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Expression of the hepatitis B virus surface antigen gene in cell culture by using a simian virus 40 vector

(recombinant DNA/animal virus/gene expression/antigen characterization)

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ABSTRACT **We have constructed a simian virus 40 recombinant carrying a fragment of DNA from hepatitis B virus. Cultured monkey kidney cells infected with this recombinant produce hepatitis B surface antigen. The antigen is excreted into the culture medium as 22-nm particles with the same physical properties, antigenic composition, and constituent polypeptides as those found in the sera of patients with type B hepatitis.**

At least half of the world population shows evidence of past or present infection by hepatitis B virus (HBV), and the approximately 200 million carriers in the world are at serious risk of chronic liver disease and, possibly, primary liver cancer. The classic marker for chronic infection by this virus is the surface antigen HBsAg which circulates in the serum of HBV carriers in three forms: 22-nm spherical particles, 22-nm filaments of various lengths, and the 42-nm spherical form known as the Dane particle. The 22-nm particles and filaments are subviral forms containing two predominant polypeptides, with apparent molecular weights of about 23,000 and 29,000, together with several minor polypeptides of larger size (1, 2). The two predominant species, which are probably identical except that the larger is glycosylated, carry both the group (a) and the subtype (d/y) antigenic determinants of HBsAg (3). The Dane particle, which represents the infectious virion, consists of a lipoprotein coat (HBsAg) surrounding an internal core particle which contains a DNA polymerase and the 3200-base pair (bp) circular DNA genome. The 22-nm particle is the predominant form in the sera of chronic carriers and circulates at concentrations as high as 100–200 μg/ml.

Characterization of the life cycle and biology of HBV has been hampered by its narrow host range, which is restricted to humans and a few other primates, and by its inability to grow in cultured cells. Recently, however, several groups have succeeded in cloning the viral genome in *Escherichia coli* phage λ (4) and plasmid vectors (5, 6) and in determining its primary structure (7–9). This has allowed the identification of a continuous 892-bp sequence that could encode surface antigen (7), a 549-bp sequence that may specify the core antigen (8), and several additional open sequences of unknown function (9).

Although the DNA sequence provides crucial structural information, it clearly is not sufficient to establish all of the HBV gene products or to indicate how these products interact during infection of the target cell. For this purpose it would be useful to develop a system for introducing defined portions of the viral genome into cultured cells. Simian virus 40 (SV40), a small DNA tumor virus that can lytically infect cultured monkey cells, provides a useful vector for this purpose. In this paper we describe the construction and propagation of a SV40 recombinant carrying a 1350-bp fragment of HBV DNA that includes the structural sequences for surface antigen. We show that monkey kidney cells infected with this recombinant synthesize surface antigen that is excreted into the culture medium as 22-nm particles. These results set an upper limit on the amount of HBV genetic information required for 22-nm particle formation and demonstrate the feasibility of using SV40 recombinants to study HBV gene expression in cultured primate cells.

MATERIALS AND METHODS

The following methods have been described: general procedures for the construction of recombinant plasmids and viruses (10); growth of African green monkey kidney cells and propagation of virus stocks (11); preparation of plasmid (12) and intracellular SV40 DNA (13); analysis of DNA by restriction endonuclease cleavage (11) and agarose gel electrophoresis (14, 15); and transformation of EK2 *Escherichia coli* strain HB101 (16). Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories (Rockville, MD) and reaction conditions were according to the supplier.

The source of HBV DNA was plasma, subtype *adw*, from an HBsAg-positive donor. Dane particles were purified by the method of Robinson (17) and incubated in the endogenous DNA polymerase reaction (18) with all four deoxynucleotide triphosphates prior to DNA extraction.

The 22-nm form of HBsAg was purified from the plasma of chronic carriers as described (19). Hyperimmune guinea pig antiserum to HBsAg/*ad* (V801-502-058) was from Research Resources Branch (National Institute of Allergy and Infectious Diseases) and monospecific antibodies to the HBs/*a* and HBs/*d* determinants were prepared from this serum by affinity chromatography (3). Fluorescein isothiocyanate-conjugated rabbit anti-guinea pig IgG was obtained from Cappel Laboratories (Cochranville, PA). Radioimmunoassays for hepatitis B core antigen (20), δ antigen (21), and *e* antigen (HBeAg test kit, Abbott Laboratories) have been described. HBsAg was detected by the Ausria II radioimmunoassay (Abbott Laboratories) and quantitated by a parallel-line assay with a known standard (BoB HBsAg/*adw* vaccine, reference lot 1, 40 μg/ml). The *d*/*y* subtype of HBsAg was determined by the competition radioimmunoassay method of Hoofnagle (22).

