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# Enhanced Production of Hepatitis B Surface Antigen in NIH 3T3 Mouse Fibroblasts by Using Extrachromosomally Replicating Bovine Papillomavirus Vector

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We have constructed a recombinant pBR322 plasmid composed of a subgenomic transforming fragment of bovine papillomavirus DNA and the hepatitis B surface antigen gene from cloned hepatitis B virus DNA and used it for transfection of NIH 3T3 mouse fibroblasts. The transformed cells retain the plasmids in extrachromosomal form with a copy number of about 50 to 100 per cell. Expression of the hepatitis B surface antigen gene linked to bovine papillomavirus DNA is independent of its orientation relative to the bovine papillomavirus vector. Cell lines continuously secreting high amounts of hepatitis B surface antigen into the medium could be established. The antigen is released into the culture medium as 22-nm particles, having the same physical properties and constituent polypeptides as those found in the serum of hepatitis B virus-infected patients.

Hepatitis B is a widespread viral disease (28). Due to the failure to propagate hepatitis B virus (HBV) in tissue culture, the only available source of antigenic material to prepare a vaccine against HBV is the serum of human chronic carriers of hepatitis B surface antigen (HBsAg). This protein in its particular form of 22-nm spherical particles is highly immunogenic and raises antibodies which protect against this disease. Therefore, several attempts have been made to establish mammalian cell lines that produce HBsAg. These lines are derived from human hepatocellular carcinomas (1, 2) as well as cells transfected with cloned HBV DNA sequences (4, 7, 11, 14, 19). Monkey kidney cells were infected with a simian virus 40 recombinant carrying 40% of the HBV genome (19). Head-to-tail tandems of the HBV genome have been introduced into mouse fibroblasts by cotransfection with the herpes simplex virus thymidine kinase gene (7, 11) or the methotrexate resistance dihydrofolate reductase gene (4) as selectable markers. We have recently used the integrated proviral form of Moloney mouse sarcoma virus to introduce an HBV fragment containing the HBsAg gene and its regulation signals into mouse fibroblasts (27).

Here we describe an expression system for HBsAg which is based on the bovine papillomavirus (BPV). Recently, Sarver et al. (23) have demonstrated the use of BPV DNA as a eucaryotic cloning vector for introducing the rat preproinsulin gene I into C127 mouse cells. This vector has some specific and useful properties. (i) It can induce foci in susceptible mouse cells (15, 18), thus providing a marker for selecting those cells that have incorporated the foreign gene. (ii) It exists as an episome, resulting in a high copy number per cell (17). (iii) The genomic organization is not permuted, because the circular form is maintained within the cell after transfection. (iv) Recently, DiMaio et al. (6) have shown that it can serve as a shuttle vector that replicates as a plasmid in both mouse and bacterial cells when joined to a pBR322 derivative.

Interested in these properties, we constructed a DNA hybrid molecule, BPV-HBV, consisting of a subgenomic fragment of BPV type 1 containing the transforming region of BPV as eucaryotic cloning vector and the HBsAg gene isolated from cloned HBV. Transfecting NIH 3T3 mouse fibroblasts with the BPV-HBV recombinant resulted in amplification of the HBsAg gene and thus in increased HBsAg production (6 μg per $10^7$ cells per day).

## MATERIALS AND METHODS

**Construction of plasmids.** The steps involved in the construction of recombinant plasmids are shown in Fig. 1. Plasmid pAO1-HBV (5) containing the whole HBV genome in the *Eco*RI site of pAO1 was cleaved with *Eco*RI. The *Eco*RI fragment encoding the HBV DNA was purified by preparative gel electrophoresis

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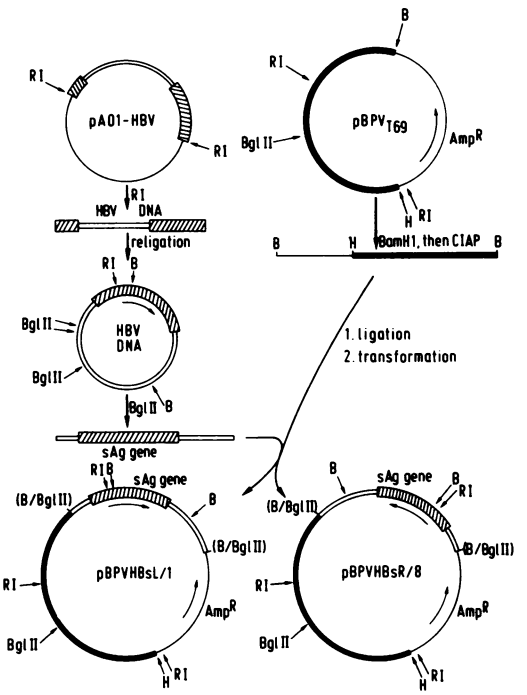


