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Coding Potential of UL/b' from the Initial Source of Rhesus Cytomegalovirus Strain 68-1

Rachel B. Gill[1,+], **J. Jason Bowman**[1,+], **Tammy Krogmann**[1], **Kurt Wollenberg**[2], **David M. Asher**[3], and **Jeffrey I. Cohen**[1,*]

[1]Medical Virology Section, Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, MD 20892 USA

[2]Bioinformatics and Computational Biosciences Branch, National Institutes of Health, Bethesda, MD 20892 USA

[3]Laboratory of Bacterial and Transmissible Spongiform Encephalopathy Agents, Division of Emerging and Transfusion-Transmitted Diseases, Food and Drug Administration, Rockville, MD 20852 USA

Abstract

Rhesus cytomegalovirus (RhCMV) 68-1 is the prototypic strain of RhCMV that has been used for pathogenesis and vaccine development. We determined the complete sequence of the RhCMV 68-1 UL/b' region directly from the original urine from which RhCMV 68-1 was isolated in 1968, and compared it to other RhCMVs. The laboratory passaged RhCMV 68-1 has inversions, deletions, and stop codons in UL/b' that are absent in the original isolate and other low passage RhCMV isolates. Fourteen of the 17 open reading frames (ORFs) in 68-1 UL/b' in the original isolate share >95% amino acid identity with low passage RhCMV. The original isolate retains 6 ORFs that encode α-chemokine-like proteins, including RhUL146 and RhUL146b that share only 92% and 81% amino acid identity, respectively, with a contemporary low passage RhCMV isolate. Identification of the original RhCMV 68-1 UL/b' sequence is important for using RhCMV 68-1 in pathogenesis and vaccine studies.

Keywords

human cytomegalovirus; rhesus cytomegalovirus; Cercopithecine herpesvirus; macacine herpesvirus 3; cynomolgus macaque cytomegalovirus; UL/b'

Introduction

Human cytomegalovirus (HCMV) causes congenital disease in infants and neonates, mononucleosis in adults, and severe disease in transplant recipients and immunocompromised patients (Mocarski et al., 2013). Currently, an effective vaccine for

*Corresponding Author. Laboratory of Infectious Diseases, National Institutes of Health, Bldg. 50, Rm. 6134, National Institutes of Health, 50 South Drive, MSC1807, Bethesda, Maryland 20892-1807. Tel: 301-496-5265, FAX 301-496-8312. jcohen@niaid.nih.gov (J.I. Cohen).

+Authors contributed equally to the manuscript.

human CMV (HCMV) has not been licensed, although it has been given a priority 1 status by the Institute of Medicine (Stratton et al., 1990). Rhesus cytomegalovirus (RhCMV) infection in rhesus macaques mimics infection of HCMV in humans, making RhCMV the most practical animal model that most closely resembles HCMV (Powers and Fruh, 2008). Like HCMV, most infections with RhCMV are asymptomatic, result from mucosal contact with bodily fluids containing the virus, and develop into a lifelong latent infection. Experimental infection of macaques with RhCMV can cause congenital disease in neonates and disseminated disease in immunocompromised animals (Yue and Barry, 2008). RhCMV, like HCMV, is accompanied by hematologic changes that may include monocytosis, neutropenia, and lymphocytosis. Both RhCMV and HCMV are common in their host populations (Andrade et al., 2003; Bate et al., 2010; Kessler et al., 1989; Vogel et al., 1994). About 80% of RhCMV open reading frames (ORFs) have orthologs in HCMV and ≥90% have orthologs at the level of protein families, strongly suggesting a common ancestor (Hansen et al., 2003; Malouli et al., 2012; Oxford et al., 2008; Rivailler et al., 2006). Comparison of the sequence of RhCMV ORFs with those from other Old World monkey CMV isolates showed a high level of conservation which was validated by identification of virion proteins using mass spectrometry (Malouli et al., 2012). Both RhCMV and HCMV contain a highly labile region labeled UL/b' that encodes proteins important in cellular tropism and immune evasion. The UL/b' region of RhCMV and HCMV frequently undergoes mutation during in vitro passage when virus is propagated in fibroblasts (Hansen et al., 2003; Oxford et al., 2008; Revello and Gerna, 2010; Rivailler et al., 2006).

