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Regular paper

Newly identified transcripts of *UL4* and *UL5* genes of human cytomegalovirus

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Human cytomegalovirus (HCMV) *UL4* and *UL5* genes are two members of the *RL11* gene family. In an earlier study, three UL4 transcripts of about 1.7, 1.5 and 1.4 kb were found in early and late classes after infection by the Towne strain by nuclease protection and primer extension analyses. In the present study, two UL4 transcripts (1.5 and 1.7 kb) were found by cDNA library screening, Northern blot, 3′ and 5′ RACE analyses to appear initially in the immediate early phase and one UL4 transcript (1.4 kb) in the late phase in a low-passage clinical isolate. Furthermore, two novel low-abundance UL5 transcripts with the same 3′ terminus as the identified UL4 transcripts in the *UL4-UL5* gene region were found in late class RNAs.

Key words: HCMV, *UL4*, *UL5*, transcript

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INTRODUCTION

Human cytomegalovirus (HCMV) contains a linear double-stranded genome of approximately 230 kilo base pair (kbp), which has a potential to encode more than 165 proteins (Murphy *et al*., 2003; Dolan *et al*., 2004; Ma *et al*., 2012). As other herpesviruses, HCMV genes are expressed in a temporal cascade comprising immediate-early (IE), early (E) and late (L) phases. The IE proteins are required for subsequent early gene expression, and the early products are required for viral DNA replication. After viral DNA replication, late gene expression occurs (Spector, 1996).

Nine multigene families have been identified in the AD169 strain of HCMV (Chee *et al*., 1990) and in three low-passage isolates (Davison *et al*., 2003a). The *RL11* family consists of 12 genes, including *RL11*, *RL12*, *RL13*, *UL1*, *UL4*, *UL5*, *UL6*, *UL7*, *UL8*, *UL9*, *UL10* and *UL11*, oriented from left to right near the left-hand terminus of the genome (Davison *et al*., 2003b).

As a member of the *RL11* family, *UL4* has been defined as an early gene encoding a 48-kDa subgenus-specific virion glycoprotein (Chang *et al*., 1989a). Two early (1.7 and 1.5 kb) and one late (1.4 kb) transcript regulated by three inducible promoters at various times have been identified in the *UL4* region of the Towne strain by nuclease protection and primer extension analyses (Chang *et al*., 1989a; 1989b). In 2007, Zhang *et al*. found three transcripts overlapping the *UL5* sequence with the 3' end at nucleotide (nt) 14748 and the 5' ends at nt13758, nt13906 and nt13925, respectively, in a late-cDNA library (Zhang *et al*., 2007). These results indicate a transcription unit in the *UL4-UL5* gene region. However, there has been no report about the transcript structures of *UL4* and *UL5* in HCMV strains isolated from patients so far. In the current study, the transcription characteristics of the *UL4-UL5* gene region were investigated in a low-passage isolate by cDNA screening, northern blotting and rapid amplification of cDNA ends (RACE). Two novel low-abundance UL5 transcripts with the same 3' terminus as the earlier UL4 transcripts identified were found in late class RNAs.

MATERIALS AND METHODS

Cell, virus and RNA preparation. HCMV low-passage strain H was isolated from a urine sample of a congenitally HCMV-infected infant in the Pediatrics Department at the Affiliated Shengjing Hospital of China Medical University. MRC-5 cells were routinely cultured in minimal essential medium (MEM) containing 15% fetal calf serum (Hyclone, USA) and penicillin-streptomycin at 37°C, 5% CO_2. After inoculation with virus, the MRC-5 cells were maintained in MEM supplemented with 2% fetal calf serum and penicillin-streptomycin at 37°C, 5% CO_2 in an incubator.

For HCMV IE infection, 100 μl/ml of cycloheximide (Sigma, USA) was added to culture medium prior to infection and the cells were harvested 24 hours post infection (hpi). For E infection, 100 μl/ml of DNA synthesis inhibitor phosphonoacetic acid (Sigma, USA) was added to the medium immediately after virus inoculation, and the infected cells were harvested at 48 hpi. For L infection, the cells were harvested at 72 hpi without any drug treatment. Total RNA was isolated from infected and uninfected MRC-5 cells using Trizol reagent (Invitrogen, CA) according to the manufacturer's instruction. Possible DNA contamination was removed from RNA preparations using DNA-free reagents (Ambion, USA). The quantity and purity of the RNA preparations were estimated by optical density measurements.

