Molecular Characterization of the Guinea Pig Cytomegalovirus UL83 (pp65) Protein Homolog

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Abstract. The tegument phosphoproteins of human cytomegalovirus (HCMV) elicit cytotoxic T-lymphocyte (CTL) responses and are hence candidates for subunit vaccine development. Little is known, however, about the tegument proteins of nonhuman cytomegaloviruses, such as guinea pig CMV (GPCMV). DNA sequence analysis of the *Eco* R I ''C'' fragment of the GPCMV genome identified an open reading frame (ORF) which is colinear with that of the HCMV tegument phosphoprotein, UL83 (pp65). This ORF was found to have identity to HCMV UL83 and was predicted to encode a 565-amino-acid (aa) protein with a molecular mass of 62.3 kDa. Transcriptional analyses revealed that a GPCMV UL83 probe hybridized with both 2.2 kb and 4.2 kb mRNA species at 48 h post-infection (p.i.); synthesis of these messages was blocked by phosphonoacetic acid (PAA), defining these as ''late'' gene transcripts. *In vitro* translation of the UL83 ORF in reticulocyte lysate resulted in synthesis of a 65 kDa protein. Immunofluorescence experiments revealed that the putative GPCMV UL83 homolog exhibited a predominantly nuclear localization pattern. Polyclonal antisera were raised against a UL83/glutathione-*S*-transferase (GST) fusion protein. This antibody identified a 70-kDa virion-associated protein, the putative GPCMV UL83 homolog, in immunoblot and radioimmunoprecipitation experiments. Labeling experiments with ^{32}P-orthophosphate indicated that the GPCMV UL83 protein is phosphorylated. Western blot analysis of glycerol tartrate gradient-purified virions and dense bodies confirmed that the putative GPCMV UL83 homolog was a constituent of both fractions.

Key words: guinea pig cytomegalovirus, UL83 gene, CMV vaccines

Introduction

Infection with human cytomegalovirus (HCMV), although usually asymptomatic in healthy individuals, is capable of causing severe disease in immunocompromized patients and newborn infants (1). Congenital HCMV infection, which occurs in 0.5–2.0% of all live births, can cause severe neurologic handicap, including sensorineural hearing loss (2). Seroepidemiologic studies indicate that congenital infections which occur in the setting of nonprimary maternal infection are only rarely associated with sequelae, suggesting that preexisting maternal immunity protects the fetus against symptomatic disease (3). Therefore, prenatal maternal immunization against CMV might substantially reduce the morbidity associated with congenital CMV infection. Ideally, vaccines for prevention of HCMV disease should undergo preclinical efficacy evaluation in animal models prior to human testing. However, the strict species-specificity of cytomegaloviruses complicates the animal model investigation of HCMV vaccines. The only relevant small animal model for the evaluation of vaccines designed to prevent congenital CMV infection is the guinea pig model. In contrast to the cytomegaloviruses of other small mammals, the guinea pig cytomegalovirus (GPCMV)

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crosses the placenta, causes disease *in utero,* and produces signs of illness in newborn guinea pigs similar to those observed in human infants (4). The structural similarities between the human and guinea pig placentas (5), coupled with the relatively long gestational period of guinea pigs, makes this model uniquely well suited to vaccine studies. Hence, the detailed molecular characterization of immunogenic GPCMV gene products may provide important insights into those aspects of the host immune response to CMV infection which are important in conferring protection against fetal damage.

Recent studies have indicated that humoral immunity against GPCMV glycoproteins appears to provide some protection to the fetus in the setting of active maternal infection. Passive administration of a high-titer anti-GPCMV polyclonal antisera to pregnant dams prior to viral challenge significantly improved pup survival in one study (6), and active immunization with a lentil-lectin column purified glycoprotein vaccine prior to conception conferred statistically significant protection against adverse pregnancy outcomes in another study (7). The major component of the protective antibody response in each of these studies appeared to target the glycoprotein B homolog of GPCMV (8). Antibodies to the recently identified GPCMV glycoprotein H (gH) were also present (9), confirming that gH may be another potential subunit candidate protein suitable for inclusion in a vaccine for prevention of congenital CMV infection.

In contrast to these detailed studies confirming the importance of humoral responses against envelope glycoproteins, relatively little information exists concerning the role of cell-mediated immune (CMI) responses in protection against GPCMV infection. The majority of HCMV-seropositive individuals possess $CD8^+$ cytotoxic T cell (CTL) responses directed against a subset of viral antigens located between the viral envelope and the nucleocapsid referred to as the viral tegument (10). The HCMV UL83 (pp65) matrix phosphoprotein is the major target of these CTLs (11,12). The UL83 protein is a major constituent of the tegument of both intact virions as well as non-infectious particles, the so-called dense bodies, which are secreted by infected cells along with virions (10). In addition to being a major CTL target, UL83 also facilitates immune evasion of cytotoxic-T-cell recognition by virtue of blocking immediate early antigen processing and presentation (13). The value of CMV-specific CTL responses which target the UL83

protein has been dramatically demonstrated in clinical studies in bone marrow transplant patients. UL83-specific CTLs can be isolated from HCMV-seropositive bone marrow donors, propagated *in vitro,* and adoptively transferred to transplant recipients, in the process conferring protection against HCMV viremia and pneumonitis (14). Other matrix phosphoproteins in the HCMV virion, including UL32 (pp150), UL82 (pp71), and UL99 (pp28), also induce strong immune responses in HCMV-infected individuals (10), although little is known about the role of CMI against these proteins in protection against disease.

The potential role which CMI responses against CMV tegument proteins may play in protecting the fetus in the setting of maternal infection during pregnancy is unknown. Conceivably, complete protection against congenital CMV disease may require the inclusion of CTL targets, and not just envelope glycoproteins, in subunit vaccines. Although subunit vaccines containing glycoproteins induce strong antibody reponses and provide some protection against adverse fetal outcome (7), this protection is likely incomplete. For example, data from clinical efficacy trials of subunit vaccines for other herpesviruses indicates that strategies which target only envelope glycoproteins fail to provide adequate protection against infection (15). Therefore, *in vivo* studies of vaccines capable of inducing CMI responses in models of congenital infection such as the GPCMV model are likely to provide important insights into the relative roles of humoral and cellular immunity in protection against disease. Unfortunately, to date there have been very few studies of GPCMV gene products, and no molecular analyses of GPCMV tegument proteins. As a necessary prerequisite to the study of the role of GPCMV tegument proteins in protection against congenital CMV infection, these studies were undertaken to test whether the GPCMV genome encodes a gene homologous to the HCMV UL83 (pp65) tegument protein. In this report, we describe the genomic location, nucleotide sequence, transcriptional analysis, and protein expression of the GPCMV UL83 homolog.

Materials and Methods

Virus and Cell Culture

Guinea pig cytomegalovirus (strain no. 22122; ATCC VR682) was propagated on embryonic guinea pig

lung cells (GPL, ATCC CCL 158). Passaged GPL cells were maintained in F-12 Nutrient medium (Gibco-BRL) supplemented with 10% fetal calf serum (FCS, HyClone Laboratories), 10,000 IU/l penicillin, 10 mg/l streptomycin, L-glutamine, and 7.5% $NaHCO_3$. Viral stock was prepared and titered by plaque assay using standard methodologies. Cell lines were screened for mycoplasma using a PCR detection system (Stratagene).

Plasmids and Sequence Analyses

The *Eco* RI ''C'' fragment of the GPCMV genome is positioned at map units 0.52–0.60 of the GPCMV genome (16), with its leftward margin (D-C junction) corresponding approximately to the position of the GPCMV gH gene (9). This fragment was purified directly from genomic DNA as previously described (8). *Eco* RI digested viral DNA was separated by agarose gel electrophoresis and the 20 kb fragment corresponding to *Eco* RI ''C'' was purified by electroelution. Multiple attempts to directly clone this fragment into a variety of plasmid vectors were unsuccessful. Therefore purified *Eco* RI ''C'' was subjected to additional restriction enzyme digestion with *Bam* HI and *Bgl* II and the resultant fragments cloned into pUC 19 (United States Biochemical). Restriction mapping and Southern blot analyses were performed to determine the orientation of the *Bam* HI and *Bgl* II inserts relative to the genomic structure. Clones were selected for DNA sequencing. One *Bam* HI subclone, pKTS 409, was predicted on the basis of mapping and sequencing studies to contain the UL83 homolog (see Fig. 1). Plasmid DNA was purified over CsCl gradients (17) and subjected to DNA sequencing. Sequencing was done with ^{35}S-dATP (New England Nuclear) using 7-deaza-dG labeling and termination reactions with Sequenase® 2.0 (United States Biochemical), or by automated sequencing using an ABI/Prism automated DNA sequencer. Both strands of this 3466 bp *Bam* HI fragment were sequenced (Fig. 2). Oligonucleotide primers were synthesized every 200–300 base pairs as needed to complete the sequence analyses.

RNA and Northern Blot Analyses

GPL cells were inoculated with GPCMV at a multiplicity of infection (MOI) of 5 pfu/cell. At multiple time points post-inoculation RNA was solubilized in guanidinium isothiocyanate and purified over CsCl gradients (17). For analysis of ''early'' RNA, phosphonoacetic acid (PAA, Sigma Chemical) was included in the media at a concentration of 200 μg/ml (18). For Northern blot analyses, 10 μg of total cellular RNA was fractionated by gel electrophoresis in 1% formaldehyde/agarose gels, blotted to nitrocelluose filters, hybridized to probes, washed, and subjected to autoradiography essentially as previously described (8). Hybridizations were performed using [α-^{32}P] dCTP labeled double stranded probes corresponding to the putative GPCMV UL83 and UL82 coding sequences (Figs. 1, 3).

In Vitro Translation, UL83 Antisera,
Immunofluorescence, Immunoblot and
Immunoprecipitation Analyses

In order to evaluate the primary translation product of the putative UL83 ORF, *in vitro* translation analyses were performed. A *Bsi* WI fragment containing the entire GPCMV UL83 ORF (Fig. 1) was cloned into the expression vector pcDNA 3 (Invitrogen), yielding plasmid pKTS 437. Coupled *in vitro* transcription/translation reactions were performed using the T_NT® lysate reaction (Promega laboratories) according to the manufacturer's specifications. Briefly, 1 μg of template DNA was transcribed using T7 RNA polymerase, and *in vitro* translation was performed in rabbit reticulocyte lysate labeled with ^{35}S methionine (1,000 Ci/mmol, New England Nuclear) at 10 mCi/ml, 30°C, for 1 h. As controls, a luciferase-expressing plasmid was also expressed (p-luciferase), as well as a GPCMV gB-expressing construct (pKTS 404). The *in vitro* translation products were suspended in Laemmli sample buffer and were separated by electrophoresis on 10% SDS-polyacrylamide gels. Following staining with Coomassie blue, the gels were dried under vacuum and subjected to autoradiography (Fig. 5a).

