JOURNAL OF VIROLOGY, Jan. 1990, p. 431–436
0022-538X/90/010431-06$02.00/0

Vol. 64, No. 1

Protection from Herpes Simplex Virus Type 1 Lethal and Latent Infections by Secreted Recombinant Glycoprotein B Constitutively Expressed in Human Cells with a BK Virus Episomal Vector

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Received 9 June 1989/Accepted 18 September 1989

The herpes simplex virus type 1 (HSV-1) glycoprotein B (gB-1) gene, deleted of 639 nucleotides that encode the transmembrane anchor sequence and reconstructed with the extramembrane and intracytoplasmic domains, was cloned under control of the Rous sarcoma virus long terminal repeat in the episomal replicating vector pRP-RSV, which contains the origin of replication and early region of the human papovavirus BK as well as a cDNA for a mutant mouse dihydrofolate reductase that is resistant to methotrexate. gB-1 (0.15 to 0.25 pg per cell per 24 h) was constitutively secreted into the culture medium of pRP-RSV-gBs-transformed human 293 cells. Treatment of transformed cells with methotrexate at high concentrations (0.6 to 6 μM) increased gB-1 production 10- to 100-fold, because of an amplification of the episomal recombinant. Mice immunized with secreted gB-1 produced HSV-1- and HSV-2-neutralizing antibodies and were protected against HSV-1 lethal, latent, and recurrent infections. Constitutive expression of secreted gB-1 in human cells may establish a system to develop diagnostic material and a subunit vaccine for HSV infections.

Envelope glycoproteins are the main target for neutralization of infectivity in enveloped viruses (29). In the case of herpes simplex virus type 1 (HSV-1), at least three surface components, glycoproteins B (gB-1), D, and H, have been shown to induce neutralizing antibodies and protective immunity (4, 6, 11, 12, 14, 16, 23, 26, 30, 33, 35). Although the pathways and mechanisms of HSV-1 envelope glycoprotein glycosylation have been partly clarified (9, 43), little is known about the importance of the carbohydrate moiety of viral glycoproteins in relation to their immunogenic and antigenic properties (29). Carbohydrates may directly confer antigenicity to viral glycoproteins by contributing specific antigenic determinants. They may affect availability of protein epitopes by steric hindrance or lead to charge alterations. They could also influence protein folding as a consequence of allosteric changes. This conformational effect of glycosylation may be crucial for gB-1 immunogenicity, because it was shown that gB-1 has an N-terminal domain containing discontinuous epitopes (24).

One important reason to produce foreign proteins in mammalian cells is to make an authentic product, that is, a protein as similar as possible to the natural protein (5). Glycosylation and processing of viral polypeptides are carried out by cellular factors and enzymes (9, 43). However, not all mammalian cells may glycosylate the protein exactly as the authentic protein, because the cell type plays a role in oligosaccharide processing. Indeed, proteins produced in heterologous mammalian cells are glycosylated at the correct sites but differ in oligosaccharide side chains and fine structure (38, 47). Similarly, the glycosydic composition of the HSV-1 glycoproteins depends on the species of the infected cells (8, 9). It is likely, therefore, that the antigenic structure of recombinant gB-1 would be identical to that of the virion glycoprotein produced during natural infection in humans only if the recombinant is expressed in human cells. Although gB-1 has been produced in yeast cells (31) and in mammalian cells of hamster and simian origin (1, 3, 10, 32), we considered it of interest to express gB-1 in human cells and to investigate its immunogenic and protective properties against lethal and latent HSV-1 challenges.

For construction of recombinant plasmids, standard techniques were used (25). To obtain a secreted form of gB-1, an *Esp*I fragment of 639 nucleotides, which encodes the transmembrane anchor sequence (7), was excised from the gB-1 gene of HSV-1 strain F. The deleted gB-1 gene was then reconstructed by self-ligation to put in frame sequences coding for the extramembrane and C-terminal intracytoplasmic domains (Fig. 1). For constitutive expression in human cells, we prepared the vector pRP-RSV-gBs, in which the deleted gB-1-coding sequences were inserted under the control of the Rous sarcoma virus long terminal repeat and of the polyadenylation signals from the HSV-1 thymidine kinase gene. The origin of replication and early region of the human papovavirus BK (50) allow the vector to persist episomally and replicate to a high copy number in human cells (27). A mouse dihydrofolate reductase (DHFR) cDNA expressing a mutant enzyme resistant to methotrexate (MTX) (39) was also included in the construction (Fig. 1).