FIG. 1. Construction of BPV recombinants containing the HBsAg gene in both of the two possible orientations. Symbols: thin lines, pBR322 sequences; thick lines, BPV sequences; double lines, HBV sequences; cross-hatched area, HBsAg gene. Cleavage sites: RI, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

through a 1% agarose gel and religated in dilute solution (10 μg of DNA per ml). Plasmid vector pBPV$_{T69}$ (18), the 69% *Hin*dIII-*Bam*HI transforming fragment of BPV type 1 cloned in plasmid pBR322 (a gift from P. Howley), was digested with *Bam*HI and treated with calf intestine alkaline phosphatase (CIAP grad 1, 1 U incubated with 2 μg of DNA at 65°C for 30 min in 400 μl of 10 mM Tris, pH 9.0; Boehringer Mannheim). The restored HBV genome was cleaved with *Bgl*II and ligated with *Bam*HI-cleaved and CIAP-digested pBPV$_{T69}$, using T4 DNA ligase (Boehringer Mannheim). The ligation mixture was used to transform *Escherichia coli* HB101, and ampicillin-resistant clones were screened for recombinant plasmids by the method of Birnboim and Doly (3). By cleavage with *Bam*HI and *Eco*RI, recombinant plasmids could be recognized. *Bam*HI cleavage could show that the HBsAg gene is inserted into the vector DNA. By cleavage with *Eco*RI, the orientation of the inserted HBsAg gene relative to the vector could be identified. These plasmids, pBPVHBsL/1 and pBPVHBsR/8, containing the insert in opposite orientations, were used to transform mouse cells.

For additional experiments, plasmid pBPVHBsR/8 was either linearized with *Hin*dIII or digested with *Hin*dIII and *Bgl*II before transfection. In the latter case, the large DNA fragment containing the HBsAg gene was separated by preparative agarose gel chromatography and recovered by electrophoresis.

Propagation of recombinant DNA molecules was conducted in facilities classified L3, following the recommendation of the Zentrale Kommission fuer die Biologische Sicherheit.

**Cell culture.** NIH 3T3 cells were maintained in Dulbecco modified Eagle minimal (DME) medium supplemented with heat inactivated 10% calf serum (GIBCO) and kanamycin at 50 μg/ml. Cellular clones were free of mycoplasma contamination (22).

**Transfection of cells.** Transfections were carried out essentially as described by Graham and van der Eb (12). Three different transfection experiments were performed as follows. Briefly, 2 μg of plasmid DNA and 10 μg of calf thymus DNA as carrier were added to $5 \times 10^5$ cells per 6-cm petri dish. After 6 h of incubation at 37°C, cells were washed once with DME medium and then refed with fresh medium. Foci visible 3 weeks after transfection were picked at about 5 weeks and grown in mass cultures for further study.

**Analysis of cellular DNA.** Total cellular DNA was extracted from confluent monolayers of transformed cells as described by Gross-Bellard et al. (13). DNA (20 μg) was digested to completion with the appropriate restriction endonucleases and separated on 0.7% agarose gels by horizontal electrophoresis. The DNA was denatured in situ and transferred to nitrocellulose filters (BA85; Schleicher & Schuell Co.) by the method of Southern (25). Filters were prehybridized and then hybridized with $^{32}$P-labeled DNA as described by Wahl et al. (29). After washing in three changes of 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) and 0.1% sodium dodecyl sulfate for 15 min and in two changes of 0.1× SSC for 15 min at 50°C, filters were dried and exposed to Kodak XAR-5 film with Du Pont Lightning Plus intensifying screens at −70°C.