RhCMV was first isolated from the urine of healthy rhesus macaques (*Macaca mulatta*) in 1968, propagated in fibroblasts, and the resulting strain was termed 68-1 (Asher et al., 1969; Asher et al., 1974). The cytopathic effects observed in vitro were similar to those seen with HCMV and African green monkey CMV strains, with refractile rounding and sloughing of infected human fetal lung fibroblasts. RhCMV 68-1, deposited in the American Type Culture Collection after subsequent passages in primary rhesus fibroblasts, was completely sequenced in 2003 (Hansen et al., 2003). Interestingly, after propagation in fibroblasts, RhCMV 68-1 was reported to replicate poorly in endothelial and epithelial cells, which mimicked the in vitro effects of highly-passaged HCMV laboratory strains like AD169 (Lilja and Shenk, 2008). Subsequently, it was determined that the UL/b' region of laboratory passaged strains of HCMV and RhCMV (namely 68-1 and 180.92) have large deletions compared with low passage (or unpassaged) isolates (Cha et al., 1996; Dolin et al., 2004; Hansen et al., 2003; Murphy and Shenk, 2003; Rivailler et al., 2006). The UL/b' region of CMV contains UL128, UL130, and UL131A, which are required for infection of endothelial cells and epithelial cells (Hahn et al., 2004; Lilja and Shenk, 2008; Ryckman et al., 2006; Wang and Shenk, 2005) as well as other viral proteins which are likely important for virus structure (Spaderna et al., 2005), signaling (Cheung et al., 2005), virus spread (Penfold et al., 1999), immune evasion (Wills et al., 2005), and latency (Goodrum et al., 2007). With the exception of replication of virus in endothelial and epithelial cells, the other functions of the UL/b' region have not been formally shown to be conserved in RhCMV. Only RhCMV laboratory strains that retain a fully intact UL/b' set of genes are shed at high levels and are easily transmitted from animal to animal in primate colonies (Oxford et al., 2011).

RhCMV68-1 has been used to study RhCMV pathogenesis (Lockridge et al. 1999), as a model for CMV vaccine development (Yue et al. 2007), and most recently as a vector to induce long-term (Hansen et al 2011) and very broad CD8 T cell responses to SIV (Hansen et al. 2013). To better understand the evolution of the prototype strain of RhCMV68-1, we determined the sequence of the UL/b' region of the virus directly from the original urine obtained in 1968 by Asher et al. and compared it with the sequence of other strains of RhCMV, other primate CMVs, and HCMV. We found that the 68-1 UL/b' ORF organization and sequence closely aligns with sequences from low passage contemporary RhCMV isolates, and that the inversions and deletions in the laboratory strain of 68-1 are notably absent. Analysis of the original sequence of the UL/b' region of 68-1 suggests that the regions important for cellular tropism and immune evasion are highly sensitive to in vitro selection, and that the greatest variance occurs amongst the CXC-chemokine-like genes within the region.

