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Structure characterization of human cytomegalovirus UL131A, UL130 and UL128 genes in clinical strains in China

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ABSTRACT. Human cytomegalovirus (HCMV) genetic determinants of endothelial cell tropism, leukocytes and dendritic cells have been identified in the genes UL131A, UL130, and UL128. We examined the structure of these three genes in HCMV. Eighteen low-passage clinical isolates and five non-passage strains from congenitally HCMV-infected infants in China were used to assess the structures of the UL131A, UL130, and UL128 genes and to find possible relationships between sequence polymorphism and different signs of HCMV disease. Comparisons were made between the UL131A, UL130, and UL128 genes of clinical strains and published sequences of Towne and Merlin strains. The UL131A coding region in the clinical strains was similar to that of Towne and Merlin strains, while UL130, and UL128 coding regions in the clinical strains were parallel with those of Towne and Merlin, respectively. Sequence comparison indicated that the UL130, and UL128 genes encode chemokine-like proteins in the clinical strain; the transmembrane regions of UL131A, and UL130 were conserved in all clinical and reference strains. The three genes of clinical strains from infants with different signs of HCMV disease had similar structure characterization. We conclude that the UL131A, UL130, and UL128 genes are highly conserved in these clinical strains. No correlation was found between the

structure of the three genes and variations in HCMV disease. The finding of chemokine-like domains in UL130, and UL128 putative proteins suggests that the predicted products play a role in HCMV infectivity.

Key words: Human cytomegalovirus; Structure characterization; UL131A, UL130, and UL128 genes

INTRODUCTION

Human cytomegalovirus (HCMV) is the main cause of congenital viral infection (Ljungman et al., 2002; Sissons and Carmichael, 2002). The HCMV genome is one of the largest and most complex genomes known, and many regions of variation have been identified, more so in clinical isolates than in laboratory-adapted strains (Cha et al., 1996; Prichard et al., 2001). Human cytomegalovirus can replicate in several cell types, including endothelial cells, which is important in intrauterine infections as well as in associations with cardiovascular disease (Jarvis and Nelson, 2002; Maidji et al., 2002).

Human cytomegalovirus clinical strains display genetic polymorphisms, possibly related to strain-specific tissue-tropism and HCMV-induced immunopathogenesis. A substantial portion of the HCMV genome encodes proteins with the potential to affect virulence through cell tropism, immune evasion, molecular mimicry, or interference with host chemokines (Hahn et al., 2004). The UL/b' region of HCMV genome was found in Toledo and several other low-passage clinical isolates, but was not found in laboratory AD169 strain (X17403) (Cha et al., 1996). The fact that AD169 has attenuated virulence and different tropism for endothelial cell than low-passage isolates leads us to consider the possibility that the predicted products of UL/b' genes determine the manifestation of HCMV infection *in vivo*. Several genes have been analyzed to look for genomic variation in clinical isolates in China, such as UL139, UL141, and UL149 open reading frames (ORF) (Qi et al., 2006; Ma et al., 2006; Ji et al., 2006).

It has been reported that HCMV genetic determinants of endothelia cell tropism and virus transfer to leukocytes are the UL131A, UL130, and UL128 genes, which are located in the UL/b' region (Hahn et al., 2004). Later, it was documented that the same genes are responsible for monocyte-derived dendritic-cell tropism (Gerna et al., 2005). The ability to infect endothelial cells and leukocytes is a nonessential virus-encoded function and is characteristic of clinical HCMV isolates, but it is not found in reference laboratory strains, such as AD169, Towne, and Davis (Gerna et al., 2002). In AD169 and Towne, the loss of endothelial cell tropism and leukocyte transmissibility is associated with mutation of the UL131A, UL130, and UL128 region (Hahn et al., 2004); extensive fibroblast propagation of an endothelial cell tropic clinical isolate (VR6110) is associated with selection of a tropism-deficient variant showing a possible deletion of UL132-130 within the UL/b' region (Grazia Revello et al., 2001). It has been indicated, by complexing with gH-gL, pUL128, pUL130, and pUL131, that they likely participate in the entry of HCMV into epithelial and endothelial cells, because mutations in any of the three ORFs can abolish epithelial and endothelial cell tropism (Hahn et al., 2004; Wang and Shenk, 2005). Ryckman et al. (2008a,b) showed that the gH/gL/UL128-131 complex can mediate entry into epithelial and endothelial cells. These facts lead to the hypothesis that HCMV disease and tissue tropism may be related to variations of UL131A, UL130, and UL128 genes among strains. This prompted us to study

