

Expression of Cell-Associated and Secreted Forms of Herpes Simplex Virus Type 1 Glycoprotein gB in Mammalian Cells

CAROL PACHL,* RAE LYN BURKE, LAURA L. STUVE, LISA SANCHEZ-PESCADOR, GARY VAN NEST, FRANK MASIARZ, AND DINO DINA

Chiron Corporation, Emeryville, California 94608

Received 26 June 1986/Accepted 23 October 1986

The gene for glycoprotein gB1 of herpes simplex virus type 1 strain Patton was expressed in stable Chinese hamster ovary cell lines. Expression vectors containing the dihydrofolate reductase (*dhfr*) cDNA plus the complete gB1 gene or a truncated gene lacking the 194 carboxyl-terminal amino acids of gB1 were transfected into CHO DHFR-deficient cells. Radioimmunoprecipitation demonstrated that the complete gB1 protein expressed in CHO cell lines was cell associated, whereas the truncated protein was secreted from the cells due to deletion of the transmembrane and C-terminal domains of gB1. Cells expressing the truncated gB1 protein were subjected to stepwise methotrexate selection, and a cell line was isolated in which the gB1 gene copy number had been amplified 10-fold and the level of expression of gB1 had increased over 60-fold. The truncated gB1 protein was purified from medium conditioned by the amplified cell line. N-terminal amino acid sequence analysis of this purified protein identified the signal peptide cleavage site and predicted the cleavage of a 30-amino-acid signal sequence from the primary protein. The immunogenicity of the truncated gB1 protein was also tested in mice, and high levels of antibody and protection from virus challenge were observed.

The herpes simplex virus type 1 (HSV-1) genome encodes four major glycoproteins, gB1, gC1, gD1, and gE1, which are found in the viral envelope as well as on the surface of infected cells (reviewed in reference 53). Glycoprotein gB1 is one of the more abundant viral glycoproteins and is essential for entry of the virus into cells. Studies with temperature-sensitive (*ts*) mutants of gB1 indicate that this glycoprotein is involved in a membrane fusion activity which is required for penetration (34, 49). In addition, a mutation which alters the rate of virus entry into the cell has also been mapped in gB1 (5). Further evidence that gB1 promotes membrane fusion comes from the mapping of syncytial (*syn*) mutations to the gB1 locus (5, 9, 35). Glycoprotein gB1, as well as the other viral glycoproteins, also plays an important role in stimulating the host immune response (8, 11, 38). Epitopes which elicit the production of neutralizing antibodies have been shown to reside in each of the four glycoproteins (reviewed in reference 53). There is also evidence that these glycoproteins stimulate cell-mediated immunity to HSV in the mouse (6, 31, 50) and in humans (62).

We have chosen to produce sufficient amounts of gB1 to aid in elucidating the immune response of the HSV-infected host and to test as a subunit vaccine. In this report we describe the expression of cell-associated and secreted forms of gB1 from stable mammalian cell lines. We also present the purification and N-terminal amino acid sequence of the secreted form of gB1 and show that this purified gB1 is immunogenic in mice. In the accompanying manuscript (54), we present the sequence and expression of the analogous HSV-2 glycoprotein, gB2.

MATERIALS AND METHODS

Cells, virus, and reagents. A dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary (CHO) cell line (57) obtained from Y. W. Kan, University of California, San Francisco, was grown in Ham F-12 medium (Gibco Labora-

tories) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 150 µg of L-proline per ml. The *EcoRI* F fragment of HSV-1 (strain Patton) cloned into plasmid pBR322 (29) was obtained from Richard Hyman, Hershey Medical Center, Hershey, Pa.

For preparation of HSV-1 strain Patton virus stocks, a monolayer of Vero cells grown to 95% confluency in an 830-cm² roller bottle was rinsed with serum-free medium prior to the addition of virus at a multiplicity of infection of 0.1 in 5 ml of serum-free medium. The virus was absorbed for 1 h at 37°C, and then 100 ml of medium containing 12% fetal calf serum was added. Infected cultures were incubated at 37°C for 24 h, and then the medium was replaced with 40 ml of fresh medium for an additional 22 h. The cells were gently shaken into the medium, and the suspension was clarified by centrifugation for 15 min at 4°C at 1,590 × *g*. The supernatant was stored on ice, and the cell pellet was suspended in 5 ml of the supernatant and alternately frozen and thawed three times. Cell disruption was completed by sonication for 2 min, and the suspension was clarified as before. The supernatants were pooled, divided into portions, and stored at -70°C. Normally the virus stock had a titer of 10⁸ to 10⁹ PFU/ml.

Restriction enzymes, T4 DNA ligase, *Escherichia coli* DNA polymerase I Klenow fragment, and other biological reagents were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and were used as specified by the manufacturer. Standard DNA manipulations such as agarose gel electrophoresis, restriction analysis, fragment isolation, ligation, and bacterial transformation followed procedures outlined by Maniatis et al. (37).

Plasmid constructions. Plasmid pHS112 (Fig. 1C), which encodes the complete gB1 gene, was constructed in two steps. First, the 3.3-kilobase (kb) *KpnI-XhoI* gB1 fragment was inserted into plasmid pSV1dhfr (Fig. 1B) in place of the *dhfr* gene by ligation of the gB1 fragment to the *HindIII-BglII* fragment of the vector after all four restriction sites had been repaired to blunt ends with the Klenow fragment of DNA polymerase I. The resulting plasmid, pHS111, was

* Corresponding author.

