JOURNAL OF VIROLOGY, Nov. 1992, p. 6784–6787
0022-538X/92/116784-04$02.00/0

Vol. 66, No. 11

Construction and Characterization of a Human Cytomegalovirus Mutant with the UL18 (Class I Homolog) Gene Deleted

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Received 17 June 1992/Accepted 10 August 1992

The UL18 open reading frame of human cytomegalovirus (HCMV) (which encodes a product homologous to major histocompatibility complex class I heavy chains) has been disrupted by insertion of the β-galactosidase gene under control of the major HCMV early promoter. The recombinant virus ΔUL18 showed no phenotypic differences from wild-type HCMV in terms of single-step growth curves or particle/infectivity ratios, indicating that the UL18 gene product is dispensable for the growth of HCMV in human fibroblasts in vitro. The synthesis of the mature cellular class I heterodimer is shut down in cells infected at a high multiplicity with wild-type HCMV, and a similar effect was seen in ΔUL18-infected fibroblasts, suggesting that although the UL18 gene product can associate with β_2 microglobulin, it is not directly involved in the disruption of class I assembly.

The 72-kDa product of the immediate-early I gene of human cytomegalovirus (HCMV) is known to be recognized well by $CD8^+$ T lymphocytes of asymptomatic persistently HCMV-infected individuals, while proteins made later in infection appear to be relatively poor targets (6), an observation which may be related to the finding that synthesis of the mature major histocompatibility complex class I heterodimer is shut down in HCMV-infected cells (8). The mechanisms involved in the ability of CMV-infected cells to evade lysis by cytotoxic T lymphocytes are poorly defined, but it has been suggested (8) that the virus may accomplish such a shutdown of cellular class I synthesis together with subsequent effects on antigen presentation by virtue of the expression of a virally encoded homolog of the cellular class I heavy chain. A model which envisages that the CMV class I homolog, the product of the UL18 gene (3), sequesters host β_2 microglobulin (β_2m), thereby disrupting assembly of the cellular class I heterodimer, has been proposed. In support of this model, it has been shown that the UL18 product will associate with human β_2m when both proteins are coexpressed by recombinant vaccinia virus (8). However, it is not possible to ascribe a definitive functional role to the UL18 gene product in CMV-infected cells without a UL18-deficient mutant. The potential of HCMV to express the *Escherichia coli lacZ* gene under control of a CMV early (β) gene promoter has previously been demonstrated (19), and we have adopted a similar strategy for the targeted insertion of the *lacZ* gene into the CMV genome at the UL18 locus.

The plasmid pATH30 (18), which contains the 8.67-kb *Hin*dIII O fragment of HCMV strain AD169 cloned in pAT153, was digested with *Nde*I, which cleaves at one site in this plasmid, 107 bp 3′ of the ATG of the UL18 open reading frame. A synthetic linker was constructed by annealing oligonucleotides 5′ TATGAGATCTAGTACTAAGCT TCA 3′ and 5′ TATGAAGCTTAGTACTAGATCTCA 3′, and this linker was ligated into *Nde*I-digested and phosphatased pATH30 to give pATH30N. The *Bgl*II site present in the linker is unique in pATH30N, and a 3.8-kb *Bam*HI fragment containing *E. coli lacZ* sequences downstream of the major CMV early promoter (12), derived from the plasmid pMV1 (supplied by G. Wilkinson, PHLS, Centre for Applied Microbiological Research, Salisbury, United Kingdom), was ligated into *Bgl*II-digested and phosphatased pATH30N to give pUL18ΔgalE. This plasmid contains the *lacZ* gene in the direction opposite to that of UL18 transcription, and although there are no transcription termination signals present at the direct 3′ end of the *lacZ* gene in pUL18ΔgalE, there is a site for poly(A) addition in the flanking CMV DNA 1.5 kb 5′ of the *Nde*I insertion site. Since the *lacZ* gene in this orientation contains translation termination codons in all three reading frames, a transcript derived from the UL18 promoter in pUL18ΔgalE would be translated to give the first 37 amino acids of UL18, 18 of which constitute the signal peptide and 19 of which correspond to residues with homology to the α1 domain of the cellular class I molecule HLA-A2 (5). Linearized pUL18ΔgalE (2.5 μg) was cotransfected with 20 μg of AD169-infected cell DNA into subconfluent monolayers of Flow 2002 human fibroblasts by the modified calcium phosphate precipitation method of Chen and Okayama (10), with a 20% dimethyl sulfoxide boost 4 h after transfection. Transfection progeny were harvested after 11 days and replated onto fresh cell monolayers. Ten days later, the medium was removed and plaques were overlaid with medium containing 1% low-melting-temperature agarose, 300 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml, and 10% fetal calf serum. Transfection efficiencies were routinely low, with between 0 and 10 plaques produced after transfection of 20 μg of CMV DNA, and cotransfection of pUL18ΔgalE with CMV DNA gave rise to progeny virus containing potential recombinant virus expressing β-galactosidase at a frequency of approximately 0.1%. Blue plaques were picked into 0.5 ml of medium containing 10% fetal calf serum, serially diluted, and replated onto fresh monolayers, and then they underwent a limiting dilution on 24-well trays of MRC5 cells. At this stage, to identify wells containing recombinant virus, the monolayers were fixed with 0.5% glutaraldehyde 10 days after infection (while the supernatants were retained as a virus stock), permeabilized with a solution containing 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 2 mM