**Protein analysis.** The presence of HBsAg in the culture medium and in CsCl gradient fractions was detected with a radioimmunoassay (AUSRIA II, Abbott Laboratories) and quantitated by a parallel-line assay with a known standard. To determine the polypeptide composition, proteins were biosynthetically labeled and analyzed on sodium dodecyl sulfate polyacrylamide gels. Cells were grown to about 80% confluency. The culture medium was then replaced with 5 ml of methionine-free medium containing 400 μCi of L-[$^{35}$S]methionine and 400 μCi of L-[$^{35}$S]cysteine (New England Nuclear Corp.) for overnight incubation at 37°C. Medium was concentrated 10-fold by fractionated precipitation with polyethylene glycol (26). Proteins ($10^6$ cpm) were incubated with 10 μl of preimmune guinea pig serum or with 0.1, 1, and 10 μl of anti-HBsAg serum from guinea pigs in 50 μl of TEN (10 mM Tris-hydrochloride [pH7.4], 1 mM EDTA, 0.13 M NaCl)–0.5% Tween 20 for 1 h at 37°C. The immune globulins were bound to 20 μl of protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) for 1 h at 37°C and washed with TEN-Tween 20, with 0.5 M LiCl, and again with TEN-Tween 20. Samples were made 5% in sodium dodecyl sulfate and 0.5 M in dithiothreitol in 30 μl, boiled for 4 min, and electrophoresed in 0.75-mm-thick slabs of 12.5% polyacrylamide–0.4% bisacrylamide, using Laemmli buffer system (16, 26). Gels were fixed for 60 min in a solution containing 10% trichloroacetic acid, 10% glacial acetic acid, and 30% methanol and impregnated with Enlightning (New England Nuclear Corp.) for 30 min.

TABLE 1. Focus formation in NIH 3T3 cells transformed by plasmid pBPVHBsR/8

| Treatment of cells with: | No. of foci per dish | HBsAg-positive foci[a] |
|---|---|---|
| Circular pBPVHBsR/8 | 20 | 7 of 10 |
| Linear pBPVHBsR/8[b] | 20 | 3 of 6 |
| HBsAg gene fragment[c] | 2–3 | 0 |
| Control (pBR322) | 0 | 0 |

[a] Tested by AUSRIA II radioimmunoassay.
[b] Plasmid was linearized with *Hin*dIII.
[c] Plasmid was cleaved with *Hin*dIII and *Bgl*II, and the HBsAg gene-containing fragment was isolated by agarose gel chromatography.

Gels were dried and autoradiographed with Kodak XAR-5 film.

**Immunogenicity studies.** Four guinea pigs were injected subcutaneously with 20 μg of purified HBsAg, using aluminum hydroxide as adjuvant. Inoculation was repeated after 2 weeks. At different times, the guinea pigs were bled. The presence of anti-HBsAg in the sera of guinea pigs was tested by AUSAB radioimmunoassay (Abbott). Anti-HBsAg titers were expressed in radioimmunoassay units calculated according to the specifications of the manufacturer.

## RESULTS

**Construction of the BPV-HBV recombinants.** As shown in Fig. 1, cloned HBV DNA was isolated from the plasmid pAO1-HBV and recircularized to restore its genomic organization. By cleavage of the circular HBV genome with *Bgl*II the complete HBsAg gene, including the promoter and polyadenylation site necessary for transcription, was obtained (21). This fragment was shown to express HBsAg (30). It was ligated into the *Bam*HI site of the plasmid vector $pBPV_{T69}$, containing the 69% transforming region of BPV, in either orientation.

**Transformation of mouse cells with the recombinant plasmids.** Transfection of NIH 3T3 mouse fibroblasts by the DNA hybrids was done as described above. Three different forms of the recombinant plasmid pBPVHBsR/8 were used for transfection: (i) its circular form, (ii) the linear form derived from *Hin*dIII digestion, and (iii) the *Bgl*II-*Hin*dIII fragment still containing the complete HBsAg gene but only part of the 69% BPV fragment, which was shown to have lost transforming activity (18). Focus formation in cultures transfected with circular and intact linear forms of the recombinant DNA became apparent after 3 weeks. At week 4 the foci were counted. When the uncut or linear recombinant plasmid was used, approximately 50% of the picked foci were found to be positive for HBsAg. In the case where the transforming activity of BPV is destroyed by deletion, the few foci detected were found to be negative for HBsAg expression. These foci may have arisen spontaneously (Table 1).

