Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 5675–5679, August 1987
Biochemistry

Expression and nuclear envelope localization of biologically active fusion glycoprotein gB of herpes simplex virus in mammalian cells using cloned DNA

(protein targeting/glycoprotein transport/nuclear budding/glycosylation and secretion/high-expression shuttle vector)

MIR AHMED ALI, MARTIN BUTCHER, AND HARA P. GHOSH*

Department of Biochemistry, McMaster University, 1200 Main Street West, Hamilton, ON L8N 3Z5, Canada

Communicated by H. Gobind Khorana, May 7, 1987 (received for review November 24, 1986)

ABSTRACT **Herpes simplex virus (HSV) is known to bud from the inner membrane of the nuclear envelope. The structural gene for the glycoprotein gB, which is essential for virus entry and cell fusion induced by HSV type 1, has been cloned in a transient expression vector containing the adenovirus major late promoter, tripartite leader sequence, and VARNA (a special RNA in adenovirus-infected cells) genes. Synthesis and glycosylation of glycoprotein gB was observed in COS-1 cells transfected with the vector containing the *gB* gene. Removal of a 3′ fragment of the cloned gene resulted in the synthesis and secretion of a truncated gB glycoprotein. Immunofluorescence studies revealed that the expressed glycoprotein was localized in the nuclear envelope as well as in the cell surface. The expressed gB-1 glycoprotein was biologically active and induced fusion of cells to produce polykaryons. These data show that HSV glycoprotein gB expressed from cloned gene can be used as a model to study targeting of proteins into the nuclear envelope as well as cell fusion induced by the virus.**

A central question in cell biology is how proteins are sorted and transported from the site of synthesis to their specific location within a eukaryotic cell. Glycoproteins of enveloped animal viruses have been used extensively as model systems to study the biogenesis, insertion into membranes, and transport to the cell surface of membrane glycoproteins (1–3). Recently, cloned genes of membrane glycoproteins of enveloped viruses that bud from the plasma membrane have been used to study possible transport signals involved in directing proteins to the cell surface (2, 3). However, very little is known about the transport and localization of proteins into intracellular membrane locations such as the Golgi complex or nuclear envelope.

The DNA-containing enveloped herpes simplex virus (HSV) assembles and buds from the inner membrane of the nuclear envelope into the perinuclear space (4, 5). The genome of HSV type 1 (HSV-1) encodes at least five antigenically distinct glycoproteins—gB-1, gC-1, gD-1, gE-1, and gH-1—which are incorporated into the viral envelope (5). In HSV-1-infected cells, precursor forms of gB-1, gC-1, and gD-1 glycoproteins containing high levels of mannose are present predominantly in the nuclear fraction (6), suggesting that the newly synthesized glycoproteins migrate to the inner nuclear membrane for virus envelopment and budding (5). The nucleotide sequence of the HSV-1 gene encoding gB-1 glycoprotein (7, 8) has been recently elucidated. An examination of the deduced polypeptide sequence of gB-1 shows that, in addition to a hydrophobic amino-terminal signal sequence, the protein contains at the carboxyl terminus a hydrophobic domain of 69 amino acids with three segments that could span the membrane and a charged cytoplasmic domain of 109 amino acids (7, 8). The glycoprotein gB is essential for viral replication (9) and is involved in viral entry (10, 11) and cell fusion (12). Thus, the glycoprotein gB-1 could be used as a model to study targeting of proteins into nuclear membranes, interaction of the membrane-anchoring sequences in multispanning proteins with membranes, and molecular mechanism of virus entry and cell fusion.

As an initial step to study the transport of proteins into the nuclear membrane and the role of the different domains of this multifunctional HSV-1 glycoprotein, we have expressed gB-1 in mammalian cells using cloned DNA and determined the intracellular localization and induction of cell fusion by the expressed gB-1 protein.

MATERIALS AND METHODS

All restriction enzymes and DNA-modifying enzymes were from either Bethesda Research Laboratories or Boehringer Mannheim and were used according to manufacturer's specifications. A cloned *Bgl* II "I" fragment of HSV-1 (KOS) was obtained from J. Smiley (McMaster University). The eukaryotic expression vectors p91023 and p91023(B) (13) were provided by R. Kaufman (Genetics Institute, Cambridge, MA). Antiserum to HSV-1 was from M. Suh (Montreal Cancer Institute and DAKO Laboratories). 3S and 35S monoclonal antibodies to HSV-1 gB were obtained from M. Zweig (National Cancer Institute, Frederick, MD). Anti-ENV-1 antiserum corresponding to the HSV-1 envelope proteins was obtained from G. Cohen and R. Eisenberg (University of Pennsylvania, Philadelphia). Monoclonal and polyclonal antibody to lamin proteins were obtained from Y. Raymond (Montreal Cancer Institute). COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with a high level of glucose supplemented with 7% fetal bovine serum.