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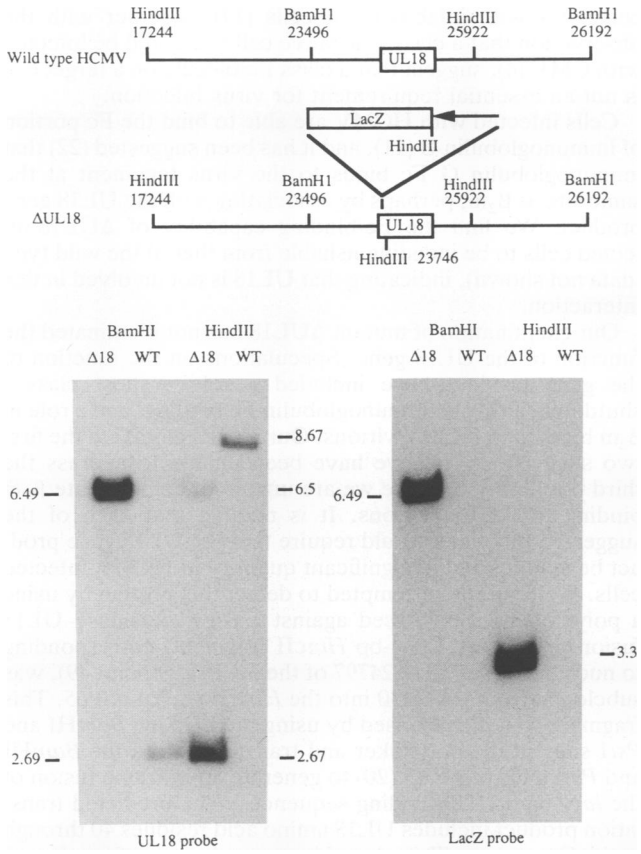

FIG. 1. Southern hybridization analysis of ΔUL18 DNA and wild-type (WT) CMV DNA following *Bam*HI or *Hin*dIII digestion.

$MgCl_2$, and stained with 1 mg of X-Gal per ml in 5 mM potassium ferricyanide-potassium ferrocyanide. Supernatants from wells which appeared to contain single blue plaques were used to infect MRC5 cells from which DNA was extracted for analysis.

