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Characterization of human cytomegalovirus UL146 transcripts

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ABSTRACT

Background: The genome of human cytomegalovirus (HCMV) has been studied extensively, particularly in the UL/b′ region. In this study, transcripts of one of the UL/b′ genes, UL146, were identified in 3 HCMV isolates obtained from urine samples of congenitally infected infants.
Methods: Northern blot hybridization, cDNA library screening, and RACE-PCR were used.
Results: All sequences of clones from a cDNA library were about 3225 bp in length with the same 5′ and 3′ ends. The results were accordant with that analyzed by Northen-blot and RACE-PCR in three HCMV clinical isolates. The transcript initiated from the region upstream of UL146 flanking region and terminated just downstream of UL132 including UL146, UL147, UL147A, UL148, and UL132 ORFs. Treatment of the infected cells with phosphonoacetic acid inhibited its transcription.
Conclusions: UL146 ORF was transcribed with 4 downstream ORFs from UL147 to UL132 at true late kinetics. The transcript of UL146 initiated at 73 nt upstream of UL146 and terminated just downstream of UL132 in the 3 clinical isolates.

1. Introduction

Cytomegaloviruses (CMVs) are members of the Betaherpesvirinae subfamily of the Herpesviridae family. Human cytomegalovirus (HCMV) is a common cause of congenital viral infections and a frequent opportunistic pathogen in transplant recipients and AIDS patients (Alford et al., 1990; Ho, 1991; Pass, 2005). Like other CMVs, HCMV has a very specific host range, but within a permissive host it enters and replicates in a wide variety of cell types (Ho, 1991; Sinzger et al., 1995).

The HCMV genome consists of 230–235 kb of double stranded DNA and more than 160 predicted protein coding open reading frames (ORFs) (Davison et al., 2003b; Dolan et al., 2004). The overall nucleotide sequence of strains isolated from unrelated sources is relatively conserved. However, sequences of specific ORFs can be highly variable, including RL6, RL12, UL4, UL18, UL55 (gB), UL73 (gN), UL74 (gO), UL139, UL144, and UL146 (Bale et al., 2001; Bar et al., 2001; Chou and Dennison, 1991; Davison et al., 2003a; Dolan et al., 2004; Hassan-Walker et al., 2004; Lurain et al., 1999; Pignatelli et al., 2001; Prichard et al., 2001; Rasmussen et al., 2003; Valés-Gómez et al., 2005).

The HCMV UL146 ORF encodes a protein with sequence characteristics of CXC (α) chemokines (Montgomery et al., 1996), suggesting that it can influence the behavior of neutrophils during infection. The predicted UL146 gene lies in the UL/b′ region between UL145 and UL147. Considerable sequence divergence has been observed in UL146 of clinical isolates (Arav-Boger et al., 2005; Dolan et al., 2004; Hassan-Walker et al., 2004; Lurain et al., 2006; Prichard et al., 2001; Stanton et al., 2005). Based on the diversity of UL146 sequences, 40 strains sequenced by Dolan et al. (2004) and by Prichard et al. (2001) respectively fall into 14 clades. The nucleotide sequence identity of the UL146 genes was below 70% for most of these strains. But no detailed analysis of its transcripts has been reported. In the current study, the UL146 transcripts were characterized in 3 clinical strains.

2. Materials and methods

2.1. Cells and virus

Human embryonic lung fibroblast (HELF) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. Three HCMV clinical isolates, named Han, Ch, and Xu were categorized as the UL146 genotypes of Group 1 and 12, according to the schema of Dolan et al. (2004). The isolates were derived from urine samples of three congenitally HCMV-infected

Abbreviations: HCMV, human cytomegalovirus; ORF, open reading frame; HELF, human embryonic lung fibroblast; PFU, plaque forming units; IE, immediate early; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; IPTG, isopropyl-β-D-thio-galactoside.

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children from the Pediatrics Department at the Affiliated Shengjing Hospital of China Medical University. All samples were collected with the permission of the infants' parents and the research had been approved by the Ethical Committee of Shengjing Hospital. HELF cells were infected with the virus at a multiplicity of infection of approximately 10 plaque forming units (PFU) per cell.

The reference strains used in this study are Toledo, TB/40, Merlin, Davis, Towne, 6397, and Han20 reported by Cunningham et al. (2010) (GenBank accession numbers AY446871, AY446866, AY446894, AY446868, AY446869, AY446870 and GQ396663, respectively).

2.2. RNA preparation

Total RNA was extracted from uninfected and HCMV-infected HELF cells using a standard guanidium isothyocyanate and phenol:chloroform method (Chomczynski and Sacchi, 1987). The extracts were treated with DNase I (Ambion, USA). Immediate early (IE) RNA was prepared in the presence of 100 μg/mL cycloheximide (Sigma, USA) at 24 h post-infection (hpi), early (E) RNA was extracted in the presence of 100 μg/mL phosphonoacetic acid (Sigma) at 48 hpi, late RNA was extracted in the absence of any blocking agents at 96 hpi, and control RNA was extracted from uninfected cells (Scott et al., 2002). Meanwhile, RNAs of infected cells treated without any blocking agents were prepared at 24, 48, 72, and 96 hpi.

2.3. Screening of the cDNA library

A cDNA library of the HCMV clinical strain Han had been previously constructed using the pBluescript II SK vector (Ma et al., 2011). The primary library consisted of 1.12×10^6 recombinant clones/mL. Nearly 95% of the vectors contained inserts, as determined by blue-white plaque screening on NZY agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thio-galactoside (IPTG). The average length of the inserts was 1.2 kb as determined by PCR with M13 primers and the Gel Image System.