The ability of pRP-RSV-gBs to express gB-1 was tested by a transient assay with human HeLa and 293 cells (17) as well as with simian COS-7 cells (15). Analysis of gB-1s expression in cells transfected with pRP-RSV-gBs is shown in Fig. 2. For radioimmunoprecipitation, cells were labeled in serum-free medium. [^{14}C]glucosamine (5 μCi/ml; specific activity, >200 mCi/mmol) was added to medium containing 1% of the normal concentration of glucose, and [^{35}S]methionine (50 μCi/ml; specific activity, >1,000 Ci/mmol) was added to methionine-free medium. Before addition of the radioactive precursor, cells were incubated for 4 h in depleted medium. Cells were labeled for 18 h (Fig. 2A, B, and D) or for various times (Fig. 2C). In all cases, cells and culture medium were harvested at the end of the labeling period. Cell extracts

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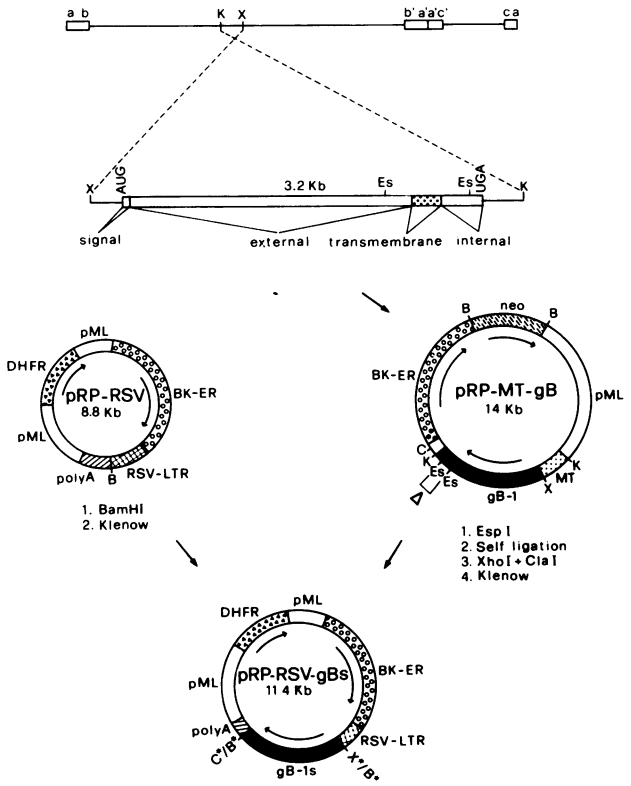

FIG. 1. Construction of recombinant plasmids to express gB-1s. In the upper part of the figure, the genome of HSV-1 strain F is represented schematically. The *Xho*I-*Kpn*I fragment, containing gB-1-coding sequences, was inserted into pRP-MT to produce the vector pRP-MT-gB (26). A fragment of 639 nucleotides, comprising the region encoding the transmembrane anchor sequence (7), was deleted (Δ) by *Esp*I digestion followed by self-ligation. The reconstructed gB-1 gene was excised by a double digestion with *Xho*I and *Cla*I and inserted by blunt-end ligation into the unique *Bam*HI site of pRP-RSV. Direction of transcription is indicated by arrows. Abbreviations: pML, bacterial plasmid pML; MT, mouse metallothionein I gene promoter; gB-1, intact gB-1-coding sequences; gB-1s, gB-1 sequences with the *Esp*I transmembrane anchor fragment deleted; BK-ER, human papovavirus BK early region and origin of replication; neo, aminoglycoside phosphotransferase gene; RSV-LTR, Rous sarcoma virus long terminal repeat promoter-enhancer sequences; polyA, polyadenylation signals from HSV-1 thymidine kinase gene; DHFR, mouse DHFR cDNA under the control of the simian virus 40 early promoter and the hepatitis B surface antigen gene polyadenylation sequences (39). B, *Bam*HI; C, *Cla*I; Es, *Esp*I; K, *Kpn*I; X, *Xho*I. Asterisks mark restriction sites lost during construction.