The correct insertion of *lacZ* sequences within the UL18 gene in recombinant virus and ΔUL18 and the absence of contaminating wild-type virus were established by Southern hybridization (Fig. 1). DNA was digested with either *Hin*dIII or *Bam*HI and, after transfer to nitrocellulose, was hybridized to a 2.5-kb fragment of the *lacZ* gene or to a 1.2-kb fragment containing the entire UL18 coding sequence. Figure 1 shows that DNA from ΔUL18 contained a 3.3-kb *Hin*dIII fragment which hybridized to the *lacZ* probe, as predicted. The UL18 gene lies in the 8.67-kb *Hin*dIII O fragment of the AD169 genome, and insertion of the *lacZ*-HCMV early-promoter cassette at this locus would disrupt this fragment, since there is a *Hin*dIII site at the early-promoter–*lacZ* junction. DNA from ΔUL18 gave rise to the 6.5-kb and 2.67-kb *Hin*dIII fragments expected after correct insertion of early-promoter and *lacZ* sequences. The 2.69-kb *Bam*HI fragment containing UL18 which is detected in wild-type virus DNA was replaced by a 6.49-kb fragment in the DNA of ΔUL18 which also hybridized to the *lacZ* probe as predicted. No evidence of wild-type virus DNA was found in stocks of ΔUL18; however, approximately 1 in 300 plaques produced by the recombinant does not stain blue with X-Gal, presumably as a result of point mutations in the

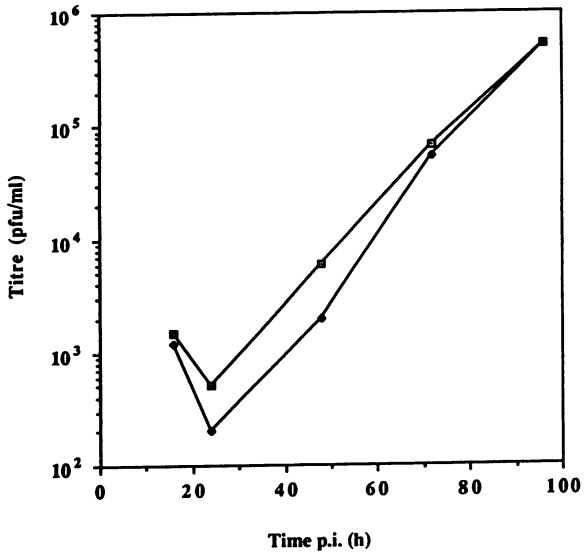

FIG. 2. Single-step growth curves of ΔUL18 (◆) and wild-type HCMV (□) on MRC5 cells. Supernatant virus was assayed for infectivity by plating serial dilutions onto monolayers of MRC5 cells, overlaying with modified Eagle's medium containing 10% fetal calf serum and carboxymethyl cellulose, and incubating at 37°C for 10 days. Plaques were then fixed, stained with toluidine blue, and counted. p.i., postinfection.

3.4-kb β-galactosidase gene. The isolation of a CMV mutant in which the UL18 gene is disrupted by *lacZ* sequences confirms that the UL18 gene product is not required for HCMV to grow in tissue culture and shows that the UL18 gene, like UL16 (15b), UL20 (15a), UL33 (7a), and US1 through US5 and the US6 family (15), is a site which may be useful for insertion of other genes into the HCMV genome. When the β-galactosidase gene was used by Spaete and Mocarski (19) to select a deletion mutant of HCMV, it was noted that sequences flanking the site of insertion were deleted, perhaps because of packaging limits of the virion. Although we have not performed a detailed restriction analysis of DNA from ΔUL18, it seems from Southern hybridization that the 8.6-kb sequences flanking the *lacZ* cassette remain unaffected by recombination events.

Initial characterization of ΔUL18 involved comparisons with wild-type CMV in terms of particle/infectivity ratios and single-step growth curves. The numbers of enveloped virus particles in stocks of AD169 and ΔUL18 were estimated by comparison with latex beads of known concentration by electron microscopy (24). Particle/infectivity ratios of 180:1 and 300:1 were calculated for AD169 and ΔUL18, respectively, and these values were not considered significantly different given the errors in particle counting and plaque assays. To compare single-step growth curves of the wild type and ΔUL18, monolayers of MRC5 cells were infected at a multiplicity of infection of 5 and, after absorption, were washed three times with medium before being overlaid with medium containing 10% fetal calf serum. Supernatant virus was harvested at 16, 24, 48, 72, and 96 h after infection, stored at −70°C in aliquots, and assayed for infectivity by plating onto monolayers of MRC5 cells. Figure 2 shows that the viruses grew to equivalent titers with comparable kinetics.