All experiments requiring physical containment level P3 were performed at the certified P3 facility of the Georgetown University Division of Molecular Virology and Immunology

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Abbreviations: SV40, simian virus 40; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; SVHBV, SV40–hepatitis B virus recombinant; SVHBV-HBsAg, hepatitis B surface antigen produced by cells infected with SVHBV; bp, base pair(s).

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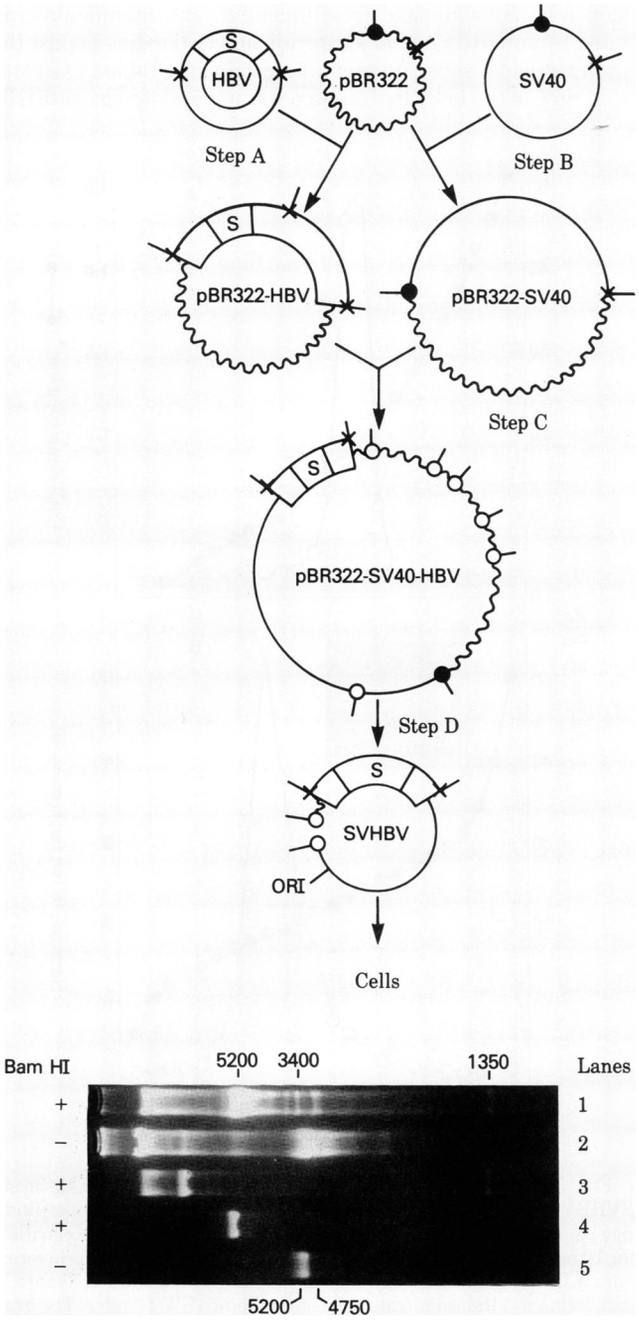