were prepared by lysing cells in 10 mM sodium phosphate buffer (pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 10^{-4} M tosyl-L-phenylalanyl-chloromethyl ketone, and 10^{-3} M phenylmethylsulfonyl fluoride as protease inhibitors. Immunoprecipitation with anti-gB-1 monoclonal antibody I-144, electrophoresis in polyacrylamide gels, and autoradiography were carried out as previously described (26). Exposure of autoradiograms was for 48 h (Fig. 2A through C) or 2 h (Fig. 2D). Since the mature, completely glycosylated form of full-length gB-1, made up of 903 amino acids, is 120 kilodaltons (kDa) (9, 34, 42) (Fig. 2A and B), the 690-amino-acid deleted protein with the same degree of glycosylation is 91.6 kDa. Cells were transfected by the calcium phosphate coprecipitation technique (18, 49) with 10 μg of pRP-RSV-gBs, and the product was detected 48 h after transfection by immunoprecipitation of the culture medium and cell lysate with anti-gB-1 monoclonal antibody I-144 (Fig. 2A). 293 cells produced a protein of about 90 kDa, whereas COS-7 cells showed a barely detectable band and HeLa cells were negative. Densitometric analysis showed that more than 95% of the product was secreted into the culture medium of 293 cells, while a negligible quantity remained cell associated. The amount of secreted gB-1 (gB-1s) was comparable to that of full-size gB-1 detected in the cell homogenate of 293 cells lytically infected by HSV-1 F. 293 cells were killed slowly by treatment with MTX. To avoid amplification of the endogenous DHFR gene during MTX selection, 293 cells were cotransfected with pRP-RSV-gBs (10 μg) and pSV2-neo (1 μg) (41). Several clones growing in medium with G418 (500 μg/ml) were isolated, expanded into mass cultures, and analyzed by immunoprecipitation with monoclonal antibody I-144. 293 cell clones 9, 13, and 20 expressed the 90-kDa deleted form of gB-1 in the supernatant medium of cell cultures, while the cell lysates were negative (Fig. 2B). When cells were pulse-labeled for 1 h with [^{35}S]methionine, unglycosylated and partially glycosylated precursors were detected in the cell lysate. After a 6-h chase, more than 90% of the products had disappeared from the cell lysate, while the 90-kDa secreted form increased correspondingly in the culture medium (data not shown). Cell lysates of both HSV-1 lytically infected 293 cells and the 293 cell clone (3C4) expressing full-length, membrane-bound gB-1 (26) yielded a substantial amount of gB-1 dimer when the sample was electrophoresed without previous heating (Fig. 2B). On the other hand, the 90-kDa deleted gB-1s did not form the dimer, suggesting that the transmembrane region contains amino acid sequences necessary for interaction of the monomers during dimerization. Kinetic analysis of gB-1 production, performed on 293 cell clone 20, showed that gB-1s secretion started between 1 and 6 h, reached a peak at 24 h, and remained constant for up to 72 h after the start of the labeling period (Fig. 2C). gB-1s was the only protein regularly detected in considerable quantities in the serum-free culture media of pRP-RSV-gBs-transformed cells that were analyzed without immunoprecipitation. The amount of secreted gB-1s was then determined by densitometric analysis of polyacrylamide gels processed by a highly sensitive, quantitative silver stain method (44). Different clones were found to secrete gB-1s at 0.15 to 0.25 μg/ml per 10^6 cells per 24 h. Since cotransfected sequences can be amplified by using DHFR vectors under MTX treatment (2, 22), an attempt was made to increase gB-1s production by propagation of pRP-RSV-gBs-transformed clones in the presence of MTX. Taking advantage of the MTX resistance of the mutant exogenous DHFR cDNA, cell clones were treated with high concentrations of MTX, above those sufficient to inhibit the endogenous DHFR activity (39). After cultivation for several passages in the presence of 0.6 or 6 μM MTX, gB-1s production increased 10 to 100 times in different stable cell clones (Fig. 2D).

