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Reassessing the Organization of the UL42 – UL43 Region of the Human Cytomegalovirus Strain AD169 Genome

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Abstract

A polymorphism in the UL42 – UL43 region of the human cytomegalovirus genome has been characterized by nucleotide sequence analysis, revealing a 929-bp insertion following nt 54,612 relative to the published strain AD169-UK genome sequence (M. S. Chee *et al.*, 1990, *Curr. Top. Microbiol. Immunol.* 154, 125 – 170). Although AD169-UK exhibited polymorphism in this genomic region, other CMV strains (Towne, Toledo, and AD169-ATCC) carried only the newly characterized longer form. The additional sequence altered the assignment of UL42 and UL43 open reading frames. UL42 decreased in size from 157 to 125 codons, retaining 76 of the previously reported carboxyl terminal codons, and UL43 increased in size from 187 to 423 codons, retaining 185 of the previously reported amino terminal codons. This additional sequence makes UL43 a more conserved betaherpesvirus US22 family member. Only AD169-UK exhibited restriction fragment length polymorphism in this region, suggesting that a deletion occurred during the propagation of this strain in cell culture. The additional sequence should be considered a bona fide part of the cytomegalovirus genome and the AD169 genome size should be corrected to 230,283 bp.

Introduction

Human cytomegalovirus (CMV) is a ubiquitous pathogen causing a variety of diseases primarily in immuno-compromised hosts and in the fetus (Alford and Britt, 1995). This virus exhibits a complex genome organization (Mocarski, 1995) and carries a linear double stranded DNA genome 229,354 bp in size based on the nucleotide sequence analysis of a set of *Hin*dIII clones derived from the AD169 genome (Oram *et al.*, 1982) and limited analysis of cosmid clones (Fleckenstein *et al.*, 1982) across *Hin*dIII sites (Chee *et al.*, 1990) (EMBL Accession No. X17403). AD169 was isolated from cultured tissue taken from adenoids of a 7-year-old girl (Rowe *et al.*, 1956); however, early passage samples of this widely used strain or descriptions of early passage history are not available. The AD169 genome sequence has been the basis for studies on CMV (Mocarski, 1995) despite the fact that it had been derived laboratory-propagated strain. Single nucleotide corrections to the original

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AD169 genome sequence have been reported in the UL102 (Smith and Pari, 1995) and US28 (Neote *et al.*, 1993) genes. Recently, the genomes of strains AD169 and Towne have both been shown to contain sequence rearrangements and to be much less complex than low-passage strains (Cha *et al.*, 1996).

We had previously observed very marked differences in the ability of CMV strains Towne, Toledo, and AD169 to replicate efficiently in thymic medullary epithelial cells in human thymus– liver implants carried by SCID-hu mice. We noted differences in the replication efficiency of the AD169 strain from two sources (ATCC vs UK) (Brown *et al.*, 1995). We noticed that strain AD169-UK, which replicated several orders of magnitude better than AD169-ATCC, exhibited a restriction fragment length polymorphism within the *Hin*dIII M region of the viral genome that had been noted in one earlier analysis (Oram *et al.*, 1982) but had not been noticed by others (Fleckenstein *et al.*, 1982; Spector *et al.*, 1982; Weststrate *et al.*, 1983). Although two species of *Hin*dIII M fragment were observed in the work by Oram and colleagues, the smaller and more abundant fragment was cloned as representative of the viral genome (Oram *et al.*, 1982) and was subsequently sequenced as a part of the strain AD169 genome analysis which led to the assignment of ORFs UL41 through UL48 (Chee *et al.*, 1990).

Materials and Methods

Virus and cells

CMV strain AD169-ATCC, obtained from the American Type Culture Collection (ATCC VR 538), AD169-UK, obtained from H. Browne (Cambridge, United Kingdom), Toledo, obtained from S. Plotkin (Pasteur Merieux, Lyon, France), and Towne, passage 134, obtained from S. Plotkin were grown in low passage (4 – 20) human foreskin fibroblasts (HFs) using Dulbecco's minimal essential medium (Gibco-BRL) supplemented with 10% NuSerum (Collaborative Biomedical), amino acids, and antibiotics as previously described (Brown *et al.*, 1995). Viral DNA was prepared as previously described (Spaete and Mocarski, 1985).

