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# Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus

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**A human cytomegalovirus (HCMV) glycoprotein gene with homology to glycoprotein B (gB) of herpes simplex virus and Epstein-Barr virus and gpII of varicella zoster virus has been identified by nucleotide sequencing. The gene has been expressed in recombinant vaccinia virus and the gene product recognized by monoclonal antibodies and human immune sera. Rabbits immunized with the recombinant vaccinia virus produced antibodies that immunoprecipitate gB from HCMV-infected cells and neutralize HCMV infectivity *in vitro*. These data demonstrate a role for this protein in future HCMV vaccines.**

*Key words:* human cytomegalovirus/glycoprotein B gene/nucleotide sequencing/monoclonal antibodies

## Introduction

Human cytomegalovirus (HCMV) is a member of the herpes virus family and like other human herpes viruses is a ubiquitous agent in human populations. In the majority of cases HCMV infection does not lead to any apparent disease, but under certain circumstances the virus is known to cause serious illness. Firstly, HCMV is a major cause of congenital disease (Weller, 1981), characterized by a large and varied range of disorders. Secondly, the virus can cause severe problems in immunocompromised individuals with complications due to HCMV infection often hampering the success of transplant surgery (Glen, 1981).

Vaccines that prevent or reduce CMV-associated disease are clearly needed. At present our poor understanding of the biology and pathology of HCMV does not allow a rational approach to the construction of live, avirulent vaccines. However, experimental live attenuated viruses have been produced and their efficacy and safety are under long-term evaluation (Plotkin, 1985). Careful consideration will need to be given to the widespread use of such live vaccines derived originally from a virus that may cause latent and persistent infections, and which is potentially oncogenic. A more attractive approach is the development of sub-unit vaccines based upon the surface glycoproteins of the virus.

Like other herpes viruses HCMV specifies multiple glycoproteins (Stinski, 1976; Pereira *et al.*, 1984). Characterization of these have involved studies of CMV-infected cells and purified virions using polyclonal sera and monoclonal antibodies (Pereira *et al.*, 1982a,b, 1984; Britt, 1984; Nowak *et al.*, 1984; Law *et al.*, 1985; Rasmussen *et al.*, 1985a,b; Britt and Auger, 1986). One glycoprotein has been partially purified and shown to elicit a neutralizing response in guinea pigs (Rasmussen *et al.*, 1985b). However, the total number of CMV-specified glycoproteins remains uncertain and the vaccine potential of individual glycoproteins is unknown. Purification of individual glycoproteins from HCMV-infected cells is a daunting prospect because the virus grows slowly and fails to shut down host cell protein synthesis during infection.

The approach we are using to identity HCMV glycoproteins which elict HCMV-neutralizing antibodies is to identify HCMV genes possessing glycoprotein characteristics by nucleotide sequencing. These genes are then cloned and expressed in vaccinia virus. The live recombinant virus is used to immunize animals and raise monospecific antisera which are tested for their ability to neutralize HCMV infectivity *in vitro*. Using this approach we have identified and expressed a glycoprotein gene that is shown to be conserved throughout the human herpes viruses. This antigen is a target for antibody-mediated virus neutralization *in vitro*. The implications for future HCMV vaccines are discussed.

## Results

### *Identification of CMV glycoprotein gene(s)*

Genes coding for putative glycoproteins in the HCMV genome were sought by sequencing cloned restriction fragments (Oram *et al.*, 1982) using the M13/dideoxy nucleotide chain termination method (Sanger *et al.*, 1980) (strategy and methods described by Bankier and Barrell, 1983). These sequences were analysed for open reading frames and RNA polymerase II transcriptional control signals. Open reading frames were then further examined for the presence of glycoprotein characteristics, namely, an amino-terminal hydrophobic signal peptide, a hydrophobic transmembrane sequence close to the carboxy terminus and potential N-linked glycosylation sites in the external domain.

Using these criteria we identified a putative glycoprotein gene within the 20-kb *Hin*dIII F fragment of HCMV. The primary translation product of this gene (open reading frame HFLF2) is a 906 amino acid polypeptide containing 16 potential N-linked glycosylation sites. Hydrophobicity plots of this protein generated using the program ANALYSEP (Staden, 1984) identify a hydrophobic sequence close to the N terminus (which may function as a signal sequence) and stretches of hydrophobic amino acids at its C terminus (which may function as anchor sequences). Using the program DIAGON (Staden, 1982) we compared the predicted translation product of this gene with glycoprotein genes of other human herpes viruses. The search revealed homology with glycoprotein B (gB) of herpes simplex virus (HSV) (Bzik *et al.*, 1984; Pellet *et al.*, 1985a) and Epstein-Barr virus (EBV) (Pellet *et al.*, 1985b); varicella zoster virus (VZV) also possesses a glycoprotein gene with homology (Davison *et al.*, 1986; Keller *et al.*, 1986). For this reason the protein encoded by reading frame HFLF2 is subsequently referred to as HCMV gB.