To determine the state of the recombinant DNA, total cellular DNA from clones 9, 13, and 20 was analyzed by Southern blot hybridization (40) after digestion with *Eco*RI, which cuts pRP-RSV-gBs once. A prominent band, comigrating with full-length control linear DNA, appeared in all three clones (Fig. 3A), suggesting that the bulk of the recombinant DNA is in a free state. Analysis of uncut cellular DNA confirmed the presence of episomal DNA (data not shown). Minor additional bands in clones 9 and 20 may indicate either integrations or rearrangements of part of the episomal molecules. Vector DNA was completely cleaved

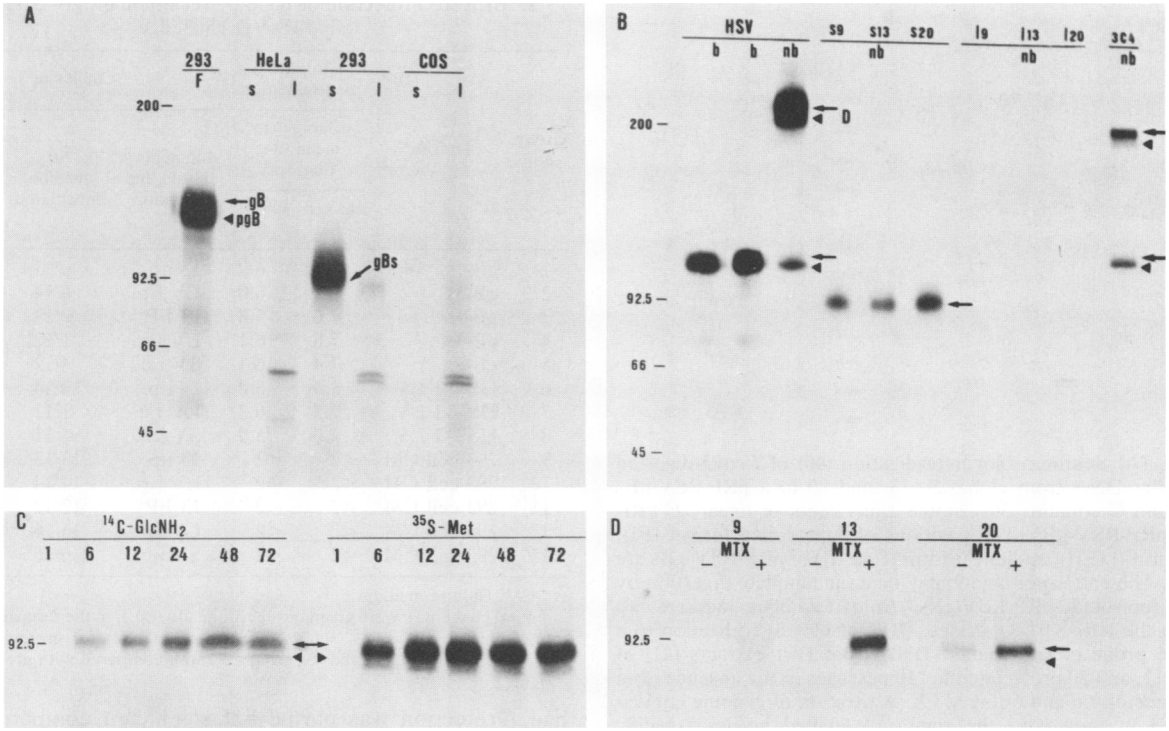

FIG. 2. Analysis of gB-1s expression in cells transfected with pRP-RSV-gBs. Arrows indicate the fully glycosylated form (gB), and arrowheads indicate the incompletely glycosylated precursor (pgB) of full-size gB-1 and of its dimer as well as that of deleted gB-1s (gBs). Molecular sizes (kilodaltons) and electrophoretic mobilities of the protein standards myosin (200), phosphorylase B (92.5), bovine serum albumin (66), and ovalbumin (45) are indicated on the left of each panel. Full-length gB-1 is 120 kDa, and deleted gB-1s is about 90 kDa. (A) HeLa, 293, and COS-7 cells were transfected with pRP-RSV-gBs and assayed for gB-1s at 48 h after transfection. Lane F contains a cell lysate of 293 cells lytically infected with HSV-1 strain F. s, Supernatant culture medium; l, cell lysate. (B) Clones 9, 13, and 20 of 293 cells stably transformed by pRP-RSV-gBs were analyzed for gB-1s expression in the supernatant culture medium (s9, s13, and s20) and cell lysate (l9, l13, and l20). Lanes HSV contain a cell lysate of 293 cells infected by HSV-1 strain F and harvested 18 and 24 h after infection. 3C4 is a cell lysate of a 293 cell clone expressing a cell-associated recombinant gB-1 (26). b and nb, Samples boiled or not boiled before electrophoresis; D, gB-1 dimer. (C) Kinetics of secretion of gB-1s produced by clone 20 after labeling with [^{14}C]glucosamine (^{14}C-GlcNH$_2$) or [^{35}S]methionine (^{35}S-Met). Culture medium was analyzed by immunoprecipitation 1, 6, 12, 24, 48, and 72 h after the start of the labeling period. (D) Immunoprecipitation of gB-1s in the culture medium of clones 9, 13, and 20 propagated for 10 passages in the absence (−) or presence (+) of 6 μM MTX. Densitometric analysis was carried out with an LKB XL laser densitometer.