the structures of these genes in strains from infants with congenital HCMV infection. We investigated the sequences and the gene content of UL131A, UL130, and UL128 genes in 23 clinical strains from congenitally infected infants in China.

MATERIAL AND METHODS

HCMV strains

The study population consisted of 23 infants with HCMV infection who were identified in the Shengjing Hospital of China Medical University through a screening program (He et al., 2001), including 18 low-passage isolates and five non-passage strains. Briefly, the 23 HCMV clinical strains included 12 from infants with jaundice; eight from infants with neurological handicaps and three from infants with congenital megacolon (Hirschsprung's disease, HD). The main manifestation of infants in the jaundice group was hepatic disfunction, including rise direct bilirubin concentrations, associated with an increase in transaminase. Besides the specific clinical manifestation, the infants in the neurological handicaps and the HD group had only increased indirect bilirubin. Strains from infants with HD were recovered from the abnormal colon tissue, and the other strains were recovered from urine samples. The infants ranged in age from one to 14 months; the mean age was 3.5 months. Clinical isolates were passaged less than 10 times through human embryonic lung fibroblasts (HELF). All clinical strains were found to contain detectable HCMV DNA by the fluorescent-quantified polymerase chain reaction (PCR) diagnostic method (He et al., 2001). The clinical strains were stored at -70°C until use.

Preparation and amplification of UL131A, UL130, and UL128 genes

Virus DNA of HCMV strains was exposed by boiling the infected cells with lysis buffer (10 mM EDTA, 10 mM Tris-HCl, 1% (w/v) SDS, 100 µg/mL proteinase K) for 15 min. All primers designed for PCR amplification of UL131A, UL130, and UL128 genes were based on the Toledo sequence (Cha et al., 1996) and the Merlin sequence (Dolan et al., 2004). To ensure sequencing of the junction and to obtain the entire sequence of the genes, neighboring primers were designed to produce sequences with 150 bp overlap. The most suitable sets of primers that we used are given in Table 1. PCR was conducted in 50-µL reactions containing 200 µM of each dNTP, 0.5 U of Taq DNA polymerase in 1X Taq reaction buffer (Promega), 150 ng of each primer, and 1.5 mM $MgCl_2$. The condition for amplification with the primers were 95°C for 4 min, followed by 35 cycles at 94°C for 45 s, 55°C for 1 min and 72°C for 1 min; this was followed by a single extension cycle at 72°C for 10 min. Each sequence was verified in a separate amplification with negative control of water.

Table 1. Primers used for polymerase chain reaction amplification of HCMV UL131A, UL130, and UL128 genes.

Fragment coordinates	Left primers sequence [5'-3']	Right primers sequence [5'-3']
Toledo strain		
12143-13237	CCCTTATCAGCGGTTGGA	TGCGGTTTGGAATACGTCAGT
13097-13969	ACGGGAAATAATATGCT	CCACAGAACGCAGACAATCAT
13908-15188	CGCCGGAACGCTGTTAC	CTTCCACCATCAGGCCAACGA
Merlin strain		
175585-176352	ATGGGCTATGAACTAATGACC	CGGCAAAGTAAACGACAA

DNA sequencing

PCR products were gel-purified using a Promega extraction kit and then sequenced directly, using the same primers, with the BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems). The sequencing reactions were performed with a PE Applied Biosystems Geneamp PCR System 2400 at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min for 30 cycles. The sequencing products were analyzed on an ABI 3770 automated sequencer (Applied Biosystems). All UL131A, UL130, and UL128 genes were sequenced bilaterally to ensure each nucleotide.