A total of 8000 single clones were randomly picked and inoculated in LB medium. In order to identify gene specific clones, aliquots of bacteria culture of the 8000 clones were mixed step by step as follows: a mixture of every 10 individual clones was designated as the first grade of colonies, a mixture of every 10 of the first grade of colonies as the second grade of colonies, and a mixture of every 10 of the second grade of colonies as the third grade of colonies, equivalent to 1000 clones. Each grade of the colonies was inoculated into fresh medium. Cells were lysed using a cell lysis buffer (50 mM Tris–HCl, pH 6.8, 15 mM NaCl, 5 mM EDTA, 0.5% NP-40). The UL146-containing cDNA clones were identified by PCR using a set of primers, 146For1 and 146Rev1 (Table 1), covering the whole UL146 ORF. Each of the DNA preparations from the third, second, and first grade of colonies and single clones were used as templates, sequentially. The PCR condition was an initial denaturation step at 94 °C, 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. Identified clones were sequenced using T7 primer, which can bind the corresponding sequence of the pBluescript II SK vector, with an ABI PRISM 3730 DNA analyzer. Only sequences with additional polyadenylated residues at their 3′ terminal ends were believed as entire cDNA sequences.

A complete list of all primers is provided in Table 1.

2.4. Northern blot hybridization

To identify transcripts of UL146, RNAs from uninfected cells and cells infected with HCMV clinical isolates Han, Ch, and Xu at IE, E, and L phases were analyzed by Northern blot. Meanwhile, RNA preparations from the three isolates at 24, 48, 72, and 96 hpi were detected to show the time course of UL146 expression after initial infection.

Five micrograms of RNA was size-fractionated in a 1.2% denaturing agarose gel and transferred onto a membrane (N-Hybond, Amersham Biosciences) by capillary blotting. A UL146 gene-specific probe was labeled with digoxigenin-dUTP, hybridized to the membrane and detected by its NBT/BCIP chromogenic signal according to the manufacturer's instructions (DIG Northern starter kit, Roche Diagnostics, Mannheim, Germany). RNA representing the noncoding strand of UL146 gene was generated using T7 RNA polymerase and labeled by incorporation of digoxigenin-dUTP. Three probes were produced according to the different sequences of isolates Han, Ch, and Xu using the primers 146For1, 146Prob and 146Prob-Xu, respectively (Table 1). The labeled probes were specifically antisense to the 354 bp of the complete UL146 protein coding sequences (nucleotides 13028–13381, GQ981646 as reference) of the three isolates. Hybridization to the RNA blots was carried out at 68 °C overnight. After reaction with an anti-digoxigenin antibody labeled with alkaline phosphatase, the blots were visualized by addition of the substrate of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). DIG-labeled RNA Molecular Weight Marker I 0.31–6.95 kb (Roche Diagnostics) was used to estimate the size of the bands by the logarithmic relationship between molecular weight and distance migrated.

2.5. Rapid amplification of cDNA 5′ ends (5′ RACE)

To define the 5′ ends of transcripts in the UL146 intergenic region, RNA preparations of the isolates Han, Ch, and Xu were analyzed by 5′ RACE using a 5′-Full RACE kit (Takara, Japan) according to the manufacturer's instructions. Briefly, the RNA preparations were treated with calf intestine alkaline phosphatase (CIAP, 4.8 U/μg RNA) for 1 h at 50 °C. Then the purified RNAs were treated with tobacco acid pyrophosphorylase (TAP, 0.25 U/μg RNA) for 1 h at 37 °C. The CIAP/TAP-treated RNAs were ligated to the supplied 5′ RACE adaptor (1 μL of 15 μM adaptor/2 μg RNA) with bacteriophage T4 RNA ligase (20 U/μg RNA) for 1 h at 16 °C. Reverse transcription was carried out at 42 °C for 1 h with 5 units of M-MLV reverse transcriptase and 5 μM of random 9-mer primers. Control reactions were carried out in parallel using RNAs treated without M-MLV [M-MLV(−)]or TAP [TAP(−)]. All reactions were performed in the presence of 10 units of RNase Inhibitor. cDNA sequence of UL146 was amplified by nested PCR using 1.25 units of LA Taq DNA polymerase (Takara) in a 50 μL reaction mixture including LA PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 1.5 mM $MgCl_2$, and 2.5 mM of each of the four deoxynucleoside triphosphates. The primers used in the primary PCR reaction were the 5′ RACE outer primer and 146Rev1 (Table 1) and those in the second PCR reaction were the 5′ RACE inner primer and 146Rev2. The PCR conditions included an initial denaturation step at 94 °C for 3 min, 20–25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min, followed by a final extension step at 72 °C for 10 min.

2.6. Rapid amplification of cDNA 3′ ends (3′ RACE)

3′ RACE was carried out with the 3′-Full RACE Core Set Ver. 2.0 (Takara) following the manufacturer's instructions. Briefly, 500 ng of RNA was used as a template to synthesize cDNA with AMV Reverse transcriptase for 30 min at 50 °C, 5 min at 99 °C and 5 min at 5 °C. The external reverse primer, oligo dT-adaptor primer, was used to prime cDNA synthesis. cDNA sequences of the 3 clinical isolates, including isolates Han, Ch, and Xu, were then amplified by nested PCR with an adaptor primer and the forward primer