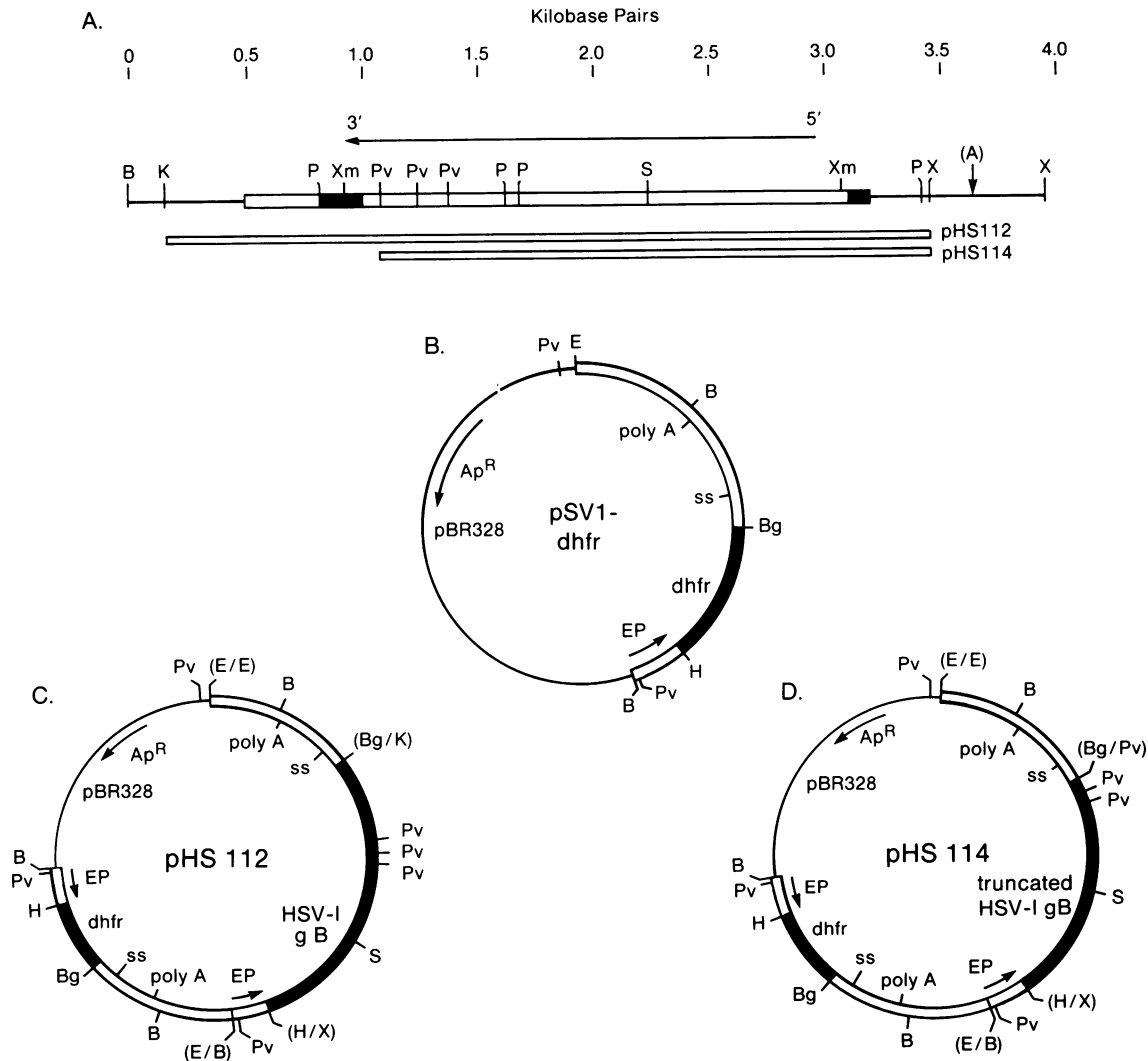


FIG. 1. Restriction map of the HSV-1 gB1 coding region and structure of gB1 expression plasmids. (A) Partial restriction map of the gB1 gene of HSV-1 strain Patton in the prototype orientation (46). The open reading frame for gB1 spans map coordinates 0.348 to 0.367 as indicated by the bar on the restriction map. The direction of transcription is from right to left. The DNA sequence has been determined (54) from the *Bam*HI site (0.345) on the left to a nonunique *Alu*I site (0.370), which is indicated by an arrow. The hydrophobic signal and transmembrane regions within the protein are indicated by solid bars. The open bars below the restriction map indicate the gB1 sequences which were inserted into expression vectors pHS112 and pHS114. (B) Structure of plasmid pSV1dhfr. Plasmid pSV1dhfr (5.99 kb) consists of the ampicillin resistance gene (*Ap*^R) and origin of replication of pBR328 (52), as a 3.32-kb *Eco*RI-*Bam*HI fragment, followed by the SV40 early promoter and origin of replication (EP; map units 0.71 to 0.65, 0.325 kb) (32) fused to the murine *dhfr* cDNA as a 0.72-kb *Hind*III-*Bgl*II fragment (39). *dhfr* is followed by 0.630 kb of SV40 sequences between map units 0.56 and 0.44, which include the small T splice donor-splice acceptor site (SS), and 0.998 kb of sequences between map units 0.19 and 0.00 (*Eco*RI), which include the SV40 early region polyadenylation signal (polyA) (32, 33). The open bars indicate SV40 sequences, the solid bars refer to *dhfr*, and the narrow line is the pBR328 sequence. (C) Structure of expression plasmid pHS 112. Plasmid pHS112 (11.24 kb) consists of plasmid pSV1dhfr plus a second copy of the SV40 early promoter linked to the entire HSV-1 gB1 (3.30-kb *Kpn*I-*Xho*I fragment, solid bar) and the gB1 gene is followed by the SV40 small T splice site and polyadenylation site. The SV40-gB1 cassette has been inserted into pSV1dhfr at the *Eco*RI site. (D) Structure of expression plasmid pHS114. Plasmid pHS114 (10.30 kb) is identical to pHS112 except that a smaller HSV-1 segment, a 2.36-kb *Pvu*II-*Xho*I fragment which encodes a truncated gB1 lacking 194 carboxyl-terminal amino acids, is inserted. Restriction sites: B, *Bam*HI; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; X, *Xho*I; Xm, *Xma*3; Bg, *Bgl*II; E, *Eco*RI; A, *Alu*I; H, *Hind*III. Restriction sites in parentheses were used in the construction of the plasmids but were not regenerated.

then digested with *Eco*RI plus *Bam*HI (partial) to excise a 5.25-kb fragment which encoded the gB1 gene flanked by the simian virus 40 (SV40) early promoter and the 3' SV40 sequences. This 5.25-kb *Eco*RI-*Bam*HI fragment was then ligated into *Eco*RI-cut plasmid pSV1dhfr after all fragment ends were again made blunt. The resulting plasmid, pHS112 (Fig. 1C), encodes the gB1 gene directed by the SV40 early promoter as well as the *dhfr* gene driven by a second copy of

the SV40 promoter. The construction of plasmid pHS114 was similar to that described for plasmid pHS112 and also involved two steps. First, the 2.36-kb *Pvu*II-*Xho*I fragment of HSV-1 (Fig. 1A), which encodes the 3'-truncated gB1 protein, was inserted into plasmid pSV1dhfr in place of the *dhfr* gene by blunt end ligation as described above. The resulting plasmid, pHS113, was digested with *Eco*RI and *Bam*HI (partial) to excise the 4.4-kb *Bam*HI-*Eco*RI SV40-

gB1 cassette, which was ligated into pSV1dhfr as described for pHS112. The resulting plasmid pHS114 is identical to plasmid pHS112, except that the gB1 protein encoded lacks the transmembrane and cytoplasmic sequences of the complete gB1 encoded in pHS112. Translation of the truncated gB in pHS114 terminates within SV40 sequences downstream from gB1. DNA sequence analysis (data not shown) indicated that termination occurred at the SV40 small T stop codon, resulting in the fusion of the C-terminal 24 amino acids of small T antigen to the carboxyl terminus of gB1.

DNA transfections and MTX selection. CHO cells deficient in DHFR were transfected as described previously (40, 58) except that carrier DNA was omitted. A calcium phosphate precipitate of DNA was prepared by mixing a 0.5-ml portion containing 15 μ g of plasmid DNA in 250 mM CaCl₂ with 0.5 ml of twofold-concentrated HEPES-buffered saline (0.14 M NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 2.8 mM glucose, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.0) added dropwise. After a 30-min incubation at room temperature, the DNA mixture was added to a 10-cm dish of cells at 50% confluency in 10 ml of F-12 medium. After incubation at 37°C for 6 to 8 h, the medium was removed from the cells and replaced with 5 ml of 15% glycerol in HEPES-buffered saline. After incubation for 4 min at room temperature, the glycerol was removed and the cells were washed with medium, and then growth was continued in the same medium (F-12). After 2 days the cells were subcultured into selective medium, Dulbecco modified Eagle medium (DME) with 10% dialyzed fetal calf serum plus the supplements described for F-12 medium. Colonies of DHFR-positive cells appeared after 10 to 14 days and were isolated by aspiration with a Pasteur pipette and transfer to a 24-well dish for propagation.

For methotrexate (MTX) selection (1), 10⁵ cells were seeded on 10-cm plates and grown in DME containing MTX (obtained from Lederle Laboratories). After 1 to 3 weeks colonies were picked and propagated as described above. The MTX solution was prepared as a 100-fold-concentrated stock in water and was stored at -20°C. MTX-containing medium was used within 2 weeks.

Immunofluorescence. To analyze gB1 synthesis in CHO cells, subconfluent cell monolayers grown in slide wells were washed three times with phosphate-buffered saline (PBS; 0.15 M NaCl, 2.7 mM KCl, 15.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄) prior to fixation with 100% methanol at -20°C for 10 min. After three additional washes with PBS followed by one wash with PBS containing 1% goat serum (PBS+GS), the fixed cells were incubated for 30 min at 37°C with the primary antibody, an anti-HSV-1 rabbit polyclonal antibody (obtained from DAKO, Santa Barbara, Calif.) diluted 1:100 in PBS+GS. The cells were then washed three times in PBS+GS, followed by incubation at 37°C for 30 min with the secondary antibody, a fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) (Cappel Laboratories, Malvern, Pa.), diluted 1:10 in PBS+GS. After four washes with PBS+GS, the slides were mounted on cover slips with 50% glycerol in 100 mM Tris hydrochloride, pH 8, as the mounting buffer. The fluorescent cells were observed in a Leitz microscope equipped with epifluorescence optics and a 63 \times phase objective. Live-cell immunofluorescence assays were carried out as described above except that the cells were initially washed once in PBS+GS, followed directly by incubation with the primary antibody at 4°C for 30 min. Before mounting with cover slips, the live cells were fixed with 5% formaldehyde in PBS.

In vivo labeling of cells and immunoprecipitation. To label

cellular proteins with [³⁵S]methionine, a confluent monolayer of cells in 3.5-cm dishes was washed once with PBS and then incubated at 37°C for 1 h with 1.0 ml of DME without methionine supplemented with 10% dialyzed fetal calf serum and 100 μ Ci of [³⁵S]methionine (>1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). For a cold methionine chase, the labeling medium was replaced with 1 ml of DME containing 2.5 mM methionine, and the cells were incubated for an additional 5 h at 37°C. After the appropriate labeling period, the medium was removed and clarified by centrifugation, followed by the addition of 0.1 ml of 10 \times lysis buffer (LB) (1 \times LB is 20 mM Tris hydrochloride, pH 8, 100 mM NaCl, 1 mM trisodium EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1.0% bovine serum albumin [BSA], 0.1% sodium dodecyl sulfate [SDS], 1.0 mM phenylmethylsulfonyl fluoride [PMSF], 10 mM benzamide, 0.2 U of aprotinin per ml). To prepare cell lysate, the labeled cell monolayer was washed in PBS followed by lysis with 0.1 ml of LB. The lysate was then scraped into tubes, vortexed briefly, and held at 4°C for 10 min. The cell debris was then removed by centrifugation at 14,000 \times g for 5 min at 4°C. The clarified lysates and media were stored at -70°C.

Prior to immunoprecipitation, nonspecific precipitates were precleared from a 0.1-ml portion of cell lysate or 0.2 ml of cell medium by incubation with 5 μ l of rabbit serum for 30 min at 4°C, followed by the addition of one-half volume of a 20% solution of protein A-Sepharose (PAS) in LB. After incubation for 30 min at 4°C with gentle rocking, the PAS complex was removed by centrifugation at 14,000 \times g for 1 min, and the supernatant was then incubated at 4°C for 30 min with 5 μ l (for cell lysates) or 10 μ l (for cell medium) of a rabbit anti-HSV-1 antibody. A cell lysate portion was also immunoprecipitated with a gB1-specific monoclonal antibody (5 μ l) designated F3AB, obtained from John Oakes and Robert Lausch, University of South Alabama, Mobile (44). SDS was omitted from the LB when this antibody was used. After incubation with antibodies, PAS suspension was added to the samples and incubated as above. The PAS-antibody-antigen complex was pelleted by centrifugation and washed three times with 1 ml of LB lacking BSA and protease inhibitors and then once with 0.12 M Tris hydrochloride, pH 7. Immunoprecipitated proteins were released from the PAS by boiling for 5 min in SDS sample buffer (30), followed by analysis on 10% SDS-polyacrylamide gels.