Plasmids and sequence analysis

Cosmids pCM1017 and pCM1049 have been described (Fleckenstein *et al.*, 1982). pON2201 carries the *Hin*dIII M fragment from cosmid pCM1049 (Fleckenstein *et al.*, 1982) cloned into pGEM3Zf/(Promega). pON2202 carries sequence between the *Hin*dIII Z/M site (nt 54,144) and a *Hpa*I site (nt 54,813; codon 118 of UL43) within *Hin*dIII M and was prepared by deleting sequences from the *Hpa*I site through the *Hin*dIII M/F site (nt 64,521) of pON2201. pON2128 carries a *Pst*I fragment representing sequences at the left end of *Hin*dIII M between nt 54,767 and 60,045 of AD169 in pGEM3Zf/.

Nucleotide sequence of pON2202 was determined on both strands by automated dideoxy sequence analysis (Lagenaur *et al.*, 1994; Sanger *et al.*, 1977) at the Stanford PAN facility using the following primers: 5′ CAGGAAACAGCTATGACC 3′, 5′ GGCGACGGCGAACTAAC 3′, 5′ CTGGCTCCCGTTCAAGAC 3′, 5′ GGCAAACGACGGCTT-TTC 3′, 5′ CGCAGCACAGGAACCTCT 3′, 5′

TGTAAAACGACGGCCAGT 3′, 5′ CGTGGTGGAATCGCTGTG 3′, 5′ TGGTGAACGAGAGCGGCG 3′, 5′ CACTGCCACCGACATGGA 3′, and 5′ CGGGGACGCAAGCATCAT 3′. All alignments were performed with the MCB Search Launcher program (Human Genome Center, Baylor College of Medicine) and assembled using SeqVu 1.0.1 (Garvan Institute of Medical Research, Sydney, Australia).

Results and Discussion

We compared the *Hin*dIII digestion patterns of AD169 DNA from virus preparations at different passage levels and from two different sources. Figure 1 shows a comparison of *Hin*dIII digests of AD169-UK and AD169-ATCC DNA from different lots of virus stock (2, 4, 16, and 22). The *Hin*dIII M region was detected by hybridization with a specific probe, pON2128 (Fig. 1A). The *Hin*dIII digests were also hybridized with a pCM1017 probe to detect *Hin*dIII fragments Y, N, J, Z, and M (Fleckenstein *et al.*, 1982) (data not shown). AD169-UK DNA contained two *Hin*dIII fragments spanning this region, one approximately 10 kbp which has been denoted *Hin*dIII M (Oram *et al.*, 1982) and the other approximately 11 kbp, which we denote here as modified *Hin*dIII M (M_m). All lots of AD169-ATCC that were analyzed contained only the 11-kbp *Hin*dIII M_m fragment. Serial propagation of AD169-UK appears to have resulted in a deletion not observed in AD169-ATCC or in other strains of virus.

We next compared the organization of this region in AD169-UK with other laboratory-propagated (Towne, passage 134) as well as low passage level (Toledo, passage 12) strains of CMV (Plotkin *et al.*, 1989) and found that these strains contained only the *Hin*dIII M_m fragment. Cosmids pCM1017, used as a probe here, and pCM1049, used to clone *Hin*dIII M_m, were derived from AD169 (Fleckenstein *et al.*, 1982) and also contained only the *Hin*dIII M_m fragment (data not shown). Toledo and AD169-ATCC were also shown to contain an *Xba*I fragment, denoted *Xba*I R_m, that was approximately 1 kbp larger in size than the major *Xba*I R fragment in AD169-UK (Oram *et al.*, 1982). This localized the region of polymorphism to between the *Hin*dIII site at nt 54,144 and the *Xba*I site at nt 56,067 on the strain AD169 genome. The Towne genome has a single restriction fragment representing a fusion of AD169 *Xba*I R and T (LaFemina and Hayward, 1980) which precludes direct comparison to Toledo or AD169. In order to further map the region of additional sequence, *Hin*dIII plus *Xba*I, *Hin*dIII plus *Eco*RI (nt 55,904), and *Hin*dIII plus *Hpa*I (nt 54,813) digests of Toledo, Towne, AD169-ATCC, and AD169-UK DNA were blotted and hybridized with the pCM1017 probe (data not shown). The additional sequence mapped to the leftmost portion of *Hin*dIII M_m within a region that should have exhibited a 669-bp *Hin*dIII– *Hpa*I fragment based on the published sequence. Instead, this fragment was approximately 1.5 kbp in size and was conserved in all strains tested. Thus, the region of variability mapped to the UL42 – UL43 region of the AD169 genome (nt 54144 to 54813) as shown in Fig. 2A. AD169-UK was the only strain exhibiting both short and long fragments.