Construction of gB Expression Plasmid. The 3.4-kilobase (kb) *Xho* I/*Bam*HI and the 3.3-kb *Xho* I/*Kpn* I fragments (Fig. 1*a*) containing the structural gene of HSV-1 gB (7) were cloned in the *Xho* I/*Bam*HI sites of pKC7 and *Sal* I/*Kpn* I sites of M13mp19, respectively. We first cloned a 1.8-kb *Pst* I/*Pst* I fragment of the *gB* gene into the *Pst* I site of p91023 (13). This was designated p9-tgB and directed the synthesis of the secreted form of gB. To generate a full-length gB expression plasmid (p9-gB), the *Bgl* II/*Sal* I 1.3-kb fragment from the plasmid p9-tgB was ligated to a 2.1-kb *Sal* I/*Eco*RI fragment from M13mp19 gB and ligated to *Eco*RI- and *Bgl* II-digested p91023(B). The detailed scheme of the construction of the gB expression plasmids is given in Fig. 1.

Transfection of Cells with Plasmids and Immunoprecipitation of Labeled Proteins. Transfection of COS-1 cells using

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: HSV-1, herpes simplex virus type 1.
*To whom reprint requests should be addressed.

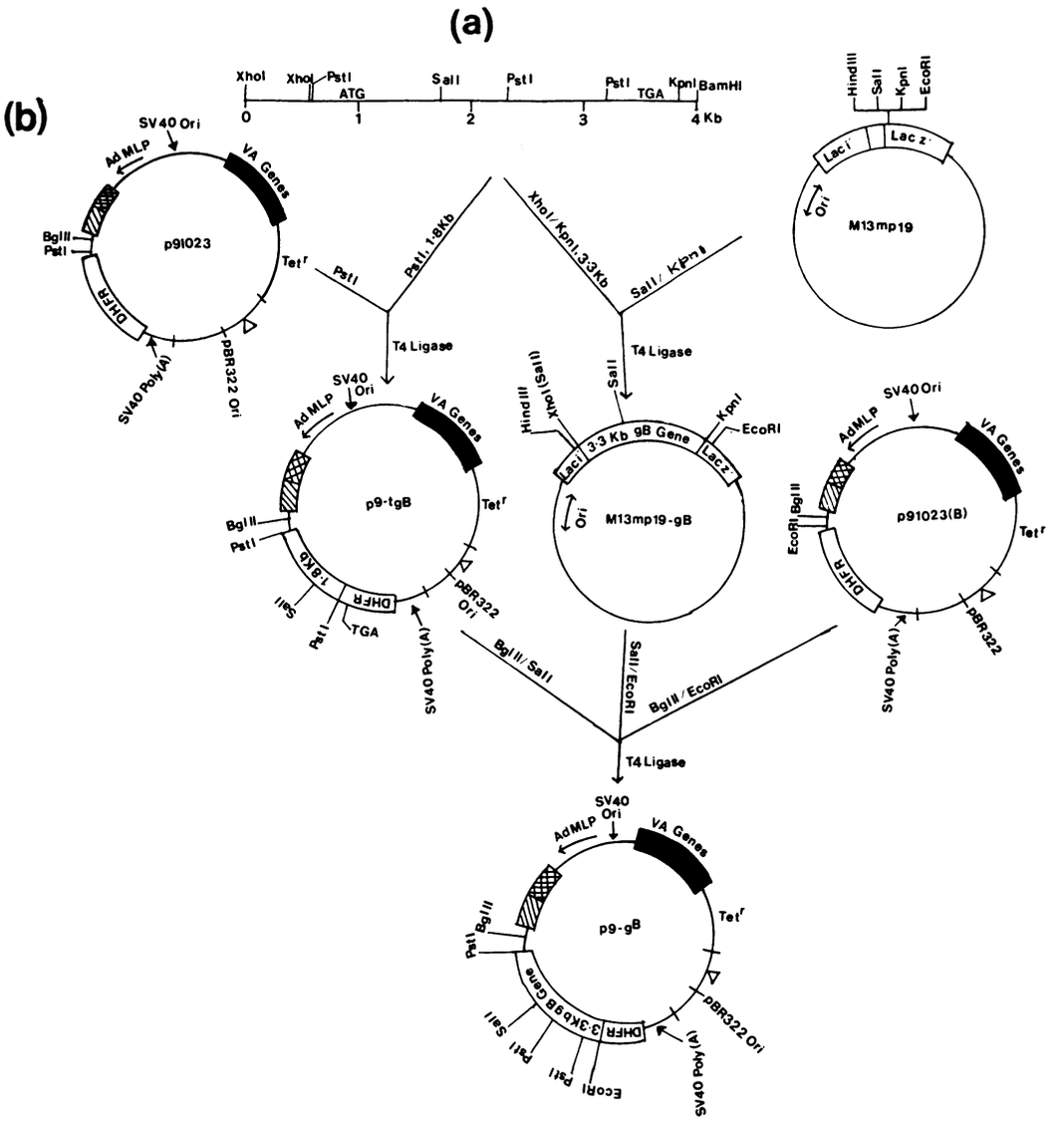

FIG. 1. (*a*) A partial restriction map of HSV-1 (KOS) DNA encoding *gB* gene between 0.345 and 0.372 map units. The map is complete to *Bam*HI, *Kpn* I, *Pst* I, *Sal* I, and *Xho* I. (*b*) Construction of gB and tgB-1 expression plasmids.

DEAE-dextran and chloroquine, labeling, and immunoprecipitation of the proteins was according to published methods (14). The secreted form of gB (tgB) was immunoprecipitated from the medium after removal of particulate material by centrifugation. The antigen–antibody complex isolated using protein A-Sepharose was analyzed on 7.5% or 10% $NaDodSO_4$/polyacrylamide gels (15). For tunicamycin treatment, cells were preincubated with 2 μg of tunicamycin per ml for 1 hr, then [^{35}S]methionine was added (40–50 μCi/ml; 1 Ci = 37 GBq) and labeling was continued for a further 2 hr in the presence of tunicamycin (15).