The nucleotide sequence and deduced amino acid sequence of HCMV gB is shown in Figure 1. The complete sequence and analysis of the *Hin*dIII F fragment will be presented elsewhere. Figure 2 shows an alignment of the gB proteins of HSV-1, EBV and HCMV and demonstrates that the proteins are homologous

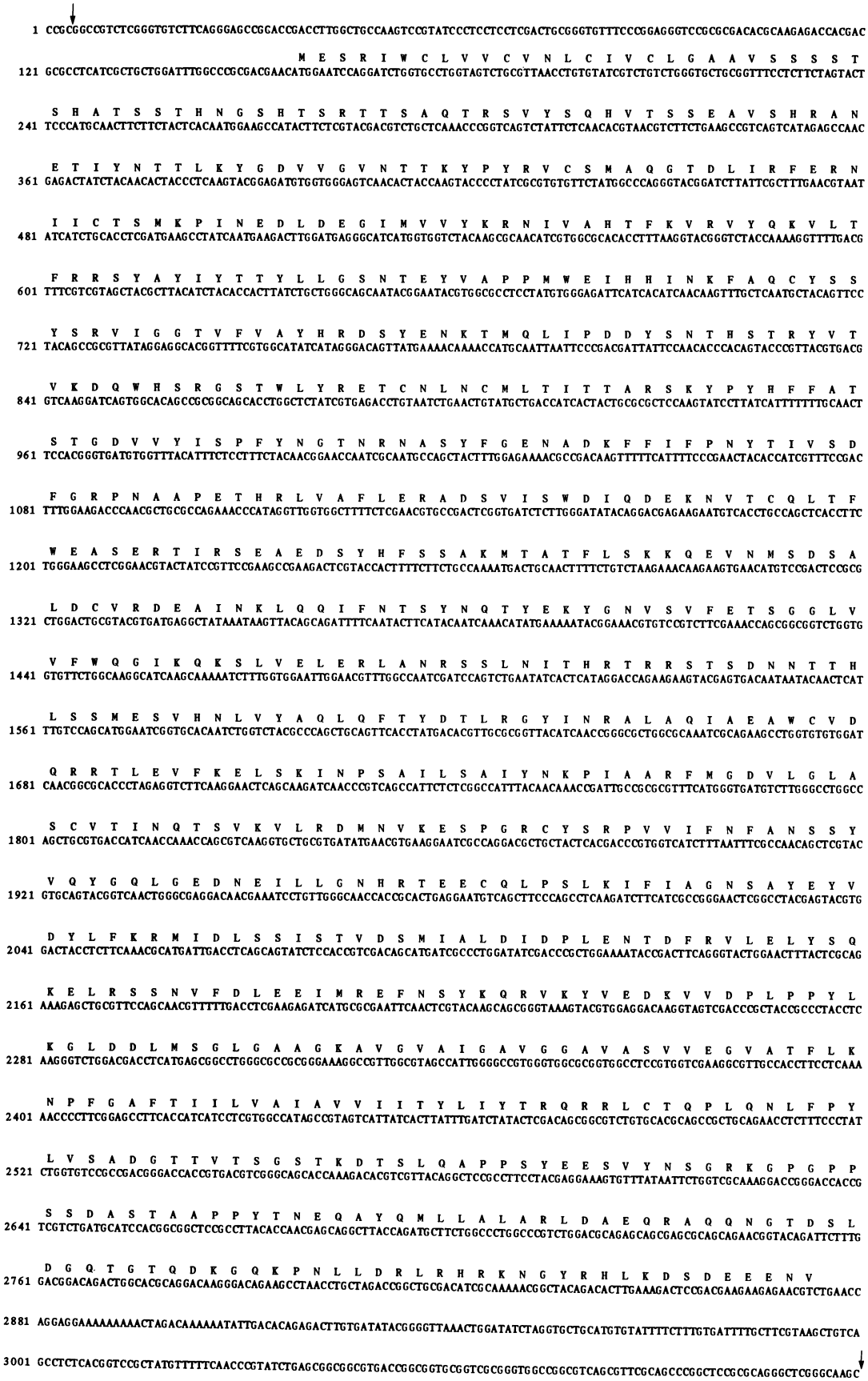


**Fig. 1.** Nucleotide sequence of a 3125-bp fragment containing the coding region for the HCMV gB. The amino acid sequence of the gB polypeptide is represented in the one-letter code above the nucleotide sequence. The *Xma*III sites used to clone the gB gene into vaccinia virus are marked by arrows.