Purification of gB1 from CHO medium. The gB1 protein was purified from medium conditioned by the growth of cells (CHO clone pHS114-C6) in roller bottles by sequential steps of lentil lectin chromatography, immunoaffinity chromatography, and concentration by ultrafiltration. For the first step, 2 liters of conditioned medium was supplemented with 1 mM PMSF and 0.5% aprotinin and then loaded onto a 30-ml column of lentil lectin-Sepharose-4B (Sigma Chemical Co., St. Louis, Mo.) at a flow rate of 50 ml/h. The column was washed sequentially with 100 ml of PBS and 100 ml of PBS containing 0.5 M NaCl. The bound fraction was eluted with PBS containing 0.5 M NaCl, 0.5 M α -methylmannoside, 0.1% Triton X-100, and 0.5% aprotinin, and fractions were assayed for gB1 by enzyme-linked immunosorbent assay (ELISA).

The peak column fractions were pooled and applied to a 10-ml immunoaffinity column prepared by linking 70 mg of a rabbit anti-gB1 polyclonal antibody to cyanogen bromide-activated Sepharose 4B. The gB1-specific rabbit antiserum was raised against gB1 protein, which was purified by preparative SDS-polyacrylamide gel electrophoresis from HSV-1-infected Vero cell lysates. Prior to coupling, an

IgG-enriched fraction was prepared from the gB1-specific rabbit antiserum by precipitation with 33% saturated ammonium sulfate. Following application of the lectin column eluate to the immunoaffinity column, the column was washed consecutively with 20 ml of 10 mM Tris hydrochloride, pH 7.5, and 10 ml of LB without SDS and BSA and then with 30 ml of 10 mM Tris hydrochloride, pH 7.5–0.5 M NaCl. The bound fraction was eluted with 3 M ammonium thiocyanate, pH 7.5, and the gB1 protein peak was detected by ELISA and Western analysis (56). The peak fractions were concentrated and equilibrated in storage buffer (100 mM NaCl, 10 mM Tris hydrochloride, pH 7.5, 1 mM EDTA, 7.5% glycerol) by ultrafiltration with a PM10 membrane (Amicon Corp., Danvers, Mass.). To remove protein adsorbed to the membrane surface, the membrane was washed with storage buffer plus 0.1% Triton X-100, and this wash was then combined with the initial concentrated fraction. The total protein concentration at each step of the gB1 purification was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Preparation of HSV-1 glycoprotein mixture. Vero cells were infected with HSV-1 strain Patton and processed as described above for preparation of virus stock. After the clarification step, the infected-cell suspension was pelleted at 4°C for 3 min at $121 \times g$. This pellet was washed once with 5 ml of PBS containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$, and the cells were lysed in 5 ml of LB without BSA and supplemented with 1 mM PMSF, 0.5% aprotinin, and 0.1 μg of pepstatin per ml. The cell lysate was then adjusted to 5 mM $CaCl_2$ and 10 μg of micrococcal nuclease per ml, and the mixture was incubated for 30 min at room temperature with gentle mixing, followed by centrifugation at $12,100 \times g$ at 4°C for 30 min. The supernatant was immediately applied to a 10-ml column of lentil lectin-Sepharose 4B, and the column was washed with 15 ml of buffer A (0.25 M NaCl, 20 mM Tris hydrochloride, pH 8.1, 0.5% Nonidet P-40, 0.1 mM $MnCl_2$, 0.1 mM $CaCl_2$). Bound glycoproteins were eluted with buffer A containing 0.2 M α -D-methylmannoside, and the glycoprotein peak was monitored by SDS-polyacrylamide gel electrophoresis. The appropriate fractions were pooled and stored at -70°C. On average, 2 to 4 mg of total protein was purified from one roller bottle of virus-infected cells. This protein mixture contained approximately 26% gB1 and 4.5% gD as well as high concentrations of gC, lower amounts of gE and gG, and a mixture of unidentified HSV-1 and Vero cell proteins.

ELISAs. The concentration of gB1 protein in CHO cell-conditioned medium was measured by an indirect ELISA, with a preparation of purified recombinant gB1 in which the concentration of gB1 had been determined by amino acid analysis used as the standard. Portions (50 μl) of F3AB, a gB1-specific monoclonal antibody (44), diluted 1:1,000 in PBS were adsorbed to the wells of a 96-well polyvinylchloride plate (Dynatech Laboratories, Inc.) by incubation for 1 h at room temperature. Excess antibody was removed by three washes with PBS containing 5% goat serum, and 50- μl portions of medium samples or the gB1 protein standard diluted in PBS+GS were added to the wells and incubated for 1 h at room temperature. The plates were then washed three times with PBS+GS, followed by a third 1-h incubation with 50 μl of rabbit anti-HSV-1 polyclonal antibody diluted 1:100 in the same buffer. Excess secondary antibody was removed by three washes with PBS+GS. Finally, 50 μl of horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Boehringer Mannheim) diluted 1:500 in PBS+GS was added to each well and incubated for

1 h. The wells were then washed once with PBS+GS and five times with PBS and then developed with 50 μl of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate(6)]; Boehringer Mannheim, Indianapolis, Ind.) at a concentration of 1 mg/ml in 0.1 M citric acid, pH 4.0, plus 0.003% H_2O_2 . The color reaction was stopped after 5 min by the addition of 50 μl of 10% SDS, and the absorbance was read at 414 nm in a microtiter plate reader.

HSV-specific antibodies were measured by a direct ELISA, with the HSV-1 glycoprotein mixture described above as the antigen. All incubations were carried out in 50- μl total volumes for 1 h at room temperature. The antigen was diluted to 10 $\mu g/ml$ in PBS and adsorbed to the wells of a 96-well polyvinylchloride plate. The plate was then washed three times with PBS containing 5% goat serum prior to the addition of test serum dilutions in PBS+GS. The wells were washed three times with PBS+GS, followed by the addition of horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel) diluted 1:200 in the same buffer. The wells were washed five times with PBS and developed with ABTS as described above. The reported titer corresponds to the serum dilution which produced an absorbance at 414 nm equal to 50% of the maximum value.

Amino acid composition and N-terminal sequence analysis. The concentration of the purified gB1 protein used as a standard for the ELISA was measured by amino acid analysis with the Pico-Tag system of Waters Associates (2). The N-terminal sequence was determined by Edman degradation with a gas phase protein sequencer (Applied Biosystems, Foster City, Calif.) (19). Phenylthiohydantoin amino acids were identified by C18 reverse-phase high-pressure liquid chromatography (17).

Isolation of DNA and RNA and determination of copy number. Cellular DNA was isolated as described previously (36) except the pronase was replaced by protease K. For determination of DNA copy number, the DNA was digested to completion with *Bam*HI, subjected to electrophoresis in a 1% agarose gel, and analyzed by Southern blot hybridization as described previously (37). The blot was probed with the 2.38-kb *Pvu*II-*Xho*I fragment of gB1 which had been labeled by nick translation (45). The hybridization was done at 45°C for 20 h in $5 \times$ SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–1 \times Denhardt solution–10% dextran sulfate–0.1% SDS–0.1% sodium PP₃–100 μg of denatured salmon sperm DNA per ml–50 mM $NaPO_4$, pH 6.8, followed by three 30-min washes at 50°C in $2 \times$ SSC–0.1% SDS. Known amounts of *Bam*HI-digested plasmid pHS114 were also analyzed on the same blot as a copy number standard. The amount of gB1-specific DNA per cell was determined by densitometric scanning of autoradiograms and comparison with the gB1 DNA standard. The number of copies of DNA per cell was calculated by assuming that the average CHO cell contains approximately 10 pg of DNA (51).

Total cellular RNA was isolated from confluent monolayers of cells (7×10^6 cells per 10-cm dish) by first rinsing the cells in PBS and then scraping the cells into 1 ml of PBS per 10-cm dish. The cells were pelleted and suspended in 0.5 ml of RNA lysis buffer (150 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA, 1% Nonidet P-40, 1,000 U of RNasin [Promega Biotech, Madison, Wis.] per ml) per 10-cm dish. The cells were vortexed for 1 min in RNA lysis buffer, or until lysis was complete, and the nuclei were then pelleted by centrifugation at $2,000 \times g$ for 5 min at 4°C. The supernatant was then adjusted to 0.5% in SDS, extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and

once with chloroform-isoamyl alcohol, followed by ethanol precipitation. The RNA copy number was determined by a quantitative dot blot, with linearized and nick translated plasmid pHS113 as the probe. A gB1 DNA standard of known concentration was also analyzed on the same dot blot. The gB1 mRNA was sized by Northern analysis of polyadenylated RNA (37), and the number of copies of gB1 mRNA per cell was calculated from the dot blot analysis from the size of the gB1 (3.9 kb) and assuming that the total amount of RNA per cell is twice the DNA content, or 20 pg. The dot blot and Northern hybridizations were done at 42°C under the conditions described above for the Southern blot.