Sequence analysis

Resulting sequences were visualized as computer traces using Chromas 1.62. The nucleotide and amino acid sequences were edited and analyzed by DNAClub. Alignment of UL131A, UL130, and UL128 genes was performed with the BioEdit 7.0 software. Comparisons were carried out with published sequences of Merlin and Towne strains (Dolan et al., 2004). Similarity was calculated by scoring for both identical amino acids and conservative amino acid substitutions. To generate the phylogenetic trees, a neighbor-joining bootstrap analysis was undertaken with the PHYLIP software package; the final nucleotide tree was rendered with Treeview. Bootstrap calculations were based on 1000 repetitions. Hydrophobic domains were predicted by the DNAstar software package. Signal peptides were predicted by SignalP 3.0. Transmembrane regions were predicted by TMpred.

Statistical analysis

Descriptive statistics and analysis of proportions were carried out by using contingency tables and the chi-square test. Only P values of <0.05 were considered to be significant.

RESULTS

Presence of UL131A, UL130, and UL128 genes in clinical strains

UL131A, UL130, and UL128 genes were successfully amplified and sequenced from the 23 HCMV clinical strains. The laboratory strain Towne and wild-type Merlin strains were analyzed in parallel with the clinical strains. Based on the comparison of UL131A, UL130, and UL128 genes to their homologues in Merlin and Towne strains, these genes were present in all low-passage isolates and non-passage strains. The sequences that we obtained have been deposited with Genbank under accession numbers DQ011966-DQ011969, DQ208232-DQ208294, DQ507864, and DQ507865.

Analysis of the UL131A, UL130, and UL128 genes from clinical strains

Compared with those of Towne and Merlin strain, the coding region of the UL131A genes in all the clinical strains contained 390 bp. The nucleotide diversity of UL131A in clinical strains was 0.8-6.3% relative to Merlin and Towne strains. Sporadic nucleotide mutations concentrated on the 5' region of UL131A, and other regions were conserved.

Except for amino acid positions 2 and 4, the sequences of the UL131A putative protein from different clinical strains were identical.

UL130 is the largest gene of the UL131A, UL130, and UL128 locus; it is the only one that is not interrupted by introns. The coding region of UL130 genes in all clinical strains and Merlin strain are 645 bp in length, whereas that in the Towne strain is 690 bp. UL130 gene in Towne strain has a frameshift near the 3' end of the gene since double T insertion. It was observed that a cysteine codon, which was conserved in both clinical strains and Merlin strain, was substituted by serine codon at the 3' end of UL130 in Towne strain. Compared with Towne strain, most of the clinical strains and Merlin strain had a C to T transition at positions 25 and 79, and a T to C transition at positions 206 and 233. This nucleotide transition is a non-synonymous substitution. Nucleotide mutations were distributed throughout the UL130 region in the clinical strains; the rate of non-synonymous mutation was 32.8% compared to that of Towne strain. Due to the change in the nucleotide length of UL130 ORF in the Towne strain, the nucleotide diversity of the gene in clinical strains was 12.7-13.6% related to Towne strain, but was 0.5-1.5% related to Merlin strain.

The coding region of UL128 gene in the clinical strains and reference strains comprises three exons. The UL128 gene in the clinical strains and Towne strain are 516 bp in length, whereas that in Merlin strain is 393 bp, due to a C to T transition in exon 3 that introduces a stop codon and causes premature translational termination. Compared with Towne strain, the UL128 gene in clinical strains and Merlin strain had an A to G transition at position 13 and an A to T transversion at position 123; all clinical strains had a T to C transition at position 306. The rate of non-synonymous mutation to that of Towne strain was 52.6%. The content of UL128 gene in clinical strains is more like that of Merlin strain than that of Towne strain, except for premature translational termination. Gene maps of UL131A, UL130, and UL128 genes in Merlin, Towne and the clinical strains are shown in Figure 1. As a result of the change of the nucleotide length of UL128 gene in Merlin strain, the nucleotide diversity of the gene in clinical strains was 24.6-25.8% compared with Merlin strain, but was 0.6-3% related to Towne strain.