Indirect Immunofluorescence. Transfected COS cells grown on coverslips were fixed in methanol at −20°C for 10 min (16) and then treated with anti-HSV-1 antiserum or anti-gB-1 monoclonal antibodies followed by incubation with fluorescein-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (16). The nuclear envelope lamina was stained with an anti-lamin monoclonal antibody (16, 17). In this case, the cells were first incubated with anti-HSV antiserum and then with rhodamine-conjugated goat anti-rabbit IgG to stain the gB proteins. The cells were then incubated with nonimmune rabbit serum. The nuclear envelope lamina was stained by incubating with a mouse monoclonal anti-lamin antibody followed by staining with fluorescein-conjugated rabbit anti-mouse IgG.

Cell Fusion. The fusogenic activity of the expressed gB-1 was monitored by exposing the transfected cells to pH 5.0–6.0 for a very short period and observing the formation of polykaryons (18).

RESULTS

Expression and Glycosylation of gB-1 Glycoprotein in COS Cells. COS cells were transfected with p9-gB plasmid, labeled with [^{35}S]methionine, and the intracellular proteins were immunoprecipitated with either anti-HSV-1 antiserum or anti-Env-1 antiserum (19) or monoclonal 3S or 35S antibodies, which recognize HSV-1 glycoprotein gB-1 (20). As shown in Fig. 2*A* each of these antibodies recognized a protein of M_r 110,000 synthesized in the COS cells transfected with p9-gB. The recognition of this M_r 110,000 protein by the monoclonal antibodies specific for gB-1 glycoprotein identifies it as gB glycoprotein, which has a M_r of 110,000–115,000 (5). The fast-moving protein band observed in lane b may correspond to prematurely terminated or proteolytic fragment of gB-1 protein immunoprecipitated by the anti-HSV-1 antiserum (see also Fig. 3, lanes g and i).