RESULTS

Construction of CHO cell lines expressing membrane-bound and secreted forms of HSV-1 glycoprotein gB1. The HSV-1 gB1 gene was located between 0.345 and 0.400 map units on the HSV-1 genome by the mapping of mutations affecting the kinetics of virus uptake, cell fusion, and reactivity with monoclonal antibodies (9, 23) and by mapping the transcript encoding gB1 (22, 43). The nucleotide sequence analysis of this region for HSV-1 strains KOS (4), F (41), and Patton (54) reveals a protein of 903 amino acids (strains KOS and F) or 904 amino acids (strain Patton) with the structure of a membrane glycoprotein, including a hydrophobic region of 29 amino acids (strains F and KOS) or 30 amino acids (strain Patton) at the N terminus, which was predicted to be a cleavable signal sequence (41). This sequence is followed by a 696-amino-acid hydrophilic domain and then a hydrophobic region of 69 amino acids that has the characteristics of a transmembrane domain, finally followed by a C-terminal domain of 109 amino acids.

To establish permanent CHO cell lines expressing either a membrane-bound or secreted form of gB1, portions of the gB1 gene from HSV-1 strain Patton were inserted into an expression vector suitable for mammalian cells. The gB1 gene was subcloned from the *EcoRI* F fragment (46) of HSV-1 strain Patton as an 8.9-kb *BamHI* fragment (map units 0.345 to 0.403). The complete gB1 coding sequences were excised from this fragment as a 3.3-kb *KpnI-XhoI* fragment which also included 250 base pairs of 5' untranslated mRNA leader sequence and 287 base pairs of 3' untranslated sequence, including two polyadenylation signals located 17 and 258 bp downstream from the translation termination codon (54) (Fig. 1A). A 2.36-kb *PvuII-XhoI* fragment of gB1 was also isolated (Fig. 1A), which would encode a gB1 protein which was truncated at the carboxyl terminus by the deletion of 194 amino acids, resulting in removal of the transmembrane and cytoplasmic domains of gB1.

These gB1 genes were inserted into the mammalian expression vector pSV1dhfr (Fig. 1B), which is a modification of plasmid pSV2dhfr (33, 55). Plasmid pSV1dhfr includes the SV40 early promoter, which directs transcription of the selectable marker *dhfr* (39). The *dhfr* cDNA is followed by SV40 sequences which include the small T splice site and the early-region polyadenylation signal. Two derivatives of plasmid pSV1dhfr were constructed: plasmid pHS112 (Fig. 1C), which encodes the complete gB gene, and plasmid pHS114 (Fig. 1D), which encodes a 3'-truncated gB protein. As suggested by previous studies (12, 47), the truncated gB1 protein should be secreted when expressed in mammalian cells due to removal of the transmembrane region of the protein. The truncated gB1 encoded by plasmid pHS114 also contains an additional 24 amino acids of the SV40 small T

antigen fused to the carboxyl terminus of the truncated gB1 protein.

CHO cells deficient in DHFR (57) were transfected with plasmid pHS112 or pHS114. Several DHFR-positive clones were isolated from each transfection by propagation in selective medium. These clones were analyzed for the synthesis of gB1 by immunofluorescence, and two CHO clones, pHS112-1 and pHS114-A7, were chosen for further analysis. When clone pHS112-1, which should express the complete gB1 protein, was analyzed by immunofluorescence of fixed cells, gB1 was detected in the cytoplasm and appeared to be associated with particulate structures (Fig. 2a). In contrast, when clone pHS114-A7, which was transfected with the truncated gB1, was analyzed in the same manner, diffuse cytoplasmic staining of gB1 was detected and no association with subcellular structures was apparent (Fig. 2b). These results suggest that in clone pHS112-1 gB1 may be membrane associated, which would be expected due to the presence of the transmembrane domain. This hypothesis is supported by the observation that when cells with intact cytoplasmic membranes were analyzed by immunofluorescence, membrane association of gB1 was detected in cells from clone pHS112-1 but not from clone pHS114-A7 (data not shown).

To analyze the gB1 proteins produced by the CHO clones, cells were labeled with [³⁵S]methionine, followed by radioimmunoprecipitation with an anti-HSV-1 rabbit polyclonal antibody. When clone pHS112-1, which expresses the full-length gB1 protein, was pulsed for 1 h, the major gB1 proteins detected in cell lysates were polypeptides of 92 and 105 kilodaltons (kDa), plus a minor band of approximately 115 kDa (Fig. 3A, lane 1). The 92-kDa polypeptide probably represents the primary gB1 translation product after signal peptide cleavage, although the apparent size is smaller than the 97-kDa polypeptide that would be predicted from the amino acid sequence. The larger 105- and 115-kDa polypeptides are probably glycosylated forms of gB1 derived from the 92-kDa precursor. To characterize further these gB1 proteins, the CHO clone was pulsed for 1 h with [³⁵S]methionine, followed by a 5-h chase. Under these conditions a considerable increase in the 115-kDa polypeptide was detected in the cell lysate, and the amount of the 92-kDa polypeptide was considerably reduced (Fig. 3A, lane 2), suggesting that the 115-kDa band may represent the mature, fully glycosylated form of gB1 produced in this cell line. This 115-kDa protein is similar in size to the gB1 polypeptides detected in HSV-1-infected cells (61) and HSV-1 virions (49). No gB1 polypeptides were detected in the cell medium after the chase (data not shown), verifying that this gB1 protein remains cell associated.

When the expression of clone pHS114-A7, encoding truncated gB1, was analyzed after a 1-h pulse, the predominant gB1 band detected intracellularly was an 88-kDa polypeptide (Fig. 3B, lane 1). This protein was also immunoprecipitated by the gB1-specific monoclonal antibody F3AB (44) (data not shown). This 88-kDa polypeptide is larger than the 78-kDa polypeptide expected for the primary translational product of the truncated gene and may represent a partially glycosylated precursor. A reduced amount of the 88-kDa polypeptide was detected intracellularly after the 5-h chase (Fig. 3B, lane 2), as was a new polypeptide of 100 kDa. When conditioned medium was analyzed after the chase, only the 100-kDa polypeptide was detected, indicating that this protein is secreted and may be a more highly glycosylated form of the 88-kDa intracellular precursor (Fig. 3B, lane 3). The efficiency of secretion of the 100-kDa gB1 protein

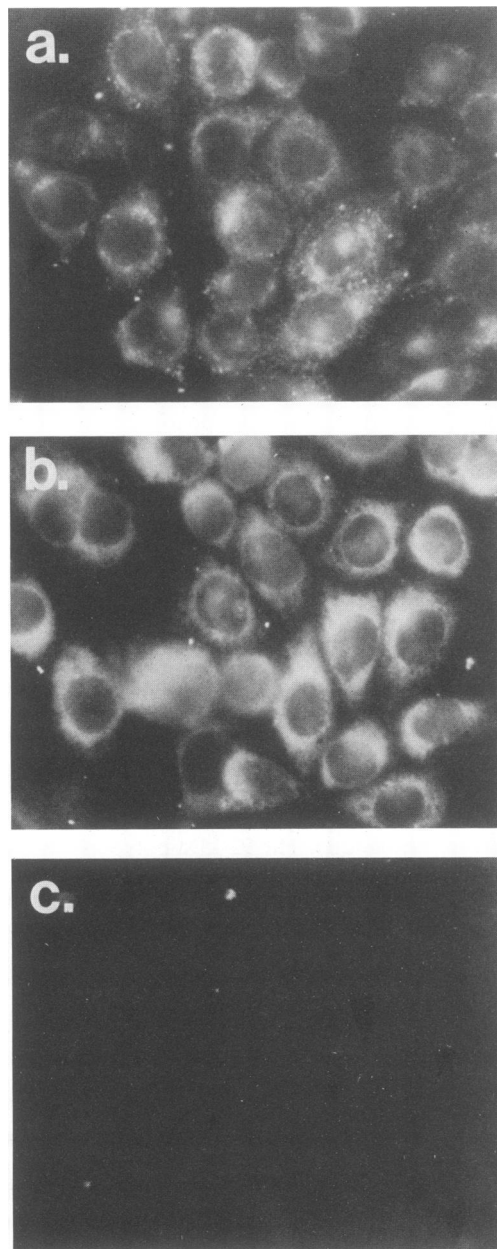


FIG. 2. Visualization by immunofluorescence of gB1 synthesized in CHO cells transfected with plasmid pHS112 or pHS114. CHO cells were transfected with plasmid pHS112, encoding intact gB1 (a), plasmid pHS114, encoding 3'-truncated gB1 (b), or the control plasmid pSV1dhfr (c). Fixed cells were treated with a rabbit anti-HSV-1 antibody, followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Magnification, $\times 1,575$.

was quite low, and from lower exposures of the gel shown in Fig. 3B, it appeared that less than 10% of the gB1 detected intracellularly as an 88-kDa polypeptide after the pulse labeling was actually secreted as the 100-kDa extracellular protein.

