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Expression of hepatitis B virus *S* gene by herpes simplex virus type 1 vectors carrying *α*- and *β*-regulated gene chimeras

(eukaryotic expression vector/foreign genes)

MENG-FU SHIH*, MINAS ARSENAKIS*, PIERRE TIOLLAIS†, AND BERNARD ROIZMAN*

*Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 East 58th Street, Chicago, IL 60637; and †Unité de Recombinaison et Expression Genetique, Institut National de la Santé et de la Recherche Médicale U-163, Institut Pasteur, Paris, France

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ABSTRACT **The domain of the hepatitis B virus (HBV) *S* gene specifying the HBV surface antigen (HBsAg) and comprising 25 base pairs of the 5′-transcribed noncoding region, the structural gene sequences, and the 3′-noncoding gene sequences including the polyadenylylation site was fused to the promoter–regulatory regions of the *β*-thymidine kinase and of the *α4* gene of herpes simplex virus type 1 (HSV-1). The chimeric constructs were then inserted into the HSV-1 genome and specifically into the thymidine kinase gene by homologous recombination through flanking sequences. Cells infected with recombinants carrying the chimeric genes produced and excreted the HBsAg into the extracellular medium for at least 12 hr concurrently with the multiplication of the HSV-1 vector. The temporal patterns of expression and the observation that HBV *S* gene linked to the HSV-1 *α* promoter–regulatory region was regulated as an HSV-1 *α* gene indicate that the HBsAg gene chimeras inserted into the virus were regulated as viral genes. The HBsAg banded in isopycnic CsCl density gradients at a density of 1.17 g/cm^3. Electron microscopic studies revealed that HBsAg harvested from the extracellular medium and banded in CsCl density gradients contained spherical particles 15–22 nm in diameter, characteristic of empty HBV envelopes. The results indicate that HSV-1 is a suitable vector for the expression of foreign genes placed under the control of HSV promoter–regulatory regions.**

In this paper we report that the herpes simplex virus type 1 (HSV-1) genome is a suitable vector for expression of foreign genes as exemplified by the gene *S* specifying the hepatitis B virus (HBV) surface antigen (HBsAg) (1). To regulate its expression, we placed the *S* gene of HBV under the control promoter–regulatory regions of HSV-1 genes. Relevant to this report are the following:

(*i*) Infection of susceptible cells with HSV-1 results in shutoff of host protein synthesis (2–4). The shutoff occurs in two stages. The initial stage is very likely caused by a structural protein of the virus (5), and genetic studies indicate that this activity is not essential for virus growth (6). A second, irreversible inhibition occurs during the viral reproductive cycle as a consequence of expression of viral gene products (7, 8). Available data based on chemical enucleation with actinomycin D or physical enucleation with the aid of cytochalasin B suggest that the inhibition is at least in part at the translational level (6, 7, 9). Although HSV-1 was shown previously to induce the expression of some host genes (10) and particularly foreign genes (e.g., ovalbumin) placed under control of viral regulatory regions and introduced into cells by transfection (11, 12), the induction is selective and transient. A central question is whether the inhibitory machinery of wild-type virus permits sustained expression of a foreign gene introduced into the HSV genome.

(*ii*) HSV genes form three major groups designated as *α*, *β*, and *γ*, whose expression is coordinately regulated and sequentially ordered in a cascade fashion (7). Previous studies have shown that for most *α* (13–16) and some *β* genes (17, 18) the promoter and regulatory domains are located upstream from the site of initiation of transcription. Specifically, chimeric genes constructed by fusion of promoter–regulatory domains of *α* gene [e.g., the gene specifying infected cell protein (ICP) no. 0, 4, 27, or 22] to the 5′-transcribed noncoding and coding sequences of other genes are regulated as *α* or *β* genes, respectively (11, 13–16, 19). In these studies we placed the HBV *S* gene under the control of the *α* promoter of *ICP4* and the *β* promoter of the viral thymidine kinase (*TK*) genes.