In order to examine the intracellular pattern of expression of the GPCMV UL83 protein, plasmid pKTS 437 was used in a transfection/immunofluorescence experiment using a methodology described elsewhere (19). Briefly, 1 μg of plasmid pKTS 437 (GPCMV UL83 construct) was transfected using Lipofectin® (Gibco-BRL) into GPL cells and, 48 h following transfection, the monolayer was incubated with a polyclonal anti-GPCMV antibody (1:1000
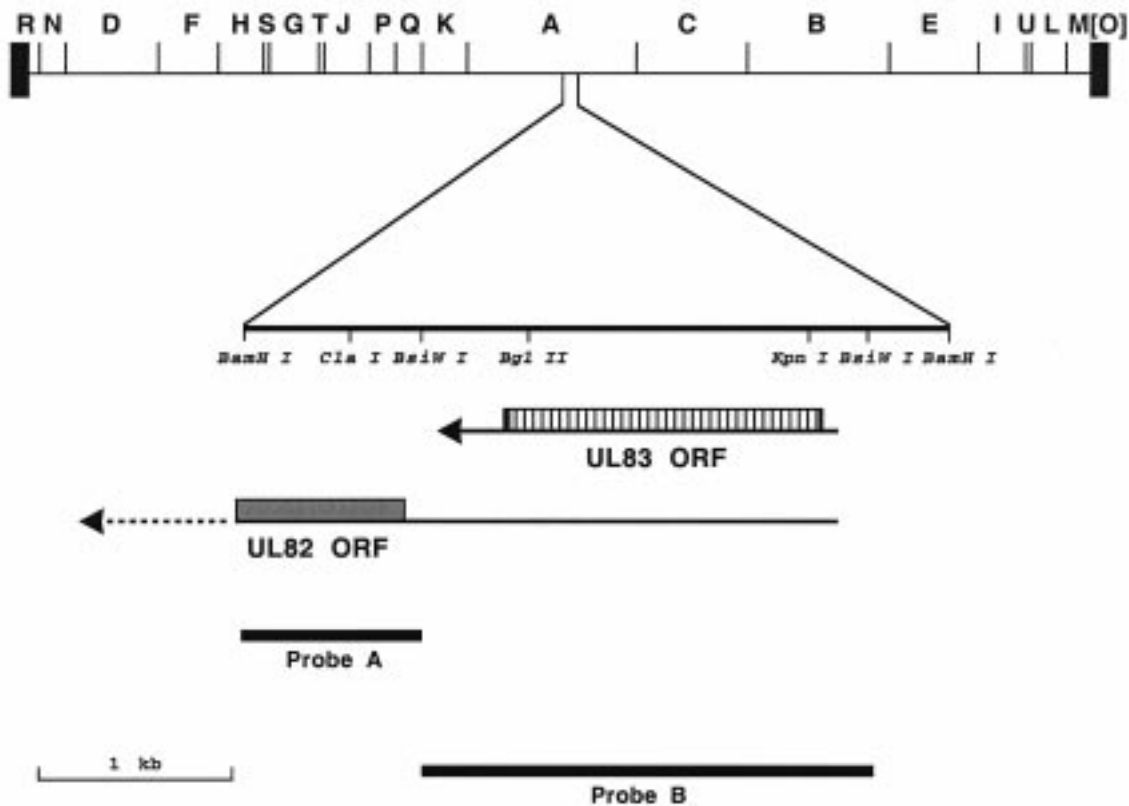
Fig. 1. Genomic location of GPCMV UL82–83 homologs. *Hin* d III restriction map of GPCMV genome with detailed restriction map of *Bam* HI subfragment (pKTS 409) is indicated. The GPCMV UL83 maps within this *Bam* HI subfragment of the *Hin* d III A fragment and is transcribed from right-to-left in this orientation of the viral genome. Arrows specify position and orientation of putative GPCMV UL83 and GPCMV UL82 transcripts. Boxes indicate coding sequences: GPCMV UL83, cross-hatched, GPCMV UL82, solid gray. UL82 ORF coding sequences continue to left of *Bam* HI site. Positions of probes used for Northern blot analyses of UL83 and UL82 transcription are indicated (see Fig. 3). Termination and polyadenylation of UL82 mRNA presumably occurs downstream of *Bam* HI site as indicated by dashed line.

dilution) followed by incubation with FITC-conjugated goat anti-guinea pig secondary antibody (Sigma) at a 1:300 dilution. As a negative control, immunofluorescence was also performed using vector DNA (pcDNA 3) alone. Monolayers were subjected to microscopic analysis using a Zeiss Axiphot 2 photomicroscope, FITC filter, 40x objective (see Fig. 7).

To generate a GPCMV UL83-specific antisera, a GST fusion protein (20) was generated by cloning a *Bgl* II-*Bsi* WI fragment into the vector pGEX-3X, yielding pKTS 333. Following IPTG induction, fusion protein was purified by a modification of a technique described elsewhere (21). Briefly, the *E. coli* pellet obtained following sonication and centrifugation (containing insoluble GST-UL83 inclusion bodies) was solubi-

lized in 8 M urea, diluted 1:3 in PBS, and, following extensive dialysis at 4°C, the fusion protein was bound to glutathione agarose. Following elution of the fusion protein with 50 mM glutathione, adult GPCMV-seronegative Hartley guinea pigs were immunized with 100 µg of fusion protein emulsified with an equal volume of complete Freund's adjuvant, and boosted every 3–4 weeks with 50 µg of fusion protein in incomplete Freund's adjuvant. For immunoblot analysis, virions were purified from late time point (96 h post-inoculation) tissue culture supernatants by purification over a 20–70% sucrose gradient as previously described (21). Virions and mock-infected GPL cells were solubilized [2% SDS, 0.0625 M Tris base (pH 6.8), 10% glycerol, 0.001% bromophenol blue, and 5% 2-ß-mercaptoethanol] and