To determine whether the UL18 gene product is involved in the shutdown of cellular class I expression, lysates of cells

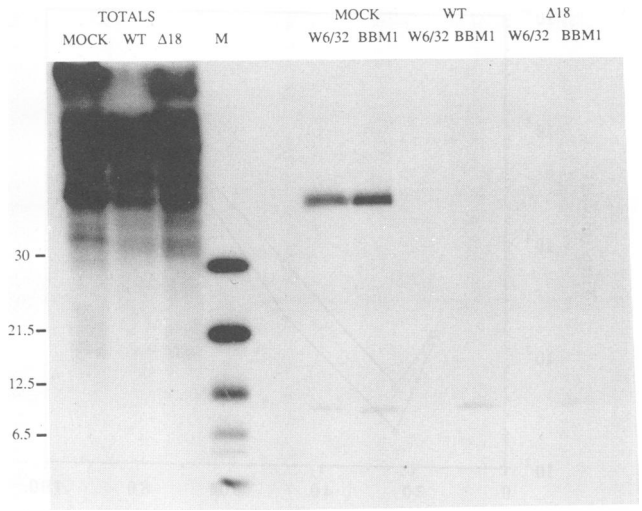

FIG. 3. Analysis of cellular class I synthesis in wild-type- and ΔUL18-infected MRC5 cells. Lysates of [^{35}S]methionine-labelled cells that were infected with wild-type CMV (WT) or ΔUL18 (Δ18) or that were mock infected (MOCK) were immunoprecipitated with one of two monoclonal antibodies, W6/32 and BBM1. M, molecular weight markers (molecular weights, in thousands, are shown on the left).

infected at 10 PFU per cell with either AD169 or ΔUL18 and labelled with 100 μCi of [^{35}S]methionine per ml in methionine-free medium 72 to 76 h postinfection were immunoprecipitated with W6/32 (2), a monoclonal antibody which recognizes the mature class I heterodimer, and with monoclonal antibody BBM1 (7), which precipitates both free and heavy-chain-associated β_2m. Figure 3 shows that the 46-kDa heavy chain could be precipitated together with the 11-kDa β_2m from uninfected cells by both W6/32 and BBM1. This complex could not be detected in cells infected with AD169 or ΔUL18, although free-β_2m synthesis appeared unaffected by infection with either virus, an observation which argues against the view that the UL18 gene product interferes with class I assembly and which leaves the function of the CMV class I homolog open to speculation.

It has been tempting to suggest a link between the finding of a class I-like gene in CMV and the observations of Grundy et al. (13) and McKeating et al. (16) that CMV virions, purified either from urine or from tissue culture supernatants, will bind human and bovine β_2m. Stannard has also demonstrated β_2m binding to the tegument of AD169 particles (21). We hoped to address this issue, having constructed a UL18 mutant; however, we have been unable to demonstrate binding of β_2m to 10^{10} purified AD169 particles incubated with either human urine or purified human β_2m, under conditions in which 100 molecules of β_2m per virion can be detected. Grundy et al. (14) have proposed that β_2m-coated virus is rendered more infectious, being able to use the cellular class I molecule as a receptor on which β_2m exchange may occur; our data which show that a UL18-deficient virus grows to titers equivalent to those of wild-type HCMV and shows similar particle/infectivity ratios would argue that if UL18 is responsible for β_2m binding, this phenomenon is not related to virus infectivity in vitro. Furthermore, the identification of a fibroblast membrane glycoprotein(s) of 32 to 34 kDa to which HCMV specifically binds (1, 23) and the finding that expression of the molecule correlates with infectivity of cells (17), together with the observation that a class I-negative cell line could be infected with CMV (4), suggest that a class I molecule on a target cell is not an essential requirement for virus infection.

Cells infected with HCMV are able to bind the Fc portion of immunoglobulin G (11), and it has been suggested (22) that immunoglobulin G Fc binds to the virus tegument at the same site as β_2m, perhaps by associating with the UL18 gene product. We find the Fc-binding capability of ΔUL18-infected cells to be indistinguishable from that of the wild type (data not shown), indicating that UL18 is not involved in this interaction.

