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Direct Expression of Hepatitis B Surface Antigen in Monkey Cells from an SV40 Vector

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ABSTRACT

We have developed an SV40-based vector that can be used for the efficient direct expression of foreign genes in permissive monkey cells. The vector lacks the coding sequences for the major SV40 late protein (VP-1) and possesses unique *Eco* RI and *Bam* HI restriction sites so that DNA fragments containing the coding sequences of foreign genes bounded by these two restriction sites can be conveniently inserted and directly expressed under the control of the VP-1 transcriptional unit. We have inserted into this vector the gene encoding the surface antigen of hepatitis B virus and observed the synthesis of this protein in monkey cells infected with this vector at a level comparable with that of VP-1. Furthermore, we have shown that the HBsAg synthesized is assembled into and secreted as a complex structure (22-nm particle) indistinguishable from that formed naturally during human infection. These observations enable us to conclude that HBsAg is the only component encoded by HBV that is required for the secretion and assembly of the 22-nm particle and that this process can occur without the involvement of a potential signal peptide suggested by the DNA sequence which precedes the coding region of mature HBsAg.

INTRODUCTION

HEPATITIS B virus (HBV) is transmitted among humans as a chronically debilitating infection which can result progressively in high incidence of chronic liver disease or liver cancer (Tiollais *et al.*, 1981). The viral surface antigen (HBsAg) is a glycosylated protein of 25,400 daltons which represents the major envelope antigen of the 42-nm particles (Dane particle) of hepatitis B virus and is thought to be the major target of neutralizing antibody (Tiollais *et al.*, 1981). HBsAg is found in the plasma of HBV carriers and is most often assembled with lipid and other proteins into either 22-nm spherical particles (22-nm particles) or filaments (Tiollais *et al.*, 1981). Similar HBsAg particles can also be purified from the culture medium of a hepatoma cell line (Marion *et al.*, 1979), indicating that the processes of assembly and secretion can occur in the absence of productive viral infection. To characterize further the processes involved in the glycosylation, assembly, and secretion of HBsAg, we sought to construct SV40 vectors suitable for the direct expression of genes from hepatitis virus in monkey cells.

SV40 virus has commonly been employed as a transducing vector for the expression of foreign genes in permissive monkey cells (Gething and Sambrook, 1981; Gruss and Khoury, 1981; Hamer *et al.*, 1979; Mulligan *et al.*, 1979). The virus is well suited for this purpose, having a small genome of double-stranded DNA whose sequence has been determined and an extensively characterized life cycle. In addition, the virus replicates efficiently in permissive cells, expressing high levels of viral proteins during the process (Acheson, 1980; Griffin, 1980). However, in most of the studies reported heretofore, the restriction enzyme recognition sites that were used to construct the recombinant virus removed some of the presumed transcriptional as well as translational controls of SV40 (Hamer *et al.*, 1979; Gething and Sambrook, 1981; Gruss and Khoury, 1981), perhaps accounting for the poor expression of the foreign gene. In this report, we describe the construction of an SV40 vector that contains unique restriction sites, between which a foreign gene can be inserted and expressed in place of the structural gene for the major capsid protein of SV40 (VP-1), preserving all known control elements of SV40.

When the recombinant SV40-HBV DNA is introduced into permissive monkey cells by DNA transfection in the presence of helper virus, HBsAg is synthesized at a level comparable with that of the major SV40 late protein (VP-1). Furthermore, HBsAg so produced is assembled into a complex particle structure indistinguishable from that observed in human serum following secretion from the liver during the infectious cycle. As such, the insertion of the HBsAg gene into this vector affords the opportunity to study not only factors controlling the expression of a foreign gene in mammalian cells, but also provides as well a convenient experimental system permitting the study of the processes involved in the posttranslational modification,

assembly, and secretion of complex macromolecular aggregates in such cells.

MATERIALS AND METHODS

All restriction enzymes, *Escherichia coli* DNA polymerase large fragment (Klenow polymerase I) (Jacobsen *et al.*, 1974), T4 polynucleotide kinase, T4 DNA ligase, and λ exonuclease were products of either BRL or New England Biolabs and were used according to the prescribed reaction conditions. Synthetic oligonucleotides were synthesized according to the method described previously (Crea *et al.*, 1978).

pHBV-T-1A is a plasmid in which HBV DNA was cloned into the *Eco* RI site of pBR322 according to published procedures (Charnay *et al.*, 1979; Valenzuela *et al.*, 1980). Plasmids pHS42 (D. Yansura and D. Kleid, unpublished result) is a derivative of pHBV-T-1A and pGH6 (Goeddel *et al.*, 1979a). This plasmid contains the structural gene for HBsAg from the ATG codon (constructed in a manner similar to that described in Fig. 2) through the HBsAg termination codon continuing to the *Hpa* I site in HBV DNA. This sequence is inserted into the filled-in *Eco* RI site of pGH6 in two separate steps. Plasmid pNCV is a derivative of pBR322 and its structure has been described (Goeddel *et al.*, 1980a).