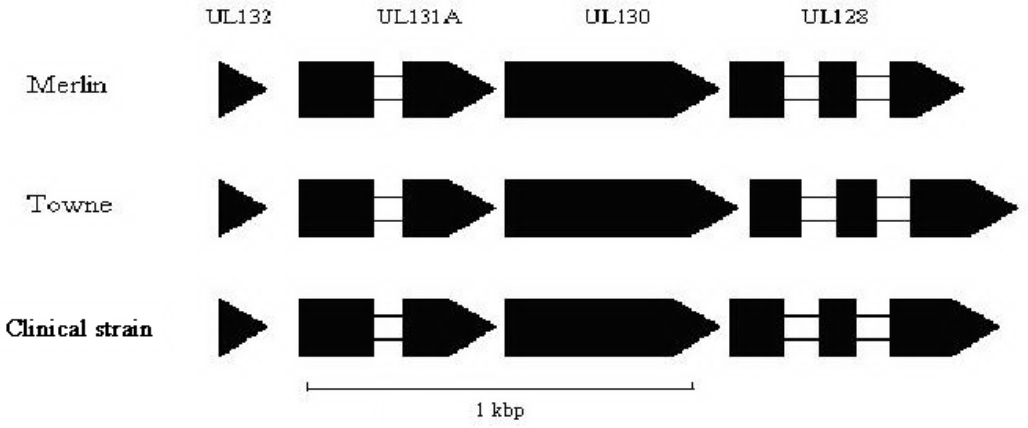

Figure 1. Coding regions of the UL131A-UL128 genes in 23 HCMV clinical strains, in comparison with those of the Merlin and Towne strains. The scale is in kbp. Protein-coding regions are shaded and introns are depicted as white horizontal bars.

Characterization of the UL131A, UL130 and UL128 genes in clinical strains and relationship between the three genes and signs of HCMV infection

Detailed sequence analysis showed that UL131A, UL130 and UL128 genes were highly conserved in all clinical strains. The nucleotide identity among all strains was 96.2, 95.8, 96.0%, respectively. Similarly, the amino acid identity scored 97.2, 96.6, 96.9%, respectively. Analysis of the relationship between the UL131A, UL130, and UL128 genes and HCMV disease showed that the clinical strains from jaundice, neurological handicaps and HD infants distributed randomly in the three genes. When the signs of HCMV infections and the UL131A, UL130, and UL128 genes of strains were considered, no significant difference was detected ($P > 0.05$).

Results of phylogenetic trees of UL131A, UL130, and UL128 genes showed no preferential clustering of clinical strains, and the five unpassaged clinical strains dispersed randomly. The Cluster dendrogram revealed that the clustering of the amino acid sequences did not correlate with propagation *in vitro*. Based on the observed collinearity between 18 low-passage isolates and five unpassaged strains, no obvious gene mutations were found in UL131A, UL130, and UL128 genes of the low-passage isolates.

Analysis of the predicted UL131A, UL130, and UL128 proteins

Alignment of the UL131A, UL130, and UL128 encoded proteins was made between reference strains and clinical strains. Given the tight conservation of posttranslational modification sites, only the motif of UL130 encoded proteins in clinical strains exchanged. The UL130 putative protein in Towne strain contains an additional casein kinase II phosphorylation site and an N-myristoylation site, which were absent in clinical strains and Merlin strain. Those in clinical strains and reference strains contain three potential N-linked glycosylation sites (Asn85, Asn118, and Asn201) (Figure 2).

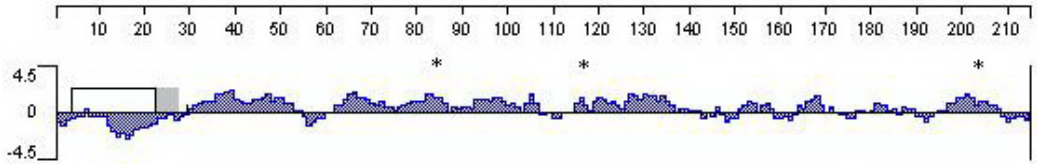

Figure 2. Computer prediction of the hydrophilicity and potential N-linked glycosylation sites (asterisks) of the predicted UL130 protein. The potential signal and membrane anchor sequence are indicated by boxes.