**Time course of HBsAg release after transfection.** To test whether HBsAg expression is correlated with the appearance of foci, we checked a cell culture continuously after transfection by radioimmunoassay until a cell line was established. The results for supernatant medium from cells transfected with the three different forms of plasmid pBPVHBsR/8 are shown in Fig. 2. In all three cases the amount of HBsAg secreted into the culture medium reached a peak value after 1 week. In the case of the HBsAg gene fragment without the transforming region of BPV, only transient expression of HBsAg was observed (30), whereas the production rate with both the circular and linear forms of pBPVHBsR/8 remained constant at an intermediate level for another 2 weeks and then rose again, coinciding with the appearance of foci.

There was no difference in the production rate of HBsAg between cells transfected with plasmid pBPVHBsR/8 or with pBPVHBsL/1 ($P/N$ = 18.8 versus $P/N$ = 18.6 after 6 days, where $P$ is the cpm of the samples and $N$ is the cpm of the negative control). This indicates that the orientation of the HBsAg gene with respect to the BPV

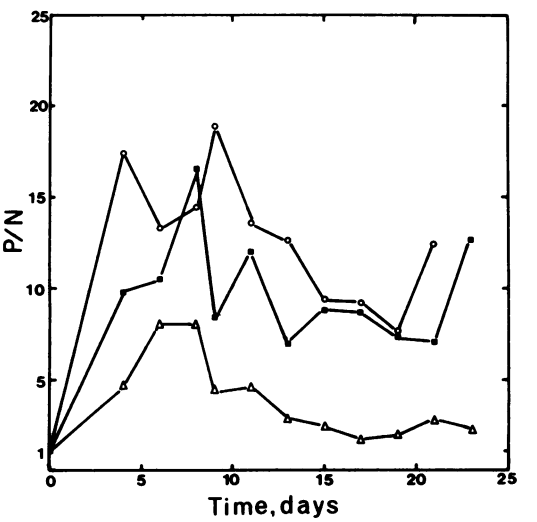


FIG. 2. Detection of HBsAg immediately after transfection of cells. Cells were grown in 6-cm petri dishes in 5 ml of DME medium–10% newborn calf serum containing kanamycin at 50 μg/ml. At time 0, 5 × $10^5$ cells were transfected with plasmid pBPVHBsR/8, either in its circular form (○) or linear form (■), or with the *Hin*dIII-*Bgl*II fragment (△). At the indicated times, media were changed and assayed for HBsAg by AUSRIA II. A P/N value >2.1 is assumed to be positive (P, cpm of samples; N, cpm of negative control).

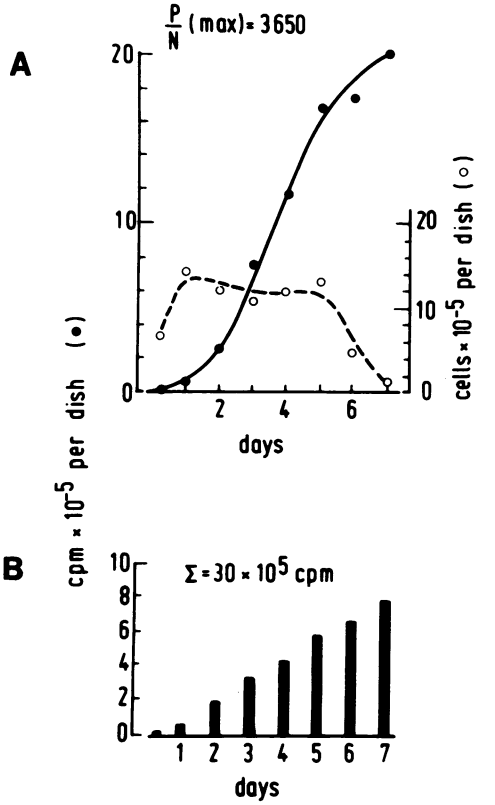


FIG. 3. Production kinetics for cell line Y1. (A) Cells were seeded at $5 \times 10^5$ per 35-mm petri dish in DME medium–1% newborn calf serum. Cells were fed with 2 ml of medium after overnight cultivation (time 0). At the indicated times, medium was removed and the volume was measured, and the medium was assayed for HBsAg. Cells were removed from the same dish by trypsinization and counted with a hemacytometer. Values shown are the average of two determinations. (B) Cells were seeded as described above. Medium was changed every day and assayed for HBsAg.