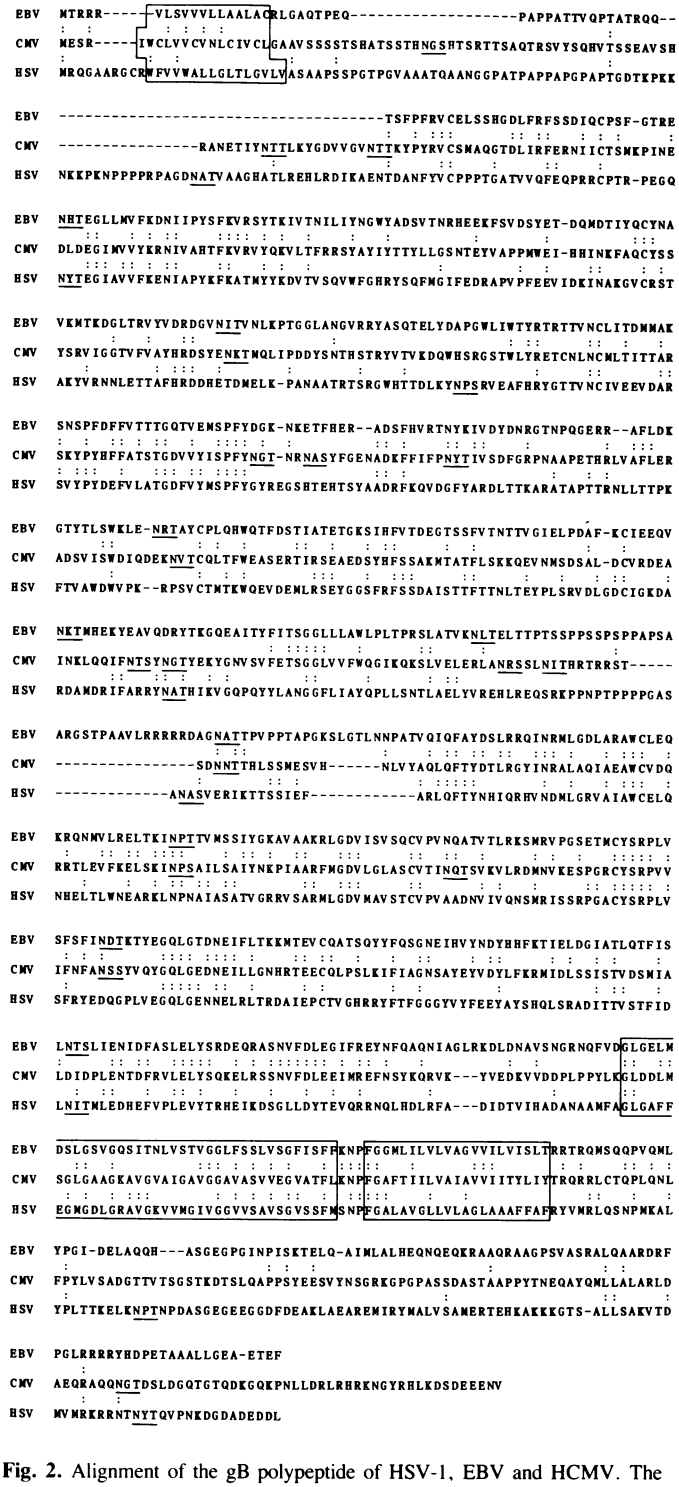

**Fig. 2.** Alignment of the gB polypeptide of HSV-1, EBV and HCMV. The sequences are displayed in the one-letter amino acid code. Dashes have been introduced into the sequence to generate maximum alignment of homologous amino acids. Identical amino acids are marked : and potential N-linked glycosylation sequences are underlined. A stretch of hydrophobic amino acids at the N terminus (potential signal sequence) and stretches of hydrophobic amino acids at the C terminus (potential transmembrane regions) are boxed.

along a large proportion of their length with the N and C termini showing least conservation. It can be seen that at 121 positions there is an identically matched amino acid in all three proteins. Taken as a proportion of EBV gB this means that over 14% of the protein is perfectly conserved. Furthermore, all 10 cysteine residues present between the putative signal and anchor sequences

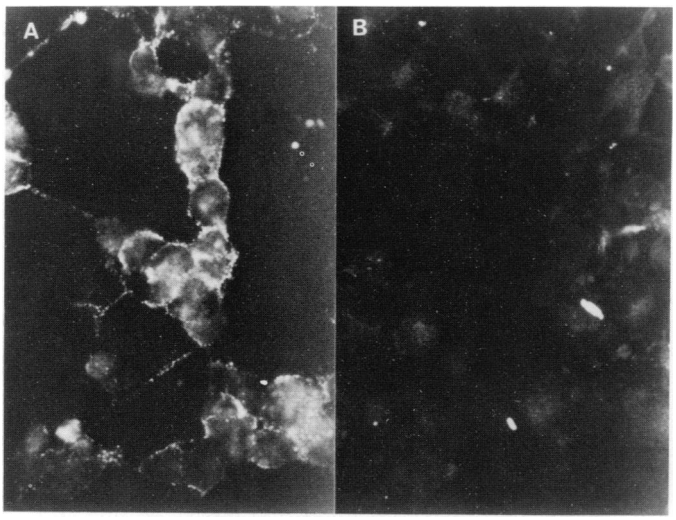


**Fig. 3.** Expression of HCMV gB on the surface of HCMV gB-VAC recombinant virus infected cells. Monoclonal antibody 39 was reacted with CV-1 cells infected either with HCMV gB-VAC **(A)** or WT vaccinia virus **(B)**. Bound antibody was detected with fluorescein-conjugated anti-mouse immunoglobulin under u.v. illumination at ×400.

are perfectly aligned, suggesting that the extracellular portion of the proteins may possess a similar overall structure. The extent of the homology between the three viral proteins provides convincing evidence that the putative HCMV glycoprotein shown in Figure 1 is that of gB.

To investigate the nature of the HCMV gB and to raise antisera against this protein the gene was excised from the *Hin*dIII F fragment of the HCMV genome and expressed in recombinant vaccinia virus. This vector system is suitable for the expression of eukaryotic virus glycoprotein genes because the proteins are correctly processed and inserted into the infected cell membrane (Mackett and Arrand, 1985; Mackett *et al.*, 1985a; Panicali *et al.*, 1983; Paoletti *et al.*, 1984; Smith *et al.*, 1983a,b; Wiktor *et al.*, 1984). In addition, the infectious recombinant virus may be used to raise monospecific antisera against the foreign protein in vaccinated animals.

