been more modest. Although many eucaryotic proteins can be synthesized using bacterial expression vectors there are certain limitations connected with the use of a bacterial host. For instance, glycosylation and processing will not occur properly in bacteria. Moreover, *Escherichia coli* does not release proteins into the growth medium. The development of eucaryotic host/vector systems is therefore needed. Most eucaryotic vectors already described are based on the SV40 replicon. One limitation of this vector type is related to the fact that SV40 causes lytic infections in cells which are permissive for replication. It is thus difficult to establish cell lines for continuous synthesis of polypeptides using such vectors. Sarver *et al.* (1981) have recently reported that the bovine papilloma virus (BPV) genome can be used as a non-lytic eucaryotic vector since the viral DNA establishes itself as an extra-chromosomal element in mouse cells. In many respects, this system resembles the procaryotic plasmids.

The 22 nm particles, present in the serum of chronic carriers of hepatitis B virus (HBV) correspond to empty envelopes of the virus. These particles carrying the hepatitis B surface antigen (HBsAg) contain one major polypeptide which is present in two forms: glycosylated and unglycosylated (for review, see Tiollais *et al.*, 1981). When integrated in the genome of animal cells, the coding sequence for this polypeptide (gene S) is able to direct the synthesis, assembly and excretion of viral envelopes (Dubois *et al.*, 1980; Hirschman *et al.*, 1980; Moriarty *et al.*, 1981). The surface antigen of HBV is therefore a convenient model for studying the capacity of a host/vector system to synthesize and maturate a complex gene product. Moreover, HBsAg is a biological product of great medical importance.

In the present study we describe the construction of recombinant molecules, based on the BPV replicon, which in mouse cells are able to direct the synthesis of HBV envelopes secreted into the cell supernatant.

Results

Construction of recombinants

The purpose of this investigation was to construct a eucaryotic recombinant, based on the BPV1 replicon, carrying the complete transcription unit of the HBsAg. The recombinants were constructed as described in Materials and methods (Figure 1). Restriction enzyme cleavage of DNA from the recombinants propagated in *E. coli* revealed that two types of plasmids were obtained which had the BPV genome inserted in opposite directions. These clones were designated pMZ-6V3 and pMZ-6V4 and were used to transfect C127 mouse cells.

Isolation of transformed clones

Lowy *et al.* (1980) have shown that C127 cells can be transformed by DNA fragments representing 69% or more of the BPV1 genome. It has also been shown that sequences in the pBR322 plasmid vector appear to impair the replication of the BPV DNA (Binétruy *et al.*, 1982). Since it was unclear whether the pML2 vector contained the same inhibitory sequences, we decided to remove as much as possible of the bacterial sequences from the pMZ recombinants before transfection. This was achieved by cleavage with a mixture of endonucleases *Pvu*I and *Sal*I which will remove ~1.5 kb of the pML vector (Figure 1). C127 mouse cells were transfected with the recombinants after cleavage but without religation. Approximately 10 foci/μg of recombinant DNA were obtained 10–14 days after transfection and individual foci were cloned in soft agar. The clones were then propagated in microtiter plates and the supernatants were assayed for the presence of HBsAg, using a radioimmunoassay. Fifteen out of 21 clones tested were found to be positive in the radioimmunoassay, thus demonstrating that the HBsAg is expressed at a high frequency in the mouse cells which were transformed by the HBV-containing recombinants. Recombinants

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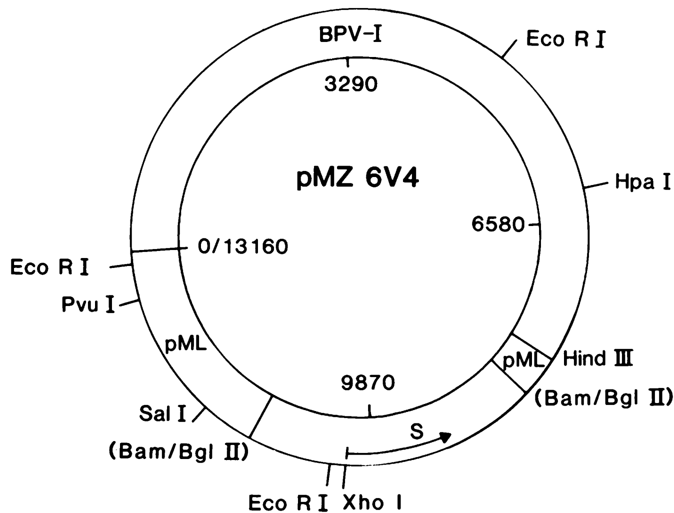