(*iii*) The procedure used for the insertion of HBV *S* gene followed that described by Mocarski *et al.* (20) and Post *et al.* (13). Specifically, both the *β-TK-* and the *α-ICP4-*regulated HBsAg were inserted into the *Bgl* II site of the *TK* gene interrupting the 5′-transcribed noncoding region of that gene. The chimeric fragments were then cotransfected with intact HSV DNA, and TK$^-$ recombinants carrying the HBsAg gene produced by homologous recombination through flanking sequences were then selected by plating on TK$^-$ cells in the presence of bromodeoxyuridine, which inactivated the TK$^+$ progeny. Because the DNA fragment carrying the HBV *S* gene appeared to contain at its terminus 3′ to the gene a promoter that substituted for the TK promoter and maintained the TK$^+$ phenotype, it was necessary to inactivate the *TK* gene in the chimeric construct by a small deletion at the *Sac* I site of that gene.

MATERIALS AND METHODS

Virus and Cells. The F strain of HSV-1 [HSV-1(F)] (21) and all recombinants derived in this study were grown and titered on Vero or HEp-2 cell lines obtained from the American Type Culture Collection. Rabbit skin cells obtained from J. McLaren were used for transfection with viral DNA. The human 143 tk$^-$ cell line (22) was used for selection of TK$^-$ recombinants.

The relevant properties of pCP10 carrying the HBV *S* gene were reported elsewhere (23). The cloning and properties of plasmid pRB103 consisting of pBR322 containing the *Bam*HI Q fragment of HSV-1(F) were described (24).

Enzymes and Radioisotopes. Restriction enzymes, T4 DNA ligase, polynucleotide kinase, T4 DNA polymerase, exonuclease BAL-31, and restriction enzyme linkers were purchased from New England Biolabs and used as directed by the manufacturer. DNA probes for screening *Escherichia coli* colonies for desired recombinant plasmids as described (24) were labeled by nick-translation with [*α*-^{32}P]dCTP ob-

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen(s); HSV-1, herpes simplex virus 1; TK, thymidine kinase; ICP, infected cell protein; bp, base pair(s).

tained from New England Nuclear.

DNA Preparation. Details of the procedures or references for cloning and preparation of plasmid and viral DNAs were listed elsewhere (24).

Transfection. Approximately 0.1–0.5 μg of recombinant plasmid DNA was cotransfected with 0.5 μg of HSV-1(F) DNA into rabbit skin cells as described by Ruyechan *et al.* (25). Plaque purifications of recombinant viruses were done as described (25).

Assay for HBsAg. Extracellular medium and lysates from recombinant or parent virus [HSV-1(F)]-infected cells were mixed with beads coated with guinea pig antibody to HBsAg (Abbott). After incubation, the beads were allowed to react with peroxidase-conjugated antibody to HBsAg. The presence of HBsAg was measured spectrophotometrically at 492 nm.

