Transcription pattern of *UL131A-128* mRNA in clinical strains of human cytomegalovirus

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Human cytomegalovirus (HCMV) mRNA was obtained from human embryonic lung fibroblast cells infected by HCMV clinical strains from urine samples of infants at different kinetic periods. The cDNA of *UL131A-128* mRNAs was amplified using reverse transcription-polymerase chain reaction (RT-PCR) and analysed by sequencing. Meanwhile, clones containing *UL131A-128* transcripts in an HCMV cDNA library of a clinical strain were selected and sequenced. It was demonstrated that *UL131A-128* mRNA was expressed with immediately early, early and late kinetics. Sequences obtained by RT-PCR showed that the *UL131A* gene consisted of two exons and the coding region of the *UL130* gene was not interrupted by any intron in the region as reported earlier. However, the transcript of the *UL128* gene showed two patterns: one pattern consisted of three exons as reported earlier; the other contained the three exons and also the first intron. Moreover, the above characteristics of *UL131A-128* spliced transcripts were confirmed by the sequences of clones selected from the HCMV cDNA library. Our results demonstrated that the *UL131A, UL130* and *UL128* genes were transcribed with the 3′-coterminal, although the initiation points of their mRNA may be different. The variation in the transcripts found in our study indicated the complex nature of transcription of *UL131A-128* genes in clinical strains of HCMV.

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1. Introduction

Human cytomegalovirus (HCMV) causes substantial morbidity and mortality in immunologically naive children, immunosuppressed transplant patients, and immunodeficient AIDS patients. Congenital HCMV infection is common and can lead to serious developmental defects involving the central nervous system and often leads to deafness (Britt *et al.* 2006; Pass *et al.* 2001). *In vivo*, HCMV can infect a broad range of cell types, including endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, macrophages and monocytes (Plachter *et al.* 1996). However, the adaptation of clinical isolates to fibroblasts results in the inability to replicate efficiently in endothelial cells or monocytes (Grazia Revello *et al.* 2001) and to transfer virus from virus-infected cells to leukocytes (Hahn *et al.* 2004). Three open reading frames (ORFs) of the *UL131A-128* locus are indispensable for virus growth in endothelial, epithelial and dendritic cells (Grazia Revello *et al.* 2001; Hahn *et al.* 2004; Gerna *et al.* 2005; Wang and Shenk 2005a). All non-endotheliotropic laboratory strains tested show mutations in the *UL131A-128* locus affecting at least one of the genes (Akter *et al.* 2003; Hahn *et al.* 2004). By forming a complex with gH–gL, it has been indicated that pUL128, pUL130 and pUL131 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; Dai and Thomas 2005). Ryckman

Keywords. cDNA library; HCMV UL131A-128; mRNA

Abbreviations used: CHX, cycloheximide; E, early; HCMV, human cytomegalovirus; HELF, human embryonic lung fibroblasts; IE, immediately early; L, late; MOI, multiplicity of infection; ORF, open reading frame; PAA, phosphonoacetic acid; RT-PCR, reverse transcriptase-polymerase chain reaction

et al. (2008a, b) have shown that the gH/gL/UL128-131 complex can mediate entry of HCMV into epithelial and endothelial cells. Thus, an intact/wild-type *UL131A-128* locus is essential for HCMV infection of most host cells except for fibroblasts.

By using mRNA mapping experiments on the laboratory strain AD169, Akter *et al.* (2003) have confirmed that the *UL131A* and *UL128* genes comprise two and three exons, respectively. Meanwhile, it has been claimed that *UL131A-128* genes are transcribed with late kinetics (Akter *et al.* 2003). In our previous study, we provided evidence that the *UL131A, UL130,* and *UL128* genes of HCMV are present in all of the 23 clinical strains from congenitally infected infants (Sun *et al.* 2009). However, there have been no reports of the structure of *UL131A-128* mRNA in HCMV clinical strains so far. In this study, we identified the mRNA structure and the transcription pattern of *UL131A-128* in clinical strains of HCMV.