To increase the level of expression of the secreted form of gB1, cells derived from clone pHS114-A7 were selected stepwise in increasing concentrations of MTX. This selection procedure has been shown to induce amplification of *dhfr* (1) and adjacent sequences (18, 24). The pHS114-A7 cell

line was initially selected in 0.3 μM MTX, and resistant clones which produced higher levels of gB1, as determined by immunoprecipitation and Western analysis (data not shown), were further amplified in 10 μM MTX. The expression level of the highest-producing clones from each amplification step is shown in Table 1; the level of gB1 production was measured by an ELISA. A 3.5-fold increase in gB1 expression was detected in the first round of amplification in 0.3 μM MTX (clone pHS114-B25), followed by an additional 18-fold increase in the second round of amplification at 10 μM MTX (clone pHS114-C6). The highest-producing clone, pHS114-C6, secreted about 2.2 pg of gB1 per cell in 24 h (Table 1), a 62-fold increase above the original parent clone, pHS114-A7. When gB1 synthesis in the pHS114-C6 clone was analyzed by radioimmunoprecipitation (Fig. 3C), the 88-kDa intracellular and 100-kDa extracellular gB1 proteins described above for the unamplified clone (pHS114-A7, Fig. 3B) were detected, and the efficiency of secretion was again quite low. Densitometric analysis (data not shown) of autoradiographs of gB1 proteins immunoprecipitated from pHS114-C6 cell lysates and media indicated that only 8% of the radioactivity incorporated into the 88-kDa polypeptide after a 1-h pulse (Fig. 3C, lane 1) could be chased into the 100-kDa secreted polypeptide after 5 h (Fig. 3C, lane 2). About 1.4% of the immunoreactive gB1 remained in the cell lysate after the 5-h chase, and about 30% of this material was in lower-molecular-weight bands (Fig. 3C, lane 4), suggesting that intracellular protein degradation was occurring.

To determine the level of amplification of gB1 sequences after MTX selection, the gB1 DNA copy number of clones pHS114-A7 (unamplified), pHS114-B25 (0.3 μM MTX), and pHS114-C6 (10 μM MTX) was determined by Southern blot analysis. The cellular DNAs were digested with *Bam*HI, which should release the gB1 sequences plus flanking SV40 sequences as a 4.4-kb fragment, and these digests were then probed with the 2.38-kb *Pvu*II-*Xho*I fragment of gB1 (Fig. 4A). The number of copies of the gB1 gene was estimated by densitometric comparison with known amounts of *Bam*HI-digested plasmid pHS114 (Fig. 4A, lanes 1 to 5). From this analysis it appears that the pHS114-A7 clone (Fig. 4A, lane 6) had eight copies of gB1 distributed among four fragments as follows: one copy at 16 kb, three copies at 8 kb, and two copies each at 4.4 and 3.9 kb. The clone from the first round of MTX amplification, pHS114-B25 (0.3 μM MTX), lost the 3.9-kb and 8-kb bands and did not amplify the 4.4-kb and 16-kb fragments (Fig. 4A, lane 7). This result indicates that the 3.5-fold increase in gB1 expression observed in these cells compared with the parental pHS114-A7 clone (Table 1) occurred without a concomitant increase in DNA copy number. The 4.4-kb and 16-kb bands were retained in the pHS114-C6 (10 μM MTX) clone; the gB1 sequences in both bands were amplified to about five copies each, and an additional copy of a 3-kb gB1 fragment was also detected (Fig. 4A, lane 8). This increase in DNA copy number of the 4.4-kb and 16-kb gB1 fragments in clone pHS114-C6 accounted for part of the 62-fold increase in gB1 expression observed when this clone was compared with the unamplified pHS114-A7 clone (Table 1). The preferential amplification of specific gB1 sequences observed in these clones is consistent with previous studies of gene amplification (25, 60) which have shown that chromosomal location determines the amplification frequency and stability of a transfected gene.

The gB1 mRNA size and copy number from the amplified clone pHS114-C6 (10 μM MTX) was also determined. By Northern analysis, more than 90% of the gB1 mRNA was

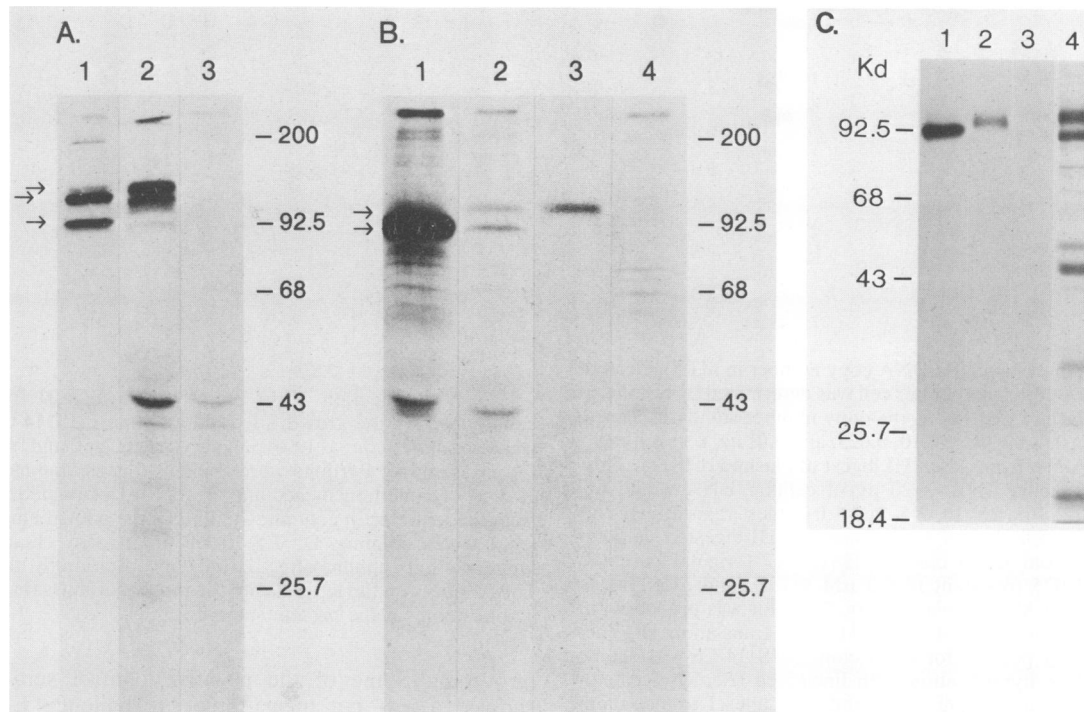


FIG. 3. Radioimmunoprecipitation of gB1 from CHO cells transfected with plasmids encoding intact or truncated forms of HSV-1 gB1. [35 S]methionine-labeled cell lysates and medium were immunoprecipitated with rabbit anti-HSV-1 antibody, separated by SDS-polyacrylamide gel electrophoresis, and detected by autoradiography. (A) Cell lysate from CHO clone pHS112-1 (expressing full-length gB1) pulsed for 1 h with [35 S]methionine (lane 1); lysate from pHS112-1 cells pulsed for 1 h followed by a 5-h chase (lane 2); lysate from control (pSV1dhfr-transfected) CHO cells after a 1-h pulse (lane 3). (B) Lysate from CHO clone pHS114-A7 (expressing truncated gB1) after a 1-h pulse (lane 1); lysate from pHS114-A7 cells pulsed for 1 h followed by a 5-h chase (lane 2); conditioned medium from pHS114-A7 cells after the 5-h chase (lane 3) (this lane is a fourfold longer exposure of the autoradiogram than lanes 1, 2, and 4); lysate from control cells after a 1-h pulse (lane 4). (C) Lysate from CHO clone pHS114-C6 (selected in 10 μ M MTX) after a 1-h chase (lane 1); conditioned medium from pHS114-C6 after a 5-h chase (lane 2); lysate from pHS114-C6 after a 5-h chase (lane 3); lane 4, a 10-fold longer exposure of lane 3. The molecular mass (in kilodaltons) and electrophoretic mobility of the protein standards, myosin (200), phosphorylase B (92.5), BSA (68), ovalbumin (43), and α -chymotrypsinogen (25.7), are shown. Arrows in panels A and B indicate major gB1-specific bands.

detected as a 3.9-kb mRNA (Fig. 4C, lanes 1 and 2), which is the size expected from the gB1 transcription unit in the plasmid pHS114 (Fig. 1D). Minor RNA species of 2.9, 2.3, and 1.9 kb were also detected. The gB1 mRNA copy number per cell was determined by a quantitative dot blot comparing gB1-specific sequences in total cellular RNA with a gB1 DNA standard (Fig. 4B). Assuming that the dot blot signal was due entirely to hybridization with the 3.9-kb mRNA, approximately 4,400 copies of gB1 mRNA per cell were detected.

Purification of recombinant gB1 and N-terminal amino acid analysis. The secreted form of gB1 was purified from the medium of clone pHS114-C6 grown in roller bottles. The conditioned medium (Fig. 5, lane A) was first fractionated by lentil lectin chromatography (lane C), which removed most

of the serum protein contaminants (lane B), followed by immunoaffinity chromatography with a gB1-specific rabbit polyclonal antibody linked to Sepharose 4B. After desalting and concentration by ultrafiltration, the gB1 preparation was about 70% pure as determined by gel analysis (Fig. 5, lane D). The overall recovery of gB1 from a typical preparation was 15% (Table 2), with about a 50% recovery at each step of the purification.