Fig. 1. Structure of the pMZ 6V4 recombinant. The *PvuI* and *SalI* restriction sites which were used for cleavage prior to transfection are indicated. Arrow indicates location of S gene. The total length of the uncleaved recombinant is 13.2 kb.

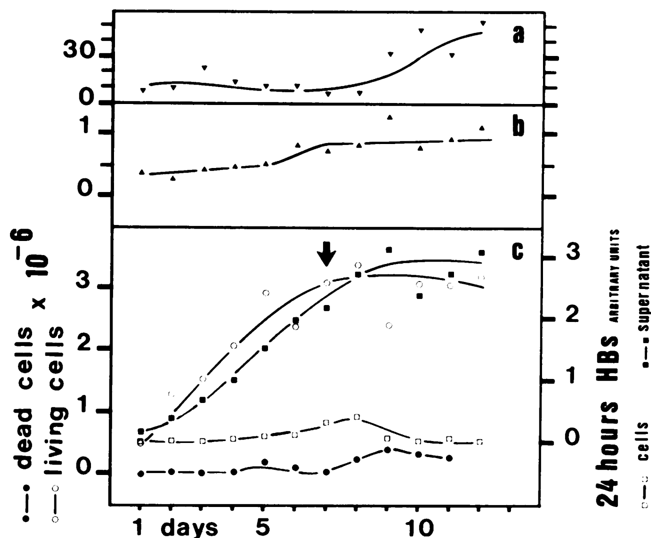

Fig. 2. Kinetics of HBsAg synthesis by a pMZ-transformed cell line. HBsAg concentration was measured as described in Materials and methods. The arrow indicates the time point when the cells became confluent. (**a**): ratio between HBsAg in supernatant and intracellular HBsAg. (**b**): amount of HBsAg in supernatant, calculated per cell equivalent. (**c**): a comparison between cell proliferation and HBsAg synthesis.

having the BPV fragment inserted in the two orientations were equally efficient in expressing the HBsAg. Growth medium from untransformed C127 cells used as a control, was negative for HBsAg.

Properties of the secreted HBsAg

The kinetics of HBsAg synthesis was first examined; transformed cells were plated at a low density and growth medium was collected at regular intervals even long after the cultures had reached confluency. The results, which are summarized in Figure 2, show that maximum synthesis was achieved after the cultures became confluent. Small amounts of intracellular HBsAg were also found which seemed to decrease after the cells became confluent. The amount of antigen secreted from different transformed cell lines ranged from 50 to 200 ng/10^6 cells/24 h.