Results and Discussion

Sequence of the UL/b' Region from RhCMV 68-1-P0

Asher originally obtained urine from 9 juvenile rhesus macaques in 1968 (Asher 1969). Animals were hydrated with intravenous fluid and given mannitol to induce diuresis. Penicillin and streptomycin were added to the urine and a portion was frozen at −70°C. Urine that had not been frozen was serially diluted, and 0.2 ml aliquots of undiluted or diluted urine were used to inoculate stationary WI38 cell (fetal human lung diploid fibroblast) tube cultures. CPE was first noted in cells inoculated with diluted urine from a female macaque on day 17 and virus from undiluted urine from the same animal was passaged in WI38 cell tube cultures and deposited in the ATCC as RhCMV 68-1 (Asher et al., 1974). Despite repeated attempts we were unable to isolate infectious virus from the urine. However, sufficient virus DNA remained in the urine sample to amplify by PCR; the copy number of RhCMV DNA in the urine was 18.3 copies of RhCMV DNA/uL by real time-PCR. We isolated DNA from the urine and obtained overlapping PCR products that corresponded to the UL/b' region RhCMV 68-1 P0 (passage 0). The complete region of RhCMV 68-1 P0 UL/b' (GenBank accession #KF011492) is 12.9 kilobase pairs with a GC content of 46% and encodes 17 ORFs in the same orientation which are most conserved with low-passage RhCMV isolates from naturally-infected macaques (Malouli et al., 2012; Oxford et al., 2008) (Figure 1, Table 1). While RhCMV 68-1-ATCC is missing ORFs UL128, UL146a, UL146b, rh161.1 and a portion of UL130, the RhCMV 68-1-P0 strain contains full-length sequences for each of these ORFs. The 5' end of RhCMV 68-1-P0 UL/b' has a 22 nucleotide sequence repeated 18 times, 16 of which are direct repeats. These sequences are conserved throughout various strains of simian CMVs, (Supplemental Table 1, nucleotide polymorphisms underlined and bolded) and may encode regulatory miRNAs (Hancock et al., 2012).

Comparison of RhCMV 68-1-P0 with other simian CMV UL/b' ORFs

The 17 ORFs encoded in RhCMV 68-1 P0 UL/b' were compared with their orthologs in other simian CMVs based on bit scores to establish if one ORF is more closely related to a certain strain of CMV. Bit scores (calculated using NCBI BLASTp) are normalized log-

scale sequence alignment scores that compare sequence alignments taking into account maximum sequence identity (longest stretch of matching amino acids) and total sequence identity (overall number of matching amino acids); the higher the number, the less likely the alignment was to have occurred by pure chance and the more likely the sequences are to have originated from similar sources. The bit scores for RhCMV 68-1-P0 UL/b'genes show that these ORFs are most similar to those in RhCMV CNPRC (not passaged in vitro), 68-1-ATCC, and 22659 (a low passage isolate) (Supplemental Figure 1, data not shown). As reported previously, RhCMV shares greatest homology outside of its species with cynomolgus macaque cytomegalovirus (CynoCMV) and does not have as high of a degree of homology with chimpanzee CMV or HCMV (Table 1).

Amino acid alignment of ORFs truncated or missing from 68-1-ATCC UL/b'

Comparison of ORFs in UL/b' between RhCMV 68-1-P0 and 68-1-ATCC indicates that ORFs UL128, UL146a, UL146b, and rh161.1 are missing from 68-1-ATCC, while ORF UL130 (which encodes a membrane glycoprotein) is truncated. RhCMV 68-1-P0 encodes UL146, UL146a, UL146b, UL147, rh161.1 and rh161.2, which are putative CXC chemokines (Alcendor et al., 2009; Oxford et al., 2008). These genes in are thought to have arisen from a single ancestral CXCL gene that gave rise to daughter genes and subsequent gene duplications (Alcendor et al., 2009). RhCMV 68-1-ATCC, encodes only UL146, UL147, and rh161.2 (which was formerly termed rh161 [Oxford et al. 2008] in 68-1-ATCC). HCMV UL146 activates CXCR1 and CXCR2, probably to attract neutrophils that carry virus to endothelial cells (Luttichau 2010), while UL147 is also important for neutrophil chemotaxis (Penfold et al., 1999). UL146a is an ELR- CXC chemokine, which suggests that the protein is important for leukocyte migration (Ebert et al., 2005), while UL146b is an ELR+ CXC chemokine, which implies that it can interact with CXCR1 and CXCR2 and induce migration of neutrophils (Kobayashi 2008). rh161.1 and rh161.2 are thought to be the result of a gene duplication event. While most other proteins in UL/b' share >95% amino acid identity between RhCMV 68-1-P0 and other RhCMV isolates, genes encoding the putative CXC chemokines are the least conserved between different RhCMV isolates. UL146b and UL146 share only 81% and 92% amino acid identity between RhCMV 68-1-P0 and RhCMV CNPRC (Table 1), and rh161.1 in RhCMV 68-1-P0 and rh161.2 from RhCMV 68-1-ATCC share only 34% amino acid identity.