2. Materials and methods

2.1 *Cells, virus and RNA extraction*

Human embryo lung fibroblast cells (HELF) were maintained in Dulbecco modified Eagle medium supplemented with 10% foetal bovine serum. Three HCMV clinical strains, named H, X and Ch, were isolated from urine samples of neonates admitted to the Pediatrics Department, Affiliated Shengjing Hospital of China Medical University. The strains were passaged in HELF cells less than 10 times before they were used in this study. Virus DNA of HCMV H strain was extracted as described by Alderete *et al.* (1999) and its UL/b' region was sequenced using a shotgun sequencing method as described previously (Zabarovsky *et al.* 1994).

HELF cells were inoculated with the HCMV strain at a multiplicity of infection (MOI) of 5.0 and the infected cells were collected for RNA preparation when 70–100% of the cells showed a specific cytopathic effect. To classify HCMV transcripts into temporal kinetic classes, HELF cells were treated with drugs as previously described (Chinchar *et al.* 2003; Ebrahimi *et al.* 2003**)**. Briefly, the HELF monolayer was treated 1 h prior to and throughout the viral infection with either cycloheximide (CHX) or phosphonoacetic acid (PAA), which acts as inhibitors of *de novo* protein synthesis and viral DNA replication, respectively. To obtain viral immediate early (IE) transcripts, CHX (200 μg/ml) pretreated cells were mock infected or infected with HCMV, and then harvested 24 h post infection. To distinguish between viral early (E) and late (L) transcripts, cells infected with HCMV in the presence or absence of PAA (300 μg/ml) were harvested 48 h post infection. Total RNA from fresh HCMV-infected cells was isolated by using TRIzol (Life Technologies, USA). To confirm the RNA stages of HCMV transcription, a control reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using primers of the late gene *UL132* in all RNA preparations at different stages (Spaderna *et al.* 2005). To determine the quality of the cDNA/RNA samples, PCR was performed using β-actin primers.

2.2 *RNA isolation and mRNA purification*

Total RNA was isolated from approximately 10^7 HCMV-infected HELF cells using Trizol agent (QIAGEN, Germany). The integrity and size of the isolated RNA were analysed by formaldehyde agarose gel electrophoresis, and the quantity and purity of RNA were estimated by detection of the optical density (OD) value. The total RNA sample was processed according to the protocol of the mRNA purification kit (Oligotex mRNA Kits, QIAGEN, Germany). At the end of the procedure, the mRNA was dissolved in 200 μl RNase-free H_2O.

2.3 *Full-length HCMV cDNA library*

A cDNA library of the HCMV clinical strain H had been constructed previously in pBluescript II SK vector. The primary library was demonstrated to consist of 1.12×10^6 recombinant clones/ml. Nearly 95% of the bacterium contained inserts as determined by blue-white plaque screening on NZY agar plates containing X-Gal and IPTG. The average length of the inserts of cDNA clones was 1.2 kb as analysed by PCR with M13 primers and the Gel Image System.

2.4 *Amplification of UL131A-128 transcripts*

The RNAs were reverse transcribed using oligo-dT with introduction of a specific sequence and reverse transcriptase M-MLV (3′-Full Race Core Set Ver.2.0, Takara Company, Japan). Then, the cDNA was used as a template for PCR amplification of *UL131A-128* transcripts. To obtain the sequences of *UL131A-128* mRNA, specific primers to the sequence upstream and downstream of *UL131A-128* were designed and used in combination with the 3′ RACE primer that is homologous to the introduced sequence by oligo-dT in cDNA (table 1, figure 1). Half-nested PCR was done using a *UL131A-128*-specific primer and a 3′ RACE primer for the first round amplification, and gene-specific primers for the second round amplification of each transcript. To rule out possible DNA contamination, a control RT-PCR reaction without reverse transcriptase was carried out. The amplification products were cloned into the T-vector pCR2.1 and sequenced using the ABI PRISM 3730 DNA analyser (Applied Biosystems, USA). Sequences of the interesting