To verify the identity of the secreted gB1 protein and to locate the signal peptide cleavage site, the N-terminal amino acid sequence of the purified protein was determined by the method of Edman degradation (19). The sequence of the first 18 amino acids of the secreted gB1 protein (Fig. 6) was identical to residues 31 to 48 of the predicted amino acid sequence for strain Patton (54). The sequence identified the first amino acid of the mature gB1 protein as Ala-31 and predicted the cleavage of a 30-amino-acid signal sequence from the primary protein. The N-terminal Ala-31 was preceded by an alanine-rich signal peptidase recognition site, Ala-28-Ser-29-Ala-30 (42, 59). This signal peptide cleavage site was also predicted by Pellett et al. (41) by hydrophobic analysis and from the known characteristics of eucaryotic signal peptides.

Immunogenicity of the truncated gB1. It has been shown previously that immunization of mice with purified gB prepared from virus-infected cells can generate a neutralizing antibody response and can protect mice against subsequent

TABLE 1. Expression of gB1 by MTX-amplified CHO clones^a

| Clone | MTX (μ M) | gB1 (pg/cell) | Increase (fold) |
|------------|----------------|---------------|-----------------|
| pHS114-A7 | 0.0 | 0.035 | |
| pHS114-B25 | 0.3 | 0.122 | 3.5 |
| pHS114-C6 | 10.0 | 2.17 | 62.3 |

^a CHO clones were grown to confluence in T-75 flasks in DME plus 10% dialyzed fetal calf serum supplemented with the indicated concentration of MTX. Medium conditioned for 24 h was assayed for gB1 by an ELISA. The values for relative increase in gB1 expression are the averages of two determinations.

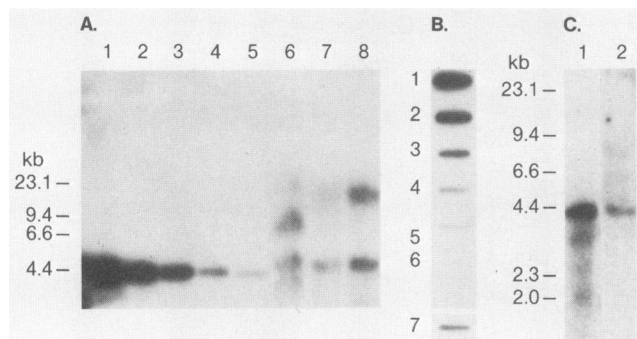


FIG. 4. (A) Analysis of gB1 DNA copy number in MTX-selected clones. The DNA copy number per cell was determined by Southern analysis with plasmid pHS114 as the copy number standard. Lanes 1 to 5 contain 5.00, 1.00, 0.25, 0.10, 0.025, and 0.01 ng, respectively, of gB1-specific DNA from a *Bam*HI digest of plasmid pHS114. This amount of gB-specific DNA in 20 µg of cellular DNA would be equivalent to 515, 103, 26, 10, 2.5, and 1.0 copies, respectively, of the gB gene per cell. Lanes 6 to 8 are *Bam*HI-digested cellular DNAs (20 µg) from CHO clones pHS114-A7 (original parental clone), pHS114-B25 (resistant to 0.3 µM MTX), and pHS114-C6 (resistant to 10 µM MTX), respectively. The blot was probed with the *Pvu*II-*Xho*I fragment of gB1 labeled by nick translation. (B) The RNA copy number per cell for CHO clone pHS114-C6 was determined by dot blot hybridization with linearized (*Eco*RI-digested) plasmid pHS113 as a copy number standard. Lanes 1 to 6 contain 6.21, 1.24, 0.31, 0.06, 0.03, and 0.01 ng, respectively, of gB1 cDNA. These amounts of gB1 cDNA in 3.0 µg of total RNA would be equivalent to 15,525, 3,100, 775, 150, 78, 20, and 2.5 copies, respectively, of gB-specific RNA per cell. Lane 7, 3.0 µg of total RNA from clone pHS114-C6. The blot was probed with plasmid pHS113 labeled by nick translation. (C) Northern analysis of polyadenylated RNA isolated from clone pHS114-C6 with the same probe as in panel B. Either 5 µg (lane 1) or 1 µg (lane 2) of polyadenylated RNA from CHO clone pHS114-6 was used.

HSV challenge (7, 10, 26). To determine whether the truncated gB1 secreted from CHO cells could also induce a protective immune response, mouse immunization experiments were done to compare the purified truncated gB1 with a mixture of glycoproteins purified from HSV-1-infected Vero cells. Similar HSV-1 or HSV-2 glycoprotein preparations have been shown to be highly immunogenic in animal models (20, 21; reviewed in reference 16). Outbred female Swiss Webster mice (20 to 25 g) were immunized on day 1 with 5 µg of truncated gB emulsified with an equal volume of complete Freund adjuvant, with one-half of the dose given intraperitoneally and the other half subcutaneously. As a positive control, a second group of mice received 20 µg of the HSV-1 glycoprotein mixture. As a negative control, a third group of mice received 23 µg of protein prepared from the medium of CHO cells transfected with plasmid pSV1dhfr (lacking gB1) by a purification procedure similar to that described for gB1. The three groups of mice were boosted on days 14 and 28 with the same immunogens formulated in incomplete Freund adjuvant and then bled on day 35 by cardiac puncture. On day 50 the mice were challenged with 3.25×10^8 PFU of HSV-1 strain Patton (approximately twice the 50% lethal dose) administered intraperitoneally, and the mice were monitored an additional 24 days for morbidity. The surviving mice were bled on day 24 postchallenge.

The prechallenge antibody titers of mice immunized with 5 µg of the recombinant gB protein (group 1) were high (Table 3) and these titers were similar to the titers for mice immunized with 20 µg of the HSV-1 glycoprotein mixture

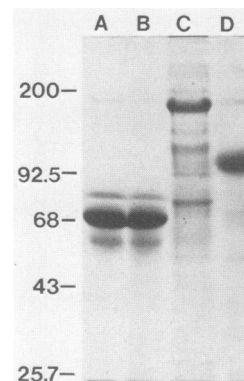


FIG. 5. Purification of the secreted form of gB1 from medium conditioned by the growth of CHO cell line pHS114-C6. Proteins were separated on a 10% polyacrylamide gel and stained with Coomassie blue. Portions were applied to each lane as follows: A, 12.5 µl of conditioned medium; B, 12.5 µl of pooled flowthrough from the lentil lectin column; C, 50 µl of the pooled eluate from the lentil lectin column; D, 7.5 µl of the pooled eluate from the immunoaffinity column after 12-fold concentration by ultrafiltration. The numbers on the left indicate the molecular mass (in kilodaltons) and positions of the protein standards.

(group 2). Some of the negative control sera (group 3) showed a low but measurable prechallenge ELISA titer which may have been the result of common cellular proteins found in the CHO medium. After challenge with HSV-1, 80% of the untreated control mice died within 7 days, whereas 80% of the mice which received recombinant gB and 100% of the mice immunized with the HSV-1 glycoprotein mixture were protected. The antibody titers in the serum of the surviving mice were also measured. For mice immunized with recombinant gB, two showed a slight reduction in titer after challenge. Mice immunized with the HSV-1 glycoprotein mixture showed little change in antibody titers after challenge, except for one mouse which showed a 6-fold increase from an initial low titer, and the one surviving control mouse also showed an increase in titer after challenge.

DISCUSSION

Stable CHO cell lines that express the complete gB1 protein or a truncated protein lacking the 194 carboxyl-terminal amino acids of gB1 have been established. The truncated protein was secreted from cells due to deletion of the transmembrane and C-terminal domains of gB1, whereas

TABLE 2. Purification of gB1 from medium conditioned by clone pHS114-C6

| Purification Step | Total vol (ml) | Total ^a gB1 (mg) | Total protein (mg) | % Recovery |
|-----------------------------------|----------------|-----------------------------|--------------------|------------|
| Conditioned medium | 8,250 | 9.3 | 24,420.0 | 100 |
| Lentil lectin column eluate | 216 | 5.2 | 173.0 | 56 |
| Immunoaffinity column eluate | 75 | 2.5 | 2.3 | 27 |
| Concentrate after ultrafiltration | 6 | 1.4 | ND ^b | 15 |

^a The concentration of gB1 was determined by ELISA with a preparation of purified gB1 in which the concentration of gB1 had been determined by amino acid analysis as the standard.

^b ND, Not determined.

| | | | | | |
|----|---|-----|-----|-----|-------------------|
| | Ala Gly | Ala | Arg | Asp | N-terminal |
| | Ala Pro Ser Ser Pro Gly Thr Pro Gly Val Ala Ala Ala Thr Gln Ala Ala Asn | | | | sequence |
| 25 | | 35 | | 45 | Predicted |
| | Val Leu Val Ala Ser Ala Ala Pro Ser Ser Pro Gly Thr Pro Gly Val Ala Ala Ala Thr Gln Ala Ala Asn | | | | sequence |

FIG. 6. Comparison of the N-terminal amino acid sequence of the secreted form of gB1 with the sequence determined by DNA sequence analysis. The N-terminal sequence of the secreted form of gB1, which was purified from medium conditioned by CHO clone pHS114-C6, was determined by Edman degradation. The predicted amino acid sequence, residues 25 to 48, for strain Patton is taken from Stuve et al. (54) and is identical to residues 24 to 47 described by Pellett et al. (41) for strain F. For five of the residues determined by Edman degradation, the identity of the residue was ambiguous, and both possible amino acids are shown.

the complete protein was cell associated. The level of expression of the truncated gB1 protein in CHO cells was increased at least 60-fold by MTX selection, and this selection was also shown to result in a 10-fold amplification in gB1 DNA copy number. The truncated form of gB1 was also purified from medium conditioned by growth of these cells, and the identity of this recombinant protein was verified by N-terminal sequence analysis. This analysis identified the signal peptide cleavage site of gB1 and predicted the cleavage of a 30-amino-acid signal sequence from the primary protein.