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         BamH I
   1     GGATCCGCCACGAAAAACGCGCTCATGTCGCGGGACCGCAACGGGCACTACGGCTTCATCAAGATGGTCAACAGGTCGCA
  81     CGACTCGTGCCGCATCTCCAAGGCTTTCCTGAGCTCCGATACGTTGTGCGTGTACGTCTCCTCGACGGCCGTGCAGAAAT
 161     ACGTCCCCCGGGAGCTGGAGCTCACCGTGAGCCTCTACCACGCGCCGACTAACCTGCCGTATCTGTCCGGTTCCATGACG
 241     CCGTACTTCATCCAGGACGCGATGACCGGCCACTTGATCGTGACGGCGCCCGTCGACATAGAGATCCCCTTCGACACCGC
 321     GTACGAGCTCAACGTCCAGCGCAAGCACTACGGTCGCCACCTGGGGCTCTTCGTCCCGTGGCACAGACCGGACGGCCTGC
 401     AGGTGACGGTGGGCACGTGGCATCCCAGAGAGTGGCTCACCGTGCGGCTCTACGCGTCCAGGTGCGGCGTTCGGCTGCAG
 481     CGCGGGGACGGGCTCGGAAAGGTCTACTTCGTCCCCACGGCGGTCACGCGGGACCGCGTCTACAGGGGAGCCCCAGTACGG
 561     CAGCACCATCAGGTCGATCCTGACCGGGTTCGACGACGAGAACCTCAGACTGGCCGGCCTCAAGATACCGACGCGTAACC
         SP-1
 641     TACCCAGTCTCCTGCTGCCCGGCAAGAAGACCCGCGCCCAGTATCGACGACTACGACGCTATGGGCGTAGACTACCCGTG
                                                              SP-1
 721     GTACGAAAACCTGAAGCAGGGAATCCCTTATATCGTGACGGCCCGCTTTTACGGAAGCGATCCCGCGCCCAAATCCGTGT
         TATA Box            PolyA                                        TATA Box
 801     AAGATATAAATCGTAACGGCGGTTCTAATAAAAACGATCGTGAACGGTATCCGTGTCTCGTGTTCGTTTAATAAAATTTT
 881     TCTCGCCCGCGCGCCCCGATCGCCCGCCCCTGCCTCTATCCGTCCCCACAGCAAACAAAACGCGCCCCCATCACATTGTA
                                            BsiW I
 961     TTTATTCGCTCGCGCGTGTCCCGTCCCCCGATCGTCCCCGTACGTTGGCACTCGGGCGGCGCGGAGAGAGAGGGGCCGGA
         Kpn I
1041     ATC ATG GAG CGG TAC GTG GGT CTC GGT ACC ACC TTG CAC CTG GCC CTC CAG GTC GAG GGT
             M   E   R   Y   V   G   L   G   T   T   L   H   L   A   L   Q   V   E   G  19
1101     CCG TTC GGG CCC CAC GAG ACC AGA TTC GTA CAC TTC GAC GCC ACC TTC ACG GTT CCC AGG
          P   F   G   P   H   E   T   R   F   V   H   F   D   A   T   F   T   V   P   R  39
1161     ACG CCG TCC GTG GTG ATC GTG GCC GAG GAG CAG GTA GGC TCT CAC CTG CCG CCT TCC AGT
          T   P   S   V   V   I   V   A   E   E   Q   V   G   S   H   L   P   P   S   S  59
1221     CCC CTG CGG ATG AAG TTC AGG GCG ATC CAC AGC CAA GAA GAA CTG GAT CTG CTC AAC CTG
          P   L   R   M   K   F   R   A   I   H   S   Q   E   E   L   D   L   L   N   L  79
1281     GAG GTA CGC AAC GCG TCG GAC CAG CCC CTG CCG GCG AAG AGC TTC TAC GAT CTC AAC GTC
          E   V   R   N   A   S   D   Q   P   L   P   A   K   S   F   Y   D   L   N   V  99
1341     GTG GTC TTC GCC CTG CCG CTG CCC AGG GTC CAC GCG GCT CCT CTA CAC ATA TTC TTC AAC
          V   V   F   A   L   P   L   P   R   V   H   A   A   P   L   H   I   F   F   N 119
1401     AGC GCC CTG AAA CCC AGC AGG GAG ACG TTC CCC ACC TGC AGC AAG ACC GTC GTG CGT CGC
          S   A   L   K   P   S   R   E   T   F   P   T   C   S   K   T   V   V   R   R 139
1461     GCC TGC GGG GCC ATC TGG GGC GTG AGG ACC GCG CTC AGC AAC ATG GCG TGG ACG GAA GGC
          A   C   G   A   I   W   G   V   R   T   A   L   S   N   M   A   W   T   E   G 159
1521     GTC GGA AAC AGG GCG CAC GTC GAC CGA GCC CTG ACC GTG ACC GTC CTC CTG TCG GTC AAA
          V   G   N   R   A   H   V   D   R   A   L   T   V   T   V   L   L   S   V   K 179
1581     CCC GCC TGC ATG ACT CAC GTC GAC TCC CTC ACG GAG ATC AAC TGT TCT CAC GAA GAC ATT
          P   A   C   M   T   H   V   D   S   L   T   E   I   N   C   S   H   E   D   I 199
1641     CAG GTG TTC AAG GCG GAG GTC TTC CAG AAG GGC AGA CCC AAC GTT CTC GGC CTG ACG TTA
          Q   V   F   K   A   E   V   F   Q   K   G   R   P   N   V   L   G   L   T   L 219
1701     CAG ACG ACC GCC AAA CCC CCG CCT AAG AAA CTG ACG TTG TTC TTC CAG CTC CTG GCC AGT
          Q   T   T   A   K   P   P   P   K   K   L   T   L   F   F   Q   L   L   A   S 239
1761     CAC GCT CAG GTC GTC ATG AGG CAC AAT CCG TAC CCG GCG CTG CAG AGT CAT CCC TCT AAC
          H   A   Q   V   V   M   R   H   N   P   Y   P   A   L   Q   S   H   P   S   N 259
1821     GGT TTC ACG ATC CAC TGC CCT GGC GAC ATT CGA TTA CAG TCT GGG CAG ACG TAC CGT CTG
          G   F   T   I   H   C   P   G   D   I   R   L   Q   S   G   Q   T   Y   R   L 279
1881     ACC CTC AGA AAC GGT TTC GAC AGT ACC TCT CAC GCC GCC CTG TTC TTC CCC GCC GAC TTC
          T   L   R   N   G   F   D   S   T   S   H   A   A   L   F   F   P   A   D   F 299
1941     CCC AAC GCC GAC ATC TCG GGC GGG CAG TGG AAA GCG CGT CAC AAC ATG GAC ATC GTC ATC
          P   N   A   D   I   S   G   G   Q   W   K   A   R   H   N   M   D   I   V   I 319
2001     CGC TCG CAC GGC GAG ACG ACC GTG CGC AAG GAC GAA GTC CTC GGA ATC GTC CAC TTC TTC
          R   S   H   G   E   T   T   V   R   K   D   E   V   L   G   I   V   H   F   F 339
2061     GAC AAC GAC CTG CTC ACC TTC CAC CGC GTG GCC GGC GTC ATA GAC ACC TGC ATG ATG GGA
          D   N   D   L   L   T   F   H   R   V   A   G   V   I   D   T   C   M   M   G 359
2121     AAA CAG TTC GAG ACC AGA GTC AGA CGT TGC TCG GAG TCC TGT CAA GAG CAG GTG TTC GTG
          K   Q   F   E   T   R   V   R   R   C   S   E   S   C   Q   E   Q   V   F   V 379
2181     AAG AGC GGC GGA CGT CGG ACC GGC AAC GCG GAT CGA CAC CGG AGA GAC AGA GAC GGC GGC
          K   S   G   G   R   R   T   G   N   A   D   R   H   R   R   D   R   D   G   G 399
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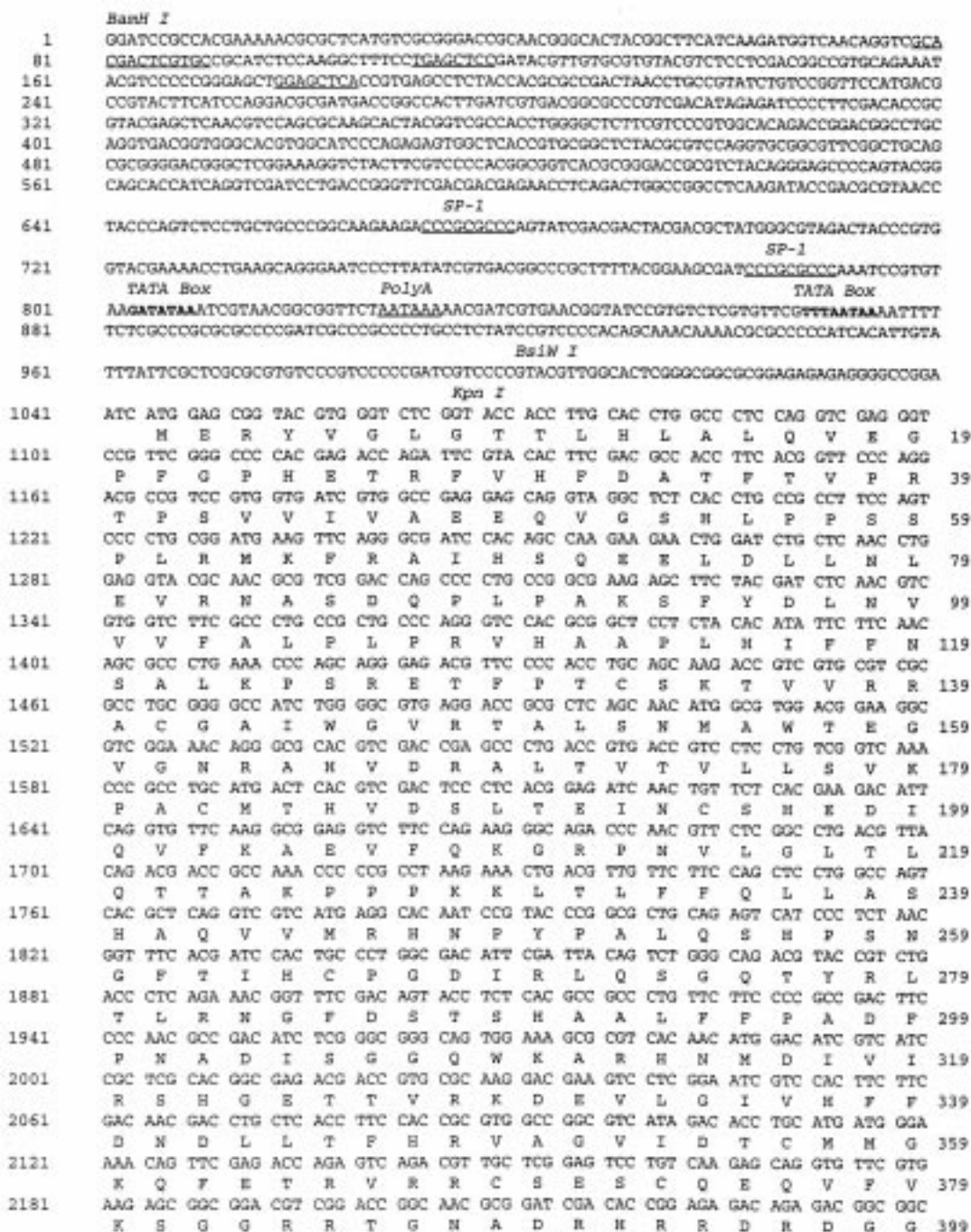
Fig. 2. Nucleotide sequence of 3466 bp region encoding the GPCMV UL83 gene. Restriction endonuclease sites are indicated above the DNA sequence for orientation purposes. The nucleotide sequence is numbered relative to *Bam* HI site (+1) with coordinates shown on left; numbering of deduced amino acid sequence of UL83 ORF indicated on the right. Potential TATA boxes are indicated in bold type. Potential *cis*-acting promoter sequences and polyadenylation sequences are underlined (see text). Charged, basic domains with homology to HCMV UL83 bipartite nuclear targeting regions are boxed. Deduced amino acid sequence of (partial) GPCMV UL82 ORF is also indicated.

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2241   GAC GAC GAC GAT GAC GAA AAC GAA GAC GGG GAA GAA GGC GAA GAA GAC GGC GAG GAA GAC
        D   D   D   D   D   E   N   E   D   G   E   E   G   E   E   D   G   E   E   D  419
2301   GTC GGG GAC GCC AAA GAC GAC GGA TCG GAA TCT AGC TCG GAA TCC GAG TTA GGC AGC GGC
        V   G   D   A   K   D   D   G   S   E   S   S   S   E   S   E   L   G   S   G  439
2361   GAG GAC AAC GAC GGG GAC GAC GAC GTG TTC GAA TGC GAG CGG CCG CTC GCT CGG GAA GAC
        E   D   N   D   G   D   D   D   V   F   E   C   E   R   P   L   A   R   E   D  459
2421   GGC GCG TCC GGA AGC GCG GAG CGC GGA ACT CTC AAC GAG TCG GAA GAC CCT TCG TTG AGA
        G   A   S   G   S   A   E   R   G   T   L   N   E   S   E   D   P   S   L   R  479
                                   Bgl II
2481   CCG AGG AGG GTC TCC GAA GAG ATC TTC CCC AGC GTG CTC TTC TAC CCG TGG GCC CTC AGC
        P   R   R   V   S   E   E   I   F   P   S   V   L   F   Y   P   W   A   L   S  499
                                                                       Sph I
2541   ATA CCG ACC GGG TTC TGC GCG TAC ATA CAC TAC AAC GTG GTG GCA TGC AGC AGC GAA CAC
        I   P   T   G   F   C   A   Y   I   H   Y   N   V   V   A   C   S   S   E   H  519
2601   TCG TCC GGC GAG GTG CAG GAC GGT TCG GTG TGG TTC GAC GGC GTA CCC ACC AGG CCC GCG
        S   S   G   E   V   Q   D   G   S   V   W   F   D   G   V   P   T   R   P   A  539
2661   TCG CGC GCG TGT TCG AGG ACG CGG CGT GAC GAC GAC GGC GGC GCC GGC ACC TCC CGT AGG
        S   R   A   C   S   R   T   R   R   D   D   D   G   G   A   G   T   S   R   R  559
                                                                             PolyA
2721   AGC CAT CGC GGG GCA CAG TAG GCTCCGCCGGCACCGCCGCGCGCGTCTCGTCCCCTGTACTTCCCGAAATAAA
        S   H   R   G   A   Q   *
2795   AAAACGGTTCCCGTAAAAACACACGCGCGTACACGTCCCGTGTTTTGATTCCGTCACGAATTACGGTATATTCATATGCGGA
2876   TAGGTCGCAACGGGTTCTCAGATCCGTCCCGACGACGCGAACCTCCCTCTCCTCCGTCCGTCCCCGCGACTCCGGATCGTA
            BsiW I
2957   GAAC ATG GCG TAC GTG GGG CTG GCG ACC CGC ATC CAC CTC GGT GGC GTA GTC GTC GGG GAA
             M   A   Y   V   G   L   A   T   R   I   H   L   G   C   V   V   V   G   E  19
                                                                             Pvu II
3018   CCG CTG TCG CCC GGA GAG AGA CGC AGG GTA CGT CTC CAG TCA CTG TCC CTG AAA CAG CTG
        P   L   S   P   G   E   R   R   V   R   L   Q   S   L   S   L   K   Q   L  39
3078   ACC GCG CCG TCC GTC GTA TGC GTG GCG CAA GAT ACC ATC GAC AGG CAA TGC GCC GTC AGA
        T   A   P   S   V   V   C   V   A   Q   D   T   I   D   R   Q   C   A   V   R  59
3138   GTC AGG TTC GAA GTG CTC TAC GAC ACG GAC GCC CTG GAG CAA CCC CAC GTC ACG ATA CAG
        V   R   F   E   V   L   Y   D   T   D   A   L   E   Q   P   H   V   T   I   Q  79
3198   AAC GTG ACG TCC AGG CGC GTC TCA CTC TCG AAA GAA GAC CTG GTG AAC CTC ATC GTC TTC
        N   V   T   S   R   R   V   S   L   S   K   E   D   L   V   N   L   I   V   F  99
                   Cla I
3258   GCC ATA CCG GTT CCC AGG ATA TCG ATC GAA GGC CTC CGG CTC GTT CAC GGC GTC AGG CCC
        A   I   P   V   P   R   I   S   I   E   G   L   R   L   V   H   G   V   R   P  109
3318   GCC CGC GGC CAT CGC CTG ATA CGC GCG GGA GAA CCT CTC GTC GAC GTG GAA CGT CTC
        A   R   G   H   R   L   I   R   A   H   G   E   P   L   V   D   V   E   R   L  129
3378   TGC GAC GAC CGC TGG ATG CTC TGC ACC GTG GTC ACC AGC ATC CGT TGG GAG ACC TAC AAG
        C   D   D   R   W   M   L   C   T   V   V   T   S   I   R   W   E   T   Y   K  149
                                                                BamH I
3438   TGC GAC AAG CAC TCC GAT CGC AGG GAT CC
        C   D   K   H   S   D   R   R   D   P
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Fig. 2. (Continued)