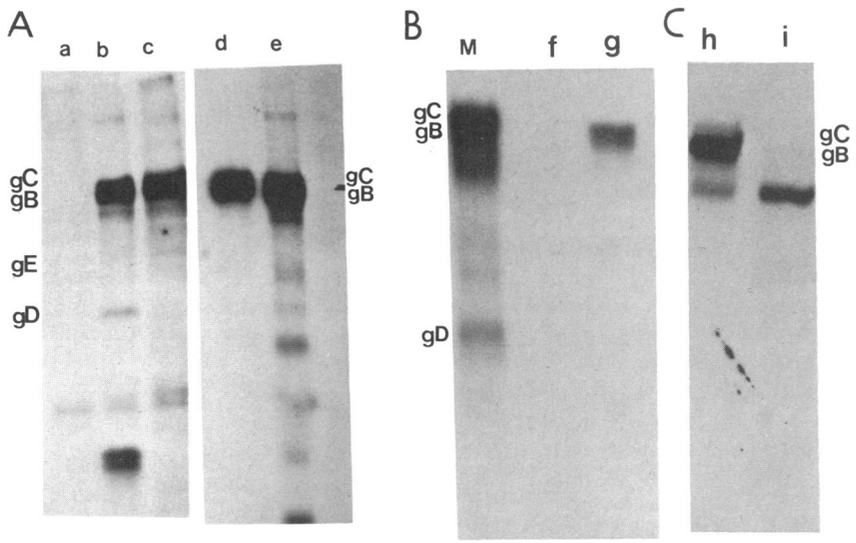

FIG. 2. Expression and glycosylation of gB protein in COS cells transfected with the p9-gB plasmid containing the complete gB-coding gene. [^{35}S]Methionine-labeled cell extracts immunoprecipitated with nonimmune serum (lane a), anti HSV-1 (lanes b and c), anti Env-1 antiserum (lane c), and 3S monoclonal antibodies (lane d). Lanes f and g, [^{3}H]mannose-labeled cell extracts immunoprecipitated with nonimmune and anti-HSV-1 antiserum, respectively; lane M, [^{3}H]glucosamine-labeled HSV-1-infected cell extracts precipitated with anti-HSV-1 antiserum. Lanes h and i, [^{35}S]methionine-labeled p9-gB-transfected cells grown in the absence and in the presence of tunicamycin, respectively. The positions of the HSV-1 proteins gB, gC, gD, and gE were determined by immunoprecipitation of [^{35}S]methionine-labeled HSV-1-infected cells with monoclonal antibodies. *A*, *B*, and *C* derived from separate experiments.

Labeling the transfected COS cells with [^{3}H]mannose followed by immunoprecipitation of the synthesized protein showed that the M_r 110,000 protein was glycosylated (Fig. 2*B*). As expected of an N-linked glycoprotein, the presence of tunicamycin reduced the size of the gB-1 protein to M_r 98,000 (Fig. 2*C*), which corresponded to the molecular weight predicted from the deduced sequence (7, 8). A small amount of unglycosylated gB is also present in the absence of tunicamycin (lane h).

Secretion of Truncated Glycoprotein tgB. The construct p9-tgB lacked the 69 amino acids containing the putative hydrophobic membrane-anchoring domain as well as the 109 amino acids containing the cytoplasmic carboxyl-terminal domain. It contained 505 amino acids from the NH_2 terminus of gB and 17 amino acids specified by the *DHFR* gene (13) at the COOH terminus. Immunoprecipitation of the extracellular medium from COS cells transfected with p9-tgB plasmid and labeled with [^{35}S]methionine showed the presence of significant amounts of a protein of M_r ≈75,000, which was recognized by anti-HSV-1 antiserum (Fig. 3, lane c). In contrast, the cytoplasmic extract of p9-tgB transfected COS cells contained a markedly decreased amount of a protein of M_r 70,000 (lane g). Trace amounts of M_r 75,000 protein were also present in the cell extract. Labeling with [^{3}H]mannose further showed that the secreted form of tgB was glycosylated (data not shown). These data show that removal of the carboxyl-terminal domain of the gB-1 glycoprotein allows the tgB-1 glycoprotein to be secreted into the medium. Removal of the carboxyl-terminal region of other viral membrane glycoproteins also resulted in their secretion into the medium (3, 21). Inhibition of glycosylation of the secretory tgB glycoprotein with tunicamycin showed that the appearance of the tgB protein in the extracellular medium was totally abolished in the presence of the inhibitor (lane e). In contrast, the intracellular form of tgB showed a decrease in size, to M_r ≈60,000, in the presence of tunicamycin (lane i). Thus, the nonglycosylated tgB protein was either not transported to the cell surface for secretion or it was rapidly degraded within the cell.