*Construction of recombinant vaccinia virus expressing HCMV gB*

Recombinant vaccinia viruses expressing foreign genes are constructed in two steps. Firstly, the foreign protein coding sequences are cloned into a specially designed plasmid vector downstream of a vaccinia virus promoter (Mackett *et al.*, 1984; Boyle *et al.*, 1985; Chakrabarti *et al.*, 1985). Secondly, the gene is inserted into the vaccinia virus genome by homologous recombination in cells infected with vaccinia virus and transfected with plasmid DNA (for review, see Smith and Moss, 1984). Analysis of the nucleotide sequence of the HCMV gB gene showed *Xma*III restriction endonculease sites 148 nucleotides upstream and 251 nucleotides downstream of the gB protein coding sequence. There were no ATG codons between the upstream *Xma*III site and the codon initiating translation of the gB open reading frame. Therefore this *Xma*III fragment was cloned downstream of a vaccinia promoter in plasmid pGS62 (Materials and methods) and the resulting plasmid pSB2 used to generate recombinant virus HCMV gB-VAC by established methods (Mackett *et al.*, 1984). The $TK^-$ recombinant virus was purified and its genome analysed by restriction endonuclease digestion and Southern blotting (data not shown). The results confirmed that the HCMV gB gene had been inserted into the vaccinia TK gene and no other genomic alterations had occurred.