UL128 is a glycoprotein, derived from exon splicing, that is one of the components of the CMV pentameric complex (consisting of UL128, UL130, UL131A, gH and gL) important for epithelial and endothelial cell tropism (Lilja and Shenk 2008; Ryckman et al., 2006). Antibody to the pentameric complex is the predominant component of CMV neutralizing antibody in human sera (Fouts et al., 2012; Genini et al., 2011; Macagno et al. 2010), and immunization of monkeys with the pentameric complex induces broadly neutralizing antibody in the animals (Wussow et al., 2013). The RhCMV 68-1-P0 UL/b' region contains a full-length copy of UL128, which includes all three exons, and its amino acid sequence is highly conserved among other RhCMV and CynoCMV isolates (Supplemental Figure 2). While the initial description of the CynoCMV Ottawa strain did not report having all three exons (Marsh et al., 2011), analysis of the nucleotide sequence revealed that CynoCMV does have a potential third exon in UL128 (Supplemental Figure 2, gray highlight).

UL130 is also a glycoprotein produced from exon splicing that is part of the pentameric complex. While the spliced version of UL130 from RhCMV 68-1-P0 aligns well with other RhCMV and CynoCMV sequences (Supplemental Figure 3), RhCMV 68-1-P0 has a deletion of 16 amino acids compared with other isolates, which likely is a result of a duplication event. RhCMV 68-1-ATCC only has the first exon of UL130, and is missing the second exon which has been termed rh157.4 (Oxford et al. 2008). Although not reported in the initial publications (Marsh et al., 2011; Rivailler et al., 2006), analysis of both RhCMV 180.92 and CynoCMV nucleotide sequences reveals that each contains the second exon of UL130 (Supplemental Figure 3, gray highlight).

UL131A is another glycoprotein within the pentameric complex. Of the three glycoproteins in this complex encoded by the UL/b' region, UL131A shares the least homology between RhCMV 68-1-P0 and other RhCMV isolates ranging from 91-95% amino acid identity.

Conclusions

The organization and sequence of the UL/b' region from the original isolate of RhCMV 68-1 (RhCMV 68-1-P0) closely aligns with contemporary wild-type isolates of RhCMV, and inversions and deletions in the laboratory strain of 68-1-ATCC relative to contemporary isolates are absent. The regions in the UL/b' region of RhCMV 68-1-P0 important for cellular tropism and immune evasion are highly sensitive to in vitro selection and the greatest divergence occurs in the CXC-chemokine-like genes.

Materials and methods

RhCMV viral DNA was isolated from urine using the QIAamp ® Viral RNA Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. RhCMV genome copy number in the urine was determined by real-time PCR using TaqMan® FAM(6-carboxyfluorescein)/TAMRA(tetramethylrhodamine) technology (Applied Biosystems, Foster City, CA), using primers 5'-TGCGTACTATGGAAGAGACAATGC (gB RT Forward) and 5'-ACATCTGGCCGTTCAAAAAAAC (gB RT Reverse) and a FAM-tagged probe 5'-FAM/ CCAGAAGTTGCGCATCCGCTT to determine RhCMV gB copy number.