DNA fragment has no significant influence on expression of HBsAg.

**Time kinetics of HBsAg expression by selected foci.** To confirm that the late HBsAg release is associated with the appearance of transformed cells, individual foci were picked (see Table 1). Those found positive for HBsAg were grown in mass culture, and the rate of HBsAg production was determined. One of the derived cell lines, Y1, was studied in detail for HBsAg production. The results are shown in Fig. 3. In maintenance medium, cells produce constant amounts of HBsAg per day at cell saturation density (see days 2 to 5 in Fig. 3a). Surface antigen released into the medium was assayed by radioimmunoassay, and measured counting rates were converted into nanograms of HBsAg per milliliter by comparison with a known standard (HBsAg/adw from human serum, 17 μg/ml; Max von Pettenkofer-Institut, Munich, W. Germany). Independently, portions were assayed by the Paul-Ehrlich-Institut, Frankfurt, W. Germany (national reference center), and these values are given in national units (1 U = 1 ng of HBsAg). Quantitation yields a production rate of 420 ng/ml per day (400 U/ml per day) at cell saturation density. The total yield after 7 days is 1.7 μg/ml (1,600 U/ml); i.e., the cell line Y1 produces about 6 μg of HBsAg per $10^7$ cells per day.

The influence of cultivation conditions was checked by feeding a culture each day (Fig. 3b). Medium was scored daily for HBsAg expression. After 7 days, the accumulated amount of HBsAg from daily collected media was 1.3 μg/ml. That is 39 μg per $10^7$ cells per 7 days.

**Characterization of the gene product.** The predominant form of HBsAg in human serum is the so-called 22-nm particle. Its biophysical properties are well documented (9, 10). To examine the form of the HBsAg produced by the transformed mouse fibroblasts, supernatant was centrifuged, and the pellet was subjected to equilibrium sedimentation through a discontinuous CsCl gradient. As seen in Fig. 4, HBsAg is present in a fraction corresponding to a buoyant density of 1.2 g/ml, which is identical to the value found for

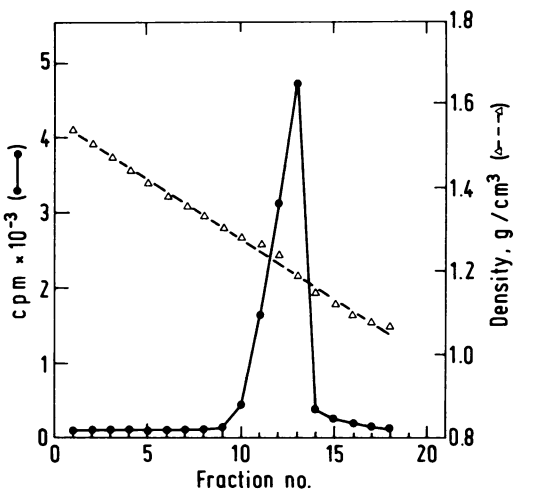


FIG. 4. CsCl gradient sedimentation of HBsAg from medium of Y1 cells. After low-speed centrifugation to remove cell debris, HBsAg was pelleted at 73,000 × *g* for 23 h at 4°C and suspended in 0.5 ml of 10 mM Tris (pH 7.5). A 200-μl portion was layered onto a 12-ml discontinuous 1.1 to 1.6 g/cm³ CsCl gradient in 10 mM Tris (pH 7.5) and run at 34,000 rpm at 14°C for 20 h in a Beckman SW41 rotor. Fractions were collected by bottom puncture, and HBsAg activity was measured by radioimmunoassay.