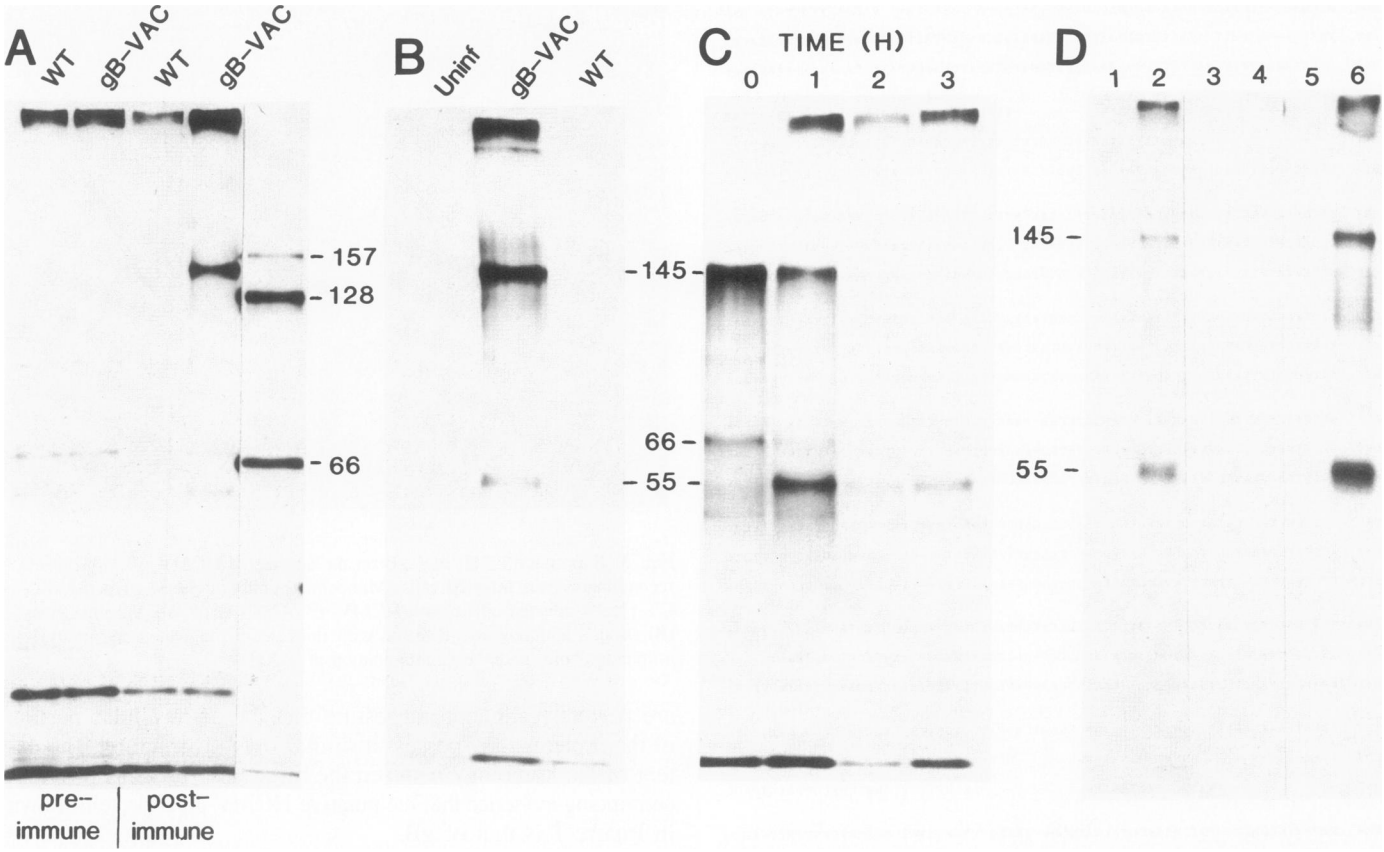


**Fig. 4.** Analysis of HCMV gB polypeptides. **(A)** Lysates from CV-1 cells infected with WT vaccinia or HCMV gB-VAC were reacted with either pre-immune sera or sera from a rabbit hyperimmunized with HCMV (kindly provided by K.Powell). Protein mol. wt markers are the HSV-1 major capsid antigen (157 kd), gB (128 kd) and VP16 (66 kd). **(B)** Lysates from CV-1 cells infected with WT vaccinia, HCMV gB-VAC or uninfected were immunoprecipitated with anti-HCMV monoclonal antibody 37. **(C)** Pulse-chase analysis. HCMV gB-VAC-infected CV-1 cells were pulsed labelled with [$^{35}$S]methionine from 4.5 to 5 h post-infection and then chased in medium containing 0.1 mM methionine for 1, 2 or 3 h. Cell lysates were immunoprecipitated with a pool of monoclonal antibodies (34, 37 and 39) that recognize HCMV gB. **(D)** Lysates from MRC-5 cells infected with HCMV (**lanes 2, 4** and **6**) or uninfected (**lanes 1, 3** and **5**) were immunoprecipitated with either pooled monoclonal antibodies (34, 37 and 39) (**lanes 5** and **6**), or pre-immune rabbit (**lanes 3** and **4**) or sera taken from a rabbit immunized with HCMV gB-VAC (**lanes 1** and **2**).

*Expression of HCMV gB by recombinant vaccinia virus*

A series of murine monoclonal antibodies raised against HCMV and shown to have virus neutralizing activity *in vitro* (H.Hart, unpublished observations) were tested for their ability to bind to cells infected with the HCMV gB-VAC. Virus plaques containing antigen recognized by the monoclonal antibody were visualized as black dots on autoradiographs in the case of [$^{125}$I]-protein A binding antibodies, or as red plaques where peroxidase-conjugated antiglobulin, substrate and chromogen were added to cell monolayers. Four out of 10 monoclonal antibodies bound to plaques formed by HCMV gB-VAC but not wild-type (WT) vaccinia virus (data not shown). It was noted that all the plaques formed by HCMV gB-VAC bound the monoclonal antibody indicating the virus was pure stock not contaminated with WT vaccinia. Similar conclusions were reached by plaquing the virus on TK$^-$ 143 cells in the presence or absence of BUdR and by analysis of the virus genomic DNA by Southern blotting.

To examine if the HCMV gB synthesized in cells infected with the recombinant virus is transported to the cell surface, indirect immunofluorescence studies were performed on non-permeabilized cells using monoclonal antibodies that were positive in binding to HCMV gB-VAC-infected cells. As shown in Figure 3, HCMV gB-VAC-infected cells showed positive surface membrane fluorescence compared with the WT-infected control. The pattern of staining on the infected cell membrane was unusual showing a granular appearance, suggesting clustering or aggregation of HCMV gB in the cell membrane.

*Analyses of gB protein*

The gB polypeptide(s) synthesized within HCMV gB-VAC-infected cells were characterized by immunoprecipitation of [$^{35}$S]-methionine-labelled cell lysates with either a polyvalent rabbit sera raised against purified HCMV or anti-HCMV gB monoclonal antibodies. The polyvalent rabbit sera immunoprecipitated a polypeptide of 145 kd from HCMV gB-VAC-infected cells but not WT vaccinia-infected cells (Figure 4A). Other polypeptides were non-specifically precipitated as shown by their reactivity with pre-immune rabbit sera.

Anti-HCMV monoclonal antibody 37 also precipitated the 145-kd protein and a further polypeptide of 55 kd (Figure 4B). Similar data were obtained with other monoclonal antibodies (34 and 39) that bound to HCMV gB-VAC-infected cells and all these monoclonal antibodies were unable to immunoprecipitate similar bands from either WT vaccinia-infected or uninfected cells. Since each monoclonal antibody was able to immunoprecipitate both the 145-kd and 55-kd species it suggests that either the same epitopes are present on both polypeptides or that these proteins are physically associated and consequently co-precipitate. The 55-kd protein is probably also present in Figure 4A but the presence of other co-migrating bands made identification uncertain.

To investigate the relationship between the 145- and 55-kd species a pulse chase experiment was performed (Figure 4C). After a pulse-labelling period (30 min) the majority of $^{35}$S-labelled protein recognized by a pool of anti-HCMV monoclonal antibodies (34, 37 and 39) was present as a 145-kd protein, although smaller species of 66 kd and possibly 55 kd were also detectable (lane 0). On chase the majority of the radioactivity shifted from the 145- to the 55-kd protein. The 66-kd band was also greatly reduced on chase suggesting that it may be unstable or may also be a precursor to the 55-kd species. The remainder of the 145-kd precursor, a 90-kd fragment, is presumably either not recognized by these antibodies or it may be rapidly degraded. The latter possibility is supported by the observation that polyclonal anti-HCMV sera also fail to detect a 90-kd fragment.