The efficiency of secretion of the truncated gB1 protein from CHO cells was low, with less than 10% of the [³⁵S]methionine-labeled gB1 being detected in the medium after a chase. The presence of lower-molecular-weight gB1 fragments in cell lysates suggests that intracellular degradation of gB1 is occurring, perhaps due to deletion of the 194 carboxyl-terminal amino acids. It is also possible that the 24-amino-acid SV40 small T fusion protein at the 3' end of the truncated gB1 protein may be responsible for the poor secretion of this protein. However, we recently constructed a gB1 expression vector similar to plasmid pHS114, except that a stop codon was introduced at the 3' end of the truncated gB1, eliminating the SV40 fusion protein. A CHO clone expressing this truncated gB1 was analyzed by radioimmunoprecipitation, and the secretion efficiency and intracellular degradation pattern of gB1 were similar to those described for the pHS114-C6 clone (Fig. 3C). These results indicate that the SV40 fusion protein was not responsible for

the poor secretion of truncated gB1 observed in CHO cells and that some other property of the gB1 protein may be involved. Using a plasmid construction similar to plasmid pHS114 for expression of a truncated HSV-2 gB protein (gB2) in CHO cells, we also observed a low secretion efficiency for gB2 (54). These results suggest that sequences necessary to direct gB1 and gB2 to the cell surface may have been deleted from these proteins by removal of the transmembrane and cytoplasmic domains. Alternatively, it is possible that deletion of the C-terminal domain of gB altered the tertiary or quaternary structure of the protein in such a way that efficient transport is blocked. Recent studies with the vesicular stomatitis virus glycoprotein G (28) and the influenza virus hemagglutinin protein (13) have suggested that for these proteins only those molecules which can assemble to form native oligomers are efficiently transported to the Golgi apparatus and the cell surface. Proteins which are transported slowly or not at all have also been found to be associated with a 77-kDa cellular protein, BiP (3, 14). We do not know whether the truncated gB1 is associated with BiP intracellularly; however, it is likely that the truncated gB1 is correctly folded because this protein is recognized by two monoclonal antibodies, F3A/B and D8AB (44), which only recognize native gB. Even though the secretion efficiency of truncated gB1 from CHO cells was low, these cell lines were more useful as a source of gB1 protein than virus-infected cells. From Vero cells lytically infected with HSV-1 we obtained about 1.0 pg of gB1 protein per cell after a 48-h lytic cycle. Alternatively, the CHO cell line pHS114-

TABLE 3. Protection of mice against virus challenge

| Group | Animal | Immunogen | Dose (µg) | ELISA titer ^a (prechallenge) | Survival (days postchallenge) | % Surviving ^b | ELISA titer (survivors) |
|-------|--------|--------------------------|-----------|---|-------------------------------|--------------------------|-------------------------|
| 1 | A | Recombinant gB1 | 5 | 1:1,928 | 10 | 80 | |
| | B | | | 1:1,097 | >24 | | 1:2,660 |
| | C | | | 1:2,660 | >24 | | 1:1,539 |
| | D | | | 1:1,641 | >24 | | 1:9,641 |
| | E | | | 1:13,737 | >24 | | 1:7,573 |
| 2 | A | HSV glycoprotein mixture | 20 | 1:2,339 | >24 | 100 | 1:1,928 |
| | B | | | 1:172 | >24 | | 1:965 |
| | C | | | 1:2,660 | >24 | | 1:5,488 |
| | D | | | 1:2,883 | >24 | | 1:3,026 |
| 3 | A | Control cell extracts | 23 | <1:25 | >24 | 20 | 1:734 |
| | B | | | 1:85 | 3 | | |
| | C | | | 1:51 | 4 | | |
| | D | | | <1:25 | 7 | | |
| | E | | | <1:25 | 4 | | |

^a The antigen for the ELISA was the HSV-1 (Patton) glycoprotein mixture. The titer is the 50% endpoint.

^b Percentage of mice surviving 14 days after challenge.

C6 secreted about 2.2 pg of truncated gB1 per cell in 24 h, and this level of production could be maintained continuously for at least 2 months.

The truncated gB1 protein may be useful for mapping the site responsible for the formation of SDS-stable, heat-dissociable dimers of gB1 which have been isolated from virions and infected cells (49). A mutant gB1 protein, *tsB5*, fails to produce dimers, migrating as a monomer in the absence of heating (15, 48). It has more recently been shown that the *tsB5* mutant contains multiple mutations (5, 9): an amino acid substitution at residue 552 which alters the rate of virus entry into the cell, a second substitution at residue 857 which causes syncytial plaque formation (*syn*), and a *ts* lesion which has not been precisely mapped but is located within the amino-terminal residues. To determine whether the truncated gB1 protein retained the dimerization site, the electrophoretic mobility of this protein in the presence of SDS was determined before and after heating. This analysis (data not shown) indicated that the truncated protein behaved exactly like the intact gB1 protein from infected cells and retained the dimerization site. Thus, the carboxyl-terminal 194 amino acids of gB1, which include the cell fusion determinant (*syn*) at residue 857, are apparently not required for dimerization. However, it is possible that the 24-amino-acid SV40 small T fusion protein at the C terminus of truncated gB1 may alter the structure so as to generate dimers by a different interaction. An analysis of the truncated gB1 protein without the terminal fusion peptide may help locate the dimerization site.

The truncated gB1 protein will also be useful for analyzing the immune response of the HSV-infected host. In addition to the dimerization site, this protein retains other important structural features of the complete protein, including 9 of the 11 potential N-linked glycosylation sites of gB1 (4, 41, 54) and the epitopic sites for two type-common neutralizing monoclonal antibodies (41). Results presented in this report indicate that the truncated gB1 protein is immunogenic in mice and elicits levels of antibody similar to those induced by a natural antigen comprising a mixture of HSV-1 glycoproteins purified from infected cells. The truncated gB1 protein and the viral glycoprotein mixture also protected mice against a lethal HSV-1 challenge and induced neutralizing antibodies in mice (data not shown) and in guinea pigs (L. R. Stanberry, D. I. Bernstein, R. L. Burke, C. Pachl, and M. G. Myers, *J. Infect. Dis.*, in press). In addition, Stanberry et al. have also shown that immunization with the truncated gB1 can greatly reduce the severity of primary and recurrent HSV-2 disease in guinea pigs. These results, together with recent observations that human HSV-specific T cells can recognize and be activated by this recombinant gB1 (62), suggest that a gB1 subunit vaccine may be effective in humans.

ACKNOWLEDGMENTS

We thank Dana Topping and Pauline Montes for careful preparation of this manuscript.