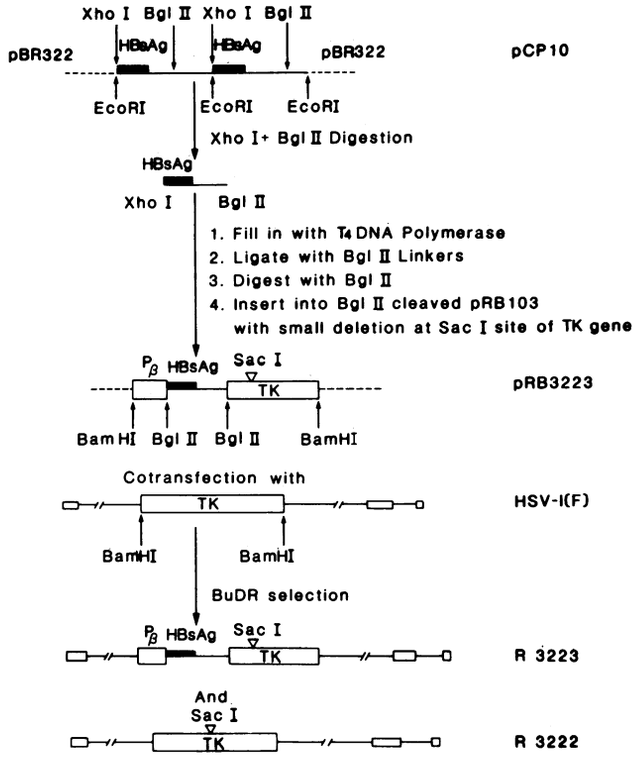

FIG. 1. Construction of an HSV-1 recombinant containing a chimeric *β-TK* promoter-regulated HBV *S* gene. pCP10 DNA was cleaved with *Xho* I and *Bgl* II and the digest was subjected to electrophoresis in a 5% polyacrylamide gel. The *Xho* I–*Bgl* II fragment containing the coding sequence for HBsAg was then purified from the polyacrylamide gel, and the termini of DNA fragments were filled with T4 DNA polymerase, ligated to *Bgl* II linkers, cleaved with *Bgl* II to produce cohesive ends, and cloned into the *Bgl* II site of pBR3222. The plasmid containing the HBV *S* gene in the correct transcriptional orientation relative to the TK promoter–regulatory region as determined from the *Bam*HI DNA restriction pattern was designated as pRB3223. The pRB3222 was made from plasmid pRB103 carrying the *Bam*HI Q fragment of HSV-1(F) (24) by *BAL*-31 digestion to remove ≈200 bp at the unique *Sac* I site in order to inactivate the *TK* gene. pRB3223 was linearized with *Pvu* II prior to cotransfection with HSV-1(F) DNA. The TK⁻ progeny was selected by plating on 143tk⁻ cells overlaid with medium containing mixture 199 lacking thymine but supplemented with 3% calf serum and bromodeoxyuridine (BuDR, 40 μg/ml). As predicted, the TK⁻ progeny contained recombinants that recombined only the deletion in the *Sac* I site (R3222) and those that recombined both the deletion and the HBsAg gene insert (R3223). R3223 was differentiated from R3222 by digestion of recombinant virus DNAs with *Eco*RI restriction endonuclease.

RESULTS

Construction of HSV-1 Vectors Carrying α- and β-Regulated HBV *S* Genes. The construction of the plasmids pRB3223 and pRB3225 carrying the α- and β-regulated HBV *S* gene, respectively, and the selection of HSV-1 recombinants R3223 and R3225 carrying these chimeric genes are shown in

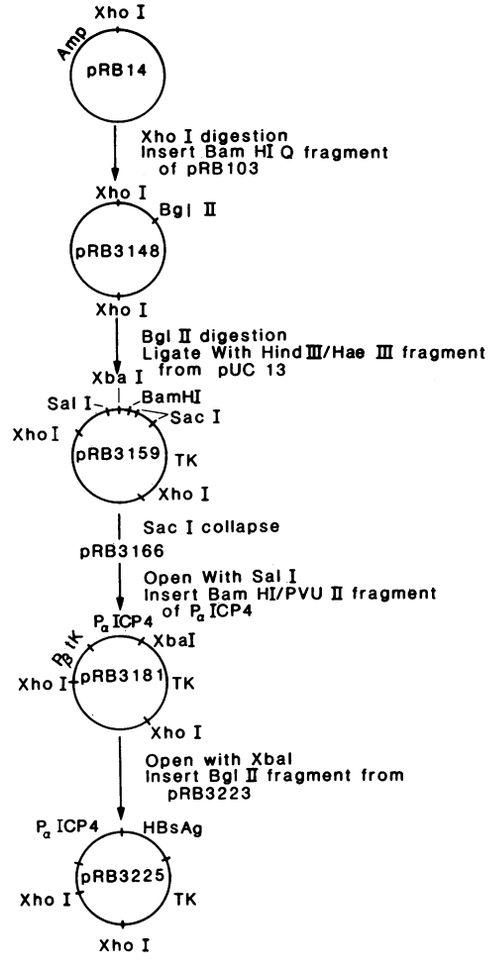

FIG. 2. Construction of an HSV-1 recombinant containing a chimeric *αICP4*–HBV *S* gene. The *Bam*HI Q fragment containing the HSV-1(F) *β-TK* gene from pRB103 was inserted into the *Xho* I site of plasmid pRB14. This plasmid was constructed from pRB322 by replacement of the *Eco*RI–*Pvu* II fragment with *Xho* I linker (New England Biolabs). Plasmid pRB3159 was then constructed by cloning the *Hin*dIII–*Hae* III fragment containing the polylinker from pUC13 into the unique *Bgl* II site of the HSV-1(F) *Bam*HI Q fragment such that the *Bam*HI site was closest to the structural sequences of the *TK* gene, whereas the *Sal* I site was closest to the transcription initiation site of that gene. The properties of this plasmid (unpublished data) will be reported elsewhere. Plasmid pRB3166 was constructed from pRB3156 by digesting with *Sac* I and religating to delete the *Sac* I fragment containing the *Bgl* II–*Sac* I fragment of *Bam*HI Q fragment. In the last step, two fragments were cloned into pRB3166 to yield pRB3225. First, the *Bam*HI–*Pvu* II fragment from pRB403 (11) containing the promoter–regulatory domain of the *α-ICP4* gene was cloned into the *Sal* I site of the polylinker sequence such that the transcription initiation site of *α-ICP4* in the *Bam*HI–*Pvu* II fragment was close to the *Xba* I site. Lastly, the *Bgl* II fragment containing the HBV *S* gene from pRB3223 was cloned into the *Xba* I site such that the *α-ICP4* promoter and the structural sequences of the HBsAg gene were in the same transcriptional orientation as determined from *Eco*RI DNA restriction endonuclease patterns. R3225 was selected from the TK progeny of a transfection of 143tk⁻ cells with pRB3225 DNA as described in the legend to Fig. 1.