The non-glycosylated precursor to the 145-kd polypeptide was identified by pulse-labelling HCMV gB-VAC-infected cells in the presence of tunicamycin. Following immunoprecipitation with anti-gB monoclonal antibodies the 145- and 55-kd proteins were greatly reduced and were replaced by a 108-kd polypeptide (data not shown). This size is consistent with the primary translation product of the HCMV gB gene (102 kd).

Sera taken from a cardiac transplant patient before and after he experienced a primary HCMV infection were tested for the ability to recognize HCMV gB synthesized by recombinant vaccinia virus. Only the serum taken after the HCMV infection was able to immunoprecipitate a 145-kd polypeptide from $^{35}$S-labelled HCMV gB-VAC-infected cell lysates (data not shown). In contrast both serum samples were able to precipitate the influenza virus haemagglutinin from lysates of cells infected with another recombinant vaccinia virus expressing the haemagglutinin of influenza A/NT/60/68 (G.L.Smith and K.Gould, unpublished data). Serum samples from other cardiac transplant patients who experienced HCMV infections during immunosuppression also immunoprecipitated HCMV gB synthesized by recombinant vaccinia virus.

*Vaccination of rabbits with HCMV gB-VAC*

Two rabbits were vaccinated with purified HCMV gB-VAC and a third with another TK$^-$ vaccinia recombinant expressing the influenza virus nucleoprotein (Yewdell *et al.*, 1985). Sera taken from both animals immunized with the HCMV gB-VAC were able to immunoprecipitate polypeptides of 145 and 55 kd from MRC-5 cells infected with HCMV but not uninfected cells (Figure 4D, lanes 1 and 2; data shown for only one animal). These bands co-migrated with polypeptides immunoprecipitated by monoclonal antibodies against HCMV gB (Figure 4D, lane 6). Pre-immune rabbit serum was unable to immunoprecipitate these polypeptides (Figure 4D, lanes 3 and 4).

Sera from all three rabbits were next tested for their ability to neutralize HCMV infectivity *in vitro* (Table I). Both rabbits immunized with HCMV gB-VAC produced antibodies which could neutralize HCMV infectivity while the third rabbit immunized with a TK$^-$ recombinant vaccinia virus expressing the influenza *NP* gene could not. All neutralization was dependent upon addition of fresh complement. Revaccination with purified virus and subsequent intramuscular injection of rabbit cells infected with HCMV gB-VAC in incomplete Freund's adjuvant resulted in significant boosting of antibody titres. Higher dilutions of sera (1:250) taken 151 days after vaccination of rabbits 1 and 2 reduced HCMV plaque formation by 79% and 52% respectively.

The restricted host range of HCMV means that no animal model system is available for HCMV protection experiments.

**Table I.** Neutralization of HCMV by rabbit sera

| Day post vaccination | HCMV plaque reduction (%) | | | |
|---|---|---|---|---|
| | Rabbit 1 | | Rabbit 2 | |
| | 1/10 | 1/50 | 1/10 | 1/50 |
| 0 | 0 | 0 | 0 | 0 |
| 46 | 54 | 30 | 40 | 16 |
| 59 | 69 | 60 | 57 | 76 |
| 76 | 70 | 61 | 48 | 30 |
| 116 | 67 | 68 | 55 | 25 |
| 151 | 97 | 87 | 77 | 77 |

Rabbits were vaccinated intradermally with $10^8$ p.f.u. of purified HCMV gB-VAC at one site on each flank. Forty-six days later animals were revaccinated similarly; 132 days post primary vaccination animals were injected intramuscularly with rabbit kidney cells infected with HCMV gB-VAC emulsified in incomplete Freund's adjuvant. A third rabbit was similarly vaccinated and revaccinated with TK$^-$ recombinant virus NP-VAC (Yewdell *et al.*, 1985). Dilutions of rabbit sera were assayed for HCMV neutralization as described in Materials and methods. Sera taken from rabbit 3 days before, or 93 days after vaccination with NP-VAC gave no reduction in HCMV plaque numbers.

In view of this, *in vitro* neutralization experiments are all that can reasonably be accomplished short of clinical trials. Clearly, however, the potential for developing an anti-HCMV vaccine based upon gB is supported by these data reported here.

## Discussion

Nucleotide sequencing of the *Hin*dIII F region of the HCMV genome has identified an open reading frame with characteristics typical of a glycoprotein gene. The deduced amino acid sequence of this glycoprotein gene has extensive homology with gB of HSV (Bzik *et al.*, 1984; Pellet *et al.*, 1985a), EBV (Pellet *et al.*, 1985b) and a glycoprotein of VZV recently termed gpII (Davison *et al.*, 1986; Keller *et al.*, 1986). The gB from HSV, EBV and HCMV are of a similar overall size and also probably have external domains with similar structure due to the conserved number and position of cysteine residues. Furthermore, analyses of the sequence on both sides of HCMV gB have revealed that this gene is present within a section of the HCMV genome conserved throughout the human herpes viruses (T.Kouzarides, in preparation). Consequently this gene has been termed HCMV gB. Previously, Pereira *et al.* (1984) named a HCMV glycoprotein 'gB' based upon its electrophoretic mobility in polyacrylamide gels with respect to other HCMV glycoproteins. It is probable that this is not the same protein as that identified here.