FIG. 1. Construction and analysis of the SV40–HBV recombinant. (*Upper*) *Step A*. A 120-μl reaction mixture containing 200 ng of HBV DNA and 1.4 μg of plasmid pBR322 DNA was digested with 9 units of *Bam*HI for 2 hr at 37°C, heat inactivated at 70°C for 10 min, extracted with $CHCl_3$, precipitated with ethanol, resuspended in 20 μl of ligation assay buffer, and treated with 4 units of T4 DNA ligase for 5 hr at 14°C. This mixture was used to transform *E. coli* HB101, and a pBR322–HBV clone was identified by colony hybridization (23). DNA from this clone was cleaved with *Bam*HI and the 1350 bp fragment containing the HBsAg coding region was purified by preparative electrophoresis through a 0.8% agarose gel (15); subcloned in pBR322 and reisolated by electrophoresis. *Step B*. pBR322–SV40 was prepared, as described (24), by cleavage with *Bam*HI/*Eco*RI, treatment with ligase, and cloning in *E. coli*. *Step C*. pBR322–HBV was then ligated to *Bam*HI-cleaved pBR322–SV40 DNA and cloned in *E. coli* as described above. *Step D*. *Hae* II cleavage of the resulting pBR322–SV40–HBV "double recombinant" DNA generated the 4950-bp linear SVHBV DNA fragment that was used to infect monkey kidney cells. Cleavage sites: X, *Bam*HI; ● *Eco*RI; ○, *Hae* II. (*Lower*) Ethidium bromide-stained 1% agarose gel containing intracellular viral DNA from cells infected with the SVHBV plus SV40*ts*A_{239} stock (lanes 1 and 2), plasmid pBR322–SV40–HBV DNA (lane 3), and purified SV40 DNA (lanes 4 and 5). The numbers above represent the lengths of linear molecules; the numbers below refer to the lengths of covalently closed circular DNAs. The uncleaved SVHBV plus SV40-*ts*A_{239} DNA (lane 2) shows a predominant band of helper DNA at 5200 bp, a band of SVHBV DNA at 4750 bp, and a heterogeneous collection of shorter DNAs; the more slowly migrating bands represent nicked circular and host cell DNA. *Bam*HI cleavage of this DNA (lane 1) generated a predominant 5200-bp band of helper DNA, a 3400-bp band of SV40 vector DNA, a 1350-bp band of inserted HBV DNA that comigrated with its authentic counterpart from *Bam*HI-cleaved pBR322–SV40–HBV DNA (lane 3), and several other bands of unknown origin.

(Rockville, MD) according to the National Institutes of Health recombinant DNA research guidelines.

RESULTS

Construction and Propagation of the SV40-HBV Recombinant. The SV40–HBV recombinant described here carries a 1350-bp fragment of HBV DNA, representing about 40% of the HBV genome, inserted into the late gene region of SV40. The first step in the construction of this recombinant was to amplify the HBV genome by cloning it in an *E. coli* plasmid vector (Fig. 1). Dane particles were purified from the serum of a chronic HBsAg carrier, subtype *adw*, and the partially single-stranded viral genome was repaired by an endogenous DNA polymerase reaction. Two fragments, 1350 and 1850 bp, were obtained after cleavage of this DNA with *Bam*HI. Partial digestion with *Bam*HI generated a full HBV genome which was ligated to *Bam*HI-cleaved plasmid pBR322 DNA and cloned in *E. coli*.

From the published sequence data (7–9) we anticipated that the HBsAg coding sequence would be located within the 1350-bp *Bam*HI fragment. This fragment was purified by electrophoresis, subcloned in pBR322, isolated, ligated to a *Bam*HI-cleaved pBR322–SV40 vector plasmid, and recloned in *E. coli*. *Hae* II digestion of the resultant pBR322–SV40–HBV "double recombinant" plasmid which retained only the 1350-bp HBV fragment removed all but 143 bp of the pBR322 DNA and yielded a homogenous preparation of 4950-bp SV40–HBV linear recombinant molecules. These molecules retained the SV40 origin of DNA replication and the complete SV40 early gene region but lacked most of the SV40 late gene region and hence were defective. Nevertheless, they could be packaged into SV40 coats and propagated as virions by making a mixed DNA infection of monkey kidney cells with an SV40 temperature-sensitive early gene mutant (SV40*ts*A_{239}) as helper. This mixed infection was performed at the nonpermissive temperature (39°C) to ensure that progeny virions would be produced only by cells doubly infected with the SV40–HBV recombinant, which supplies functional SV40 early gene products, and with the helper, supplies all of the required SV40 late gene products (11, 25, 26).

To determine if the SV40–HBV recombinant was encapsidated into SV40 virions, we took advantage of the fact that only those genomes incorporated into viral particles during the original DNA infection will be transferred and replicated in a subsequent viral infection (27). Accordingly, we infected a fresh culture of monkey cells with the virus stock from the DNA infection, prepared intracellular viral DNA 3 days later, and examined it by restriction endonuclease cleavage and agarose gel electrophoresis (Fig. 1). This showed that the stock contained approximately 75% helper genomes and 5% SV40–HBV recombinant genomes retaining the complete 1350-bp HBV fragment. The remaining 20% of the DNA was found in a heterogeneous collection of genomes with lengths ranging from about 3000 to 4900 bp; although not examined in detail, these molecules contained no more HBV genetic information than present in the

original subcloned 1350-bp fragment. We refer to this complex mixture of virus as "SVHBV."