proteins were separated on 10% SDS-polyacrylamide gels (22). In some experiments, additional purification and separation was performed to separately isolate virions and dense bodies from infected tissue culture lysates (see below and Fig. 6) prior to SDS-PAGE and immunoblot analysis. Following electrophoresis, proteins were electroblotted to Nytran® (Schleicher & Schuell) membranes. After transfer, the membranes were washed in Tris-buffered saline (TBS, 0.2 M Tris hydrochloride, pH 7.5, 0.5 M NaCl) and subsequently were incubated with either guinea pig preimmune serum or guinea pig anti-GEX/UL83 antiserum (1:50 dilution), or a polyclonal convalescent anti-GPCMV antisera (1:100 dilution) in TBS, overnight, at 4°C.

Following extensive washing in TTBS (TBS containing 0.5% Tween-20), the blots were incubated with a horseradish-peroxidase conjugated rabbit anti-guinea pig antiserum (Accurate Chemical & Scientific Co.) at a 1:1000 dilution for 120 min at 25°C. The blot was then washed in TTBS and then developed with 4 chloro-1-Napthyl phosphate (30 mg in 10 ml methanol, 4°) and 30 μl H_2O_2 in 50 ml TBS.

Immunoprecipitation was used to identify the putative GPCMV homolog of UL83 in infected tissue culture lysates. Briefly, GPL cells were infected with GPCMV (MOI of 5) and 96 h post-inoculation were washed and incubated for an additional 8 h with cysteine/methionine-free media supplemented with
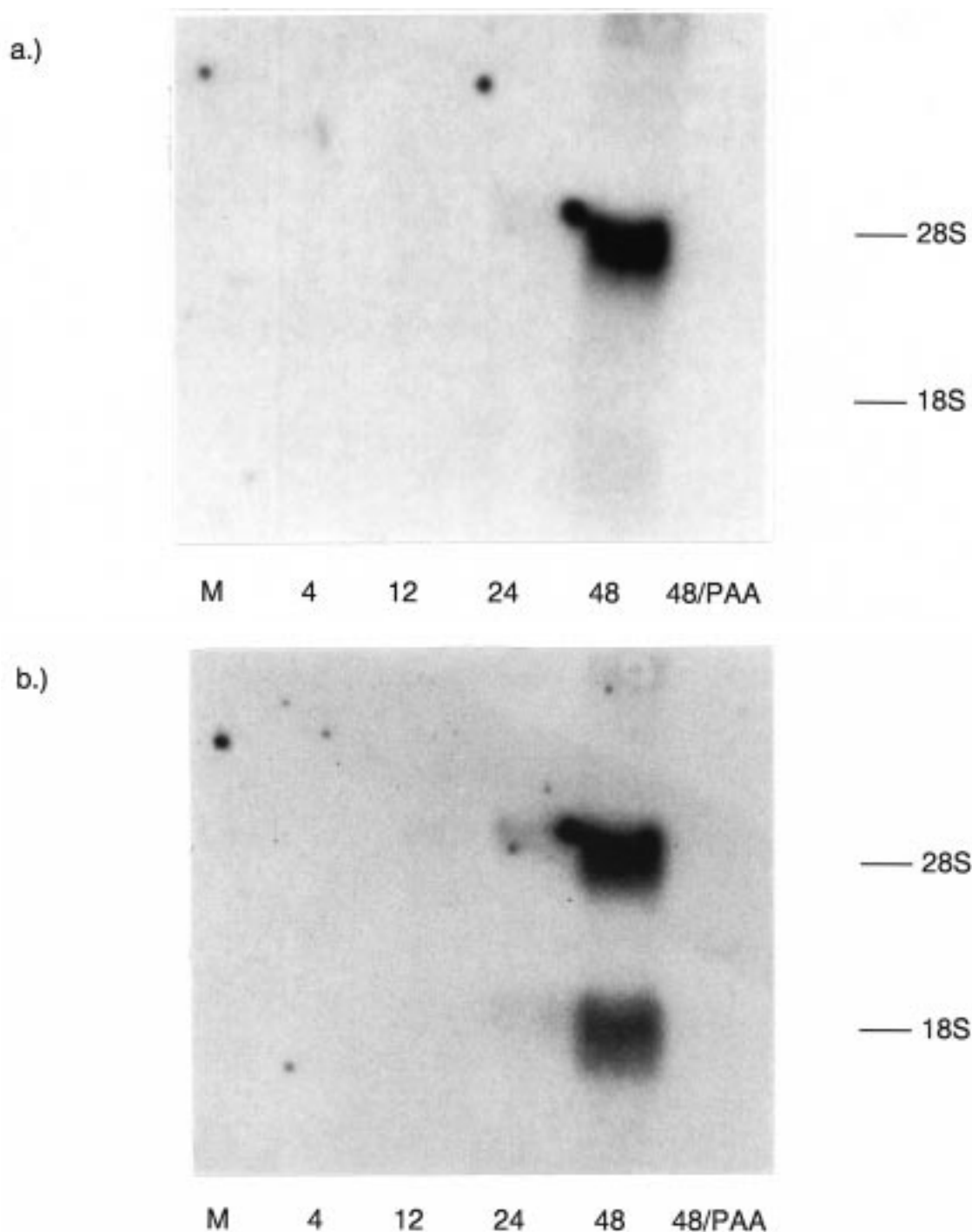
Fig. 3. Northern blot analyses of GPCMV UL82 and UL83 transcription. Whole cell RNA (10 µg/lane) was harvested from mock-infected (lane 1, M) or GPCMV-infected cells at the indicated times post-infection and following electrophoresis and transfer to nitrocellulose membranes was hybridized with [α-^{32}P] dCTP-labeled probes (3a, probe ''A'', Fig. 1; 3b, probe ''B'', Fig. 1). For analysis of transcription under ''early'' gene conditions, phosphonoacetic acid (PAA) was included at a concentration of 200 µg/ml (PAA). The positions of 28S and 18S ribosomal subunits are indicated. Both probes identify a 4.2 kb mRNA. However only the upstream probe (probe ''B'') hybridizes with the 2.2 kb mRNA. GPCMV UL83 RNA could be translated from either mRNA species, but the 4.2 kb message is deduced to be the UL82 message. Neither RNA species is discernable with RNA harvested from lysates with PAA present in the media, defining GPCMV UL82 and GPCMV UL83 as ''late'' genes.

50 µCi/ml of ^{35}S-Translabel (ICN Radiochemicals, Costa Mesa, CA). In some experiments, cells were washed at 96 h post-inoculation with phosphate-free DMEM (Gibco-BRL) and were incubated for an additional 8 h in phosphate-free DMEM supplemented with 10% FCS and 200 µCi of [^{32}P]-orthophosphate (ICN) per dish. Lysates were solubilized in 1 ml of RIPA buffer (10 mM Tris pH 7.4, 0.15 M NaCl, 1% NP-40, 1% DOC, 0.1% SDS, 0.5% aprotinin) and approximately 250 µl of lysate was precleared by additional of 10 µl of guinea pig preimmune sera and 50 µl of 10% fixed *Staphylococcus* protein A. Following centrifugation, either pooled anti-GPCMV antibody or guinea pig immune GST-GPCMV UL83 antisera was next mixed with the labeled precleared lysate and incubated overnight at 4°C. The immune complexes were then collected by addition of *Staphylococcus* protein A, washed twice in RIPA buffer, once in high salt buffer (2 M NaCl, 10 mM Tris pH 7.4, 1% NP-40, 0.5% DOC) and, following a final wash in RIPA buffer, the immune complexes were collected, Laemmli sample buffer was added, and the proteins were electrophoresed in 10% polyacrylamide gels. Following electrophoresis, the gels were impregnated in a radiographic enhancement solution (Enlightening®, NEN Research Products, Wilmington, DE) and autoradiographs were prepared.

Gradient Purification of Virions and Viral Dense Bodies

In some experiments, virus isolated by sucrose gradient purification was subjected to an additional density gradient centrifugation step on glycerol tartrate gradients using a protocol described elsewhere (23). Briefly, material collected by banding over the sucrose gradient was dialyzed overnight at 4°C against TN buffer (0.05 M Tris HCl, pH 7.5, 0.1 M NaCl) and was layered onto a preformed gradient in a Beckman SW41 ultracentrifuge tube which was prepared using a standard two-chamber gradient maker, with 5 ml of 30% (w/w) glycerol in TN buffer containing 15% (w/w) potassium tartrate in the feeder chamber and 4 ml of 35% (w/w) potassium tartrate in TN buffer in the mixing chamber. The gradient was centrifuged overnight at 25,000 RPM, 10°C in a Beckman SW41 rotor. When the gradient was illuminated from above, discrete, well-separated bands were noted, an upper band containing virions and a lower band containing dense bodies (Fig 6). These respective bands were removed by puncturing the side of the ultracentrifuge tube with a syringe and needle and were subjected to quantification of total protein and to SDS-PAGE and immunoblot analyses as described above (see Fig. 6).

Results

Identification of GPCMV UL83 Homolog Coding Sequences in the GPCMV Genome

The GPCMV and HCMV genomes have been shown in previous studies to exhibit striking conservation in the relative positions of structural glycoprotein genes such as gB and gH (8,9). Assuming approximate colinearity of the HCMV and GPCMV matrix phosphoprotein genes, it was therefore predicted that the GPCMV UL83 homolog would be encoded within the *Eco* RI ''C'' subfragment of the *Hin*d III ''A'' region of the viral genome. Attempts to directly clone the *Eco* RI ''C'' region of the GPCMV genome were unsuccessful. Therefore a library of *Bam* HI and *Bgl* II subclones of *Eco* RI ''C'' was generated. Sequence analyses of one *Bam* HI subclone, pKTS 393, identified ORFs with identity to the HCMV UL84, UL85 and UL86 proteins; similarly, DNA sequencing of a second *Bam* HI subclone, pKTS 414, revealed identity to the UL80 ORF of HCMV (24). Therefore additional sequence analysis was undertaken of another *Bam* HI subclone of *Eco* RI ''C'', plasmid pKTS 409. Southern mapping and PCR analyses confirmed that this *Bam* HI fragment was positioned between the *Bam* HI fragments corresponding to pKTS 414 and pKTS 393 in the context of the viral genome (data not shown). Within this *Bam* HI fragment were identified sequences with deduced open reading frames which had identity to the UL82 and UL83 proteins of HCMV. Hence, these data indicate conservation in the GPCMV genome of the block of HCMV gene products from UL80, the CMV protease, through UL86, the major capsid protein (10,24). These relationships are reviewed in Fig. 1. The relative position and direction of transcription of the GPCMV UL83 gene is similar to that of HCMV: the UL83 and UL82 homologs are transcribed from right-to-left and are located at approximate map unit positions 0.58–0.55, based on the orientation as originally defined by Isom (25).