Figs. 1 and 2 and detailed in the respective legends. The HBV *S* gene was obtained as a 1.7-kilobase-pair (kbp) *Xho* I–*Bgl* II fragment from plasmid pCP10. The initiation codon of HBsAg is located 25 bp downstream from the *Xho* I site. In our constructs, the initiation codon of the HBV *S* gene in the β-regulated gene was therefore ≈80 bp downstream from the transcription-initiation site derived from the *β-TK* gene, whereas the initiation codon for HBV *S* gene in the α-regulated gene was 60 bp from the transcription initiation derived from the *α-ICP4* gene. Recombinants R3223 and R3225 could not be differentiated from other HSV-1 recombinants carrying insertions or deletions in the *TK* gene (13, 20).

Synthesis of HBsAg in Cells Infected with Recombinant Viruses Carrying the HBsAg Gene. Three series of experiments indicated that cells infected with recombinants R3223 and R3225 carrying α- and β-regulated HBsAg genes, respectively, produce HBsAg.

The results of the first series of experiments (Table 1) show that Vero cells infected with R3223 and R3225 produced and excreted the HBsAg into the extracellular medium. The amounts of HBsAg accumulating in the infected cells reached peak levels ≈15-fold higher than the background levels measured in medium and lysates of cells infected with HSV-1(F) at or before 8 hr after infection and did not significantly increase thereafter. The amounts of HBsAg detected in the extracellular medium increased with time, indicating that HBsAg was excreted and accumulated outside the infected cell. The patterns of accumulation of α- and β-regulated HBsAg were similar and in accord with the observation that the kinetics of accumulation of α- and β-regulated *TK* genes expressed by viruses carrying these genes were also similar (13).

The second series of experiments (Table 2) was designed to determine whether the α-chimeric HBV *S* gene was regulated as an α gene. A characteristic of HSV-1 α genes is that they are the only viral genes transcribed in cells exposed during and after infection to inhibitors of protein synthesis (6). As shown in Table 2, only the HBV *S* gene contained in R3225 was expressed in cells infected and maintained in the presence of cycloheximide and then released from the inhibitory effects of cycloheximide in the presence of actinomycin D to preclude the transcription of β genes dependent on the synthesis of the *α-ICP4* gene product.

The third series of experiments concerned the characteristics of the HBsAg produced in cells infected with R3223. As shown in Fig. 3, HBsAg accumulated in the extracellular medium banded in isopycnic CsCl density gradients at a density of 1.17 g/cm^3. Electron microscopic examination (Fig. 4) of the banded material revealed the presence of particles 15–22 nm in diameter, characteristic of empty viral envelopes of HBV (26).

Table 1. Expression of α- and β-regulated HBV *S* gene in cells infected with recombinant and parent viruses

Time after infection, hr	HSV-1(F)		R3223		R3225	
	Medium	Cell lysate	Medium	Cell lysate	Medium	Cell lysate
4	0.08	0.07	0.42	0.57	0.58	0.56
8	0.08	0.07	3.69	0.95	3.31	1.06
12	0.09	0.07	6.33	1.26	5.06	1.20