To further evaluate the arrangement of sequences in the *Hin*dIII M region, a plasmid clone, pON2202, was subjected to nucleotide sequence analysis with oligonucleotide primers indicated in Fig. 2 and an automated sequencer (Lagenaur *et al.*, 1994; Sanger *et al.*, 1977). nstead of the expected 669-bp based on published sequence, an insertion of 929 bp was

found at nt 54,612, resulting in a total fragment size of 1599 bp (GenBank Accession No. AF01963).

The presence of this region in all CMV strains surveyed reinforced the notion that the longer fragment represented an authentic segment of CMV genome. The sequence was found to alter previously predicted ORFs, UL42 and UL43 (Chee *et al.*, 1990). Modified UL43 ($UL43_{mod}$) shared the amino terminal 185 codons with previously published UL43 (Chee *et al.*, 1990), but had an additional 238 carboxyl-terminal codons. Thus, a 423-aa protein would be encoded by $UL43_{mod}$. $UL43_{mod}$ is a much more convincing US22 family member that includes a complete set of conserved motifs (Chee *et al.*, 1990; Efstathiou *et al.*, 1992; Kouzarides *et al.*, 1988; Nicholas and Martin, 1994), as shown in Fig. 2B. Modified UL42 ($UL42_{mod}$) is 125 codons in size, sharing 76 carboxyl terminal codons with previously published UL42 (Chee *et al.*, 1990) and is predicted to be a transmembrane protein due to the previously identified hydrophobic run near the carboxyl terminus. $UL42_{mod}$ starts at a strong consensus initiation codon and is more homologous (17% identity, 61% similar) to M42 of murine CMV (Rawlinson *et al.*, 1996) than previously identified UL42, as depicted in Fig. 2C. A BLASTP search (Altschul *et al.*, 1990) revealed no close relatives of $UL42_{mod}$ in the Swissprot database or in any other herpesvirus. Although $UL42_{mod}$ and $UL43_{mod}$ appeared to be the most likely protein-coding sequences in this region based on their size and the presence of initiation codons, other ORFs ranging in size from 33 to 205 codons (all from initiation codons) were also found in this region. These additional ORFs were not similar to any of the ORFs in murine CMV, HHV-6 or HHV-7, and were not considered further. The additional sequence also revealed a 63-bp region between the stop codon of UL43 and the initiation codon of UL42.

$UL43_{mod}$ shows much greater similarity to betaherpesvirus US22 family members (Chee *et al.*, 1990; Efstathiou *et al.*, 1992; Kouzarides *et al.*, 1988; Nicholas and Martin, 1994) and includes not only a US22 family motif I (GxxoxoxWP; where “x” is any aa and “o” is a hydrophobic aa) previously described for UL43, but also complete motifs II (ooCCxxxLxxoG), III, and IV (Fig. 2B). A BLASTP search of the Swissprot database revealed $UL43_{mod}$ similarity with M43 (Rawlinson *et al.*, 1996) in the murine CMV genome (24% identity and 58% homology over the entire 423 aa) as well as U25 ORFs of HHV-6 and HHV-7 (Fig. 2B). These $UL43_{mod}$ homologs align throughout their carboxyl termini but have divergent amino termini. M43 shows similarity to the predicted amino terminus of $UL43_{mod}$ beginning at M43 aa 140. $UL43_{mod}$ is similar to M43 over its entire length. Similarity to other US22 family members begins at motif I ($UL43_{mod}$ aa 142), which represents the amino terminus of many US22 family members including U25 of HHV-6 or HHV-7 (Gompels *et al.*, 1995; Nicholas, 1996; Nicholas and Martin, 1994). Like other members of the human CMV US22 family members, $UL43_{mod}$ is more similar to US22 family members UL24, UL29, and UL36. After UL region family members, $UL43_{mod}$ appears more similar to TRS1/IRS1 than to other U_s region family members. Similarity to U_s region members is restricted to a small number of amino acids (aa) within the conserved motifs (Chee *et al.*, 1990; Efstathiou *et al.*, 1992; Kouzarides *et al.*, 1988; Nicholas and Martin, 1994). Unlike other US22 family members, $UL43_{mod}$ exhibits a striking cluster of basic aa at the extreme carboxyl terminus (8 of the 13 carboxyl terminal residues are K or