The hepatoma cell line used as a source of HBsAg is the Alexander cell line (designated pLCWIII in the text) described previously (Macnab *et al.*, 1976; Marion *et al.*, 1979) which was passaged in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. HBsAg was assayed by the Austria II radioimmunoassay kit (Abbott Laboratories) and quantitated by serial dilution of the unknown sample and comparison to the positive control (20 ng/ml) supplied in the assay kit.

The procedures for isolating plasmid DNA, performing electrophoresis, transforming bacteria, and other related methods were as described (Goeddel *et al.*, 1979b; Davis *et al.*, 1980). Growth of the CV-1 cell line and the propagation of virus stock (tsA28 or recombinants) were performed according to published procedures (Mertz and Berg, 1974).

RESULTS

Construction of SV-40 DNA lacking the gene encoding VP-1

Examination of the nucleotide sequence of SV40 encompassing the coding region of VP-1 protein indicated two well-placed restriction endonuclease cleavage sites (Fig. 1) which could be used to generate a vector lacking the VP-1 coding sequences (Mulligan *et al.*, 1979). The first is a *Hind* III cleavage site at nucleotide position 1493, 6 nucleotides 5′ to the initiation codon for the VP-1 protein. The second, a *Bam* HI cleavage site at nucleotide 2533, is 50 nucleotides 5′ to the termination codon of the VP-1 gene. To obtain SV40 DNA with a deletion between these two sites, we carried out experiments outlined in Fig. 1. Briefly, wild-type SV40 DNA was first cleaved with *Bam* HI to obtain full-under conditions such that eacn DNA molecule was cleaved approximately once (there are six *Hind* III cleavage sites in the SV40 DNA). Subsequently, a synthetic decanucleotide, dAGCTGAATTC, was ligated to the digested DNA through cohesive ends generated by the *Hind* III cleavage (-TCGA). The mixture was then digested with *Eco* RI (to generate a cohesive end from the *Eco* RI site present within the added decanucleotide) and cloned into pBR322 between the *Bam* HI and *Eco* RI sites. Plasmids containing SV40 sequences were screened and the one with the stipulated deletion was isolated and designated as pSVR. Large quantities of this SV40 DNA vector were prepared by the propagation of pSVR plasmid in *E. coli* followed by cleavage of the plasmid DNA with *Eco* RI and *Bam* HI.

The purpose for the added synthetic decanucleotide is the introduction of an unique *Eco* RI site which is absent in this part of SV40 DNA. In addition, the added decanucleotide restores the original physical distance between the *Hind* III cleavage site and initiation codon for VP-1 protein when a DNA fragment containing the coding sequences of a foreign gene is constructed as described below and ligated to this vector through the *Eco* RI cleavage site (see below and Fig. 3B for more details). This may be an important consideration, as the spacing between the ribosome binding site and the initiation codon of a gene has been shown to affect the translational efficiency in prokaryotes profoundly (Goeddel *et al.*, 1980b; Guarente *et al.*, 1980).

Construction of recombinant SV40 DNA capable of synthesizing HBsAg

For the direct expression of HBsAg in pSVR, the following modifications of the HBsAg gene are required (i) an *Eco* RI site located immediately 5′ to an initiation codon, (ii) a *Bam* HI site located distal to the coding sequences, and (iii) a size comparable with the VP-1 gene of SV40 (900 bp) to ensure efficient packaging of the recombinant SV40-HBV molecule into virus particles. To meet these requirements, we carried out a series of experiments detailed in Fig. 2. An important step in this construction is the creation of an *Eco* RI restriction site immediately 5′ to a Met codon (ATG) of HBsAg. This was achieved by using a synthetic 12-mer (dATGGAGAACATC) as a site-specific primer; this sequence represents codons of the first four amino acids of the mature HBsAg (Met-Glu-Asn-Ile) (Valenzuela *et al.*, 1980). The primer was elongated by *E. coli* DNA polymerase large fragment (Klenow fragment), resulting in a blund-ended DNA molecule at precisely the Met codon of HBsAg (Goeddel *et al.*, 1980b). Following ligation into a suitable vector, an *Eco* RI site is generated immediately proximal to an initiation codon.