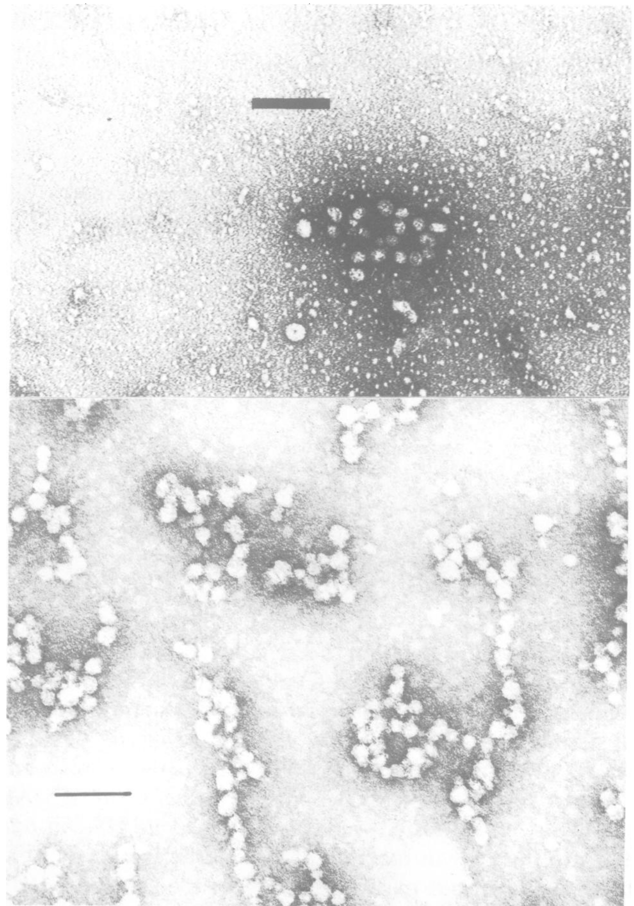

Fig. 3. Electron micrographs showing particles, secreted into the growth medium of a pMZ-transformed cell line. The particles were purified by repeated cycles of CsCl gradient centrifugation and incubated with a monoclonal antiserum against HBsAg before being examined in the electron microscope. The magnification bar indicates 100 nm.

To demonstrate that the secreted HBsAg formed particles similar to those known to be present in the serum of HBV chronic carriers, isopycnic CsCl gradient centrifugation and electron microscopic observations were carried out. The results showed that HBsAg material having a density of 1.20 g/cm^3 was present in the medium, i.e., having the same density as is characteristic for lipid-containing HBsAg particles of human origin. Figure 3 shows an electron micrograph of particles which had been purified by several cycles of isopycnic CsCl gradient centrifugation. These spherical particles had an average diameter of 22 nm with a morphology similar to that of HBsAg particles from human serum.

To examine the polypeptides present in the HBsAg material, the particles were purified by CsCl gradient centrifugation followed by *in vitro* labeling with ^{125}I. An anti-HBsAg serum was used to immunoprecipitate the HBV-specific proteins before analysis by SDS-polyacrylamide gel electrophoresis. The two prominent bands, corresponding to polypeptides with estimated mol. wts. of 20 K and 24 K (Figure 4) are likely to represent the glycosylated and the unglycosylated forms of the major HBsAg polypeptide. The antiserum also precipitated larger polypeptides in the size range 45 – 70 K, as has been noticed in previous studies (Pourcel *et al.*, 1982).

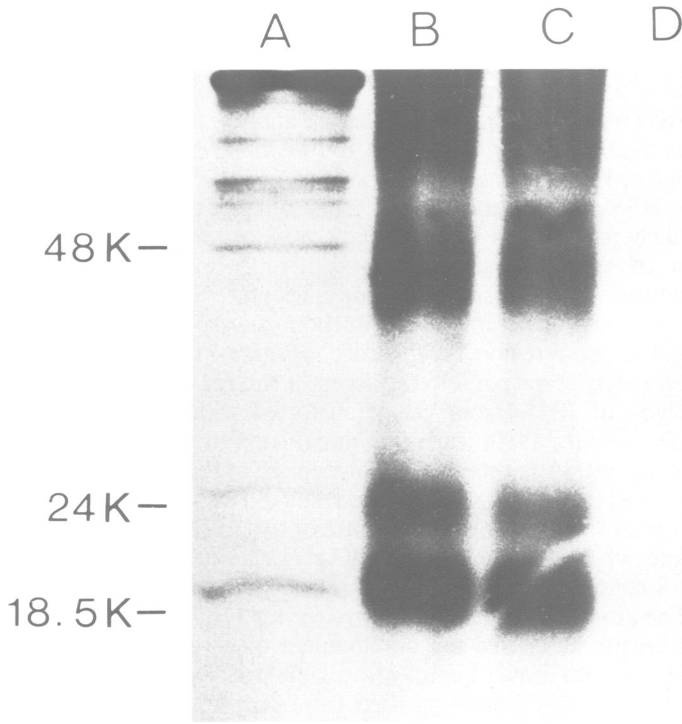

Fig.4 . Analysis by SDS-polyacrylamide gel electrophoresis of HBsAg polypeptides secreted from pMZ-transformed C127 cells. The antigen was labeled *in vitro* with ^{125}I and precipitated with anti-HBsAg serum at 1.50 (**B**) and 1:100 (**C**) dilutions and with a preimmune serum (**D**) before analysis in a 13% polyacrylamide gel. The figure shows an autoradiogram. ^{35}S-Labeled adenovirus type 2 polypeptides (**A**) were used as size markers and the estimated mol. wts. of representative bands are indicated.