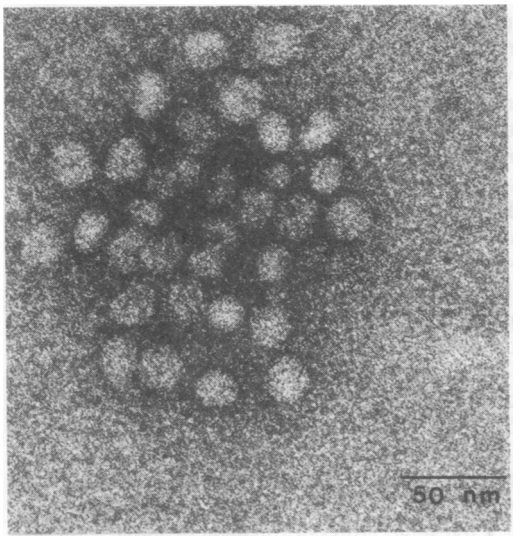


FIG. 5. Electron micrograph of 22-nm spherical HBsAg particles produced by Y1 cells. The peak fraction from CsCl density gradient was dialyzed and concentrated. Particles were visualized by negative staining with 1% phosphotungstic acid (×320,000).

the 22-nm particles in human serum (9). After concentrating the peak fractions of the CsCl gradient, the particles were further characterized by electron microscopy. Particles with a mean diameter of 22 nm could be visualized (Fig. 5). No filamentous structures were detected.

To determine the polypeptide composition, HBsAg from labeled cells was immunoprecipitated and analyzed by electrophoresis on a polyacrylamide gel. As seen in Fig. 6, there are four polypeptides with molecular weights of 24,000 (24K), 28K, 34K, and 37K, which are not found in the supernatant from untransfected NIH 3T3 cells. These results are in agreement with recently published data for purified HBsAg from human serum (26).

To determine the immunogenicity of the gene product, we injected guinea pigs twice with purified HBsAg from cell line Y1 at a 2-week interval. The anti-HBsAg levels detected in the serum of guinea pigs at various times after inoculation are summarized in Table 2. Increasing antibody titers were observed in all guinea pigs after HBsAg injection.

**State of recombinant DNA in transformed cells.** To examine whether the BPV-HBV recombinant DNA replicates extrachromosomally in the transformed cells or is integrated into the host genome, we analyzed total DNA by blot analysis (25). Total cellular DNA from one clone producing high amounts of HBsAg, cell line Y1 (lanes a to f in Fig. 7), was either left untreated or treated

TABLE 2. Anti-HBsAg titer obtained in sera of guinea pigs immunized with HBsAg particles from Y1 cells

| Wk[a] | Titer[b] in guinea pig: | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 0 | 0 | 0 | 0 | 0 |
| 2 | 96 | 100 | 62 | 76 |
| 4 | 352 | 20,800 | 6,120 | 6,480 |
| 6 | 88 | 35,200 | 5,760 | 7,200 |
| 8 | 100 | 56,000 | 5,400 | 12,100 |
| 12 | 800 | 70,000 | | 11,200 |
| 18 | 1,000 | 200,000 | | 34,600 |

[a] The first injection of HBsAg was at time 0, the second 2 weeks later.

[b] Titers are in radioimmunoassay units calculated according to the specifications of the manufacturer.

with a restriction endonuclease that recognizes no site (*Sac*I), a single site (*Hin*dIII, *Bgl*II), two sites (*Bam*HI), or three sites (*Eco*RI) within the plasmid pBPVHBsR/8. As a control, the recom-

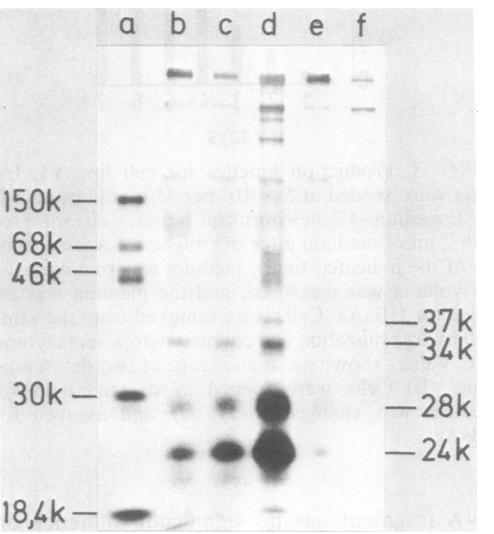


FIG. 6. Electrophoretic analysis of immunoprecipitated HBsAg polypeptides. Labeled proteins ($10^6$ cpm) from cell line Y1 and from untransfected NIH 3T3 cells were incubated with preimmune guinea pig serum or with high-titered anti-HBsAg serum from guinea pigs and precipitated as described in the text. Proteins were analyzed by polyacrylamide gel electrophoresis and autoradiography. Lane a, $^{14}$C-labeled protein standards (globulins [150K], bovine serum albumin [68K], ovalbumin [46K], carbonic anhydrase [30K], and lactoglobulin A [18.4K]); lanes b through d, proteins from cell line Y1 incubated with 0.1 μl (lane b), 1 μl (lane c), and 10 μl (lane d) of anti-HBsAg serum; lane e, proteins from cell line Y1 incubated with 10 μl of preimmune serum; lane f, proteins from untransfected NIH 3T3 cells incubated with 10 μl of anti-HBsAg serum.