by *Mbo*I and resistant to digestion by *Dpn*I (data not shown), demonstrating that episomal recombinant molecules replicate in human cells. Quantitation was carried out by dot blot hybridization of supernatant DNA from Hirt extracts (21) of stable cell clones to a gB-1 DNA probe. The results show that different clones contain from 10 to 70 copies of pRP-RSV-gBs per cell. Propagation of cell cultures in the presence of 0.6 or 6 μM MTX increased DNA copy number fourfold in clone 9, eightfold in clone 13, and twofold in clone 20, as determined by densitometric scanning of the autoradiograms (Fig. 3B). Hybridization to a DHFR cDNA probe gave the same results, which indicates that the gB-1 gene and the DHFR cDNA were coamplified in the episomal vector. Comparable quantitative data were obtained when hybridization to total cellular DNA was performed with either a gB-1 DNA or a DHFR cDNA probe, suggesting that the endogenous DHFR sequences were not amplified. These results were confirmed by RNA analysis. Dot blot hybridization of cytoplasmic RNA (48) showed an increase of gB-1 and DHFR transcripts in pRP-RSV-gBS-transformed clones after addition of MTX (Fig. 4A and B). In normal 293 cells, DHFR RNA was slightly increased at 0.6 μM MTX, but cells did not survive selection in 6 μM MTX and died after a few passages at this MTX concentration (Fig. 4B).

Two-month-old female BALB/c mice (cAnNCrlBR; Charles River Breeding Laboratories, Inc.) were immunized on day 1 by intraperitoneal inoculation with serum-free cell culture medium from pRP-RSV-gBs-transformed 293 cells, which contained 10 μg of gB-1s and which was emulsified with an equal volume of complete Freund adjuvant. On day 25, immunization was repeated with the same amount of gB-1s formulated in incomplete Freund adjuvant. Control animals were injected intraperitoneally with culture medium of normal 293 cells in the same way as animals receiving gB-1s or were immunized on day 1 by inoculation of a nonlethal dose (1/10th the 50% lethal dose [LD_{50}]) of HSV-1 LV into the footpad. One week before challenge, animals were bled from the tail and serum antibody titers against HSV-1 F and HSV-2 strain G were measured by plaque reduction tests with 100 PFU of virus (26). The 100% endpoint neutralization titer for each serum sample was determined, and the geometric mean titers were calculated. Lethal challenges were given on day 50. HSV-1 13 or LV (100 LD_{50}s) were inoculated intraperitoneally or in the footpad. The neurovirulent strains (45) LV (20 LD_{50}s) and SV (5 LD_{50}s) were inoculated intracerebrally. Mortality was observed for 20 days. For latent infection, mice were inoculated in the right rear footpad with a sublethal dose (one-