Our examination of mutant ΔUL18 has not illuminated the function of the UL18 gene. Speculations on the function of the gene product have included a role in host class I shutdown, a role in immunoglobulin Fc binding, and a role in β_2m binding to HCMV virions. Our results eliminate the first two suggestions, but we have been unable to address the third possibility because we are unable to demonstrate β_2m binding to AD169 virions. It is notable that each of the suggested functions would require that the UL18 gene product be synthesized in significant quantity in HCMV-infected cells. We therefore attempted to detect this protein by using a polyclonal serum raised against a β-galactosidase–UL18 fusion protein. A 1,044-bp *Hin*cII fragment, corresponding to nucleotides 23753 to 24797 of the HCMV genome (9), was subcloned from pATH30 into the *Hin*cII site of pSP65. This fragment was then excised by using the flanking *Bam*HI and *Pst*I sites in the polylinker and transferred into the *Bam*HI and *Pst*I sites of pEX3 (20) to generate an in-frame fusion of the *lacZ* and UL18 coding sequences. The predicted translation product includes UL18 amino acid residues 40 through to the C terminus. This plasmid was used to transform *E. coli* POP2136, and a 150-kDa fusion protein was induced by heat shock. The protein was purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and used to immunize rabbits by subcutaneous and intramuscular routes. Sera were screened by Western blotting (immunoblotting) against lysates of cells infected with recombinant vaccinia virus expressing the UL18 gene (8). Serum obtained after four immunizations was affinity purified by a two-step process in which antibodies against β-galactosidase were first removed by using β-galactosidase linked to cyanogen bromide-activated Sepharose, and antibodies against the UL18 gene product were selected by using the purified *lacZ*-UL18 fusion protein linked to the same support. These affinity-purified antibodies detected a 68-kDa protein in lysates of cells infected with a recombinant vaccinia virus expressing the UL18 gene and failed to recognize any products in control vaccinia virus-infected cell lysates (Fig. 4a), confirming the specificity of the sera for the UL18 product. This protein has previously been shown to form a heterodimer with human β_2m (8). However, we were unable to detect this protein in lysates prepared from equivalent numbers of cells infected with HCMV at a multiplicity of infection of 10 (Fig. 4b). It is clear that if the UL18 gene is expressed during HCMV infection of fibroblasts, the amount of protein produced must be very low, and this result reinforces the view that the UL18 gene product plays no significant role in the permissive infection of fibroblasts by HCMV. Although we have been unable to discern any phenotypic differences between AD169 and ΔUL18, it should perhaps be noted that we have examined ΔUL18's properties only in human diploid fibroblast cell lines, and analysis of the ability of this mutant to infect a range of primary human cell types may shed light on the function of the UL18 gene product in vivo.

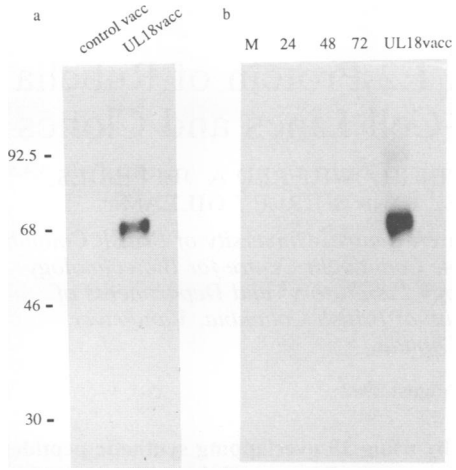

FIG. 4. Western blot of vaccinia virus (vacc)-infected cell lysates (a) and CMV-infected cell lysates (b) incubated with affinity-purified polyclonal antibodies to a UL18–β-galactosidase fusion protein. Lysates from BHK cells infected with recombinant vaccinia viruses (UL18vacc) and from MRC5 cells that were either infected with AD169 and incubated for 24, 48, and 72 h or mock infected (M) were electrophoresed in a 10% polyacrylamide gel and transferred to nitrocellulose. The filters were incubated with affinity-purified anti-UL18 serum, and the incubation was followed by detection of bound antibodies with ^{125}I-protein A. Molecular weight markers (in thousands) are shown on the left.

This work was supported by the Medical Research Council, United Kingdom.

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