Prediction of signal peptide by signal PV3.0 showed that all clinical strains contained an N-terminal signal peptide in the UL131A, UL130, and UL128 predicted proteins. Most likely, the signal peptide cleavage site of UL131A putative protein in the clinical strains was within 18 to 19 amino acids; that of UL130 was located between 25 and 26 amino acids, and that of UL128 was at 27 to 28 amino acids. The UL130 putative protein of clinical strains contains a putative chemokine domain from amino acids 46 to 120, although it lacks two of the four cysteines that are strictly conserved in a chemokine of this type (Figure 3). The UL128 proteins share four conserved cysteine residues near their N termini that are characteristic of

β- (or CC-) chemokines; a putative CC chemokine domain was independently noticed in the UL128 ORF (Figure 4). The CC chemokine motif was conserved among all tested strains.

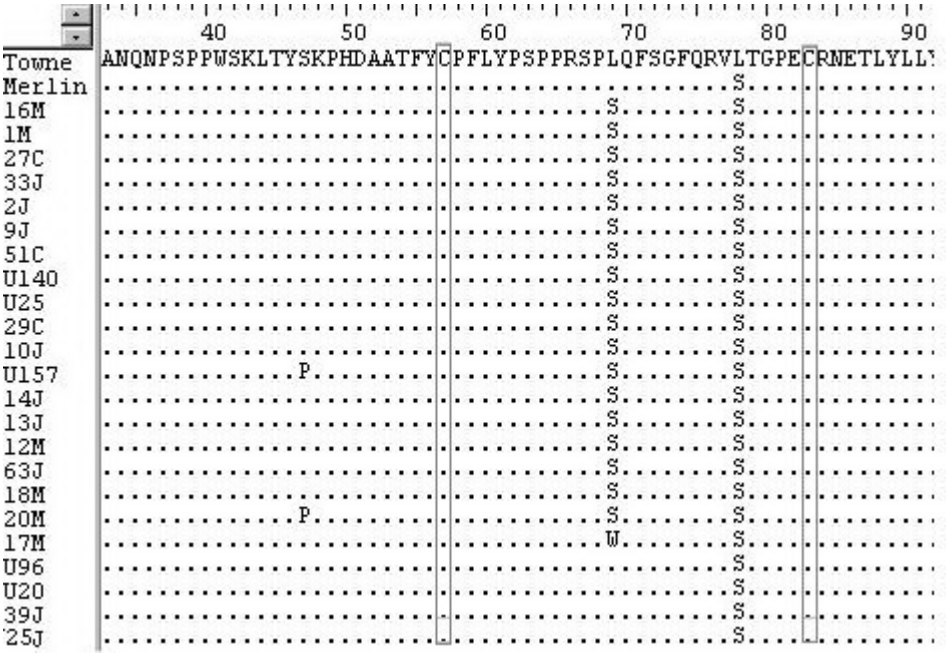

Figure 3. Cysteines in HCMV UL130 protein that are suggested to be important for CXC chemokine structure. The two conserved cysteine residues are in box.