Nucleic Acid Sequence Analysis of the Putative GPCMV UL83 Homolog

Convenient restriction sites were used for subcloning to facilitate sequence analyses. Both strands of the 3466 base pair *Bam* HI fragment were subjected to DNA sequencing (Fig. 2). Analysis of the DNA sequence identified an ORF of 1695 nucleotides capable of encoding a protein of 565 amino acids. Two potential TATA boxes were noted 241 and 177 bp, respectively, upstream of the first of two putative ATG codon. A consensus polyadenylation signal sequence (AATAAA) was noted 48 bases downstream of the putative stop UL83 codon, and is followed by a GT rich sequence characteristic of a transcriptional termination signal (26). A second ORF initiates at a favorable Kozak-consensus ATG 220 nucleotides downstream of the UL83 stop codon; this ORF has identity to the UL82 protein of HCMV (27).

The protein predicted from the putative GPCMV UL83 homolog has characteristics similar to the tegument phosphoproteins of other cytomegaloviruses. Computer-based search of GenBank sequences using the MacVector® 5.0 software analysis program revealed that this ORF shares significant identity with both the HCMV UL82 and UL83 genes and the MCMV M82-M84 genes (28,29). Sequence analysis also identified an ORF downstream of the GPCMV UL83 gene which had identity to the UL82 gene of HCMV (Fig. 1). The putative protein product of the GPCMV UL83 homolog has a calculated M_r of 62.43 kDa, much smaller than that of the murine cytomegalovirus (MCMV) M83 homolog, which is 90.7 kDa, but comparable to the HCMV UL83, which is calculated to be 62.9 kDa. The protein is highly charged (14.4% acidic residues, 14.7% basic residues), with a predicted pI of 5.41. Overall the GPCMV UL83 protein has 23% identity and 42% homology with the HCMV UL83 (global alignment score of 322), and 23% identity and 35% homology with the MCMV M83 (global alignment score of 168). Clustal alignment of the amino acid sequence of HCMV UL83 and GPCMV UL83 revealed that the areas of strongest identity are limited to the amino terminal 400 aa of the respective molecules (Fig. 4a). Alignment of hydrophilicity profiles, however, (Kyte-Doolittle scale, Fig. 4b) revealed that these proteins share highly charged, hydrophilic domains in the carboxy-terminal 200 aa, suggesting a high

degree of functional homology even in the absence of sequence homology.

Transcription of the GPCMV UL82 and UL83 Homologs

To analyze transcription of the putative GPCMV UL82 and 83 homologs, radiolabeled probes corresponding to respective coding sequences were prepared and Northern blot hybridizations were performed. RNA was purified from tissue culture at various time points post-inoculation with GPCMV. For analysis of transcription under ''early'' gene conditions, phosphonoacetic acid (PAA) was included at a concentration of 200 µg/ml (18). The UL83-specific probe hybridized with abundant 2.2 and 4.2 kb transcripts which were detectable by 48 h post-viral inoculation. In contrast, the GPCMV UL82-specific probe hybridized with only the 4.2 kb message (Fig. 3). These transcripts were not detected in RNA obtained from infected cells when PAA was included in the media, suggesting that transcription from this region of the genome appears to be limited to the ''late'' phase of infection. Since the 4.2 kb mRNA identified by the GPCMV UL82 probe is larger than can be explained by coding sequences alone (data not shown), and since no consensus TATA signal is present between the GPCMV UL83 stop codon and the putative GPCMV UL82 start codon (Fig. 2), it is likely that the UL82 protein is encoded by the second cistron of a ''bicistronic'' mRNA which initiates at the UL83 TATA sequence. The GPCMV UL83 protein could be synthesized by translation of either the first cistron of this message, or from the 2.2 kb species: this 2.2 kb message presumably polyadenylates at the consensus poly A signal immediately downstream of the UL83 stop codon (Fig. 2).

Analysis of the GPCMV UL83 Protein

In order to examine the primary translation product of the GPCMV UL83 homolog, *in vitro* transcription/ translation analyses were conducted. The putative GPCMV UL83 ORF was cloned into the eucaryotic expression vector pcDNA 3, generating plasmid pKTS 437, and plasmid DNA was subjected to coupled *in vitro* transcription/translation in reticulocyte lysate in the presence of ^{35}S-labeled methionine. As shown in Fig. 5a, *in vitro* translation resulted in
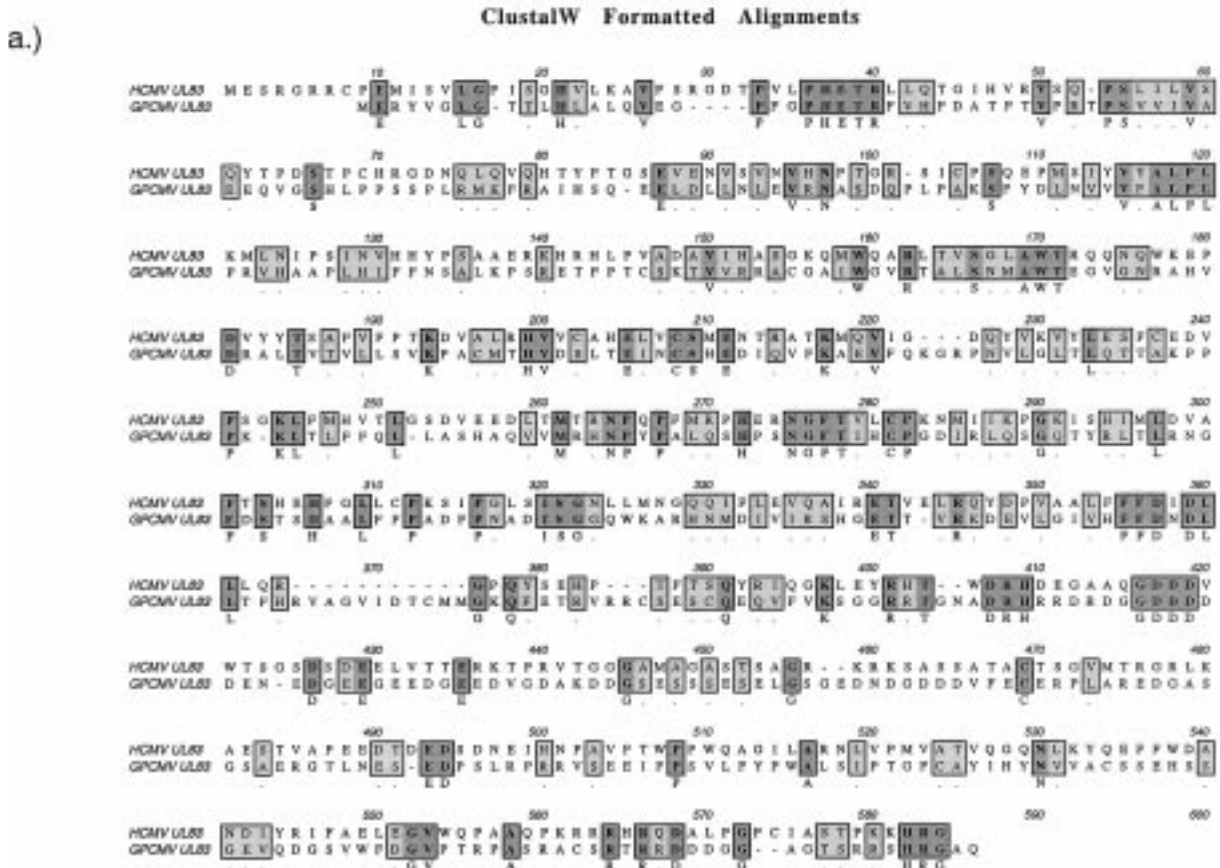
Fig. 4. Comparison of the amino acid sequences of GPCMV UL83 and HCMV (AD169) UL83 proteins. A) Clustal alignment produced by MacVector 5.0 protein sequence analysis software (BLOSUM matrix): gap separation distance 8, delay divergence 30%. Amino acid similarity is noted to be strongest in amino terminal 2/3 of protein coding sequence. B) Hydrophilicity profiles (Hopp-Woods scale) of respective proteins. Although COOH-terminal coding sequences are relatively divergent, strong similarity in hydrophilicity profiles is noted, suggesting significant functional homology.

synthesis of a protein species of approximately 65 kDa, in good agreement with the M_r of the GPCMV UL83 protein predicted by the deduced amino acid sequence. To analyze the intracellular trafficking of the GPCMV UL83 protein, pKTS 437 plasmid DNA was transfected into GPL cells and polyclonal antisera used in an immunofluorescence analysis. These experiments revealed that the subcellular localization of *de novo* synthesized GPCMV UL83 was virtually exclusively nuclear (Fig. 7), consistent with a nuclear targeting function of the putative protein.