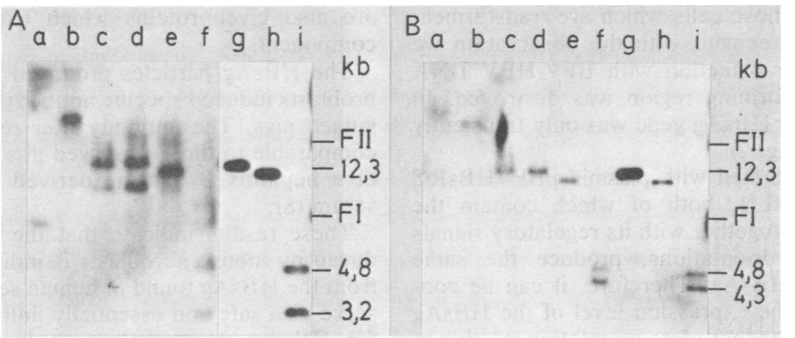


FIG. 7. Blot hybridization of $^{32}$P-labeled BPV DNA and HBV DNA to cellular DNA purified from cell line Y1. DNA (20 μg) was cleaved to completion with *Sac*I (lane b), *Hin*dIII (lanes c and g), *Bgl*II (lane d), *Bam*HI (lanes e and h), and *Eco*RI (lanes f and i) and electrophoresed through a 0.7% agarose gel (lane a contains uncleaved Y1 DNA). DNA from cell line Y1 is shown in lanes a through f, and DNA from plasmid pBPVHBsR/8 is shown in lanes g through i. Fragment sizes are indicated. (A) Blot after annealing with $^{32}$P-labeled BPV DNA. (B) The same blot after annealing with $^{32}$P-labeled HBV DNA.

binant plasmid itself was cleaved with *Hin*dIII, *Bam*HI, and *Eco*RI (lanes g, h, and i). Hybridization was done either with the $^{32}$P-labeled BPV DNA fragment (Fig. 7A) or the HBsAg gene (Fig. 7B). The bands obtained after cleavage of total cellular DNA with *Hin*dIII, *Bgl*II, *Bam*HI, and *Eco*RI (lanes c to f) are of identical size compared with those of the control (lanes g, h, and i), indicating the extrachromosomal status of the plasmid in the cells. The second *Bam*HI fragment is too small to be seen on the gel. Although *Eco*RI cleaves the plasmid three times, hybridization to HBV DNA or to the BPV DNA fragment results in only two bands, since in both cases there is one fragment which does not hybridize to the labeled probe (compare the restriction maps in Fig. 1). The autoradiograph after annealing with BPV DNA shows additional bands for the cleavage products of *Hin*dIII, *Bgl*II, and *Eco*RI. However, since the size of these bands is the same after cleavage with *Hin*dIII or *Bgl*II, an integrated status may be excluded. Such minor DNA species were also observed by Sarver et al. (23). It is believed that they belong to deletion mutants of the episome. Since hybridization to HBV DNA does not show these additional bands and no additional bands can be seen when the *Bam*HI-cleaved DNA is hybridized to BPV (*Bam*HI cleaves only in the HBV part of the episome), these results indicate that the part of the episome containing the HBsAg gene is lost. DNA isolated from untransformed cells did not hybridize with any BPV or HBV DNA sequences (data not shown).

The supercoiled form of the plasmid can only be seen for undigested cell line Y1 hybridized with BPV DNA (Fig. 7A, lane a). Contrary to this result, a slow migrating DNA species, as seen in some BPV transformants reported by Law et al. (17) and Sarver et al. (23), can be seen here in undigested and *Sac*I-treated DNA probes (lanes a and b). Since cleavage with single-cut restriction endonucleases converted the slow migrating complex into linear form III molecules, it was concluded that this form probably represented complexes of circular units (17, 23). This may be an explanation of why the supercoiled form cannot be seen in all of these lanes.