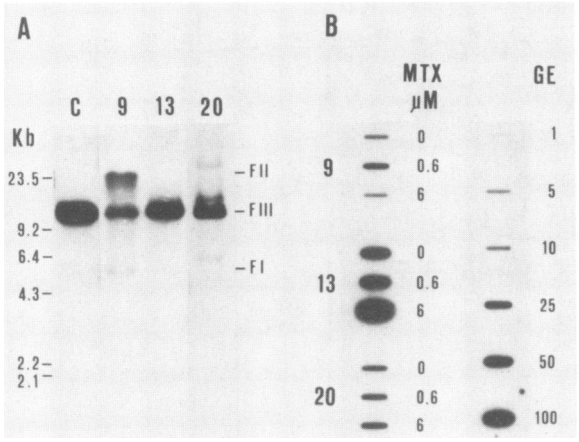

FIG. 3. (A) Southern blot hybridization (40) of *Eco*RI-digested total cellular DNA from clones 9, 13, and 20 to a pRP-RSV-gBs probe. Lane C contains 50 genome equivalents of linear, *Eco*RI-digested pRP-RSV-gBs. The positions of supercoiled form I (FI), circular form II (FII), and linear form III (FIII) of pRP-RSV-gBs are indicated. The pattern of lane 9 may be an incomplete digestion by *Eco*RI of episomal pRP-RSV-gBs. *Hin*dIII lambda markers are shown on the left. Kb, Kilobases. (B) Dot blot hybridization to a gB-1 gene probe of supernatant DNA from Hirt extracts (21) of clones 9, 13, and 20 propagated for 10 passages in the absence or in the presence of 0.6 and 6 μM MTX. A titration of genome equivalents (GE) is shown on the right. ^{32}P-labeled probes (specific activity, 3×10^8 to 9×10^8 cpm/μg) were produced by random primer extension (13). DNA extraction, electrophoresis in agarose gels, transfer, and hybridization were performed as described previously (26).

half the LD_{50}) of HSV-1 F. After 6 weeks, half of the animals were killed. To test for viral latency, the right lumbosacral root ganglia, associated with the sciatic nerve, were explanted onto indicator Vero cells as described previously (36) and observed for cytopathic effect characteristic of HSV-1. The remaining animals were kept and observed for 6 months for the appearance and outcome of recurrent infections in the inoculated leg.

Mice immunized with recombinant gB-1s achieved complete protection against a lethal challenge with different HSV-1 strains administered intraperitoneally or in the footpad. Protection was partial but significant compared with that in nonimmunized controls when mice were challenged intracerebrally with the neurovirulent strains LV and SV (Table 1). The lower survival rate after the SV challenge compared with that after the LV challenge may be due to the excess of PFU (1.5×10^6 in 5 LD_{50}s) of the SV variant administered to mice compared with the LV variant (1.8×10^2 in 20 LD_{50}s). Immunization with gB-1s was as efficient in protection of mice as was immunization with HSV-1. Mice inoculated by any route (intraperitoneally, intracerebrally, or in the footpad) that survived the challenge were kept for 6 months and remained healthy, without signs of paralysis. Infectious virus was not detected in their livers, spleens, or brains, which were analyzed at the end of the observation period. Since it was reported that inoculation with HSV-1 structural proteins protects mice from an intraperitoneal or intracerebral viral challenge administered 24 to 72 h later (37), mice were inoculated intraperitoneally with 10 μg of gB-1s and challenged intracerebrally with HSV-1 LV or SV 24 and 72 h later. All mice died after the 24-h challenge, and 90% of them died after the 72-h challenge (data not shown), which indicates that protection by gB-1s is due to long-lasting active immunization and not to some short-term effect. Before challenge, geometric mean neutralizing antibody titers in sera of mice immunized with gB-1s were greater than 128 and comparable to antibody titers of mice immunized with infectious virus (Table 1). Because of common antigenic determinants in gB-1 and gB-2 (34, 43), neutralizing antibody titers greater than 32 were also detected against HSV-2. In the latency experiments, mice immunized with gB-1s were significantly protected against ganglionic infection, compared with nonimmunized controls (Table 2). All the nonimmunized mice had a severe, often necrotizing primary infection in the inoculated leg. In agreement with another mouse model (20), they showed several spontaneous recurrences during an observation period of 6 months. On the other hand, no lesions appeared in the inoculated legs of immunized mice and no recurrences of the latent infection were detected during the observation period.

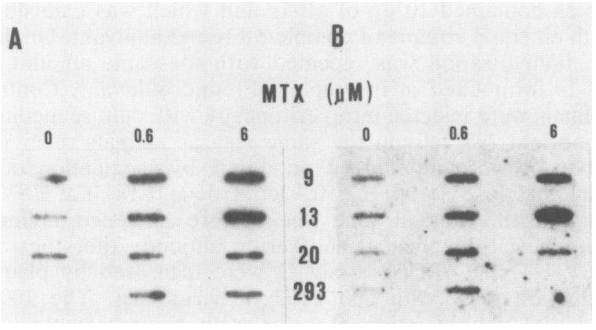

FIG. 4. Dot blot hybridization of cytoplasmic RNA from clones 9, 13, and 20 to gB-1 (A) and DHFR (B) probes. Cells were propagated for 10 passages in the absence or presence of 0.6 and 6 μM MTX. The fourth rows (293) in panels A and B contain RNA from normal 293 cells. In panel A, normal cells were not treated with MTX and were uninfected (left) and infected with HSV-1 for 18 (middle) and 24 h (right). Radiolabeled probes were prepared as described in the legend to Fig. 3.