Monkey Kidney Cells Infected with SVHBV Synthesize HBsAg. We used specific immunological assays to show that monkey kidney cells infected with SVHBV synthesized HBsAg but no other established HBV antigens. Immunofluorescence analysis revealed that approximately 45% of the cells infected with SVHBV expressed cytoplasmic HBsAg by 72 hr after infection (Fig. 2), whereas uninfected cells and cells infected with wild-type SV40 were negative. Quantitative radioimmunoassays showed that a culture of 2×10^7 cells produced a total of 2.5 μg of HBsAg. Of this, 40% was found in the medium and 60% was released from the cells by freeze-thawing and sonication. Subtype analysis (22) showed that SVHBV-HBsAg had the same antigenic composition (d+, y−) as the antigen from the original donor of the HBV DNA. SVHBV-infected cells were negative for HBcAg and δ antigen by immunofluorescence. The culture medium was negative (P/N<2.1) for HBcAg, HBeAg, and δ antigen by solid-phase radioimmunoassay.

The HBsAg Is Secreted from Monkey Kidney Cells as a Particle. SVHBV-HBsAg found in the tissue culture fluid is a 22-nm particle whose physical characteristics are the same as those of the particles found in human serum. Isopycnic banding in CsCl of SVHBV-HBsAg revealed a buoyant density value (1.2 g/cm^3) identical to that of purified HBsAg run in parallel (Fig. 3*A*). The SVHBV-HBsAg material was further characterized by rate zonal centrifugation (Fig. 3*B*). Most of the SVHBV-specific antigen sedimented as a particle indistinguishable from the 22-nm particles found in human sera. Examination of the antigen by electron microscopy of the pooled, concentrated peak fractions from the sucrose gradient (Fig. 3 Inset) revealed 22-nm spherical particles with the same appearance as the predominant form of HBsAg from human sera. Examination of the less-highly purified material from the CsCl gradient also showed 22-nm filaments of variable lengths (results not shown). No Dane particles were observed.

HBsAg Polypeptides. The polypeptide composition of the excreted SVHBV-HBsAg was analyzed by immunoprecipitation and gel electrophoresis of both chemically and biosynthetically labeled antigen. SVHBV-HBsAg from the rate sedimentation step was radioiodinated, resedimented, and immunoprecipitated by anti-HBs Ag/*ad* antiserum and antibodies against the group (*a*) and *d*-subtype specific determinants (Table 1). Greater than 60% of the iodinated antigen was precipitated by anti-HBs

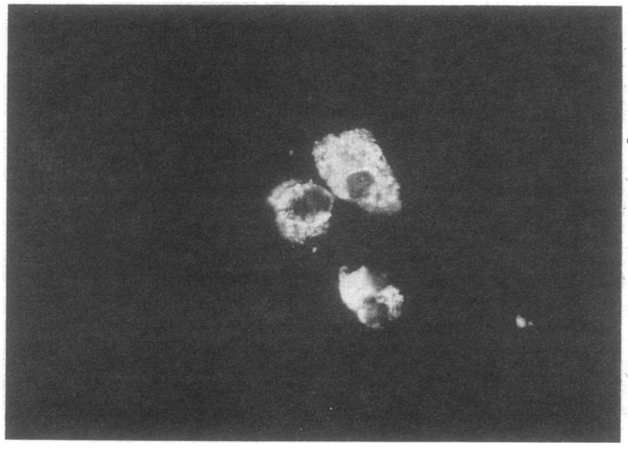

FIG. 2. Indirect immunofluorescent staining of SVHBV-infected monkey kidney cells for HBsAg. (×640.) Culture medium (2 ml) from SVHBV-infected monkey kidney cells was removed 72 hr after infection. The cells were pelleted from the medium, fixed for 5 min with ether, and incubated with guinea pig anti-HBs/*ad* serum. The cells were further incubated with fluorescein-conjugated rabbit anti-guinea pig IgG and examined under a Zeiss photomicroscope III with epi-fluorescence. Uninfected cells or cells infected with wild-type SV40 showed no fluorescent staining.