R), a characteristic that is also present in M43 (Fig. 2B). Like many US22 family members, $UL43_{mod}$ exhibits several consensus N-linked glycosylation sites (NxT/S) located at aa 26, 118, 299, 345 but has no hydrophobic stretch sufficiently long to span a membrane. $UL43_{mod}$ and M43 contain conserved cysteines at aa 23, 180, 181, 383, 404, 410. The $UL43_{mod}$ homolog U25 of HHV-6 has been shown to be a transactivator of gene expression (Nicholas and Martin, 1994), a characteristic that was first noted for other US22 family members (Cardin *et al.*, 1993; Colberg-Poley *et al.*, 1992; Geng *et al.*, 1992; Stasiak and Mocarski, 1992).

This report has identified additional nucleotide sequence in the UL42 – UL43 region of the CMV genome. The additional sequence is present in all AD169 strain variants and was apparently overlooked during the selection of plasmid clones for sequencing in previous work. The overall identity of all other regions of the AD169-ATCC and AD169-UK genomes can be deduced from previous restriction enzyme mapping (Fleckenstein *et al.*, 1982; Greenaway *et al.*, 1982; Oram *et al.*, 1982; Spector *et al.*, 1982; Weststrate *et al.*, 1980, 1983) as well as the absolute identity of many individual genes that have been sequenced (Boshart *et al.*, 1985; Heilbronn *et al.*, 1987; Jahn *et al.*, 1987; Mach *et al.*, 1986; Ruger *et al.*, 1987) and compared to the complete genome sequence (Chee *et al.*, 1990). Direct comparison of AD169-UK and AD169-ATCC restriction digests in our hands has not revealed any other differences (Fig. 1A and data not shown). We believe that strain AD169-UK may have developed a deletion in the UL42 – UL43 region during serial passage which was performed in a manner to attenuate virulence (Elek and Stern, 1974); however, the presence of the deletion does not correlate with reduced growth in thymic epithelial cells in SCID-hu mice (Brown *et al.*, 1995). When AD169-UK was subjected to additional plaque purification, the two variants were able to grow independently and to similar levels, indicating that the 929-bp sequence is dispensable for growth in cultured fibroblasts (Dargan *et al.*, 1997). Defective CMV genomes are known to be generated by purposeful high multiplicity passage (Stinski *et al.*, 1979) and a similar process may lead to the loss of regions of the CMV genome that are dispensable for growth in cultured fibroblasts. Limited homology of a similar nature has been noticed at the borders of herpes simplex virus defective genomes which arise by a recombination mechanism during high multiplicity passage (Mocarski *et al.*, 1985). The flanking regions matched the published sequence (Chee *et al.*, 1990) for this region with one copy of the sequence 5′ GCAG 3′ at both ends of the insertion. It is possible that the 4-bp repeat (GCAG) bracketing the region played some role in recombination because one copy of this tetranucleotide remained in place following deletion. Although the AD169-UK strain is polymorphic, other strains, including the AD169 strain from ATCC lack evidence of polymorphism in this region. We propose that the additional 929 bp and ORFs $UL42_{mod}$ and $UL43_{mod}$ should be considered *bona fide* constituents of the CMV genome and the estimated size of the strain AD169 genome should be increased to 230,283 bp.

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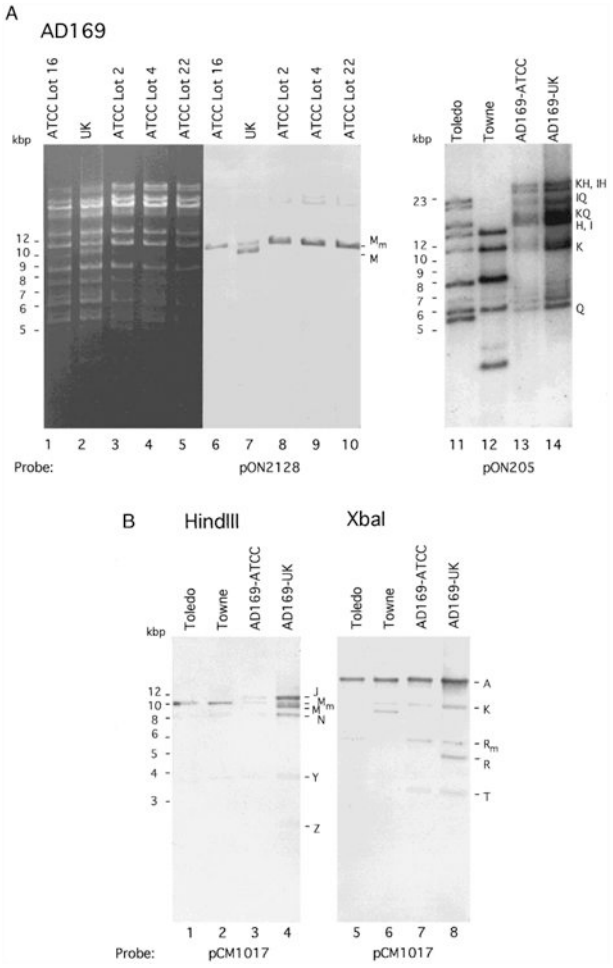