Northern blotting. Northern blot analysis was carried out according to a standard procedure established in

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Abbreviations: CHX, cycloheximide; DIG, digoxigenin; E, early; HCMV, human cytomegalovirus; hpi, hours post infection; IE, immediate early; L, late; MEM, minimal essential medium; nt, nucleotide; ORF, open reading frame; PAA, phosphonoacetic acid; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; TAP, tobacco acid pyrophosphatase

our laboratory. Aliquots of 5 μg of RNA per lane from IE, E and L infected and mock-infected MRC-5 cells were subjected to denaturing agarose gel (1.5% [wt/vol]) electrophoresis in the presence of 5.4% formaldehyde, alongside digoxigenin-labeled RNA molecular weight marker I (Roche, USA). Probes were labeled with digoxigenin using a DIG Northern Starter kit (Roche, USA). Primers used to produce the RNA probes are listed in Table 1. In order to evaluate the effects of cycloheximide and phosphonoacetic acid on HCMV replication, transcripts of *UL123*, *UL34* and *UL99*, which are IE, E and L genes, respectively, were detected in the same RNA preparations by Northern Blot. The separated RNA was transferred onto a positively charged nylon membrane by capillary transfer. Then, the membrane was baked at 80°C for 2 hours followed by pre-hybridization for 30 min at 60°C using the Dig EasyHyb buffer (Roche, USA) and hybridization with RNA probes for 16 hours at 60°C. Following washing twice with 2% SSC, 0.1% SDS buffer and 0.1% SSC, 0.1 % SDS buffer at 50°C under constant agitation, the membrane was incubated with anti-digoxigenin antibodies conjugated to alkaline phosphatase and probes were visualized with the chemiluminescence substrate CDP-Star (Roche, USA) using ChemiDocTM XRS+ (Bio RAD, USA). To ensure equal RNA loading, the RNA preparations were adjusted basing on 28S and 18S rRNA levels estimated by electrophoresis and ethidium bromide staining.

Screening of cDNA library. A full-length cDNA library of HCMV H strain has been constructed in pBluescript SK vector before (Ma *et al.*, 2011). Recombinants of the cDNA library were transferred into JM109 (Promega, USA). A total of 8600 clones were randomly picked up and inoculated into LB medium. A pair of *UL5* gene specific primers (Table 1) were used to screen for *UL5* clones from the cDNA library by graded polymerase chain reaction (PCR) as described before (Sun *et al.*, 2010; Qi *et al.*, 2011). The PCR reaction conditions were as follows: 95°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a final elongation at 72°C for 10 min. Inserts of the selected clones were sequenced using vector primers M13F and M13R on an ABI PRISM 3730 DNA analyzer (Applied Biosystems, CA).

5' RACE (rapid amplification of cDNA 5'ends). For mapping 5' ends of transcripts, 5' RACE was performed using the 5'-full RACE Kit (TaKaRa, China) according to the manufacturer's instructions. First-strand cDNA was synthesized with M-MLV reverse transcriptase using random 9-mer primers provided in the kit.Reactions without TAP (to account for the interference caused by 5' phosphate of tRNA, rRNA and incomplete mRNA) and M-MLV (to determine the interference by contaminating DNA) were performed as two controls. *UL4-UL5* gene specific cDNA sequences were amplified by nested PCR using specific primers R_1 and R_2, together with the 5' RACE adaptor primer provided in the kit (Table 1). The PCR conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by final elongation at 72°C for 10 min.

3' RACE. For mapping 3' end of transcripts, 3' RACE was performed using 3'-full RACE Core Set Ver.2.0 (TaKaRa, China) according to the manufacturer's instructions. First-strand cDNA was prepared using oligo-dT-adaptor primer and M-MLV. Nested PCR was then performed using specific primers F_1 and F_2, together with the oligo-dT-adaptor primer provided in the kit.