To prepare homogeneous recombinant SV40-HBV DNA for transfecting monkey cells, the *Bam* HI–*Eco* RI fragment containing SV40 DNA from pSVR was first ligated to the *Eco* RI–*Bam* HI fragment encoding HBsAg from pHS94 (Fig. 2) and the ligated fragment was subsequently cloned in the *Bam* HI site of a pBR322 derivative (derived from pHBV-T-1A, Fig. 2) as shown in Fig. 3A. The cloned DNA, pSVHBSA, could then be cleaved with *Bam* HI to generate a DNA fragment of 5382 nucleotides which

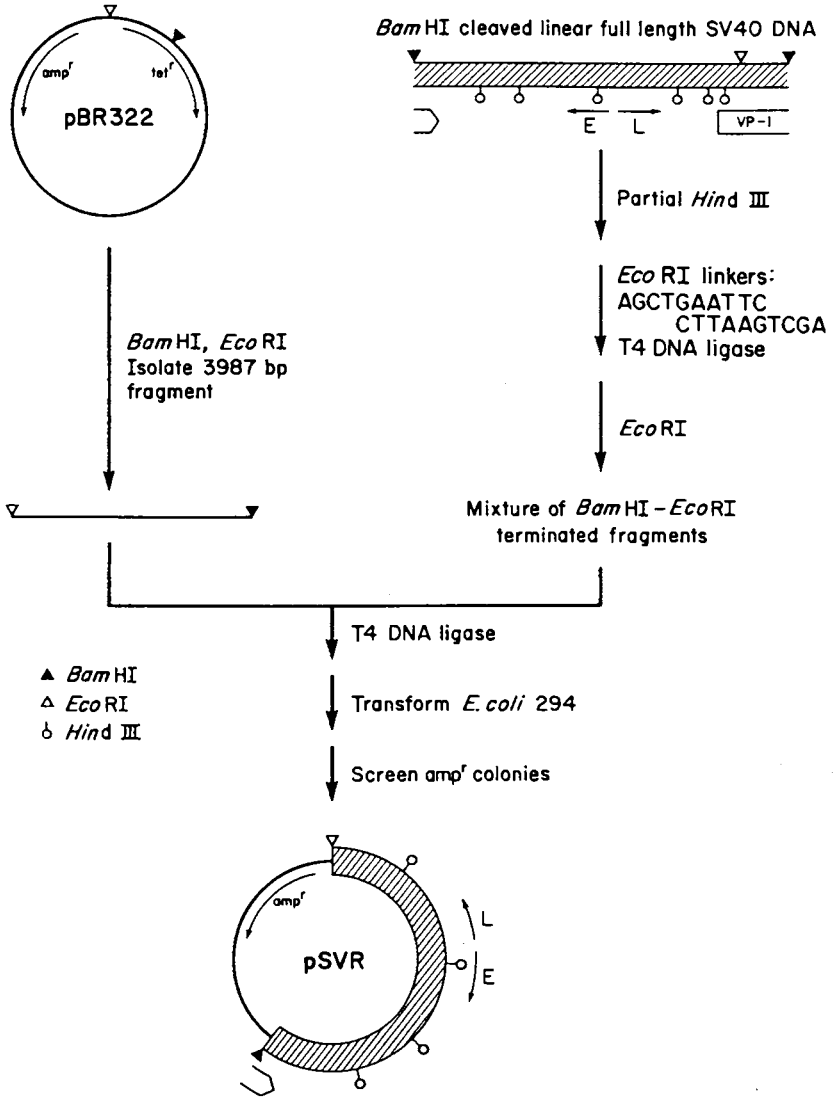


FIG. 1. Construction of an SV-40 DNA without the coding region of VP-1 protein. Eight μg of *Bam* HI-cleaved, linear, full-length SV40 DNA (obtained by either cleaving wild-type SV40 DNA or SV40 DNA cloned at the *Bam* HI site of pBR322) were digested with two units of *Hind* III (BRL) in a 50-μl reaction mixture. After incubation at 37°C for 30 min, the reaction was stopped by the addition of excess EDTA and the reaction mixture was deproteinized by phenol extraction followed by ethanol precipitation. The partially digested DNA was then resuspended in 10 μl of TE buffer (10 m*M* Tris-Cl pH 7.5, 1 m*M* EDTA). Synthetic decanucleotide dAGCTGAATTC (0.1 nmole) was phosphorylated with ATP by polynucleotide kinase in 10 μl reaction mixture containing 10 units of kinase. Incubation was at 37°C for 1 hr. Then an aliquot (3 μl) of the kinase reaction mixture was