To generate a monospecific antisera to the putative GPCMV UL83 homolog, a UL83-glutathione-*S*-transferase (GST) fusion protein was generated. A hydrophilic 49 amino acid region spanning Ile_{486} through Gln_{565} of the GPCMV UL83 ORF was subcloned as a *Bgl* II—*Bsi* WI fragment into the vector pGEX-3X (20) and, following IPTG induction, fusion protein was purified from transformed *E. coli*. To examine for expression of the putative GPCMV UL83 protein in infected cells, monospecific GPCMV UL83 antibodies were generated using the GST-GPCMV UL83 fusion protein to immunize adult seronegative Hartley guinea pigs. Purified virions were immunoblotted to nitrocellulose and blots were then incubated with either guinea pig preimmune serum or guinea pig anti-GST/UL83 serum. Blots were also incubated with a control high-titer anti-GPCMV polyclonal guinea pig serum (6). An
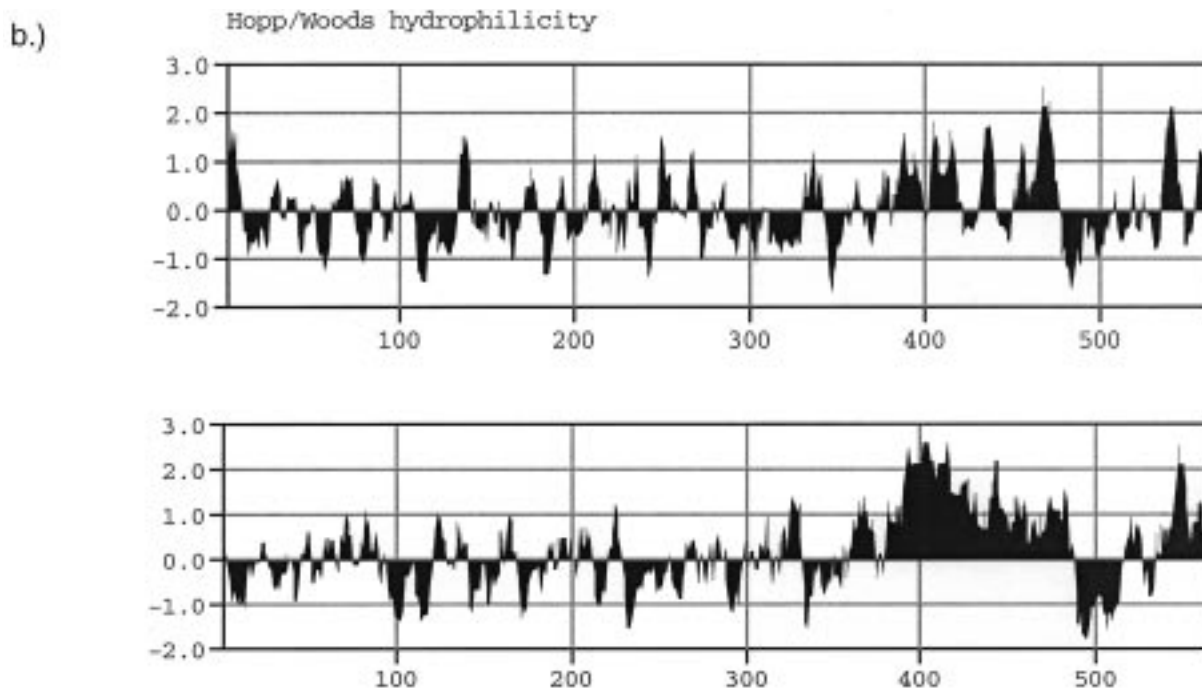
Fig. 4. (Continued)

immunoreactive protein of approximately 70 kDa was noted using each of two independently derived monospecific GST/GPCMV UL83 antisera (Fig. 5b, arrow). No signal was obtained using immunoblotted proteins obtained from uninfected cell lysates (data not shown). A protein of approximately the same molecular weight was also identified in a Western blot assay using a polyclonal anti-GPCMV convalescent guinea pig serum. When purified virus particles were subjected to further separation into virion and dense body fractions by glycerol tartrate ultracentrifugation, the GPCMV UL83 antisera was found to be immunoreactive with both fractions (Fig. 6), confirming that the GPCMV UL83 homolog is present in both virions and dense bodies. GST/GPCMV UL83 antibodies were next used in immunoprecipitation assays. *Staphylococcus* protein A was used with anti-GST/GPCMV UL83 antisera to precipitate virion proteins which had been radiolabeled using ^{35}S-cysteine/methionine. Lysates of GPCMV-infected cells were harvested at late time points (96 h) post-viral inoculation. These assays also identified a protein species of approximately 70 kDa, the putative GPCMV UL83 protein, in infected cell lysates (Fig. 5c). Similarly, [32P]-orthophosphate labeling of infected tissue culture lysates resulted in labeling of

a 70 kDa species which was also immunoprecipitated by the GPCMV UL83 monospecific sera, indicating that the GPCMV homolog of UL83 is, like the HCMV pp65, a phosphoprotein (Fig. 5d). This 70 kDa species was also immune precipitated by polyclonal anti-GPCMV antisera, indicating that the GPCMV UL83 is immunogenic in the context of natural guinea pig infection.

Discussion

Although the guinea pig provides the only small animal model of transplacental CMV infection, vaccine and pathogenesis studies have been limited to date because of the lack of molecular characterization of the GPCMV genome. However, information about the molecular biology of specific GPCMV envelope glycoproteins has recently become available, specifically for the glycoprotein B and H homologs (8,9). In this paper, we report the first molecular characterization of a GPCMV tegument protein, the UL83 (pp65) gene homolog, show that this protein is a phosphoprotein which targets the nucleus, and further demonstrate the presence of the protein in GPCMV virions and dense bodies.
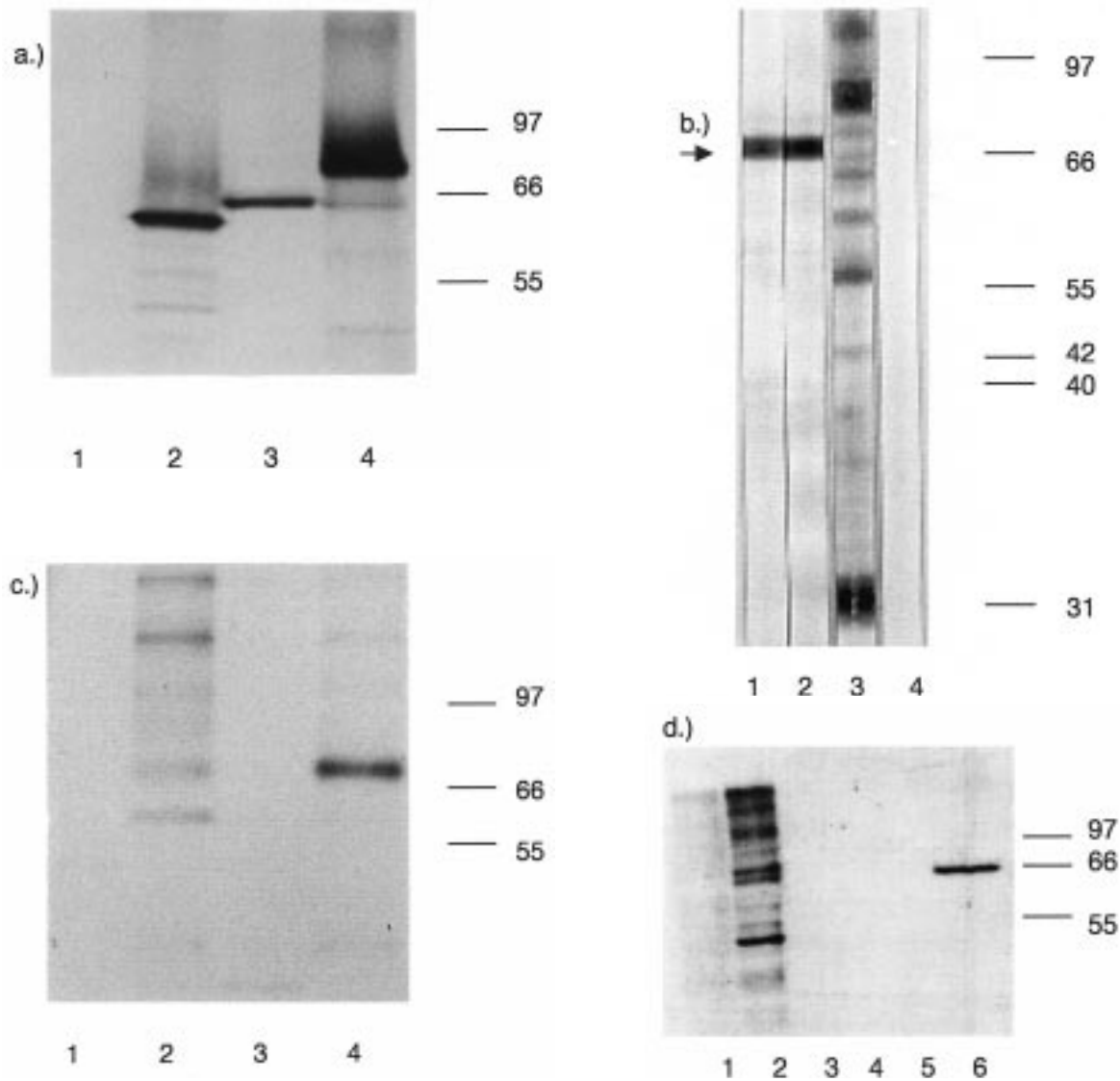
Fig. 5. Characterization of GPCMV UL83 Protein. A) Coupled *in vitro* transcription/translation analysis of plasmid pKTS 437 (containing GPCMV UL83 coding sequences under control of HCMV major immediate earlier promoter) expressed in ^{35}S-labeled rabbit reticulocyte lysate. Lane 1, no plasmid control; lane 2, p-luciferase positive control; lane 3, pKTS 437; lane 4, GPCMV gB-expressing clone pKTS 404. Single *in vitro* translation product of approximately 65 kDa is observed using this expression construct, in good agreement with the deduced M_r of 62.3 kDa. B) Detection of GPCMV UL83 protein in virus particles by Western blot. Virus particles were purified over 20–70% sucrose gradients and, following SDS-PAGE, proteins were immunoblotted to nitrocellulose membranes and probed with guinea pig preimmune serum or a GPCMV UL83-specific polyclonal guinea pig antisera raised against a GST-GPCMV UL83 fusion protein, administered to guinea pigs with Freund's adjuvant. The position of molecular weight size markers is indicated. Lane 1 and lane 2 represent independently derived sera from two adult Hartley GPCMV seronegative guinea pigs separately immunized with GEX-UL83; lane 3, polyclonal anti-GPCMV antisera from seropositive animal; lane 4, seronegative preimmune sera. A virion-associated protein of approximately 70 kDa is identified (arrow) representing the GPCMV UL83 protein. A protein of similar size is also identified by a polyclonal convalescent guinea pig serum from a GPCMV-infected animal. C) RIP-PAGE analysis of ^{35}S methionine/cysteine-labeled lysates. Same antisera used for Western analyses (B) were used to immunoprecipitate radiolabeled proteins from ^{35}S-labeled lysates at late time point (96 h) post-viral infection. After preclearing lysates with preimmune sera, proteins were immunoprecipitated with either antisera from mock-infected animals (lane 1), polyclonal anti-GPCMV antisera (lane 2), preimmune guinea pig antisera (lane 3), or antisera from a GEX-GPCMV UL83 immunized animal (lane 4) in the presence of *Staphylococcus* protein A. GEX-UL83 antisera specifically immune precipitates a 70 kDa band, the putative GPCMV homolog of the UL83 protein. D) RIP-PAGE analysis of ^{32}P orthophosphate-labeled lysates.
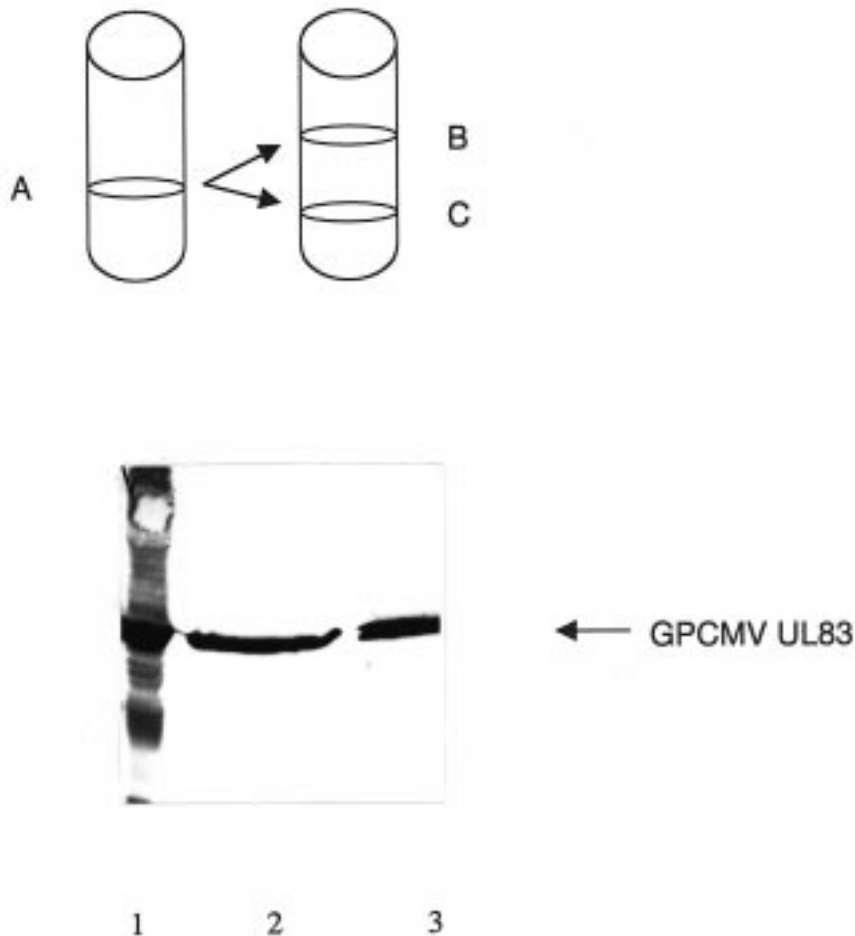
Fig. 6. Western analysis of gradient purified virions and dense bodies. Virus particles purified by sucrose gradient purification (band ''A'') were subjected to dialysis and subsequent banding over negative viscosity/positive density glycerol-tartrate gradients as described in text. Upper band containing virion particles (band ''B'') and lower band containing dense bodies (band ''C'') were rebanded, dialyzed, and subjected to SDS-PAGE. Western blot analysis using GST-UL83 specific antisera indicates that sucrose banded fraction (lane 1), virion fraction (lane 2), and dense body fraction (lane 3) all are immunoreactive, confirming presence of GPCMV UL83 protein in virion and dense body fractions.