Cloning and sequencing. RACE products were gel-purified and inserted into PCR2.1 vector PCR2.1 (Invitrogen, China) with T4 ligase at 14°C overnight. The ligation products were transformed into *E. coli* DH5α competent cells. Ten to twenty clones of each purified PCR product were selected randomly and identified by PCR. Then, the insert sequences of the clones were confirmed by DNA sequencing on an ABI PRISM 3730 DNA analyzer (Applied Biosystems, USA). The nucleotide positions referred to in this study are in reference to the sequence of the HCMV Towne strain (GenBank: FJ616285.1). Open reading frame (ORF) was predicted using Editseq program of the DNA star package.

Table 1. Gene specific primers used in this study

Primer	Sequence (5'-3')	Genomic coordinates
Northern Blot		
UL4-NF	AGAGTATCGTTATGATGCTTAGAA	13446-13469
UL4-NR	ATTATTTAGGACACGGTTAGGTTG	13898-13875
UL5-NF	TCGCCCGTCCTCTGAAATACC	14445-14465
UL5-NR	CAAGCACACCCCAGCCCAC	14631-14613
UL123-NF	GCCTTCCCTAAGACCACCAAT	172316-172296
UL123-NR	ATTTTCTGGGCATAAGCCATAATC	172216-172239
UL34-NF	TCGGTCATACGGACTCGGTGT	45494-45514
UL34-NR	AATGCTGCGGTTGCCTTGC	44758-45740
UL99-NF	CTGGGCTGCGAGTTGCTGGC	144702-144721
UL99-NR	GATGGTGGTGATGTTTTGAGGGTT	145235-145212
cDNA library screening		
WS	TTGTCTTTTGTGTGGTTGGTCG	14072-14093
WR	CCGTAGAACTGCTTAGTGATGATG	14319-14296
5′ RACE		
R_1	CCAGCCCACCCGTTCATCTTA	14621-14601
R_2	ACTACCCGCAAAAGTCCCTCCC	14594-14573
3′ RACE		
F_1	GTTGTATGGTTGGTCGGTTTGC	14078-14100
F_2	GACATCACCGAGGCGGAGAG	14138-14158

Note: Sequence positions are of the Towne strain (GenBank: FJ616285.1). Primer sequences for synthesis of UL123 probe were the same as those used in the reference (Stamminger *et al.*, 1991).

RESULTS

UL4-UL5 transcripts detected by Northern blot

Northern blots were repeated three times with different RNA preparations of IE, E and L viral expression phases. The RNA blots were first hybridized with

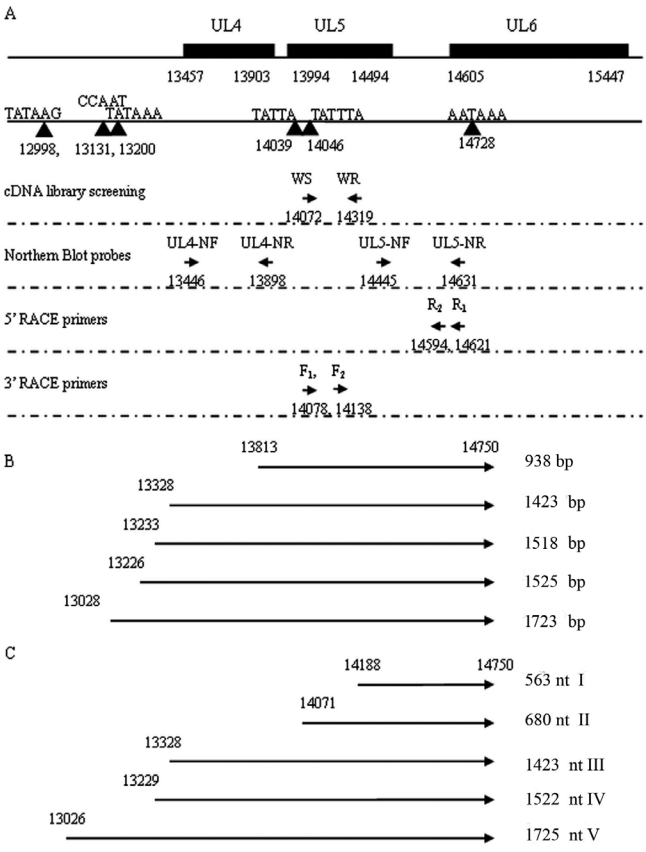

Figure 1. Genome structure of UL4-UL6 gene region of HCMV H strain.
(**A**) Black boxes indicate open reading frames of transcripts from this region. The positions of the transcripts are referred to the sequence of Towne strain (GenBank FJ616285). The TATA, CAAT and poly(A) signal are represented by black triangles. 5′ ends of primers for cDNA library screening, synthesis of RNA probe, 5′ RACE and 3′ RACE experiments are marked in four lines, respectively. (**B**) UL4-UL5 transcripts identified by cDNA library sceening. (**C**) UL4-UL5 transcripts identified by RACE experiments. The length of each transcript is indicated on its right, and the 5′ and 3′ ends are marked on its left and right, respectively.