Table 1. Primers used for PCR amplification of HCMV *UL131A*, *UL130* and *UL128* transcripts and selection of cDNA clones

Primer	Sequence (5′-3′)
3′ RACE primer	CGCGGATCCTCCACTAGTGATTTCAC TATAGG
UL131A primer	CCGGAATTCCGGCTGTGTCGGGTGT GGCT
	CGCGTCGACCTAGTTGGCAAAGAGC CGCA
UL130 primer	CCGGAATTCTTGTCGACCCTGCGGCTT CTGCTTCGTCAC
	CGCGGATCCGGTACCTCAAACGATGA GATTGGGATG
UL128 primer	CCGCCCGGGCAGTCCCAAAAACCTG ACGCC
	CGCGTCGACTCACTGCAGCATATAGC CCA

genes were analysed using DNA Star and BioEdit Software (TA Hall Software Company, USA).

2.5 *Selection of clones containing HCMV UL131A-128 cDNA in the cDNA library by PCR amplification*

Original recombinants of the cDNA library were electro-transferred into *Escherichia coli* DH5*α*. A total of 4000 single clones were randomly picked up and inoculated into LB medium. To identify gene-specific clones, templates of PCR were prepared as follows: the aliquots of every 10 single clones were mixed together as the first grade of colonies, and so were the secondary and third grade of colonies. *E. coli* from each grade of colony was inoculated into fresh medium. The DNA of the propagated *E. coli* was exposed by using cell lysis buffer (50 mM TrisCl pH 6.8, 15 mM NaCl, 5 mM EDTA, 0.5% NP-40). PCR was then performed using each of the DNA preparations sequentially from the third, second, first grades of colonies and single clones as templates. The gene-specific primers are listed in table 1. The PCR conditions were initial denaturation at 94°C, 30 cycles of 94°C for 30 s, 50°C (depending of the primers used) for 30 s, and 72°C for 1 min, followed by final elongation at 72°C for 5 min. Identified clones were sequenced using the T7 primer of the pBluescript II SK vector, using the ABI PRISM 3730 DNA analyser. Only sequences with additional polyadenylated residues at the 3′ terminus, which did not exist in the genome, were taken to be entire mRNA sequences.

3. Results

3.1 *Sequencing of UL/b' region of HCMV H strain DNA*

A sequence of 18132 nucleotides was obtained from the H strain *UL/b'* region and submitted to GenBank (GenBank no. GQ981646).

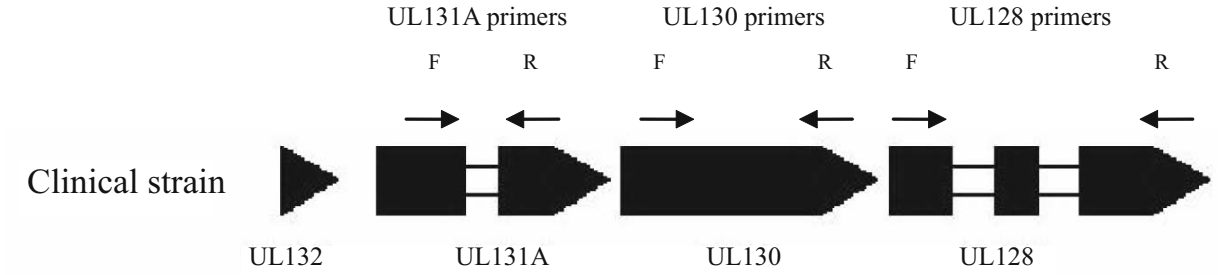

Figure 1. Relative positions of the primers for the *UL131A-128* genes in the study

3.2 *Analysis of RNA preparations*

The total RNA appeared as a long smear with clear bands of 28 S and 18 S. The ratio of OD260/OD280 of the total RNA was 1.82, and the concentration was 4.62 μg/μl. Therefore, we could conclude that the total RNA obtained from the HELF cells infected with the HCMV clinical strain had obviously not been degraded and that the purity was high enough for further use. As a control reaction for confirming the HCMV RNA stages, a transcript of the HCMV late gene *UL132* with 813 nt was found only in late RNA (figure 2).