added to a ligation mixture (20 μl) containing 66 m*M* Trs-HCl pH 7.5, 6.6 m*M* $MgCl_2$, 10 m*M* DTT, 0.05 mg/ml BSA, 0.5 m*M* ATP, 4 μg of the partially *Hind* III-digested SV40 DNA (above), and 10 units of T4 DNA ligase. The ligation reaction was incubated at 20°C for approximately 16 hr. The ligated DNA was treated with restriction endonuclease *Eco* RI. The fragments, now containing *Eco* RI ends were ligated to the *Bam* HI–*Eco* RI fragment of pBR322 (0.5 μg) in a 15-μl ligation mixture and used to transform *E. coli* 294. Plasmid DNA from various transformants was screened by various restriction enzyme digestions for the presence of the SV40 DNA fragment of interest. The plasmid was designated pSVR. The relative positions of 5′ termini of both early and late transcripts are indicated by E and L.

VP-1 protein replaced by that of HBsAg and with the approximate position of the ATG of VP-1 protein restored (Fig. 3B). After *in vivo* ligation through the *Bam* HI sites (see below), the poly(A) site and the transcriptional termination site of the late transcripts will be contiguous to the coding sequence of HBsAg, and the VP-1 transcrip-

Propagation of recombinant SV40 virus and expression of HBsAg in monkey cells

To test the efficiency of HBsAg synthesis in such a vector system, we introduced *Bam* HI-cleaved pSVHBSA DNA into a monolayer of CV-1 cells by DEAE-dextran (McCut-