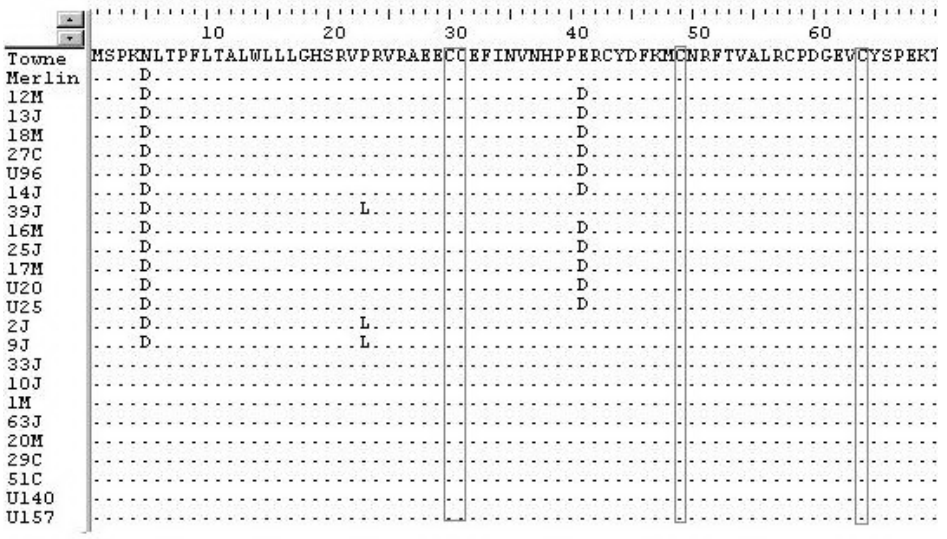

Figure 4. Cysteines in HCMV UL128 protein that are suggested to be important for CC chemokine structure. The four conserved cysteine residues are in box.

Analysis of a transmembrane region in UL131A, UL130, and UL128 putative proteins

Rigoutsos et al. (2003) predicted that UL131A and UL128 genes encode transmembrane (TM) protein. In our study, we predicted that the putative TM domains were within 10-20 residues of UL131A putative protein and 8-25 residues of UL130 putative protein in the clinical strains and reference strains (Figure 2). Based on our analysis, UL128 putative protein did not contain the TM domain. The TM domain of UL131A, and UL130 was conserved in all clinical strains and reference strains.

DISCUSSION

The UL131A-UL128 locus contains three genes: UL131A, UL130, and UL128. A mutation in any one of these three genes can abolish endothelial cell tropism (Hahn et al., 2004). Laboratory-adapted strains, which do not efficiently infect endothelial cells, consistently contain mutations in this region. When we compare UL131A, UL130, and UL128 genes in AD169, Towne and clinical strains, AD169 contains a single nucleotide insertion in UL131A exon 1, and Towne has a frameshift mutation in the carboxy-terminal region of UL130. Neither the Towne nor the AD169 strain can efficiently infect human umbilical vein endothelial cells (HUVECs). We provide evidence that the UL131A, UL130, and UL128 genes of HCMV were present in all clinical strains found in the 23 congenitally infected infants that we examined. Detailed sequence analysis in all clinical strains showed that the UL131A, UL130, and UL128 genes are highly conserved, similar to the findings of Baldanti et al. (2006). No clustering of viral strains on the basis of UL131A, UL130, and UL128 sequences related to the different clinical signs of HCMV disease was observed. Our study provides basic data on the genetic structure of HCMV UL131A, UL130, and UL128 genes in an Asian population.

Strains that have been passaged in HELFs were previously noted to contain mutations in one or more of UL131A, UL130, and UL128 (Dolan et al., 2004). Dolan found that all passaged strains had visibly disabling mutations in these genes in comparison with two unpassaged strains. It has been demonstrated that passage of HCMV strains in human fibroblasts is associated with loss of ability to grow in endothelial cells (Sinzger et al., 1999; Grazia Revello et al., 2001). We found that the five unpassaged clinical strains dispersed in different branches of the phylogenetic tree. Based on the observed collinearity between five unpassaged strains and 18 low-passaged isolates, all passaged isolates contain no obvious gene mutations in any of the three genes. It seems likely that limited passage of HCMV in HELF is not enough to change the gene content of the HCMV UL131A, UL130, and UL128 regions. The UL131A, UL130, and UL128 gene map derived from low passage isolates accurately represents that of wild-type HCMV.