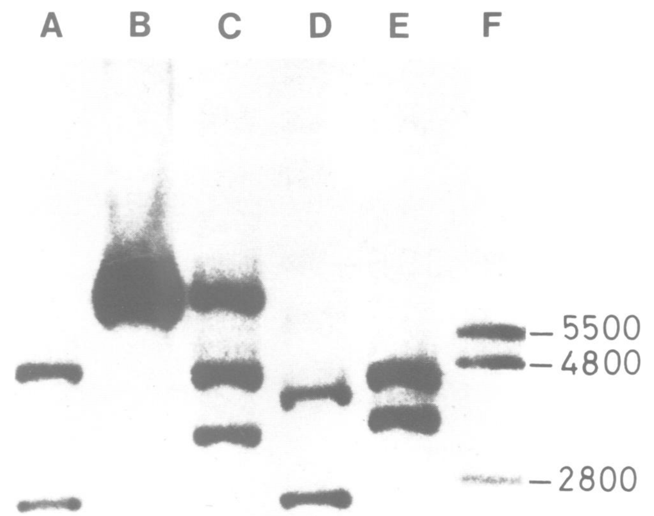

Fig. 5. Analysis of DNA from five different clones of pMZ-transformed cells. The DNA was digested with *Eco*RI, transferred to nitrocellulose by the method of Southern (1975) and hybridized to ^{32}P-labeled pMZ 6V3 DNA. **Lane F** represents 10 copies of *Eco*RI-digested pMZ 6V4 plasmid DNA per genome equivalent. The *Sal*I/*Pvu*I fragment was not removed from the pMZ recombinant in **lane F** before analysis.

Viral DNA sequences in transformed cells

The total cellular DNA was extracted from several clones of mouse cells transformed by the pMZ recombinants. The DNA was cleaved with different restriction enzymes, separated by electrophoresis in a 1% agarose gel and transferred to nitrocellulose by the method of Southern (1975). Viral sequences were demonstrated using the ^{32}P-labeled pMZ plasmid as a probe and Figure 5 shows some representative results. Reconstruction experiments, using artificial mixtures of known amounts of pMZ DNA and cellular DNA, showed that the clones contained 20–200 copies of viral DNA. From the cleavage pattern obtained with restriction enzymes which only cut the recombinant once, it was possible to conclude that the viral DNA is present in an extra-chromosomal state (data not shown). A surprising finding was that all clones gave rise to different cleavage patterns (Figure 5), none of which was identical to that of the recombinant, used for transfection. This observation suggests that the recombinants have suffered rearrangement during or after transfection. Analysis with different sets of restriction enzymes revealed that the rearrangements involved deletions as well as insertions (data not shown).

Analysis of HBV-specific RNA in the transformed cells

The synthesis of HBV-specific RNA was studied by S1 nuclease analysis. A probe, 5′ end-labeled in the *Xho*I site of the HBV genome (Figure 1; nucleotide 127 according to the nomenclature of Tiollais *et al.*, 1981), was used for the analysis (Figure 6). A promient, ~160 nucleotides long, double band corresponding to 5′ ends located around position 3150 in the HBV genome was observed in the electropherogram. Several shorter less intensive bands were also present in the electropherogram. These could either represent alternative cap sites or possibly acceptor sites for splicing (see below). Similar S1 nuclease cleavage patterns were obtained when RNA from different clones was analyzed including those which had the BPV genome in opposite orientations.

Discussion

The HBsAg particles, present in the supernatant of the pMZ-transformed cells seem to be identical to the 22 nm particles present in the serum of HBV DNA (Dubois *et al.*, 1980; Hirschman *et al.*, 1980; Moriarty *et al.*, 1981). This result demonstrates that the BPV vector can be used to accomplish synthesis, glycosylation, assembly and secretion of a eucaryotic lipoprotein complex. These are properties which a useful eucaryotic vector should have.