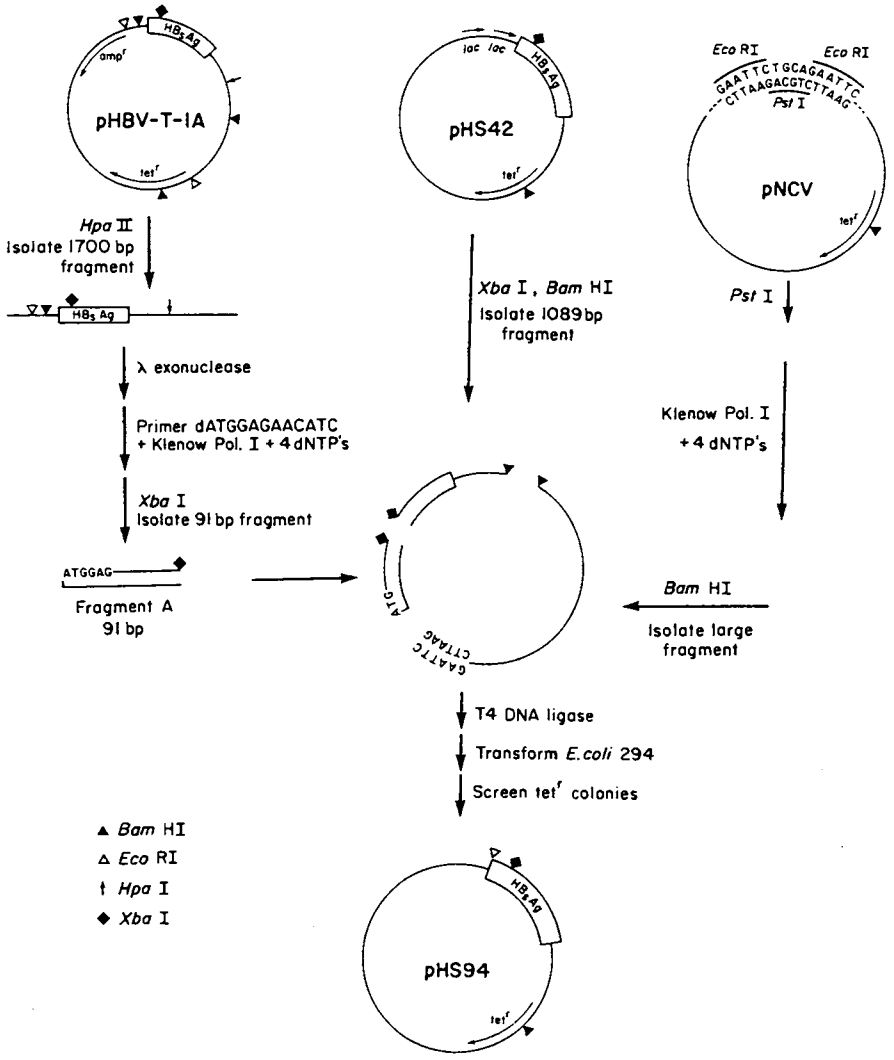
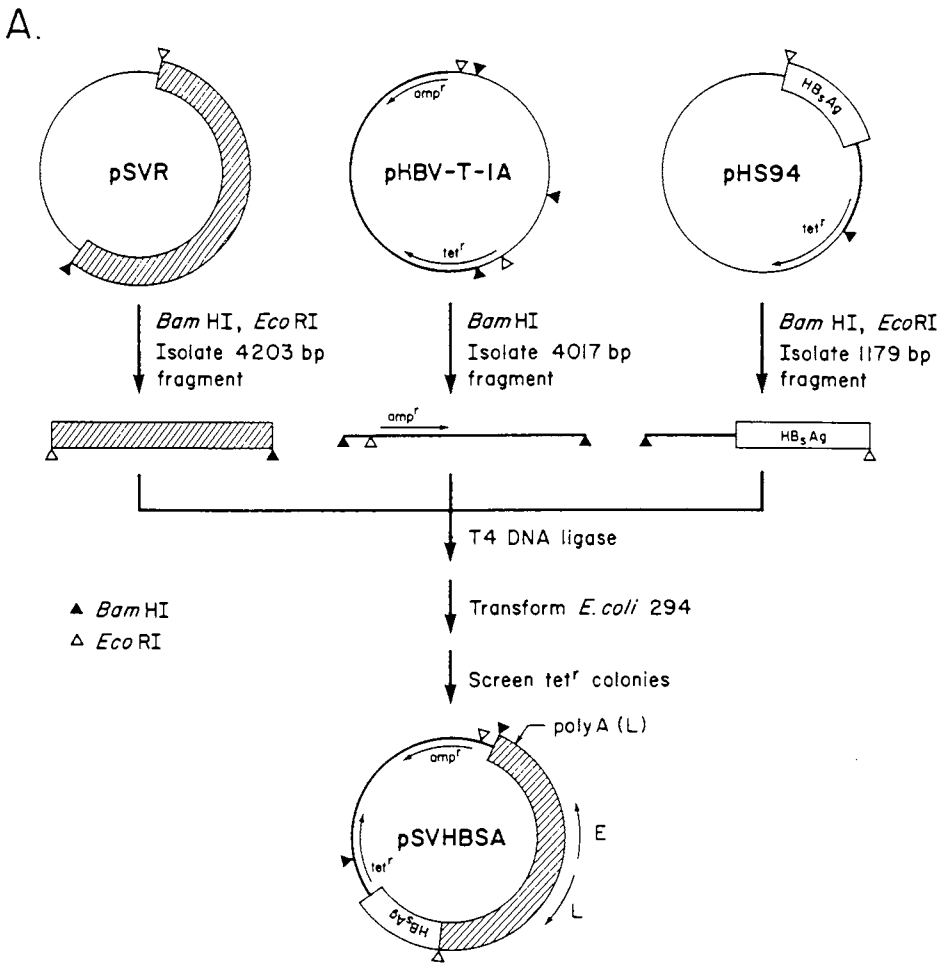