Intracellular Localization of gB-1 Glycoprotein. The intracellular location of the synthesized gB-1 glycoprotein was determined by indirect immunofluorescence (16) of COS cells transfected with p9-gB plasmid. The gB-1-specific proteins were located by immunofluorescence after staining with fluorescein-conjugated antibodies. To determine the position of the nuclei, the slides were also examined under a phase contrast microscope. Photographs of typical patterns of fluorescence observed are shown in Fig. 4. For cells expressing the gB-1 glycoprotein, an intense distinct ring of fluorescent staining around each nucleus (short arrow) was observed (Fig. 4 *A* and *C*). This kind of fluorescence pattern has been attributed to staining of the nuclear envelope (16, 21–23). Results of double immunofluorescence of the cells with anti-HSV and anti-lamin antibody confirmed that the gB-1 glycoprotein was localized in the nuclear envelope (Fig. 4 *C*

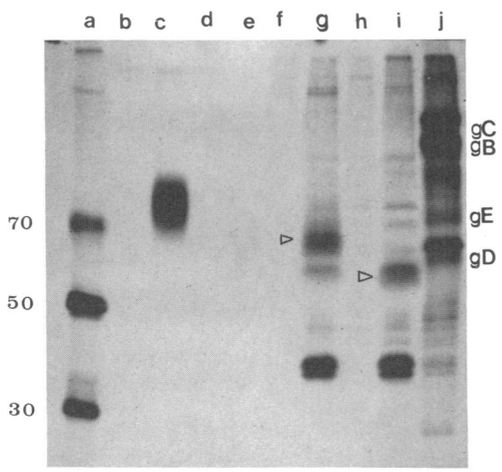

FIG. 3. Expression, glycosylation, and secretion of truncated tgB protein. COS cells transfected with p9-tgB vector and cultured in the absence (lanes b, c, f, and g) or in the presence (lanes d, e, h, and i) of tunicamycin were labeled with [^{35}S]methionine. Lane a, vesicular stomatitus virus marker; lanes b–e, radioactivity in the medium; lanes f–i, intracellular radioactivity. Lanes, b, d, f, and h, immunoprecipitation with nonimmune serum; lanes c, e, g, and i, immunoprecipitation with anti-HSV-1 antiserum. Lane j, ^{35}S-labeled HSV-1-infected cells immunoprecipitated with anti-HSV-1 antiserum. Numbers on left represent $M_r \times 10^{-3}$.