The pMZ recombinants exist in the transformed mouse cells as plasmid-like elements. With regard to copy number (20–200 copies) and the absence of integrated sequences, our results are in agreement with those reported for the BPV vector system by other investigators (Sarver *et al.*, 1981). A disadvantage of the BPV vector system is that the recombinants often seem to become rearranged. This phenomenon

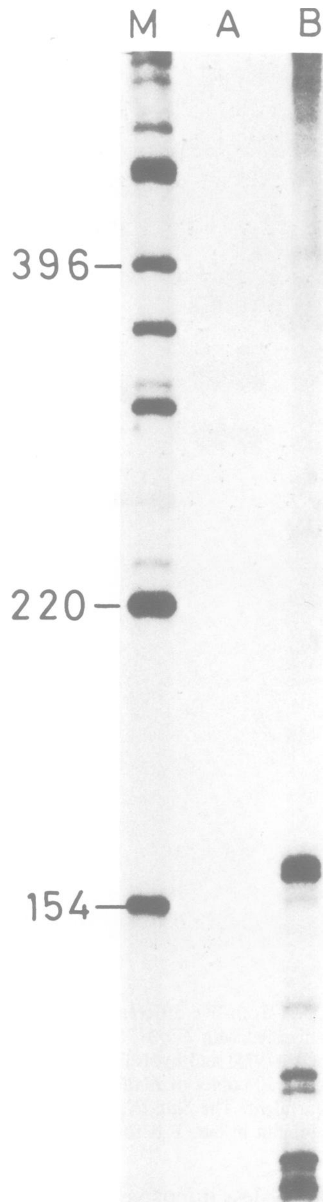

Fig. 6. S1 nuclease analysis of RNA from pMZ transformed cells. pMZ 6V4 DNA, cleaved with *Xho*I and 5′-labeled was used as the probe for hybridization which was carried out at 53°C. Protected fragments obtained with **(B)** and without RNA **(A)** are shown. pBR322 marker fragments are present in **lane M**.

has also been observed previously and is thought to be caused by inhibitory sequences present in the bacterial vector (Binétruy *et al.*, 1982). Therefore, we removed most of the bacterial sequences from our recombinants by restriction enzyme cleavage prior to transfection. This did not, however, eliminate the problem. It is conceivable that the variation in amounts of antigen secreted from different clones could be related to changes in the region of the template which controls the expression of gene S.

Since clones which have the BPV genome inserted in the two orientations synthesize HBsAg and express HBV-specific RNAs of similar structure, it appears likely that the HBV-specific transcripts are controlled by a HBV encoded promoter. This is expected from the localization of the promoter region for the S gene, i.e., between positions 2423 and 2837 with a TATA-box at position 2776 (Pourcel *et al.*, 1982). Results obtained from the S1 nuclease analysis raise questions as to the structure of the mRNA for the major polypeptide of HBsAg. The results identified position 3160 as the 5′ end of the HBV-specific transcripts. This position is, however, unlikely to represent the cap site because of the known location of the HBV encoded promoter. A more probable hypothesis is that a splicing event takes place during mRNA maturation which eliminates sequences corresponding to the so-called pre-S region. In this case, position 3160 would approximately correspond to the acceptor site for the splice. An analysis of RNA from cells transfected with HBV DNA (Dubois *et al.*, 1980) lends additional support to the notion that the mRNA for the major polypeptide of the HBsAg may be spliced (Stenlund, unpublished results). Further studies are necessary to determine the structure of this mRNA and to examine whether additional mRNAs exists from which the pre-S region could be translated.