The orientation and position of conserved gene products among the animal cytomegaloviruses is striking. Based on the prediction of genome co-linearity observed for other highly conserved genes, we predicted that the GPCMV UL83 homolog would map to the *Eco* RI ''C'' region of the viral genome.

Detailed DNA sequence analysis identified an ORF with identity to the HCMV UL83 mapping to a *Bam* HI subclone of *Eco* RI ''C''. The deduced amino acid sequence of this protein was 565 amino acids, similar to than that observed for the HCMV UL83 protein. BLAST sequence analysis assigned an overall

Fig. 5. (Continued). UL83-specific sera were used to immunoprecipitate radiolabeled proteins from ^{32}P-labeled lysates. After preclearing lysates with preimmune sera, phosphoproteins were immunoprecipitated using a polyclonal anti-GPCMV antisera either from mock-infected, ^{32}P-labeled GPL cells (lane 1), or from a lysate pulse-labeled at a late time point (96 h) post-inoculation with GPCMV (lane 2). Preimmune guinea pig antisera (lane 3), or antisera from a GEX-UL83 immunized animal (lane 4) were used to immune precipitate phosphoproteins from mock-infected, ^{32}P-labeled GPL cells or from a lysate pulse-labeled at a late time point (96 h) post-inoculation with GPCMV (lane 5, preimmune, lane 6, GST-UL83 immune sera). All immune precipitations were performed using *Staphylococcus* protein A. GEX-UL83 antisera specifically immune precipitates a 70 kDa phosphoprotein, confirming phosphorylation of GPCMV UL83 homolog.
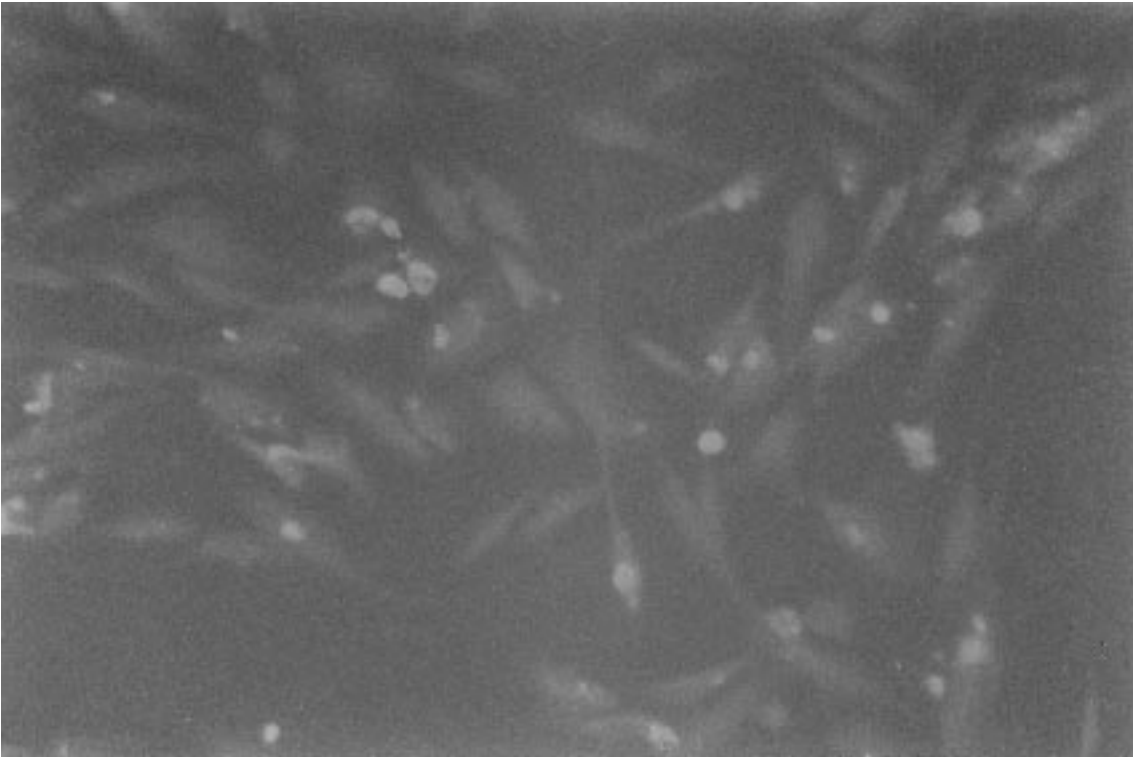
Fig. 7. Immunofluorescence analysis of GPCMV UL83 expression. Plasmid pKTS 437 (see text) contains GPCMV UL83 coding sequences under control of HCMV immediate early promoter. Following transfection into GPL cells, polyclonal anti-GPCMV antibody was used for immunofluorescence analysis of intracellular UL83 expression. Preimmune sera revealed no fluorescence, and transfection of vector plasmid only (pcDNA 3) also was negative (data not shown). Pattern of fluorescence is nuclear in distribution, suggesting that GPCMV UL83 homolog has functional nuclear targeting signal. Fluorescence obtained with FITC-conjugated goat anti-guinea pig secondary antibody (Sigma) at a 1:300 dilution. Monolayers were subjected to microscopic analysis using a Zeiss Axiphot 2 photomicroscope, FITC filter, 40x objective.

similarity of 42% and amino acid identity of 23% between the GPCMV UL83 homolog and the HCMV UL83. Conservation of sequence was most remarkable in the amino terminal 2/3 of the molecule (Fig. 4). Athough there was sequence divergence in the COOH-terminal 1/3 of the molecule, the hydropathy profile was similar, suggesting conservation of function: furthermore, the GPCMV UL83 homolog contains strongly basic motifs in this region of the molecule (Fig. 2), suggesting conservation of the bipartite, basic nuclear localization motif which has been defined for the HCMV UL83 (30,31). Immunofluorescence assays using monospecific anti-GPCMV UL83 antisera in cells transfected with a GPCMV UL83 expression plasmid identified a predominantly nuclear pattern of fluorescence (Fig. 7), suggesting that the potential nuclear targeting

domains of the GPCMV homolog may be functioning in a manner analogous to the HCMV UL83 protein.

Transcription of the GPCMV UL83 could initiate at either of two potential TATA elements 241 and 177 bp bases upstream of the putative ATG translation initiation codon. Examination of the UL83 promoter for *cis*-acting sequences identified the sequences CCCGCGCCC and CCCGCGCCC, which bear homology to the consensus sequence for the SP-1 family of transcription binding factors (32). Novel sequences of interest in the GPCMV UL83 promoter included two 13 base pair palindromic repeats GCACGACTCGTGC and an octameric inverted repeat, TGAGCTCC. The 8-nucleotide repeat sequence is of considerable interest in view of the observation that inverted repeat octamer sequences have been shown to confer responsiveness to viral

immediate-early gene products in the transcriptional regulation of the HCMV UL83 gene (33). The role these *cis*-sequences may play in transcriptional regulation of the GPCMV UL83 promoter is currently under investigation. Examination of sequences downstream of the GPCMV UL83 stop codon revealed an ORF with identity to the HCMV UL82 (pp71) homolog. Northern blot experiments using a probe corresponding to the GPCMV UL83 ORF revealed two mRNA species of approximately 4.2 and 2.2 kb, whereas a probe corresponding to the GPCMV UL82 coding sequences identified only the 4.2 kb band (Fig. 3). Since the UL82 probe hybridized only with the 4.2 kb RNA, we conclude that the GPCMV UL82 is most likely encoded by a bicistronic ''read-through'' transcript which likely initiates at the same TATA box element as the smaller UL83-specific species. Similar bicistronic transcripts have also been described for the HCMV UL82/83 transcription unit (28,30,33,34).

The pattern of transcription of the GPCMV UL83/ UL82 transcription unit appears to have some significant differences from that of the HCMV UL83. Previous work on HCMV UL83 transcription revealed that HCMV UL83 is an ''early-late'' gene, with maximal levels of transcription occurring at late time points, but with a modest amount of transcription occurring at early time points (33,34). In the report published by Depto and Stenberg, however, it is noteworthy that, in the presence of PAA, UL83 transcription was dramatically diminished (33). These authors concluded that, although some UL83 transcription occurs at an early time points, that transcription is ''greatly amplified'' following the initiation of DNA replication. In the description of transcription of the MCMV UL83 homolog (M83), metabolic blockade with PAA was not performed; however, Western blot analysis of protein synthesis in the presence and absence of PAA demonstrated unequivocally that the M83 protein is a ''late'' protein (29). The transcription of the GPCMV UL83 homolog appears to be PAA-sensitive, suggesting that GPCMV UL83 is a true ''late'' gene. As noted above, these findings are clearly consistent with analyses of UL83 homolog genes from other cytomegaloviruses. More detailed analyses (RNAse protection analysis and single-stranded DNA probe analysis) are in progress to test whether there is a small ''early'' component of GPCMV UL83 transcription. However, our Northern data suggest that the GPCMV UL83 RNA is predominantly a ''late'' transcript.