digoxigenin-labeled UL5 RNA probe (Fig. 1a). The results showed that besides the major transcript of 1600~1800 nt detected in all three expression phases, a weak transcript of about 650 nt was detected by the UL5-specific probe in the L RNA preparation. No band was found in RNA preparation from mock-infected cells. Transcripts of *UL123*, *UL34* and *UL99* genes, which are IE, E and L genes (Welch *et al.*, 1991; Stamminger *et al.*, 1991; Adam *et al.*, 1995), respectively, were detected in the corresponding RNA preparations (Fig. 2a). To determine whether the 1600~1800 nt transcript contains *UL4* sequence or not, UL4-specific RNA probe was used. As shown in Fig. 2b, only the 1600~1800 nt transcript was detected by the UL4 probe.

UL4-UL5 transcripts screened from the HCMV cDNA library

Five cDNA clones selected by graded PCR were identified to contain the sequence congruent with the *UL4-UL5* gene region. Sequencing results showed that all the five cDNA sequences were unspliced and had the same 3' end but different 5' ends. The 3' terminus was at nt 14750, which is downstream of a poly(A) signal (AATAAA) at nt 14728-14733, by comparing with the sequence of HCMV Towne strain. The 5'ends of the cDNA sequences were at nt 13028, 13226, 13233, 13328 and 13813, respectively. The corresponding lengths of the cDNA sequences were 1723 bp, 1525 bp, 1518 bp, 1423 bp and 938 bp, respectively (Fig. 1b).

5′ and 3′ termini of UL4-UL5 transcripts identified by RACE

In order to further identify the 5' and 3' termini of the UL4-UL5 transcripts detected by cDNA library screening and Northern blot, RACE experiments were employed with IE, E and L class RNAs of HCMV H. In 5' RACE experiments, a product of 1600 bp was obtained in all three classes of RNAs and three additional products of about 1300, 500 and 400 bp were amplified in the L class RNA using specific primers R_1 and R_2 (Fig. 3a). Sequencing results

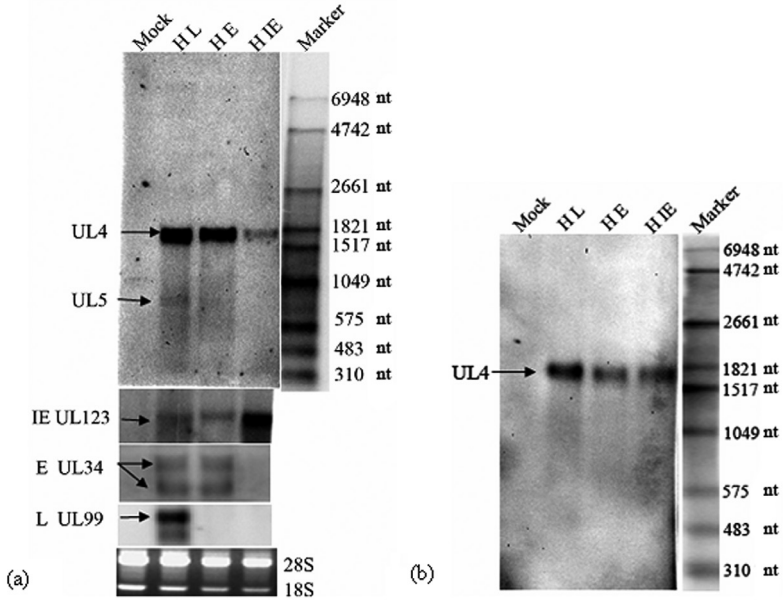

Figure 2. Northern blot analysis of UL4-UL5 transcripts in HCMV H strain.
(**A**) Northern blot was performed with *UL5*, *UL123*, *UL34* and *UL99* specific probes, respectively, using 5 μg of total RNA harvested from HCMV H infected MRC5 cells at IE, E and L phases. RNA from mock-infected cells (Mock) was used as a control. Sizes of molecular weight markers (300 to 6900 nt) are shown at the right. Judging from the amounts of 28S and 18S rRNAs in each RNA preparation estimated by ethidium bromide staining, equal amounts of RNA were loaded in different lanes. (**B**) Northern blot was performed with UL4 specific RNA probe using 5 μg of total RNA harvested from infected MRC5 cells at IE, E and L