Replicate Vero cell cultures in 25-cm^2 flasks were exposed to R3223, R3225, and parent [HSV-1(F)] viruses, respectively, at 2 plaque-forming units per cell for 1 hr and then incubated at 37°C in maintenance medium consisting of mixture 199 supplemented with 1% calf serum. At the times indicated, the maintenance medium from one set of flask (5 ml) was removed, and the cells were washed three times with 5 ml each of phosphate-buffered saline (0.15 M NaCl/8.2 mM Na_2HPO_4/1.5 mM KH_2PO_4/2.5 mM KCl), harvested in 1 ml of phosphate-buffered saline, freeze–thawed three times, and centrifuged in an Eppendorf microcentrifuge. The supernatant fluid containing the cell lysate was then brought to 5 ml with phosphate-buffered saline. Portions containing 200 μl of medium and infected cells were assayed for the presence of HBsAg with the Abbott AUSZYME II diagnostic kit according to the procedure recommended by the manufacturer. Values are given in optical density units.

Table 2. Regulation of HBsAg synthesis in cells infected with recombinant viruses

Cycloheximide	Material tested	HSV-1(F)	R3222	R3223	R3225
Absent	Medium	<0.06	<0.06	5.22	2.34
Absent	Cell lysate	<0.06	<0.06	3.09	1.04
Present	Medium	<0.06	<0.06	<0.06	0.77
Present	Cell lysate	<0.06	<0.06	<0.06	0.80

Replicate HEp-2 cell cultures in 25-cm^2 flasks were preincubated for 1 hr in maintenance medium containing 50 μg of cycloheximide per ml (Sigma) and then infected with R3222, R3223, R3225, and HSV-1(F) at 20 plaque-forming units per cell, respectively. At 5 hr after infection, the medium containing cycloheximide was removed, and the cells were washed extensively and then incubated in medium containing 10 μg of actinomycin D (Sigma) per ml. The cells and medium were harvested after 90 min of additional incubation as described in the legend to Table 1. Values are given in optical density units.

DISCUSSION

In this report we demonstrate that the HSV-1 genome can act as an expression vector for foreign genes, as exemplified by the *S* gene of HBV. The salient features and significance of the results presented in this report are as follows:

(*i*) Although HSV-1 shuts off host macromolecular metabolism and especially host protein synthesis, it appears not to affect the expression of foreign genes, as exemplified by HBV *S* genes inserted into the viral genome and regulated by

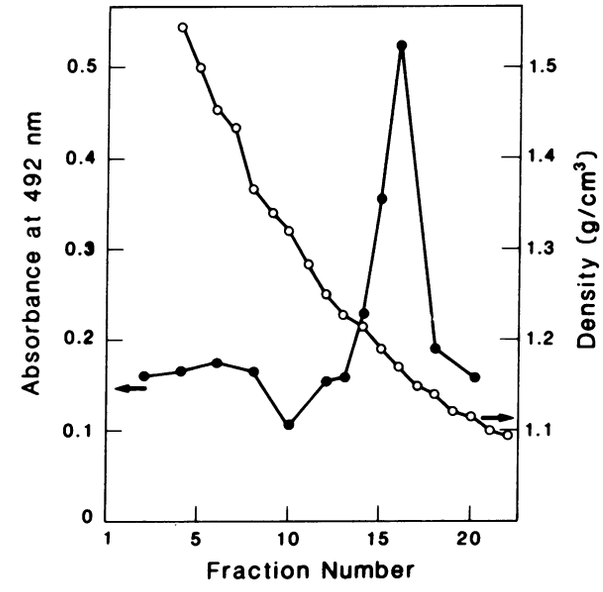

FIG. 3. Buoyant density of purified HBsAg in CsCl gradient. Vero cells were infected with R3223 at 2 plaque-forming units per cell. At 12 hr after infection, 9 ml of maintenance medium was harvested and centrifuged at 36,000 rpm for 20 hr at 4°C in a Beckman SW41 rotor. The pellet was suspended in 0.5 ml of the same medium and then 200 μl was layered on top of a CsCl (1.1–1.5 g/ml) density gradient and centrifuged at 36,000 rpm for 36 hr at 25°C in a Beckman SW41 rotor. Fractions (0.5 ml) were collected from the bottom of the centrifuge tube and diluted 1:10 with phosphate-buffered saline. The fractions were assayed for the presence of HBsAg as described in the legend to Table 1.