LITERATURE CITED

- Alt, F. W., R. E. Kellems, J. R. Bertino, and R. T. Schimke. 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J. Biol. Chem.* **253**:1357-1370.
- Bidlingmeyer, B. A., S. A. Cohen, and T. L. Tarvin. 1984. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* **336**:93-104.
- Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Post-translational association of immunoglobulin heavy chain binding protein with nascent chains in nonsecreting and secreting hybridomas. *J. Cell. Biol.* **102**:1558-1566.
- Bzik, D. J., B. A. Fok, N. A. DeLuca, and S. Person. 1984. Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. *Virology* **133**:301-314.
- Bzik, D. J., B. A. Fok, N. A. DeLuca, and S. Person. 1984. Nucleotide sequence of a region of herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. *Virology* **137**:185-190.
- Carter, V. C., P. A. Schaffer, and S. S. Tevethia. 1981. The involvement of herpes simplex virus type 1 glycoproteins in cell-mediated immunity. *J. Immunol.* **126**:1655-1660.
- Chan, W. L. 1983. Protective immunization of mice with specific HSV-1 glycoproteins. *Immunology* **49**:343-352.
- Cory, L., and P. G. Spear. 1986. Infections with herpes simplex viruses. *N. Engl. J. Med.* **314**:686-691.
- DeLuca, N., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes. 1982. Nucleotide sequences of herpes simplex virus type 1 affecting virus entry, cell fusion and production of glycoprotein gB (VP7). *Virology* **122**:411-423.
- Dix, R. D., and J. Mills. 1985. Acute and latent herpes simplex virus neurological disease in mice immunized with purified virus-specific glycoproteins gB or gD. *J. Med. Virol.* **17**:9-18.
- Eberle, R., and S. W. Mou. 1983. Relative titers of antibodies to individual polypeptide antigens of herpes simplex virus type 1 in human sera. *J. Infect. Dis.* **148**:436-444.
- Gething, M.-J., and J. Sambrook. 1982. Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein. *Nature (London)* **300**:598-603.
- Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell* **46**:939-950.
- Haas, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature (London)* **306**:387-389.
- Haffey, M. L., and P. G. Spear. 1980. Alterations in gB specified by mutants and their partial revertants in herpes simplex virus type 1 and relationship to other mutant phenotypes. *J. Virol.* **35**:114-128.
- Hall, M. J., and K. Katrak. 1986. The quest for a herpes simplex virus vaccine: background and recent developments. *Vaccine* **4**:138-150.
- Hawke, D., P.-M. Yuan, and J. E. Shively. 1982. Microsequence analysis of peptides and proteins. II. Separation of amino acid phenylthiodydantoin derivatives by high performance liquid chromatography. *Anal. Biochem.* **120**:302-311.
- Haynes, J., and C. Weissmann. 1983. Constitutive, long-term production of human interferons by hamster cells containing multiple copies of a cloned interferon gene. *Nucleic Acids Res.* **11**:687-706.
- Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer. 1981. A gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* **256**:7990-7997.
- Hilfenhaus, J., H. Moser, A. Herrmann, and R. Mauler. 1981. Herpes simplex virus subunit vaccine: characterization of the virus strain used and testing of the vaccine. *Dev. Biol. Stand.* **52**:321-331.
- Hilleman, M. R., V. M. Larson, E. D. Lehman, R. A. Salerno, P. C. Conrad, and A. A. McLean. 1981. Subunit herpes simplex virus-2 vaccine, p. 503-506. *In* A. J. Nahmias, W. R. Dowdle, and R. F. Schinazi (ed.), *The human herpesviruses: an interdisciplinary perspective*. Elsevier, New York.
- Holland, L. E., R. M. Sandri-Goldin, A. L. Goldin, J. C. Glorioso, and M. Levine. 1984. Transcriptional and genetic analyses of the herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. *J. Virol.* **49**:947-959.
- Holland, T. C., R. M. Sandri-Goldin, L. E. Holland, S. D. Marlin, M. Levine, and J. C. Glorioso. 1983. Physical mapping of the mutation in an antigenic variant of herpes simplex type 1 by use of an immunoreactive plaque assay. *J. Virol.* **46**:649-652.
- Kaufman, R. J., and P. A. Sharp. 1982. Amplification and expression of sequences cotransfected with a modular dihydrofolate reductase complementary DNA gene. *J. Mol.*

- Biol. 159:601-621.
25. Kaufman, R. J., L. C. Wasley, A. J. Spiliotes, S. D. Grossels, S. A. Latt, G. R. Larsen, and R. M. Kay. 1985. Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. *Mol. Cell. Biol.* 5:1750-1759.
 26. Kino, Y., T. Eto, K. Nishiyama, N. Ohtomo, and R. Mori. 1986. Immunogenicity of purified glycoprotein gB of herpes simplex virus. *Arch. Virol.* 89:69-80.
 27. Kousoulas, K. G., P. E. Pellett, L. Pereira, and B. Roizman. 1984. Mutations affecting conformation or sequence of neutralizing epitopes identified by reactivity of viable plaques segregated from *syn* and *ts* domains of HSV-1 (F) gB gene. *Virology* 135:379-394.
 28. Kreis, T. E., and H. F. Lodish. 1986. Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. *Cell* 46:926-937.
 29. Kudler, L., T. R. Jones, R. J. Russel, and R. W. Hyman. 1983. Heteroduplex analysis of cloned fragments of herpes simplex virus DNAs. *Virology* 124:86-99.
 30. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
 31. Lawman, M. J. P., R. J. Courtney, R. Eberle, P. A. Schaffer, M. K. O'Hara, and B. J. Rouse. 1980. Cell-mediated immunity to herpes simplex virus: specificity of cytotoxic T cells. *Infect. Immun.* 30:451-461.
 32. Lebowitz, P., and S. M. Weissman. 1979. Organization and transcription of the SV40 genome. *Curr. Top. Microbiol. Immunol.* 87:43-172.
 33. Lee, F., R. Mulligan, P. Berg, and G. Ringold. 1981. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumor virus chimaeric plasmids. *Nature (London)* 294:228-232.
 34. Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer. 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. *Virology* 115:149-160.
 35. Little, S. P., and P. A. Schaffer. 1981. Expression of the syncytial (*syn*) phenotype in HSV-1 strain KOS: genetic and phenotypic studies of mutants in two *syn* loci. *Virology* 112:686-702.
 36. Luciw, P. A., H. Opperman, J. M. Bishop, and H. E. Varmus. 1984. Integration and expression of several forms of Rous sarcoma virus DNA used for transfection of mouse cells. *Mol. Cell. Biol.* 4:1260-1269.
 37. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 38. Norrild, B., S. L. Shore, T. L. Cromeans, and A. J. Nahmias. 1980. Participation of three major glycoprotein antigens of herpes simplex virus type 1 early in the infectious cycle as determined by antibody-dependent cell-mediated cytotoxicity. *Infect. Immun.* 28:38-44.
 39. Nunberg, J. H., R. J. Kaufman, A. C. Y. Chang, S. N. Cohen, and R. T. Schimke. 1980. Structure and organization of the mouse dihydrofolate reductase gene. *Cell* 19:355-364.
 40. Parker, B. A., and G. R. Stark. 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infection by virus or viral DNA. *J. Virol.* 31:360-369.
 41. Pellett, P. E., K. G. Kousoulas, L. Pereira, and B. Roizman. 1985. Analysis of the herpes simplex virus strain F glycoprotein gB gene: primary sequence and predicted protein structure of the wild-type and monoclonal antibody-resistant mutants. *J. Virol.* 53:243-253.
 42. Perlman, D., and H. O. Halvorson. 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J. Mol. Biol.* 167:391-409.
 43. Rafield, L. F., and D. M. Knipe. 1984. Characterization of the major mRNAs transcribed from the genes for glycoprotein B and DNA-binding protein ICP8 of herpes simplex virus type 1. *J. Virol.* 49:960-969.
 44. Rector, J. T., R. N. Lausch, and J. E. Oakes. 1982. Use of monoclonal antibodies for analysis of antibody-dependent immunity to ocular herpes simplex virus type 1 infection. *Infect. Immun.* 38:168-174.
 45. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237-251.
 46. Roizman, B. 1979. The organization of the herpes simplex virus genomes. *Annu. Rev. Genet.* 13:25-57.
 47. Rose, J. K., and J. E. Bergmann. 1982. Expression from cloned cDNA of cell-surface and secreted forms of the glycoprotein of vesicular stomatitis virus in eucaryotic cells. *Cell* 30:753-762.
 48. Sarmiento, M., M. Haffey, and P. G. Spear. 1979. Membrane proteins specified by herpes simplex virus. III. Role of glycoprotein VP7 (B₂) in virion infectivity. *J. Virol.* 29:1149-1158.
 49. Sarmiento, M., and P. G. Spear. 1979. Membrane proteins specified by herpes simplex virus. IV. Conformation of the virion glycoprotein designated VP7 (B₂). *J. Virol.* 29:1159-1167.
 50. Schrier, R. D., L. I. Pizer, and J. W. Moorhead. 1983. Type-specific delayed hypersensitivity and protective immunity induced by isolated herpes simplex virus glycoprotein. *J. Immunol.* 130:1413-1418.
 51. Sober, H. A. 1970. *Handbook of biochemistry*, 2nd ed., p. H-112. The Chemical Rubber Co., Cleveland, Ohio.
 52. Soberon, X., L. Covarrubias, and F. Bolivar. 1980. Construction and characterization of new cloning vehicles. I. Deletion derivatives of pBR322 and pBR325. *Gene* 9:287-305.
 53. Spear, P. G. 1985. Glycoproteins specified by herpes simplex viruses, p. 315-356. *In* B. Roizman (ed.), *The herpesviruses*, vol. 3. Plenum Publishing Corp., New York.
 54. Stuve, L. L., S. Brown-Shimer, C. Pacht, R. Najarian, D. Dina, and R. L. Burke. 1987. Structure and expression of the herpes simplex virus type 2 glycoprotein gB gene. *J. Virol.* 61:326-335.
 55. Subramani, S., R. Mulligan, and P. Berg. 1981. Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors. *Mol. Cell. Biol.* 1:854-864.
 56. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.
 57. Urlaub, G., and A. Chasin. 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc. Natl. Acad. Sci. USA* 77:4216-4220.
 58. Van der Eb, A. J., and F. L. Graham. 1980. Assay of transforming activity of tumor virus DNA. *Methods Enzymol.* 65:826-839.
 59. von Hejne, G. 1984. How signal sequences maintain cleavage specificity. *J. Mol. Biol.* 173:243-251.
 60. Wahl, G. M., B. R. de Saint Vincent, and M. L. de Rose. 1984. Effect of chromosomal position on amplification of transfected genes in animal cells. *Nature (London)* 307:516-520.
 61. Wenske, E. A., M. W. Bratton, and R. J. Courtney. 1982. Endo- β -N-acetylglucosaminidase H sensitivity of precursors to herpes simplex virus type 1 glycoproteins gB and gC. *J. Virol.* 44:241-248.
 62. Zarling, J. M., P. A. Moran, R. L. Burke, C. Pacht, P. W. Berman, and L. L. Laskey. 1986. Human cytotoxic T cell clones directed against herpes simplex virus infected cells. IV. Recognition and activation by cloned glycoproteins gB and gD. *J. Immunol.* 136:4669-4673.