The UL/b' region of RhCMV 68-1 was amplified by PCR (primers listed in Supplemental Table 2). Overlapping PCR products obtained by using the HotStarTaq® DNA polymerase kit (QIAGEN Inc.) were gel purified (GenEluteTM Agarose Spin Column, Sigma-Aldrich, St. Louis, MO). Amplicons were cloned using the Promega pGEM®-T Easy Vector System (Madison, WI) and sequenced using either external T7 and SP6 or internal primers. At least two clones for each amplicon were sequenced. If clones from amplicons were not obtained, the isolated fragments were sequenced directly. Sequencing reactions were assembled using Lasergene SeqMan Pro (DNASTAR, Inc., Madison, WI). The UL/b' sequence from RhCMV 68-1-P0 has been deposited in Genbank (accession number KF011492).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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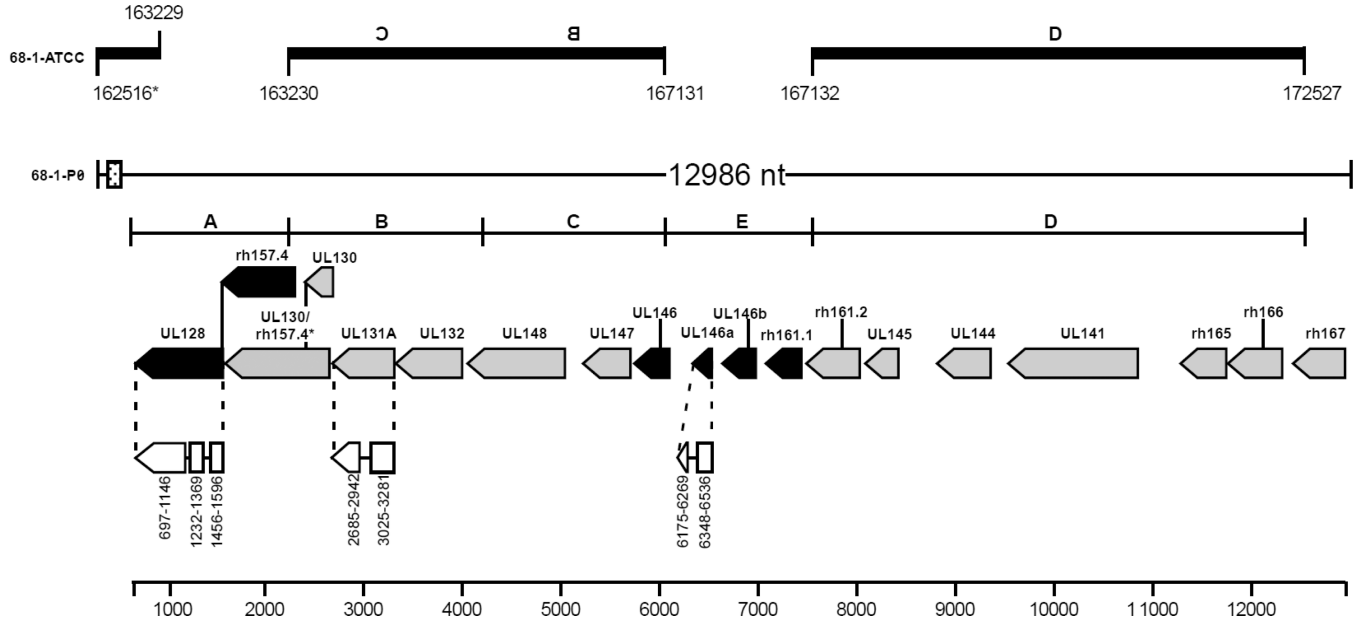

Fig. 1.

Genomic arrangement of UL/b' open reading frames (ORFs) sequenced from the original source of RhCMV 68-1-P0 compared with passaged RhCMV 68-1 deposited in the ATCC (68-1-ATCC). Nucleotide numbering of RhCMV 68-1-ATCC (based on Rivailler et al., 2006) is shown at the top for comparison. The genomic structures, consensus regions (A-D), and predicted ORFs are depicted based on previously reported sequences of RhCMVs from low passage or unpassaged isolates (Rivailler et al., 2006; Oxford et al., 2008). RhCMV 68-1-P0 ORFs are on the complementary strand. Gray shaded ORFs are present or have a highly homologous region in 68-1-ATCC, while black shaded ORFs are absent from 68-1-ATCC. White regions joined by lines are proposed of exons. The splice variant for UL130 is indicated as UL130/rh157.4. A putative microRNA region (22-mer repeated 18 times, dotted box) is highlighted at the 5' end of the contig and is also present in the 68-1-ATCC strain. The figure is based on that from Oxford et al. 2008.