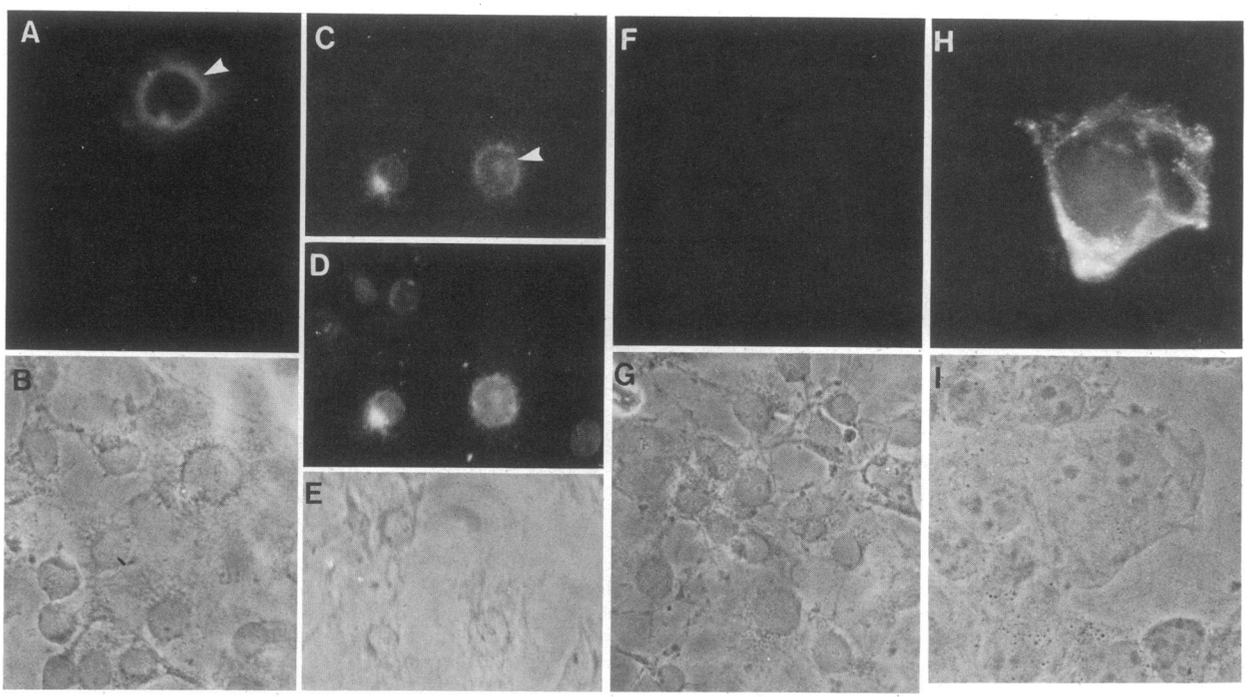

FIG. 4. Indirect immunofluorescence staining of transfected COS cells. Cells were transfected, fixed, and stained as described. The internal staining of COS cells transfected with p9-gB-1 DNA (*A*) and p91023 DNA (*F*) are shown. (*C* and *D*) Double immunofluorescence staining of COS cells with anti-HSV and anti-lamin antibodies, respectively. Arrowheads indicate nuclear membrane staining. (*G*) Surface staining of COS cells transfected with p9-gB plasmid. (*B*, *E*, *G*, and *I*) Phase contrast photomicrograph corresponding to *A*, *C*, *F*, and *H*, respectively.

and *D*). Perinuclear staining corresponding to Golgi apparatus and weak and diffused cytoplasmic fluorescent staining of the endoplasmic reticulum was also observed. Biochemical fractionation of the cells expressing gB-1 proteins and $NaDodSO_4$/polyacrylamide gel analysis of labeled proteins present in the fractions showed that ≈20% of the total gB-1 protein was localized in the nuclear fraction (data not shown). For virus envelopment to occur in the inner membrane of the