3.3 *Temporal kinetic classes of UL131A-128 transcript*

Drug inhibitors were used to map the *UL131A-128* transcripts into temporal kinetic classes during the infection *in vitro* as outlined in Materials and methods. Specific transcripts of HCMV *UL131A-128* genes were obtained from cells infected by clinical strains H, X and Ch at immediate early, early and late times by RT-PCR (figure 3, data of *UL131A* and *UL130* not shown). Sequencing results showed that the two bands

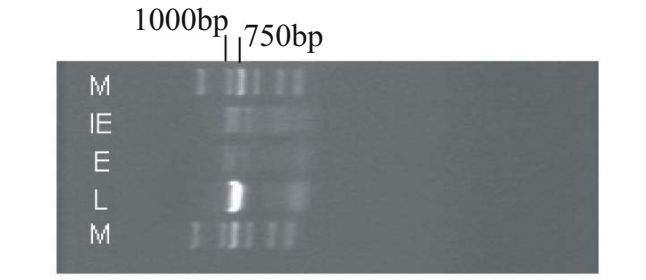

Figure 2. Transcripts of HCMV *UL132* in clinical strains amplified by RT-PCR with UL132-specific primers in the immediately early (IE), early (E) and late (L) kinetic classes. M, DL2000 DNA molecular weight marker; IE, H strain in the IE kinetic classes; E, H strain in the E kinetic classes; L, H strain in the L kinetic classes.

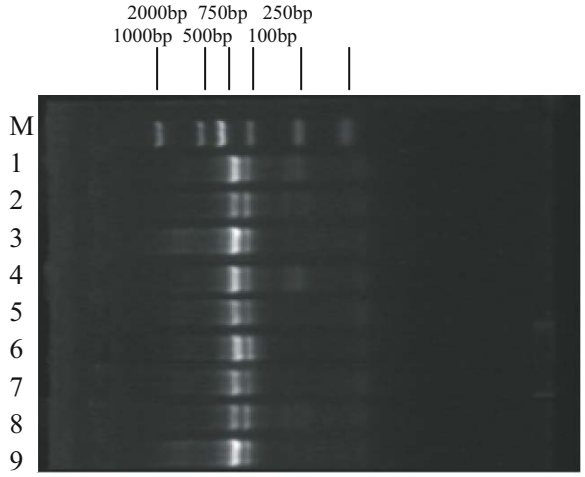

Figure 3. Transcripts of HCMV *UL128* in clinical strains amplified by RT-PCR with UL128-specific primers in the immediately early (IE), early (E) and late (L) kinetic classes. M, DL2000 DNA molecular weight marker; 1–3, Han strain in the IE, E and L kinetic classes, respectively; 4–6, Chen strain in the IE, E and L kinetic classes, respectively; 7–9: Xu strain in the IE, E and L kinetic classes, respectively.

of the *UL128* transcripts were all specific transcripts, one consisted of the three exons 519 nt in length as reported earlier and the other one consisted of three exons and the sequence of the first intron 642 nt in length. The quantities of *UL128* transcript containing the sequence of the first intron were higher than those containing only the three exons in the clinical strains studied in all kinetic classes.

3.4 *Structure of HCMV UL131A-128 transcript*

Sequencing results of *UL131A-128* transcripts showed that the transcript sequence of *UL131A* consisted of two exons 390 nt long, and *UL130*, which was 645 nt in length. Interestingly, the transcript of the *UL128* gene showed two transcription patterns, one form consisted of three exons 519 nt long and the other form consisted of three exons and the sequence of the first intron 642 nt long.