By comparing the hybridization signals of the digested cellular DNA with the known amount of plasmid DNA, an average number of 50 to 100 plasmid copies per cell was estimated.

## DISCUSSION

We have described the construction of a BPV-HBV recombinant plasmid that retains the 69% transforming fragment of BPV as a dominant selective marker for stable HBsAg-expressing cell lines. The transformation efficiency of NIH 3T3 mouse fibroblasts by the BPV-HBV recombinant plasmid was 10 focus-forming units per μg of plasmid DNA, which is threefold lower than that described for the unrecombined BPV transforming fragment (18).

From blot analysis we conclude that the BPV-HBV recombinant plasmid exists predominantly as a free extrachromosomal episome within the transformed cells. The HBsAg gene is coamplified with the BPV DNA (6, 17, 23). Thus, the high production of HBsAg, up to 420 ng/ml per day, may be explained by the special mode of the replicative cycle of the BPV vector.

The transient expression of HBsAg during the 10 days after transfection may reflect the fact that a large proportion of the cells take up the BPV-HBV DNA but only some of them retain the transfected DNA. Stable expression is only

achieved in those cells which are transformed. This is in agreement with the observation we made after transfection with BPV-HBV DNA whose transforming region was destroyed. In that case, the HBsAg gene was only transiently expressed (Fig. 2).

Cells transfected with plasmid pBPVHBsR/8 or pBPVHBsL/1, both of which contain the HBsAg gene together with its regulatory signals in opposite orientations, produce the same amounts of HBsAg. Therefore, it can be concluded that the expression level of the HBsAg gene is independent of its orientation relative to the BPV vector. Additional influence of BPV sequences on expression of HBsAg, e.g., enhancing sequences, cannot be discounted.

From one high-producing cell line, Y1, propagated from a single transformed focus, production kinetics were analyzed under conditions where cells were kept in maintenance medium (Fig. 3a), in accordance with the conditions of Skelly et al. (24) for the production rate of the well-established human hepatoma cell line PLC/PRF/5 (2). A production rate for HBsAg of 420 ng/ml per day (400 U/ml per day) was determined. This level has been maintained, so far, for 6 months (more than 100 generations). Expressed in terms of cell numbers, the total yield is approximately 6 μg per $10^7$ cells per day. Production kinetics for PLC/PRF/5 cells (2) were made in parallel and under identical conditions. After 7 days an amount of 80 ng of HBsAg per ml was found (27). This is about 20 times lower than the yield of 1.7 μg/ml obtained from Y1 cells after 7 days. Moreover, the Y1 cells have an advantage in that they grow faster than the hepatocarcinoma cells, they can be kept alive in a higher density for longer time, and they do not contain the complete HBV genome. Therefore, no infectious HBV particles can arise. Production rates of HBsAg with orders of magnitude similar to that for Y1 cells have been reported only by Christman et al. (4) for NIH 3T3 cells where they have amplified HBV sequences intrachromosomally by increasing the concentration of methotrexate after cotransfection with the dihydrofolate reductase gene.

The biophysical properties of the HBsAg secreted into culture medium were the same as those reported for that of human serum (9). Even the polypeptide composition compares favorably with that from HBsAg purified from human plasma (26). As shown for HBsAg from human plasma, four polypeptides were found with molecular weights of about 24K, 28K, 34K, and 37K. The major components seen in Fig. 6 are the 24K and 28K forms. The appearance of the 28K form indicates glycosylation of the 24K form of HBsAg (20). Stibbe and Gerlich (26) were able to show that the 34K and 37K forms are also glycoproteins which exist as minor components.

The HBsAg particles produced by mouse fibroblasts induced specific antibody formation in guinea pigs. The antibody titer reached levels comparable to those observed in a potency test of a hepatitis B vaccine derived from human serum (8).

These results indicate that the HBsAg produced by mouse fibroblasts is indistinguishable from the HBsAg found in human serum and can serve as a safe and essentially unlimited source for HBsAg. Nevertheless, it has still to be demonstrated that it induces antibodies in chimpanzees which protect against HBV infection.

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