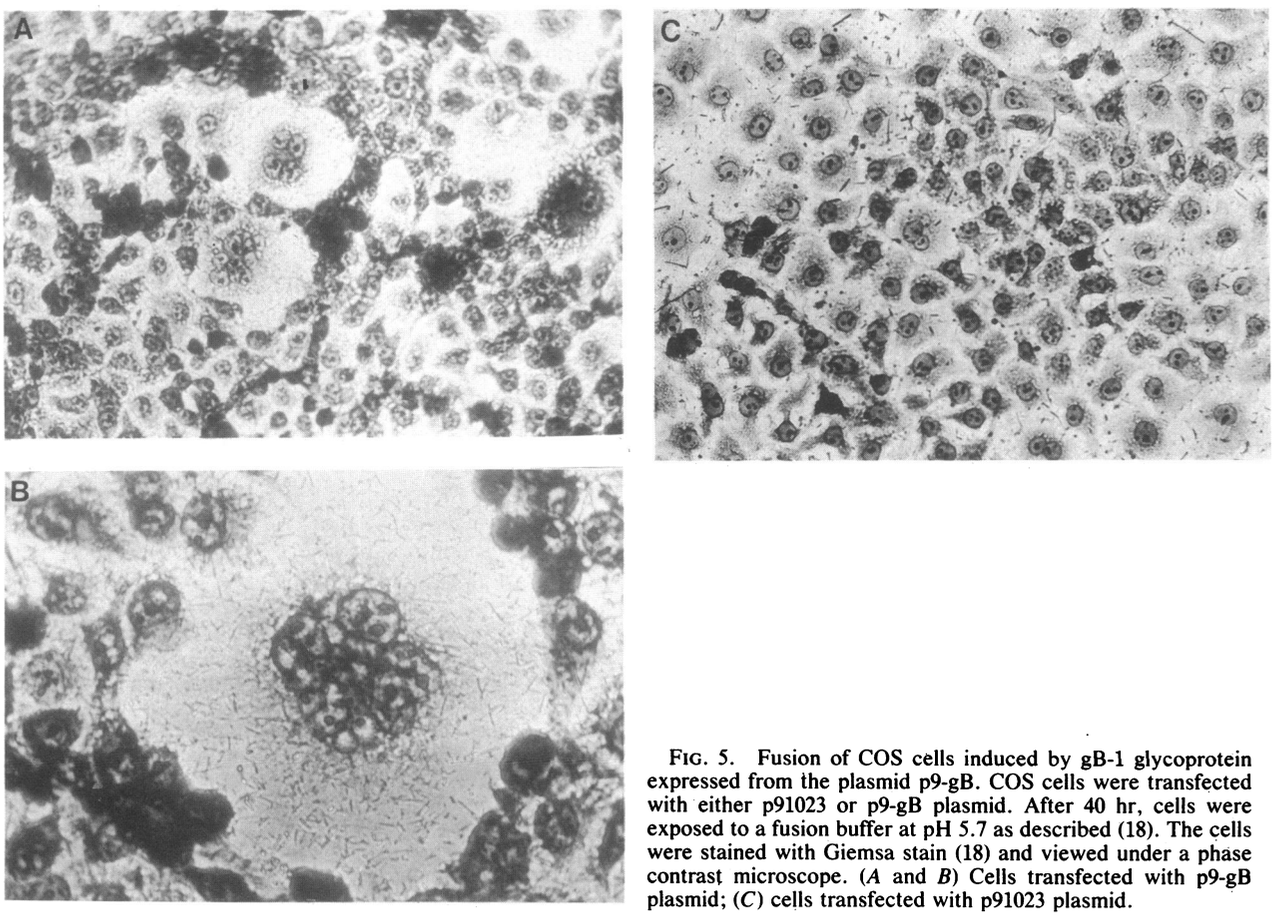

FIG. 5. Fusion of COS cells induced by gB-1 glycoprotein expressed from the plasmid p9-gB. COS cells were transfected with either p91023 or p9-gB plasmid. After 40 hr, cells were exposed to a fusion buffer at pH 5.7 as described (18). The cells were stained with Giemsa stain (18) and viewed under a phase contrast microscope. (*A* and *B*) Cells transfected with p9-gB plasmid; (*C*) cells transfected with p91023 plasmid.

nuclear envelope, gB protein must be transported to the inner nuclear membrane from the endoplasmic reticulum, its site of synthesis, presumably via the outer nuclear membrane. However, the resolution of the immunofluorescence technique could not distinguish between the two nuclear membranes. The expressed gB-1 protein was also detected at the cell surface (Fig. 4*H*). The localization of gB-1 protein in the cell surface is essential for the biological activity of the gB-1 protein involving antigenicity and cell fusion (5).

Cell Fusion Induced by Expressed gB-1 Glycoproteins. The biological activity of the gB-1 protein expressed by the transfected cells was determined by formation of polykaryons containing four to six nuclei as a result of cell fusion induced by exposing the cells to a low pH of 5.7 for a short period of time. Polykaryons were observed only with cells transfected with p9-gB plasmid (Fig. 5 *A* and *B*) but not with cells transfected with the p91023 vector (Fig. 5*C*). Very few polykaryons were observed below pH 5.7.

DISCUSSION

Our results show that glycosylated gB-1 protein can be synthesized in mammalian cells, and a truncated gB-1 protein is also secreted from the cell. The secretion of tgB-1 is dependent on glycosylation, suggesting that transport to the cell surface of tgB-1 requires glycosylation. A similar requirement of glycosylation for migration of the vesicular stomatitus virus G protein to the cell surface has also been reported recently (14, 15). Both gB-1 and tgB-1 were immunologically active and were recognized by anti-gB or anti-HSV antibodies. Recently, *gB* gene has also been expressed in yeast (24) and in *Escherichia coli* (25) as a nonglycosylated but immunologically active protein.

Examination of cells expressing gB-1 protein by immunofluorescence staining shows that gB-1 is localized in the nuclear envelope membrane. Since HSV buds from the inner nuclear membrane, viral glycoproteins synthesized in the endoplasmic reticulum must be transported to the inner nuclear membrane for assembly of the virion. The mechanism of transport of membrane proteins to the nuclear envelope is, however, currently not known. In the case of the nonglycosylated and cytoplasmically synthesized T antigen of simian virus 40, and the nucleoprotein NP of influenza virus, short internal peptides rich in basic amino acids have been reported as a nuclear localization signal (26, 27). In addition to the nuclear envelope, the gB-1 glycoprotein was also localized in the endoplasmic reticulum and the Golgi apparatus. Since the rough endoplasmic reticulum is continuous with the outer nuclear membrane (28), the gB-1 protein may remain attached to the cytoplasmic membranes during its transport from the endoplasmic reticulum to the nuclear envelope. Immunofluorescence analysis of the cell surface of transfected COS cells shows that gB is also transported to the plasma membrane. The presence of gB-1 at the cell surface is essential for the immunological response of HSV-1-infected cells as well as cell fusion induced by the glycoprotein (5). Although the glycoprotein gB has been shown to be essential for virus entry and cell fusion (5, 9–12), other HSV-1-specific glycoproteins such as gC, gD, and gE have also been implicated to play a role in the process of cell fusion induced by HSV (5). The precise roles that these glycoproteins play in HSV-induced cell fusion is, however, not understood. Our results demonstrate that gB protein can induce cell fusion in the absence of any other HSV glycoprotein. The roles that other HSV glycoproteins play in the cell fusion process can thus be studied.