FIG. 2. Construction of a "HBsAg expression cassette." Plasmid pHS94 was constructed through the ligation of three DNA fragments: (i) fragment A, which contains the DNA sequences from the Met codon of matured HBsAg (Valenzuela *et al.*, 1980) to the unique *Xba* I site; (ii) the *Xba* I–*Bam* HI fragment from pHS42 which contains the remaining coding sequence of HBsAg from the unique *Xba* I plus some 3′ untranslated sequences up to the *Hpa* I site followed by the 375 bp *Eco* RI–*Bam* HI fragment of pBR322 (linked through the *Hpa* I and blunted *Eco* RI sites); and (iii) the treated vector DNA from pNCV. To obtain fragment A, 50 μg of pHBV-T-IA DNA was first digested with *Hpa* II (80 units) in a 200-μl reaction mixture and fractionated by polyacrylamide gel electrophoresis to obtain a 1.7-kb DNA, in which the presumed initiation codon for the coding sequences of HBsAg was located closer to the 5′ end of the sense strand (about 400 bp). The purified *Hpa* II fragment was then treated with λ exonuclease (2 units) in 100-μl reaction mixture (New England BioLab) for 30 min at 37°C. The λ exonuclease will degrade DNA from the 5′ end of the sense strand from HBsAg-coding sequences and expose the antisense strand for pairing with added primer. The λ exonuclease-treated DNA was then deproteinized and resuspended in 50 μl of *E. coli* DNA polymerase reaction mixture containing 40 m*M* potassium phosphate buffer pH 7.4, 1 m*M* DTT, 6m*M* $MgCl_2$, 0.5 m*M* each of dNTPs, and 0.2 nmole of dATGGAGAACATC (^{32}P-labeled at 5′ end by T4 polynucleotide kinase). The mixture was first heated at 90°C for 1 min, annealed at 0°C for 30 min, and then incubated at 37°C for 3 hr in the presence of 2 units of *E. coli* DNA polymerase Klenow fragment. The DNA polymerase will synthesize DNA primed by the added primer and degrade any unpaired template to create a blunt-ended DNA molecule. The resultant DNA was then deproteinized, digested with *Xba* I (45 units) in a 100-μl reaction mixture, and fractionated by electrophoresis in 5% polyacrylamide gel. The 91-bp DNA (fragment A) was isolated after autoradiographic detection. To prepare the third fragment, the vector DNA, pNCV DNA was first cut with 24 units of *Pst* I enzyme in a 100-μl reaction mixture and then treated with 2 units *E. coli* DNA polymerase large fragment in a 50-μl reaction mixture (see above for details) at 8°C for 1 hr. The DNA polymerase treatment removed the 4 unpaired nucleotides created by *Pst* I digestion and left a blunt end after an intact *Eco* RI restriction site. The DNA was subsequently digested with *Bam* HI to obtain the large fragment which contains part of Tet^R gene and the pBR322 replication origin. Final ligation mixture (30 μl) containing 300 ng of treated pNCV DNA, 200 ng of *Xba* I–*Bam* HI fragment derived from pHS42, and approximately 10 ng of the fragment A was incubated at room temperature for 16 hr and used to transform *E. coli* 294. Plasmid DNA from the Tet^R transformants was screened with restriction enzyme digestion, and the DNA sequence of one



B.

SV40 (-VP-1) ···TCTAAAAGCTTATGAAGATG···
SV40 (-HB_sAg) ···TCTAAAAGCTGAATTCATG···

FIG. 3. Construction of recombinant SV40 DNA capable of synthesizing HBsAg. A. The construction of pSVHBSA DNA. Contained in this construction are three DNA fragments: (i) SV40 DNA from the *Bam* HI + *Eco* RI-digested pSVR DNA; (ii) pBR322 DNA from *Bam* HI-digested pHBV-T-IA DNA (see Fig. 2 for detailed structure); and (iii) HBsAg coding sequence containing DNA from *Bam* HI + *Eco* RI-digested pHS94. The three fragments were ligated by T4 DNA ligase through their respective *Bam* HI and *Eco* RI sites in a 15-μl ligation mixture. One class of transformants should contain a plasmid which has reconstituted an intact Tet^R gene of pBR322 and, therefore, can be selected by Tet^R and separated from the large number of Tet^S transformants resulting from self-ligation of vector DNA. Plasmid from such a transformant, pSVHBSA, was obtained and its DNA construction was verified by restriction enzyme digestion. The early and late transcriptional starts of SV40 are indicated by E and L respectively. The poly(A) addition site of SV40 late mRNA is also indicated. B. DNA sequence surrounding the ATG initiation codon of VP-1 protein (boxed ATG in top line) (Acheson, 1980) is compared with that of HBsAg created in pSVHBSA DNA (boxed ATG in bottom line). The *Hind* III site which was changed to an *Eco* RI site is underlined. The ATG 6 nucleotide 5′ to the boxed ATG is also a possible initiation codon of VP-1 protein.

virus (tsA28) at 41°C. The tsA28 virus encodes a temperature-sensitive SV40 T antigen (Tegtmeyer and Ozer, 1971) and is therefore replication defective at 41°C. *Bam* HI-

VP-1 protein. As expected, a cytopathic effect was observed in CV-1 cells which received both tsA28 and the pSVHBSA DNA, with complete lysis observed after 2

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