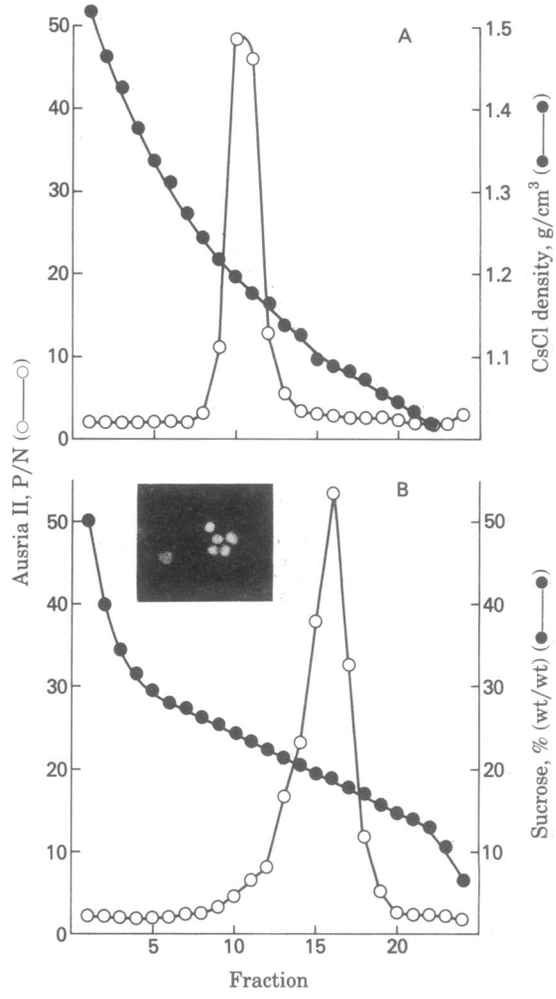

FIG. 3. Biophysical properties and appearance of HBsAg from SVHBV-infected monkey kidney cells. (*A*) The medium of monkey kidney cells, harvested 72 hr after infection with SVHBV, was clarified for 10 min at 1000 × *g* to remove cells and a 0.5-ml sample was layered onto a five-step gradient (1.1–1.6 g/cm^3) of CsCl in 0.01 M Tris·HCl (pH 7.4) in a cellulose nitrate tube of the Spinco SW 41 rotor. The gradient was centrifuged for 18 hr at 4°C and 34,000 rpm. Fractions (0.5 ml) were collected by bottom puncture and assayed for HBsAg by a commercial radioimmunoassay (Ausria II); results are expressed as the ratio of ^{125}I cpm in the sample (P) to the negative control (N; 93 cpm). CsCl density was determined by refractometry. (*B*) A 7-ml sample of medium from the SVHBV-infected cells was layered onto a 5-ml two-step gradient (1.2 and 1.5 g/cm^3) of CsCl in 0.01 M Tris·HCl (pH 7.4) and centrifuged for 18 hr at 4°C and 34,000 rpm in an SW 41 rotor. Fractions (1 ml) were collected by bottom puncture and assayed for HBsAg (at 1:10 dilution) by Ausria II; CsCl density was measured by refractometry. HBsAg was recovered in a single fraction at approximately 1.2 g/cm^3 (P/N = 65 at 1:10 dilution). This fraction was dialyzed extensively against 0.85% NaCl/0.01 M phosphate, pH 7.4, and a 0.5-ml sample was layered onto an 11-ml linear gradient of 10–30% (wt/wt) sucrose in the buffered saline with a cushion of 0.5 ml of 66% (wt/wt) sucrose in buffered saline. After centrifugation for 4.5 hr at 35,000 rpm and 4°C in an SW 41 rotor ($22 \times 10^{10}\ \omega^2 t$), 0.5-ml fractions were collected by bottom puncture and assayed for HBsAg (at 1:10 dilution) by Ausria II and for sucrose concentration by refractometry. (*Inset*) Electron microscopy of particles stained with 1% phosphotungstic acid; these particles are from fractions 15–17 (*B*) and are 20–24 nm in diameter. (×100,000.)