We thank Drs. R. Kaufman, J. Smiley, M. Suh, M. Zweig, G. Cohen, Y. Gluzman, and Y. Raymond for kindly providing materials without which this study was not possible, and Mrs. Dale Tomlinson for typing the manuscript. This work was supported by the Medical Research Council of Canada and National Cancer Institute of Canada.

1. Ghosh, H. P. (1980) *Rev. Infect. Dis.* **2,** 26–39.
2. Gething, M.-J. (1985) in *Protein Transport and Secretion*, ed. Gething, M.-J. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 1–20.
3. Garoff, H. (1985) *Annu. Rev. Cell Biol.* **1,** 403–445.
4. Darlington, R. W. & Moss, L. H., III (1968) *J. Virol.* **2,** 48–55.
5. Spear, P. G. (1984) in *The Herpes Viruses*, ed. Roizman, B. (Plenum, New York), Vol. 3, pp. 315–356.
6. Compton, T. & Courtney, R. J. (1984) *J. Virol.* **49,** 594–597.
7. Bzik, D. J., Fox, B. A., DeLuca, N. A. & Person, S. (1984) *Virology* **133,** 301–314.
8. Pellet, P. E., Kousoulas, K. G., Pereira, L. & Roizman, B. (1985) *J. Virol.* **53,** 243–253.
9. Little, S. P., Jofre, J. T., Courtney, R. J. & Schaffer, P. A. (1981) *Virology* **115,** 149–160.
10. Sarmiento, M., Haffey, M. & Spear, P. G. (1979) *J. Virol.* **29,** 1149–1158.
11. DeLuca, N. A., Bzik, D. J., Bond, V. C., Person, S. & Snipes, W. (1982) *Virology* **122,** 411–423.
12. Manservigi, R., Spear, P. G. & Buchan, A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3913–3917.
13. Kaufman, R. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 689–693.
14. Machamer, C. E., Florkiewiez, R. Z. & Rose, J. K. (1985) *Mol. Cell. Biol.* **5,** 3074–3083.
15. Kotwal, G., Buller, M., Wunner, W., Pringle, C. & Ghosh, H. P. (1986) *J. Biol. Chem.* **259,** 4699–4701.
16. White, E., Blose, S. H. & Stillman, B. W. (1984) *Mol. Cell. Biol.* **4,** 2865–2875.
17. Lebel, S. & Raymond, Y. (1984) *J. Biol. Chem.* **259,** 2693–2696.
18. White, J., Matlin, K. & Helenius, A. (1981) *J. Cell Biol.* **89,** 674–679.
19. Eisenberg, R. J., Hydrean-Stern, C. & Cohen, G. H. (1979) *J. Virol.* **31,** 608–620.
20. Showalter, S. D., Zweig, M. & Hampar, B. (1981) *Infect. Immun.* **34,** 684–692.
21. Rose, J. K. & Bergmann, J. E. (1983) *Cell* **34,** 513–524.
22. Shidar, H. & Matsumoto, S. (1983) *Cell* **33,** 423–434.
23. Wills, J. W., Srinivas, R. V. & Hunter, E. (1984) *J. Cell Biol.* **99,** 2011–2023.
24. Nozaki, C., Makizumi, K., Kino, Y., Nakatake, H., Eto, T., Mizuno, K., Hamada, F. & Ohtomo, N. (1985) *Virus Res.* **4,** 107–113.
25. Person, S., Warner, S. C., Bzik, D. J., Debroy, C. & Fox, B. A. (1985) *Gene* **35,** 279–287.
26. Kalderon, D., Roberts, B. L., Richardson, W. D. & Smith, A. E. (1984) *Cell* **39,** 499–509.
27. Davey, J., Dimmock, N. J. & Colman, A. (1985) *Cell* **40,** 667–675.
28. Franke, W. W., Scheer, U., Krohne, G. & Jarasch, E.-D. (1981) *J. Cell Biol.* **91,** 39s–50s.