In order to confirm the structure of the *UL131A-128* transcript, 6 specific clones with two forms of inserts (figure 4) were selected from the full-length cDNA library. The sequences obtained have been deposited with Genbank under accession numbers GU568339-GU568344. As was found during the sequencing of the *UL131A-128* transcripts, sequences of 4 clones contained the sequence of the first intron of *UL128*, and 2 clones lacked the sequence of the first intron. Furthermore, the sequence of the 6 clones demonstrated that the UL*131A-128* gene locus shared the same determination site of their transcript although their initiation point may be different (figure 5). Compared to the sequence of the Merlin strain, the clones D947 and B428 lacking the sequence of the first intron of *UL128* originated from the *UL131A* transcript initiation point and the second exon of *UL131A*, respectively, but the other 4 clones containing the sequence of the first intron of *UL128* originated from different points of the *UL130* region, respectively.

4. Discussion

A series of studies have demonstrated that the genetic determinants of endothelial cell tropism, virus transfer to leukocytes and monocyte-derived dendritic-cell tropism of HCMV are the *UL131A, UL130* and *UL128* genes, which are located in the UL/b' region (Hahn *et al.* 2004; Gerna *et al.* 2005; Wang and Shenk 2005b). The ability to infect endothelial cells and leukocytes is a non-essential virus-

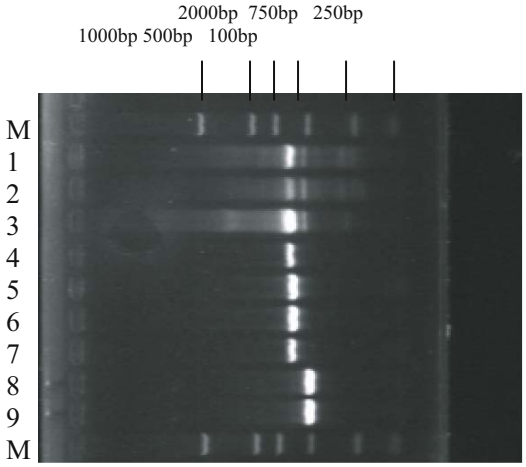

Figure 4. Analysis of the clones from the cDNA library by PCR with UL128-specific primers, compared with HCMV *UL128* transcripts of the HCMV clinical strains. M, DL2000 DNA molecular weight marker; 1–3: Han strain in the IE, E and L kinetic classes; 4–7, clones of B270, C666, C868, B753, respectively; 8–9, clones of B428 and D947, respectively.

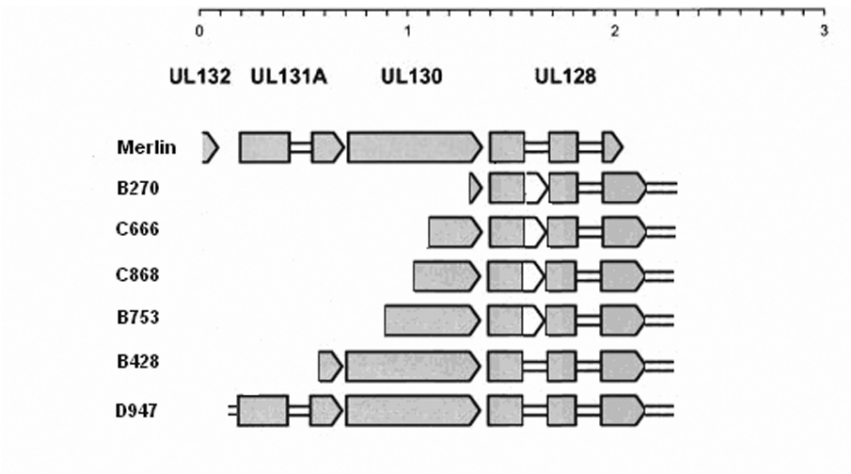

Figure 5. Structure of the transcripts obtained from the 6 selected HCMV cDNA clones. The termination sites of HCMV cDNA clones are in the 176164 nucleotides of the Merlin sequences. The initiation sites of the HCMV cDNA clones are between the 176999 and 178177 nucleotides of the Merlin sequences (Dolan *et al.* 2004). *Note*: White arrow indicates the first intron transcribed in the HCMV *UL128* gene. Among the 6 selected clones, 4 contain the sequence of the first intron of the *UL128* gene.