The GPCMV UL83 protein generated by *in vitro* translation was approximately 65 kDa, in agreement with the size deduced from the ORF identified by DNA sequencing. Immunofluorescence analysis of a transfected plasmid expressing the GPCMV UL83 protein identified a predominantly nuclear pattern of fluorescence (Fig. 7). A GPCMV UL83-specific antisera generated against a GST fusion protein recognized a 70 kDa virion-associated band in Western blot, the putative GPCMV homolog of the HCMV pp65 protein. Similarly, immunoprecipitation analysis using specific antisera also identified a 70 kDa band in ^{35}S-labeled infected tissue culture lysates. Since the HCMV UL83 (pp65) is extensively phosphorylated at casein kinase II (CKII) sites clustered within the highly hydrophilic region approximately spanning amino acids 360–480 (35), we next examined whether the GPCMV UL83 homolog is similarly phosphorylated. Using [^{32}P]-orthophosphate labeled tissue culture lysates, we identified a single phosphorylated species of approximately 70 kDa using our GPCMV UL83-specific antisera (Fig. 5d), indicating that the GPCMV homolog is phosphorylated. Monospecific antisera which targets the murine cytomegalovirus homolog of UL83, the M83 protein, is immunoreactive with three substantially different size classes of proteins, identifying species of 125, 105 and 70 kDa by Western blot (29). In contrast, we could only identify a single UL83 species by Western blot and RIP-PAGE analyses. Presumably, the migration of the GPCMV UL83 homolog at 70 kDa (compared to the predicted M_r of 62.3 kDa) reflects an alteration in migration induced by phosphorylation, although further experiments will be necessary to define which potential CKII sites are specifically utilized. Inspection of the amino acid sequence reveals ten consensus CKII sites, with seven of these being present between aa residues 430–521, including a predicted site (Ser_{473}) which is co-linear with a known phosphorylation site for the HCMV pp65 (35). In addition to being phosphorylated at CKII sites, the HCMV pp65 itself has been regarded as a serine threonine protein kinase, since anti-pp65 antibodies immunoprecipitate a CKII-like activity (36,37). Recent evidence suggests that this kinase activity may represent a ''captured'' cellular kinase, polo-like kinase 1 (38). Whether the GPCMV UL83 protein contains endogenous kinase activity or co-immunoprecipitates with endogenous cellular kinases will require additional investigation: however,

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based on its amino acid sequence homology, its presence in dense bodies, its nuclear localization pattern, and its ability to be phosphorylated, it is clear that this protein is the homolog of the HCMV pp65.

The identification of the UL83 homolog of GPCMV should facilitate the development and testing of subunit vaccines in the GPCMV model of congenital infection. Since the major target of CTL responses in the setting of HCMV infection is the UL83 protein, the identification of a conserved homolog in GPCMV will allow the testing of a mixed glycoprotein/phosphoprotein vaccine in the only available animal model of congenital CMV infection. Indeed, our preliminary experiments have suggested the immunogenicity of a subunit GPCMV UL83 DNA vaccine (39). Work by other groups has demonstrated the immunogenicity of a HCMV UL83 DNA vaccine in mice (40), but the species-specificity of CMV precluded any subsequent challenge or protection experiments. The protective efficacy of maternal immune responses to potential CTL targets in limiting placental and fetal infection with CMV has to date not been experimentally evaluated. The cloning and successful subunit expression of the UL83 gene product of GPCMV will allow the testing of the hypothesis that preconceptual immunity against CMI targets may be useful in improving fetal outcomes in the setting of maternal CMV challenge.

Insights derived from the study of the GPCMV UL83 protein homolog may also be germane to the study of the role of this protein in the pathogenesis of *in utero* infection. HCMV UL83 (pp65) is efficiently targeted to the cell nucleus, both as a cytoplasmically injected viral particle early in infection, and as a newly synthesized protein at later time points in infection. This activity has been mapped to a bipartite nuclear localization signal (30,31). Once inside the nucleus, pp65 has a strong affinity for nuclear lamins (41). In transfected astrocytoma cells, a recombinant form of pp65 was found to form a stable association with metaphase chromosomes (42). These observations regarding the complex trafficking of the UL83 protein within the nucleus suggest that pp65 may conceivably play a role in teratogenesis, potentially by contributing to chromosomal injury in mitotically active fetal cells. The GPCMV UL83 homolog has similar functional nuclear localization signals (43). Since the UL83 gene is known to be dispensable for the growth of HCMV in tissue culture (44), the generation of GPCMV UL83 deletion mutants could allow the testing of the role of this protein in both the transplacental transfer of virus as well as the damage to the developing fetus in the guinea pig model of congenital CMV infection.

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References

1. Alford C.A. and Britt W.J., in: Fields B.N. and Knipe D.M. (ed), *Virology* second edition. Raven Press, New York, 1981–2010, 1990.
2. Boppana S.B., Pass R.F., Britt W.J., Stagno S., and Alford C.A., Pediatr Infect Dis J *11*, 93–99, 1992.
3. Fowler K.B., Stagno S., Pass R.F., Britt W.J., Boll T.J., and Alford C.A., N Engl J Med *326*, 663–667, 1992.
4. Griffith B.P. and Aquino-De Jesus M.J.C., Transplant Proc *23*, 29–31, 1991.
5. Griffith B.P., McCormick S.R., Fong C.K.Y., Lavallee J.T., Lucia H.L., and Goff E., J Virol *55*, 402–409, 1985.
6. Bratcher D.F., Bourne N., Bravo F.J., Schleiss M.R., Slaoui M., Myers M.G., and Bernstein D.I., J Infect Dis *172*, 944–950, 1995.
7. Schleiss M.R., Bourne N., and Bernstein D.I., Pediatr Res *43*, 252A, 1998.
8. Schleiss M.R., Virology *202*, 173–185, 1994.
9. Brady R.C. and Schleiss M.R., Arch Virol *141*, 2409–2424, 1996.
10. Spaete R.R., Gehrz R.C., and Landini M.P., J Gen Virol *75*, 3287–3308, 1994.
11. McLaughlin-Taylor E., Pande H., Forman S.J., Tanamachi B., Li C.R., Zaia J.A., Greenberg P.D., and Riddell S.R., J Med Virol *43*, 103–110, 1994.
12. Wills M.R., Carmichael A.J., Mynard K., Jin X., Weekes M.P., Plachter B., and Sissons J.G., J Virol *70*, 7569–7579, 1996.
13. Gilbert M.J., Riddell S.R., Plachter B., and Greenberg P.D., Nature *383*, 720–722, 1996.
14. Walter E.A., Greenberg P.D., Gilbert M.J., Finch R.J., Watanabe K.S., Thomas E.D., and Riddell S.R., N Engl J Med *333*, 1038–1044, 1995.
15. Bishopp F., Bioworld Today *7*(231), 1, 4, 1996.
16. Gao M. and Isom H.C., J Virol *52*, 436–447, 1984.
17. Maniatis T., Fritsch E.F., and Sambrook J., *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

18. Yin C.-Y., Gao M., and Isom H.C., J Virol *64*, 1537–1548, 1990.

19. Rixon F.J., Addison C., McGregor A., Macnab S.J., Nicholson P., Preston V.G., and Tatman J.D., J Gen Virol *77*, 2251–2260, 1996.

20. Smith D.B. and Johnson K.S., Gene *67*, 31–40, 1988.

21. Paglino J.C., Brady R.C., and Schleiss M.R., Arch Virol *144*, 447–462, 1999.

22. Laemmli U.K., Nature *227*, 680–685, 1970.

23. Talbot P. and Almeida J.D., J Gen Virol *36*, 345–349, 1977.

24. Schleiss M.R., Aktan L., and Erdem G., Abstracts of the XXII International Hervesvirus Workshop, Abstract #434, 1997.

25. Isom H.C., Gao M., and Wigdahl B., J Virol *49*, 426–436, 1984.

26. McLauchlan J., Gaffney D., Whitton J.L., and Clements J.B., Nucleic Acids Res *13*, 1347–1366, 1985.

27. Chee M.S., Bankier A.T., Beck S., Bohni R., Brown C.M., Cerney R., Horsnell T., Hutchinson C.A., Kouzarides T., Martignetti J.A., Preddie E., Satchwell S.C., Tomlinson P., Weston K.M., and Barrell B.G., Curr Top Microbiol Immunol *154*, 125–169, 1990.

28. Rüger B., Klages S., Walla B., Albrecht J., Fleckenstein B., Tomlinson P., and Barrell B., J Virol *61*, 446–453, 1987.

29. Cranmer L.D., Clark C.L., Morello C.S., Farrell H.E., Rawlinson W.D., and Spector D.H., J Virol *70*, 7929–7939, 1996.

30. Schmolke S., Drescher P., Jahn G., and Plachter B., J Virol *69*, 1071–1078, 1995.

31. Gallina A., Percivalle E., Simoncini L., Revello M.G., Gerna G., and Milanesi G., J Gen Virol *77*, 1151–1157, 1996.

32. Faisst S. and Meyer S., Nucleic Acids Res *20*, 3–26, 1992.

33. Depto A.S. and Stenberg R.M., J Virol *63*, 1232–1238, 1989.

34. Geballe A.P., Leach F.S., and Mocarski E.S., J Virol *57*, 864–874, 1986.

35. Pande H., Lee T.D., Churchill M.A., and Zaia J.A., Virology *178*, 6–14.

36. Michelson S., Tardy-Panit M., and Barzu O., J Biochem *149*, 393–399, 1985.

37. Somogyi T., Michelson S., and Masse M.J., Virology *174*, 276–285, 1990.

38. Gallina A., Simoncini L., Garbelli S., Percivalle E., Pedrali-Noy G., Lee K.S., Erikson R.L., Plachter B., Gerna G., and Milanesi G., J Virol *73*, 1468–1478, 1999.

39. Schleiss M., Bourne N., Jensen N., Bravo F., Reece A., Erdem G., and Bernstein D., Pediatr Res *45*, 173A, 1999.

40. Pande H., Campo K., Tanamachi B., Forman S.J., and Zaia J.A., Scand J Infect Dis (Suppl. 99), 117–120, 1995.

41. Sanchez V., Angeletti P.C., Engler J.A., and Britt W.J., J Virol *72*, 3321–3329.

42. Dal Monte P., Bessia C., Landini M.P., and Michelson S., J Gen Virol *77*, 2591–2596, 1996.

43. McGregor A. and Schleiss M.R., unpublished data.

44. Schmolke S., Kern H.F., Drescher P., Jahn G., and Plachter B., J Virol *69*, 5959–5968, 1995.