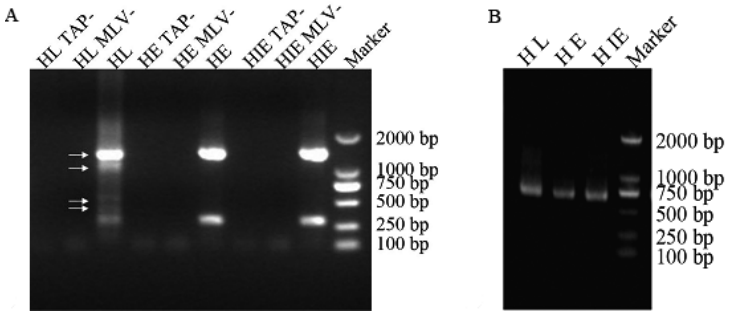

Figure 3. RACE results for UL4 and UL5 transcripts.
IE, E and L RNA preparations from HCMV H infected MRC5 cells were used as template. (**A**) 5′ RACE using specific primers R_1 and R_2. TAP (-) and MLV (-) are negative controls. (**B**) 3′ RACE using gene specific primers F_1 and F_2. Arrows indicate specific bands.

showed that two 5' ends, at nt 13229 and 13026, were obtained in all three classes of RNAs, and three other 5' ends at nt 13328, 14071 and 14188 were detected in the L RNA only.

In 3' RACE experiments, a band of about 700 bp was obtained from all RNA preparations using specific primers F_1 and F_2 (Fig. 3b). Consistent with the results obtained by cDNA screening, sequencing results of the band demonstrated a 3' end located at nt 14750 downstream of the consensus poly(A) signal (AATAAA) at nt 14728-14733.

Sequence analysis of the UL4 and UL5 transcripts

To obtain the complete sequence of the UL4 and UL5 transcripts, the sequences obtained by 5' RACE and 3' RACE were linked together according to their overlapping sequences. Detailed information of the linked transcripts is showed in Fig. 1c. The full lengths of the UL4 transcripts were 1423, 1522 and 1725 nt with the 5' ends located at nt 13328, 13229 and 13026, respectively. The UL5 transcripts, which initiated at nt 14188 and 14750, comprised 563 and 680 nt, respectively. The lengths of the UL4 transcripts were consistent with those identified in the Northern blots, and their sequences were the same as those obtained by cDNA library screening. However, the lengths of the UL5 transcripts were only in accord with those found in the Northern blots. Based on the DNA sequence, two non-conventional potential TATA promoter elements (TATTA and TATTTA) were predicted at nt 14039 and 14046, upstream of the identified UL5 transcripts (Fig. 4). The UL5 transcripts have a potential to encode an 81-amino-acid protein with a calculated molecular mass of 9 kDa.

TTTGCTGTG**TATTA**TG**TATTTA**GATCACGCTTAACGCTCGTATTTGTTGTATGGTTGG
TATA box I RNA initiation
TCGGTTTGCGTCTCCATGATTGTGCCACGTTCGAGTCCTGCTGTTACGACATCACCG
II RNA initiation
AGGCGGAGAGTAACAAGGCTATATCAAGAGACGAAGCGGCATTGACCTCCAGCGT
ORF
GAGCGCCCGTACACCGTCCCTGGCGATCGCGCCTCCTCCTGACCGATCG***ATGCTGT***
TGTCGCGAGAGGAAGAACTCGTTCCGTGGAGTCGTCTCATCATCACTAAGCAGTTCT
ACGGAGGCCTGATTTTCCACACCACCTGGGTCACCGGCTTCGTCCTGCTAGGACTCT
TGACGCTTTTCGCCAGCCTGTTTCGCGTACCGCAATCCATCTGTCGTTTCTGCATAGA
CCGTCTCCGGGACATCGCCCGTCCTCTGAAATACCGCTATCAACGTCTTGTCGCTAC
CGTGTAGCTAGTTAGCCAGCTGTGTGTAGTTGTGTTTTGTTTTTGCATATTTGTTTTC
AGTCAGAGAGTCTGAAACGGGGTGGGAGGGACTTTTGCGGGTAGTGCATGCTAAG
ATGAACGGGTGGGCTGGGGTGTGCTTGATAACTCACTGTTTGAATACGCGCTCACG
CACTTATGTAGTACTCAACATGTTAGCTTTTGCCCGCACGCCCCGGGGCGTGCCGA
GCTGCCTTTTTAATAAAGTCTGGGTTTCCCGATACGCGCTGG
Poly A signal