Fig. 1.

Size variation in the *Hin*dIII M region of CMV strain AD169-UK compared to AD169-ATCC, Towne, and Toledo. (A) Electrophoretically separated (0.5% agarose gel) *Hin*dIII-digested DNAs from AD169-ATCC (Lot 16), AD169-UK, AD169-ATCC (Lot 2), AD169-ATCC (Lot 4), and AD169-ATCC (Lot 22) were visualized by staining with ethidium bromide (lanes 1 – 5) and by subsequent DNA blot hybridization (lanes 5 – 10) with a fluorescein-11-dUTP (Amersham) random-primed labeled pON2128 probe detected by ECL (Amersham). Lots 2 and 4 of AD169 are the earliest stocks grown from material that was deposited with ATCC by the Rowe laboratory. Lot 16 represents material obtained from ATCC by our laboratory in 1987, and Lot 22, which has sustained 80 to 90 passages in culture, is a more recent (1995) preparation. Although early passages of AD169-UK were not examined, the strain we obtained from H. Browne (Cambridge) generated a pattern previously reported (Oram *et al.*, 1982). This strain had been obtained from the Rowe laboratory in 1960 in its 14th passage and was passaged 54 times by Stern and colleagues (Elek and Stern, 1974) and was provided to J. Oram and H. Browne by J. Booth (St. Georges Hospital Medical School, London). (B) Electrophoretically separated (0.7% agarose gel) *Hin*dIII-digested (lanes 1 – 4) or *Xba*I-digested (lanes 5 – 8) strain Toledo, Towne, AD169-ATCC (Lot 16), and AD169-UK DNA fragments hybridized with a

fluorescein-11-dUTP random-primed labeled pCM1017 probe (Fleckenstein *et al.*, 1982) and detected by ECL. Note that Towne lacks the *Xba*I R/T site present at nt 56,067 in the AD169 genome and therefore exhibits a 9.5-kbp fragment instead of *Xba*I fragments Rm (6 kbp) and T (3.5 kbp) that are observed in the other strains. Size markers are given on the left and AD169 genome restriction fragment names (with subscript m indicating the modified fragments identified here) are indicated to the right of each set of lanes.