Table 1

Amino acid percent maximum identity comparison of RhCMV 68-1-P0 UL/b' open reading frames to other CMV isolates.

			% AA Maximum Identity of 68-1-P0 vs. Other CMV Isolates						
RhCMV ORF	**Protein Class**	**Proposed Function**	**RhCMV 68-1 (ATCC)**	**RhCMV CNPRC**	**RhCMV 22659**	**RhCMV 180.92**	**Cyno CMV (Ottawa)**	**Chimp CMV**	**HCMV (Merlin)**
UL128	Membrane glycoprotein	Pentamer envelope complex Essential for endo/epithelial cell tropism	NH	99	100	99	97	36	42[1]
UL130	Membrane glycoprotein	Pentamer envelope complex Essential for endo/epithelial cell tropism	100[2]	98	99	99	87	80	80
UL131A	Membrane glycoprotein	Pentamer envelope complex Essential for endo/epithelial cell tropism	100[2]	93	91	95	97	78	60
UL132 (rh160)	Membrane glycoprotein	Essential for epithelial cell tropism	99	99	99	99	95	35	40
UL148 (rh159)	Potential CXC (α) Chemokine	Potentially encoded as a large transcript with UL146-UL132	100	99	99	100[3]	89	55	42
UL147 (rh158)	Potential CXC Chemokine	Homology to HCMV vCXCL2α-chemokine like protein	100	97	99	NH	98	44	41
UL146	Potential CXC Chemokine	Homology to HCMV vCXCL1 α-chemokine like protein	100	92	100	NH	79	UL146: 35 UL146A: 56	26
UL146a	Potential CXC Chemokine	α-chemokine like protein	NH	98	99	NH	90	33	UL146: 57
UL146b	Potential CXC Chemokine	α-chemokine like protein	NH	81	100	NH	80	NH	NH
rh161.1	Potential CXC Chemokine	Possible duplication of rh161.2 α-chemokine like protein	34[4]	98	100	NH	93	NH	NH
rh161.2[4]	Potential CXC Chemokine	α-chemokine like protein	100[4]	98	100	NH	97	NH	NH
UL145 (rh162)	Potential kinase	Highly conserved with putative PKC and CK II sites	100	100	99	NH	99	65	65
UL144 (rh163)	Membrane Receptor	TNFR-like protein	100	97	99	NH	99	43	63
UL141 (rh164)	Membrane glycoprotein	Downregulate cell surface expression of CD155 and CD112	100	99	99	NH	97	47	67
rh165	Membrane glycoprotein	Possible duplication of tandem ORFs (rh165, 166, 167)	100	99	96	NH	95	NH	NH
rh166	Membrane glycoprotein	Possible duplication of tandem ORFs (rh165, 166, 167)	100	99	98	NH	96	NH	NH

			% AA Maximum Identity of 68-1-P0 vs. Other CMV Isolates						
RhCMV ORF	**Protein Class**	**Proposed Function**	**RhCMV 68-1 (ATCC)**	**RhCMV CNPRC**	**RhCMV 22659**	**RhCMV 180.92**	**Cyno CMV (Ottawa)**	**Chimp CMV**	**HCMV (Merlin)**
rh167	Membrane glycoprotein	Possible duplication of tandem ORFs (rh165, 166, 167)	100	98	NH	97[3]	95	NH	NH

GenBank numbers: RhCMV 68-1-P0 (KF011492); RhCMV 68-1-ATCC (AY186194.1); RhCMV CNPRC (EF990255.1); RhCMV 22659 (EU130540.1); RhCMV 180.92 (DQ120516.1); CynoCMV-Ottawa (JN227533.1); Chimp CMV (AF480884.1); HCMV-Merlin (AY446894.2)

[1] ADB84700.1 (low-passage clinical isolate used due to mutation in UL128 of Merlin.)

[2] Comparison made using nucleotide alignment

[3] ORF truncated in comparison sequence

[4] Comparison made using rh161.2 (which was formerly termed rh161 in 68-1-ATCC [Oxford et al. 2008])

NH=Homolog not found in comparison sequence