TABLE 1. Protection of mice by recombinant gB-1s against lethal HSV-1 challenge

Group no.	Antigen[a]	In vitro neutralization: Geometric mean titer (log_2) of: HSV-1 F	HSV-2 G	Challenge[b]: Inoculation strain and location	No. dead/no. inoculated	Protection (%)
1	gB-1s	8.1	6.2	13 i.p.	0/13	100
2	gB-1s	7.2	6.0	LV i.p.	0/14	100
3	gB-1s	7.6	5.8	13 FP	0/13	100
4	gB-1s	7.8	6.1	LV i.c.	1/12	91.7
5	gB-1s	7.4	6.1	SV i.c.	4/12	66.7
6	HSV-1 LV	8.4	5.8	13 i.p.	0/14	100
7	HSV-1 LV	7.5	6.3	LV i.c.	1/11	90.9
8	HSV-1 LV	7.7	5.9	SV i.c.	4/11	63.6
9	293 cell CM	<2	<2	13 i.p.	13/13	0
10	293 cell CM	<2	<2	LV i.p.	14/14	0
11	293 cell CM	<2	<2	13 FP	12/12	0
12	293 cell CM	<2	<2	LV i.c.	16/16	0
13	293 cell CM	<2	<2	SV i.c.	16/16	0

[a] CM, Culture medium.

[b] Viral challenges were given intraperitoneally (i.p.), in the footpad (FP), or intracerebrally (i.c.). $P < 0.001$ (Fisher's exact test) for groups 4 and 7 compared with group 12 and for groups 5 and 8 compared with group 13.

TABLE 2. Protection of mice by recombinant gB-1s against latent and recurrent HSV-1 infections

Antigen[a]	Challenge				
	No. of survivors/no. inoculated	Latent infection		Recurrent infections[b]	
		No. positive/no. tested	Protection (%)	No. positive/no. observed	Protection (%)
gB-1s	20/20	2/10	80	0/10	100
293 cell CM	16/20	8/8	0	8/8	0

[a] CM, Culture medium.

[b] In animals that tested positive, more than five vesicles were observed in the diseased leg at each recurrence. Often, the vesicles fused into one large, extended lesion. $P < 0.001$ by Fisher's exact test for group 1 compared with group 2 for the results of both latent and recurrent infections.

Infectious virus was isolated from the legs of diseased mice both in the acute phase of the latency experiments and during the recurrences.

In this system, a considerable expression of gB-1s was obtained through replication of the episomal recombinant vector. After MTX treatment, a remarkable rise in gB-1s production was accompanied by an increased copy number of the episomal vector, indicating that MTX amplification of genes coselected with DHFR cDNA acts not only on chromosomal sequences (2, 22) but also on episomal molecules. gB-1s production was remarkable in 293 cells, in which endogenous adenovirus *trans*-acting factors (17), which stimulate transcription from eucaryotic cellular and viral promoters (19, 28, 46), may enhance the activity of the Rous sarcoma virus long terminal repeat, which drives expression of gB-1s sequences. The strategy used to delete the transmembrane anchor sequence may be related to immunogenicity and protection induced by gB-1s against lethal and latent infections. In fact, reconstruction of the protein with its external and cytoplasmic domains may facilitate appropriate folding to form native antigenic determinants better than truncation which eliminates the carboxy terminus. Recently, protection of rabbits against HSV-1-induced keratitis, encephalitis, and death was obtained by immunization with recombinant secreted gB-1 (R. Manservigi and E. Cassai, submitted for publication). Constitutive production of a substantial amount of secreted gB-1 in human cells may provide useful material for diagnostic purposes and for the preparation of a subunit vaccine against HSV infections.

This work was supported by the Italian National Research Council (Progetti Finalizzati Biotecnologie e Biostrumentazione, Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie, e Oncologia), by North Atlantic Treaty Organization research grant 86-170, and by Associazione Italiana per la Ricerca sul Cancro.

We thank A. Bevilacqua, M. Bonazzi, and P. Zucchini for excellent technical assistance and for preparing the manuscript.

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