Table 1. Immunoprecipitation of ^{125}I-labeled HBsAg from SVHBV-infected cells

Sample	% precipitation by antiserum			
	Pre	HBs/*ad*	HBs/*a*	HBs/*d*
Serum HBsAg/*ad*	1.2	83.5	76.6	82.8
SVHBV-HBsAg	5.9	64.1	62.2	59.1

The peak fractions (Fig. 3*B*, fractions 15–17, 300 μl each) were pooled and concentrated 15-fold by pelleting. The concentrate was iodinated by the chloramine-T procedure (28) and further purified by rate zonal centrifugation in sucrose. A standard HBsAg/*ad* preparation isolated from human serum was iodinated and purified in parallel. Radioiodinated SVHBV-HBsAg and HBsAg/*ad* (100 μl each) were incubated with 10 μl of guinea pig antiserum at room temperature for 2 hr and precipitated with rabbit anti-guinea pig IgG (140 μl) in 0.05% Tween 20 in phosphate-buffered saline. After further incubation at 4°C for 18 hr, the mixture was centrifuged at 2300 × *g* for 30 min and the precipitates were washed twice with 300 μl of Tween 20 in phosphate-buffered saline. The supernatants were pooled and the percentage precipitation was calculated (29).

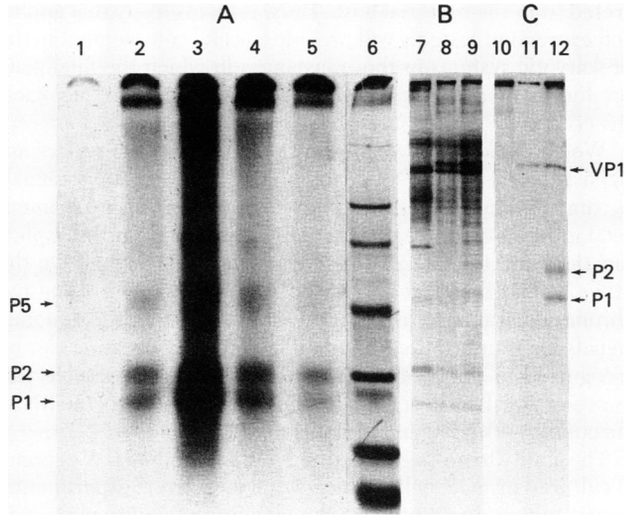

FIG. 4. Electrophoretic analysis of SVHBV-HBsAg polypeptides. (*A*) Immunoprecipitation of ^{125}I-labeled SVHBV-HBsAg and human HBsAg/*ad*. The antigens were radioiodinated and precipitated as described in Table 1, solubilized in 2% NaDodSO$_4$/2% 2-mercaptoethanol, and electrophoresed through a NaDodSO$_4$/7.5% acrylamide gel (25). Lanes: 1, SVHBV-HBsAg precipitated with preserum; 2, SVHBV-HBsAg precipitated with anti-*ad*; 3, HBsAg precipitated with anti-*ad*; 4, SVHBV-HBsAg precipitated with anti-*d*; 5, SVHBV-HBsAg precipitated with anti-*a*; 6, ^{14}C-labeled protein standards [myosin (200,000) phosphorylase *b* (92,500), bovine serum albumin (68,000), ovalbumin (45,000), chymotrypsinogen A (25,700), β-lactoglobulin (18,400), and cytochrome *c* (11,700)]. (*B*) Total medium from [^{35}S]methionine-labeled cells. Confluent monolayers of about 2 × 10^7 monkey kidney cells were incubated with SVHBV (lane 9) or with wild-type SV40 (lane 8) or without virus as controls (lane 7). At 68 hr after infection, the medium was replaced with 5 ml of methionine-free medium containing 200 μCi of [^{35}S]methionine per ml. Five hours later the medium was removed and an aliquot was treated with 2% NaDodSO$_4$/2% 2-mercaptoethanol and electrophoresed through a NaDodSO$_4$/20% polyacrylamide gel. (*C*) Immunoprecipitation of [^{35}S]methionine-labeled peptides. The medium from *B* was treated with anti-*ad* antiserum followed by formalin-fixed *Staphylococcus aureus* Cowan I bacteria (30) and the precipitates were solubilized and electrophoresed through the same NaDodSO$_4$/20% polyacrylamide gel as in *B*. Lanes 10, uninfected; 11, wild-type SV40; 12, SVHBV. The positions of HBsAg polypeptides P1 and P2 and of the SV40 structural protein VP1 are indicated.

and by both of the monospecific antibodies. NaDodSO$_4$ gel electrophoresis of the denatured and reduced precipitates revealed two predominant polypeptides (P1 and P2) with the same mobilities as those isolated from human serum (Fig. 4*A*). These proteins are thought to be identical except that P2 is glycosylated. In addition, the iodinated SVHBV-HBsAg contained at least one of the minor polypeptides of higher molecular weight (P5).