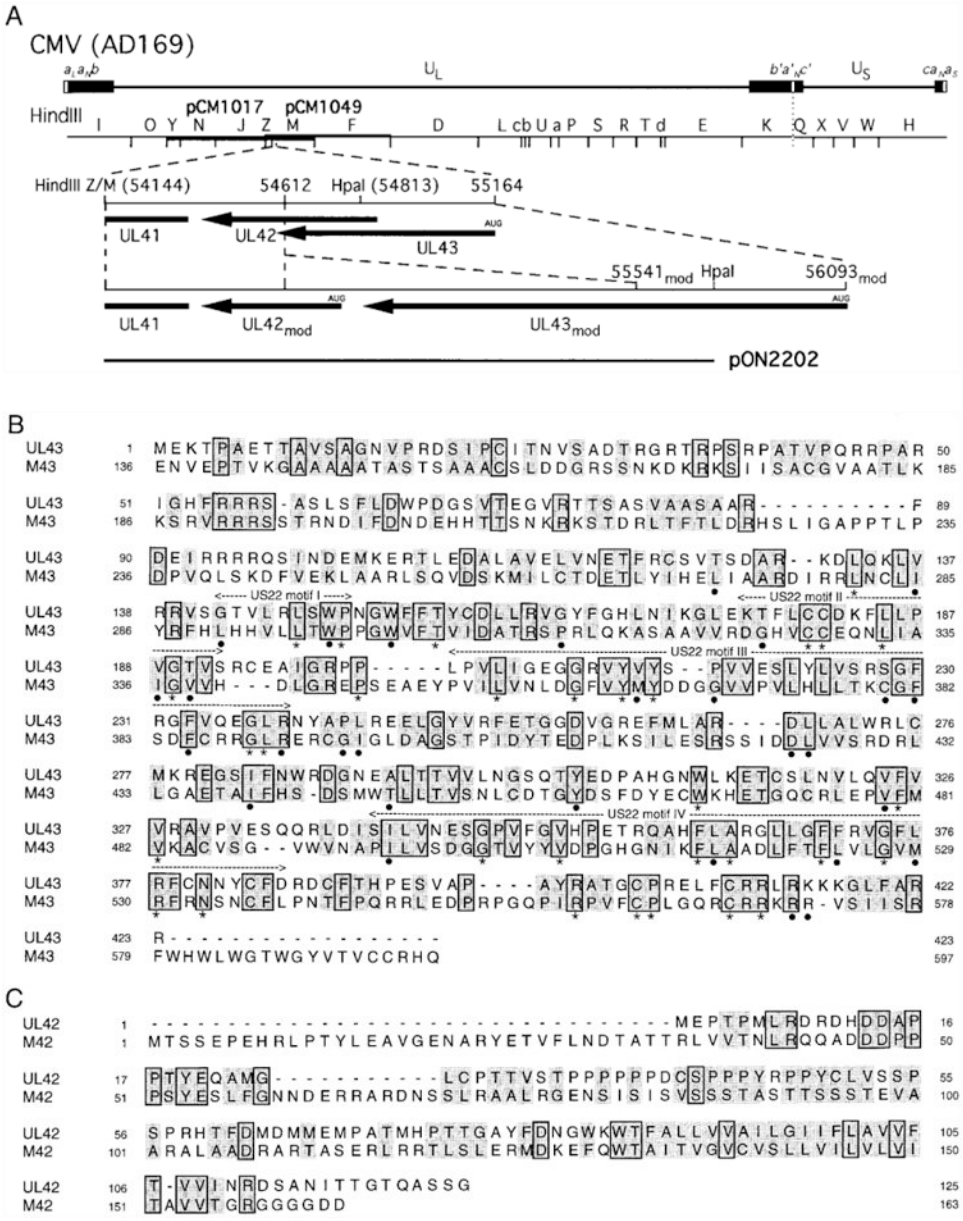

Fig. 2.

Nucleotide sequence arrangement and predicted aa sequence of $UL42_{mod}$ and $UL43_{mod.}$ (A) The top line shows the CMV strain AD169 genome with the thickened blocks representing repeated sequences (Mocarski, 1995). A *Hin*dIII map of the prototype genome is shown with the region carried by pCM1017 (nt 25921 to 64521) and the region carried by pCM1049 (nt 51805 to 84864) depicted by thickened lines below and above the *Hin*dIII map, respectively (Fleckenstein *et al.*, 1982). The expanded region shows the published ORF arrangement of the region between nt 54,144 and 55,164 on the AD169 genome (Chee *et al.*, 1990) and the modified region containing a 929-bp insert at nt 54,612 that alters the predicted size and aa sequence of UL42 and UL43. The plasmid clone pON2202, which carries 1599 bp of sequence aligning with a *Hin*dIII site at 54,144 and a *Hpa*I site at 54,813 within UL43 (Chee *et al.*, 1990). (B) Deduced aa sequence of $UL43_{mod}$ and alignment with aa 136 to 597 of

murine CMV M43 (Rawlinson *et al.*, 1996). Areas with US22 family motifs (I – IV) are shown above. Identical aa are boxed, conserved aa are shaded, and gaps are indicated by a dash. Identity across all four UL43 homologs ($UL43_{mod}$, M43, U25 of HHV-6, and U25 of HHV-7) are denoted by an asterisk and identity in three of the four homologs along with a conservative substitution in the fourth are denoted by a dot below the aa. (C) Deduced aa sequence of $UL42_{mod}$ and alignment with murine CMV M42 (Rawlinson *et al.*, 1996). The GenBank Accession No. for the 1602-bp region (including the complete *Hin*dIII and *Hpa*I sites) is AF01963.