Previously observations indicate that two novel spliced genes UL131A and UL128 comprise two and three exons, respectively (Akter et al., 2003); the splicing patterns were confirmed by mRNA mapping experiments. Protein-coding regions of all clinical strains were proposed based on conservation of encoded amino acid sequences and conceptually linked together via candidate splice donor and accepter sites. In our study, UL131A and UL128 flanking UL130 in clinical strains were predicted by sequence comparisons between clinical strains and reference strains. UL131A and UL128 in all clinical strains comprise two and three exons, respectively. The coding regions of UL130 in clinical strains and reference strains were similar, but a cysteine codon, which is conserved in clinical strains and Merlin strain, is substituted

by serine codon at 3' end of UL130 in Towne strain. All clinical strains and reference strains contain three potential N-linked glycosylation sites; Towne strain contains an additional casein kinase II phosphorylation site and an N-myristoylation site, which were deleted in clinical strains and Merlin strain. It is possible that this difference modifies the function of UL130 in a way that retains the ability of the clinical strains to grow in HUVEC and HELF cells. UL128 in Merlin strain is truncated by a single nucleotide substitution that introduces a premature in-frame termination codon into the third exon. The mutation is included in the GenBank entry, but it was absent from the clinical sample from which Merlin was derived. In our study, UL128 genes of the clinical strains and Towne strain had no nucleotide substitution, different from what was found in the third exon of Merlin strain.

The UL131A, UL130, and UL128 putative protein of HCMV, CCMV, and SCMV commences with a predicted signal peptide, suggesting that they are secreted proteins (Akter et al., 2003). Specifically, the 46-120 tract of UL130 protein can be modeled on CXC chemokine, although it lacks two of four cysteines that are strictly conserved in chemokines of this type. The UL128 proteins share four conserved cysteine residues near their N termini that are characteristic of β-chemokine, which are considered necessary for functional activity of chemokines (Novotny et al., 2001). In addition, UL131A, UL130, and UL128 gene products may be involved in attraction-adhesion of leukocytes to endothelial cells (Hahn et al., 2004). In our study, the signal peptide cleavage site of putative protein in HCMV UL131A, UL130, and UL128 in all clinical strains was conserved. The UL130 proteins were indeed predicted to have a chemokine-like domain. The four cysteine residues in UL128 putative protein were conserved among all clinical strains. The conservation of chemokine-like domains in UL130 and UL128 putative proteins of all the clinical strains provides evidence for a role in leukocyte-endothelial cell interaction. The chemotactic activity of each individual gene product of the UL131A-UL128 locus, as well as the potential cooperation with other viral or cellular gene signaling molecules, remain to be further elucidated.

Transmembrane protein is an important type of functional protein. It plays important biological roles, such as serving as a highly active mediator between the cell and its environment, catalyzing specific transport of metabolites and ions across membrane barriers. Recent studies have revealed that the fraction of TM proteins in the proteome is almost always 20-30%, irrespective of the diverse range of organism and genome sizes. Rigoutsos et al. (2003) predicted that HCMV UL131A and UL128 putative proteins were TM protein; this prediction is supported by the TM domain in regions 10-28 amino acid of UL131A, and in regions 25-40, and 85-100 of UL128. We predicted that UL131A, and UL130 putative proteins were TM protein with TM domains in regions 10-20 and 8-25 residues, respectively. This indicated that the TM domains of UL131A and UL130 could be important for maintaining the functional structure of the proteins.

In conclusion, the UL131A, UL130, and UL128 genes were highly conserved in all clinical strains. The conservation of the coding potential of UL131A, UL130, and UL128 appears to be a stable characteristic of all field strains, which is associated with conservation of endothelial and dendritic cell tropism as well as transfer to leukocytes. On the other hand, no link was observed between structure of the three genes and different signs of HCMV disease. Again, this observation would point to an *in vivo* strong positive pressure towards maintenance of these genes, would appear essential for HCMV replication and spread in body tissues and for various clinical signs of HCMV disease. The presence of chemokine-like do-

mains in UL130, and UL128 putative proteins suggests that the predicted products play a role in HCMV infectivity for cells. Further studies will be aimed at clarifying the function of UL131A, UL130, and UL128 protein during the replication of HCMV.

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