In a companion experiment, SVHBV-specific proteins were biosynthetically labeled by incubating the infected cells with [^{35}S]methionine for 4 hr late in the lytic cycle. Direct examination of the medium from SVHBV-infected cells revealed two proteins with the same mobilities as P1 and P2 (Fig. 4*B*), both of which were precipitated by anti-HBs antiserum (Fig. 4*C*). In contrast, no such proteins were found in the medium from uninfected or wild-type SV40-infected cells. This experiment also demonstrated that HBsAg is actively excreted rather than merely released by cell lysis because only a subpopulation of the total cell proteins was found in the medium.

DISCUSSION

We have described the construction of an SV40–HBV recombinant that retains about 70% of the SV40 genome and 40% of the HBV genome. This hybrid virus is replicated and packaged into SV40 virions in cultured monkey kidney cells, the permissive host for SV40, that have been coinfected with a complementing SV40 helper virus. The expression of the inserted HBV sequences was examined by both immunological and biochemical techniques. This showed that the recombinant directs the synthesis of surface antigen but no other known HBV-specific antigens. Furthermore, blocking assays and immunoprecipitation with monospecific antibodies demonstrated that the SVHBV-derived HBsAg had the same subtype (*ad*) as the antigen from the original donor of the HBV DNA. Because the SVHBV recombinant was derived from a cloned plasmid containing only the 1350-bp *Bam*HI fragment of HBV, these observations demonstrate that both group- and subtype-specific determinants of HBsAg are encoded within a limited portion of the viral genome.

HBsAg from monkey cells contains the same major polypeptides (P1 and P2), with the same P1-to-P2 ratio, as HBsAg derived from human serum. Because P2 represents a glycosylated form of P1, it appears that the posttranslational modification of this HBV gene product is not unique to liver cells. At least one minor polypeptide of higher molecular weight (P5) was also seen in extrinsically labeled HBsAg but not in biosynthetically labeled HBsAg. Interestingly, the size of this polypeptide (49,000) represents the upper limit of the coding capacity of the inserted HBV fragment.

The HBsAg encoded by SVHBV is excreted into the culture medium as 22-nm particles with the same buoyant density, sedimentation properties, and appearance as the particles from human serum. These particles are produced at a rate of 2.5 μg/10^7 cells per 2 days or approximately 3 × 10^4 particles per infected cell per day. This compares favorably with HBsAg production by the Alexander cell line (31) derived from a human hepatocellular carcinoma. We conclude that monkey kidney cells possess all of the functions required for particle formation and that the failure of HBV to grow in tissue culture is not due to a block at this stage of the viral life cycle.

Recently, Dubois *et al.* (32) used the thymidine kinase cotransformation technique to obtain mouse L cell lines that excrete 22-nm particles. However, in those experiments, HBsAg expression depended upon the presence of two complete tandem copies of the HBV genome rather than upon a defined subfragment. The HBV surface antigen gene has also been expressed in *E. coli* as part of a 138,000-dalton HBsAg–β-galactosidase fusion polypeptide (33). Not surprisingly, this hybrid antigen is not glycosylated, assembled into particles, or ex-

creted from the bacterial host. Thus, it seems likely that animal cell expression systems will provide a useful complement to the prokaryotic systems in those instances in which the final gene product is modified and assembled into a complex biological structure.

We do not know to what extent expression of the surface antigen gene in SVHBV depends upon SV40 regulatory signals as compared to HBV signals. The vector used in this experiment retains the SV40 late region promoter, the late 19S mRNA splice junction, and the late region polyadenylylation signal. On the other hand, Hamer *et al.* (24, 34, 35) have shown, using the chromosomal mouse α- and β-globin genes, that the genomic signals for transcription initiation and RNA processing can be recognized in the SV40–monkey cell system. To resolve this question, it will be necessary to study the structures of the RNAs encoded by SVHBV and mutants derived from it.

The ability to propagate defined portions of the HBV genome in cultured primate cells raises several interesting experimental opportunities. In addition to the obvious possibility of vaccine production, it should be feasible to construct viruses that encode useful diagnostic reagents such as surface antigen peptides bearing single, highly specific immunological determinants. SV40–HBV hybrids might also be useful for identifying new HBV gene products—e.g., nonstructural proteins that are not excreted into the serum. Finally, the availability of SV40–HBV recombinants provides an experimental system with which to investigate various mechanisms for the persistence and pathogenicity of HBV in liver cells.

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