Figure 4. Nucleotide sequences of UL5 transcripts.
Two possible TATA boxes are marked in bold. Sequence of the transcript is underlined, and the predicted open reading frame is in bold italics. Positions are shown on the genomic, sequence of Towne strain (GenBank: FJ616285.1). The poly (A) signal is shown in box.

DISCUSSION

It has been reported that the *UL4* gene is transcribed into three transcripts of 1700, 1500 and 1400 nt, initiated at nt 13026, 13229 and 13313, respectively, and terminated at nt 14750 (GenBank: FJ616285.1). The two longer transcripts are transcribed in E infection phase and the shortest one in L infection phase (Chang *et al.*, 1989a; 1989b). In the present study, the structures of the UL4 transcripts were confirmed in a low-passage HCMV strain. Except for the 1400 nt transcript, the structures of the other two UL4 transcripts were completely consistent with those found in the previous studies (Chang *et al.*, 1989a; 1989b).

HCMV IE genes are expressed as the critical first step in virus infection. Only a few genes, including *UL36-UL38, UL115-UL119, UL122-UL123, US3* and *IRS1/TRS1,* within the ~230-kbp HCMV genome have been found to be transcribed at the IE phase of infection up to now (Oduro *et al.*, 2012). IE proteins impair many cellular functions to facilitate later phases of infection, including cellular DNA synthesis, STAT signaling, apoptosis, and so on (Murphy *et al.*, 2000; Wiebusch *et al.*, 2001; Skaletskaya *et al.*, 2001; Child *et al.*, 2004; Paulus *et al.*, 2006; Marshall *et al.*, 2009; Mccormick *et al.*, 2010). The *UL4* gene has been defined as an early gene according to its transcription being detected primarily in E infection phase (Chang *et al.*, 1989a). The UL4 transcripts are regulated differentially by three promoters during the course of infection (Chang *et al.*, 1989b). However, the 1725 and 1522 nt transcripts of *UL4* gene, initiated at nt 13026 and 13229, were detected initially in the IE infection phase by 3' RACE, 5' RACE and Northern blot in the present study. This result indicated that transcription of *UL4* gene may be active as early as in the IE infection phase. The meaning of the *UL4* gene expression during IE phase needs to be investigated further.

In the present study, one monocistron containing the *UL5* sequence was confirmed by Northern blot and RACE. These results showed that, in addition to transcription together with the *UL4* gene, *UL5* can be transcribed independently in late infection phase. The monocistron originates from two different sites and has the same 3' end with that of the UL4 transcripts. However, no UL5 cDNA clone was found in the HCMV cDNA library. The reason for the failure to detect UL5 transcripts in the cDNA library could be the lower abundance in the infected

MRC5 cells. Northern blot was performed several times. Although the RNA preparations used in these experiments were not from the same batch of cells, the UL5 transcripts were always detected in L class RNAs. These results indicated that the *UL5* gene is a real late class gene. Murphy *et al.* have identified that *UL5* has a potential to encode protein (2003). Existence of two non-canonical TATA elements (TATTA and TATTTA) upstream of the mapped initiation sites provided evidence for the UL5 transcripts to be conventional mRNAs.

In the present study, two UL4 transcripts were found to be transcribed initially in the IE phase in a low passage HCMV strain, and two UL5 transcripts with the same 3' termini as the UL4 transcripts were identified during the late phase at a relatively low level. Detailed information on HCMV transcripts may benefit understanding HCMV pathogenesis, finding new diagnostic targets and establishing new strategies for prevention of HCMV diseases.

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