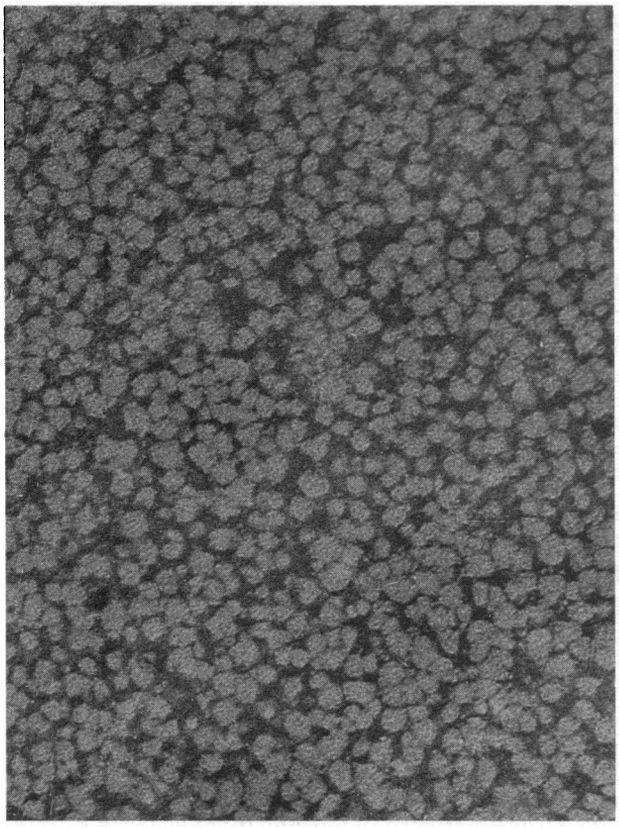

FIG. 4. Electron micrograph of purified HBsAg. The HBsAg collected from peak CsCl density gradient fractions were negatively stained with 2% phosphotungstic acid. The particle size ranges from 18 to 25 nm.

HSV promoter–regulatory regions. The antigenicity of the gene product, its buoyant density in CsCl density gradients, and the characteristic 15- to 22-nm particles present in the banded preparations suggest that the product of the gene carried by HSV-1 is an authentic product of HBV *S* gene.

(*ii*) The results presented in this report indicate that both the *α-ICP4-* and the *β-TK*-linked HBsAg genes expressed the antigen for at least 12 hr. The patterns of synthesis of the HBsAg and the observation that *α-ICP4*-linked gene was regulated as an *α* gene indicate that the chimeric HBV *S* genes in the HSV-1 vector were regulated as viral genes. It should be noted, however, that R3223 and R3225 recombinants multiply in cultured cells and that the production and accumulation of HBsAg is a by-product of lytic infections. The production of HBsAg could be heightened significantly by insertion of the HBV *S* gene regulated by an *α* promoter–regulatory region into the genome of temperature-sensitive mutants in the *ICP4* gene, inasmuch as such mutants have been shown to express *α* genes continuously in cells infected and maintained at the nonpermissive temperature (27–29).

(*iii*) This report demonstrates that the HSV genome can be used as an expression vector for foreign genes and therefore is similar in this respect to vaccinia virus genome (30, 31). HSV has a very broad cell type and species host range; it can be produced in large amounts, stored without significant loss in potency, and used to infect synchronously large-scale cultures of cells from a variety of sources. HSV-1 expression vectors would be particularly useful for the biosynthesis in human cells and characterization of products of (*a*) genes of viruses whose growth is restricted in cell culture (e.g., HBV), (*b*) genes of infectious agents that are particularly hazardous for humans, and (*c*) cellular genes expressed at very low levels or not at all in cultured cells. HSV-1 expression vectors would also be useful for the analyses of gene regulation, especially at the translational level.

(*iv*) In this study the HBV-1 *S* gene was inserted into wild-type genomes modified at the site of insertion. Although as much as 7 kbp of DNA has been inserted (27), the capacity of wild-type HSV-1 DNA to carry additional gene products might be limited. This laboratory has reported previously the construction of mutant viral genome HSV-1(F)I358 from which ≈14 kbp of DNA contained within the internal reiterated sequences had been replaced with a 2-kbp insert (32). By replacing the insert and expanding the genome to its known maximal capacity, the I358 mutant could carry as much as 23 kbp of foreign DNA. Therefore, HSV-1(F)I358 has the capacity to serve as a vector of several genes specifying antigens from a variety of human infectious agents for immunoprophylaxis. Expression of foreign genes by the I358 vector will be reported elsewhere.

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