The absence of a cell culture system for HBV propagation has greatly hampered the development of a vaccine against HBV. Up to now the only available vaccine consists of HBsAg 22 nm particles purified from sera of HBV chronic carriers (Szmuness *et al.*, 1980; Crosnier *et al.*, 1981). Recombinant DNA technology is therefore an attractive alternative to produce HBsAg material for vaccine purposes. To be a strong immunogen HBsAg has to be in the form of particles (Morein *et al.*, 1978). Animal cells and yeast are apparently both able to assemble HBsAg and are therefore worth consideration (Alexander *et al.*, 1976; Dubois *et al.*, 1980; Hirschman *et al.*, 1980; Moriarty *et al.*, 1981; Valenzuela *et al.*, 1982). In yeast, however, the particles are neither secreted nor properly glycosylated. The BPV vector system which makes it possible to introduce a large number of gene S copies in animal cells is therefore of particular interest. The amounts of HBsAg obtained at present using the BPV vector (50–200 ng/10^6 cells) are similar to those obtained both from cells carrying integrated copies of the HBV genome (50 ng/10^6 cells, Dubois *et al.*, 1980) and from cells transfected with a SV40/HBV recombinant (125 ng/10^6 cells, Moriarty *et al.*, 1981). It should be possible to increase the production of HBsAg from the multicopy vector by replacing the HBV promoter with a more potent one and by introducing sequences which enhance expression.

The pMZ recombinants may furthermore provide the basis for the construction of a eucaryotic vector that can be used for synthesis and export of proteins.

Materials and methods

Cloning and transfection procedures

Plasmid pMA (kindly provided by M.A. Auger) contains a 2743 bp long subgenomic *Bgl*II fragment (positions 2423–1984) of HBV DNA carrying the coding sequences for the pre-S and S genes and their putative promoter (Pourcel *et al.*, 1982) inserted in the *Bam*HI site of the pML2 vector (Lusky and Botchan, 1981). The BPV1 genome was cloned in the *Hind*III site of pBR322 vector as described in a separate communication (Ahola *et al.*, in preparation). The entire BPV genome was inserted in the *Hind*III site of the pMA plasmid (Figure 1). The resulting recombinants were propagated in the HB 101 strain of *E. coli.* C127 cells, kindly obtained from Peter Howley (NIH), were used as the eucaryotic host for the clones. Transfection was carried out according to Wigler *et al.* (1978).

Subcloning of recombinant foci

Cells were subcloned in soft agar using the method of MacPherson and Montagnier (1964).

S1 analysis

The method of Favoloro *et al.* (1980) was followed. Cytoplasmic RNA was isolated by the method of Brawerman *et al.* (1972).

Viral DNA in transformed cells

The total DNA was extracted from the transformed cells according to the method of Steffen and Weinberg (1978) and cleaved with the appropriate restriction enzymes before separation in 1% agarose gels. The fragments were transferred to a nitrocellulose sheet by Southern's (1975) method and viral DNA was identified using a ^{32}P-labeled probe (Rigby *et al.*, 1977).

Antigen determination

The commercially available kit for surface antigen detection ('Ausria II' Abbot) was used for radioimmunoassays. Quantitative estimates were made by performing serial dilutions using 0.2 ng/ml as the detection threshold.

Isopycnic centrifugation

Centrifugation was carried out for 55 h at 272 000 *g* and 4°C as described by Pourcel *et al.* (1982).

Electron microscopy

Samples were incubated with an anti-HBs monoclonal antibody, kindly provided by G. Kalil before being adsorbed on carbon formvar coated grids and stained using the negative staining technique. The grids were examined using a Philips EM 201 transmission electron microscope.

Protein analysis

HBsAg particles, purified by CsCl gradient centrifugation, were labeled *in vitro* with ^{125}I. SDS-polyacrylamide gel electrophoresis was carried out according to Persson *et al.* (1980). Immunoprecipitation was performed as described by Persson *et al.* (1980) using a rabbit antiserum prepared against HBsAg (Pourcel *et al.*, 1982).

Kinetics of HBsAg synthesis

C127 cells were plated at a concentration of 5 x 10^5 cells/20 cm^2 and 1 ml of Dulbecco's modified Eagles' medium supplemented with 5% foetal calf serum was added and subsequently changed every 3 days or 24 h before counting of cells and determination of HBsAg concentration.

Three Petri dishes were assayed every day and the average result was used for plotting. Intracellular HBsAg was determined after repeated cycles of freezing and thawing of the cells in 1 ml of fresh medium.

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Note added in proof

After this manuscript was submitted for publication we learnt that Stratowa *et al.* (EMBO J., **1**, 1573-1578) have used a retrovirus vector to achieve expression of HBsAg. We have recently obtained cell lines which produce higher amounts of HBsAg (~600 ng/10^6 cells/24 h). The production level remains stable for at least 1 month