The HCMV gB gene has been engineered into vaccinia virus under control of a vaccinia promoter that is active throughout the virus infection. The gene product has been identified within cells infected with this virus by immunoprecipitation with rabbit serum raised against HCMV particles and with murine monoclonal antibodies against HCMV. Two major glycosylated forms of gB, a 145- and 55-kd polypeptide, were identified. Pulse chase experiments demonstrated that the 55-kd protein is derived from a 145-kd precursor and immunofluorescence of non-permeabilized infected cells showed that the gene product is expressed on the cell surface. These properties of HCMV gB expressed via recombinant vaccinia virus are indistinguishable from gB synthesized in HCMV-infected cells. Since vaccinia virus mRNAs are non-spliced it would seem likely that the gB mRNA in HCMV-infected cells would also be unspliced and the contiguous protein coding sequences in HCMV DNA are consistent with this.

Other investigators have obtained murine monoclonal antibodies that recognize HCMV proteins in virions or infected cells that have similar electrophoretic mobilities to gB identified here (Pereira *et al.*, 1984; Nowak *et al.*, 1984). Britt (1984) reported disulphide-linked polypeptides of 160, 116 and 55 kd that were co-precipitated by monoclonal antibody and have recently reported that HCMV envelopes contain only the 116- and 55-kd forms derived from the 160-kd precursor (Britt and Auger, 1986). Similarly, Rasmussen *et al.* (1985a) reported a 55-kd product to be derived from a 130-kd precursor. It seems likely that these proteins and the family of HCMV glycoproteins described as glycoprotein A (Pereira *et al.*, 1984) may be the same as the gB gene products identified here. This may now be directly tested by using the gB product expressed from vaccinia or other vectors. The exact relationship between the 55-kd protein and its 145-kd precursor will require amino acid sequence analyses of the termini of the 55-kd species.

Rabbits vaccinated intradermally with the live vaccinia recombinant HCMV gB-VAC developed antibodies that immunoprecipitated both the 145- and 55-kd species from HCMV-infected cells. These sera and anti-gB monoclonal antibodies should prove useful in characterizing the expression of gB within HCMV-infected cells. Importantly, HCMV infectivity was neutralized by sera from vaccinated rabbits indicating that gB is the target of a neutralizing antibody response. Four anti-HCMV gB monoclonal antibodies used here were also able to neutralize efficiently HCMV infectivity *in vitro* in the presence of complement. Monoclonal antibodies described in other studies against HCMV glycoproteins of similar electrophoretic mobilities to gB described here, have also neutralized HCMV infectivity in the presence of complement. Similarly guinea pig sera raised against immunoaffinity-purified HCMV 130/55-kd proteins also neutralized HCMV *in vitro* in the presence of complement (Rasmussen *et al.*, 1985b).

Immunity to HCMV involves both antibody and cellular immune mechanisms (Quinnan *et al.*, 1982). The investigations of cell-mediated immune reactions to HCMV are severely hampered by the restricted tissue tropism of the virus. The ability of vaccinia recombinants to stimulate cytotoxic T lymphocyte (CTL) responses to cloned gene products in vaccinated animals (Bennink *et al.*, 1984, 1986; Wiktor *et al.*, 1984; Yewdell *et al.*, 1985) and to infect a broad range of target cells for cytotoxicity assays makes these viruses versatile tools for studying cellular immune responses. This approach has been used to identify influenza virus antigens recognized by CTL in mouse (Bennink *et al.*, 1984, 1986; Yewdell *et al.*, 1985) and man (McMichael *et al.*, 1986) and experiments are in progress to see if HCMV gB is recognized by CTL taken from humans after HCMV infections.

The role of HCMV gB in virus infection is unknown; however HSV gB is a major component of the virion envelope, is a target for antibody-mediated neutralization and has also been implicated in the penetration of the virus into cells (Spear, 1985). Similarly, VZV gB is an abundant virion protein and is the target for neutralizing antibodies (Davison *et al.*, 1986). The fact that four out of 10 anti-HCMV monoclonals that are able to neutralize HCMV infectivity recognize HCMV gB suggests this protein is highly immunogenic or abundant in virions or both. We have demonstrated here that presentation of HCMV gB to the rabbit immune system through its expression in vaccinia virus raises antibodies which neutralize virus infectivity *in vitro*. Sera taken from humans following HCMV infection also contain antibodies that recognize this protein. Taken together these data strongly support the inclusion of this protein in future HCMV vaccines. Vaccinia virus has itself been proposed as a vector system for the delivery of foreign antigens to the immune system (Mackett and Arrand, 1985; Mackett *et al.*, 1985a; Panicali *et al.*, 1983; Paoletti *et al.*, 1984; Moss *et al.*, 1984; Smith *et al.*, 1983a,b, 1984; Wiktor *et al.*, 1984). Whilst immunization of immunocompromised individuals, who are at high risk from HCMV-related disease, with a live vaccinia virus vaccine is contraindicated, the possibility of using such a vaccine in other circumstances should not be excluded. Alternatively, this gene could be expressed in other vector systems towards the production of a subunit vaccine. Here we have used the vaccinia vector system to express the first HCMV glycoprotein gene to be identified and sequenced and to demonstrate the vaccine potential of its gene product.