encoded function and is characteristic of clinical isolates of HCMV, but it is not found in reference laboratory strains, such as AD169, Towne and Davis (Gerna *et al.* 2002). Extensive fibroblast propagation of an endothelial cell tropic clinical isolate (VR6110) is associated with the selection of a tropism-deficient variant showing a possible deletion of *UL132-130* within the *UL/b'* region (Grazia Revello *et al.* 2001). Ryckman *et al.* (2008a, b) showed that the gH/gL/UL128-131 complex can mediate entry of HCMV into epithelial and endothelial cells. These facts lead to the hypothesis that HCMV disease and tissue tropism may be related to variations in the *UL131A, UL130* and *UL128* genes among strains.

By using RT-PCR, 5′- and 3′-RACE, and northern blot, Akter *et al.* (2003) found that *UL131A* and *UL128* of the laboratory strain AD169 comprised two and three exons, respectively. Meanwhile, partially spliced RNA of *UL128* was found to contain the sequence of the first intron. It has been claimed that the *UL131A-128* genes are transcribed with late kinetics (Akter *et al.* 2003). However, no evidence has been reported so far on the *UL131A-128* mRNA structure in HCMV clinical strains.

In our study, clinical strains from infected infants were used. By using RT-PCR, 3′-RACE, selection of cDNA library clones and sequencing techniques, the structures of HCMV *UL 131A, UL130* and *UL128* in clinical strains were demonstrated to be the same as those found by Akter *et al.* (2003). Six clones were selected from the cDNA library. Four of them contained partially spliced RNA of *UL128* with the sequence of the first intron. Transcripts from the 6 clones shared the same determination site, although their initiation points were different. The length of the 4 transcripts were 600–1100 nt, which are somewhat the same as those found by Akter *et al.* (2003) by northern blot, a *UL128* probe hybridized to major RNAs of 0.5–1.0 kb. But based on the genomic sequence, no TATAA box and regulation sequences were found upstream of the corresponding gene region.

Among the 6 clones selected from cDNA library, 4 clones contained the partially spliced RNA of *UL128* with the sequence of the first intron. Many more transcripts containing the first intron were found by using RT-PCR in different kinetic classes of the clinical strains. It seems that the transcript of *UL128* containing the sequence of the first intron is the major form of *UL128* mRNA. Our results are different from those of Akter *et al.* (2003), and indicate that besides late time, transcripts of the *UL131A-128* genes were also found in the immediately early and early periods by RT-PCR. This implied that the *UL131A-128* genes may be transcribed earlier than has been reported.

In transcribing HCMV, some genes have been demonstrated to be more complex, such as *UL146-147A* (Lurain *et al.* 2006), which include overlapping transcripts with different 5-prime termini, co-terminal 3' ends and different temporal expression of the transcripts. Transcripts of the *UL131A-128* genes obtained from the cDNA library showed similar transcriptional patterns and complexities. All the sequences of the 6 clones had the same 3' end and various initiation sites among the genes. The single poly A signal, which is downstream of the *UL128* stop codon, supports the possibility of a common 3'-terminus for all of these transcripts (Cha *et al.* 1996; Dolan *et al.* 2004). Regulation at the level of translational initiation has been described for several cellular and viral genes (e.g. herpes simplex virus thymidine kinase gene) as a means to increase the diversity of protein products (Liu *et al.* 2000; Stamminger *et al.*

2002). Whether the two isoforms of *UL128* transcripts differ in function remains to be studied.

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