## Materials and methods

*Cells and viruses*

HCMV strain AD169 was grown in MRC-5 cells in Glasgow's modified minimal essential medium (GMEM) containing 10% fetal bovine serum (FBS). Virus infectivity was measured by plaque assay on MRC-5 cell monolayers. Infected cells were overlaid with GMEM containing 10% FBS and 0.64% carboxymethyl cellulose and incubated at 37°C for 10 days. Cells were fixed in 10% formol saline, stained with 0.1% toluidene blue and plaques visualized microscopically. Vaccinia virus strain WR was grown in HeLa cells and infectivity titres determined on CV-1 cell monolayers previously described (Mackett *et al.*, 1985b). A $TK^-$ recombinant vaccinia virus that contained the HCMV gB gene under control of a vaccinia promoter was constructed from plasmid pSB2 and wild-type (WT) vaccinia virus using previously described methods (Mackett *et al.*, 1984).

*Plasmid constructions*

The HCMV gB gene was isolated from plasmid pAT153 that contained the 20-kb *Hin*dIII F fragment of the HCMV genome (Oram *et al.*, 1982). pAT153/HCMV *Hin*dIII F was digested with *Bam*HI and a 8.5-kb fragment isolated and self-ligated to form pSB1. pSB1 was digested with *Hin*dIII and *Bam*HI and a 5-kb *Hin*dIII–*Bam*HI fragment isolated that contained the HCMV gB gene. This fragment was further digested with *Xma*III and a 3.1-kb fragment isolated and its termini filled in by incubation with Klenow DNA polymerase. Plasmid pGS62 [derived from pGS20 (Mackett *et al.*, 1984) by deletion of the *Eco*RI site upstream of the translocated vaccinia virus promoter] was cut with *Sma*I, its termini were dephosphorylated and it was then ligated with the 3.1-kb DNA fragment containing the HCMV gB gene to form pSB2. The orientation of the gB gene with respect to the vaccinia promoter was checked by digestion of pSB2 with *Eco*RI which cuts pGS62 once and the HCMV gB gene once asymmetrically.

*Identification of anti-HCMV gB monoclonal antibodies*

Monoclonal antibodies against HCMV were screened for reactivity against gB by binding to plaques formed on CV-1 cell monolayers by either WT vaccinia virus or recombinant HCMV gB-VAC. The procedure was essentially as described (Smith *et al.*, 1983b; Mackett *et al.*, 1985a,b) in which bound antibody was detected using $^{125}$I-labelled Staph A protein. Alternatively, anti-HCMV gB antibodies that did not bind Staph A protein were detected by incubation with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako) for 1 h at room temperature. After washing 10 times in PBS the cell monolayers were incubated with $H_2O_2$ and 3-amino-9-ethylcarbazole as chromogenic substrate.

*Immunoprecipitation*

CV-1 cells infected at 30 plaque-forming units (p.f.u.)/cell with either WT vaccinia virus or recombinant HCMV gB-VAC or mock-infected were incubated in methionine free medium for 3–3.5 h post-infection and then radiolabelled by incubation in 100 μCi/ml of [$^{35}$S]methionine for 2.5 h. MRC-5 cells infected at 5 p.f.u./cell with HCMV or mock-infected were labelled with [$^{35}$S]methionine (28 μCi/ml) 72–96 h post-infection. Cells were scraped from the bottles, collected by centrifugation, washed in PBS and then lysed in RIPA buffer (0.05 M Tris–HCl pH 7.2, 0.15 M NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 μg/ml DNase, 2 mM phenylmethylsulphonyl fluoride) for 10 min on ice. Lysates were centrifuged (30 000 r.p.m., 60 min, 4°C, Beckman SW 50.1 rotor) and supernatants incubated for 20 min at room temperature with rabbit serum, human sera or anti-HCMV monoclonal antibodies. Immune complexes were precipitated by 50 μl of a 50% suspension of Protein A Sepharose for 2 h at 4°C, the beads washed three times in RIPA and bound material eluted in 10% SDS, 1 M Tris pH 6.8, 50% glycerol, 0.2% bromophenol blue and 1.5% dithiothreitol. Samples were boiled for 3 min and electrophoresed through 10% polyacrylamide gels. Gels were fixed in methanol/acetic acid, impregnated with fluorographic enhancer (Amplify, Amersham) and autoradiographs prepared.

*Membrane immunofluorescence*

CV-1 cells were grown on glass coverslips and infected with WT vaccinia virus

or HCMV gB-VAC at 30 p.f.u./cell. Thirteen hours later cells were washed in PBS, fixed in an isotonic solution of 2% paraformaldehyde for 30 min at room temperature and then incubated in PBS containing 1% BSA. Cell monolayers were then incubated with monoclonal antibodies diluted in PBS/1% BSA for 1 h at room temperature, washed extensively in PBS, and bound antibody detected by incubation with fluorescein-conjugated rabbit anti-mouse immunoglobulin (Dako) diluted 1:20 in PBS containing 1% BSA and 2% normal rabbit serum. Fluorescence was observed with u.v. illumination ×400.

*HCMV neutralization assays*

Serum samples were heated at 56°C for 30 min to inactivate the complement. Serum dilutions were then incubated with an equal volume of HCMV (750 p.f.u.) for 30 min at 37°C. Fresh non-immune rabbit serum was then added to a final concentration of 5% as a source of complement and the mixture incubated for 30 min at 37°C before assay of residual virus on MRC-5 cells. Plaques were counted 10 days later and data expressed as percentage reduction in plaque numbers.

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